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REVIEW

Antiviral Approaches against Influenza Virus

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SUMMARY Preventing and controlling influenza virus infection remains a global public health challenge, as it causes seasonal epidemics to unexpected pandemics. These infections are responsible for high morbidity, mortality, and substantial economic impact. Vaccines are the prophylaxis mainstay in the fight against influenza. However, vaccination fails to confer complete protection due to inadequate vaccination coverages, vaccine shortages, and mismatches with circulating strains. Antivirals represent an important prophylactic and therapeutic measure to reduce influenza-associated morbidity and mortality, particularly in high-risk populations. Here, we review current FDA-approved influenza antivirals with their mechanisms of action, and different viral- and host-directed influenza antiviral approaches, including immunomodulatory interventions in clinical development. Furthermore, we also illustrate the potential utility of machine learning in developing next-generation antivirals against influenza.

KEYWORDS antiviral agents, drug resistance mechanisms, influenza, machine learning, monoclonal antibodies

INTRODUCTION

The public health measures intended to curtail SARS-CoV-2 have suppressed the circulation of influenza viruses for the 2020–2021 season (1). However, as coronavirus disease 2019 (COVID-19) restrictions relax worldwide, influenza is reemerging in the United States (US) (2) and globally (1). An estimate of past seasons places the number of deaths between ~291,000 and 646,000 globally in typical years (3), and ~12,000 to 51,000 in the US (4). The individuals with a greater risk of severe disease from influenza include people >65 years of age, children <2 years of age, individuals with comorbidities (i.e., asthma, heart, liver, kidney disease, obesity, etc.), and immunocompromised people (i.e., HIV, leukemia, and others on immunosuppressants) (5).

Influenza is primarily a respiratory disease, and organ systems outside the lungs represent an underappreciated aspect of influenza pathogenesis. Some extrapulmonary complications reported in influenza infection include renal (6), neurological (7), and cardiac (8). In addition, myocarditis, a rare but substantial side effect resulting from mRNA SARS-CoV-2 vaccination (9, 10) and SARS-CoV-2 infection (10), occurs during influenza infections (11).

The health impacts of influenza also have far-reaching effects on the economy. One way to estimate the economic impact of influenza is to assess both direct and indirect costs. These influenza-associated costs include medical care expenses and lost earnings. The estimated economic burden of influenza in the US alone is between 6.3 and 25.3 billion US dollars annually, with the most significant percentage impacting ages 18 to 49 (12).

Effective treatments and preventive measures, including vaccines and antivirals, can reduce health and economic burdens. However, the substantial diversity of influenza viruses impacts these measures. Influenza viruses belong to *Orthomyxoviridae* and are classified into A, B, C, and D types. Influenza A, B, and C viruses can infect humans. Types A and B cocirculate as the primary seasonal strains causing mild to severe

respiratory infections and other complications in humans. Yearly vaccine formulations thus incorporate both types. Influenza viruses are further subdivided into subtypes and lineages based on antigenic characteristics and genetic sequences of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) (13). Currently, 18 HA and 11 NA (14) subtypes are found in nature for influenza A virus (IAV). Based on its HA, IAV can be categorized into group 1 and group 2.

In contrast, influenza B viruses (IBVs) do not belong to groups or subtypes but are classified into two major lineages, B/Yamagata and B/Victoria. The naming conventions for influenza viruses hint at their diversity. For instance, an IAV designated A/Tasmania/503/2020 is an H3N2 component in the Flucelvax quadrivalent vaccine product for 2021 to 2022 in the US. It is so named because it was the 503rd human isolate from the island state of Tasmania, Australia, possessing an H3 HA and N2 NA subtype isolated in 2020.

Influenza diversification occurs by two main mechanisms, antigenic shift and antigenic drift. When two different influenza viruses within an influenza type coinfect the same cells within an individual, the mixing and matching of viral genome segments occur. A change in HA and NA antigenic characteristics can occur due to this reassortment, and this process is called antigenic shift. For example, the 2009 pandemic virus, initially known as "swine" flu, is a triple-reassortant virus because it contains gene segments of avian-, human-, and swine-origin IAVs (15). Pandemic influenza strains, including the 1918 Spanish flu A (H1N1), 1957 Asian influenza A (H2N2), 1968 Hong Kong influenza A (H3N2), and the 2009 pandemic influenza A (H1N1)pdm09, arose due to antigenic shifts. Antigenic drift, a much slower process, refers to the accumulated genetic mutations within the viral genome over time. Antigenic drift and shift have implications for the genesis of an epidemic, pandemic, and drug-resistant influenza viruses. Such a dynamic viral diversity is also the reason why it is necessary to update vaccines annually.

Vaccination is currently the best method to protect against morbidity and mortality from influenza infection. However, vaccine effectiveness varies by year, population under study, and strain. Overall, the vaccine effectiveness ranged from 10% to 60% in the US from 2004 to 2021 (16). The factors involved include vaccine mismatch (17–19), preexisting influenza immunity, age, weight, biological sex, and immune status (20–25). Efforts to improve vaccine efficacy are a complex problem of urgent concern, as both host and viral factors play crucial roles. Meanwhile, alternatives are needed to fill the gaps caused by vaccine-related issues for prophylactic and therapeutic interventions.

Antiviral drugs have been critical tools in the struggle against influenza viruses. The Centers for Disease Control and Prevention (CDC) recommends four antiviral drugs to treat influenza, Oseltamivir phosphate, Zanamivir, Peramivir, and Baloxavir marboxil (BXM). Oseltamivir, Zanamivir, and Peramivir are NA inhibitors that block NA activity and viral egress from cells, while Baloxavir inhibits viral replication by inhibiting the polymerase acidic protein (PA) (26). These drugs are not a cure but can reduce the time to clinical resolution (27, 28). NA inhibitors and Baloxavir are also recommended for treating humans infected with avian influenza viruses, A(H5N1), A(H7N9), and A(H5N6) (29).

The emergence of drug-resistant influenza strains can render antivirals ineffective (30). The matrix protein 2 (M2) inhibitors, like Amantadine used since the 1960s (31) against seasonal influenza, are no longer recommended. The widespread prevalence of M2 mutations conferring resistance—occurring initially in A(H3N2) viruses between 2003 and 2006 and A(H1N1) viruses in 2009—resulted in the discontinuation of M2 inhibitors in treating influenza (32–36). In addition, NA inhibitor-resistant (36) and Baloxavir-resistant (27) strains have emerged, though their prevalence can change rapidly depending on the specific drug in question and the background strain where the mutation occurs (30, 37). Improving therapeutics against influenza can fill in gaps in vaccine efficacy that are likely to persist in the near and long term, considering the unpredictable nature of seasonal and pandemic influenza outbreaks. This review

focuses on antiviral and immunomodulatory drugs that are US Food and Drug Adminstration (FDA)-approved or in clinical development against the influenza virus.

INFLUENZA VIRION AND REPLICATION IN HOST CELLS

Influenza is a negative-sense single-stranded RNA (ssRNA) virus with segmented genome encapsidated by the viral nucleoprotein (NP) (38). The virion is usually spherical or elliptical, ranging in size from 80 to 120 nm in diameter, and sometimes filamentous, reaching more than 20 μm in length (39). The host plasma membrane forms the lipid envelope protecting the viral genome. Two viral glycoproteins, HA and NA, along with small amounts of matrix protein 2 (M2), are present on the surface of the virions (40). M2 forms ion channels through the lipid layers. The matrix protein 1 (M1) forms a lining underneath the lipid bilayer to maintain the morphology of the virion (41). The envelope, along with the viral proteins, encloses and protects the virion core, which contains the viral ribonucleoprotein (vRNP) complexes and the nuclear export protein (NEP) (42). Each viral RNA (vRNA) contains conserved sequences at their 3′ and 5′ ends and ranges in size from 890 bp to 2,341 bp (43). NP wraps around each of the genomic RNA segments that also contains a copy of the RNA-dependent RNA polymerase (RdRp) consisting of polymerase acidic protein (PA), polymerase basic protein 1 (PB1), and polymerase basic protein 2 (PB2) subunits (39). The IAV and IBV viruses carry eight negative-strand RNA segments, whereas the C and D viruses contain seven segments each (44, 45). The genome of the IAV encodes 12 proteins essential for efficient replication in host cells and the release of mature virions. These proteins consist of the three proteins that make up the vRNA polymerase subunits (PA, PB1, and PB2), the viral glycoproteins HA and NA, M1, M2, NP, PB1-F2, and nonstructural proteins 1 (NS1) and 2 (NS2) or NEP (46, 47).

Figure 1 shows the life cycle of the virus in host cells. The influenza virus enters the host by infecting the epithelial cells in the respiratory tract (in humans) or the intestinal tract (in birds) (48). The binding of the viral HA protein to *N*-acetylneuraminic (sialic) acid expressed on the host cell surface facilitates the virus attachment to the host cells. Sialic acids are nine-carbon acidic monosaccharides found at the termini of many glycoproteins. The binding of the sialic acid to either carbon 3 or carbon 6 of the galactose results in forming either α-2,3 or α-2,6 configurations. The HA protein has specificity for either α-2,3 or α-2,6 linkages (49). After binding to the host receptors, the virus gets internalized by the receptor-mediated endocytosis in the endosome. M2 ion channel proteins facilitate proton transport, resulting in the acidification of the virion. Acidification triggers a conformational change in the HA protein and induces fusion of the viral envelope with the endosome and disrupts the internal protein-protein interactions in the virions, thus releasing the viral RNPs into the host cytoplasm (50). The viral genome is imported into the nucleus for replication. The NP and all three viral RNA polymerase subunits carry the nuclear localization signals (NLSs). In addition, the importin α/β-dependent nuclear import pathway also takes part in the nuclear import of vRNPs (51–55). After importing into the nucleus, the viral RNA's transcription for viral protein synthesis and replication occurs. The RNA polymerase complex of vRNP initiates transcription and replication (45, 56). The primer-independent replication of the virus occurs *de novo* and goes through a cRNA intermediate to create more vRNA copies (45, 56).

The influenza virus NP monomers encapsulate the genome to form a double-helix structure capped by the subunits of the RNA polymerase components PA, PB1, and PB2 at one end. The newly formed viral RNA and polymerase complexes (RNPs) traffic to the plasma membrane in a rat sarcoma virus (Ras)-related Rab GTPase protein 11-dependent manner for assembling new virions (57). While the replication of the viral genome is a primer-independent process, transcription of the genome is a primer-dependent process. Since the viral RNA polymerase (RNAP) lacks capping ability, the RNAP uses a process termed "cap snatching" for generating 5′ capped RNA primers used for mRNA synthesis (58). The viral RNAP binding to a serine 5′ phosphorylated host RNA polymerase II C-terminal domain (CTD) initiates the cap-snatching process

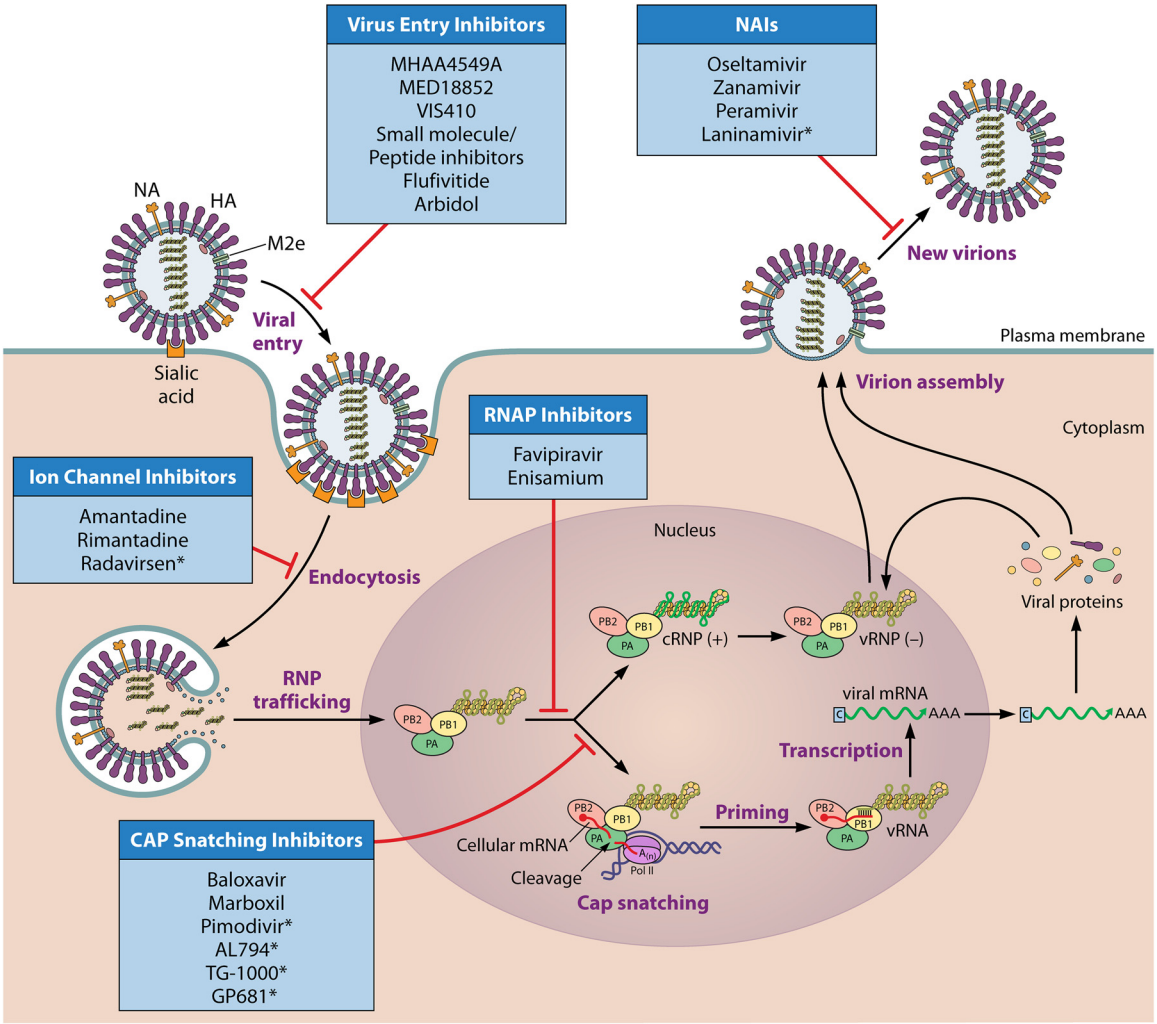

FIG 1 Schematics showing the life cycle of the influenza virus and sites of action of anti-influenza drugs. Influenza virus enters the cell by attaching to the epithelial cell surface by binding the viral hemagglutinin (HA) to sialic acid receptors expressed on the surface of host cells. Once the virus is internalized through endocytosis and fusion, the viral M2 protein forms a channel facilitating the release of the viral genome in the cytoplasm. The viral RNPs are imported into the nucleus, where they undergo the process of cap snatching, as viral RNAP lacks capping abilities. Further, it is replicated and transcribed into mRNA to facilitate the viral protein expression and participate in synthesizing the genomic RNA for incorporation into new progeny viruses. The release of new virus particles to the extracellular milieu is promoted by the viral neuraminidase (NA). Steps at which different antiviral drugs target the virus life cycle are shown in blue boxes. RNA polymerase (RNAP) inhibitors either inhibit the RNA replication or cap-snatching properties of RNAP. The amantadine class of drugs blocks the internalization and uncoating of the virus. However, neuraminidase inhibitors prevent viruses from budding and dispersing. Similarly, as indicated in the figure, mAb and small-molecule inhibitors also potentially inhibit the entry of the virus. Drugs in clinical trials are denoted by asterisk.

(59). The PB2 subunit binds to the host RNAs containing a cap 1 structure, and the PA endonuclease cleaves approximately 9 to 14 nucleotides downstream of the 5′ cap, resulting in generation of primers with the 3′-hydroxyl group (56, 60, 61). The PB1 subunit plays a crucial role in the RNA polymerase's assembly and catalytic function. The RNAP reaches a uridine-rich sequence at about 16 to 17 nucleotides from the 5′ end of the vRNA template, triggering termination and polyadenylation. This results in the generation of viral mRNA transcripts identical to the cellular transcripts, thus enabling the viral mRNAs to evade the host's innate immune recognition.

Further, it also enables the nuclear export of mRNA into the cytoplasms of infected cells, leading to their translation for the production of viral proteins (62). The cytosolic ribosomes and the endoplasmic reticulum-associated ribosomes translate the viral mRNAs (NP, PB1, PB2, PA, NS1, NS2, and M1) and viral glycoproteins-associated mRNA (HA, NA, M2), respectively (63). The importin α/β proteins shuttle the newly synthesized

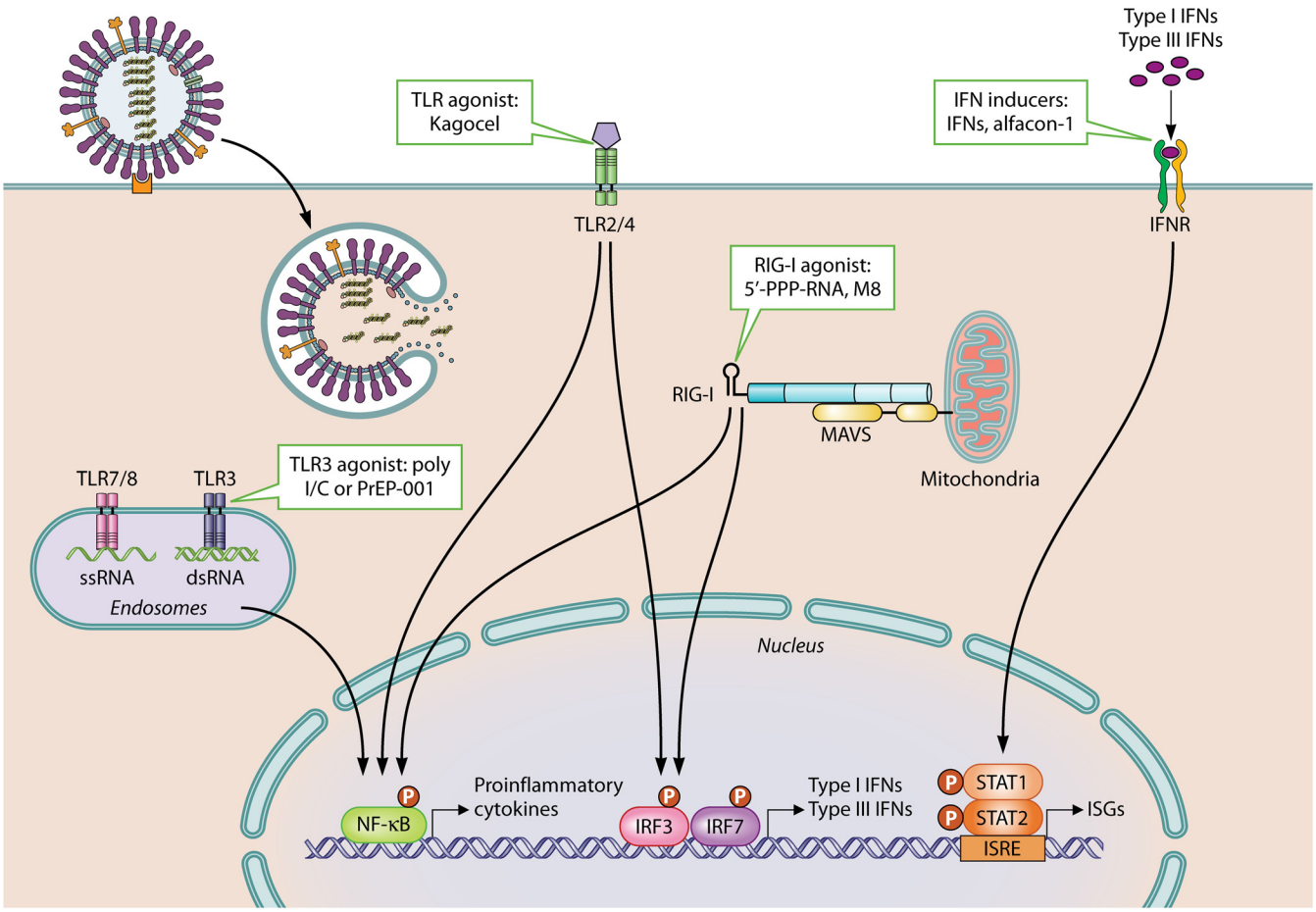

FIG 2 Host innate immune sensing of the influenza virus by RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs). The cytosolic and endosomal RNA sensors, RLRs and TLRs, can detect genomic RNA, dsRNA, and small RNA molecules produced during viral replication. An activated RLR, RIG-I, undergoes a conformational change that allows the RLRs to recruit other proteins and trigger the IFN-signaling pathway. On the other hand, TLR3 and TLR7/8 initiate the antiviral IFN program by recruiting the signal adaptor molecules that subsequently activate downstream kinases and transcription factors to elicit the production of the IFNs and proinflammatory cytokines. Agonists and inducers as potent stimulators of the innate antiviral response in pipeline as antiviral drugs are indicated.

NP and polymerase subunits from the cytosol to the nucleus. These imported viral polymerases increase the rate of viral RNA synthesis. Subsequently, the viral proteins HA, NA, and M2 traffic to the host cell membrane to assemble the mature virion, and the viral M1 and NEP proteins facilitate this process (44). Further, the sialidase activity of the NA removes the sialic acid residues from the N-linked glycans present on the HA and NA of the viral envelope, leading to the release of the newly assembled virions (44, 64).

INNATE IMMUNE RECOGNITION AND VIRAL EVASION

To invade the host tissues, the virus must overcome the mucus layer lining the respiratory and oral mucosa (65). Once the virus enters the host cells, the host activates cellular defenses to clear the infection. Initial recognition of the influenza virus involves the Pattern recognition receptors (PRRs) recognizing the components of the pathogen called pathogen-associated molecular patterns (PAMPs) or components of the cells referred to as damage-associated molecular patterns (DAMPs). The three main classes of PRRs involved in the recognition of the influenza virus are Toll-like receptors (TLRs), the retinoic acid-inducible gene-like (RIG-I) receptors (RLRs), and the nucleotide oligomerization domain (NOD)-like receptor family (NLRs) pyrin-containing 3 (NLRP3) (Fig. 2) (66–68). The RLR family sensors, consisting of RIG-I, melanoma differentiation factor 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2), recognize single- or double-stranded RNA (dsRNA) molecules (69). The most studied NLR is NLRP3,

which leads to the activation of the inflammasome pathway, which plays a vital role during influenza virus infections (70, 71). Monocytes, macrophages, dendritic cells (DCs), neutrophils, and epithelial cells express NLRP3. NLRP3 is activated by several PAMPs and DAMPs, including dsRNA, the viral M2 protein, and the reactive oxygen species (71). In addition to the cytoplasmic receptors, TLRs on the cell membrane are also crucial for antiviral response (69, 72). For influenza virus recognition, TLR3, TLR7/8, and RIG-I, which recognize dsRNA and ssRNA, play vital roles (73, 74).

The recognition of PAMPs by PRRs activates several transcription factors, including interferon regulatory factors 3 and 7 (IRF3 and IRF7) and nuclear kappa light chain enhancer of activated B cell (NF-κB), resulting in type I and type III interferon (IFN) secretion (75). These IFNs bind to their receptors and induce the expression of interferon-stimulated genes (ISGs) that mediate various antiviral functions. IFNs induction also leads to the release of several proinflammatory cytokines and chemokines, the activation and modulation of antigen-specific T-cell and B-cell responses, and the recruitment of cells such as neutrophils, monocytes, and natural killer cells to the sites of infection. The dendritic cells (DCs) in the lungs are classified as migratory conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs, which play an important role after exposure to influenza virus infection (76, 77). The lung-migratory cDCs, following uptake of viral antigens, migrate to secondary lymphoid organs, where they present viral antigens to the naive B and T cells to induce adaptive immune responses. Both innate and adaptive responses contribute to the dampening of influenza virus infection, leading to protection and viral clearance (78, 79).

While the host mounts a defense response to clear the virus infection, the virus has also developed strategies to overcome the host immune system. Understanding these pathways provides insights into building better therapeutic interventions or targeting the host's signaling pathways to overcome the viral evasive mechanisms. The viral NS1 protein is the leading player in overcoming the host response. In infected cells, NS1 is rapidly expressed to high levels and interferes with the host's pathways at multiple levels to antagonize the host innate immune response (80). Specifically, NS1 inhibits the induction of type I IFNs by inhibiting the RIG-I-mediated antiviral signaling pathway (81–84). In addition, NS1 can bind to host protein kinase R (PKR) (85) and eukaryotic translation initiation factor 4G (eIF4G) and promotes the translation of mRNA (82, 86). Segment 3 of the influenza genome encodes protein PA-X, which also blocks the antiviral responses. In infected cells, PA-X is known to selectively degrade the host's mRNAs and noncoding RNAs, which is beneficial for viral replication due to the attenuation of the antiviral responses (87). Thus, downregulating the activity of viral NS-1 or PA-X or enhancing the expression of the host RIG-I signaling pathway offers opportunities to develop intervention strategies to combat influenza infections.

FDA-APPROVED ANTIVIRAL DRUGS FOR INFLUENZA

Anti-influenza compounds can be effective in controlling viral infections during epidemics or pandemics. The critical events in the viral life cycle (viral binding to cellular receptors, viral entry, fusion and release of RNP, transcription, translation of viral proteins, assembly, and viral budding) can be hindered by antiviral compounds, as shown in Fig. 1. M2 ion channel inhibitors or adamantanes (not recommended anymore), NA inhibitors (sialic acid analogs), and a polymerase inhibitor (metal-chelating compound), which target viral entry, replication, release, and spread, are three classes of antiviral drugs approved by the FDA to control influenza virus infections. In Table 1, currently used FDA-approved drugs and their mode of action are summarized, and Fig. 3 shows their structures.

M2 Ion Channel Inhibitors

Both Amantadine and Rimantadine belong to the adamantane class of drugs (88). The FDA approved Amantadine (Symmetrel) in 1976 and Rimantadine (Flumadine) in 1994 (89) to prevent and treat IAV (90) in the US. Adamantanes served as the first-choice antivirals against IAV outbreaks for many years (91, 92). Both Amantadine

TABLE 1 FDA-approved antivirals currently being used for the treatment of influenza

Characteristic	Oseltamivir	Zanamivir	Peramivir	Baloxavir
Activity	IAV and IBV	IAV and IBV	IAV and IBV	IAV and IBV, including NAI-resistant strains
Antiviral target	Neuraminidase	Neuraminidase	Neuraminidase	Endonuclease
Mechanism of action	Impairs virus release	Impairs virus release	Impairs virus release	Blocks viral transcription
Administration/duration	Oral	Inhaled	Intravenous	Oral
Treatment	Any age	7 yrs and older	6 mo and older	12 yrs and older
Prophylaxis	3 mo and older	5 yrs and older	Not recommended	Postexposure prophylaxis 12 yrs and older
Plasma half-life (h)	6–10	2.5–5.1	12–24	79.1 h
Peak time (h)	3–4	1–2	2–4	3.5–4 h
C_{max}[a] (ng/mL)	259	39	34	68.9 for 40 mg, 82.5 for 80 mg
Protein binding (%)	3	<10	<30	93–94
Clearance	99% kidneys	Urine and feces, unabsorbed	90% renal	Feces and urine
Pro(s)	Impairs release of both IAV and IVB	Impairs release of both IAV and IVB, can be inhaled	Impairs release of both IAV and IVB	Blocks transcription from IAV and IVB
Cons	Should be given within the first 48 h of symptom onset	Should be given within the first 48 h of symptom onset	Should be given within the first 48 h of symptom onset	Cannot be given with dairy products, calcium-fortified beverages, or laxatives and antacids
	Dose adjustment is required for renal dysfunction	Not to be used in patients with allergies to lactose, asthma, or COPD	Diarrhea, bronchitis, nausea, and sinusitis	Diarrhea, bronchitis, nausea, and sinusitis
	Nausea, vomiting, and dizziness	Bronchospasm, bronchitis, cough, sinusitis		
Resistance marker(s)	H275Y (H1N1, H1N1 pandemic), R292K (H7N9)	R294K (H7N9)	H275Y (H1N1)	I38T (H1N1 pandemic, H3N2)
References	152, 418–420	419, 421	152, 419	192, 422, 423

[a]C_{max}, maximum concentration of drug in serum.

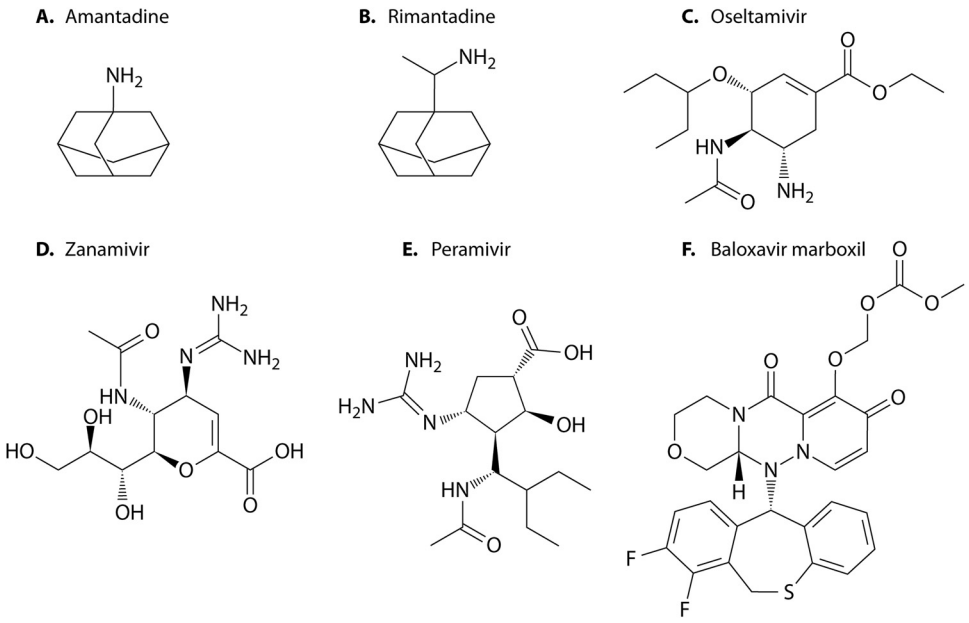

FIG 3 Structures of FDA-approved antivirals for influenza. (A) Amantadine (IUPAC name, adamantan-1-amine). (B) Rimantadine (IUPAC name, 1-(1-adamantyl)ethanamine). (C) Oseltamivir (IUPAC name, ethyl (3R,4R,5S)-4-acetamido-5-amino-3-pentan-3-yloxycyclohexene-1-carboxylate). (D) Zanamivir (IUPAC name, (2R,3R,4S)-3-acetamido-4-(diaminomethylideneamino)-2-[(1R,2R)-1,2,3-trihydroxypropyl]-3,4-dihydro-2H-pyran-6-carboxylic acid). (E) Peramivir (IUPAC name, (1S,2S,3S,4R)-3-[(1S)-1-acetamido-2-ethylbutyl]-4-(diaminomethylideneamino)-2-hydroxycyclopentane-1-carboxylic acid). (F) Baloxavir marboxil (IUPAC name, [(3R)-2-[(11S)-7,8-difluoro-6,11-dihydrobenzo[c][1]benzothiepin-11-yl]-9,12-dioxo-5-oxa-1,2,8-triazatricyclo[8.4.0.03,8]tetradeca-10,13-dien-11-yl]oxymethyl methyl carbonate).

and its derivative, Rimantadine, target and inhibit the function of IAV M2. However, the IBV M2 ion channel is not sensitive to the adamantanes class of drugs (93). The most common side effects of these M2 inhibitors are anxiety, insomnia, dizziness, headache, and gastrointestinal upset (94, 95).

Mechanism of action. The tetrameric M2 protein belongs to type III transmembrane (TM) proteins known as viroporins (96, 97). Embedded in the viral membrane, M2 forms an ion channel (98). There are ~14 to 68 molecules of M2 per virion (99). The M2 protein allows acidification and uncoating, which is necessary to import vRNPs into the nucleus. It also plays an important role in viral assembly (100) and budding (101). The Amantadine-sensitive ion channel from IAV is well characterized, with several X-ray and nuclear magnetic resonance (NMR) structures (102–105). IAV M2 consists of three regions, the extracellular domain, the transmembrane domain (TMD), and the cytosolic tail. The N-terminal domain from amino acid residues 1 to 22 is required for M2 incorporation into virion (103, 106). The M2 TMD region is conserved in all the human, swine, equine, and avian strains of IAV (107–109). The minimal functional IAV M2 TMD comprised of amino acid residues from 22 to 46 is essential for tetramerization and retaining conductance features like a full-length protein (96, 110). Moreover, the M2 ion channel is affected by both the pH and the surrounding membrane environment (102). The wild-type (WT) M2 ion channel of IAV has proton/cation exchange activity and is selective for protons over Na^+ and K^+ ions (111, 112).

After viral entry, the ion channel allows protons from host cell endosomes to enter the virion core. The influx of protons makes the core acidic, allowing M1 and vRNPs to dissociate (113). Blocking the M2 protein ion channel activity with Amantadine and Rimantadine can prevent the uncoating step (114). M2 also plays an essential role during the transport of newly synthesized HA protein across the *trans*-Golgi network (TGN) to the cell surface. M2 equilibrates the pH of the TGN with that of the host cell cytoplasm and prevents premature conformational rearrangement of HA (115, 116). Blocking this activity of the IAV M2 protein ion channel with Amantadine and

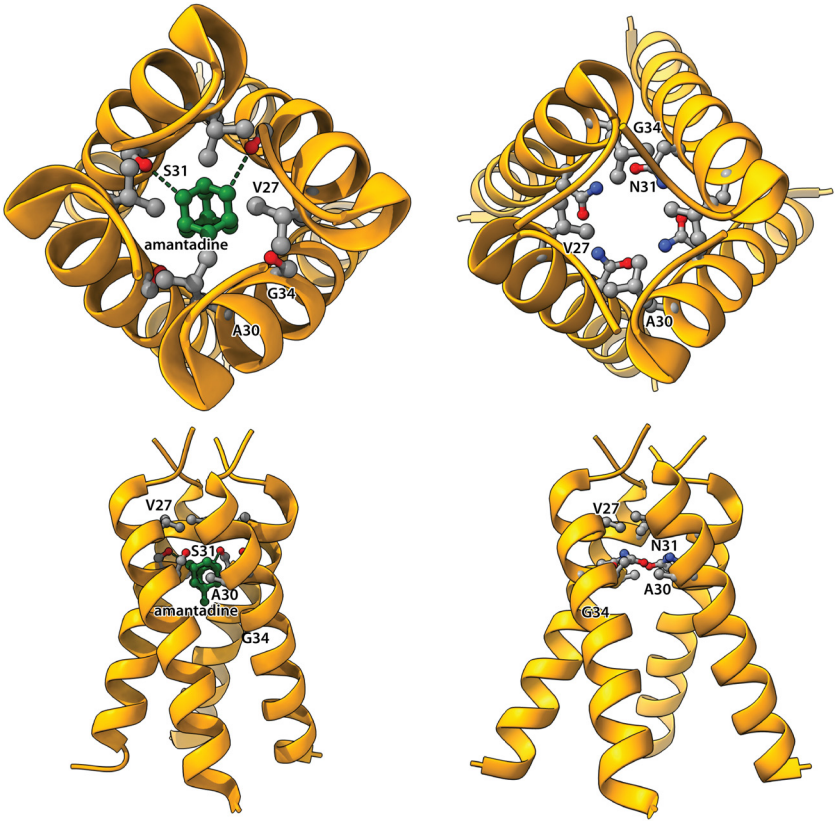

FIG 4 Solved structures of IAV M2 wild type (PDB accession no. 6BKK) with Amantadine bound (left) and the Ser31Asn mutant (PDB accession no. 6MJH), which blocks Amantadine binding (right). The top left structure depicts the IAV M2 wild type (orange) from a top-down perspective, i.e., looking through the transmembrane channel. The Ser31 residues from all four chains in the quaternary structure interact with the bound Amantadine (green). The bottom left vignette depicts a 90°rotation of the structure to show the overall structural geometry of the wild-type IAV M2. The top right structure represents the Ser31Asn mutant IAV M2, which blocks Amantadine binding. The asparagines from each chain interact with one another to cause a structural change to the protein, which prevents the Amantadine to bind yet maintains protein function. The Ser31Asn mutation in the IAV M2 protein renders the antiviral Amantadine ineffective.

Rimantadine drugs can prevent the fusion of the virus and host-cell membranes (117). The adamantane class of drugs targets the TMD of IAV M2 (108). Amantadine (100 μM) treatment can block 90% of the A(H3N2) M2 channel activity (107, 118). Structural studies revealed that the TM pore contains the drug-binding site (119–122). Several studies confirmed that the pore-binding site is Amantadine's drug-binding site in M2 (123–125). Crystal structures of M2 further showed that the His37 and Trp41 residues were most conserved in the the IAV M2 TMD (109) and essential for ion channel activity. His37 is required for high proton selectivity, and Trp41 is needed for opening and closing the pore, acting as the channel gate (126, 127). Mutating His37 abolishes proton selectivity and low pH activation properties of M2 (128). Several other amino acid residues critical for ion channel activity were also identified by site-directed mutagenesis (129, 130). The crystal structure of M2 in complex with the inhibitor further showed that the drug molecule interacted with Val27, Ala30, Ser31 (Fig. 4, left), and Gly34 residues (108).

Solid-state NMR spectroscopy demonstrated a low- and high-affinity drug-binding location in the IAV M2 TMD channel. The M2 channel gets occluded when the drugs bind to these sites (119). Another study suggested inhibitor binding outside the M2 channel (117). A recent study investigated the oligomerization of the full-length IAV M2 TMD with various concentrations of Amantadine. The authors observed that IAV M2 could assemble into a range of oligomeric states and Amantadine perturbed

oligomerization. These findings suggest that Amantadine could directly affect ion channel activity or induce conformational changes in the M2 ion channel (102).

Resistance to M2 ion channel inhibitors. Drug resistance-conferring substitutions occur in the TM region of the M2 molecule. Influenza viruses with Ser31Asn (Fig. 4, right), a mutation of M2 started circulating before the wide use of Amantadine. In clinical isolates, even a single substitution in the amino acid at position 26, 27, 30, 31, or 34 of the M2 protein conferred drug resistance (34, 131–133). Recombinant A(H1N1) viruses engineered with Leu26Phe, Val27Ala, Ala30Thr, Ser31Asn, Gly34Glu, and Val27Ala/Ser31Asn mutations in the M2 gene also showed that these mutations could cause Amantadine resistance (32). M2 mutants retained virulence and remained transmissible between humans. Amantadine inhibited the wild-type IAV M2 channel activity but not the Ser31Asn IAV M2 channel (134), which are two mechanisms by which IAV avoids blockage of its M2 ion channel function. Stouffer et al. proposed a model where drug binding physically blocks the channel's pore, halting proton flow (108). The drug-resistant mutations in the IAV M2 TMD do not permit the binding of drugs to the channel. Another study showed that proton flow continued through the channel despite the drug binding to M2 TMD. Regardless of the drug binding in the channel pore, an increase in the channel diameter retained function (135). The resistance to adamantanes can also emerge during treatment (133, 136). Surveillance studies worldwide reported increased M2 resistance to adamantanes in A(H3N2) and A(H1N1) viruses (33, 34), and almost 95% of resistant viruses had the Ser31Asn change, whereas only about 1% had the Val27Ala substitution. Leu26Phe, Ala30Thr, Gly34Glu, and Lys38Phe were extremely rare ($<$0.2%) (133). These resistant variants displayed no reduction in replicative fitness or transmissibility (137).

Virus strains from human, avian, and swine reservoirs have M2 ion channel mutations. A majority of H5N1 double mutants, reported between 1996 and 2005 from Vietnam, Cambodia, Malaysia, and Thailand, carried Ser31Asn and Leu26Ile M2 substitutions (138). M2 mutations with Ser31Asn and Val27Ala were also dominant among circulating H5N1 viruses (139). In summary, resistance to this class of drugs severely impacted their effectiveness against many influenza strains, including the 2009 A(H1N1)pdm09 pandemic strain (34, 90). In 2006, the CDC recommended discontinuing the use of both Amantadine and Rimantadine for the treatment of influenza (140).

The IBV M2 structure became available recently (141). The closed and open IBV M2 channels differ significantly from the closed and open IAV M2 channels. Also, IBV M2 has polar pore-facing residues and conducts protons inward and outward. In contrast, IAV M2 has a hydrophobic pore and conducts protons inward. Further research and development of next-generation antiviral agents that target both IAV and IBV M2 channels with the least potential to generate resistant strains are needed.

NA Inhibitors

The NA enzyme comprises four identical subunits (each ~470-amino-acid [aa] residues), with discrete structural domains comprising the cytoplasmic tail, the TM region, the stalk, and the catalytic head (142, 143). The NA enzyme cuts α-ketosidically linked Neu5Ac residues on the ends of various glycoproteins or glycolipids (144). NA inhibitors (NAIs), the first structure-based rationally designed inhibitors for the influenza virus, specifically target the active site of NA (145, 146). Stalling the catalytic activity of the NA enzyme by NAIs results in the formation of virus aggregates and prevents virus release, thus effectively limiting reinfection (147).

Several *in vitro* studies conducted in the 1970s demonstrated the ability of sialic acid analogs to inhibit NA activity (147–149). Further advances in the field came when the crystal structure of the NA molecule became available in the early 1980s. These studies paved the way for the discovery of several effective NA inhibitors, including three (Zanamivir, Oseltamivir, and Peramivir) approved in the US for clinical use (Table 1). The FDA approved Zanamivir (Relenza Diskhaler) and Oseltamivir (Tamiflu) in 1999 for the treatment of IAV and IBV in the US (150, 151). In addition, the FDA approved

Peramivir (Rapivab) for treatment of influenza infections in adults in 2014 (26). NAIs shorten the length of treatment, decrease hospitalization time in intensive care unit patients, and reduce the risk of death (152).

The prodrug Oseltamivir phosphate is a sialic acid analog and is hydrolyzed to its active form, Oseltamivir carboxylate. It is an oral antiviral treatment for acute influenza in adults and children over 2 weeks old (up to 48 h after the onset of symptoms) and for chemoprophylaxis in adults and children over 1 year old. The CDC recommends the use of oral Oseltamivir for the treatment of influenza in infants (<14 days old). Although not part of the FDA-approved indications, the CDC and the American Academy of Pediatrics recommend Oseltamivir for chemoprophylaxis in 3- to 12-month-olds and Oseltamivir in liquid form is approved to treat infants over 2 weeks of age or older (153, 154). Adverse effects reported for Oseltamivir include vomiting, nausea, abdominal pain, delirium, and anemia (26, 28). Zanamivir (inhaled) is used for the treatment of uncomplicated acute influenza infection (both A and B) within 2 days after the onset of symptoms (adults and children aged over 7 years old). Each Relenza Rotadisk (GSK) contains four blisters. Each blister holds a powder mixture of 5 mg of Zanamivir and 20 mg of lactose (155). Early treatment of uncomplicated influenza with Zanamivir leads to a reduction in severity of symptoms. It is also recommended for chemoprophylaxis of influenza in adults and children over 5 years old. Its use is contraindicated for individuals with severe milk protein allergy since each blister contains 20 mg of lactose or milk protein.

Adverse effects of Zanamivir include fever, myalgia, cough, headache, bronchospasm, nausea, and vomiting (26). Peramivir is approved for treating uncomplicated acute influenza within 48 h of symptoms onset (patients aged over 18 years old). However, it is not approved for use in children or for prophylaxis. Adverse drug-related outcomes include diarrhea, constipation, insomnia, and hypertension (26). Recently, a new preparation of Zanamivir, as a solution for intravenous administration, called Dectova, was authorized for use in the European Union. Intravenous Zanamivir, or Dectova, is used in seriously ill and hospitalized patients infected with IAV or IBV (156). It allows the treatment of patients whose medical condition does not allow the use of medications suitable for oral administration or inhalation (157).

Mechanism of action. The current understanding of NA's active site comes from several structural and biochemical studies (150, 158, 159). There are several catalytic residues (Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371, and Tyr406; N2 numbering) which interact with sialic acids and participate in catalysis. These framework residues (Glu119, Arg156, Trp178, Ser179, Asp198, Ile222, Glu227, His274, Glu277, Asn294, and Glu425; N2 numbering) stabilize and maintain the functional structure of the active site by an extensive network of hydrogen bonds (160–162). These framework residues that do not interact with sialic acid support the catalytic residues. The active site of the NA enzyme consists of five regions based on the binding environment, S1 (Arg118, Arg292, and Arg371 positively charged electrostatic region), S2 (Glu119 and Glu227 negatively charged region), S3 (Trp178 and Ile222 hydrophobic region), S4 (a hydrophobic region derived from the side chains of Ile222, Ala246, and the hydrophobic face of Arg224), and S5 (mixed polarity, Glu276 and Ala246) (159). The left structure in Fig. 5 shows Oseltamivir (green) firmly bound to the NA wild-type protein (orange) through interactions with numerous residues, including Arg224, Glu276, Asn294, Tyr406, Arg371, and Arg292. Based on crystal structures, group 1 and group 2 NAs exist in at least two conformations due to orientation of "loop 150." Structurally, the group 1 NAs show a cavity next to the catalytic active site formed due to the movement of loop 150 because of the binding of the substrate within the active site. The NAs from group 2 lack this cavity (163, 164).

NA inhibitors (Oseltamivir, Zanamivir, and Peramivir) mimic the enzyme's natural substrates. These sialic acid analogs competitively inhibit NA activity by binding to conserved residues in its active site (144, 159, 165). The crystal structure of Neu5Ac2en or DANA (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid), a transition-state analog of sialic acid, in complex with NA, allowed further characterization of the catalytic site

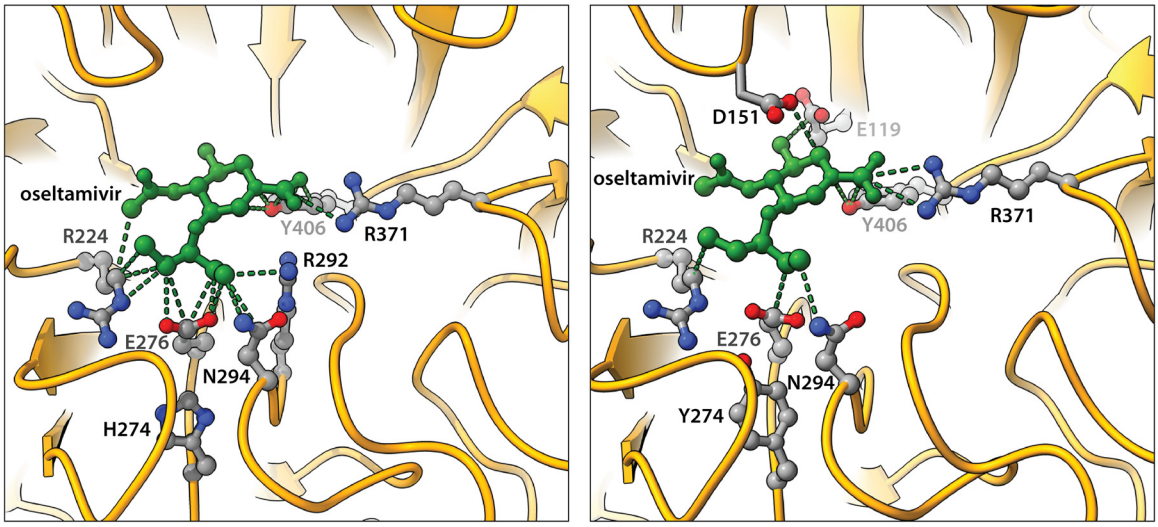

FIG 5 N1 neuraminidase (NA) wild type (PDB accession no. 2HU0) with bound Oseltamivir (left) and NA H274Y mutant (PDB accession no. 3CL0) with bound Oseltamivir (right). The left structure shows Oseltamivir (green) firmly bound to the NA wild-type protein (orange) through interactions with numerous residues, including Arg224, Glu276, Asn294, Tyr406, Arg371, and Arg292. The right structure shows the H274Y mutant, which results in a modified binding pocket. While the residue at 274 does not directly interact with the bound antiviral molecule, the mutation from histidine to tyrosine negatively affects the binding affinity of the drug through changes in the overall binding pocket, as seen by the reduced number of interactions on the right. The H274Y mutation in the NA protein renders the antiviral Oseltamivir less effective.

(166). The NA inhibitor complex showed several amino acid residues that directly bind DANA (167). In summary, NAIs closely mimic their natural substrate or enzyme transition-state complex and fit in the active site pockets due to an energetically favorable interaction (168).

Resistance to NAIs. NA inhibitors, especially Oseltamivir, are widely used in the US and have demonstrated clinical effectiveness against influenza viruses (seasonal and emerging) (169, 170). Since NAIs resemble the natural substrate (sialic acid), it was reasoned that developing drug-resistant viable mutant viruses would be difficult. However, drug-resistant mutants were reported *in vitro* (171) and in the clinic (172, 173). A(H1N1) viruses resistant to Oseltamivir were detected worldwide during the 2007 to 2008 influenza season (174). By 2009, most circulating seasonal A(H1N1) viruses were resistant to Oseltamivir. These were eventually displaced by the emerging A(H1N1)pdm09 viruses. A(H1N1) virus with a single His275Tyr amino acid change resulted in resistance to Oseltamivir (162). The His275Tyr mutation near the drug binding site of NA (163) perturbed the conformation of NA, allowing a decrease in the binding free energy of the His275Tyr NA-Oseltamivir complex (162, 175–179). The structure in Fig. 5, right, shows the His274Tyr mutant with a modified binding pocket. While the residue at 274 does not directly interact with the bound antiviral molecule, the mutation from histidine to tyrosine negatively affects the binding affinity of the drug through changes in the overall binding pocket. Several NA substitutions or deletions have been associated with reduced inhibition by one or more NAIs (summarized on the WHO global influenza surveillance and response system website) (180). Resistant H5N1 viruses with substitutions His275Tyr (His274Tyr) and/or Asn294Ser (Asn295Ser) were isolated from humans and bird species (181). There is a difference in the ability of NAIs, especially Oseltamivir, to inhibit the enzyme activity of H5N1 viruses from two clades, 1 and 2, as there are amino acid differences (e.g., Tyr252) which have been shown to affect the baseline susceptibility in neuraminidase inhibitor (NI) assay (182, 183).

Global surveillance of influenza viruses' susceptibility to NAI was conducted by the Neuraminidase Inhibitor Susceptibility Network (NISN) since Oseltamivir and Zanamivir entered the global markets in 1999. Since 2012 to 2013, the Antiviral Working Group

(AVWG) of the Global Influenza Surveillance and Response System (GISRS) of WHO has been performing this activity (184). WHO Collaborating Centers collected a total of 10,641 influenza viruses worldwide between 2013 and 2014. Around 2% of the viruses showed reduced inhibition to at least one NA inhibitor (185). Surveillance conducted in 2017 to 2018 assessing 15,409 viruses reported the occurrence of viruses with reduced susceptibility to NAIs being 0.8% (186).

The Oseltamivir-resistant strains (His274Tyr, Ile117Val, Glu119Ala, and Arg292Lys) have emerged and have an impact on the use and effectiveness of NAIs (187). These Oseltamivir-resistant strains remain sensitive to Zanamivir (156). Therefore, NA inhibitors remain an important option for treating influenza virus-infected subjects.

Inhibitors of Viral Polymerase

Influenza virus RdRp is a heterotrimeric complex with three subunits, PA, PB1, and PB2, (60), which perform both RNA transcription and replication processes. Therefore, viral polymerase remains a very attractive target for the design of new anti-influenza therapeutics (188). Baloxavir marboxil (BXM) (Table 1) is a novel inhibitor of the viral RNA polymerase. BXM got its first approval in Japan (February 2018), followed by the US (October 2018) and Europe (January 2021), for treating IAV and IBV infections (27, 189). Since influenza viruses lack their capping enzymes, they snatch the cap from nascent host RNAs. This strategy also makes viral mRNAs structurally indistinguishable from the host's mRNAs and exploits the host's cellular pathways for nuclear export and further processing of its genetic components (178). BXM is a prodrug of Baloxavir acid (BXA), which can specifically target the cap-dependent endonuclease activity of the PA subunit of the influenza virus. It also inhibits viral strains resistant to NAIs (29, 177). BXA is the first anti-influenza drug that inhibits the viral polymerase activity from several subtypes of influenza A viruses [A(H1N2), A(H5N1), A(H5N2), A(H5N6), A(H7N9), and A(H9N2)] (190). An *in vitro* study by Mishin et al. showed that BXA inhibitor has broad activity and inhibits replication of type A, B, C, and D viruses (191).

A recent multicenter, randomized, placebo-controlled trial further evaluated the clinical outcomes of this inhibitor during the 2018–2019 flu season. The study assessed the post-exposure efficacy of a single-dose BXM in preventing influenza infection in household contacts. The study found a significant decrease in clinical influenza cases among BXM treated versus the placebo group (13.6% placebo group versus 1.9% BXM group) (192). In addition, the risk of viral infection, irrespective of indications, was lower with BXM than with placebo. Based on these outcomes, in 2020, the FDA further expanded the use of BXM as post-exposure intervention, which allows treatment of those 12 years and older who come in contact with the infected individuals (193). The data from clinical trials showed that a single dose of BXM can reduce viral titers and lower disease symptoms (194). The most common adverse effects reported were headache, hematuria, pharyngitis, and increased alanine aminotransferase level (28, 192).

Mechanism of action. In brief, the influenza PB2 subunit first captures a nascent host RNA using its m7G cap-binding motif. Next, the PA endonuclease activity hydrolyzes the phosphodiester bond 8 to 14 nucleotides downstream of the 5′ cap (195). This process generates capped RNA primers to initiate transcription by the PB1 subunit (196). The crystal structure of PA endonuclease displays a (Pro)Asp...X_n.(Asp/Glu)X Lys) where X is any amino acid) motif, required for metal ion coordination, like other known endonucleases (60). The active site of PA harbors a cluster of three conserved acidic residues (Gln80, Asp108, and Gln119) and His41 residue involved in binding to divalent metal ions. Baloxavir exhibits its inhibitory activity by binding to these catalytic metal ions in the enzyme's active site (60, 197, 198). The left structure of Fig. 6 depicts the binding mode of Baloxavir (green) in the binding pocket of the wild-type PA protein (orange). Baloxavir efficiently targets influenza virus replication by inhibiting the initiation of mRNA synthesis.

Resistance to Baloxavir. Growing viruses in the presence of drugs can result in the generation of drug-resistant variants. *In vitro* passages of influenza A(H1N1) virus in

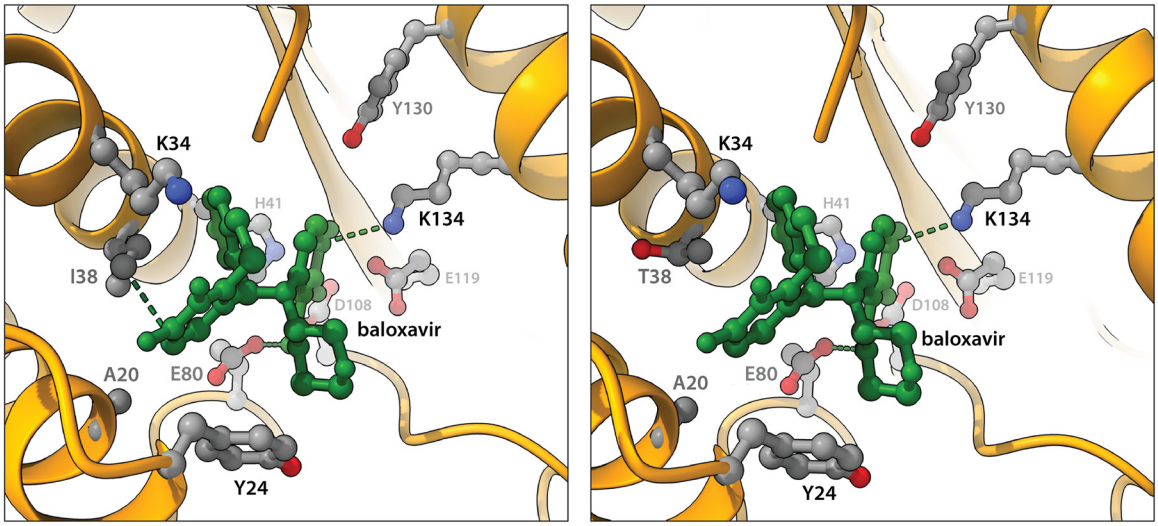

FIG 6 Polymerase acidic (PA) protein wild type (PDB accession no. 6FS6) bound with Baloxavir (left) and PA Ile38Thr mutant (PDB accession no. 6FS7) bound with Baloxavir (right). The left structure depicts the binding mode of Baloxavir (green) in the binding pocket of the wild-type PA protein (orange). The right structure shows Baloxavir bound to the mutant PA protein, where the Threonine does not interact with the Baloxavir, reducing the binding affinity of the antiviral to the mutated binding pocket. In addition, the I38T mutation in the PA protein renders the antiviral Baloxavir less effective.

Madin-Darby bovine kidney (MDBK) cells treated with BXA resulted in Ile38Thr PA mutant virus (190). The Ile38Thr substitution did not significantly alter the replication kinetics of rescued (H1N1) Ile38Thr and (H3N2) Ile38Thr PA variants in Madin-Darby canine kidney (MDCK) cells (199). Mutant viruses of Ile38 residue in the PA active site were isolated clinically (27, 192, 194). The Ile38Thr substitution also reduced antiviral effects of Baloxavir in clinical trials (194, 200, 201). Crystal structures of the PA-Baloxavir complex revealed several active site residues involved in interacting with the drug (Fig. 6, right) (194). The most prevalent substitutions resulting in reductions in Baloxavir activity involved changes from Ile at residue 38 in the wild-type viruses to Thr, Phe, or Met. In addition, the Ile38Thr mutation perturbed van der Waals interactions with the inhibitor in the binding pocket (191, 194, 202). Ile38Thr does not interact with Baloxavir; it reduces the binding affinity of the antiviral to the mutated binding pocket and renders the antiviral Baloxavir less effective.

In patients treated with Baloxavir, during the 2018–2019 season, mutations (Ile38Thr, Ile38Phe, and Ile38Met) in the PA active site from A(H1N1)pdm09 and A (H3N2) influenza viruses were detected (203, 204). A single dose of BXM was compared to placebo or Oseltamivir in a phase 3 randomized, double-blind study. This study found that 9.7% of viruses isolated (36/370) developed mutations of Ile38 residue 3 to 9 days after treatment (201). Eleven cases infected with influenza A(H3N2) viruses, including one patient untreated with Baloxavir, had the PA Ile38Thr substitution (205). Another A(H1N1)pdm09 virus with the PA Glu23Lys mutation detected in a pediatric patient without Baloxavir treatment showed reduced susceptibility to Baloxavir (206). Such human-to-human transmission events of resistant variants are concerning and require monitoring and surveillance (203, 207). In Japan, Baloxavir is approved for treatment of children less than 12 years old. However, there is a high rate of resistance emergence in children treated with Baloxavir (208). Ile38Thr and Ile38Met in A(H3N2) variants detected during post-treatment monitoring showed almost 10-fold reductions in BXA susceptibility in pediatric patients (194). Treatment-emergent substitutions [Ile38Asn from A(H1N1)pdm09 and Ile38Arg in A(H3N2)] were also detected from the CAPSTONE-2 study (209). PA mutations at Ala37Thr and Glu199Gly had relatively minor effects compared to mutations at residue 38 (194). In addition, 13,523 PA sequences deposited to the Global Initiative on Sharing All Influenza Data (GISAID) or NCBI Influenza Virus Resource (NCBI-IVR) databases (2017 to 2018) were screened for

mutations associated with reduced Baloxavir susceptibility (186). This analysis showed that the frequency of viruses with reduced susceptibility to PA inhibitors was low (0.08%). Another study determined BXA 50% effective concentration (EC_{50}) values by plaque reduction assays in MDCK cells and reported EC_{50} values for A(H3N2) virus with PA Glu23Arg (EC_{50}, 5.7 nM) and PA-Ile38Thr (EC_{50}, 25 nM) mutations. They found a steep increase in EC_{50} of the drug from 5.7 nM to 975 nM for the double mutant Glu23Arg/Ile38Thr (210), thus potentially impacting the treatment efficacy.

WHO's AVWG summarizes and posts updates on PA mutations associated with decreased susceptibility *in vitro* (211). Regular surveillance and monitoring for the emergence of seasonal influenza (both A and B variants) and tracking changes in the susceptibility of new viral mutants to inhibitors are crucial to ensure the accuracy of treatment guidelines.

STRATEGIES TO OVERCOME DRUG RESISTANCE WITH NEXT-GENERATION ANTIVIRALS

Significant progress has been made in addressing M2 resistance with the newer generation of inhibitors (212, 213). DeGrado and coworkers used the power of molecular dynamics (MD) simulations to solve the resistance problem and developed a novel inhibitor called spiro-adamantyl amine (214). The inhibitor blocks proton conductance in the WT and Val27Ala mutant M2 channels (215). This is a very promising discovery given the fact that the M2 Val27Ala mutation became transmissible and provided proof of concept that MD simulations can be used for the discovery of inhibitors of targets (104, 105, 216, 217). The drug resistance mechanism of M2-Ser31Asn channel blockers has been extensively studied using multiple viruses in different cell lines (218). The Leu46Pro (134) and Arg45His (219) mutations have been identified from serial viral passage experiments *in vitro*. Compounds such as isoxazole-conjugated Adamantane bind AM2 with the Ser31Asn mutation and inhibit channel activity (134). Wang and coworkers discovered three compounds, (3s,5s,7s)-*N*-((5-(3-methoxythiophen-2-yl)isoxazole-3-yl) methyl)Adamantane-1-amine, (1s,3r,5R,7S)-3-(((5-(3-methoxythiophen-2-yl)isoxazole-3-yl) methyl)amino)Adamantane-1-ol, and (1s,3r,5R,7S)-3-(((5-(2-(methylthio)phenyl)isoxazole-3-yl)methyl)amino)Adamantane-1-ol), which significantly inhibited single (M2-Ser31Asn) and double (M2-Ser31Asn Leu26Ile and M2-Ser31Asn Val27Ala M2) ion channel mutants (139). The significant outcome of inhibition of the drug-resistant M2 double mutants is remarkable. These studies further advance the field and provide insights for developing novel antivirals.

Oseltamivir treatment can also result in resistant viruses (NA-His275Tyr, Arg292Lys, and Asn294Ser), although these Oseltamivir-resistant strains remain sensitive to Zanamivir. Several groups are working to develop a newer generation of inhibitors. Naturally occurring compounds from several microbes and medicinal plants have been used to refine and develop synthetic influenza antivirals (152). For example, a flavonoid compound, Kaempferol-3-0-[(4″5″-0-isopropylidene)-*a*-L-rhamnopyranoside], isolated from cassia trees, showed NA inhibitory activity (50% inhibitory concentration [IC_{50}] value of 187.40 μM which can serve as a potential lead for synthesizing novel NA inhibitors (187).

Baloxavir binds to the endonuclease of both IAV and IBV and is a critical drug in fighting IAV infection. However, Baloxavir-treated subjects with resistant viruses carrying Ile38Thr and Glu23Lys substitutions in PA have been sequenced (209). The mutation at position 38 in the PA has been shown to have a major impact on viral susceptibility to the inhibitor and is therefore also the most well studied (194). In addition, the PA double mutant Glu23Arg Ile38Thr can severely compromise the utility of BXA (210). Currently, there are no drugs that target and inhibit PA-resistant mutants. Therefore, next generation drugs need to be developed that are also effective against mutant strains.

ANTIVIRALS IN CLINICAL DEVELOPMENT OR APPROVED FOR USE IN DIFFERENT PARTS OF THE WORLD

As discussed earlier, influenza viruses are resistant to currently used antiviral drugs (26, 220). Therefore, developing new influenza drugs to work against drug-resistant

strains is a public health need. An ideal influenza drug should have a broad range of activity against different strains, ease of administration, flexibility in the timing of administration during infection, improved effectiveness, and fewer chances of development of drug-resistant viral strains (221). Here, we summarize some promising antivirals either approved for use in different parts of the world or currently in various phases of clinical trials that can fulfill the need for new influenza drugs (Fig. 7 and Table 2).

NA Inhibitors

NAIs are the most prescribed antiviral drugs against influenza, with proven effectiveness in shortening the duration of clinical illness and virus clearance (28, 152, 161, 222). Therefore, different chemical approaches have been applied in search of novel neuraminidase inhibitors (187). Laninamivir is one such drug that inhibits NA activity and has been used in Japan for several years.

Laninamivir. Laninamivir octanoate, or CS-8958, is an octanoyl ester is a prodrug for Laninamivir. Upon administration, it changes to its active form in the respiratory tract, as shown in Fig. 7A. Laninamivir exhibits a broad inhibitory activity against influenza A and B viruses, including Oseltamivir-resistant A(H5N1) influenza viruses *in vitro* and *in vivo* (223). A clinical study investigating the pharmacokinetics of 40 mg of Laninamivir administered as an inhaled prodrug in healthy individuals indicates maintenance of 50% inhibitory concentrations in epithelial lining fluids for 10 days (224). Different mechanisms, such as increased lipophilicity and hydrolysis leading to the generation of a highly hydrophilic form, have been proposed for this long-lasting ability of the drug (225, 226). The clinical efficacy of Laninamivir was evaluated in a double-blind noninferiority trial in which 334 and 326 patients exhibiting febrile influenza symptoms were administered a single inhalation of 40 mg or 20 mg Laninamivir prodrug, respectively. As a control, 336 patients received an oral dosage of 75 mg of Oseltamivir twice daily for 5 days.

Laninamivir at a 40-mg dose reduced the number of patients shedding the viruses on day 3 and was also effective against Oseltamivir-resistant A(H1N1) and A(H1N1) pdm09 viruses (227). All three groups had similar time courses for the alleviation of influenza symptoms. The most common side effects included diarrhea, vomiting, and nausea. The incidence of these mild to moderate side effects was similar among the three groups. Mild to moderate dizziness was reported only by the patients receiving 40 mg or 20 mg of Laninamivir octanoate.

Furthermore, a safety evaluation study of Laninamivir octanoate hydrate treatment of 567 patients revealed that abnormal behavior/delirium and syncope were primarily associated with influenza infection and not due to the treatment. However, the incidence of adverse events and their potential causal relationship with Laninamivir are similar to those caused by other NAI treatments (228). Hence, patients need to be closely monitored for behavioral changes when taking this drug. A clinical trial with 803 patients evaluated the potential of Laninamivir octanoate as a preexposure prophylactic treatment strategy. The results indicated that a single administration of 40 mg or 20 mg of the drug effectively prevented the development of clinical influenza (229). Recently, a meta-analysis of nine studies involving treatment and three studies of prophylactic treatment revealed that during an A (H3N2) influenza infection, Laninamivir octanoate treatment was associated with a longer duration of fever than Oseltamivir treatment (230). Thus far, isolation of viruses with mutations causing resistance to Laninamivir has not been reported following treatment.

Viral Polymerase Complex Inhibitors

For nearly two decades, influenza antiviral therapy has been based mainly on adamantanes and NAIs. However, the emergence of resistant viral strains and suboptimal clinical effectiveness with these drugs warrant a search for alternative targets for drug development. Along with its indispensable role in the influenza virus replication cycle and highly conserved sequence among different influenza viruses, the polymerase complex seems

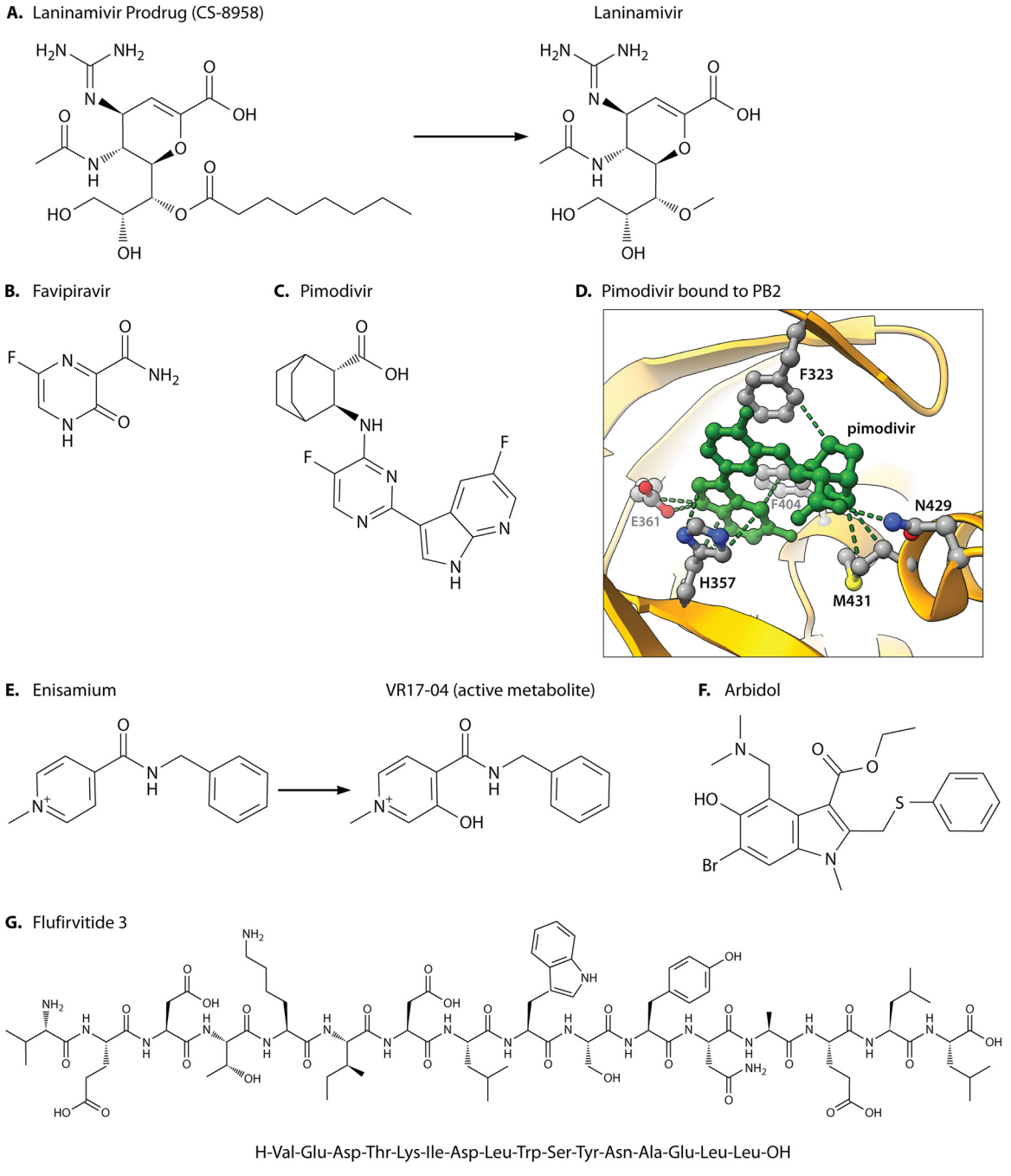

FIG 7 Structures of influenza antivirals in clinical trials. (A) Laninamivir (IUPAC name, (2R,3R,4S)-3-acetamido-4-(diaminomethylideneamino)-2-[(1R,2R)-2,3-dihydroxy-1-methoxypropyl]-3,4-dihydro-2H-pyran-6-carboxylic acid) and its prodrug (CS-8958). (B) Favipiravir (IUPAC name, 5-fluoro-2-oxo-1H-pyrazine-3-carboxamide). (C) Pimodivir (IUPAC name, (2S,3S)-3-[[5-fluoro-2-(5-fluoro-1H-pyrrolo[2,3-b]pyridin-3-yl)pyrimidin-4-yl]amino]bicyclo[2.2.2]octane-2-carboxylic acid). (D) Polymerase basic protein 2 (PB2) wild type (PDB accession no. 7AS0) bound with pimodivir (JNJ-63623872). This structure depicts the strong binding mode of the antiviral pimodivir (green) in the binding pocket of PB2 (orange). Studying mutant variations of the residues in direct contact with pimodivir could be informative as to possible escape mutants for this investigational therapeutic. (E) Enisamium (IUPAC name, *N*-benzyl-1-methylpyridin-1-ium-4-carboxamide) and its active metabolite. (F) Arbidol (IUPAC name, ethyl 6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2-(phenylsulfanylmethyl)indole-3-carboxylate. (G) Flufirvitide 3 (IUPAC name, (2S)-2-[[(2S)-2-[[(2S)-2-[[(2S)-2-[[(2S)-4-amino-2-[[(2S)-2-[[(2S)-2-[[(2S)-2-[[(2S)-2-[[(2S)-2-[[(2S,3S)-2-[[(2S)-6-amino-2-[[(2S,3R)-2-[[(2S)-2-[[(2S)-2-[[(2S)-2-amino-3-methylbutanoyl]amino]-4-carboxybutanoyl]amino]-3-carboxypropanoyl]amino]-3-hydroxybutanoyl]amino]hexanoyl]amino]-3-methylpentanoyl]amino]-3-carboxypropanoyl]amino]-4-methylpentanoyl]amino]-3-(1H-indol-3-yl)propanoyl]amino]-3-hydroxypropanoyl]amino]-3-(4-hydroxyphenyl)propanoyl]amino]-4-oxobutanoyl]amino]propanoyl]amino]-4-carboxybutanoyl]amino]-4-methylpentanoyl]amino]-4-methylpentanoic acid).

TABLE 2 Antivirals in clinical development or approved in different parts of the world

Target molecule	Nature of compound	Drug	Mode of action	Effectiveness	Side effect(s)	Reference(s)
NA Inhibitors	Chemical	Laninamivir	Inhibits neuraminidase activity	Broad range of influenza inhibitory activity, including influenza B- and Oseltamivir-resistant strains	Diarrhea, vomiting, nausea	224, 227
Viral polymerase inhibitors	Chemical	Favipiravir or T-705	Acts as purine nucleoside, can induce chain termination and lethal mutagenesis	Activity against novel and reemerging influenza virus	Teratogenicity, embryotoxicity, gastrointestinal symptoms, increased uric acid	234, 235
		Pimodivir or JNJ-63623872	Blocks cap binding functions of PB2 subunit of RNA polymerase	Discontinued, as it failed to demonstrate benefit over standard of care	Dose-related diarrhea, decreased neutrophils	243, 244
		AL794	Binds selectively to endonuclease domain of PA subunit	Discontinued due to failure to establish a single safe and effective dose and variability in drug exposure	Headache, dizziness	248, 249
		ZSP1273	Inhibits PB2 subunit of RNA polymerase	Results of clinical trials awaited for antiviral efficacy	Diarrhea, leucopenia, neutropenia	251
		Enisamium iodide or Amizon	Inhibition of RNA synthesis	Faster patient recovery and reduced symptoms	Bitter taste in mouth and burning sensation in throat	256
		TG1000	Inhibition of cap-dependent endonuclease activity of PA	Results awaited from clinical trial	Results on safety awaited	257
		GP681	Inhibition of PA subunit	Clinical trial ongoing	Results on safety awaited	259
Ion channel inhibitor	Si-RNA	AVI-700 or radavirsen	Targets both M1 and M2 translation	Well tolerated but clinical efficacy studies awaited	Headache and Proteinuria	279
HA inhibitors	Peptide	Flufirvitide 3	Broad spectrum entry inhibitor	Results not posted	Results not posted	268
	Chemical	Arbidol or umifenovir	Inhibition of HA-induced membrane fusion	Reduction in time taken for resolution of clinical symptoms	Not known so far	269, 273
	MAb	MHAA4549A	Targets highly conserved epitope on HA and neutralizes virus	No significant difference in viral load	Not known	322
	MAb	MED18852	Broad-spectrum neutralizing activity	No significant advantage found in treated groups	Adverse side effects Including bronchitis	327
	MAb	VIS410	Broad-spectrum neutralizing activity	Provides therapeutic benefit with reduced viral load	Generally safe	330, 331

to be a target for antiviral development (188, 231, 232). Several new compounds targeting different subunits of influenza virus polymerase showed effectiveness. Here, we have discussed new compounds which target influenza polymerase complex.

Favipiravir. Favipiravir, also known as T-705, exhibits a broad-spectrum antiviral activity against influenza and flavi-, arena-, bunya-, noro-, and alpha-RNA viruses (233, 234). Favipiravir has anti-influenza activity against different virus subtypes, including A, B, and C. It acts as a purine nucleoside and can induce chain termination and lethal mutagenesis. Viral RNA polymerases recognize it as an alternative substrate, primarily as guanosine and secondarily as an adenine analog, resulting in errors during viral RNA synthesis (235, 236). A dose-finding, placebo-controlled, double-blind study with three groups of patients was conducted to assess the appropriate dose regimen and pharmacokinetics of Favipiravir for treating uncomplicated influenza. Two groups were treated for 4 days with either a high dose administered to 195 patients or a low dose of Favipiravir administered to 134 patients. Additionally, 201 patients served as a placebo control. Results from this study suggest that Favipiravir is well tolerated (237); however, the time to resolve symptoms was only 6.1 h shorter than the placebo (238). Recently, Wang et al. compared the effectiveness of the combination therapy of Favipiravir and Oseltamivir with the monotherapy of Oseltamivir in 128 and 40 critically ill influenza patients, respectively. Favipiravir and Oseltamivir therapy, compared to Oseltamivir therapy alone, accelerated clinical recovery. In addition, only 21.9%, compared to 67.5%, of patients demonstrated undetectable influenza viral RNA on day 10 (239). Although Favipiravir was found to be safe in clinical studies, due to concerns of teratogenicity and embryotoxicity, it was approved for conditional marketing in Japan for limited usage involving treatment for novel or reemerging influenza viruses (234).

Moreover, the usage of Favipiravir is completely restricted for pregnant women. Females of childbearing age are directed to avoid pregnancy during treatment and 7 days after the last dose to eliminate the drug from the system (233). Besides embryotoxicity, less severe side effects from treatment with Favipiravir include gastrointestinal symptoms and increased uric acid levels (240). Finally, although Favipiravir-resistant influenza viruses have been reported from an *in vitro* study (241), viruses with such mutations have not been isolated from the patients treated with this drug.

Pimodivir. Pimodivir is also known as VX-787 or JNJ-63623872. This orally administered nonnucleoside inhibitor suppresses the early stages of viral RNA transcription by blocking the cap-binding function of the PB2 subunit of RdRp (242). Pimodivir interacts with the m7 GTP guanine base by occupying the central cap-binding domain of PB2 (243). In a phase IIa study, 104 healthy subjects were challenged with A/Wisconsin/67/2005 A(H3N2) influenza virus, and 72 subjects were given Pimodivir at 100 mg, 400 mg, a loading dose of 900/600 mg, and a loading dose of 1,200/600 mg once daily for 5 days, and 32 subjects served as placebo controls. Interestingly, all the Pimodivir-treated groups showed a reduction in viral shedding and clinical symptoms compared to the control group (244). A double-blind, placebo-controlled phase IIb study assessing the clinical efficacy of Pimodivir reported dose-related diarrhea, nausea, and decreased neutrophils as a side effect (245). In this study consisting of 293 influenza patients between the ages of 18 to 64, patients received a twice-daily oral dose of Pimodivir alone (300 mg or 600 mg) or in combination with Oseltamivir (600 mg Pimodivir and 75 mg Oseltamivir) for 5 days. Pimodivir in combination with Oseltamivir resulted in lower viral titers and a shorter time to the resolution of symptoms. Another phase II clinical trial with a combination therapy of 600 mg Pimodivir with 75 mg Oseltamivir administered twice daily for 7 days was carried out to assess Pimodivir pharmacokinetics in influenza virus-infected 25 elderly patients between the ages of 65 to 85 years old compared to 38 nonelderly adults aged 18 to 65 years old. The primary outcome was a comparison of the time taken for the resolution of symptoms (246).

Interestingly, the two age groups of patients did not show any meaningful differences in pharmacokinetics or efficacy between them. The Pimodivir-Oseltamivir versus Oseltamivir groups did not show any differences in time to discharge. The viruses

isolated from Pimodivir-treated groups did not show any mutations in PB2. However, in other clinical studies, the emergence of mutations in the PB2 protein of the virus replicating in the presence of Pimodivir has been reported (244, 245). These include PB2 substitutions Met431Ile, Ser324Lys/Asn/Arg, Phe325Leu, Ser337Pro, Lys376Asn/Arg, Thr378Ser, and Asn510Lys, which decreases the susceptibility to the drug by several fold (245). Despite some promising initial results in the clinical setting, the clinical developmental program for Pimodivir was discontinued by Janssen, as the drug failed to demonstrate a benefit over standard of care (SOC) in influenza-infected patients (247).

AL-794. AL-794, an ester prodrug of ALS-033719, is orally active and inhibits both IAV and IAB viruses by binding selectively to the endonuclease domain of the PA subunit (240). In a double-blind phase I clinical study, the drug's pharmacokinetics, safety, and tolerability were evaluated in healthy individuals. The findings indicated that a twice-daily dosage of AL-794 up to 200 mg is well tolerated and generally achieved the expected efficacious levels of ALS-033719 in plasma (248). Further, to characterize the safety and antiviral activity of AL-794, a challenge study was conducted in which healthy subjects were intranasally inoculated with influenza A/Perth/16/2009 A(H3N2) virus and were subsequently treated with AL-794 50 mg and 150 mg twice daily for 5 days. AL-794 treatment reduced symptoms, viral load, and mucus weight, with a more significant decrease in these parameters at 150 mg without any safety concerns (249). The most common side effects associated with the oral administration of this drug were headache and dizziness (248). AL-794 was discontinued because, unlike another PA inhibitor, Baloxavir, a single dose that was effective and tolerated was not established in the clinical studies, along with significant variability in the exposure levels of drugs based on gender and prior food consumption (240).

ZSP1273. ZSP1273 is an orally administered small-molecule inhibitor that possesses anti-influenza activity because of its capability to inhibit the PB2 subunit of RdRp. A phase I clinical trial with 100 healthy individuals was conducted in China to assess the safety, tolerability, pharmacokinetics, and food-drug interactions of ZSP1273 (250). The study's findings revealed that the bioavailability of the drug was not affected by food intake, and high-fat diet had a limited effect on the drug's pharmacokinetics. The most common side effects were diarrhea, leukopenia, and neutropenia. The study recommended a dose of $\geq$200 mg for influenza patients (251). In addition, a phase III clinical study that compares the antiviral efficacy of ZSP1273 to placebo or Oseltamivir in uncomplicated IAV patients is expected to be completed by 2022 (252).

Enisamium iodide. Enisamium is an isonicotinic acid derivative, a low-molecular-weight compound known by the trade name Amizon or lab code FAV00A and is currently marketed in former Soviet Union countries and Mongolia as an antiviral agent against influenza and other viruses. A study by Boltz et al. showed a broad range of antiviral activity of Enisamium iodide against multiple strains of influenza A and influenza B viruses in primary normal human bronchial epithelial (NHBE) cells at 23- to 64-fold-lower doses than the cytotoxic concentration (253). In a follow-up study, the antiviral effects of the drug were reported in the ferret model of influenza infection. The authors also suggested that the mode of action of this compound is the inhibition of RNA synthesis of influenza A viruses (254). To evaluate the safety and clinical efficacy of Enisamium iodide, a randomized single-blind clinical study was conducted in patients aged 18 to 60 years with confirmed influenza and other respiratory viruses (255). The study reported faster patient recovery with reduced disease symptoms and viral shedding in the treatment group of 60 subjects than in the placebo group of 40 subjects (256). The adverse events included a bitter taste in the mouth, a burning sensation in the throat, and minor gastrointestinal side effects. Furthermore, the authors also confirmed that the mode of action of Enisamium iodide is through the inhibition of viral RNA polymerase by a hydroxylated metabolite of VRI7-04, as shown in Fig. 4D.

TG-1000. Developed by Tiagen Biotechnology company in Taiwan, TG-1000 is a novel influenza drug that shows inhibitory activity against IAV, IBV, and Oseltamivir-resistant viruses via inhibition of cap-dependent endonuclease activity of the PA subunit. The FDA

approved the investigational new drug application for TG-1000 in 2020. A phase II double-blind dose-ranging study to investigate the clinical efficacy and safety of TG-1000 is underway in adult patients with uncomplicated influenza (257).

GP681. GP681 is a prodrug that gives rise to GP1707007, a metabolite that inhibits the PA subunit of RdRp. GP681 was effective in preclinical studies; hence, it is being evaluated in clinical trials. A phase I clinical trial to assess the safety and tolerance of GP681 tablets in healthy adults has been completed (258). The results from a phase I clinical study are awaited. Currently, a phase II clinical trial to evaluate the safety and antiviral efficacy of GP681 tablets in the treatment of uncomplicated influenza is underway (259).

Antivirals Targeting the HA Protein

HA, the surface glycoprotein of the influenza virus, is a preferred target for developing antivirals, therapeutic antibodies, and vaccines. HA is translated from mRNA as a precursor protein HA0, which, upon proteolytic cleavage, gets converted to HA1 and HA2 subunits linked by a disulfide linkage (260, 261). Structurally, it possesses two distinct domains, a globular head that varies greatly among the different strains of influenza and a helix-rich stem region, which is conserved (261). Though the field of antiviral agents targeting HA is relatively recent compared to existing antivirals targeting NA, the developments in the area are promising, with increasing numbers of HA-targeting antivirals (161, 262–265). Here, we summarize small molecules and peptide inhibitors targeting the HA protein in clinical trials.

Flufirvitide 3. Flufirvitide 3 is a peptide composed of 16 amino acids derived from a specific region of HA2 called the fusion initiation region. It shows a broad-spectrum entry inhibition against different influenza subtypes, including IBV *in vitro* (266). A randomized placebo-controlled phase I dose-escalating study for evaluation of the safety profile of Flufirvitide 3 nasal spray was conducted in healthy subjects (267). Another phase I study for safety, tolerability, and pharmacokinetics of single and repeated doses of the dry powder form of Flufirvitide 3 was done in healthy adults (268). However, to our knowledge, the results of both these clinical trials have not been posted yet.

Arbidol. Arbidol (umifenovir or DB13609), an indole derivative molecule, is the first clinically used drug to inhibit HA-induced membrane fusion (262, 263, 269). This antiviral agent is believed to also act as an immunomodulator against some other viruses (270–272). Currently, it is licensed for over-the-counter use against both IAV and IBV infections in Russia and China. Arbidol functions as a molecular glue by attaching to the hydrophobic cavity in the HA trimer stem region. This binding interferes with the conformational changes, leading to the inhibition of membrane fusion and release of viral genome into the infected cells (269).

In a clinical study of 119 patients with influenza infection, 200 mg of Arbidol was administered orally for 5 days. The efficacy of Arbidol is observed mostly in the acute stage of the disease. Furthermore, the treatment effectively reduced the time taken to resolve all symptoms of the disease, severity of the disease, and duration of virus shedding (273). A phase III clinical trial to evaluate combination antiviral therapy of Oseltamivir and Arbidol versus Oseltamivir monotherapy for the treatment of severe influenza has been completed in China, but results are not yet available (274). In Russia, a phase IV clinical study evaluating Arbidol as a therapeutic and prophylactic agent against influenza has been completed; the results of the study results are not yet available (275).

Ion Channel Inhibitors

M1 and M2 proteins of the influenza virus are splice variant products of the same gene segment, and these are also a target for therapeutic interventions, including antisense strategies. As an antiviral agent against influenza viruses, small interfering RNA (siRNA) has demonstrated utility in preclinical studies (276–278). AVI-7100 is one such drug to be tested in clinical studies.

AVI-7100 (Radavirsen). AVI-7100, or Radavirsen, a phosphorodiamidate morpholino oligomer, targets M1 and M2 mRNA translation, as these two proteins share the same translation initiation start site. Further, the oligonucleotide is designed with a unique backbone structure for increased uptake in the infected cells. A phase I clinical trial of AVI-7100 was conducted in 56 healthy subjects between 18 and 60 years old, with intravenous administration of this molecule at different dosages. The findings suggest that a single injection of AVI-7100 up to 8 mg/kg and multiple dosages at 8 mg/kg once daily for 5 days are well tolerated and safe (279). However, the study also reported headache and proteinuria as the most common side effects in the placebo group (280). Further clinical studies are required to assess the therapeutic potential of AVI-7100 against influenza.

Passive Immunization

Treatment or protection against a disease can also be achieved through passive immunization where antibodies are transferred from immunized/infected and recovered individuals or recombinant monoclonal antibodies (mAbs) to naive individuals or patients. Convalescent plasma, hyperimmune sera, and lab-generated mAbs fall under the passive immunization strategy.

Convalescent plasma and intravenous immunoglobulins. Viral challenge studies in ferret and mouse models demonstrated the utility and efficacy of convalescent plasma and intravenous immunoglobulins against influenza (281, 282). Treatment of infected patients with passive transfer of antibodies reduced the severity and duration of the disease (283–285). During the 1918 influenza pandemic, convalescent human serum was administered to patients as a treatment against influenza. A meta-analysis by Luke et al. has reported that this mode of treatment during the pandemic results in a reduced risk of death. Interestingly, the timing of administration is crucial, as the positive outcome is associated with early administration of sera after the onset of symptoms (286). However, due to the safety issues related to the use of serum, the passive immunization approach did not get much attention until recently when hyperimmune sera were tested in clinical trials. The administration of hyperimmune intravenous globulin (hIVIG) fractionated from convalescent plasma of recovered patients of the 2009 A (H1N1)pdm09 pandemic to patients with severe influenza A(H1N1)pdm09 infection reduced mortality and virus load (287). However, the efficacy of hIVIG in hospitalized influenza patients from different geographical regions over five influenza seasons was not superior to placebo when it was coadministered with Oseltamivir (288). In another clinical trial with severe influenza A patients, the high-titered anti-influenza plasma has shown no significant benefit over the standard low-titered plasma as a treatment (289). Hence, further clinical studies on the passive transfer of sera/plasma in severe influenza patients are needed to assess the safety profile and therapeutic efficacy in reducing the viral titers and time to resolution of disease symptoms and pathology.

Monoclonal antibodies. The tremendous technological advancement during the past decade has facilitated the generation of recombinant human mAbs (290, 291). Intensive studies of human B cell repertoire upon influenza virus infection and vaccination have led to the isolation of several antiviral mAbs directed against different influenza viral proteins (290, 292–295). These antibodies are beneficial for treating hospitalized influenza patients without any added drug resistance concerns, as they target the highly conserved epitopes of viral proteins. Moreover, in recent years, the cost of these antibodies has also reduced significantly due to improved production methods (296). The advances in genetic immunization and vector-based delivery of these monoclonal antibodies can reduce the cost of production (297–299). Influenza infection or vaccination induces antibodies against 5 antigenic sites of HA protein (Ca1, Ca2, Cb, Sa, and Sb in cases of group 1A viruses and A, B, C, D, and E in cases of group 2 viruses). These antibodies are directed predominantly against the highly variable strain-specific HA head region. These antibodies may inhibit hemagglutination and neutralization of the virus. Antibodies against receptor-binding sites prevent infection and neutralize the virus. mAbs that react with multiple influenza A strains, including cross-subtypes (S139/1, C05, and F045-092) and cross-subclasses

against influenza B viruses (CR8033 and C12G6), were isolated (295, 300, 301). Receptor-binding antibodies drive the evolution of influenza viruses due to immune pressure. In addition to the antibodies against the head region, antibodies are also induced against the stem region of group 1 (CR8020, C179, F10, CR6261, 3.1, 3E1, and S9-3-3), group 2 (9H10), or against both group 1 and group 2 (31.a.83, 56.a.09, FI6v3, MEDI8852, and CR9114), which demonstrated broad neutralizing activity preventing membrane fusion (295, 302). These broadly neutralizing antibodies against HA stem can either function directly by preventing membrane fusion or indirectly engage in antiviral effector function by NK cells or macrophages via antibody-dependent cell-mediated cytotoxicity (ADCC) (303, 304). Apart from HA, NA is also a target for antibody responses and antibodies induced against the catalytic site. Other regions of NA can inhibit NA catalytic activity, thus blocking the release of progeny viruses from the infected cells. NA-specific MAbs, 228-1BO3, and several anti-N1 and -N2 antibodies, HCA2, 1GO1, 2E01, 1GO4, NA-45, and Z2B3, bind to the catalytic site, while CD6, NA-63, NA-80, and NA-22 bind outside the catalytic site and inhibit NA function (295, 305–308). In addition to the surface glycoproteins HA and NA, the ectodomain of matrix protein 2 (M2e) also is a target for antibody induction by infection (309). mAbs against M2e (14C2, C40, C40G1, L66, N547, Z3, Z3G1, 391, 472, 522, 602, 770, 934, and 1191) were generated in mice and tested for their activity *in vitro* and *in vivo* in mouse models (310–312). However, the MAbs against M2e have not yet been isolated from infected or vaccinated individuals. Influenza NP is a conserved internal protein that has been shown to activate CD8 T-cell responses, aiding in heterosubtypic protection and viral clearance (313–315). Following infection, antibodies against NP are found, and their role in conferring protection is not understood (316). Animals immunized with soluble NP protein or animals that received NP-specific antibodies were protected against viral challenge (317, 318). Mice transgenic for a human mAb against H5N1 NP isolated from a patient infected with H5N1 virus conferred protection against viral challenge (319). However, there are no reports on the isolation or function of human anti-NP mAbs. The mAbs directed against the HA stem region in clinical development are discussed here.

MHAA4549A. MHAA4549A is a human anti-influenza IgG1 mAb isolated and referred to as 39.29 earlier (292). This antibody targets a highly conserved epitope on the HA stalk region of the influenza virus and can neutralize influenza viruses from both groups 1 and 2 (320). Lim et al. conducted two phase I studies to evaluate the safety and pharmacokinetics of intravenously administered MHAA4549A (321). Results from the clinical trial suggest that the study drug is safe and well tolerated, with a serum half-life of 23 days. Additionally, both these studies reported mild headaches as the most common adverse side effect. The therapeutic potential of the MHAA4549A antibody was evaluated in a challenge study where 100 healthy subjects were inoculated with A/Wisconsin/67/2005 A(H3N2) virus followed by intravenous injection with different dosages of MHAA4549A (322). This placebo-controlled phase IIa study demonstrated that the highest tested dose of 3,600 mg of MHAA4549A significantly reduced virus burden and influenza symptoms.

Furthermore, in a phase IIb randomized, double-blind, placebo-controlled study, the safety and efficacy of MHAA4549A (3,600 mg and 8,400 mg) plus a standard dose of Oseltamivir were evaluated in hospitalized influenza patients with severe infection. The median time taken to the cessation of oxygen support to maintain a stable oxygen saturation of 95% and normalization of respiratory function were considered the study's primary outcome. However, an interim analysis showed no significant difference in the time taken for normalizing respiratory functions or virus load between placebo plus Oseltamivir and MHAA4549A plus Oseltamivir (323).

MED18852. MED18852 is a broad-spectrum human mAb with potent IAV-neutralizing activity. It was first isolated and characterized by Kallewaard et al. and has shown promising results in preclinical settings (324, 325). This has led to the evaluation of its therapeutic potential in both uncomplicated and severe influenza infection in the clinical setting. MED18852 has proven safe at a dose ranging from 250 mg to 3,000 mg in

healthy adults without any antibody detection against MED18852 posttreatment (326). A follow-up study assessed the safety profile and therapeutic potential of this antibody in patients with uncomplicated influenza (327). The treatment group comprised of 126 influenza A confirmed adults receiving a single intravenous injection of MED18852 at 3,000 mg, 3,000 mg of MED18852 in combination with Oseltamivir, 750 mg of MED18852 in combination with Oseltamivir, or a placebo combined with Oseltamivir. MED18852 treatment has resulted in more adverse effects than Oseltamivir alone, with bronchitis being the most common side effect. Moreover, there has been no significant difference in all treatment groups' viral titers and influenza symptoms. Therefore, a planned clinical trial to evaluate MED18852 efficiency in hospitalized influenza patients did not move forward (328).

VIS-410. VIS-410 is an engineered human mAb known to target unique epitopes on the influenza HA stem region and possesses broad neutralizing activity against different strains of IAV. In preclinical studies, VIS-410 protected the mice from the lethal challenge of A(H7N9) influenza virus (329). Following this, a phase I safety trial has been conducted in 41 healthy adults, revealing that VIS-410 is generally safe at dose level 2 to 50 mg/kg with mild to moderate adverse effects, including diarrhea (330). Furthermore, the study has reported that a single intravenous injection of VIS-410 has a mean half-life of 12.9 days, and the concentration in the serum and respiratory tract is proportional to the dosage used. Furthermore, Hershberger et al. also have shown in a randomized double-blind, placebo-controlled, phase II study that a dose ranging from 2,000 mg to 4,000 mg per individual is safe and well tolerated in 138 healthy adults with uncomplicated influenza (331). Finally, a phase IIA challenge study with A/California/07/2009 A(H1N1)pdm09 in 46 healthy adults has demonstrated that upon treatment with VIS-410 post-inoculation with the virus, there was 76% decrease in viral load, suggesting that VIS-410 provides therapeutic benefits (332).

Combination Therapies

Antivirals against influenza can also be used in different combinations for enhanced potency and reduced emergence of drug-resistant viruses. One of the first drug combinations against the influenza virus was Amantadine with interferon (333). Subsequently, a triple combination of antivirals, including interferon alpha 2, Ribavirin, and Rimantadine, was also tested against influenza virus (334). This combination of antiviral therapy can simultaneously act on different targets and can prove beneficial for treating more severe forms of influenza. In addition, as described in previous sections, several new drugs have been tried in combination with already-established antivirals like Oseltamivir. Currently, several clinical studies are ongoing to test the pharmacokinetic interactions and tolerability of several drug combinations, Flufenamic acid-Clarithromycin-Oseltamivir, Naproxen-Clarithromycin-Oseltamivir, and ADS-8902 (335–337).

Host Protein-Targeted Therapies

The study of the interaction between the influenza virus and various host proteins is another important area of active investigation. In recent years, considerable progress has been made in deciphering the role of host factors during the influenza virus life cycle (338). Genome-wide screens using siRNA or chemical inhibitors or clustered regularly interspaced short palindromic repeats (CRISPR), along with interactome and transcriptome data using different approaches, expanded our current knowledge (339–342). These host factors can also serve as potential targets for developing antivirals, as the virus is dependent on the host cellular machinery to complete its life cycle (342). Targeting the host factors for antiviral therapy has some added advantages, including the low likelihood of the emergence of resistance, as most of these factors are evolutionarily conserved. Moreover, it can also potentially apply to multiple respiratory viruses, as these viruses mainly utilize common molecular pathways (343, 344). However, the major drawback of host-targeted antivirals is the greater risk of host toxicity, as there are safety and tolerability concerns in targeting cellular functions; hence, careful safety studies are required (345).

GSK1325756 (Danirixin). GSK1325756 is a selective and reversible small molecule and a CXCR2 antagonist that decreases neutrophil activation and migration to the area of inflammation in preclinical studies. In the respiratory tract, there is neutrophil influx during infection, and excessive neutrophil influx correlates with severe influenza symptoms (346). The Danirixin dose-dependent inhibition of agonist-induced activation of neutrophils in healthy patients suggested that the inhibitor can be beneficial for controlling inflammation (347). Additionally, the phase I study demonstrated that oral Danirixin was generally well tolerated as a single dose (up to 400 mg) or repeated treatment (50 mg daily and 200 mg for 14 days). However, the pharmacokinetics of the study drug can be affected by diet, age, and other factors (347, 348). The efficacy and safety during a small phase IIb trial evaluated the safety and efficacy of two dosages of Danirixin in combination with Oseltamivir in 10 adults hospitalized with influenza (349). Danirixin exhibited a similar safety and tolerability profile as reported previously with no serious adverse effects. However, a conclusion on the efficacy is unavailable because of the small sample size consisting of four patients in each combo treatment group and two in the placebo-Oseltamivir group (349). Additionally, 45 patients with mild-to-moderate chronic obstructive pulmonary disease (COPD) demonstrated improvement in respiratory symptoms and health status when they received Danirixin along with the standard-of-care inhalation medications compared to 48 patients who received only the standard-of-care treatment. These data indicate potential benefits of treating influenza in patients with COPD (350).

Probenecid. Probenecid is an inhibitor of the host protein organic anion transporter (OAT3) required to support IAV replication (351). Probenecid is a good candidate to be repurposed for influenza treatment, as it is already used for treating hypouricemic disorder (gout), with established safety, pharmacokinetics, and interactions with other drugs (352). Several studies demonstrated that probenecid coadministration could significantly increase Oseltamivir metabolite concentration in plasma (353, 354). Alternate-day dosing of Oseltamivir in combination with Probenecid four times daily and the conventional Oseltamivir dosing had similar levels of plasma concentration (354). As Oseltamivir is considered a standard-of-care treatment and can be limiting in the event of an influenza pandemic, additional studies on alternative dosing strategies and pharmacokinetics are needed.

Celecoxib. It is an immunomodulator that inhibits the COX-2 enzyme. Celecoxib (200 mg daily) was used with Oseltamivir and compared with oseltamivir alone as a treatment for severe influenza A infection in phase III trial (355) from 2014 to 2017. The results demonstrated that the cotreatment of celecoxib with oseltamivir significantly reduced mortality and cytokines (interleukin 6 [IL-6] and IL-10) without increased adverse effects (356).

DAS-181 (Fludase). Fludase, or DAS-181, is a recombinant fusion protein that enzymatically cleaves sialic acid receptors responsible for the binding and entering of influenza virus in respiratory epithelial cells. The interaction results in the inhibition of entry of the virus in these cells (357). Preclinical studies of this drug against influenza demonstrated its potent antiviral activity (357, 358). A phase II clinical trial evaluated multiple doses of 30 mg and a 10-mg single dose of DAS-181 along with a placebo in 56, 69, and 52 influenza-infected patients, respectively (359). In addition, three doses of 10 mg of DAS-181-F2 compared to placebo significantly reduced influenza virus shedding (359). Additional phase I trials were conducted as dry powder administered at dosages ranging from 0.5 mg to 4.5 mg (formulations DAS-181-F03 and F-04) to target the upper respiratory tract and minimize adverse events and the increased dose to 20 mg for 3 days as a single dose to improve the effectiveness in healthy adults (360). The trials in healthy adults to evaluate the safety and systemic exposure demonstrated that 1- and 3-day treatments with 20 mg of DAS-181 daily were well tolerated with no severe side effects. However, adverse respiratory symptoms were recorded during the treatment longer than 7 days with the development of anti-DAS-181 antibodies in the subjects with respiratory symptoms.

Diltiazem. Diltiazem is a licensed calcium channel blocker known to relax blood vessels and is used to treat hypertension. It was identified in a screen of host factors with influenza-inhibitory activity (361). It was demonstrated *in vitro* and *in vivo* in a mouse model that Diltiazem modulates host antiviral genes *in vitro* and *in vivo* in a mouse model, and cotreatment with Oseltamivir enhanced the antiviral efficacy (362). The results lead to rapid authorization of a phase II ongoing clinical trial to assess the efficacy of the Diltiazem-Oseltamivir combination in treating severe influenza (363).

Host Innate Immune Activators

Strategies designed to modulate and boost the innate immune response against the virus are critical in reducing the impact of influenza. During the influenza infection, different innate immune sensors get activated eventually, leading to the establishment of the antiviral state (as described in an earlier section) (66). Lee et al. studied the role of TLRs in influenza A infection and reported that TLRs play an essential role in early influenza infection with the upregulation of TLR3 with other TLRs (364). Since these innate immune sensors are evolutionarily conserved, their ligands have potential as next-generation antivirals without the associated risk of developing drug resistance.

In most cases, coinfection with other respiratory viruses occurs with influenza infection (365, 366). Therefore, targeting influenza virus with the ligands of these pathogen sensors can also target several of these coinfections, ultimately leading to containing their infection. Figure 2 shows the synthetic ligands of PRRs with their downstream cellular signaling pathways.

RIG-I agonists. The cytosolic sensor RIG-I plays an indispensable role in recognizing 5′PPP-RNA and induction of the innate immune response (367). RIG-I ligand and 5′PPP-RNA activate the innate immune response and inhibit the replication of WT and drug-resistant influenza viruses *in vitro* and *in vivo* in animal models (368). This inhibition is independent of virus subtypes, drug sensitivity status, and virulence. Furthermore, the activation of the RIG-I pathway generates a broad-spectrum antiviral response as suggested by the replication inhibition of the Ebola virus in cell culture (369). Similarly, using the systems approach, Goulet et al. showed the antiviral potential of 5′PPP-RNA *in vitro* against several viruses along with the lethal challenge of A(H1N1)pdm09 influenza virus in a murine model (370–372).

Furthermore, the length, secondary structure, and sequence of 5′PPP-RNA modulate RIG-I-mediated protective antiviral response in cell cultures and murine models (373). In some cases, small-molecule agonists could also serve as antivirals for efficient control of virus infection by inducing tissue-wide innate immunity like the IRF3 activator, which induces innate immune gene expression (374). However, inefficient cytosolic delivery hindered the efficacy of RIG-I ligands. Recently, the clinical development of RIG-I agonists has advanced by developing novel polymeric carriers, which promote endosomal escape, leading to the enhanced activity and delivery of 5′PPP-RNA to the cytosol where RIG-I is located (375).

TLR3 agonists. Polyriboinosinic:polyribocytidylic acid [polyI:C] is a double-stranded RNA analog and one of the most used TLR3 agonists in experimental settings (376). Depending on the cellular localization, polyI:C activates different PRRs. For example, in endosomes, polyI:C activates TLR3 in the endosomes and RIG-I and MDA5 in cytosol (376–378). Prophylactic treatment of mice with polyI:C has shown immediate protection against the challenge with several influenza virus strains via upregulation of TLR3 in airways (379). Furthermore, intranasal delivery of polyI:C to aged mice upon challenge with influenza virus protected them without severe side effects (379). Nonavailability of a formulation that enhances the uptake of polyI:C and protects it from the RNases hindered the clinical development. PrEP-001 is a proprietary formulation of polyI:C which is in powder form and can be delivered intranasally, eliciting innate immunity. In a placebo-controlled study, 27 subjects received two doses of 6.4 mg of Pr-EP prior to challenge with 10 50% tissue culture infective doses ($TCID_{50}$) of A/Perth/16/2009 virus (380). PrEP-001 is safe and well tolerated with reduced symptom score, peak viral titers, and duration of A(H3N2)-IAV infection (381).

Type I interferons and IFN inducers. As interferons are the key contributors in the antiviral response against influenza, any defects in their production or regulation can have devastating effects. In humans, defective type I or type III IFN amplification causes the mild disease to intensify into a severe life-threatening disease (382). *In vitro* and *in vivo* studies have suggested the effectiveness of IFN treatment in influenza infection (383). The prophylactic use of oral interferon alpha at a low dose (150 IU) for prevention of acute respiratory viral infection (ARI) and influenza resulted in reduced disease severity in those who also received influenza vaccination. However, the low-dose IFN administration did not affect the overall incidence of ARI (384). Other immune molecules like Alfacon-1, known to be potent against different viruses, were tested against influenza in hospitalized patients. However, results from clinical trials are not published yet (385). Kagocel is an immunomodulator that induces type I and III interferons and exhibits broad-spectrum activity against influenza (Fig. 2). A recent study using murine lymphocytes from Peyer's patches suggested that PRRs mediate the action of this drug (386). In a clinical study, Kagocel administration increased cytokine levels in the plasma of patients with influenza infection (387). The drug has been evaluated for preventing ARI in young adult students over 18 years of age and adult and older adult (18 to 70 years) health care workers in Russia (388, 389).

Volatile anesthetics. A prospective blind cohort study of pediatric patients with upper respiratory tract infection who underwent anesthesia with halothane demonstrated significantly shorter and fewer clinical symptoms than untreated patients (390). In addition, halothane and other volatile anesthetics exhibited antiviral responses in murine and ferret models of influenza (391–393). The mechanism by which volatile anesthetics decrease the pathogenesis of influenza virus involves the modulation of the host Th1-adaptive immune response, specifically IFN-γ activation of CD8 T cells and monocyte recruitment (394). Although we do not normally anesthetize patients to treat viral infections, this therapy was again explored in COVID-19 patients on ventilators. Additionally, this therapeutic strategy may lead to the development of new approaches.

MACHINE LEARNING AS A TOOL FOR DRUG DISCOVERY AND DEVELOPMENT

Machine learning (ML) has become increasingly popular to facilitate all stages of drug discovery and development, as it has the potential to reduce time and lower cost (395, 396). A typical application of ML is the prediction of antiviral resistance. For instance, we can use decision trees and neural networks to predict resistance to antivirals, such as the M2 ion channel inhibitor, adamantane class of drugs and the NA inhibitor, Oseltamivir (397). In addition, ML can predict the potential functional causality of putative targets based on known antiviral agents. Pang et al. (398) developed AVPIden, a two-stage classification tool for predicting antiviral peptides (AVP) and potential functional activities against eight viruses, including HIV, influenza A virus, and SARS-CoV.

Additionally, with the recent advances in natural language processing, ML algorithms such as BeFree (399) and DigSee (400) can quickly scan massive amounts of data to identify relevant gene-disease associations. Furthermore, ML can identify novel therapeutics by understanding fundamental biological mechanisms. For example, recent evidence suggests that viral-induced alternative splicing promotes influenza replication (401). Deep neural networks (DNNs) accurately predict alternate splicing signals based on genomic and cellular features (402). Another vital application of ML is to reduce the search space for druggable targets and improve the virtual screening efficiency of lead compounds. For example, support vector machine models identified five oseltamivir derivatives with potent inhibition targeting NA against H1N1 and H3N2 (403). Combined with molecular docking software, random-forest models were used to screen group 2 NAs (N2, N3, N6, N7, and N9), of the IAV(H7N9) (404) virus. In a recent study (405), SiMMap, a hierarchical clustering method (406), screened more than 200,000 public records of compounds to identify HA inhibitors for the influenza A virus. Multineural networks can identify compounds with structures similar to the currently used drugs (407, 408). In addition, ML can optimize the compound design for small-molecule and other

therapeutics. For example, AstraZeneca used deep reinforcement learning as a parameter tuning tool to find optimal values for *de novo* compound designs (409).

Moreover, ML methods can design small molecules based on multiscale behavior and interactions to selectively inhibit multiple influenza targets while mitigating interaction with host proteins to minimize adverse effects (410). Finally, ML can have application in drug repurposing for influenza (362, 411–417). With the integrated efforts to improve data quality and availability, ML is a promising approach to developing next-generation antivirals and therapeutics for infectious diseases.

CONCLUSIONS AND FUTURE PERSPECTIVES

Influenza, though a vaccine-preventable disease, remains a serious public health problem globally. Current FDA-approved therapeutics have a short treatment window. Influenza virus can overcome the effects of the antiviral agents and develop drug resistance, leading to epidemics and pandemics. Apart from developing new classes of direct-acting antiviral drugs that act on viral factors, targeting the essential host factors needed for viral entry, replication, and release is another important area for therapeutic interventions, especially when the circulating viruses are resistant to one or more currently approved antiviral drugs. Activating innate immune responses with the ligands of evolutionarily conserved pathogen sensors can be a pan-antiviral strategy against drug-resistant viruses. Treatment approaches like passive immunization using mAbs or polyclonal sera in combination with antiviral drugs can benefit severely ill influenza patients with limited treatment options. Developing potent antiviral therapeutics that target influenza is crucial for prophylactic and therapeutic interventions against seasonal and pandemic influenza viruses.

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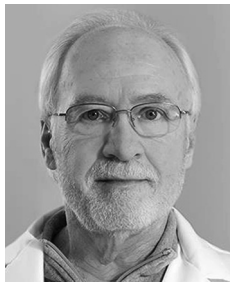
Jan Pohl, Ph.D. serves as the Chief of the Biotechnology Core Facility at the Centers for Disease and Prevention, which he joined in 2008. He received his undergraduate degree in chemistry and his Ph.D. degree in biochemistry from Charles University, Prague (1981). Between 1981 and 1986, he held research positions in protein chemistry at the Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague. He was a faculty member and directed the Microchemical and Proteomics Facility at Emory University School of Medicine in Atlanta, GA (1987 to 2008). His research focuses on protein structure elucidation and synthesis and structure-function relations of host defense proteins and peptides. He has published/host defense proteins and peptides. He has published over 150 research articles.



Paul R. Knight, M.D., Ph.D. is a Distinguished Professor of Anesthesiology at the University at Buffalo (UB). Knight was the first M.D./Ph.D. degree candidate to graduate from the Pennsylvania State College of Medicine. He completed his residency in Anesthesiology at the Milton S. Hershey Medical Center. In 1977, Knight joined the faculty of the Department of Anesthesiology at the University of Michigan, where, by 1986, he had risen to the rank of tenured professor. In 1992, he was recruited to UB as Professor of Anesthesiology and Microbiology and Chair of the Department of Anesthesiology. He served in this capacity until 1998. Knight's international prominence is largely due to his contributions to the study of various lung injuries and myocardial dynamics. He has been a pioneer in assessing how general anesthetics affect viral respiratory injury and was the first to demonstrate that these agents can modify host antiviral immunity.

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