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Focusing on the influenza virus polymerase complex: recent progress in drug discovery and assay development

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Abstract

Influenza viruses are severe human pathogens that pose persistent threat to public health. Each year more people die of influenza virus infection than that of breast cancer. Due to the limited efficacy associated with current influenza vaccines, as well as emerging drug resistance from small molecule antiviral drugs, there is a clear need to develop new antivirals with novel mechanisms of action. The influenza virus polymerase complex has become a promising target for the development of the next-generation of antivirals for several reasons. Firstly, the influenza virus polymerase, which forms a heterotrimeric complex that consists of PA, PB1, and PB2 subunits, is highly conserved. Secondly, both individual polymerase subunit (PA, PB1, and PB2) and inter-subunit interactions (PA-PB1, PB1-PB2) represent promising drug targets. Lastly, growing insight into the structure and function of the polymerase complex has spearheaded the structure-guided design of new polymerase inhibitors. In this review, we highlight recent progress in drug discovery and assay development targeting the influenza virus polymerase complex and discuss their therapeutic potentials.

Keywords

Influenza; polymerase; ribonucleoprotein; PA; PB1; PB2

1. INTRODUCTION

Influenza viruses are respiratory pathogens that affect both humans and animals. Human infection of influenza virus results in symptoms ranging from coughing, sneezing, headache, respiratory depression to pneumonia-related death [1]. In general, the disease outcomes of influenza virus infection in healthy adults are self-limiting and the patient will recover

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within a week or two without treatment. However, disease progression is drastically exacerbated in highly susceptible groups such as children, elderly, immunocompromised individuals, cancer patients, and people with chronic diseases such as diabetes, asthma, and heart disease [2]. Overall, influenza virus infection accounts for ~36,000 deaths and millions of hospitalizations in the United States during the annual influenza epidemic [3]. As a result, influenza virus infection is currently listed among the top-ten leading causes of death in the United States, and the death toll of influenza virus infection-related deaths surpasses that of breast cancer. The average annual direct medical costs are \$10.4B, and projected lost earnings due to illness and loss of life are \$16.3B [4]. Moreover, the public health and economic impacts accompanying sporadic influenza pandemics are several orders of magnitude higher [5]. The devastating effects of influenza pandemics have been documented by the 1918 H1N1 Spanish influenza, the 1957 H2N2 Asian influenza, the 1968 H3N2 Hong Kong influenza, and the recent 2009 H1N1 Swine influenza [6].

Despite the persistent public health threat posed by influenza viruses, we are currently limited by our countermeasures to prevent infection. To combat existing and emerging drug-resistant influenza viruses, there is an urgent need to develop the next-generation of influenza antivirals. Among the compounds that are currently in development, the influenza polymerase inhibitors have proven to be desired drug candidates. This review examines the limitations associated with the two classes of FDA-approved antivirals, followed by an introduction into the development of new antiviral drugs entering clinical trials. We also introduce the structure and function of the influenza virus polymerase, the drug discovery and assay development for both individual subunits and inter-subunit interactions of the polymerase, and an up-to-date summary of influenza polymerase inhibitors and their antiviral efficacy. This review is not intended to be a comprehensive review of all studies published regarding the influenza virus polymerase or inhibitors that target influenza polymerase indirectly, e.g. through host factors. Instead, our focuses are on the recent discovery of novel inhibitors targeting the polymerase complex and innovative assays for screening inhibitors targeting the influenza virus polymerase.

1.1 General overview of influenza viruses

Influenza viruses are single-strand negative-sense RNA viruses that belong to the *Orthomyxoviridae* family [7]. There are three-recognized influenza types: A, B and C, and a possible D type was also recently identified. However, due to the limited knowledge regarding influenza D virus, it will not be discussed. Influenza viruses A, B, and C all infect humans. In addition, influenza A viruses also circulate among aquatic birds, which are the natural hosts of influenza viruses. Although the species barrier between humans and avian species normally prevents the direct transmission of avian strains to humans, certain avian influenza strains such as highly pathogenic avian influenza (HPAI) viruses H5N1 and H7N9 are known to infect human and have a high mortality rate [8]. It is believed that influenza pandemic is caused by the emerging or re-emerging viruses that are produced by the re-assortment of influenza A viruses, which is also referred to antigenic shift [7]. In contrast, influenza B virus almost exclusively infects humans and seals and is generally less virulent than influenza A virus [9, 10]. Nevertheless, influenza B virus-related mortality has been frequently reported [9]. The percentage of infection caused by influenza B viruses in

seasonal influenza epidemics can range from a few percent to more than 90% [9]. In contrast to influenza A virus, influenza B virus has not been shown to lead to influenza pandemic outbreaks. Overall, influenza B viruses remain a relevant human respiratory pathogen, and both the trivalent and quadrivalent seasonal influenza vaccines contain the viral components from influenza B viruses [11]. Influenza C viruses infect humans, dogs and pigs. It usually causes mild disease in children. In summary, as prioritized by the pandemic potential and severity of disease outcomes, influenza A and B viruses are the central focus of drug discovery and mechanistic studies of viral replication, transmission, viral-host interactions, and drug resistance.

Influenza A viruses are further classified into subtypes based on their surface antigens, hemagglutinin (HA) and neuraminidase (NA). There are at least 18 HAs and 11 NAs, which can result in up to 198 different influenza A virus subtypes theoretically [12]. Majority of the subtypes have been found in aquatic birds. The common subtypes circulating among humans are, however, limited to H1N1 and H3N2 strains, although H2N2 strain was also circulating in humans from 1957 to 1968 [13]. Unlike influenza A viruses, influenza B viruses are divided into two antigenically distinct lineages: Yamagata and Victoria [9]. Influenza B viruses from both lineages currently circulate alongside influenza A viruses H1N1 and H3N2 in humans. Table 1 lists the representative influenza viruses that have been circulating among humans in recent years in the Northern Hemisphere.

Influenza A and B viruses encode at least eleven viral proteins, three of which are the viral membrane proteins, HA, NA, and M2. Inside the virion envelope are the matrix protein M1 and the viral ribonucleoprotein complex. Both influenza A and B virus genome consist eight segments of negative-sense viral RNA (vRNA). Each vRNA binds to multiple copies of nucleoprotein (NP) and a heterotrimeric RNA-dependent RNA polymerase complex, and the whole assembly is referred to as the viral RNA ribonucleoprotein (vRNP) [14]. The viral polymerase complex is composed of three subunits: polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic protein (PA) [15]. The complex is located at the partially complementary ends of the viral RNA. The nuclear export protein (NEP) (also known as NS2) is also found to be present in the virion. Other proteins such as NS1 are only expressed during the viral replication cycle and are not incorporated into the mature virion.

1.2. Influenza virus replication cycle

Influenza viral infection starts with attachment of viruses to host cells surface via the binding between viral HA protein and host receptor molecules such as sialic acid-containing glycoproteins or lipids [16]. This is followed by viral entry into host cell by endocytosis (Fig. 1). The acidic pH in the endosome triggers a conformational change in HA which lead to the fusion of the viral and endosomal membranes. During this process M2 proton channel is activated, resulting in the acidification of the viral interior causing the dissociation of vRNP complex from the matrix protein M1 [17]. The vRNPs are then transported into the nucleus, where they mediate both viral RNA transcription and replication. At the initial stage of viral replication, transcription of vRNA to mRNA is the predominant process as this step is required to synthesize additional copies of viral proteins for the subsequent vRNA

replication. Since influenza virus polymerase lacks an inherent capping activity, it initiates viral transcription using a capped primer, derived via a cap-snatching mechanism [18]. The influenza virus polymerase PB2 subunit contains a cap-binding domain, which can capture the 5'-cap of nascent host pre-mRNAs, allowing PA endonuclease domain to cleave the capped mRNA at 8–14 nucleotides downstream of the cap structure. The capped host mRNA then serves as a primer for the viral mRNA transcription. In the next step, viral mRNAs are exported to the cytoplasm for translation by hijacking host cellular protein synthesis machinery. Newly translated viral proteins, PA, PB1, PB2 and NP, are then transported back to the nucleus and assemble with cRNAs and vRNAs to form cRNPs and vRNPs, respectively. For replication of vRNA, the full-length of single-stranded negative-sense vRNA genome segments are copied into complementary RNAs (cRNAs), then cRNAs are used as templates for the production of progeny vRNAs. Progeny vRNAs then assemble with NP, PA, PB1, and PB2 to form vRNPs, which are exported from nucleus with the assistance from M1 and nuclear export protein (NS2). In the next step, the vRNPs, together with other viral proteins such as M1, M2, HA, and NA, are transported to the plasma membrane for virion assembly. Lastly, mature virions are released from the infected cells by budding and detachment, a process that involves NA-mediated cleavage of sialic acid linkage from host cell receptors (Fig. 1).

1.3. FDA-approved influenza antivirals and drugs that are at the late stage of clinical trials

Influenza vaccines remain the mainstay in preventing seasonal influenza virus infection [11]. It is recommended by the centers for disease control and prevention (CDC) that most people aged 6 months or older should get influenza vaccines [19]. Although vaccines are generally effective in preventing seasonal influenza infections with an overall of 60% effectiveness [20, 21], they are generally not available in preventing influenza pandemics, largely due to the difficulty in predicting the antigens in the emerging influenza strains [22]. In addition of influenza vaccines, small molecule antiviral drugs are also available for prophylaxis and treatment of influenza virus infection [23, 24]. Currently there are two classes of FDA-approved influenza antivirals (Table 2): 1) amantadine and rimantadine, which are M2 inhibitors that inhibit virus uncoating [25, 26]; and 2) oseltamivir, zanamivir, and peramivir, which are neuraminidase inhibitors that inhibit virus egress [27]. Another neuraminidase inhibitor, laninamivir, was approved in Japan. Similar to other anti-infective agents, resistance to both classes of influenza antivirals necessitates the development of the next generation of anti-influenza drugs. Amantadine and rimantadine are no longer recommended for use in influenza infection due to widespread drug resistance, according to the CDC. This leaves the neuraminidase inhibitors as the last choice of FDA-approved drugs. Unfortunately, resistance to the only orally available anti-influenza drug, oseltamivir, is continuously reported [28–32]. Although it was originally believed that it is unlikely for influenza viruses to develop resistance to neuraminidase inhibitors, the 2007–2008 H1N1 seasonal strain in the northern hemisphere that contains the NA-H275Y mutation was found to be completely resistant to oseltamivir [33]. This clinical observation suggests that influenza viruses are indeed capable of evolving drug resistance without compromising the fitness of transmission [34]. In addition to drug resistance, oseltamivir also has a narrow therapeutic window and is only effective when given to patients within 24 hours when symptoms appear. For critically ill patients, oseltamivir also showed disappointing efficacy

[35]. Zanamivir is administered intra-nasally, rather than orally as is oseltamivir, so its use is limited, especially in infants and critically ill patients. The recently approved peramivir is an intravenous agent and is typically restricted to hospitalized patients. In addition to M2 and neuraminidase inhibitors, viral fusion inhibitors such as arbidol have also been widely used in Russian and China [16, 36]. Arbidol targets the viral HA protein and prevents viral fusion with the host cells [16, 37]. However, the therapeutic benefit of arbidol is restricted by its narrow spectrum of antiviral activity and drug resistance [38]. Clearly, the treatment gaps associated with the current influenza therapies, coupled with existing and emerging drug resistance to the approved drugs, emphasize the urgent need to develop new therapeutic agents. The desired features for the next-generation of influenza antivirals should include but not limited to: broad-spectrum antiviral activity against both influenza A and B viruses, convenient administration to both general population and critically ill patients, high efficacy even when delivered at the late stage of disease progression, a novel mechanism of action, and a high genetic barrier to drug resistance. In response to the calling for more effective influenza antivirals, robust drug discovery programs have been launched both in academia and industry to develop either direct-acting antivirals or host-targeting antivirals [27]. These efforts result in a number of promising drug candidates that are currently at the late stages of human clinical trials (Table 2) [24]. Among the five clinical candidates, DAS-181 and nitazoxanide are host-targeting influenza antivirals. DAS-181 is a bacteria-derived sialidase which hydrolyses the Neu5Ac $\alpha(2,3)$ - and Neu5Ac $\alpha(2,6)$ -Gal linkages of sialic acid-containing receptors on the host cell surface [39]. As a result, the cellular receptor for viral entry is destroyed and no longer supports viral replication. Nitazoxanide is an antiparasitic drug that was repurposed for the treatment of influenza virus infection [40]. It represents another example of host-targeting antiviral. The mechanism of action of nitazoxanide involves impairing the maturation of viral hemagglutinin, possibly by inhibiting the post-translational glycosylation of HA [41]. The remaining three compounds, VX-787, T-705, S-033188, all of which target the viral polymerase complex, highlighting the therapeutic potential influenza virus polymerase inhibitors [42]. Specifically, VX-787 is a PB2 cap-binding inhibitor which binds to the PB2 cap-binding domain, thereby preventing the priming of viral mRNA transcription; T-705 is a prodrug which is converted to the active metabolite in vivo and gets incorporated in viral RNAs [43, 44]. As a result, the fidelity of viral replication is hampered and a high mutation rate is observed [45]. Compound S-033188 is a PA endonuclease inhibitor, which inhibits the cleavage of host mRNA [24]. As a result, viral mRNA transcription cannot be initiated. As it has been clearly demonstrated by the current influenza antiviral drug discovery paradigm, the influenza virus polymerase complex is undoubtedly a high profile drug target that is highly likely to lead to the first-in-class next-generation influenza antiviral. We shall next dissect the mechanism of action for VX-787, T-705, and S-033188, as well as highlight new polymerase inhibitors currently in early stages of development or preclinical trails.

2. INFLUENZA VIRUS POLYMERASE

2.1. Structure of influenza virus polymerase complex

Recent years have witnessed tremendous breakthroughs in structural biology of influenza virus polymerase complex [15, 42, 46]. As a result, rational design of inhibitors targeting the

polymerase complex has become a reality [42]. Structure determination of the polymerase complex has long been hampered by the difficulty in expressing sufficient quantities of pure and biologically active influenza virus polymerase complex. Gratifying, by switching to the bat-derived influenza A/H17N10 strain, the first X-ray crystal structure of the influenza A virus polymerase complex with bound vRNA promoter was solved at 2.65 Å resolution (PDB: 4WSB) (Fig. 2A) [18]. This structure offers the first glimpse into how each subunit within the heterotrimeric polymerase complex coordinates with each other to mediate vRNA transcription and translation. As elucidated by the X-ray crystal structure, PA interacts extensively with PB1 with a total buried surface area of 17,330 Å². In contrast, PA only interacts marginally with PB2, and the total buried surface area between PA and PB2 is only 2,880 Å². The PB1 subunit interacts with the PB2 subunit extensively with a total buried surface area of 14,100 Å². The N-terminus domain and C-terminus domain of PA subunit is flanked by the PB1 subunit and is connected by an extended linker. The endonuclease activity of the viral polymerase is encoded in the N-terminus domain of the PA subunit, while the C-terminus domain of PA, together with the PB1 subunit, forms a tunnel where the vRNA promoter binds. The polymerase activity is encoded in the PB1 subunit which adapts the characteristic fold similar to other viral RNA polymerase with a central active site made of the fingers, palm, and thumb domains. The X-ray crystal structure of the influenza B virus polymerase complex from the B/Memphis/13/03 strain was also solved (PDB: 4WRT and 4WSA) (Fig. 2B) [18]. Although there is only a moderate sequence conservation between the bat-derived influenza A polymerase and the B/Memphis/13/03 polymerase (36% for PA, 59.5% for PB1, and 37.0% for PB2), the overall structures are remarkably similar. Notably, the PB2 cap-binding domain in the influenza B virus polymerase is rotated 70° in respect to that in the influenza A polymerase, indicating this domain can rotate *in situ*. More detailed discussion regarding the structure and function of influenza virus polymerase complex can be found in recent reviews [15, 42, 46, 47].

3. DRUG DISCOVERY AND ASSAY DEVELOPMENT TARGETING THE INFLUENZA VIRUS POLYMERASE COMPLEX.

The high-resolution structures of the influenza A and B polymerase complexes render rational drug design a reality. The past years have witnessed a tremendous progress in developing antivirals targeting the virus polymerase complex [48, 49]. Not only each individual subunit within the polymerase complex (PA, PB1, PB2) has been validated as drug targets, the subunit interactions among viral polymerase, such as PA-PB1 and PB1-PB2, are also attractive targets for drug design. In the following sections, we focus on the recent discoveries of novel inhibitors targeting the influenza virus polymerase complex and their mechanism of inhibition. In addition, we also highlight innovative assays that have been developed for high-throughput screening and characterization of polymerase inhibitors.

3.1. Influenza virus mini-genome assay and in vitro vRNPs-mediated viral RNA synthesis assay.

Two functional assays have been developed to validate whether a compound inhibits the viral polymerase activity: the influenza virus minigenome assay and the in vitro vRNPs-mediated viral RNA synthesis assay.

3.1.1. Influenza virus mini-genome assay—The replication of influenza virus centers on the vRNPs, which are the basic genetic unit for viral transcription and replication. The influenza mini-genome assay is the gold standard assay for studying viral replication and the mechanism of antivirals [50]. The mini-genome assay was designed to specifically capture the viral transcription and replication processes during the viral replication cycle without using the infectious virus, thereby eliminating other viral replication steps such as viral entry, assembly, and budding [51, 52]. In this assay, the negative-sense of the reporter gene, firefly luciferase, flanked by the non-coding regions of one of the eight vRNA segments is used to mimic vRNA (Fig. 3). Rather than provided by the infectious virus, influenza polymerase PB1, PB2, PA, and NP are expressed from individually transfected plasmids. Therefore, the expression of the firefly luciferase reporter gene is only controlled by the transfected viral polymerase complex. In addition, a second reporter such as a constitutively expressed Renilla luciferase is normally used as an internal control to normalize the transfection efficacy. Chloramphenicol acetyltransferase (CAT) and fluorescent proteins [e.g. green fluorescent protein (GFP)] have also been used as the reporters in addition to the firefly luciferase [53, 54]. In comparison, the firefly luciferase reporter has the advantage of circumventing assay interference from intrinsic absorbance and fluorescence that may be present in certain test compounds. Recently, several stable cell lines constitutively expressing a reporter vRNP complex have been successfully generated [54–58]. This strategy removes the need for transient transfection and thus provides a rapid method amendable for high-throughput screening. The mini-genome assay is currently the standard assay used to validate the antiviral mechanism of drug candidates derived from either rational design or HTS, and is also frequently used to identify host factors involved in viral replication [59–61].

3.1.2. In vitro vRNPs-mediated viral RNA synthesis assay—In addition to the cell-based assay, an assay to measure the enzymatic activity of influenza polymerase using vRNP complexes isolated from influenza virus particles has been developed [62]. The vRNP complexes can also be expressed in insect cells using baculovirus expression vectors and be used to synthesize short stretches of vRNA and cRNA in vitro [63]. The expressed vRNPs can also be applied in a scintillation proximity assay (SPA) to examine replication initiation [64, 65]. Compared with the mini-genome assay, the vRNA synthesis assay using purified vRNP complex is more technically challenging as it requires the expression and isolation of the vRNP complex, as well as handling radio-labeled reagents. For this reason, the vRNA synthesis assay using purified vRNP complex is not routinely used in majority of the drug discovery laboratories. Despite the challenges, a fluorescence polarization-based assay has been developed recently to study the initiation of cap-primed (transcription) or unprimed (replication) RNA synthesis using recombinant influenza B polymerase [66]. This assay avoids the use of isotope and is adaptable to high-throughput screening for polymerase inhibitors. Compared to cell-based mini-genome assay, the cell free vRNA synthesis assay can provide direct evidence whether a compound interferes the vRNP-mediated vRNA replication, while the mini-genome assay is unable to differentiate protein synthesis inhibition versus inhibition of the vRNP-mediated viral RNA transcription.

In summary, the influenza mini-genome assay is more convenient than the *in vitro* vRNPs-mediated viral RNA synthesis assay and can be easily adapted by most drug discovery laboratories. However, the limitation of influenza mini-genome assay is that it cannot rule out the possibility that an active compound might target host factors that are essential for viral polymerase maturation and assembly. Therefore, additional secondary assays need to be implemented to further confirm the mechanism of action of active hits from the mini-genome assay.

3.2. Drug discovery and assay development targeting PA endonuclease activity

3.2.1. Drug discovery targeting PA endonuclease domain—The N-terminal domain of PA subunit (PA_N; residues 1–196) encodes the endonuclease activity, which is required for cleavage of host cellular pre-mRNAs for viral mRNA transcription. Among the drugs in development targeting the influenza virus polymerase complex, the PA endonuclease inhibitors are the most extensively explored [67]. More than dozens of crystal structures have been solved for the PA endonuclease domain, both in the inhibitor-free form and inhibitor-bound form, thus providing a compelling foundation for structure-based drug design and elucidation of drug inhibition mechanism [67]. The active site of the PA endonuclease domain comprises three negatively charged residues E180, D108, E119, and one H41 residue which collectively coordinate with one or two metal ions, which could be Mg²⁺ or Mn²⁺ (Fig. 4A). The metal coordination in the PA endonuclease domain is similar to that of the HIV integrase [68]. The precedent of targeting the endonuclease activity to suppress viral infection has been set by the HIV endonuclease inhibitors such as raltegravir, dolutegravir, and elvitegravir. Built upon the knowledge and expertise in developing HIV integrase inhibitors, similar strategies have been successfully applied to PA endonuclease drug discovery. Inhibitors targeting the PA endonuclease domain generally contains a metal-chelating headgroup and peripheral substitutions that interact with several other pockets in the active site. There are several sub-pockets within the active site (Fig. 4B and 4C), which accounts for its capacity to accommodate structural diverse compounds such as **L-742001**, **RO-7**, **ANA-0**, compounds **1** and **2** (Fig. 4D). Compound **L-742001** is a prototypical endonuclease inhibitor which contains the classic metal-binding motif – diketo acid, and this compound is frequently used as a positive control for PA endonuclease. To access the genetic barrier to drug resistance of **L-742001**, *in vitro* serial viral passage experiment was performed under the drug selection pressure of **L-742001** [69]. Gratifying, no resistant virus was selected after ten passages, indicating **L-742001** has a high *in vitro* genetic barrier to drug resistance. Nevertheless, when mutations were introduced by random mutagenesis, two mutants, F405S and E119D, were identified to confer drug resistance without significant loss of replication fitness. This result raised the concern that resistance might eventually emerge later one under either drug selection pressure or sporadic mutations. Indeed, resistance to HIV integrase inhibitors has been well documented [70]. It is important to emphasize the possibility that resistance may be inevitable, and it is not a question of if but a question of when [71].

Among the recently discovered PA endonuclease inhibitors, compound **RO-7** is of particular interest [72, 73]. **RO-7** can be viewed as an analog of dolutegravir (Fig. 4D), which is a HIV integrase inhibitor, as both of them contain the same metal-binding group. Since the PA

endonuclease is highly conserved among influenza A and B viruses, it is expected that PA endonuclease inhibitors should have broad-spectrum antiviral activity against both types of influenza viruses (A and B) as well as their subtypes. Indeed, compound **RO-7** was found to inhibit multiple subtypes of influenza A viruses (H1N1, H3N2), influenza B viruses from both lineages (Yamagata and Victoria), and zoonotic viruses (H5N1, H9N2, and H7N9).[73] When tested *in vivo* using influenza virus infected BALB/c mice model, **RO-7** significantly protected the mice from lethal infection of either the influenza A/California/04/2009 (H1N1)pdm09 or B/Brisbane/60/2008 virus, and no **RO-7** resistant virus was isolated from the lungs of **RO-7** treated mice [72].

Compound **ANA-0** was originally identified as a potent influenza antiviral from a high-throughput screening [75]. The antiviral mechanism of **ANA-0** was later confirmed as inhibiting the influenza PA endonuclease [75]. When tested in combination therapy, **ANA-0** was found to have a synergistic effect with zanamivir. In addition, **ANA-0** was also had *in vivo* antiviral activity in protecting mice from infection by the A/HK/415742Md/09 H1N1 virus.

Another PA endonuclease inhibitor worth highlighting is compound **1**. As a classic example of fragment-based drug design, compound **1** was designed by fragment growth and fragment merging [76]. Specifically, the pyromeconic acid scaffold emerged as a potent metal chelator for PA endonuclease from a screening a library of ~300 metal-binding fragments. Next, substitutions were first individually introduced at different positions of pyromeconic acid, then optimal substitutions were merged to yield compound **1** with potent antiviral activity and high selectivity.

Very recently, a class of N-acylhydrazones were synthesized by a one-pot condensation of hydrazide with aldehydes [74]. This effort led to the identification of compound **2**. The co-crystal structure of compound **2** with PA endonuclease revealed that the galloyl moiety from compound **2** chelates with two metal ions located at the active site of PA endonuclease.

For a complete list of PA endonuclease inhibitors, please refer to recent reviews [67, 74]. Given the precedent of clinical success of HIV integrase inhibitors, influenza PA endonuclease inhibitors are highly promising drug candidates.

3.2.2. Assays for screening and characterizing PA endonuclease inhibitors—

To date, three assays have been well widely used for screening and characterizing PA endonuclease inhibitors, namely gel-based endonuclease assay, fluorescence resonance energy transfer (FRET)-based endonuclease assay, and the fluorescence polarization assay. The PAN cleaves both RNA and DNA, but because of the higher stability of DNA than RNA, DNA substrates are more frequently used in both assays. In the gel-based endonuclease assay (Fig. 5A) [77, 78], a single-stranded circular DNA is incubated with PA or PAN at 37 °C. After the reaction is quenched, the digested DNA are separated by DNA agarose gel electrophoresis and visualized under UV light. Although the apparent limitation of the gel-based assay is that it is not amendable for high-throughput screening, it can be a powerful tool for visualizing the cleavage patterns of PA endonuclease activity.

The other assay is FRET-based endonuclease assay (Fig. 5B), in which a single-stranded DNA oligonucleotide was conjugated with a fluorophore (e.g. FAM) and a quencher (e.g. TAMRA) on its 5' and 3' termini, respectively, is used as a substrate [75, 79]. After cleavage by PA endonuclease activity, the quencher is released from the substrate and therefore the cleavage can be quantified by measurement of donor fluorophore fluorescence signal.

In addition to the endonuclease activity, a fluorescence polarization assay has also been developed to screen PA endonuclease inhibitors (Fig. 5C) [80, 81]. The fluorescent ligand was designed based on **L-742001**. The design of the fluorescent probe was guided by the structure-activity relationship (SAR) of **L-742001** as well as the co-crystal structure of **L-742000** with PA_N [69].

3.3. Drug discovery and assay development targeting PB2 cap-binding domain

3.3.1. Drug discovery targeting PB2 cap-binding domain—Influenza viral mRNAs are synthesized through a cap-snatching mechanism catalyzed by the viral polymerase [7, 14, 15, 74]. Specifically, PB2 binds to the 7-methyl GTP on the 5' end of the host pre-mRNA through its cap-binding domain, then PA cleaves the host RNA strand at a position 10-to-13-nucleotide 3' to the 7-methyl GTP through its endonuclease activity [15]. The cleaved host pre-mRNA subsequently serves as a primer for viral mRNA transcription. The cap-binding site is essential for cap-dependent transcription of viral mRNA by viral RNPs, rendering it a desire drug target for antiviral drugs [42]. Based on the X-ray crystal structure of PB2 bound to the 7-methyl GTP (PDB: 4CB4) (Fig. 6A) [82], a number of 7-methyl GTP analogs were designed and docked to the cap-binding domain of PB1 using Autodock4 [82]. Based on the co-crystal structure, the 7-methyl guanine base was kept intact as it involved in extensive hydrogen bonding with PB2, whereas the ribose and the phosphate at the N-9 position were replaced with various substituents as they are solvent-exposed (Fig. 6A). Designed compounds were subsequently synthesized and tested in the AlphaScreen assay. This effort led to the identification of one of the most potent inhibitor, **4**, which had IC₅₀ value of 7.5 μM against the PB2 cap-binding domain from a H3N2 strain. However, none of the designed compounds had cellular antiviral activity, presumably because of their poor cellular membrane permeability. Nevertheless, this study clearly demonstrated that the cap-binding domain of PB2 is a druggable target which warrants further development.

Encouraged by the preliminary results, inhibitors with more favorable drug-like properties have been developed to target the PB2 cap-binding domain [83]. One representative example is **VX-787** (JNJ-63623872) (Fig. 6B), which is currently in clinical trial. Very recently, additional analogs of **VX-787**, compounds **5** and **6**, were developed to further improve the oral bioavailability [84] and metabolic stability [85].

Recently, compound **PB2-39** was reported to act as a PB2 cap-binding inhibitor.[86] It inhibits multiple subtypes of influenza A viruses both in vitro and in vivo, including H1N1 and HPAI strains such as H5N1 and H7N9.

3.3.2. Assays for screening and characterizing PB2 cap-binding domain inhibitors.

The assays employed to characterize PB2-cap binding inhibitors include AlphaScreen assay, fluorescence polarization assay, bead-binding assay, surface plasma resonance (SPR) assay, and isothermal titration calorimetry (ITC) assay [87]. From the X-ray crystal structure of PB2 bound to 7-methyl GTP (PDB: 4CB4) [82], both the triphosphate and the ribose hydroxyl groups from 7-methyl GTP are solvent exposed (Fig. 6A), thus they represent attractive sites for probe conjugation. As shown in Fig. 7, all probes designed to bind to PB2 cap-binding domain have modifications at either the triphosphate or the ribose hydroxyl groups. Specifically, in the AlphaScreen assay, the 7-methyl GTP was labeled with a biotin to give biotin-EDA-m⁷-GTP (Fig. 7A), and PB2 protein was labeled with a His₆-tag [82]. The 6-histidine kit contains nickel chelator-coated acceptor beads and streptavidin-coated donor beads and were used to detect the interactions between biotin-EDA-m⁷-GTP and His₆-PB2. The principle of this assay is based on singlet oxygen-mediated energy transfer. Specifically, excitation of the donor beads at 680 nm produces singlet oxygens, which have a short half-life of 4 μsec and can diffuse approximately 200 nm in solution before deactivation to the triplet ground state oxygen. If receptor beads are in proximity, the singlet oxygens will transfer the energy to the thioxene derivatives within the acceptor bead, resulting in light production at 520–620 nm. Compound **4** (Fig. 6B) was characterized by the AlphaScreen assay. Fluorescence polarization (FP) assay is another method to test PB2 cap-binding inhibitors [86]. As illustrated in Fig. 7B, upon excitation by polarized light, the small fluorescent molecule rotates fast when it is free in solution and the emitted light is largely depolarized. When binding to a protein, the rotational movement of the whole complex becomes slow and, thus, the emitted light becomes polarized. Therefore, the protein binding activity can be monitored by the degree of light polarization. As a rapid and quantitative method, FP was applied in high-throughput screening to identify PB2 cap-binding inhibitors [86]. One hit compound, designated **PB2–39**, was found to inhibit multiple subtypes of influenza A viruses both *in vitro* and *in vivo* [86]. In the bead-binding assay (Fig. 7C), 7-methyl GTP is immobilized to agarose beads through a C10 linker. The amount of PB2 bound to the beads in the presence and absence of inhibitors can be quantified by western blot [88].

SPR and ITC are standard *in vitro* assays which are routinely applied to characterize small molecule binding to biological targets. ITC measures the heat released upon a small molecule interacting with a protein. Fitting the titration curve gives detailed information regarding the enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) changes for a binding event between a given small molecule and a protein. Such information is very useful in guiding the following structure-activity relationship studies. Furthermore, it also provides the binding stoichiometry, which is important for understanding the mechanism of action. For SPR, the PB2 cap-binding domain was immobilized on a carboxymethylated dextran surface. When a small molecule interacts with PB2, the overall mass increases, which is detected through the resonance. The SPR assay was applied to characterize the binding between compound **PB2–39** and the PB2 cap-binding domain [86].

In summary, a number of assays have been established for PB2 cap-binding domain. In comparison, the AlphaScreen and fluorescence polarization assays are amenable for high

throughput screening, while bead-binding assay, SPR, and ITC are more suitable as secondary assays.

3.4. Drug discovery and assay development for inhibitors targeting influenza virus polymerase subunit protein-protein interactions (PPIs)

PPIs play pivotal roles in the formation of influenza virus polymerase complex. Due to the critical function and the high conservation of protein sequence and structure among the influenza polymerase subunits (PA, PB1, and PB2) [89–93], interfering the polymerase subunit interactions could affect polymerase activity and viral replication. As elucidated by the X-ray crystal structures of the influenza virus polymerase complex [18, 94], PA interacts extensively with PB1 with a total buried surface area of 17,330 Å². In contrast, PA only interacts marginally with PB2, and the total buried surface area between PA and PB2 is only 2,880 Å². The PB1 subunit also interact with the PB2 subunit with a total buried surface area of 14,100 Å². The large binding interfaces between PA-PB1 and PB1-PB2 render them attractive targets for developing new antiviral compounds against influenza viruses [93, 95, 96].

3.4.1 Drug discovery targeting PA-PB1 interactions—Among the drugs in development targeting influenza virus polymerase subunit interactions, PA-PB1 inhibitors were the first-in-class and most extensively developed [96]. The PB1-binding pocket in PA mediates the interactions between PB1 (1–15) peptide and the PA C-terminal domain. Using structure-based virtual screening, a number of hit compounds were prioritized based on the docking score and were subsequently tested for inhibiting PA-PB1 interactions [97]. Compounds **10** and **11** were confirmed to inhibit PA-PB1 interactions in ELISA assay. Compound **10** had antiviral activity against multiple influenza A and B viruses with EC₅₀ values ranging from 12.2 μM to 22.5 μM, while compound **11** was much less potent and was only active against the A/California/7/9 and the A/Parma/24/09 strains with EC₅₀ values of 75.5 μM and 82.2 μM, respectively. Nevertheless, subsequent lead optimization based on the hit compound **11** led to compound **12** with improved antiviral activity [98]. Similarly, high-throughput docking study conducted by Botta et al identified two series of PA-PB1 inhibitors with novel chemotypes, namely 3-cyano-4,6-diphenyl-pyridine **13** and nitrobenzofurazan **15**. [99, 100] Following lead optimization of the 3-cyano-4,6-diphenyl-pyridine **13** yielded compound **14** with an EC₅₀ value of 3.5 μM against the A/PR/8/34 strain. [101] However the broad-spectrum antiviral activity and in vivo antiviral activity of these PA-PB1 inhibitors have not been reported.

Allosteric inhibitors of PA-PB1 interactions targeting a site outside of the PB1(1–15)-binding pocket were also discovered. **PAC-3** was shown to have potent inhibition of the PA-PB1 interaction in ELISA assay (IC₅₀ = 8.5 ± 2.2 μM) as well as potent antiviral activity (EC₅₀ = 0.80 ± 0.12 μM against A/HK/415742/09(H1N1)). A structural analog of **PAC-3**, **ANA-1**, was selected for further characterization of the antiviral spectrum and *in vivo* activity. It was found that **ANA-1** inhibits multiple subtypes of influenza A viruses with submicromolar efficacy in in vitro antiviral assay. In addition, **ANA-1** also significantly improved the survival rate and body weight loss for mice that were infected with lethal dose of A/HK/415742Md/09 H1N1 virus. This is the first demonstration of the *in vivo* efficacy of

PA-PB1 inhibitors. Molecular docking predicted that **ANA-1** binds to a site distinct from the PB1(1–15) binding pocket, however no further experimental data was provided to support this hypothesis.

3.4.2. Drug discovery targeting PB1-PB2 interactions—Although PB1 also forms extensive interactions with PB2 with a total buried surface area of 14,100 Å², as of date, there has only been one study reporting the discovery of small molecule inhibitors targeting PB1-PB2 interactions [103]. Using a similar approach as the discovery of **PAC-3**, Zheng et al identified two small molecules, **PP4** and **PP7**, that had potent inhibition of the PB1-PB2 interactions as well as potent antiviral activity [104]. However, unlike **PAC-3** which had broad-spectrum antiviral activity against multiple subtypes of influenza A viruses, **PP7** showed subtype- selective antiviral activity against the influenza A strains tested. The lack of broad-spectrum antiviral activity might be due to the fact that PP7 binds a site which is not highly conserved among different influenza A strains.

Although protein-protein interactions are traditionally challenging drug targets, the success of targeting PA-PB1 and PB1-PB2 interactions with the aforementioned small molecules shed light on the promise of targeting these highly conserved protein binding interfaces.

3.4.3. Assays for screening and characterizing inhibitors targeting PA-PB1 or PB1-PB2 interactions—Up to date, a number of assays had been developed and employed for studying influenza viral PPIs as well as for screening of PPIs inhibitors.

Among the reported assays, two-hybrid system in mammalian cell [105–107], co-immunoprecipitation [108, 109], pull-down assay [97, 110], BIFC [111], Split-Luciferase [112], and ELISA were most frequently used [97, 104, 113]. Among them, pull-down assay and co-Immunoprecipitation (Co-IP) assay are classical methods for detecting PPI and have very similar principles. Basically, a target protein (usually with a tag) is extracted from a mixture (e.g., cell lysate) via its affinity to another protein which is immobilized on a solid support (such as Ni-Nat beads or Glutathione Sepharose, depending on the tag used). After washing, bound proteins are eluted from the support and analyzed using western blot (Fig. 10A). When antibody is employed to bind the target protein, pull-down assay is termed as Co-IP. It should be noted that pull-down and Co-IP assays are robust methods for detecting strong interactions, but they may not be effective in detecting weak or transient protein-protein interactions. The reason for this is that both assays require cellular disruption and stringent purification steps which might perturb weak or transient protein-protein interactions.

ELISA is an established assay for protein-protein interactions and has been applied to screen inhibitors PA-PB1 and PB1-PB2 interactions [97, 104, 113]. A target protein is immobilized onto a microtiter plate, then a second binding protein was added (Fig. 10B). After blocking, incubation and washing, bound protein is detected using the protein-specific antibody conjugated with a reporter enzyme and quantified by measurement of the reporter enzyme activity. Although ELISA usually requires purified proteins, it is generally more accurate, sensitive, and specific than other immunoassays, and is suitable for high-throughput screening.

Two split-reporter systems, the bimolecular fluorescence complementation (BiFC) and split luciferase complementation assay (SLCA), which are capable of detecting protein-protein interaction in their native location, have been applied for detection of PA-PB1 and PA-PB2 interactions and screening for inhibitors [111, 112, 114]. Both BiFC and SLCA are based on the similar principle that each half of the split reporter protein is intrinsically nonfunctional on its own (e.g. no fluorescence or luminescence), however, a functional reporter protein will be formed if two halves are brought into close proximity via the interactions between the two conjugated proteins [115] (Fig. 10C). Thus, the specific protein-protein interactions can be detected quantitatively via measurement of fluorescence or luminescence. Both BiFC and SLCA have their own advantages and disadvantages. For instance, BiFC can be used to visualize the interaction within live cells [111], but it has the background interference due to the requirement of external excitation light to generate fluorescence. In contrary, SLCA offers much lower background and more sensitive readouts, thus, SLAC is a more robust method for high-throughput screening than BiFC [112]. Nevertheless, SLAC needs an exogenous substrate and is not suitable for imaging at subcellular resolution.

In addition to the above mentioned assay to detect PPI, a cell-based protein localization assay for indirect detection of PA-PB1 interaction was also reported [97]. As PA lacks the nuclear-localization signal sequence, when it is expressed alone PA locates in the cytosol. However, when co-expressed with PB, PA and PB1 forms the PA-PB1 complex and gets translocated into nucleus. Therefore, the inhibition of PA-PB1 interaction can be detected by visualization of PA localization. For this purpose, PA needs to be conjugated with a tag, which is usually a fluorescence protein such as GFP.

4. SUMMARY AND OUTLOOK

The influenza virus polymerase lacks any proof-reading function, and as a result, drug resistance can emerge either under drug selection pressure or by random mutation. Although the genetic variation of influenza viruses is advantageous for the virus survival and evolution, it creates a grand challenging in developing effective therapeutics against influenza virus infection. In response to the need of the next-generation of antiviral drugs with novel mechanisms of action and a high genetic barrier to drug resistance, influenza virus polymerase-targeting inhibitors stand out as promising drug candidates. Recent ground-breaking advancements in structure biology and mechanism of influenza polymerase complex significantly enhanced our knowledge of influenza polymerase-mediated viral replication. As a result, rational drug design targeting the influenza polymerase complex becomes a reality. Inhibitors targeting either individual polymerase subunits or the interactions between different subunits have been designed and were shown to possess potent antiviral activity both *in vitro* and *in vivo*. The expeditious drug discovery progress is undoubtedly facilitated by the innovative assays for the screening and characterizing polymerase inhibitors. It is expected that such assays can be similarly applied to other drug targets. Collectively, these efforts yield three polymerase inhibitors that are currently at late stages of human clinical trials. It is highly promising that influenza polymerase inhibitors will become the next-generation of influenza antivirals.

In summary, the therapeutic advantages of influenza virus polymerase inhibitors include: 1) no cross-resistance with existing influenza antivirals due to their novel mechanism of action; 2) high-genetic barrier to drug resistance; 3) broad-spectrum antiviral activity against different types and subtypes of influenza A and B viruses. When approved, the polymerase inhibitors can be used either alone to treat multidrug-resistant influenza viruses or used in combination with NA inhibitors to delay the evolution of drug resistance.

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Fig. 1.

Influenza virus replication cycle. The whole cycle of viral replication consists of five steps: entry, replication, translation, assembly, and release. In the entry step, influenza virus enters host cell through receptor-mediated endocytosis. In the replication step, viral RNA polymerase (PA, PB1, PB2) mediate both viral RNA transcription and replication. For viral transcription, vRNA is transcribed into 5'-capped mRNAs by viral RNA polymerase. During replication, viral RNAs (vRNAs) are first copied into complementary RNAs (cRNAs), which are sequentially used as templates for the production of vRNAs. In the translation step, the mRNAs exported from the cytoplasm are translated into viral proteins by cellular ribosomes. Newly translated proteins are transported to the nucleus (PA, PB1, PB2, NP, M1) or the plasma membrane (HA, NA, M2). In the assembly step, progeny viral RNPs, HA, NA, M2 and M1 start to bud from the cell membrane. In the release step, mature virions are cleaved from the cell surface through NA-mediated hydrolysis. HA: Hemagglutinin; M1: Matrix

protein 1; M2: Matrix 2; NA: Neuraminidase; NEP: Nuclear export protein; PB1, polymerase basic 1; PB2, polymerase basic 2; PA, polymerase acidic; NP, nucleoprotein; RNP: Ribonucleoprotein.

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Fig. 2.
X-ray crystal structures of the viral polymerase complexes from the bat influenza A/H17N10 virus and the human B/Memphis/13/03.

Fig. 3.

Influenza virus mini-genome assay. In a classic mini-genome assay, cells are transfected with a plasmid to generate virus-like RNA, in which firefly luciferase is in the reverse orientation and flanked with the 5' and 3' non-coding regions of one of the viral RNA segments on each side to mimic a viral RNA segment. When co-expressed with viral polymerase machinery (PB1, PB2, PA and NP) translated from four plasmids individually, viral polymerase recognizes the non-coding regions on the reporter transcript and initiates both replication and mRNA transcription, resulting in expression of the firefly luciferase, whose activity can be quantified using commercial substrates. A plasmid encoding Renilla luciferase is co-transfected and used to normalize variations in transfection efficiency.

Fig. 4.

Inhibitors targeting the influenza PA endonuclease domain. (A). X-ray crystal structure of influenza A virus PA endonuclease domain (PDB: 5DES).[69] (B) X-ray crystal structure of PA endonuclease domain in complex with **L-742001** (PDB: 5CGV).[69] (C) X-ray crystal structure of PA endonuclease domain in complex with N-acylhydrazones **2** (PDB: 5EGA). [74] (D) Chemical structure of representative PA endonuclease inhibitors. The common pharmacophore of **RO-7** and **dolutegravir** is highlighted in red.

Fig. 5.

Assays for the screening and characterizing PA endonuclease inhibitors. (A) Gel-based endonuclease assay. Single-stranded DNA plasmid, such as M13mp18, was incubated with PAN. After the reaction was stopped, products were separated by agarose gel electrophoresis. Left lane: DNA molecular maker; Middle lane, M13mp18 only; Right lane, M13mp18 plus PAN. (B) FRET-based endonuclease assay. Briefly, the template, a single-strand DNA oligonucleotide, is dual labeled with a donor fluorescence (Such as FAM) and a quencher (such as TAMRA) on its 5' and 3' terminals, respectively. After incubation with PAN, the increase of donor fluorescence signal due to the release of quencher by endonuclease cleavage is quantified. (C) Fluorescence polarization assay for PA endonuclease inhibitors. Compound **3**, a conjugate structure of **L-742001** with fluorescein, is used as the fluorescent probe.

Fig. 6. Inhibitors targeting the PB2 cap-binding domain. (A) Crystal structure of influenza A H5N1 PB2 cap-binding domain with bound 7-methyl GTP (PDB: 4CB4) [82]. (B) Chemical structures of 7-methyl GTP and PB2 cap-binding inhibitors.

Fig. 7.

Assays for screening and characterizing PB2 cap-binding inhibitors. (A) AlphaScreen assay. Biotin-EDA-m⁷-GTP and His-tagged PB2 protein are used as binding partners. (B) Fluorescence polarization assay. FITC-labelled 7-methyl GTP is used as a fluorescent probe. (C) Bead-binding assay. Briefly, PB2_{cap} was incubated with 7-methyl GTP-immobilized agarose. After intensive washing, PB2_{cap} was eluted from agarose beads and further analyzed by SDS- PAGE and Coomassie blue staining.

Fig. 8. Structure and inhibition of influenza polymerase PA-PB1 subunit interactions. (A). X-ray crystal structure of PA-C-terminal domain in complex with the PB1-N-terminal domain (PDB: 3CM8) [102]. PA is colored in gray and PB1 is colored in yellow. (B). Structures of PA-PB1 inhibitors reported in the literature.

PP4

PP7

Fig. 9.
Chemical structure of PB1-PB2 inhibitors.

Fig. 10.

Assays for screening and characterizing PPIs of PA-PB1 or PB1-PB2. (A) Cartoon representation of the pull-down assay. (B) Cartoon representation of the ELISA assay. (C) Cartoon representation of the split-reporter system. (D) PA nuclear localization assay. In this assay, cells are transiently transfected with plasmid(s) expressing PA-GFP only (left) or co-expressing PA-GFP and PB1 (right). After 24 h for protein expression, cells were fixed and the localization of PA-GFP was examined using confocal microscope (unpublished data).

Table 1.

Representative influenza viruses that have been circulating among humans in recent years (2009–2017) in the Northern Hemisphere.

Influenza A viruses	Influenza B viruses
A/California/7/2009 (H1N1)pdm09-like viruses	B/Brisbane/60/2008 (Victoria)-like viruses
A/Hong Kong/4801/2014 (H3N2)-like viruses	B/Phuket/60/2008 (Yamagata)-like viruses
A/Texas/50/2012 (H3N2)-like viruses	B/Massachusetts/2/2012 (Yamagata)-like viruses
A/Switzerland/9715293/2013 (H3N2)-like viruses	B/Wisconsin/1/2010 (Yamagata)-like viruses
A/Victoria/361/2011 (H3N2)-like viruses	

Table 2.

Influenza antivirals that are approved by FDA or at the late stages of clinical trials.

	Chemical Structure	Mechanism of Action	Spectrum of antiviral activity	Administration Route
Commercial available influenza antiviral	Amantadine (Symmetrel) Rimantadine (Flumadine)	Inhibits the M2 proton channel and prevents viral uncoating	Influenza A viruses	Amantadine: oral Rimantadine: oral
	Oseltamivir (Tamiflu) Zanamivir (Relenza)	Inhibits viral neuraminidase and prevents the viral egress	Influenza A and B viruses	Oseltamivir: oral Zanamivir: inhalation Peramivir: i.v. Laninamivir octanoate: inhalation
	Peramivir (Rapivab) Laninamivir octanoate (CS-8958)
	Arbidol	Inhibits viral fusion by targeting the viral hemagglutinin	Influenza A viruses with group 1 hemagglutinin	Oral
Influenza antivirals that are at the late stages of clinical trials	DAS-181	Destroys the cellular receptors of viral entry by cleaving the Neu5Ac $\alpha(2,3)$ - and Neu5Ac $\alpha(2,6)$ -Gal linkages of sialic acid (host-targeting antiviral)	Influenza A and B viruses	DAS181: oral, inhalation
	Nitazoxanide	Impairs hemagglutinin maturation	Influenza A and B viruses	Oral
	JNJ-63623872 (VX-787)	Inhibits viral polymerase PB2 subunit	Influenza A viruses	JNJ-63623872 (VX-787): oral
	Favipiravir (T-705)	Incorporates in viral RNA and increases mutation rates	Influenza A and B viruses	Favipiravir (T-705): oral
	S-033188 (structure undisclosed)	Inhibits viral polymerase PA subunit cap-dependent endonuclease activity	Influenza A and B viruses	S-033188: oral