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REVIEW

Novel antiviral drug discovery strategies to tackle drug-resistant mutants of influenza virus strains

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ABSTRACT

Introduction: The emergence of drug-resistant influenza virus strains highlights the need for new antiviral therapeutics to combat future pandemic outbreaks as well as continuing seasonal cycles of influenza.

Areas covered: This review summarizes the mechanisms of current FDA-approved anti-influenza drugs and patterns of resistance to those drugs. It also discusses potential novel targets for broad-spectrum antiviral drugs and recent progress in novel drug design to overcome drug resistance in influenza.

Expert opinion: Using the available structural information about drug-binding pockets, research is currently underway to identify molecular interactions that can be exploited to generate new antiviral drugs. Despite continued efforts, antivirals targeting viral surface proteins like HA, NA, and M2, are all susceptible to developing resistance. Structural information on the internal viral polymerase complex (PB1, PB2, and PA) provides a new avenue for influenza drug discovery. Host factors, either at the initial step of viral infection or at the later step of nuclear trafficking of viral RNP complex, are being actively pursued to generate novel drugs with new modes of action, without resulting in drug resistance.

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Influenza virus; antiviral; drug resistance; antiviral target; drug design

1. Introduction

Influenza viruses continue to change their antigenicity by successfully evading the host immunity to infections. The antigenic drift caused by genetic mutations in the influenza viral genome not only leads to the emergence of drug-resistant strains [1–3] but also evades antibody-mediated viral neutralization [4,5]. Although antivirals and vaccines are available for influenza A and B viruses, 300,000 to 500,000 individuals die of influenza infections every year worldwide [6,7]. Influenza viruses are classified based on their antigenic differences in the viral nucleoprotein (NP) and matrix protein 1 (M1). Based on the serological reactivity of their antigenic surface proteins, hemagglutinin (HA) and neuraminidase (NA), Influenza A viruses (IAVs) are further divided into 18 HA subtypes and 11 NA subtypes among the IAVs [8]. Frequent antigenic drift due to mutations is responsible for annual epidemics, whereas occasional antigenic shifts (genetic reassortments along with secondary modification, e.g., glycosylation of HA and NA) result in major pandemics such as the Spanish flu (1918), Asian flu (1957), Hong Kong flu (1968), and the most recent 2009 H1N1 pandemic [9]. The constant threat posed by influenza viruses to humans has led to continued efforts over the years to develop and deploy more effective antivirals [10] and broadly protective vaccines [11].

This present article is meant to review the strategies for designing novel antiviral agents to overcome drug resistance. Briefly, the replication cycle of influenza virus infection and various types of inhibitors targeting each step of infection are summarized in Figure 1. Currently, there are two classes of US Food and Drug Administration (FDA)-approved antivirals for influenza: M2 ion-channel blockers (amantadine and rimantadine, Figure 2(d)) [12,13], and neuraminidase (NA) inhibitors (zanamivir, oseltamivir, and peramivir, Figure 3(d)) [14]. Because of the high level of resistance to M2 ion-channel blockers among circulating IAVs, the Centers for Disease Control and Prevention (CDC) recommends against the clinical use of M2 inhibitors [15,16]. Moreover, a rapid increase in the emergence of mutants resistant to oseltamivir [17,18] highlights the urgent need for improved small molecule inhibitors against known targets, as well as novel inhibitors with new modes of action (MOAs) as shown in Figure 1. Understanding the mechanisms of action of the current anti-influenza drugs is a perquisite for designing novel antiviral agents to overcome drug resistance.

2. Antiviral drug designing and screening strategies

To accelerate the drug discovery process, against both novel targets and pre-existing targets with drug resistance problem, basic platforms for drug designing and screening are required. This section will briefly discuss each method in relation to anti-influenza drug discovery.

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Article highlights

- The emergence of NA inhibitor-resistant influenza viruses, as well as continued circulation of M2 ion channel blocker resistant mutant strains, necessitates the design of novel antivirals against both wild-type and drug-resistant viruses.
- Structure-based drug design and bioisosteric replacement strategies have made progress in the design of novel antivirals.
- Antivirals that target the conserved viral polymerase complex or proteins of host origin would reduce antiviral resistance.
- Combinational therapy with current FDA-approved drugs and drugs acting on novel targets of viral or host origin can be used not only to treat infection by drug-resistant mutants but also to lower the chance of eliciting novel drug resistance.
- Some host-targeting antivirals, along with the most conserved viral polymerases are currently in clinical trials and may provide options for tackling with drug-resistant influenza viruses

This box summarizes key points contained in the article.

2.1. Structure-based drug discovery

One of the most successful examples of pharmacophore-based drug discovery is zanamivir [19], which still receives great attention due to its exceptional antiviral activities against wild-type as well as oseltamivir-resistant variants. Pharmacophore modeling, featuring hydrophobic centroids, aromatic rings, hydrogen bond acceptors or donors, cations and anions, can be established either by structure-based modeling using pre-defined three-dimensional (3D) structure of target protein, or by ligand-based modeling based on active ligand information [10]. If the 3D structure is available, it is possible to combine pharmacophore features of potential ligands and the target protein, which is conducive in identifying hits that are more selective.

Recent advances in pharmacophore modeling and structural biology have led to the discovery of novel and potent influenza drugs [20–23], by examining the interactions of inhibitors and the protein in the enzyme-binding pocket [10]. Information on the binding pocket of a receptor for its ligand is very important for drug design, particularly for conducting mutagenesis studies [24]. In the literature, the binding pocket of a protein receptor is usually defined as those residues that have at least one heavy atom within a distance of 5Å from a heavy atom of the ligand. Such a criterion was originally used to define the binding pocket of ATP in the Cdk5-Nck5a* complex [25], which later proved quite useful in identifying the functional domains and stimulating the relevant truncation experiments. A similar approach has been used to define the binding pockets of many other receptor–ligand interactions important for drug design [26–30]. Inhibitors of influenza neuraminidase and PB2 cap binding are proven examples of

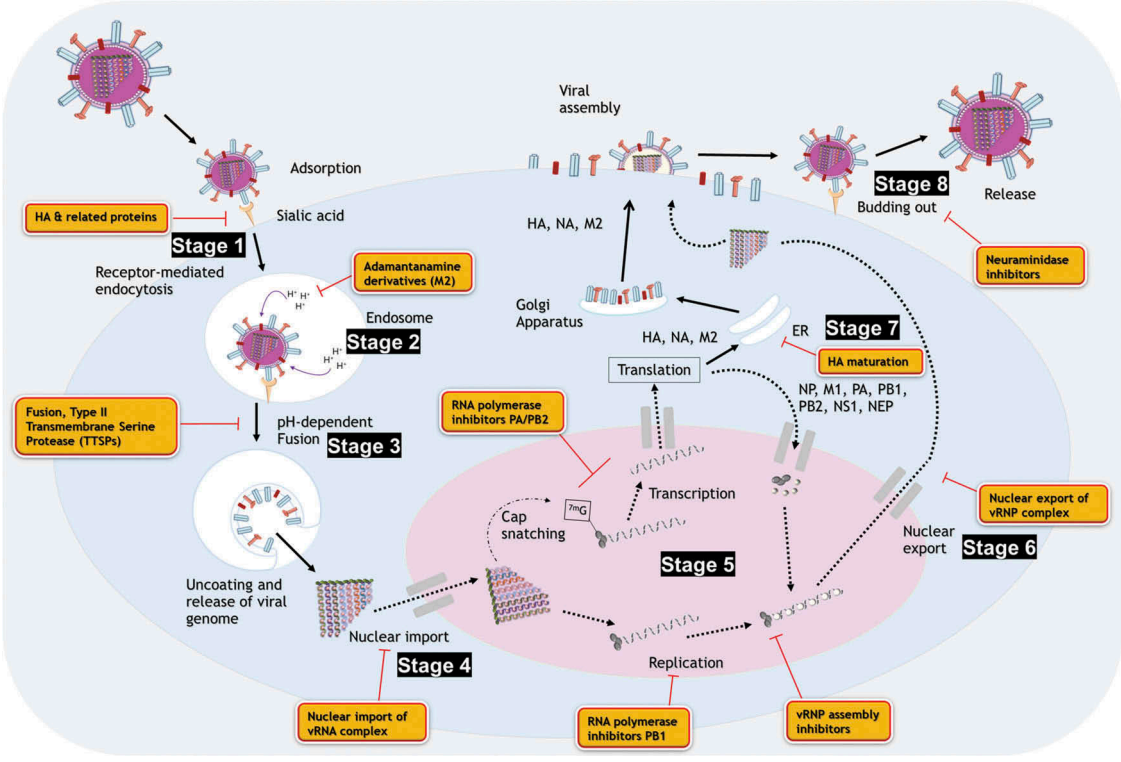


Figure 1. **Influenza virus life cycle and potential antiviral targets**. Influenza HA binds to the sialic acid of the host cell receptor, and the virus enters the cell via receptor-mediated endocytosis. Low pH in the endosome triggers a conformational change of the HA protein, leading to fusion of viral and endosomal membranes. vRNP complexes are then released into the cytoplasm and transported to the nucleus. In the nucleus, viral polymerase subunit PB2 captures the 5′ cap of host pre-mRNA. PA cleaves the pre-mRNA cap, which is then used as a primer for viral mRNA transcription. When there are enough translated PB2, PB1, PA, NP, M1, NS1, and NEP proteins in the nucleus, the viral polymerase stops transcription and initiates viral RNA replication. Newly synthesized vRNPs are transported to the cytoplasm with the aid of bound M1-NEP complex. The viral surface proteins HA and NA and matrix protein M1 are further processed in the endoplasmic reticulum (ER), glycosylated in the Golgi apparatus, and transported to the surface of the cellular membrane. vRNP complexes exported from the nucleus are then incorporated into new virions that bud out of the infected cells. When the newly formed virions interact with sialic acid at the cell surface, NA cleaves the bond between sialic acid and HA, releasing the virion from the infected cell. The newly generated virions infect neighboring cells to initiate the next cycle of infection. The stages of replication where the virus life cycle may be blocked are indicated in yellow boxes.

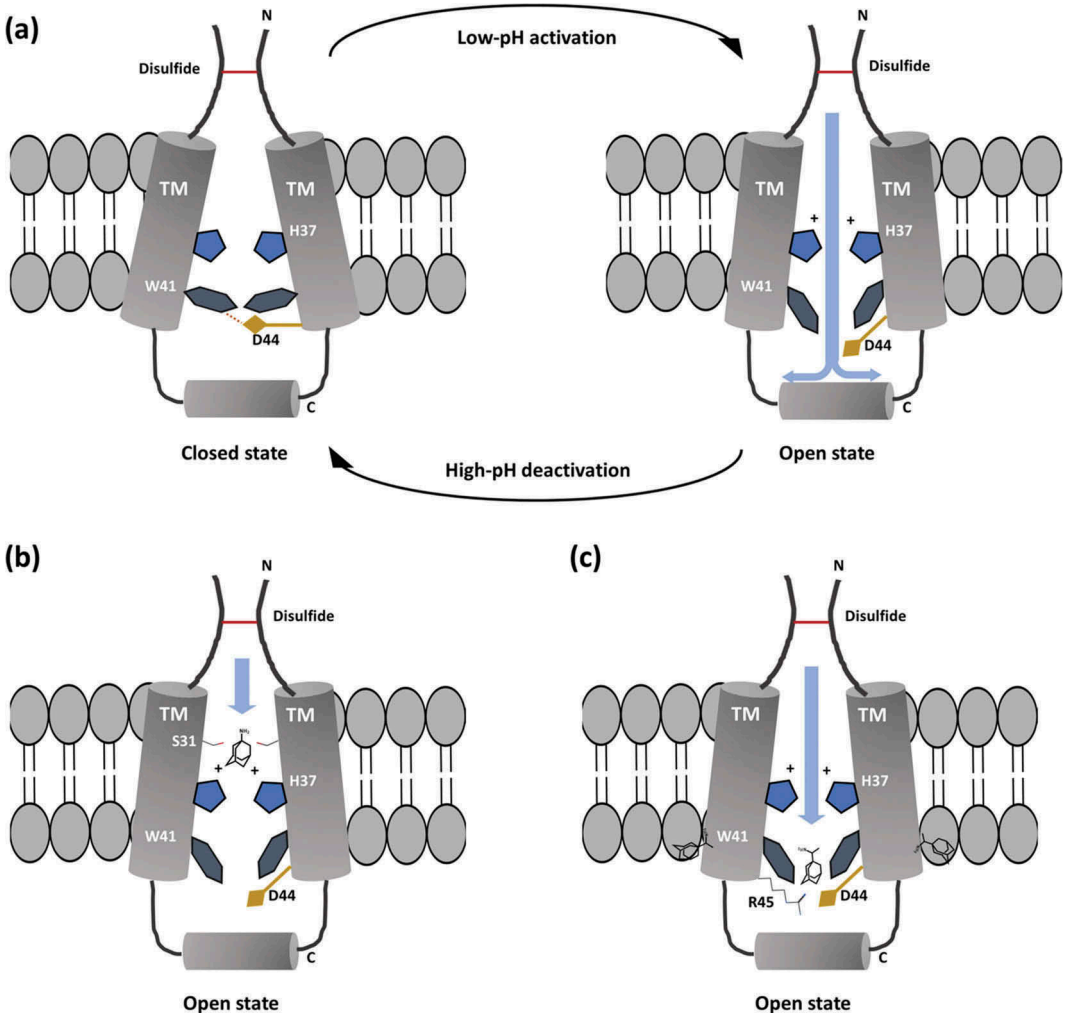


Figure 2. **Influenza M2 ion channel and mechanism of activation/inhibition**. Closed and open states of the M2 ion channel induced by low pH activation and high pH deactivation (a), Inhibition of the ion channel by amantadine (b) and rimantadine (c).

drug discovery using receptor–ligand interactions, which will be discussed later.

As mentioned earlier, knowledge on the receptor-ligand structures is vitally important in rational drug design. Although X-ray crystallography is a powerful tool in this regard, it is time-consuming and expensive, and not all protein receptors can be successfully crystallized. Recent breakthroughs indicate that NMR is indeed a very powerful tool in determining the 3D structures of, especially, membrane proteins [12,28], but it is also time-consuming and costly. To acquire the structural information in a timely manner, a series of 3D protein structures were developed using structural bioinformatics [24,29–32] and were found very useful for drug development.

2.2. High throughput screening

Although no influenza drug identified through high throughput screening (HTS) has been FDA approved, the intrinsic value of HTS should not be underestimated. In drug discovery against influenza virus, HTS can be performed with two approaches: i) biochemical assays on purified viral targets, and ii) cell-based assays. The first approach can be used when target-based drug discovery is preferred by the investigators. The main advantage of this approach is an easy adaptation into HTS format as exemplified in NA inhibition [23], PB2 cap-binding inhibition [33] or PA endonuclease inhibition assay [34]. The second approach has been used to evaluate both the compound mediated CPE inhibition and the cytotoxicity of the compound, usually in 384 well multiplate format [35]. This approach has been well optimized in several institutes, and the reagents for determining the endpoint cell viability, such as luciferase-based reagent kits, neutral red solutions, or MTT, are readily available from commercial sources [35–37]. It is worth noting that in both the assays, it is important to optimize the assay conditions to achieve a Z-factor (a screening window coefficient) value above 0.5, S/N (signal-to-noise ratio) value above 10 and S/B (signal to background) value over 5 [38] (formula for calculating Z-factor and S/N value is shown in Figure 4) to ensure the high quality of assay results.

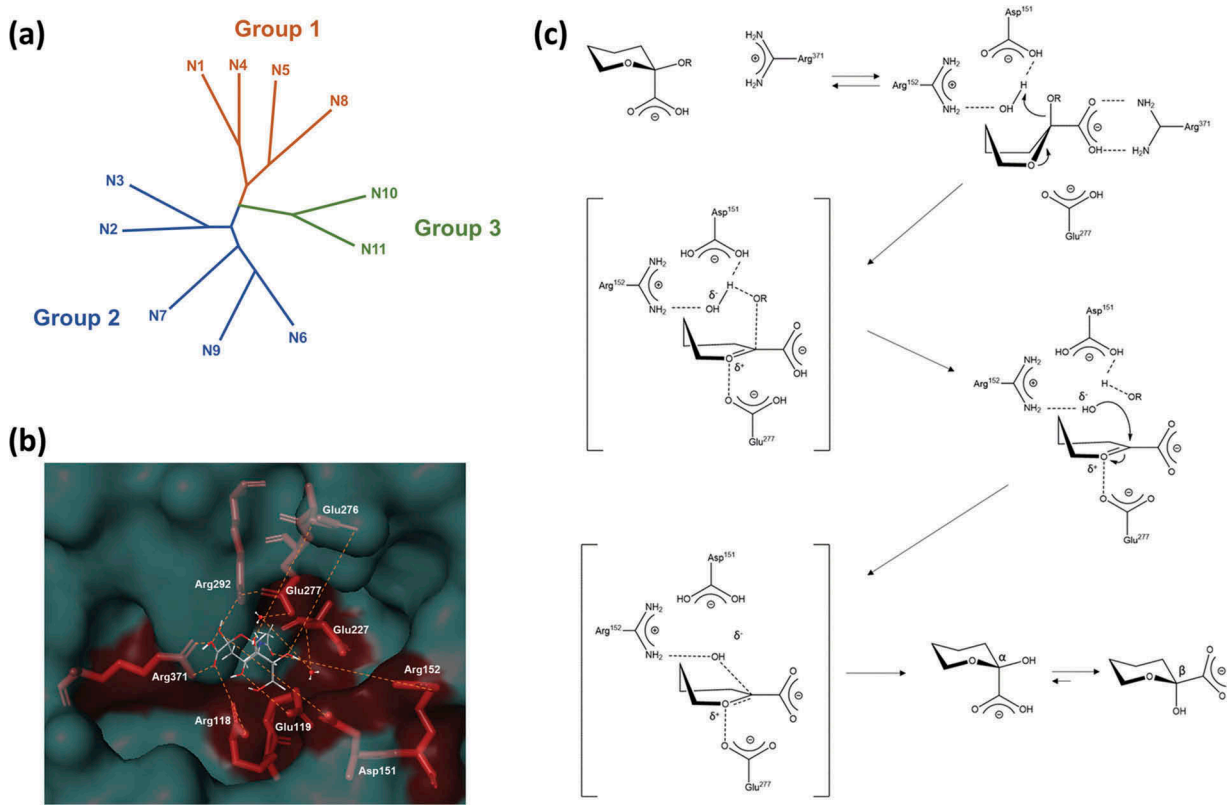


Figure 3. **Influenza neuraminidase and mechanism of activation**. Three groups of neuraminidases in influenza A viruses (a), interaction of sialic acid in neuraminidase enzyme pocket (b) and the chemical interaction of neuraminidase reaction (c).

Z' value

$$Z' = 1 - \frac{3\sigma_p + 3\sigma_n}{|\mu_p - \mu_n|}$$

μ_p: the mean of positive control

σ_p: the standard deviation of positive control

μ_n: the mean of negative control

σ_n: the standard deviation of negative control

S/N (signal-to-noise)

$$S/N = \frac{\mu_p - \mu_n}{((\sigma_p)^2 + (\sigma_n)^2)^{1/2}}$$

S/B (signal-to-background)

$$S/N = \frac{\mu_p}{\mu_c}$$

Figure 4. Formula for calculating Z' value, S/N and S/B value.

3. Targets of current FDA-approved drugs

3.1. *M2 ion channel – mechanism of inhibition*

The M2 ion channel is important in the initial phase of influenza virus infection. The channel is a homotetrameric single-pass type III integral membrane protein composed of four M2 units, each containing 97 amino acid residues [39]. After receptor-mediated endocytosis and subsequent endosome fusion, the M2 ion channel transports protons from the late endosome into the virion, leading to the acidification of the virion (Stage 2

in Figure 1). This weakens the interaction between M1 and the viral ribonucleoprotein (vRNP) complex so that the subsequent fusion of the viral and endosomal membrane releases the vRNP complex into the cytosol.

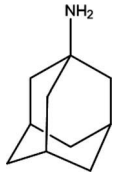
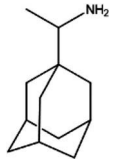
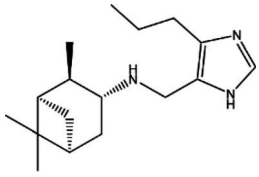
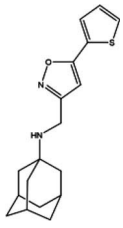
The influenza M2 ion channel is activated when the four histidine residues (His37 on each M2 unit) in the center of the channel, detect a low pH in the endosome, resulting in a proton flux into the virion. The His37 residues transfer protons across the channel by interacting with water molecules. When the pH of the virion is lowered, a tetrad of tryptophan residues near the C terminus of the channel (Trp41) closes the pore and blocks further proton flux through the channel (Figure 2(a)) [39]. The first antiviral drugs approved by the FDA for the treatment of IAV infection were adamantanes, amantadine and rimantadine (Table 1), which act by entering the barrel of the tetrameric ion channel and blocking proton translocation (Figure 2(b),c). Adamantanes work only on IAVs because of the differences in the ion-channels of IAV and influenza B virus (IBV).

3.1.1. Drug development targeting M2 mutant viruses

The emergence of drug-resistant IAV strains has rendered adamantanes largely ineffective. The major mutations responsible for the resistance are L26F, V27A, and S31N [40], all of which are located in the transmembrane region of M2. S31N is the most predominant of the resistance causing mutations and is reportedly present in over 95% of adamantane-resistant mutants. The CDC recommends against the clinical use of adamantanes because of the high global prevalence of adamantane-resistant IAVs [41].

However, there have been many efforts to develop amantadine derivatives that can inhibit both the wild-type viruses and the adamantane-resistant mutants [41] (summarized in Table 1). In 2012, Hu et al. reported that pinanamine derivatives effectively inhibit wild-type IAVs and partially resistant mutants [42]. By extensively studying the structure-activity relationships, they identified compound 33, which was active against both amantadine-sensitive viruses and amantadine-resistant viruses. This compound inhibited amantadine-sensitive viruses by blocking the function of wild-type A/M2, but it did not have the same effect on S31N mutant A/M2 in amantadine-resistant viruses, suggesting that it inhibits the mutant A/M2 viruses by a different mechanism that is yet to be elucidated. DeGrado et al. discovered an arylmethyl amantadine derivative (named M2WJ332), with high affinity for the S31N mutant that had a 50% effective concentration (EC_{50}) of 153 nM against S31N mutant viruses [43].

Table 1. Antiviral compounds targeting M2 ion-channel.

Name	Chemical structure	IC_{50}	Reference
Amantadine		0.22μg/ml*	36
Rimantadine		0.03μg/ml*	16
Compound 33		2.5 ~ 3.4μM	42
M2WJ332		153nM	43

*Against wild-type

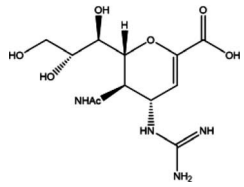
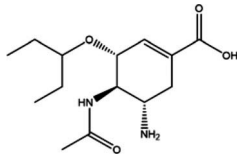
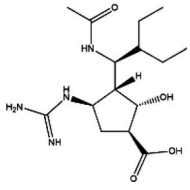
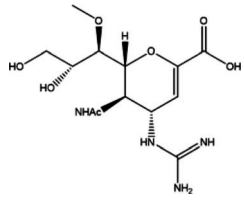
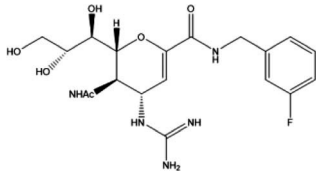
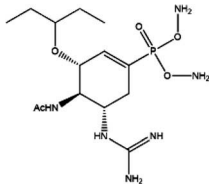
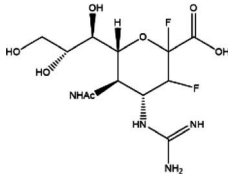
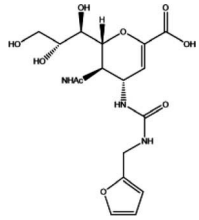
3.2. Neuraminidase (NA)

NA is a type II transmembrane protein and the second major glycoprotein of influenza viruses [44]. IAV has 11 NA subtypes that fall into three groups (Figure 3(a)). The main function of NA is to cleave sialic acid, which effectively abolishes the interaction between sialic acid and HA during the budding process of newly formed virions from the infected cells at a later step of influenza infection cycle (Stage 8 in Figure 1). Because of its important function in the virus life cycle, NA is a prominent target for influenza drug discovery.

3.2.1. Mechanism of NA inhibition and resistance

The active site pocket of influenza NA and its catalytic mechanism is described in Figure 2(b,c). The key amino acids (Asp151, Arg152, Glu277, and Arg371) that mediate the cleavage of sialic acid from HA are conserved among all influenza virus subtypes [45]. In 1993, Itzstein et al. discovered zanamivir (Table 2) [19], a potent NA inhibitor, using the crystal structure of NA, which was solved by Colman et al. [46]. Using computational chemistry techniques, they replaced the C4 hydroxyl group with a guanidine group, which increased the binding activity in the enzyme pocket by 300 fold, due to the formation of a salt bridge with the conserved Glu119 in the enzyme pocket and also a charge–charge interaction with Glu227 in the active site. Detailed information on the sialic acid binding pocket was further exploited by Kim et al. to design oseltamivir, another drug that would receive FDA approval [47]. To improve the poor oral bioavailability of zanamivir, they used a carbocyclic template and replaced a carboxylic acid group in zanamivir with an ethyl ester group, allowing the compound to work as a prodrug (oseltamivir phosphate). They also replaced a glycerol moiety with a lipophilic pentyloxy side chain based on the observation that the C7 hydroxyl of the

Table 2. Antiviral compounds targeting neuraminidase.

Name	Chemical structure	IC_{50}	Reference
Zanamivir		5 ~ 14nM*	19
Oseltamivir		1nM*	47
Peramivir		0.1 ~ 1.4nM*	49
Laninamivir		2.49 ~ 18.9nM	50
Compound 9f		1 ~ 90nM	23
Compound 13b		7.39 ~ 19.5nM	64
FeqGuDFSA		1 ~ 10nM	65
Compound D5		-	66

*Against wild-type

lipophilic side chain does not interact with the NA active site and as a result induces a repositioning of Glu276, which allows for optimal hydrophobic interactions. A carboxylate form of oseltamivir (Table 2) efficiently inhibited IAVs and IBVs [26]. Most notably, the pharmacokinetic properties were also greatly improved, making oseltamivir effective as an oral medication, as compared with zanamivir which is only effective as an inhalant [48]. Shortly after the discovery of oseltamivir, structure-activity analyses using the three-dimensional structure of influenza NA led to the identification of peramivir, a cyclopentane NA inhibitor, with EC_{50} values similar to those of oseltamivir and zanamivir [49]. In addition, laninamivir, a zanamivir analog, was designed to have potent antiviral activity similar to that of zanamivir and also to be active against oseltamivir-resistant mutants [50]. The pro-drug form of laninamivir showed a long retention profile in mouse model [51], resulting in potent antiviral activity *in vivo*, and greatly increasing the survival after a lethal challenge [50].

Although the sialic acid-binding pocket is highly conserved among influenza viruses, slight structural differences among NA inhibitors gives rise to resistant mutants in a drug-specific manner. In case of oseltamivir, the predominant mutation H274Y blocks the hydrophobic interaction of the lipophilic pentyloxy side chain by inhibiting the conformational change of Glu276, thus reducing the binding affinity between the drug and NA [52]. A similar hydrophobic interaction occurs with peramivir [53,54], which loses NA-binding affinity due to the H274Y mutation. Detailed analysis of the fitness of H274Y mutant viruses revealed that there are additional compensatory mutations that allow the virus to grow either fully (R194G, E214D) or partially (L250P, F239Y) [55]. In contrast, the hydrophilic glycerol moiety of zanamivir interacts with Glu276 via hydrogen bonding, which does not require a conformational change, allowing this drug to retain its inhibitory activity against H274Y mutants. Unlike oseltamivir, zanamivir and laninamivir are not widely reported to generate resistant influenza strains. Whether oseltamivir is intrinsically more prone to antiviral resistance remains to be determined. One possibility is that the core structures of zanamivir and laninamivir are more similar to the natural substrate than oseltamivir and are therefore less likely to induce resistance. For instance, the oxygen

atom in sialic acid sugar six-membered ring is retained in zanamivir, whereas it is replaced by an 'unnatural' carbon atom in oseltamivir (Table 2). Another possibility is that zanamivir and laninamivir have not been used frequently enough for resistance to be observed at the clinical level. It is, however, possible that the influenza variants with reduced susceptibility to both oseltamivir and zanamivir are circulating, as reported recently [56–58].

3.2.2. Drug development targeting mutant viruses

To tackle drug resistance, efforts are underway to develop new generation of NA inhibitors, either by structure-based rational drug design or by bioisosteric replacement of moieties in the current NA inhibitors.

3.2.2.1. Structure-based drug design. As one of the most successful examples of rational drug design, NA inhibitors continue to attract interest with a view to overcome drug resistance. Recent studies have shown that group 1 NAs have a '150-cavity' in the substrate-binding pocket [59] that can be accessed by the larger side chain of either Neu5Ac2en or zanamivir derivatives. Several compounds that interact with the 150-cavity have proven to be effective inhibitors of both wild-type viruses and oseltamivir-resistant mutants [60]. Early studies indicate that this approach works only for group 1 NAs because group 2 NAs do not have the 150-cavity [59]. However, a recent study showed that oseltamivir carboxylate could induce closed 150-loop of N2 NA to partially open, implying the importance of considering ligand-induced flexibility of the 150-cavity [61]. Gao et al. showed that the H1N1 strain, which caused the 2009 pandemic, which is still circulating, does not have a 150-cavity [59]. However, von Itzstein et al. found that the compound 3-(p-tolyl)allyl-Neu5Ac2en, an inhibitor specific to group 1 NAs, lock opened the 150-loop [62], Gao et al. also observed a similar interaction between the inhibitor and the active site of NA of pH1N1 [63]. In addition to the 150-cavity, the '430-loop' [59,60] near the sialic acid-binding site was proposed as a potential target for new inhibitors of both group 1 and group 2 NAs. The flexible 430-loop in both the NA groups is located close to the C-1 residue of zanamivir when that drug binds with NA. Feng et al. synthesized zanamivir derivatives with C-1 and C-4 modifications. Among them, a C-1 modified derivative (Compound 9f, Table 2) bearing a 3-fluorobenzyl amine exerted the greatest potency, with IC_{50} values comparable to zanamivir [23]. The binding model of this compound with group 1 and group 2 NAs showed that the C-1 modified side chain projected towards the 430-cavity, demonstrating the potential of this cavity as a target for future drug discovery, targeting both wild-type viruses and oseltamivir-resistant mutants.

3.2.2.2. Bioisosteric replacement design. The most successful example of the bioisosteric replacement strategy for the discovery of novel and potent NA inhibitors may be laninamivir [50] (Table 2). Replacement of the hydroxy group with a methoxy group at the seventh carbon of zanamivir was well tolerated and did not compromise the anti-NA activity against wild-type viruses or oseltamivir-resistant mutants (H274Y and R292K). Moreover, the introduction of an acyl chain at the ninth carbon of R-125,489, a metabolite form of laninamivir, prolonged the *in vivo* efficacy of that drug in the mouse model. Continued efforts to modify the structures of NA inhibitors led to a number of potent inhibitors that are active against oseltamivir-resistant mutants. For example, one of the oseltamivir phosphonate derivatives, compound 13b (Table 2), was active against two strains of H274Y mutant, with IC_{50} values of 7.39 nM and 19.5 nM, respectively, compared with 295 nM and 971 nM, respectively, for oseltamivir [64]. This is due to a stronger interaction exerted by phosphonate, compared to carboxylate, with the guanidinium ion. It should be emphasized that all previous NA inhibitors are reversible in nature. Recently, however, an attractive strategy of irreversible NA inhibition was reported [65]. A zanamivir derivative developed using that strategy, FeqGuDFSA (Table 2), had more than 100 fold greater activity against oseltamivir and zanamivir-resistant mutants than oseltamivir and zanamivir, respectively, in an enzyme inhibition assay. Moreover, this compound had EC_{50} values ranging from 1 nM to 10 nM in plaque size reduction assays and resulted in 100% survival in a mouse infection model. This designing principle involves the incorporation of fluoride atom in the skeleton as an 'electron sink' that would trap and stop the electron relays required for a successful completion of the sialidase enzyme reaction into a 'dead end' product. Ye et al. reported another example of bioisosteric zanamivir modification [66] at the C-4 position of zanamivir and showed that a furan-2-ylmethyl derivative (compound D5 in Table 2) exerted anti-influenza activity as potent as zanamivir. Importantly, the pharmacokinetic study of this compound showed an increased plasma half-life ($t_{1/2}$), compared to that of zanamivir, following either intravenous or oral administration, in clear contrast to zanamivir, which is effective only by the inhalation route.

4. Novel targets for drug design

As summarized in Figure 1, the influenza virus replication cycle is complex, involving nuclear trafficking of viral polymerase complex and different modes of RNA synthesis. In contrast to previously described drugs targeting surface NA antigen, it is not surprising that novel antivirals are being developed targeting the internal, more conserved viral proteins.

4.1. Viral polymerase complex

4.1.1. Viral polymerase

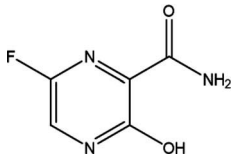
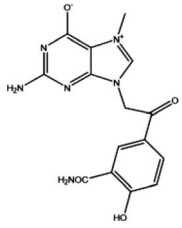
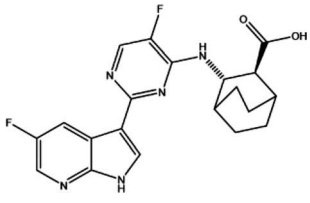
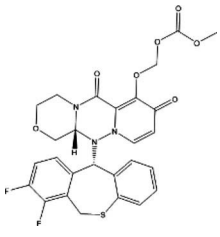
The influenza polymerase is composed of three viral proteins: PB1, PB2, and PA. As shown in Figure 1, the polymerase complex 'steals' the cap-structure from cellular mRNAs and utilizes it as a primer to initiate the transcription. In this unique cap-snatching process, PB2 binds to the cap of host pre-mRNAs, and PA cleaves at about 10–14 nucleotides from the N-terminal 5'-cap, via its endonuclease activity. Then, PB1, an RNA dependent RNA polymerase (RdRp), adds nucleotides successively to the cleaved cap primer using the vRNA as

a template [67,68]. The polymerase complex also carries out primer independent synthesis using a complementary RNA (cRNA) as a replicative intermediate. The dual action of RdRp provides a unique window for antiviral design.

The catalytic domain of RdRp is conserved among various RNA viruses and is an outstanding target for small molecule inhibitors. In 2002, Toyama Chemical Co. in Japan discovered Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide, Table 3), an antiviral drug that targets RdRp of RNA viruses (Stage 5-replication in Figure 1), which undergoes an intracellular phosphoribosylation to achieve an active form, favipiravir ribofuranosyl-5′-triphosphate [69,70]. This inhibitor is currently in the late phase of clinical trials and is effective against IAV, IBV as well as influenza C virus (ICV), having EC_{50} values between 0.063 and 22.5 μM.

The overall mechanism and the individual role of each polymerase protein have been established for the cap-dependent transcription (Stage 5-transcription in Figure 1), however, the detailed structures only became available recently for structure-based drug design [68,71–74]. Briefly, Cusack et al. first determined the X-ray structure of PB2 cap-binding domain [74] in 2008. They found that the Glu361 residue undergoes hydrogen bonding with the N1 and N2 positions of guanine of 5′ cap. Also, two highly conserved aromatic residues (His357 and Phe404) [75] form a strong π-stacking interaction with m^7-guanosine of the 5′-cap, similar to other well-characterized cap-binding proteins such as eIF4E [76]. Based on the structural information, compound 8e (Table 3), an m^7GTP analog that inhibited the cap-binding activity of PB2 with an IC_{50} of 0.6 μM was designed [33]. More recently, researchers from Vertex Pharmaceuticals discovered pimodivir, a novel azaindole inhibitor of PB2 [77]. This compound, although structurally different from m^7GTP, binds to the m^7GTP-binding pocket of PB2 and displayed potent antiviral activities against multiple IAVs including NA drug-resistant mutants. A preliminary study of the effects of selective pressure imposed by pimodivir exposure identified six PB2 variants (Q306H, S324I, S324N, S324R, F404Y, and N510T), which are rarely observed in naturally occurring human isolates. However, pimodivir is not effective against IBVs [22]. Despite the potential limitations, an ongoing endeavor found several azaindole analogs [78] with inhibitory activity in cell culture and *in vivo* mouse model [79].

Table 3. Antiviral compounds targeting viral polymerase complex.

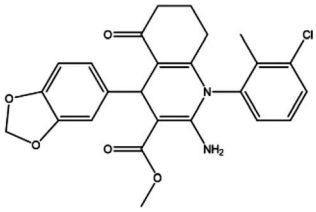
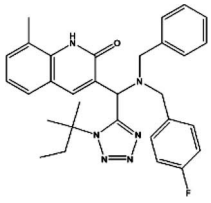
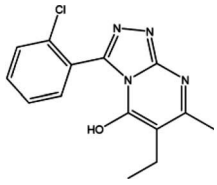
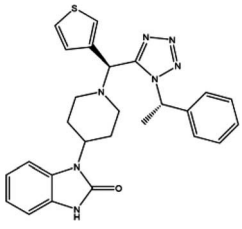
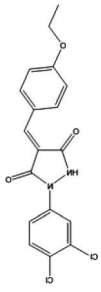
Name	Chemical structure	IC_{50}	Reference
Favipiravir		0.063 ~ 22.5μM	69
Compound 8e		0.6μM	33
Pimodivir		1.6nM	77
Baloxavir marboxil		0.46 ~ 6.48nM	20

The crystal structure of the influenza endonuclease at the N-terminus of PA (PA_N) was solved in 2009 by Cusack et al. [73], and Rao and Liu et al. [72]. The endonuclease active site core of PA_N coordinates with metal ions via the highly conserved catalytic residues His41, Glu80, Asp108, and Glu119, offering a promising target for novel drug discovery by chelating the metal essential for endonuclease activity. The influenza PA inhibitor Baloxavir marboxil, which was developed by Shionogi Co. (Table 3), was recently approved for clinical use in the US [80]. Baloxavir marboxil works on both IAV and IBV, with an EC_{90} of 0.46–0.98 nM and 2.21–6.48 nM, respectively. Moreover, this inhibitor showed significant antiviral activities against avian influenza H5N1 and H7N9 subtypes as well as oseltamivir-resistant strains [80]. A recent study showed that I38T substitution is a major drug-resistant marker for Baloxavir marboxil and not E119D substitution, which is known to be the major drug-resistant marker for previous influenza virus endonuclease inhibitors [81]. Co-crystal structures of endonucleases and Baloxavir marboxil show that Van der Walls contact is the factor that influences the activity of the drug [20]. This further demonstrates that the PA endonuclease can be an effective target for future drug discovery against NA drug-resistant strains.

4.1.2. Viral polymerase complex formation

The PB2, PB1, and PA proteins are all integral parts of the viral polymerase complex [82], and therefore, provide ample opportunities to develop novel antivirals interfering with the protein–protein interactions (Stage 5-vRNP assembly in Figure 1). The structures of the binding interfaces of PA-PB1 [83] and PB1-PB2 [84] subunits enabled the identification of potent influenza inhibitors that disrupt the interactions among polymerase complex subunits. Three α-helices of the N-terminal domain of PB1, interact with a pockets formed by four α-helices and two β-hairpin domains of the C-terminal domain of PA, providing an opportunity to identify novel inhibitors targeting protein–protein interactions (PPIs) [83]. The first PPI inhibitor (Compound 1, Table 4) against influenza virus, which disrupts the PB1-PA interactions, was discovered by Muratore et al. and found to be effective for both IAVs and IBVs [85]. Very recently, Watanabe et al. performed a structure-based virtual screening

Table 4. Antiviral compounds targeting viral polymerase subunit interaction.

Name	Chemical structure	IC_{50}	Reference
Compound 1		12.2 ± 2.6/ 22.5 ± 3.7	85
Compound PA-49		0.53 ~ 0.92μM	21
ANA-1		0.9 ~ 1.23μM	86
Compound 12a		0.6 ~ 2.7μM	87
Compound PP7		1.4μM	88

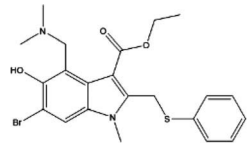
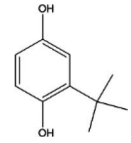
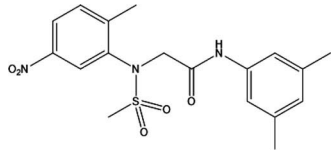
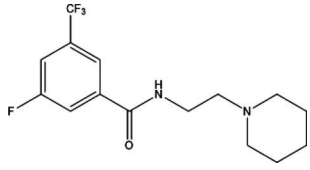
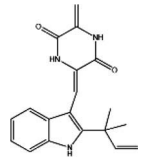
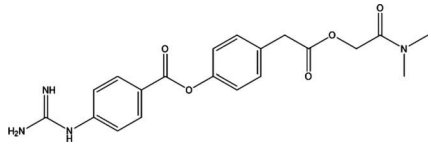
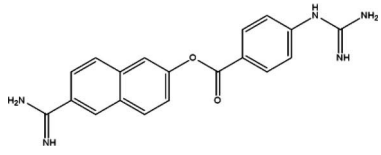
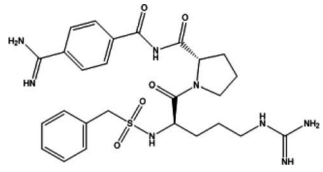
and identified a quinolinone derivative, compound PA-49, with a tetrazole moiety (Table 4) that is active against influenza viruses. Docking simulations suggest that the compound interrupts the PA-PB1-binding interface where most of the amino acids responsible for binding are conserved [21]. Moreover, the compound is active against both IAVs and IBVs with EC_{50} values in the submicromolar range and CC_{50} values > 100 μM. Yuan et al. discovered another PA-PB1 PPI inhibitor [86] by screening chemical libraries. Molecular docking simulations of the compound ANA-1 (Table 4), active against multiple IAVs, suggested multiple hydrogen bonds (Asp426, Glu427, Arg582, and Leu585) at the C-terminal domain of PA. Very recently, compound 12a (Table 4) was shown to be active against both IAVs and IBVs, including amantadine and oseltamivir-resistant mutants [87]. Of note, compound 12a did not show any sign of resistance even after 10 passages of the virus in the presence of increasing amount of the compound.

In addition to the PA-PB1 interaction, the PB1-PB2 interaction can also be a target for PPI inhibitors. The crystal structure of the PB1-PB2 interface revealed salt bridges between the three α-helices of PB1-C and helix 1 of PB2-N [84]. There are also non-polar contacts (e.g., Ile4 and Leu7), but those are hidden from the protein surface. Yuan et al. identified a small molecule that targets the PB1-PB2 interactions [88]. Optimization of this compound by chemical library screening led to the discovery of a PPI inhibitor (Compound PP7, Table 4) with an EC_{50} of 1.4 μM and a CC_{50} > 500 μM as tested by plaque reduction assay. The mode of action of PP7 was verified by *in vitro* ELISA, by detecting the interference of binding of full-length PB1 and N-terminal PB2 recombinant proteins, and mini replicon assay.

4.2. Hemagglutinin (HA) mediated viral entry and HA maturation

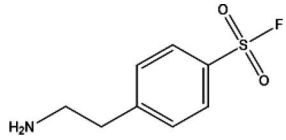
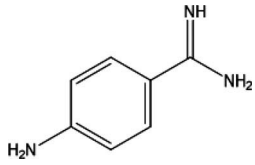
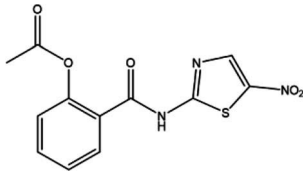
Due to high propensity of mutations leading to resistance, HA has long been dissuaded as a target for developing antivirals. Moreover, frequent antigenic drift of IAVs causing 'vaccine mismatches' urges the development of 'universal' vaccines [89]. In Recent years, novel strategies targeting HA-dependent membrane fusion events are gaining attention (Stage 3 in Figure 1). Along with NA, HA is a major influenza surface glycoprotein, and exhibits two important functions; 1) it binds to the receptor sialic acid on host cells, and 2) triggers fusion between the viral and endosomal membranes, resulting in the release of the viral genome into the cytoplasm. HA is activated via proteolytic cleavage of the precursor protein HA0 to form HA1 and HA2, which are linked by disulfide bonds, which is an obvious step for antiviral intervention [90]. Viral replication can also be efficiently blocked by inhibiting HA induced membrane fusion. The first such agent was umifenovir (Table 5), an indole derivative developed by the Center for Drug Chemistry in Russia [91,92]. Leneva et al. investigated umifenovir-resistant influenza mutants and concluded that umifenovir inhibits influenza virus replication by blocking HA-mediated membrane fusion [93]. More recently, several fusion inhibitors were found with EC_{50} values in the micromolar range. Biochemical assays and examination of the co-crystal structure with HA showed that tert-butyl hydroquinone (TBHQ) [94] (Table 5) blocks the HA fusion activity [95]. Structural studies show that both umifenovir and TBHQ binds to the hydrophobic pocket at the interface between HA protomers [91,95]. Other examples of HA fusion inhibitors summarized in Table 4 are MBX2546 [96], CL-385,319 [97] and neoechinulin B [98] having EC_{50} values of 0.3, 27.03 and 27.4 μM, respectively. Recent studies have shown that type II transmembrane serine proteases (TTSPs) play a major role in viral entry by performing the proteolytic cleavage of HA0 into HA1 and HA2 (Stage 1 in Figure 1), the active form of HA that mediates the membrane fusion event [90]. TTSP inhibitors might provide a way to overcome current drug resistance

Table 5. Antiviral compounds targeting HA-mediated viral entry and HA maturation.

Name	Chemical structure	IC_{50}[a]	Reference
Umifenovir		0.59μM	91, 92
tert-butyl hydroquinone		0.6μM	95
MBX2546		0.3μM	96
CL-385,319		27.03μM	97
neoechinulin B		27.04μM	98
Camostat mesilate		-	99
Nafamostat mesilate		-	99
Benzylsulfonyl-d-arginine-proline-4-amidinobenzylamide		-	100

(*Continued*)

Table 5. (Continued).

Name	Chemical structure	IC_{50}[a]	Reference
4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride		-	101
p-aminobenzamidine		-	101
Nitazoxanide		0.9–3.2μM	102, 103

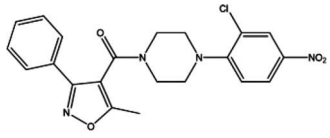
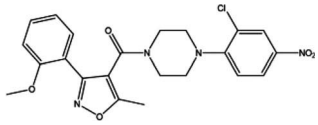
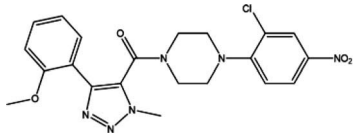
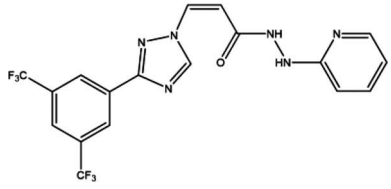
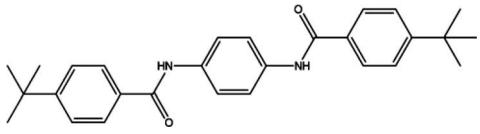
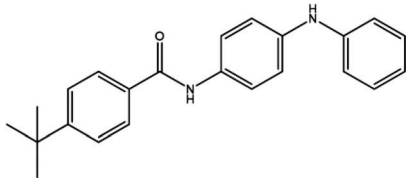
issues. Representative inhibitors for TTSPs are shown in Table 5, including camostat mesilate [99], nafamostat mesilate [99], benzylsulfonyl-d-arginine-proline-4-amidinobenzylamide [100], 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and p-aminobenzamidine [101]. A notable drug that is in phase 3 clinical trial is nitazoxanide (Table 5), which blocks the post-translational modification of HA (Stage 7 in Figure 1), active against influenza A viruses with EC_{50} values ranging 0.9 to 3.2 μM [102,103]. Furthermore, nitazoxanide showed synergistic effect when treated in combination with either zanamivir (CI = 0.3 to 0.48) or oseltamivir (CI = 0.18 to 0.31), suggesting that combination therapy regimen is another option for the control of influenza virus infections.

4.3. Nucleoprotein and viral ribonucleoprotein complex trafficking

Influenza virus genome replication and transcription occurs in the host cell nucleus, and therefore, the nuclear trafficking of RdRp provides an important avenue for the inhibition of viral replication as previously described in section 3.1. A major function of influenza NP involves the nuclear and cytoplasmic trafficking of vRNP complexes, which differs from the function of NP in other non-segmented or segmented (-) RNA viruses [104]. Importantly, NP is an integral part of the viral RNA complex (vRNPs) and provides a target independent of the polymerase complex (PB1, PB2, PA) (Section 3.1.1.) for anti-influenza drugs. Kao et al. reported the first small molecule that was shown to inhibit the function of NP in 2010 [105]. Using a forward chemical genetics approach and a library containing more than 50,000 compounds, they selected 39 compounds for further mode of action studies. They then focused on NP nuclear trafficking using fluorescence microscopy and selected five compounds, the most effective of which had anti-influenza virus activity with an EC_{50} < 1 μM as measured by plaque reduction assay. The commercially available compound nucleozin (Table 6) was identified based on structural information, and further mode of action studies showed that the compound triggers NP aggregation and inhibits NP nuclear localization (Stage 4 in Figure 1). Krystal et al. identified an analog of nucleozin, with a simple OMe substitution (compound 3, Table 6), that was almost threefold more potent than nucleozin [106]. This analog induced the formation of NP oligomers, as confirmed by dynamic light scattering analysis and by X-ray co-crystal structure of the analog. Further modification of the nucleozin resulted in identification of compound 5 (Table 5) with increased potency. There are three genera of influenza viruses (A, B and C types), as classified by antigenic differences in NP and the matrix protein. Hence, the direct targeting of NP was successful only with compounds active against IAVs.

To bypass the issue of viral type specificity, Tripp et al. looked at cellular components of the nuclear transport machinery, which would operate in the infection process for all influenza viruses. Based on the knowledge that the nuclear export (Stage 6 in Figure 1) of influenza vRNP is mediated by exportin 1 (XPO1), potential anti-influenza activity of verdinexor (Table 6), a selective antagonist of XPO1, was tested [107]. Verdinexor effectively inhibited the replication of both IAV and IBV, including the pandemic H1N1 strain, the highly pathogenic H5N1 strain, and the recently emerged H7N9 strain, with EC_{50} values far below micromolar range. Shaw et al. performed similar studies of the inhibition of vRNP localization using ultra-high throughput screening resulting in the identification of hit compound S119 (Table 6), which had an EC_{50} of 60 nM in primary screening, and further optimization of the hit compound lead to discovery of compound S119-8 (Table 6) as a potent anti-influenza agent [108]. S119

Table 6. Antiviral compounds targeting NP and vRNP trafficking.

Name	Chemical structure	IC_{50}[a]	Reference
Nucleozin		0.069 ~ 0.33μM	105
Compound 3		2μM	106
Compound 5		0.07μM	106
Verdinexor		0.01 ~ 0.42μM	107
Compound S119		27.43μM	108
Compound S119-8		5.32μM	108

was effective even after infection, indicating that S119 exerts its antiviral effect at a post-cell-entry step of the viral life cycle. Mechanistic studies showed that S119 induces NP aggregation and has a higher affinity for NP in the monomeric state than for NP in the oligomeric state within the vRNP structure. It is noteworthy that S119 causes NP aggregation by interacting with S283 of NP and therefore, has no antiviral effect against influenza viruses carrying proline at the same position. Therefore, in an attempt to develop a broad-spectrum antiviral, Shaw et al. further identified S119-8, a derivative of S119 that has an antiviral effect against viruses with NP containing either S283 or P283. Furthermore, S119-8 was effective against both IAV and IBV at low micromolar concentrations, had CC_{50} values over 40 μM, and displayed a synergistic antiviral effect when combined with oseltamivir, as demonstrated by isobolograms.

5. Conclusion

Despite continued circulation of influenza virus among human population, and the threat of zoonotic transmission of highly pathogenic avian H5N1 and H7N9 viruses, the arsenal of antivirals available for the fight against influenza infections is woefully limited. The emergence of drug-resistant strains further heightens the urgency to identify novel targets and develop new chemical entities. Recent advances in structural biology, pharmacophore modeling, and high throughput screening have provided information and tools that can be used to guide the design and discovery of novel antiviral agents against influenza. Current endeavors are divided into two different, but not mutually exclusive, approaches. The first is to devise novel or improved chemical entities against well-established targets. Ideally, the drug candidates should have

potent antiviral activity against not only multiple types of viruses, but also drug-resistant mutant viruses. They should also have a higher resistance barrier than existing drugs that act on the same targets. Novel small molecule compounds against M2 and NA drug-resistant mutants have been identified, encouraging further development of potent inhibitors of known targets. The second approach is to validate new targets (viral or host origin) and develop new classes of antivirals. Internal viral proteins such as NP or the viral polymerase complex offer new targets that can be exploited either by structure-based drug design or by *in vitro* high throughput screening. Host cell nuclear transport machineries may offer additional targets for controlling the nuclear trafficking of viral RNP complexes. Although targeting of the host machinery is unlikely to elicit viral resistance, attention should be given to potential interference with the host metabolism and consequent general toxicity. New compounds developed using either of the approaches, some of which are in clinical trials, may provide strong options for the treatment of influenza virus infections in the near future.

6. Expert opinion

The eradication of IAVs is unlikely because of its capacity for interspecies transmission, which maintains the viral reservoir in nature [1]. Therefore, the development and stockpiling of vaccines and antivirals should be continued to mitigate the seasonal circulation of IAVs and prepare for future pandemic outbreaks. At present, the therapeutic efficacy of antiviral drugs is compromised by their side effects [109] and by the emergence of drug-resistant mutants [110]. In Japan for instance, oseltamivir is contraindicated for use in children and adolescents between 10 and 19 years of age because of the concerns about neurological side effects [111,112].

The ultimate goal of anti-influenza virus research is to develop safe and effective drugs without generating drug-resistant mutants. NA and M2 ion-channel have been proven prone to generate resistance. Besides continued efforts on designing novel inhibitors against known viral targets, the discovery and validation of novel targets, of viral or host origin, is essential. Recent advances in pharmacophore modeling and structural biology, as well as ultra HTS, has led the acceleration of drug discovery against influenza polymerase complex PA endonuclease, PB2 cap-binding domain, PA-PB1-PB2 protein–protein interface and its nuclear trafficking, which hold future potential due to their uniqueness in the mode of action. As mentioned in section 2, not only the crystal structure but also the solution structure deduced form NMR, combined with structural bioinformatics, can dramatically increase the efficiency of drug discovery process. Of note, a unique cap-snatching mechanism for transcription by concerted action of the polymerase complex (PB1/PB2/PA) provides a window for novel antivirals [21,77,80,88]. Because of its conserved nature across various influenza strains and subtypes, the antiviral effect is likely to be broad-spectrum. Notably, the endonuclease inhibitor baloxavir recently received its first global approval in Japan for the treatment of influenza A or B virus infections [80]. This drug was also found to be prone to resistance during phase 2 clinical study; for instance, PA I38T/F/M mutants were observed after the drug treatment [20]. However, these mutations have not been documented among natural isolates of IAVs or IBVs according to the NCBI database. Study of viral replication kinetics *in vitro* showed that the mutations impaired replication capacity, compared to the wild type, in both canine and human cells, and the reduced fitness may not support human-to-human transmission of the resistant viruses. Nonetheless, this information will be useful for post-marketing surveillance of drug effectiveness.

It was long thought that NP is not a suitable target for drug discovery because of serological variations in the NP antigen, which was evidenced by the narrow specificity of nucleozin for IAVs but not IBVs [105]. However, NP targeting compound, e.g., S119-8 showed inhibitory effects against both IAVs and IBVs and had a synergistic effect with oseltamivir [108], further demonstrating that a combination therapy targeting both external and internal proteins could address to the issue of drug resistance. An alternative way to interfere with the replication/transcription cycle in both IAV and IBV is to target the host factors that interact with NP. As evidenced by genome-wide RNA interference screens [113–115] that identify various host factors involved in influenza virus replication [116–118], targeting host factors may yield small molecule inhibitors with higher resistance barriers than current antivirals targeting viral proteins. It is worth mentioning that umifenovir exerts its broad-spectrum antiviral effect by interfering at the step of viral host membrane fusion [102,103], but is prone to resistance because of its reliance on viral HA protein [104]. In contrast, interfering with the initial step of viral entry by targeting host-membrane resident proteases is not likely to generate resistance while providing broad-spectrum activities [71]. Even if targeting host proteins may provide a way to minimize antiviral resistance, attention should be given to general cytotoxicity, which can be a side effect of interfering with normal metabolic pathways. Nevertheless, some host-targeting antivirals, along with the most conserved viral polymerases are currently in clinical trials and may provide options for tackling drug-resistant influenza viruses [119].

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