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Influenza A virus nucleoprotein: a highly conserved multi-functional viral protein as a hot antiviral drug target

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Abstract

Prevention and treatment of influenza virus infection is an ongoing unmet medical need. Each year, thousands of deaths and millions of hospitalizations are attributed to influenza virus infection, which poses a tremendous health and economic burden to the society. Aside from the annual influenza season, influenza viruses also lead to occasional influenza pandemics as a result of emerging or re-emerging influenza strains. Influenza viruses are RNA viruses that exist in quasispecies, meaning that they have a very diverse genetic background. Such a feature creates a grand challenge in devising therapeutic intervention strategies to inhibit influenza virus replication, as a single agent might not be able to inhibit all influenza virus strains. Both classes of currently approved anti-influenza drugs have limitations: the M2 channel blockers amantadine and rimantadine are no longer recommended for use in the U.S. due to predominant drug resistance, and resistance to the neuraminidase inhibitor oseltamivir is continuously on the rise. In pursuing the next generation of antiviral drugs with broad-spectrum activity and higher genetic barrier of drug resistance, the influenza virus nucleoprotein (NP) stands out as a high-profile drug target. This review summarizes recent developments in designing inhibitors targeting influenza NP and their mechanisms of action.

Keywords

Influenza virus; nucleoprotein; antivirals; nucleozin; antiviral drug resistance

1. INTRODUCTION

The influenza A virus is the leading cause of annual influenza epidemics and occasional influenza pandemics. Each year in the United States, an estimated 36,000 deaths and millions of hospitalizations are due to influenza-related illness [1, 2]. Globally, there is an estimate of 250,000–500,000 deaths annually [3]. In the case of influenza pandemics, it causes even more catastrophic damage as witnessed with the 1918 Spanish flu and the 2011 swine flu. The pathogenicity of influenza viruses arises from the following two factors [4]: (1) the influenza virus is transmissible through both direct contact and aerosol droplets [5]

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and (2) the influenza virus exists as quasispecies [6, 7]. Influenza viruses can be transmitted through the air, which allows it to spread quickly to susceptible hosts in a short period. Because of this unique character, the influenza virus is constantly listed as one of the top pathogens that could potentially wipe out the whole human population. In fact, influenza virus is classified as a category C priority pathogen by NIAID. Regarding therapeutic intervention methods, the influenza virus is not an easy microbe to target. That is because the influenza virus is a negative sense RNA virus that exists as a quasispecies as a result of antigenic drift and antigenic shift. Due to the lack of a proof-reading function of the viral polymerase, each cycle of influenza viral replication tends to produce a mixture of viruses with different point mutations [8–10]. This is denoted *antigenic drift*. In addition, the genome of the influenza virus is arranged as eight individual segments. Thus different influenza strains can swap their genomes if they happen to infect the same host, which normally happens in swine. In such a scenario, new strains of influenza viruses will be produced. This is denoted *antigenic shift*. An influenza pandemic is often a result of antigenic shift because humans lack the pre-immunity to neutralize the emerging influenza strains. The most recent example is the 2009 H1N1 swine flu. The re-assorted viruses are normally associated with high morbidity and mortality rate such as the H5N1 and the H7N9 strains, which have mortality rates of ~60% [11, 12] and ~27% [13, 14], respectively.

The influenza virus is an enveloped virus [15]. There are three viral proteins on the viral membrane: the hemagglutinin (HA), neuraminidase (NA), and the M2 proton channel. Inside the virion are matrix protein M1, viral ribonucleoproteins (vRNPs), and nuclear export protein (NEP). There are several other viral proteins, such as the non-structured protein (NS1) and PB1–F2 that are only expressed in infected cells but are not incorporated in the mature virion. Each virion contains eight segments of negative-sense viral RNA (vRNA) assembled as individual viral ribonucleoproteins. Each viral ribonucleoprotein contains the viral polymerase, which consists PA, PB1, and PB2 subunits, the nucleoprotein (NP) and a single strand of vRNA. Upon attachment to the host cell through the interaction between HA from the virus and the sialic acid receptor on the host cell surface, the influenza virus enters host cells by endocytosis. In the late endosome, the M2 proton channel is activated by the acidic pH, allowing protons to flux into the virion from the endosome. This leads to the dissociation of vRNPs from the matrix protein M1. Concurrently, the low pH also triggers a conformational change of HA. This exposes the hydrophobic fusion peptide in the HA2 domain of HA to insert into the host cell membrane and initiates membrane fusion [16]. The released vRNPs are next translocated to the nucleus through the fusion pore. The viral polymerase then catalyzes viral genome transcription and replication. Viral mRNAs are subsequently transported back to the cytoplasm for translation to viral proteins. In the late stage of viral replication, the vRNPs are transported back to the cytoplasm to be packed into the progeny virion. Finally, the NA cleaves the host cell sialic acid linkage and releases the progeny virion to the circulating system. For a comprehensive review of the influenza virus life cycle, see references [15, 17].

NP plays a central role in viral replication [18]. As a structural protein with no intrinsic enzymatic activity [19], it is the most abundant viral protein in infected cells. NP is a critical component of the vRNP complex, and the recognized functions of NP include, but are not limited to, organization of RNA packing, nuclear trafficking [20–23], and vRNA

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transcription and replication [24, 25]. Monomeric NP is a 56 kDa protein with a patch of basic residues capable of binding to a single-strand RNA. Each NP is capable of binding to 24 bases of RNA [19, 26]. NP assembles into NP oligomers by a flexible tail loop, which can insert into the neighboring NP monomer [27]. NP also directly interacts with the PB1 and PB2 subunits of the viral polymerase [28–31]. From cryogenic electron microscopy studies of recombinant vRNPs, the minimal functional unit of RNP comprises one viral polymerase and nine NP monomers arranged in a rod-shaped structure [28, 32, 33]. Aside from providing structural support for viral RNA and polymerase, NP also contains nuclear localization signals (NLS) and nuclear export signals (NES) [34–38]. Both sequences are critical in mediating the trafficking of the vRNPs in and out of the nucleus through active transport during the viral replication cycle. The size of vRNPs (100–200Å) is too big to diffuse through the 9 Å nuclear pores [39]. The cellular protein that mediates import of vRNPs includes importin-α (or karyopherin-α), which was shown to bind to NP through the NLS sequence [21]. In the mature virion, vRNPs are attached to the inner layer of viral membrane through interaction with matrix protein M1 molecules. Dissociation of vRNP from M1 is a pre-requisite for the vRNP translocation into the nucleus as it exposes the NLS [40, 41]. In the late stage of viral replication, vRNPs are transported back to the cytoplasm to be incorporated into the progeny virion, and this process is mediated via the interaction of a protein assembly comprising cellular chromosome region maintenance 1 receptor (CRM1), M1, NEP, and vRNP [42–44]. Additionally, NP is also critical for the viral genome transcription and replication [24, 25]. The influenza virus produces three types of viral RNAs: the messenger RNA (mRNA), the viral genome RNA (vRNA), and the complementary positive-sense RNA (cRNA). The cRNA is an intermediate for the replication of vRNA. The production of all three RNA species requires a functional NP. Overall, NP is a multifunctional protein and is indispensable for viral replication. For more detailed discussion about the functions of NP and vRNPs, readers are referred to the following review articles [18, 45–49].

Currently there are two classes of FDA-approved antivirals used for the prevention and treatment of influenza virus infection: M2 channel blockers and neuraminidase inhibitors [50, 51]. M2 channel blockers such as amantadine and rimantadine inhibit the early stage of viral replication by preventing uncoating of vRNPs from the matrix protein M1 [52–54]. Both amantadine and rimantadine are no longer recommended by the Centers for Disease Control and Prevention to treat influenza infection because of predominant drug resistance. The majority of circulating human influenza viruses carry drug-resistant mutations, such as S31N, V27A, and L26F, in the M2 transmembrane pore domain, which result in amantadine resistance [55]. Additional drawbacks of adamantanes include central nervous system toxicity and lack of efficacy against the influenza B virus [56, 57]. The influenza B virus similarly encodes a proton channel called BM2 [58]. However, BM2 has completely different pore-lining residues than AM2 [59, 60], which confers the lack of binding to amantadine. Compounds targeting drug-resistant AM2 mutants have been reported recently, and these compounds are currently at the preclinical development stage [61–65]. The other class of FDA-approved influenza antivirals, including oseltamivir, zanamivir, and peramivir, inhibit progeny virion release by targeting the viral neuraminidase [66, 67]. NA is a sialidase that hydrolyzes the host cell sialic acid receptor such that the progeny virions can be

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released. NA inhibitors are currently the mainstay and also the last line of defense against influenza virus infection. Resistance to NA inhibitors was originally thought to be difficult to occur [68, 69] because NA inhibitors are close structural mimics of the sialic acid, which is the natural host receptor for NA. However, such a notion was challenged by the appearance of the seasonal H1N1 influenza viruses prior to 2009, which were completely resistant to oseltamivir due to an H275Y mutation [70, 71]. Moreover, likely due to its frequent use, resistance to oseltamivir is continuously on the rise [72–74]. It will only be a matter of time before oseltamivir-resistant influenza strains become dominant. Therefore, there is an immediate need to develop the next generation of novel antivirals [75].

Among various compounds that are currently in preclinical and clinical development stages for influenza virus infection [50, 75], NP inhibitors appear to be one of the most promising classes because of their potent antiviral efficacy and broad-spectrum antiviral activity [76, 77]. As a viral protein, NP has no host cellular counterpart, thus a high selectivity index could be achieved for NP inhibitors. Moreover, NP is highly conserved among influenza A virus strains from different species, implying that the influenza virus is less prone to develop resistance to NP inhibitors. This review highlights NP inhibitors discovered so far and delineates their mechanisms of action. Overall, NP represents a high-profile drug target with multiple druggable drug-binding sites.

2. STRUCTURES OF INFLUENZA A VIRUS NP

The first X-ray crystal structure of NP to come to light was the A/WSN/33 (H1N1) NP structure (PDB: 2IQH) [27]. This structure was solved without RNA and associated viral polymerase. NP was crystalized as a trimer (Fig. 1A), which is consistent with the results from electro microscopy and size exclusion gel filtration chromotography. The NP monomer and trimer was in equilibrium in solution. The NP-NP oligomerization was mediated via a flexible tail-loop (residues 402–428) that can insert into the body domain of a neighboring NP monomer (Fig. 1B). The ionic interaction between R416 from the tail-loop of one NP monomer and E339 from the body domain of another monomer was found to be critical for the NP oligomerization. NP mutants containing alanine substitution at either or both of these two residues failed to form trimers. The resulting NP alanine mutants were also unable to synthesize vRNA, implying that NP self-oligomerization is essential for its function [78]. A construct with the deletion of the tail-loop domain, 402–428, was also unable to oligomerize. The monomeric NP adapts a crecent shape with distinct head and body domains (Fig. 1C). In between these two domains is the RNA-binding groove, which is rich in basic residues. Later on, the H5N1 NP structure was also solved by X-ray crystallography (Fig. 1E) (PDB: 2Q06) [79]. Mutating the basic residues in the RNA-binding groove of NP significantly decreased its RNA binding ability [79]. The monomeric H5N1 NP structure was very similar to the previous H1N1 NP structure, with a root-mean-square deviation (RMSD) of only 1 Å. However, the major difference resides in the orientation of the C-termini tail-loops (Fig. 1D and Fig. 1E): in the H1N1 NP structure (Fig. 1D) the tail-loop is nearly horizontal, while in the H5N1 NP structure (Fig. 1E) it points toward the upper right at a 45-degree angle. The different orientaiton of the tail-loop is the determinant of NP oligomerizaiton. As a result, the H1N1 NP trimer could not overlay with the model of H5N1 NP trimer. The linkers between the tail-loop and the NP body/head domains (residues 397–

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401 and 429–437) were indeed very flexible, which allows the tail-loop to adopt different orientations. The NP trimers in the crystal structures are unlikely to be phsyiologically relevant to the NP oligomer, which might require a different tail-loop orientation to form larger oligomers. Most recently, the A/WSN/33 (H1N1) monomeric NP structure with an R416A mutation was also solved (PDB: 3ZDP) (Fig. 1F) [80]. Because the mutated tail-loop was unable to insert into the body domain of the neighboring NP monomer, it folds back to the head and body domains. Nevertheless, the head and body domains of this mutant NP structure were nearly identical to the previous two structures.

3. INHIBITORS TARGETING INFLUENZA A VIRUS NP

The multi-functional role of NP, coupled with the high sequence conservation, makes it an ideal drug target [45]. Inhibitors targeting NP have been identified through both forward and reverse chemical genetic methods. The following sections will review different classes of NP inhibitors and their mechanisms of action. It is intriguing to find out how a small protein such as NP (498 residues) can accommodate several drug binding pockets.

3.1. Inhibitors targeting the tail-loop binding pocket

Mutagenesis studies suggest that the E339–R416 salt bridge between two NP monomers is not only important for NP oligomerization but also essential for the vRNP activity [81]. Mutating either residue or both resulted in the inhibition of viral replication. Structurally, this ionic interaction is located in the tail-loop pocket of the NP monomer and provides a driving force for NP oligomerization. Purified wild-type (WT) NP exists predominantly as trimers, while E339A and R416A exist as monomers. Therefore, targeting the NP tail-loop binding pocket is a natural next step. A cyclic peptide (peptide **1**) comprising the tail-loop region (residues 409–418) had weak inhibition of viral replication (EC_{50} = 904 μM) (Table 1). The weak inhibition is probably the result of its poor cell penetration. Subsequent virtual screening identified several small molecule hit compounds that were shown to disrupt NP trimerization and induce formation of NP monomers. The most potent hit is compound **1**, showing an EC_{50} value of 2.7 μM against the A/WSN/33 (H1N1) virus (Table 1). Compound **1** represents the first rationally designed NP inhibitor based on the crystal structure of H1N1 NP (PDB: 2IQH).

3.2. Nucleozin and its analogs

Nucleozin was discovered as an NP inhibitor concurrently by several groups through cell-based antiviral screening [82–84]. To unveil the mechanism of action of nucleozin, the A/WSN/33 (H1N1) virus was subjected to serial passage experiments with increasing concentrations of nucleozin or its close analogues [82–84]. If resistant strains were to emerge throughout the process, this suggests that the drug might be a direct-acting antiviral. By sequencing the viral genome, the resistant mutants can be mapped to corresponding viral proteins. The resulting viral proteins might therefore be the putative drug targets. Using this standard virology technique, several groups independently identified a number of mutations in NP that render nucleozin or its analog less effective. Three mutations, Y289H, N309K/N309T, and Y52H, were identified from the drug selection passage experiments. Recombinant viruses carrying either one of these mutants or in combination in the NP

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protein were resistant to nucleozin and its analogs, suggesting that NP is the sole drug target of this class of compounds. Likewise, circulating influenza A strains, such as A/California/07/09 (H1N1), A/Solomon Islands/3/06 (H1N1), A/Brisbane/10/07 (H3N2), which carry Y289H, N309T, and Y52H, respectively, in their nucleoprotein genes, were similarly resistant to nucleozin and its analogs. Since the X-ray crystal structures of the influenza nucleoprotein were known, the pockets in which these selected mutants reside might be the corresponding drug binding sites of nucleozin [84]. However, it is intriguing to find out that these three mutations are spread to two pockets that are more than 17 Å apart (Fig. 2A), far exceeding the length of nucleozin and its analogs. These two pockets are designated as the Y52 pocket and the Y289/N309 pocket (Fig. 2A). Mutagenesis of the residues in the vicinity of these two pockets further confirmed the engagement of both pockets in nucleozin binding. Both pockets are located on the same face in the body domain of nucleoprotein, which is opposite to the RNA-binding groove. The question arose was how could nucleozin and its analogs bind to both pockets at the same time? The mystery was finally unveiled by the co-crystal structure of nucleoprotein from A/WSN/33 (H1N1) with a nucleozin analog, compound **2** (PDB: 3RO5) [83] (Fig. 2B). It has been found that compound **2** serves as a molecular glue to bridge two nucleoprotein monomers together in an antiparallel dimer configuration. Two molecules were found at the nucleoprotein dimer interface and each molecule interacts with Y52 pocket from one monomer and the Y289/N309 pocket from another monomer (Fig. 2C). Specifically, the isoxazole group resides in the Y52 pocket in NP_1, while the nitrobenzene group resides in the Y289/N309 pocket in NP_2 (Fig. 2D). Key interactions include a hydrogen bond between the side chain hydroxyl of S376 from NP_1 and the amide carbonyl from compound **2**, a **π-π** interaction between Y289 from NP_2 and the nitrobenzene ring from compound **2** (Fig. 2D). Mutating a charge neutral Y289 in NP_2 to a charged histidine residue might prevent the hydrophobic nitrobenzene group in compound **2** from binding in this pocket. Y52H mutation in NP_1 might result in a similar effect. The N309 residue sits on top of the piperazine linker and it does not directly interact with the drug; however, mutating the asparagine to threonine or lysine might partially occlude the Y289/N309 pocket. High-resolution structures of these mutants might be able to offer a more reasonable explanation for the observed drug resistance. The structure of nucleoprotein containing the Y289H was solved by X-ray crystallography (PDB: 4DYT) (Fig. 2E colored in cyan). Overlapping one of the monomers from this mutant structure with that of the compound **2**–bound WT NP (PDB: 3RO5) revealed that these two monomers are nearly superimposable, diverging only in the flexible tail-loop region (Fig. 2E). In the Y289H mutant structure, the histidine ring flips and partially occupies the Y289/N309 site, which might account for the resistance to compound **2** (Fig. 2F). A close analog of nucleozin, **FA-10**, was claimed to inhibit Y289H-containing influenza strains, A/WSN/33 (Y289H) and swine-origin influenza A H1N1 (289H), with EC_{50} values of 11.3 ± 0.9 μM and 5.0 ± 0.5 μM, respectively (Table 2) [84]. However, by comparing these with the efficacy of nucleozin against A/California/07/09 (Y289H) (EC_{50} = 17 μM), it appears that the Y289H remains resistant to **FA-10**. Therefore, further structure–activity relationship (SAR) studies are necessary to optimize the efficacy against the Y289H mutant to the similar level as nucleozin in inhibiting the A/WSN/33 (H1N1) (EC_{50} = 0.14 μM).

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Nucleozin has excellent in vitro cellular activity and submicromolar EC_{50} values against susceptible influenza A strains, such as A/WSN/33 (H1N1) and A/Gull PA/417583 (H5N1). However, nucleozin has low aqueous solubility and poor metabolic stability in mouse liver microsomes [45]. These suboptimal pharmacodynamics properties might account for the moderate efficacy of nucleozin analogs in mouse studies [84]. In an effort to optimize these properties, Krystal *et al.* designed a triazole analog of nucleozin, compound **3**, which was shown to have significantly improved solubility and stability. Compound **3** was able to fully protect mice from influenza virus–induced death when dosed above 10 mg/kg [83]. Similarly, Ding *et al.* designed several nucleozin analogs using scaffold-hopping and bioisosteric replacement strategies [85]. One of the most potent analogs, compound **4**, has similar in vitro antiviral activity as that of nucleozin. The in vivo efficacy of this molecule has not yet been reported. The co-crystal structures of H1N1 NP with several nucleozin analogs (compounds **5**–**9**) were also solved by X-ray crystallography and the coordinates were deposited in the protein data bank. These structures will greatly facilitate the rational design of the next generation of nucleozin analogs.

3.3. NP inhibitors targeting the RNA-binding groove

The first reported inhibitor targeting the NP RNA-binding groove is **F66** (Fig. 3) [86]. It was predicted to bind to the R174–K184 epitope region in the RNA-binding groove (Fig. 3). **F66** was selected from in silico screening using the H5N1 NP structure (PDB: 2Q06). It inhibits several influenza A strains, including A/California/07/09 (H1N1), A/Wisconsin/67/05 (H3N2), and A/New Caledonia/20/99 (H1N1), with low micromolar EC_{50} values in cellular antiviral assays. **F66** was not active against the B/Brisbane/60/08 strain, probably because of the sequence divergence between influenza A and B NPs. When tested in a mouse model of influenza infection, **F66** demonstrated around 40% survival protection. No further experimental evidence was provided to support the claimed mechanism of action for **F66**.

The second reported example of an inhibitor binding to the RNA-binding groove of NP is naproxen (Fig. 4) [87]. Naproxen is a known inhibitor of cyclooxygenase type 2 (COX-2) and is available as an over-the-counter anti-inflammatory drug. It was discovered to bind to the influenza A virus NP protein by *in silico* docking and molecular dynamics simulations using H1N1 NP (PDB: 2IQH) as the input structure. Three energetically similar poses of naproxen were found at the NP RNA-binding groove near residues Y148, Q149, R150, R152, F489, R355, and R361 (Fig. 4A). In all docked poses, the carboxylate from naproxen was found to form ionic interactions with the guanidine from one of the arginines (Fig. 4C). As naproxen was proposed to bind to the RNA-binding groove of NP, surface plasma resonance (SPR) and fluorescence experiments were designed to evaluate whether naproxen could compete with RNA binding to NP. Results have shown that naproxen indeed competed with RNA binding to the WT NP, but not the NP mutants, which contain an alanine mutation at the key residues at the naproxen drug binding site. Naproxen-bound NP was also more resistant to proteolytic digestion than free NP, which further supports the direct binding of naproxen to NP. The mean EC_{50} value for naproxen was 16 ± 5 μM in inhibiting the A/WSN/33 (H1N1) strain. No drug-resistant mutants were selected after six passages of drug selection. When tested in an in vivo influenza virus–infected mouse model, naproxen had a moderate effect in preventing the weight loss when dosed at 8 mg via intranasal route.

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Following this initial findings, Slama-Schwok *et al.* further designed several naproxen analogs with enhanced binding to NP by fragment extension (Fig. 4B) [88]. Unlike naproxen, which forms only one salt bridge with either R152 or R361, the designed analogs naproxen A and C0 form two salt bridges with both R152 and R355 (Fig. 4D, 4E), which accounts for the increased in vitro binding affinity. No cellular antiviral activity data have been reported for both of these designed inhibitors.

Discovery of naproxen as an antiviral drug is an interesting finding as it might serve as a drug with dual pharmacology. It is well documented that infection with certain influenza strains, such as H1N1 and H5N1, triggers an uncontrolled immune response, which is often referred to as "cytokine storm" [89, 90]. In such a scenario, naproxen can not only exert the antiviral effect by its direct viral inhibitory activity but also help suppress the proinflammatory response [87].

3.4. RK424

Using a cell-based high-throughput screening (HTS) assay, Aida *et al.* screened a library of 50,000 compounds against the A/WSN/33 (H1N1) virus [91]. Among the list of identified hits, R424 was found to have broad-spectrum antiviral activity against multiple influenza A strains with submicromolar EC_{50} values (Fig. 5) [92]. Mechanistic studies suggested that RK424 inhibited the early stage of viral replication post the viral entry, possibly affecting the viral genome replication. Indeed, RK424 was found to inhibit vRNP activity by the mini-genome assay. This observation was further confirmed by western blotting, showing significant reduction of viral protein (HA, NP, NA, M1, and M2) expression with R424 treatment. Next, fluorescence microscopy was applied to examine the effect of RK424 on the localization of NP 6 hours post-infection in HeLa cells. RK424 was found to restrict NP export from the nucleus, indicating that NP might be the potential drug target of RK424. The putative drug binding site of RK424 in NP was revealed by molecular docking using AutoDock (Fig. 5A, 5B). The pocket with the highest binding free energy comprises residues R162, S165, L264, and Y487. SAR results correlated well with the proposed mode of RK424 binding: (1) The ionic interaction between the carboxylic acid in RK424 and the R162 side chain guanidine is critical (Fig. 5B). Changing the carboxylic acid at the 4 position of quinoline to an amide (compound **10**) significantly abolished the antiviral activity (Fig. 5C); (2) The biphenyl ring at the position 2 of quilonine is also critical as it forms a π-π interaction with Y487 and hydrophobic interactions with surrounding residues (Fig. 5B). Replacing the hydrophobic benzene ring linker with a charged piperazine (compound **11**) led to decrease of activity (Fig. 5C); (3) The phenyl ring from the quilonine forms hydrophobic interactions with L264. Converting quinoline to pyridine (compound **12**) completely reduced the antiviral activity (Fig. 5C). The proposed binding mode was further confirmed by pull-down experiments using compound cross-linked beads. As the RK424 drug binding site is close to three functional domains (the RNA binding groove, the NP dimer interface, and NES3), binding of R424 to monomeric NP was shown to disrupt both NP-RNA and NP-NP interactions (Fig. 5A).

The residues surrounding the RK424 binding site appears to be functionally conserved as recombinant viruses carrying mutations at any of the four sites (R162, S165, L264, and

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Y487) were not able to be rescued. This observation is consistent with sequence alignment, which showed > 99% conservation of these residues among avian, human, and swine influenza A viruses [92].

Despite its potent cellular antiviral activity, RK424 has poor bioavailability via either intravenous or oral administration. In both cases, the $C_{free\ max}$ did not exceed the IC_{50} of RK424. When dosed intraperitoneally with 10 mg/kg, RK424 offered 25% survival protection against mice challenged with a lethal dose of A/WSN/33 (H1N1). The in vivo efficacy of RK424 is less than that of oseltamivir phosphate. The poor in vivo activity might arise from the limited aqueous solubility and high plasma protein binding (99.9%). Nevertheless, RK424 was found to have a synergistic effect when combined with oseltamivir. Thus further optimization of RK424 is needed in order to advance this compound to animal model studies.

From the chemistry perspective, quinoline appears to be a high-profile pharmacophore in influenza antivirals. Several reported quinoline-containing antivirals are structurally very similar to RK424, and it is reasonable to suspect that some of the quinoline compounds might have dual drug targets. Thus it is worthwhile to briefly discuss their mechanism of action.

One of the most well-known medicines containing quinoline is chloroquine (Table 3). Chloroquine inhibits both influenza A and B virus with micromolar EC_{50} values [93–96]. As a lysosomotropic agent, it was proposed that the neutral form could diffuse into the endosome. Once inside the endosome, the low pH protonates the quinoline nitrogen as well as the distal nitrogen at position 4 in the side chain. This double protonated species cannot readily penetrate the endosomal membrane. As a result, the endosomal pH increases [95], which impedes the conformational change of hemagglutinin for viral uncoating. Overall chloroquine acts as a proton sponge and neutralizes the endosomal pH. The efficacy of chloroquine in influenza virus–challenged mouse studies was controversial [97, 98]. As a known antimalarial drug, chloroquine was further advanced to human clinical trials to treat influenza virus–infected human patients. However, the results showed that chloroquine did not have significant therapeutic efficacy [99].

Compound **13** was identified through an HTS that was designed to monitor NS1-mediated down-regulation of host gene expression (Table 3) [100, 101]. Compound **13** was found to reverse the inhibition of host gene expression mediated by NS1 [100]. The structural similarity between compound **13** and brequinar promoted the authors to investigate the involvement of dihydroorotate dehydrogenase (DHODH), the drug target of brequinar, in influenza viral replication. DHODH is one of the key enzymes involved in the *de novo* synthesis of pyrimidine, which serves as an essential building block for DNA, RNA, and phospholipids biosynthesis. Inhibitors of *de novo* pyrimidine synthesis have been shown to inhibit influenza viral replication [102]. Further evidence of the involvement of DHODH in viral replication was provided by the rescue experiment. Pyrimidines such as uridine and cytidine were able to revert the inhibitory activity of compound **13**. Subsequent SAR studies based on compound **13** yielded compound **14**, which showed drastically increased activity against A/WSN/33 [103]. The structural similarity between compound **14** and RK424

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suggests that such compounds might target multiple targets at the same time, which explains their potent efficacy.

BPR3P0128 is a synthetic analog that was also derived from SAR studies of an HTS hit (Table 3) [104, 105]. BPR3P0128 inhibited the early stage of viral replication by targeting the PB2-associated cap-snatching function, which is required for transcribing viral mRNA from vRNA. Interestingly, BPR3P0128 not only inhibits influenza viruses (both A and B strains) but also several other RNA viruses such as EV71, CVB3, and HRV2. Host cellular factors such as heat shock proteins 70 and 90 were among the potential targets of BPR3P0128. No in vivo animal data has been reported for BPR3P0128.

Compound **15** is a synthetic analog derived from a hit compound selected from HTS (Table 3) [106]. It was found to inhibit multiple influenza A strains as well as one influenza B strain with low micromolar EC_{50} values. Although both compound **15** and RK424 inhibit the early stage of viral replication, compound **15** is structurally distinct from RK424 and compound **14** as it lacks the 2-biaryl substation as well as the 4-carboxylic acid, suggesting that NP or DHODH is unlikely to be the drug target of compound **15**.

Influenza NP contains several highly conserved tryptophans. It has been shown that binding of nucleozin to NP causes concentration-dependent quenching of tryptophan fluorescence [84, 107], possibly due to nucleozin binding induced conformational changes. Hou *et al.* adapted this assay in 96-well plate format and used it to screen a library of small molecules [107]. Not surprisingly, one nucleozin analog was shown as a positive hit. Interestingly, amodiaquine was also identified as a possible NP binder, and the activity was further confirmed in antiviral assays (Table 3). Amodiaquine is a close analog of chloroquine, thus its antiviral activity might be the result of both NP binding and endosomal pH modulator [96].

3.5. PPQ581

In the same HTS assay where nucleozin was identified [82], Cheng *et al.* also found another hit compound, PPQ581 (Fig. 6A). The EC_{50} value of PPQ581 against the A/WNS/33 (H1N1) strain was 2.3 μM [108]. Subsequent mechanistic studies revealed that PPQ581 similarly imparted the vRNP nuclear trafficking as that of nucleozin, suggesting that both compounds might exert their antiviral activity through the same target: the NP protein. Subsequent drug-resistant selection experiments resulted in resistant strains with a single mutation, S377G, in the NP protein. A recombinant virus carrying the S337G mutation WSN_NP377G was only moderately resistant to PPQ581. The EC_{50} value of PPQ581 in inhibiting WSN_NP377G was 8 μM. Moreover, the NPS377G has not been found in natural isolates, suggesting that the appearance of resistance is very rare. Modeling studies docked PPQ581 to a site composed of residues Y313, S376, E375, I316, S377, and T378 (Fig. 6B). Mutating S377 to glycine appears to abolish a critical hydrogen bonding between the O1 carbonyl and the S377 side chain hydroxyl group, explaining the observed resistance (Fig. 6C).

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3.6. NP inhibitors with unknown mechanisms

A proline-rich peptide, PPWCCCSPMKRASPPPAQSDLPATPKCPP, was identified to bind to the influenza A virus NP protein in a screening using NP as bait to select against a library of proline-rich peptides [109]. When fused to a cell-penetrating HIV-TAT protein transduction domain, the resulting conjugate inhibited the replication of A/WSN/33. No further detail was given regarding the molecular mechanism and how this peptide might bind to the NP protein.

In a similar approach, Aida et al. screened an mRFP-tagged NP against a library of small molecules that were immobilized on chemical arrays by photo cross-linking [110]. The hit compounds were further confirmed by SPR binding assay and antiviral plaque reduction assays. Several mycalamide A analogs (Fig. 7) were found to have moderate inhibition against A/WSN/33 (H1N1) in Madin-Darby canine kidney (MDCK) cells when tested at 32 μM. There was generally a good correlation between NP binding affinity and the in vitro antiviral efficacy. The most potent compound is compound **16**, which reduced the plaque numbers by 86 % at 32 μM. These compounds might bind to the N-terminal NLS (1–13aa) sequence as the truncation construct, NP14–110aa, had much weaker binding affinity toward compound **16**. However, it remains unknown whether the reduced binding is caused by the different folding of the resulting truncated protein.

Shaw *et al.* recently reported several NP inhibitors identified from a SPR screening (Fig. 8) [111]. In this study, an in-house library was screened against NP_R416A by SPR using Biacore 3000. Hit compounds from SPR screening were next tested in an RNP reconstitution reporter assay. One of the hits, compound **17**, reduced the vRNP transcriptional activity by 30% at 12.5 μM. Subsequent screening of compound **17** analogs revealed two more compounds, **18** and **19**, with enhanced vRNP inhibition. The EC_{50} values of **18** and **19** were found to be 13.01 ± 1.78 μM and 19.14 ± 9.02 μM, respectively. The detailed mechanism of how these compounds bind to NP remains unknown.

4. CONCLUSION

The influenza virus poses a global public health concern and remains a human menace that has not been fully addressed yet. Currently we are limited in the countermeasures to prevent and treat influenza virus infection. Influenza virus claims 250,000–500,000 lives annually, even with the availability and use of vaccine and antiviral drugs [3]. Influenza vaccines remain the mainstay for prevention of influenza virus infection. However, the effectiveness of influenza vaccines is limited to immune-competent individuals [112–114]. For seniors older than 65 or immunocompromised patients, influenza vaccines have little to no effect. Moreover, the influenza virus might continue to mutate during the period of vaccine production (usually six months), which results in vaccine mismatch and reduced effectiveness. This is witnessed by the last influenza season in the northern hemisphere [115, 116]. The predominant circulating strain in the 2014–2015 influenza season was the A/Switzerland/2013 (H3N2) strain; however, it was not included in the vaccine. Likewise, the current approved anti-influenza drugs also have limitations. Amantadine and rimantadine are no longer recommended for the prophylaxis and treatment of influenza virus infection, which leaves neuraminidase inhibitors as the only choice for treatment. This is not the ideal

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situation as the more frequently the NA inhibitors are used, the faster the virus might be able to mutate and become resistant to it [117, 118]. Moreover, oseltamivir has limited efficacy in inhibiting the highly pathogenic H5N1 virus infection [119]. Thus there is an urgent need to develop novel antiviral drugs with different mechanisms of action than that of NA. The lack of proof-reading function of the viral RNA polymerase, coupled with the segmented feature of viral RNA genome allowing for segmented reassortment, and the vast range of natural hosts for influenza A virus, make the influenza A virus a fast evolving species that is difficult to inhibit by a single antiviral drug. Among the list of drug targets that are currently in the pipeline of drug development [50, 120–123], influenza nucleoprotein stands out as one of the most promising drug targets. NP is highly conserved among influenza strains isolated from different species. NP is also essential for the function of vRNP and interacts with several viral (PB1, PB2 [31], vRNA, and M1 [22]) and host cell factors [49] during the viral replication cycle. The multi-functional role of NP provides a number of opportunities for therapeutic intervention. As it has already been discussed, NP inhibitors that disrupt either one or several of these interactions have shown to be potent antivirals. The advent of the X-ray crystal structures of NP has greatly facilitated the rational design of NP inhibitors, such as the tail-loop pocket binding inhibitor compound **1** [81], the RNA groove-binding inhibitor F66 [86], and naproxen [87]. The crystal structures were also very helpful in rationalizing the mechanisms of action for hits identified from HTS. It is highly promising that NP inhibitors will be added to our therapeutic armamentarium in fighting against the fast-evolving influenza virus in the near future.

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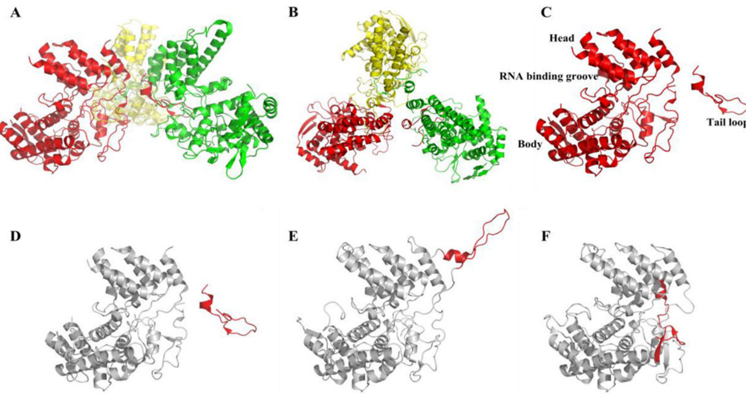

Fig. (1).

X-ray crystal structures of influenza A virus NP structures. (A) Side view of the H1N1 NP trimer (PDB: 2IQH); the monomers are colored in red, blue, and yellow. (B) Top view of the H1N1 NP trimer (PDB: 2IQH). (C) H1N1 NP monomer (PDB: 2IQH). (D) H1N1 NP monomer (PDB: 2IQH) with the tail-loop highlighted in red. (E) H5N1 NP monomer (PDB: 2Q06) with the tail-loop highlighted in red. (F) H1N1 NP_R416A mutant monomer (PDB: 3ZDP) with the tail-loop highlighted in red.

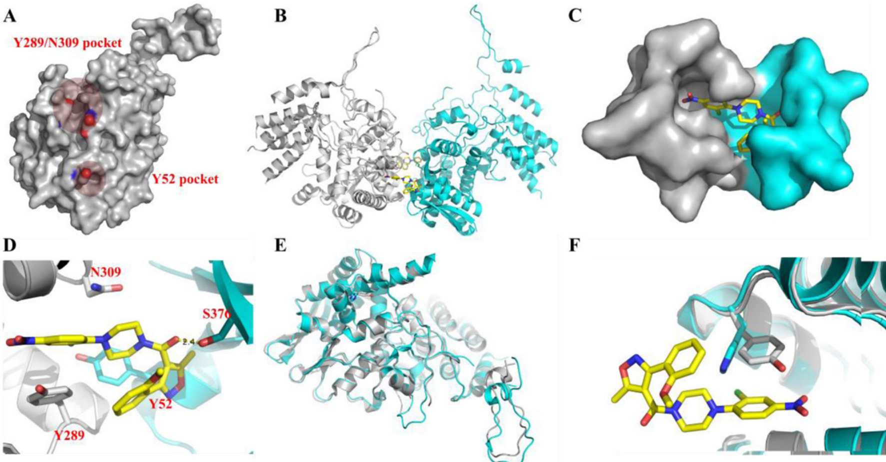

Fig. 2.

Co-crystal structures of compound **2**–bound NP. (A) Surface representation of two drug-binding pockets in the body domain of NP. These two pockets are designated the Y289/N309 pocket (top) and the Y52 pocket (bottom). (B) Dimer structure of H1N1 NP in complex with compound **2** (PDB: 3RO5). (C) Surface representation of the drug-binding pocket located at the interface of two NP monomers. (D) Interactions between compound **2** and key residues surrounding the drug binding site. (E) Overlay structures of NP_Y289H (PDB: 4DYT) with compound **2**–bound WT H1N1 NP (PDB: 3RO5). (F) Close view of the nucleozin binding pocket in the overlay structures (4DYT and 3RO5).

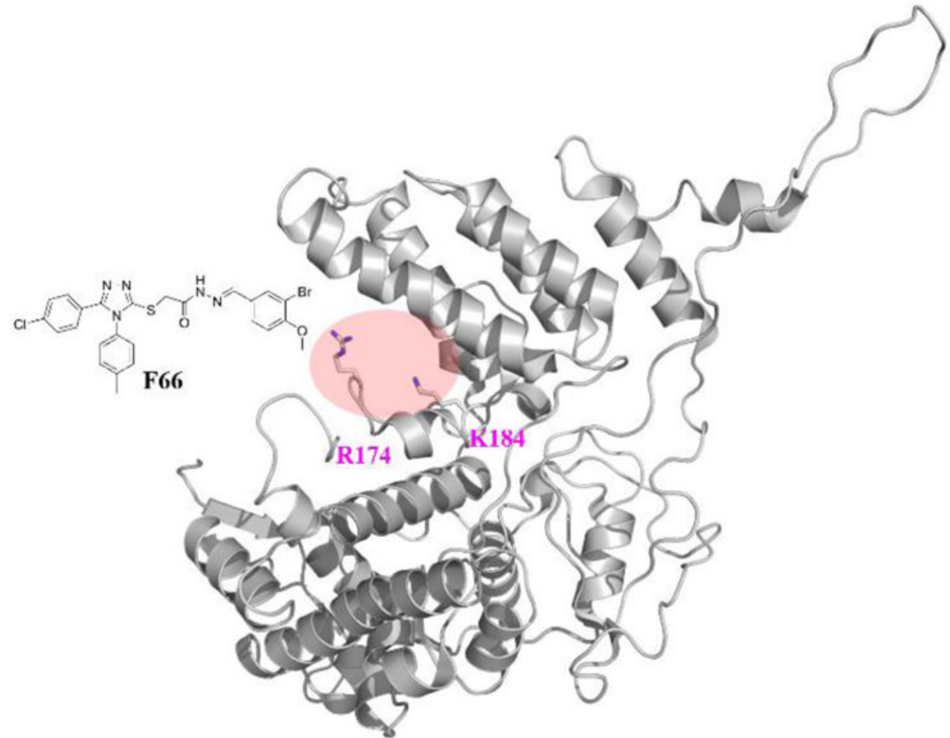

Fig. 3.

Chemical structure of **F66** and its putative binding site in the RNA-binding groove of H5N1 NP (PDB: 2Q06).

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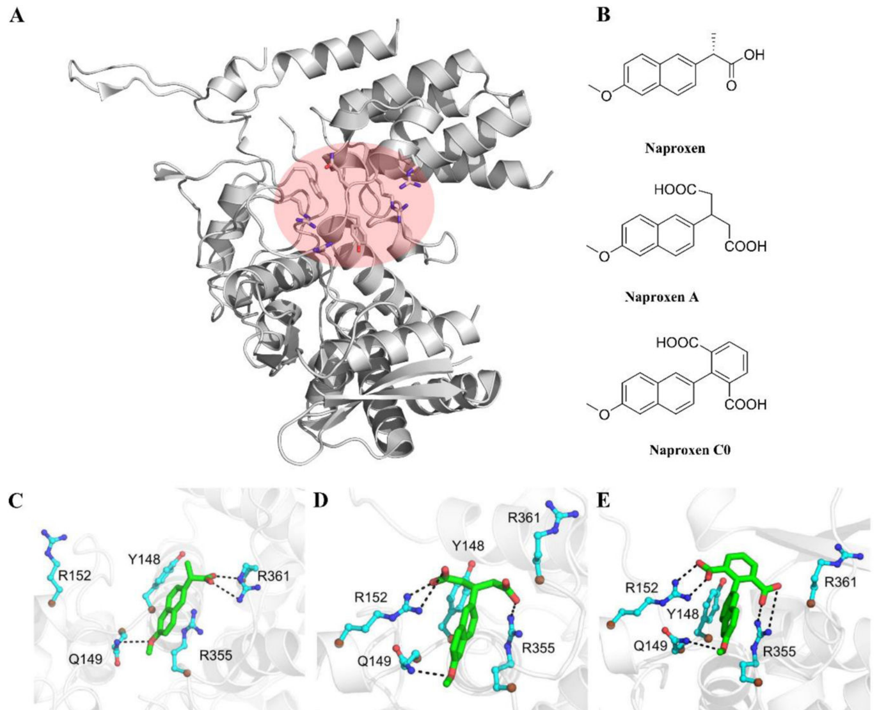

Fig. 4.

Binding of naproxen and its analogs to the H1N1 NP protein (PDB: 2IQH). (A) The drug binding site of naproxen in NP. (B) Chemical structures of naproxen and its analogs, naproxen A and naproxen C0. (C) One of the docked conformations of naproxen in the RNA-binding groove of NP. (D) Docked conformation of naproxen A in the RNA-binding groove of NP. (E) Docked conformation of naproxen C0 in the RNA-binding groove of NP. Figures 4C–E were reproduced from reference [88] with permission.

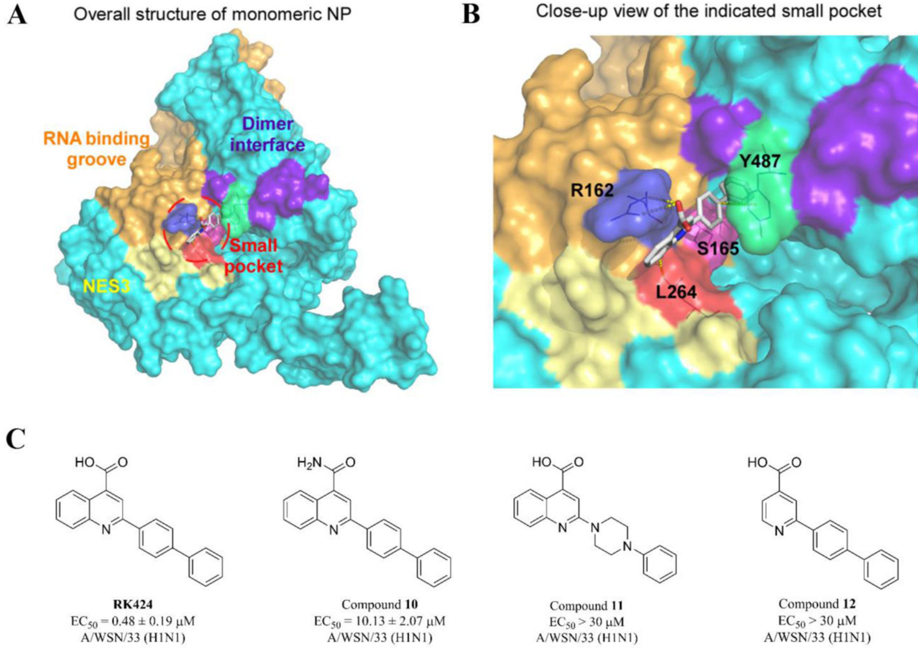

Fig. 5.

Binding mode of RK424 to H1N1 NP and structures of RK424 analogs. (A) Docked conformation of RK424 in H1N1 NP (PDB: 2IQH). The drug-binding pocket is adjacent to three critical domains: the RNA-binding groove, the dimer interface, and the NES3 domain. (B) Detailed molecular interaction between RK424 and NP. (C) Chemical structure and antiviral efficacy of RK424 and its analogs. Figures 5A–B were reproduced from reference [92] with permission.

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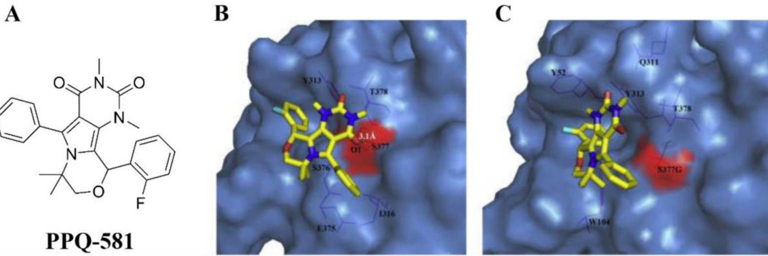

Fig. 6.

Chemical structure of PPQ-581 (A) and the docked poses of PPQ-581 in the wild-type NP (B) and the NP_S377G (C). Figures 6B–C were reproduced from reference [108], copyright © 2016 Elsevier Masson SAS. All rights reserved.

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Mycalamide A

Compound **16**

Fig. 7.

Chemical structures of mycalamide A and its analog compound **16**.

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Compound **16**   Compound **17**   Compound **18**

Fig. 8.
NP inhibitors identified from SPR screening.

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Table 1

NP tail-loop binding inhibitors.

NP tail-loop binding inhibitorsf	EC_{50} (μM) against A/WSN/33 (H1N1)
C Q P T F S V Q R N L C Peptide **1**	904
Compound **1**	2.7

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Table 2

Nucleozin and its analogs

Nucleozin and its analogs	EC_{50} (μM) against A/WSN/33 (H1N1)
Nucleozin	0.17 [83]
Compound **2** (PDB: 3RO5)	0.04 [83]
FA-10	15.3 11.3 (WSN_Y289H) 5 (S-OIV H1N1) [84]
Compound **3**	0.07 [83]
Compound **4**	0.68 [85]
Compound **5** (PDB: 3TG6)	0.07 [83]
Compound **6** (PDB: 4DYP)	N. A.

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Nucleozin and its analogs	EC_{50} (μM) against A/WSN/33 (H1N1)
Compound **7** (PDB: 4DYN)	N. A.
Compound **8** (PDB: 4DYB)	N. A.
Compound **9** (PDB: 4DYA)	N. A.

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Table 3

Quinoline analogs and their antiviral efficacy against influenza viruses.

Quinoline-based antivirals	EC50 (μM)	Mechanism of Action
Chloroquine	A/Hong Kong/X31 (H3N2) 4.31 A/Puerto Rico/8/34 (H1N1) 0.61 A/California-like/2009 (H1N1) 4.01	Elevate the endosomal pH, thus inhibiting low pH-trigged virus-endosome fusion [95, 96]
Compound **13**	A/WSN/33 (H1N1) 7.6	Interferes with *de novo* pyrimidine synthesis by inhibiting dihydroorotate dehydrogenase (DHODH) [100]
Compound **14**	A/WSN/33 (H1N1) 0.041	Interferes with *de novo* pyrimidine synthesis by inhibiting dihydroorotate dehydrogenase (DHODH) [103]
BPR3P0128	A/WSN/33 (H1N1) 0.03	Targets a cellular factor(s) (maybe HSP90 or HSP70) associated with viral PB2 cap-snatching activity [104, 105]
Compound **15**	Influenza A/Udorn/72 (H3N2) 0.10 Influenza A/PR/8/34 (H1N1) 0.07 Influenza A1/Jingfang 86-1(H1N1) 0.10 Influenza A3/Lufang 93-1(H3N2) 0.06 Influenza B/Shenzhen/747–0.05	Inhibits an early stage of viral replication [106]
Amodiaquine	Influenza A/WSN/33 (H1N1) 6.3 ± 2.4	Binds to NP and quenches tryptophan fluorescence [107]