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Recent progress in structure-based anti-influenza drug design

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Abstract

Seasonal and pandemic influenza have caused high morbidity and mortality worldwide. Recent emergence of influenza A H5N1 and H1N1 strains has heightened concern, especially as a result of their drug resistance. The life cycle of influenza viruses has been well studied and nearly all the viral proteins are becoming potential therapeutic targets. In this review, we present an overview of recent progress in structure-based anti-influenza drug design, paying close attention to the increasing role of computation and strategies for overcoming drug resistance.

Introduction

Seasonal and pandemic influenza represent one of the major threats to public health. The annual influenza epidemic results in 250 000–500 000 deaths worldwide [1]. During the past century, the 1918 Spanish flu, 1957 Asian flu and 1968 Hong Kong flu pandemics caused millions of fatalities [2]. More-recent years have seen the emergence of the 1997 H5N1 virus in Hong Kong, or ‘bird flu’, known for its high fatality rate (although low transmission in humans) [1], and the 2009 H1N1 virus in Mexico, or ‘swine flu’, which contributed to at least 16 000 deaths [1]. These two viruses have heightened concern, especially because they can carry drug-resistant mutations [3,4].

Influenza viruses are negative-sense single-stranded RNA viruses, belonging to the family *Orthomyxoviridae*. Based on the antigenic difference in their nucleoproteins and matrix proteins, the viruses are classified into three types: A, B and C [5]. Influenza A is the major pathogen for most cases of epidemic influenza, and has thus attracted the most attention. The influenza A genome is composed of eight RNA segments, five of which code for one protein each and the other three code for two proteins each [6]. The proteins (Table 1) are: hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), M2 proton channel, nucleoprotein (NP), non-structural protein 1 (NS1), nuclear export protein (NEP; formerly known as NS2), polymerase acid protein (PA), polymerase basic proteins (PB1 and PB2) and a protein named PB1-F2 which is expressed from a second reading frame (+1) of the PB1 gene [7]. PB1, PB2 and PA form the RNA polymerase. The surface glycoproteins HA

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and NA provide the viruses with distinct antigenic properties. Influenza A viruses are further organized according to HA and NA subtypes. Sixteen HA subtypes (H1 to H16) and nine NA subtypes (N1 to N9) have been identified. The subtypes of the 1997 bird flu and the 2009 swine flu viruses have been indicated above. Recent seasonal flu epidemics are dominated by the H3N2 and H1N1 subtypes (along with influenza B viruses).

Vaccines and drugs are two effective strategies against influenza. {1. AU: Because available drugs don't really provide an effective anti-influenza strategy this sentence should reflect that. Also Vaccination isn't always an option as you go onto say. Please re-word this sentence for clarification. Would something like: Vaccine and drug usage are two strategies for combating influenza infection, be ok here?} Vaccination is not a realistic plan for a rapidly spreading influenza pandemic, because of the substantial lead time for vaccine production. The antiviral drugs provide alternative options to control influenza infections. To date, four antiviral drugs have been approved by the FDA, including two NA inhibitors, oseltamivir (Tamiflu®) and zanamivir (Relenza®), and two M2 channel blockers, amantadine (Symmetrel®) and rimantadine (Flumadine®). Rapid emergence of drug-resistant viral mutations has limited the use of the NA inhibitors [3,8,9] and rendered the M2 blockers ineffective [4,10–12]. It is urgent that novel anti-influenza drugs are developed.

The life cycle of influenza viruses has been well studied and nearly all the viral proteins are becoming potential therapeutic targets [13,14]. Here, we present an overview of recent progress in structure-based anti-influenza drug design, with close attention being paid to the increasing role of computation strategies that can be used for overcoming drug resistance.

Hemagglutinin

The surface glycoprotein HA attaches the viral particle to sialic acid receptors on the host cell surface for viral entry and promotes the release of viral ribonucleoprotein complexes through membrane fusion [15,16]. HA is a trimer, commonly divided into a head region and a stem region (Figure 1a); each chain is synthesized as a precursor polypeptide and then cleaved into two fragments, HA1 (328 amino acids) and HA2 (221 amino acids), linked by a disulfide bond. The 16 subtypes of HA are phylogenetically divided into two groups [17]: group 1, composed of H1, H2, H5, H6, H8, H9, H11, H12, H13, H16 subtypes; and group 2, composed of H3, H4, H7, H10, H14, H15 subtypes.

Both roles of HA in the viral life cycle have been targeted for therapeutic designs (Figure 1a). The receptor binding site is in the head region, lined by three structural elements of HA1 within each monomer, including the 190-helix (residues 188–194), the 130-loop (residues 134–138) and the 220-loop (residues 221–228) [18–21]. Virtual screening of the ZINC database (<http://zinc.docking.org/>) against an H5-subtype HA by Nandi [22], based on docking and ligand–receptor hydrogen-bonding, identified compounds **1** and **2** (Figure 1b) as potential lead molecules.

Binding of small molecules such as tert-butylhydroquinone (TBHQ; Figure 1b) to HA was found to inhibit membrane fusion [23–25]. Recent structure determination [26] identified the TBHQ binding site in a group-2 HA at the prefusion trimeric interface of the stem region. Residues lining this site include L29 {2. AU: You have used a combination of amino acid codes in this manuscript I have changed amino acids to single letter code for consistency. Please check that I have done so accurately} of HA1 and L98 and A101 of HA2 in one monomer, and L55 and L99 of HA2 in an adjacent monomer. Apparently, TBHQ binding stabilizes the prefusion trimer conformation and prevents the pH-induced conformational changes of HA1 necessary for membrane fusion. This binding site is blocked in group-1 HAs [26], but fusion inhibitors that target group-1 HAs appear to bind at a neighboring site

at the trimeric interface [24,25]. Based on similarity to these earlier ligands, Tang *et al.* [27] screened the Roche collection of ~1 million compounds. Compound **3** (Figure 1b) was found to be a potent and selective inhibitor to H1-subtype HAs.

Seasonal vaccines elicit neutralizing antibodies that recognize epitopes on the surface of the highly variable head region of HA; vaccine components are varied each year in the hope of matching the circulating viral strains in the forthcoming flu season. Recently, neutralizing antibodies that recognize highly conserved sites in the stem region have been discovered [28–30]. These antibodies promise to withstand viral mutations and provide broad neutralization. The same consideration led Fleishman *et al.* [31] to design proteins computationally that bind to a conserved surface patch in the stem region (Figure 1a). The designed proteins bound HA with nanomolar affinity and inhibited the pH-induced fusogenic conformational changes of HA. The success of the computational design was further demonstrated by the crystal structure of the complex, with a binding interface nearly identical to that in the design.

Neuraminidase

NA is another glycoprotein, a tetramer with relatively independent monomers, expressed at the surface of the influenza virus. It is responsible for releasing progeny viral particles by cleaving the terminal sialic acid from HA receptors on cell membranes [32], and facilitates the mobility of viruses in the respiratory tract [33]. Phylogenetically the nine NA subtypes can also be divided into two groups [34]: group 1, composed of N1, N4, N5 and N8 subtypes; and group 2, composed of N2, N3, N6, N7 and N9 subtypes. Based on the structures of N2 and N9 NAs [35,36] and with 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (Neu5Ac2en; Figure 2a), a naturally occurring NA inhibitor, as the template, two inhibitors, zanamivir and oseltamivir (Figure 2a), were designed [37,38] and subsequently approved by the FDA. They were among the earliest examples of structure-based drug design and represent the most successful effort in structure-based anti-influenza drug discovery [39]. Other NA inhibitors were developed subsequently, including peramivir [40] and A315675 [41] (Figure 2a) as well as oligomeric zanamivir [42].

The most common oseltamivir-resistant mutations include H274Y in group-1 NAs and R292K in group-2 NAs [8]. The latter mutation also confers partial resistance to zanamivir [8,43]. Both of the mutated positions are near the active site of the enzymes (Figure 2b). H274Y-carrying viruses and R292K-carrying viruses are susceptible to A315675, but only partially susceptible to peramivir [8].

The residues around the active site of NAs are well-conserved among the nine subtypes, and the two FDA-approved drugs are effective against group-1 and group-2 enzymes, with similar nanomolar IC₅₀s [8]. However, structure determination for group-1 NAs in 2006 [34] showed that, contrary to group-2 NAs, the 150-loop (residues 147–152) adopts an open conformation in the apo form, opening a cavity adjacent to the active site (Figure 2b). Molecular dynamics simulations have further demonstrated considerable conformational flexibility of the 150-loop [44,45]. The 150-cavity provides opportunities for designing N1-selective inhibitors, which were exploited recently by a number of computational design studies [46–51]. Notable among these is the study of Rudrawar *et al.* [51], who introduced allyl and arylallyl substituents at the C3 position of Neu5Ac2en (compound **4**, Figure 2a). These derivatives selectively inhibited group-1 NAs over group-2 NAs, and are equally potent against wild-type NA and the H274Y mutant (with micromolar IC₅₀s). In agreement with their molecular modeling prediction, the substituents indeed occupy the 150-cavity in crystal structures of the NA-inhibitor complexes. By extending the moiety at the C4 position of zanamivir, Wen *et al.* [50] also obtained a compound with a micromolar IC₅₀.

Other computational design studies [52–57] have taken advantage of the multitude of NA structures available in the Protein Data Bank (PDB). In all of these studies, including those based on the open conformation of the 150-loop, a database of compounds or a library of derivatives of an established NA inhibitor (oseltamivir and A315675 in particular) is screened using the dock-and-score approach. This approach, although providing the necessary high throughput, has ample room for improvement in accuracy [58]. Validation such as that seen in the study of Rudrawar *et al.* [51] will continue to be important.

M2 proton channel

M2 forms a tetrameric proton-selective ion channel activated by the low pH of endosomes. Each M2 monomer has only 97 residues. The N-terminal 25 residues are exterior to the {3. AU: please confirm edit here is ok} virus; the next 21 residues form a single transmembrane helix, followed by a 16-residue amphipathic helix residing at the hydrophobic–hydrophilic interface of the viral membrane inner leaflet [59] (Figure 3a); and the C-terminal 35 residues are in the viral interior. While in the endosome, inward proton conductance by M2 mediates acidification of the viral interior and facilitates uncoating of the virus after its endocytosis into the host cells. In addition, the amphipathic helix is associated with virus budding [60] and the C-terminal segment is involved in M1 binding [61].

M2 is the target of the amantadine and rimantadine drugs (Figure 3b). After a brief period of controversy about the drug binding site [62,63], it is now established that these drugs act as channel blockers, binding at a site on {4. AU: Edit ok?} the channel pore where a number of drug-resistant mutations occur [59,63–67] (Figure 3a). Amantadine, first synthesized by Prelog and Seiwerth in 1941 [68], was approved as an anti-influenza drug by the FDA in 1966. Its derivative, rimantadine received FDA approval in 1994. Owing to their side effects on the central nervous system, these drugs were not widely used. Other M2 inhibitors, including BL1743 [69] (Figure 3b), have also been identified.

High incidence of drug-resistant mutations in recent years [10,11] has led the Centers for Disease Control and Prevention (CDC) to recommend that these drugs should not be used [70]. The most common drug-resistant mutation is S31N, but V27A and L26F are also often found. Several recent design studies have targeted these mutants, using either amantadine [71–74] or BL1743 [75–78] as the template. Studies of the former {5. AU: Edit ok?} resulted in compounds that are more-potent inhibitors than amantadine against wild-type M2 but are ineffective against the amantadine-resistant mutants. The latter studies, however, resulted in several compounds, that are potent inhibitors of the V27A and the L26F mutants. Compound **5** is an example [77]. Molecular dynamics simulations suggested that the pore site in the mutants is expanded; the bulky hydrophobic group of compound **5** appears to be well accommodated there. No effective inhibitor for the most common M2 mutant, S31N, has been found.

The M2 pore-lining H37–W41 cluster (Figure 3a), through its unique chemistry, shepherds the permeant proton through the channel [59]. H37 is responsible for channel activation by low exterior pH [79] and for proton selectivity [80], whereas W41 is the channel gate that prevents outward flow of proton current [81]. Given its essential functional role, the H37–W41 cluster seems a promising target for designing inhibitors that will withstand viral mutations.

Nucleoprotein

The primary function of NP is to encapsidate the segmented RNA and bind with the three polymerase subunits, PA, PB1 and PB2, to form ribonucleoprotein particles (RNPs) for RNA transcription, replication and packaging [82]. In each RNP, the viral RNA wraps

around individual NP molecules, which are strung together by burying the ‘tail loop’ (residues 402–428) of one NP molecule (498 residues) inside an adjacent NP molecule [83–85] (Figure 4). In mature virions and when newly assembled RNPs are ready for export from the host cell nucleus, RNPs are bound with the M1 protein [86] (Figure 4).

By random screening Kao *et al.* [87] recently discovered that a compound, nucleozin (Figure 5a), induced NP aggregation, thereby inhibiting nucleus accumulation of NP and viral replication. Subsequent structure determination for NP bound with a nucleozin analog identified a NP dimer with a back-to-back arrangement between the monomers, resulting in an interface that harbors two anti-parallel ligand molecules [88]. Nucleozin-resistant mutations around the two ligand binding sites have been found [87–89].

The NP tail-loop binding pocket and the RNA binding groove have recently been targeted for rational drug design (Figure 5b). It has been established that NP oligomerization mediated by tail-loop binding is essential for the transcription and replication activity of RNPs, and that a salt bridge between E339 lining the binding pocket and R416 on the tail loop is essential for tail-loop binding [90]. Now, Shen *et al.* [91] have shown that the tail-loop peptide can inhibit NP oligomerization and slow down viral replication. By virtual screening, they have also identified compound **6** (Figure 5a) as a potent inhibitor of NP oligomerization and replication of wild-type and nucleozin-resistant strains. The latter finding unequivocally demonstrates that compound **6** and nucleozin target different NP sites.

Targeting the RNA binding groove by the dock-and-score method, Fedichev *et al.* [92] identified compound **7** (Figure 5a) as a potential NP binder. *In vitro* and *in vivo* experiments confirmed the efficacy of this compound as an influenza A virus inhibitor.

Other influenza proteins

Structures for many of the domains of the remaining proteins (Figure 6a) have now been determined. The structures harbor numerous functional sites and protein–protein and protein–RNA binding sites. These provide new targets for structure-based drug design.

RNA polymerase

The heterotrimer of PA, PB1 and PB2 forms the RNA polymerase of influenza. Once inside the nucleus of the host cell, the polymerase (as part of an RNP) first transcribes and then replicates the viral RNA. The newly synthesized viral mRNA has a 5′-capped fragment cleaved from host pre-mRNA [93]; the cleavage is afforded by the endonuclease activity of the N-terminal domain (residues 1–256) of PA [94] (Figure 6b). The C-terminal domain (residues 257–716) harbors a ‘mouth’ to which the N-terminal region (residues 1–16) of PB1 is inserted [95,96] (Figure 6b). PB1 contains the polymerase domain, but neither the structure nor the precise boundary of this domain is known. The C-terminal region (residues 678–757) of PB1 complexes with the N-terminal region (residues 1–37) of PB2 [97]. For the remainder of PB2, residues 318–483 form the cap-binding domain [98]; residues 535–684 form a putative RNA binding domain [99]; and residues 686–759 form a domain that binds human importin α 5, the classical eukaryotic nuclear import adaptor [100].

Recent screening by Iwai *et al.* [101] of 33 macrocyclic bisbibenzyl compounds identified marchantin E as an inhibitor of PA endonuclease activity. This molecule docked well into the endonuclease active site and inhibited the growth of influenza A viruses. The many protein–protein and protein–RNA interfaces involving polymerase domains provide additional target sites for drug design. In particular, a peptide corresponding to the N-terminal 25 residues of PB1 inhibits the polymerase activity and viral replication,

presumably by blocking the assembly of the polymerase trimer [102]. Small-molecule compounds can confer a similar inhibitory effect.

An alternative reading frame within the PB1 gene produces PB1-F2 [7]. This protein (ranging from 80 to 90 residues [6]) is relatively short-lived, localized in mitochondria membranes and induces cell death, but no structural information is yet available.

Matrix protein 1

In the virion, M1 forms an intermediate layer between the membrane-bound HA, NA and M2 proteins and the eight RNPs. In the nucleus of infected cells, binding of M1 to newly assembled RNPs is essential for their export [103]. M1 is also the major driving force in virus budding [104].

The structure of M1 residues 1–164 has been determined [105,106] (Figure 6c). Residues 1–67 and residues 89–164 form four-helix bundles that are stacked side by side; the linker between them also contains a short helix. No structure is available for the C-terminal region but it is known to have a significant helix content [106]. The middle domain contains the basic motif ¹⁰¹RKLKR¹⁰⁵, which is a nuclear localization signal and essential for M1 import into the nucleus of infected cells [107]. This basic motif is also involved in the binding of M1 to NEP [108].

Nuclear export protein

NEP, produced by a spliced form of the NS1 gene, is the second adaptor protein between RNPs and the cellular protein Crm1, which mediates the nuclear export of many proteins containing a leucine-rich nuclear export signal (NES) [108] (Figure 4). The N-terminal region (residues 1–54) of NEP bears the NES motif and binds Crm1. The C-terminal domain (residues 63–116) is a helical hairpin (Figure 6d). W78 has been identified as pivotal for the M1 binding site, and is surrounded by glutamate residues. The M1–NEP and NEP–Crm1 interfaces can potentially be targets for drug design.

Non-structural protein 1

The major function of NS1 is to protect viruses against the antiviral responses mediated by interferon α/β in infected cells [109]. NS1 consists of 230 or 237 residues depending on the strain, and forms a dimer. It can be divided into two functional domains: an N-terminal domain (residues 1–73) that binds double-stranded RNA [110] and a C-terminal effector domain (residues 74–230/7) that binds multiple cellular proteins [109], including CPSF30, a protein required for the 3'-end processing of all cellular pre-mRNAs [111] (Figure 6e).

Twu *et al.* [112] found that a 61-residue fragment comprising the second and third zinc fingers of CPSF30 inhibited viral replication. This fragment binds to NS1 [109]; therefore its antiviral effect suggests that inhibiting the binding of NS1 with CPSF30 opens a route for drug design. Using a yeast-based assay, Basu *et al.* [113] screened the NCI Diversity Set Library (http://dtp.nci.nih.gov/branches/dscb/diversity_explanation.html) for compounds that inhibited the actions of NS1. Four such compounds were identified, but it was unknown where they bound to NS1, or, indeed, whether they bound to NS1 directly or to cellular factors that regulated NS1.

Recombinant viruses that express mutated or truncated viral proteins can be designed as live-attenuated virus vaccines. This has now been demonstrated for NS1 [114] and NEP [115].

Concluding remarks

The 1997 H5N1 and 2009 H1N1 influenza strains and the rapid emergence of drug-resistant mutations in pandemic and seasonal influenza viruses have heightened the urgency to develop new antivirals. Advances in viral biology and structure determination for nearly all the influenza viral proteins have provided a solid foundation. Taking advantage of that, in the past few years structure-based anti-influenza drug design has been a remarkably active area of research: on the established targets (HA, NA and M2) and on the newly identified potential drug targets (in particular NP and the RNA polymerase).

Computation is now playing an ever-growing role in structure-based drug design. Virtual screening has the throughput to handle libraries containing large numbers of compounds. However, the level of accuracy is still such that experimental validation is essential. Another contribution that computation can make is to generate structural models for viral protein domains and for complexes between viral proteins themselves and between viral proteins and cellular factors.

One can but marvel at the ingenuity with which influenza viruses evade host-cell immune responses and generate drug-resistant mutations. Experience with structure-based drug design during the past three decades has resulted in valuable lessons on overcoming drug resistance. A promising strategy is to target conserved regions of viral protein structures or sites essential for viral functions. Although a note of caution should be considered when reflecting on the experience of oseltamivir and zanamivir, which targeted the well-conserved active site of NA, conserved and essential functional sites should nevertheless be a priority for future drug design.

Winning the war against influenza will probably require combination therapies that target multiple viral protein functions. The observation that *in vitro* selection of mutant viruses resistant to NA inhibitors identified many mutations in HA demonstrates that these two viral proteins compensate for each other's functions [116]. Correlated mutations in circulating viruses [6] further suggest that such compensation occurs between other viral proteins as well. A combination therapy will render viral functional compensation ineffective, providing a strong motivation to target all the viral proteins and their interactions in future drug design.

Acknowledgments

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- All flu proteins are becoming drug targets
- Computation is playing an ever-growing role in structure-based anti-flu drug design
- Strategies for overcoming drug-resistant flu virus mutations are emerging

Figure 1.

(a) Hemagglutinin (HA) trimer, bound to a sialic acid, N-acetylneuraminic acid (Neu5Ac); tert-butylhydroquinone (TBHQ); or a designed protein, HB36 (PDB entries 3M5I, 3EYK and 3R2X, respectively). Two of the HA monomers are represented as magenta and yellow surfaces, and the third as a cartoon, with HA1 and HA2 in light and dark blue, respectively. (b) Small molecules that either target the sialic acid binding site (Neu5Ac and compounds **1** and **2**) or inhibit membrane fusion (TBHQ and compound **3**).

{8. AU: I am unable to edit Fig 1. Please follow DDT house style and use sentence case throughout: i.e. Sialic acid}

Figure 2.

(a) Neuraminidase inhibitors. **(b)** The active site of N1 (light blue) and N9 (yellow) neuraminidases (PDB entries 2HU0 and 2C4A, respectively). The N1 structure has the 150-loop in the open conformation, even with oseltamivir (shown with carbons in green) bound. The 150-loop and 430-loop of N1 and N9 are shown in dark blue and in orange, respectively. N1 sidechains interacting with oseltamivir are also shown.

{9. AU: I am unable to edit Fig 2. Please follow DDT house style and close up in-house nomenclature for compounds: i.e. A315675. Because this is DDT house style please note that I have made this change in your manuscript}

Figure 3.

(a) The M2 conductance domain, comprising the transmembrane and amphipathic helices (PDB entry 2L0J). The bound amantadine (shown with carbons in magenta) is from PDB entry 2KQT. Sidechains prone to drug-resistant mutations or functionally important are shown. Yellow shade represents the viral membrane. (b) M2 channel blockers.

{ 10. AU: I am unable to edit Fig 3. Please follow DDT house style and close up in-house compound nomenclature: i.e. BL1743, please also use the correct symbol for the minus sign on Cl⁻ }

Figure 4. M1 and nuclear export protein (NEP) as adaptor proteins between a ribonucleoprotein particle and Crm1.

Figure 5.

(a) Small molecules that induce nucleoprotein (NP) aggregation (nucleozin) and target the tail-loop binding pocket (compound **6**) or the RNA binding groove (compound **7**). (b) Structure of an NP monomer, with the tail loop (in magenta) of an adjacent NP molecule buried (PDB entry 2IQH). A salt bridge important for tail-loop binding is highlighted in the inset. Arginine sidechains defining the RNA binding groove are also shown.

{ 11. AU: I am unable to edit Fig 5. Please follow DDT house style and use sentence case throughout: i.e. Tail-loop binding pocket }

Figure 6.

(a) Domain organizations of PA, PB1, PB2, M1, NEP and NS1. (b) Left: endonuclease domain of PA (PDB entry 3HW5). The Mg²⁺ ion and the surrounding residues defining the active site are shown. Right: C-terminal domain of PA bound with the N-terminal region of PB1 (PDB entry 3CM8). (c) The N-terminal (light blue) and middle (cyan) domains of M1 (PDB entry 1EA3). Cationic residues, including those in the nuclear localization signal motif ¹⁰¹RKLKR¹⁰⁵ are displayed. These residues are involved in NEP binding. (d) The C-terminal domain of NEP (PDB entry 1PD3). W78 and surrounding anionic residues involved in M1 binding are shown. (e) Left: N-terminal domain of NS1 bound to double-stranded RNA (PDB entry 2ZKO). Two cationic residues in the binding interface are displayed. Right: effector domain of NS1 bound to two copies of the second and third zinc fingers of CPSF30 (PDB entry 2RHK). The zinc fingers are in yellow and green.

Table 1

Functions, binding sites and inhibitors of influenza proteins

Proteins	Functions	Binding sites	Inhibitors
HA	Virus attachment to sialic acid receptors on host cell surface; fusion of virus and cell membranes	Sialic acid binding site; TBHQ binding site	TBHQ
NA	Cleavage of sialic acid receptors to release progeny viruses from host cells	Active site	Zanamivir; oseltamivir
M2	Acidification and uncoating of endosome-entrapped virus; virus assembly and budding	Inside pore near Ser31	Amantadine; rimantadine
NP	Capsidation of viral RNA and binding of three polymerase subunits to form ribonucleoprotein particles	Tail-loop binding site; RNA binding site	{6. AU: are there none available? If none available please state here and hereafter for absolute clarity in Table 1}
Polymerase	Viral RNA transcription and replication	PA: endonuclease active site; binding site for PB1 PB1: polymerase active site PB2: cap binding site; importing binding site	
M1	Structural component of virion; nuclear export of ribonucleoprotein particles	NEP binding site	
NEP	Nuclear export of ribonucleoprotein particles from host-cell nucleus	Crm1 binding site; M1 binding site	
NS1	Protection against host-cell antiviral responses	Double-stranded RNA binding site; CPSF30 binding site	