ELSEVIER

Recent progress in structure-based anti-influenza drug design

Juan Du[1,3], Timothy A. Cross[2,3,4] and Huan-Xiang Zhou[1,3]

[1] Department of Physics, Florida State University, Tallahassee, FL 32306, USA
[2] Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306, USA
[3] Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306, USA
[4] National High Magnetic Field Laboratory, 1800 E. Paul Dirac Dr., Tallahassee, FL 32310, USA

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 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–H16) and nine NA subtypes (N1–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 strategies for combating influenza infection. 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

Corresponding author:. Zhou, H.-X. (hzhou4@fsu.edu)

1359-6446/06/$ - see front matter http://dx.doi.org/10.1016/j.drudis.2012.06.002

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	Neu5Ac; 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	–[a]
Polymerase	Viral RNA transcription and replication	PA: endonuclease active site; PB1 binding site 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	–

[a] For the last five entries, no inhibitors have been firmly established, but leads have been proposed in some cases (see text).

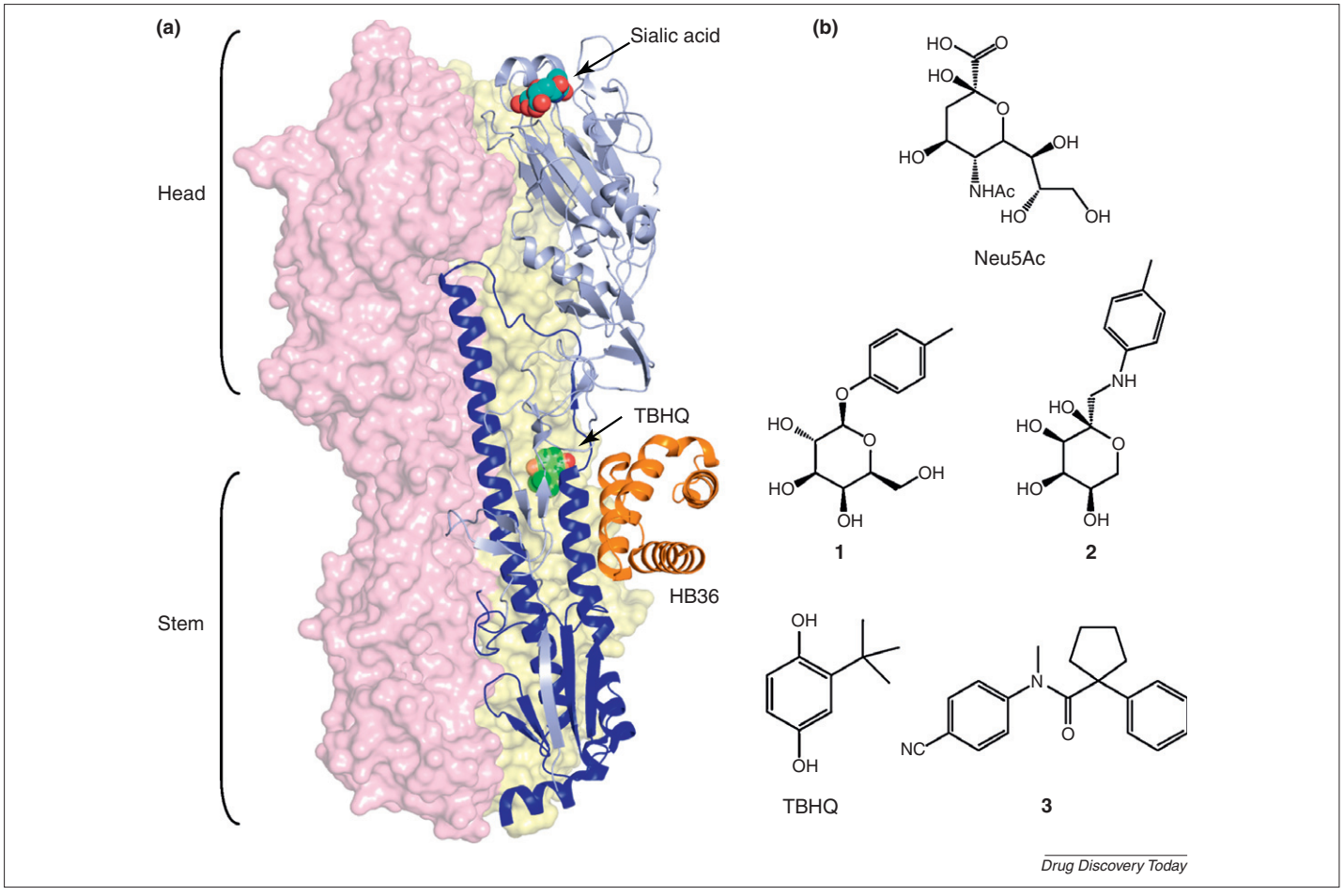

FIG. 1

Hemagglutinin (HA) and its inhibitors. **(a)** 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**).

being paid to the increasing role of computation and 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 (Fig. 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 (Fig. 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** (Fig. 1b) as potential lead molecules.

Binding of small molecules such as tert-butylhydroquinone (TBHQ; Fig. 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 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** (Fig. 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 (Fig. 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; Fig. 2a), a naturally occurring NA inhibitor, as the template, two inhibitors, zanamivir and oseltamivir (Fig. 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] (Fig. 2a) as well as oligomeric zanamivir [42].

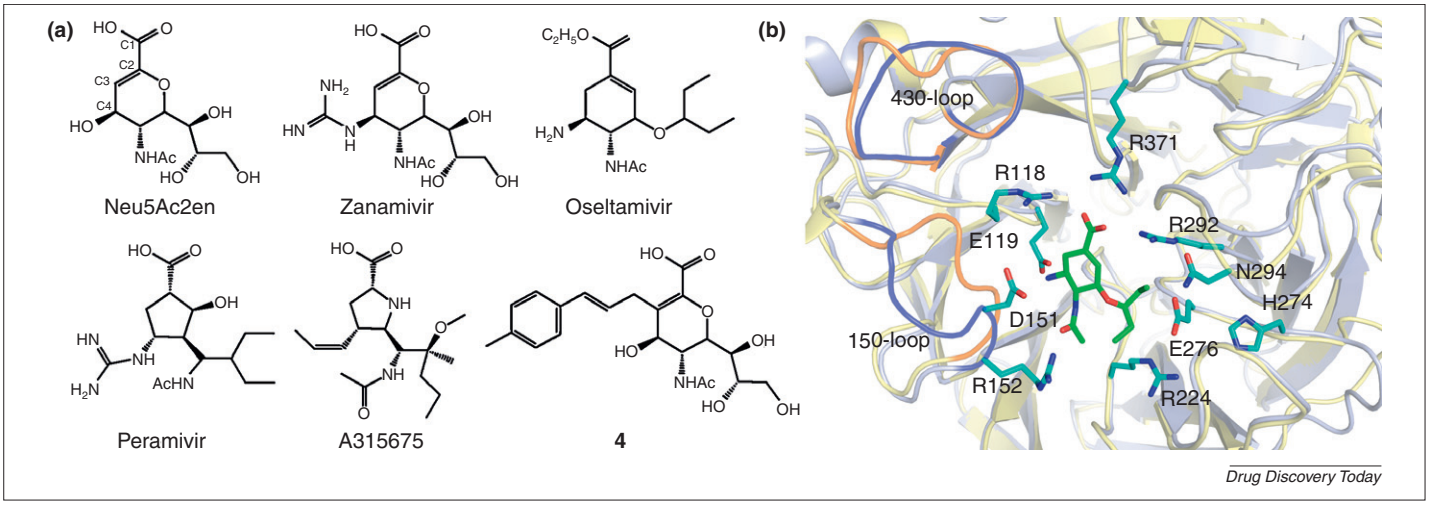

FIG. 2

Neuraminidase (NA) and its inhibitors. **(a)** NA inhibitors. **(b)** The active site of N1 (light blue) and N9 (yellow) NAs (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.

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 (Fig. 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 IC_{50}s [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 (Fig. 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 several 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**, Fig. 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 IC_{50}s). 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 IC_{50}.

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 viral membrane; 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] (Fig. 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 (Fig. 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 in the channel pore where several drug-resistant mutations occur [59,63–67] (Fig. 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] (Fig. 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 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. The former studies resulted in compounds that are more-potent inhibitors than amantadine against wild-type M2 but are ineffective against the amantadine-resistant mutants.

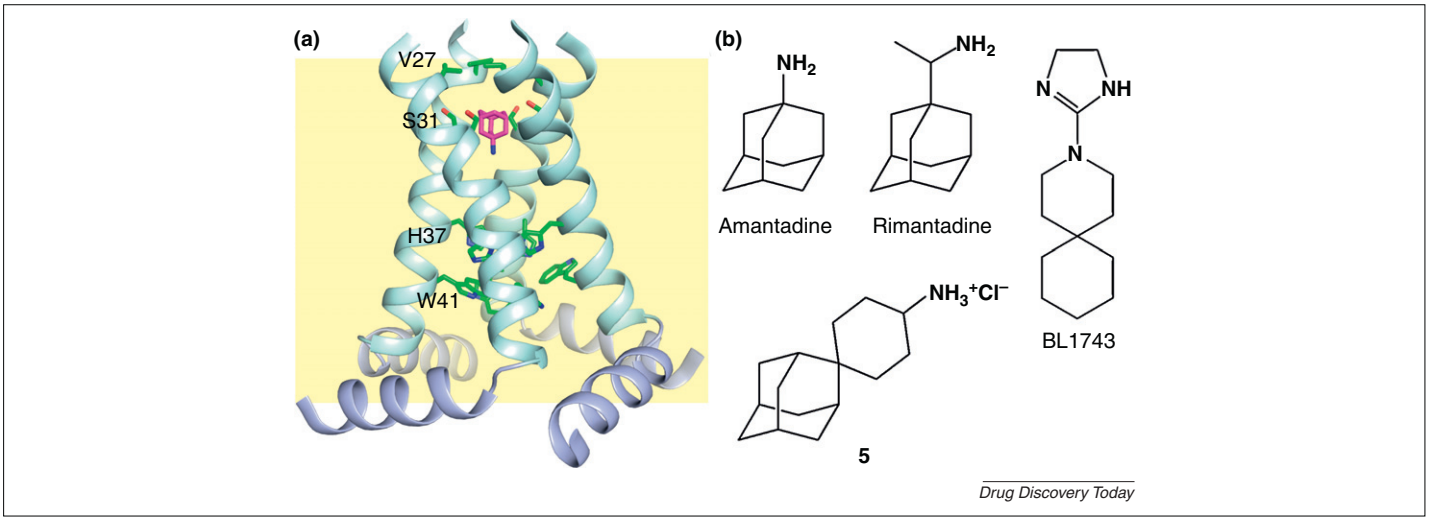

FIG. 3

M2 and its inhibitors. **(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.

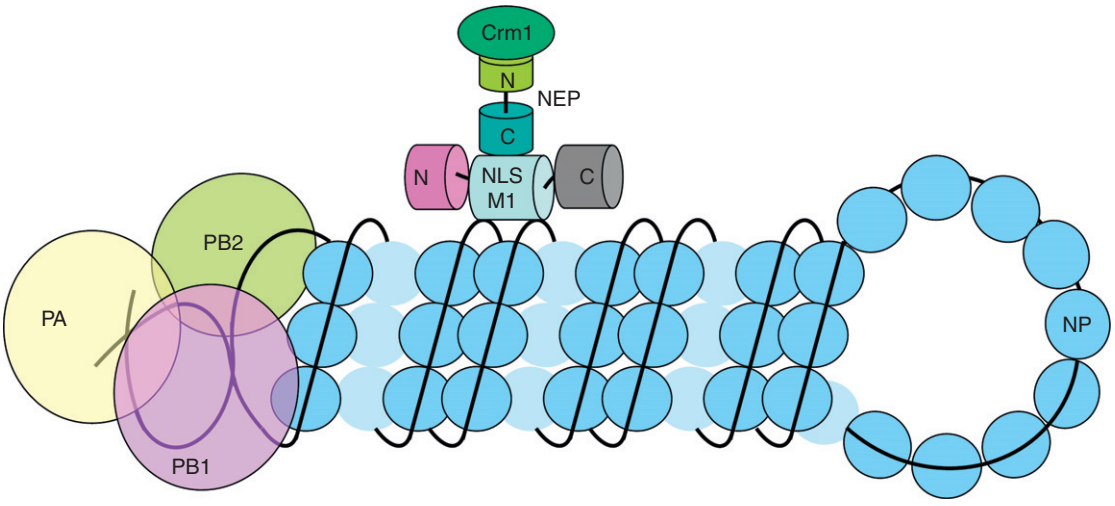

FIG. 4

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

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 (Fig. 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] (Fig. 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] (Fig. 4).

By random screening Kao *et al.* [87] recently discovered that a compound, nucleozin (Fig. 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 (Fig. 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** (Fig. 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** (Fig. 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 (Fig. 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] (Fig. 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] (Fig. 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

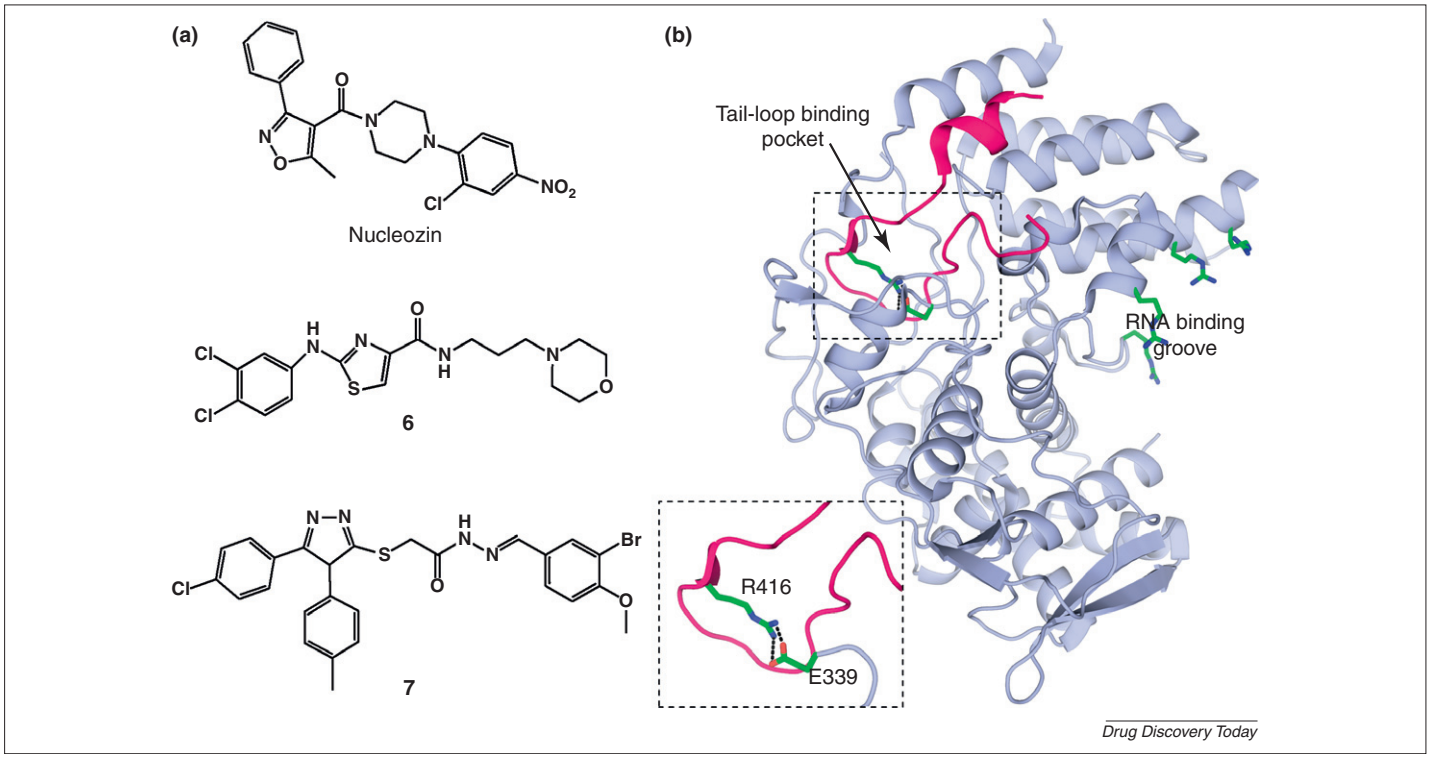

FIG. 5

Nucleoprotein (NP) and its potential inhibitors. **(a)** Small molecules that induce 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.

domain [99]; and residues 686–759 form a domain that binds human importin α5, 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] (Fig. 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 ^{101}RKLKR^{105}, 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] (Fig. 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 (Fig. 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] (Fig. 6e).

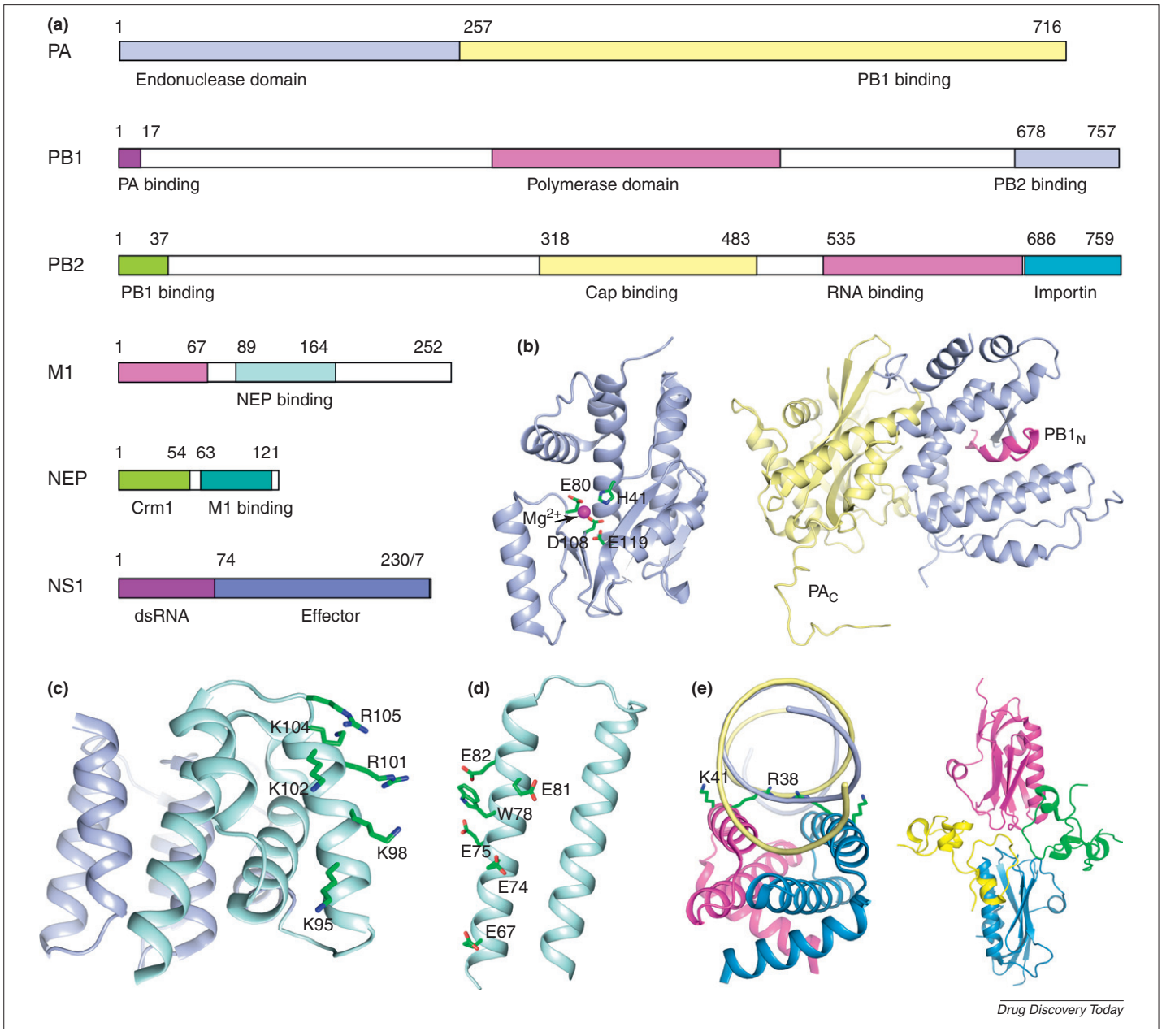

FIG. 6

Domain organizations and structures of six viral proteins. **(a)** Domain organizations of PA, PB1, PB2, M1, NEP and NS1. **(b)** Left: endonuclease domain of PA (PDB entry 3HW5). The Mg^{2+} 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 ^{101}RKLKR^{105} 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.

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, both 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

This work was supported in part by Grants GM58187 and AI23007 from the National Institutes of Health.

References

1 World Health Organization Website. http://www.who.int/
2 Cox, N.J. and Subbarao, K. (2000) Global epidemiology of influenza: past and present. *Annu. Rev. Med.* 51, 407–421
3 Gubareva, L.V. *et al.* (2001) Selection of influenza virus mutants in experimentally infected volunteers treated with oseltamivir. *J. Infect. Dis.* 183, 523–531
4 Gubareva, L. *et al.* (2009) Update: drug susceptibility of swine-origin influenza A (H1N1) viruses, April 2009. *Morb. Mortal. Wkly. Rep.* 58, 433–435
5 Palese, P. and Young, J.F. (1982) Variation of influenza A, B, and C viruses. *Science* 215, 1468–1474
6 Ghedin, E. *et al.* (2005) Large-scale sequencing of human influenza reveals the dynamic nature of viral genome evolution. *Nature* 437, 1162–1166
7 Chen, W. *et al.* (2001) A novel influenza A virus mitochondrial protein that induces cell death. *Nat. Med.* 7, 1306–1312
8 Mishin, V.P. *et al.* (2005) Susceptibilities of antiviral-resistant influenza viruses to novel neuraminidase inhibitors. *Antimicrob. Agents Chemother.* 49, 4515–4520
9 Meijer, A. *et al.* (2009) Oseltamivir-resistant influenza virus A (H1N1), Europe, 2007–08 season. *Emerg. Infect. Dis.* 15, 552–560
10 Bright, R.A. *et al.* (2006) Adamantane resistance among influenza A viruses isolated early during the 2005–2006 influenza season in the United States. *J. Am. Med. Assoc.* 295, 891–894
11 Deyde, V.M. *et al.* (2007) Surveillance of resistance to adamantanes among influenza A(H3N2) and A(H1N1) viruses isolated worldwide. *J. Infect. Dis.* 196, 249–257
12 Furuse, Y. *et al.* (2009) Large-scale sequence analysis of M gene of influenza A viruses from different species: mechanisms for emergence and spread of amantadine resistance. *Antimicrob. Agents Chemother.* 53, 4457–4463
13 Krug, R.M. and Aramini, J.M. (2009) Emerging antiviral targets for influenza A virus. *Trends Pharmacol. Sci.* 30, 269–277
14 Das, K. *et al.* (2010) Structures of influenza A proteins and insights into antiviral drug targets. *Nat. Struct. Mol. Biol.* 17, 530–538
15 Skehel, J.J. and Wiley, D.C. (2000) Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu. Rev. Biochem.* 69, 531–569
16 Harrison, S.C. (2008) Viral membrane fusion. *Nat. Struct. Mol. Biol.* 15, 690–698
17 Nobusawa, E. *et al.* (1991) Comparison of complete amino acid sequences and receptor-binding properties among 13 serotypes of hemagglutinins of influenza A viruses. *Virology* 182, 475–485
18 Ha, Y. *et al.* (2001) X-ray structures of H5 avian and H9 swine influenza virus hemagglutinins bound to avian and human receptor analogs. *Proc. Natl. Acad. Sci. U. S. A.* 98, 11181–11186
19 Gamblin, S.J. *et al.* (2004) The structure and receptor binding properties of the 1918 influenza hemagglutinin. *Science* 303, 1838–1842
20 Stevens, J. *et al.* (2006) Structure and receptor specificity of the hemagglutinin from an H5N1 influenza virus. *Science* 312, 404–410
21 Yang, H. *et al.* (2010) Structures of receptor complexes of a North American H7N2 influenza hemagglutinin with a loop deletion in the receptor binding site. *PLoS Pathog.* 6, e1001081
22 Nandi, T. (2008) Proposed lead molecules against hemagglutinin of avian influenza virus (H_5N_1). *Bioinformation* 2, 240–244
23 Bodian, D.L. *et al.* (1993) Inhibition of the fusion-inducing conformational change of influenza hemagglutinin by benzoquinones and hydroquinones. *Biochemistry* 32, 2967–2978
24 Staschke, K.A. *et al.* (1998) Inhibition of influenza virus hemagglutinin-mediated membrane fusion by a compound related to podocarpic acid. *Virology* 248, 264–274
25 Deshpande, M.S. *et al.* (2001) An approach to the identification of potent inhibitors of influenza virus fusion using parallel synthesis methodology. *Bioorg. Med. Chem. Lett.* 11, 2393–2396
26 Russell, R.J. *et al.* (2008) Structure of influenza hemagglutinin in complex with an inhibitor of membrane fusion. *Proc. Natl Acad. Sci. U. S. A.* 105, 17736–17741
27 Tang, G. *et al.* (2010) Discovery of novel 1-phenyl-cycloalkane carbamides as potent and selective influenza fusion inhibitors. *Bioorg. Med. Chem. Lett.* 20, 3507–3510
28 Ekiert, D.C. *et al.* (2009) Antibody recognition of a highly conserved influenza virus epitope. *Science* 324, 246–251
29 Sui, J. *et al.* (2009) Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat. Struct. Mol. Biol.* 16, 265–273
30 Corti, D. *et al.* (2011) A neutralizing antibody selected from plasma cells that binds to group 1 and group 2 influenza A hemagglutinins. *Science* 333, 850–856
31 Fleishman, S.J. *et al.* (2011) Computational design of proteins targeting the conserved stem region of influenza hemagglutinin. *Science* 332, 816–821
32 Palese, P. and Compans, R.W. (1976) Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA): mechanism of action. *J. Gen. Virol.* 33, 159–163
33 Klenk, H.D. and Rott, R. (1988) The molecular biology of influenza virus pathogenicity. *Adv. Virus Res.* 34, 247–281
34 Russell, R.J. *et al.* (2006) The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. *Nature* 443, 45–49
35 Varghese, J.N. *et al.* (1992) The structure of the complex between influenza virus neuraminidase and sialic acid, the viral receptor. *Proteins* 14, 327–332
36 White, C.L. *et al.* (1995) A sialic acid-derived phosphonate analog inhibits different strains of influenza virus neuraminidase with different efficiencies. *J. Mol. Biol.* 245, 623–634
37 von Itzstein, M. *et al.* (1993) Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* 363, 418–423
38 Kim, C.U. *et al.* (1997) Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: design, synthesis, and

structural analysis of carbocyclic sialic acid analogues with potent anti-influenza activity. *J. Am. Chem. Soc.* 119, 681–690
39 von Itzstein, M. (2007) The war against influenza: discovery and development of sialidase inhibitors. *Nat. Rev. Drug Discov.* 6, 967–974
40 Babu, Y.S. *et al.* (2000) BCX-1812 (RWJ-270201): discovery of a novel, highly potent, orally active, and selective influenza neuraminidase inhibitor through structure-based drug design. *J. Med. Chem.* 43, 3482–3486
41 Kati, W.M. *et al.* (2002) *In vitro* characterization of A-315675, a highly potent inhibitor of A and B strain influenza virus neuraminidases and influenza virus replication. *Antimicrob. Agents Chemother.* 46, 1014–1021
42 Watson, K.G. *et al.* (2004) Highly potent and long-acting trimeric and tetrameric inhibitors of influenza virus neuraminidase. *Bioorg. Med. Chem. Lett.* 14, 1589–1592
43 Yen, H.L. *et al.* (2005) Neuraminidase inhibitor-resistant influenza viruses may differ substantially in fitness and transmissibility. *Antimicrob. Agents Chemother.* 49, 4075–4084
44 Amaro, R.E. *et al.* (2007) Remarkable loop flexibility in avian influenza N1 and its implications for antiviral drug design. *J. Am. Chem. Soc.* 129, 7764–7765
45 Amaro, R.E. *et al.* (2011) Mechanism of 150-cavity formation in influenza neuraminidase. *Nat. Commun.* 2, 388
46 García-Sosa, A.T. *et al.* (2008) Design of multi-binding-site inhibitors, ligand efficiency, and consensus screening of avian influenza H5N1 wild-type neuraminidase and of the oseltamivir-resistant H274Y variant. *J. Chem. Inf. Model.* 48, 2074–2080
47 Cheng, L.S. *et al.* (2008) Ensemble-based virtual screening reveals potential novel antiviral compounds for avian influenza neuraminidase. *J. Med. Chem.* 51, 3878–3894
48 Li, Y. *et al.* (2009) Rational design of Tamiflu derivatives targeting at the open conformation of neuraminidase subtype 1. *J. Mol. Graph. Model.* 28, 203–219
49 Mitrasinovic, P.M. (2009) On the structure-based design of novel inhibitors of H5N1 influenza A virus neuraminidase (NA). *Biophys. Chem.* 140, 35–38
50 Wen, W-H. *et al.* (2010) Analogs of zanamivir with modified C4-substituents as the inhibitors against the group-1 neuraminidases of influenza viruses. *Bioorg. Med. Chem.* 18, 4074–4084
51 Rudrawar, S. *et al.* (2010) Novel sialic acid derivatives lock open the 150-loop of an influenza A virus group-1 sialidase. *Nat. Commun.* 1, 113
52 An, J. *et al.* (2009) A novel small-molecule inhibitor of the avian influenza H5N1 virus determined through computational screening against the neuraminidase. *J. Med. Chem.* 52, 2667–2672
53 Rungrotmongkol, T. *et al.* (2009) Design of oseltamivir analogs inhibiting neuraminidase of avian influenza virus H5N1. *Antivir. Res.* 82, 51–58
54 Park, J.W. and Jo, W.H. (2010) Computational design of novel, high-affinity neuraminidase inhibitors for H5N1 avian influenza virus. *Eur. J. Med. Chem.* 45, 536–541
55 Wang, S-Q. *et al.* (2010) Three new powerful oseltamivir derivatives for inhibiting the neuraminidase of influenza virus. *Biochem. Biophys. Res. Commun.* 401, 188–191
56 Durrant, J.D. and McCammon, J.A. (2010) Potential drug-like inhibitors of Group 1 influenza neuraminidase identified through computer-aided drug design. *Comput. Biol. Chem.* 34, 97–105
57 Rungrotmongkol, T. *et al.* (2010) Combinatorial design of avian influenza neuraminidase inhibitors containing pyrrolidine core with a reduced susceptibility to viral drug resistance. *Comb. Chem. High Throughput Screen.* 13, 268–277
58 Gilson, M.K. and Zhou, H.X. (2007) Calculation of protein–ligand binding affinities. *Annu. Rev. Biophys. Biomol. Struct.* 36, 21–42
59 Sharma, M. *et al.* (2010) Insight into the mechanism of the influenza A proton channel from a structure in a lipid bilayer. *Science* 330, 509–512
60 Rossman, J.S. and Lamb, R.A. (2011) Influenza virus assembly and budding. *Virology* 411, 229–236
61 Chen, B.J. *et al.* (2008) The influenza virus M2 protein cytoplasmic tail interacts with the M1 protein and influences virus assembly at the site of virus budding. *J. Virol.* 82, 10059–10070
62 Schnell, J.R. and Chou, J.J. (2008) Structure and mechanism of the M2 proton channel of influenza A virus. *Nature* 451, 591–595
63 Stouffer, A.L. *et al.* (2008) Structural basis for the function and inhibition of an influenza virus proton channel. *Nature* 451, 596–599
64 Hu, J. *et al.* (2007) Backbone structure of the amantadine-blocked trans-membrane domain M2 proton channel from influenza A virus. *Biophys. J.* 92, 4335–4343
65 Yi, M. *et al.* (2008) A secondary gate as a mechanism for inhibition of the M2 proton channel by amantadine. *J. Phys. Chem. B* 112, 7977–7979
66 Cady, S.D. *et al.* (2010) Structure of the amantadine binding site of influenza M2 proton channels in lipid bilayers. *Nature* 463, 689–692
67 Cross, T.A. *et al.* (2012) M2 protein from influenza A: from multiple structures to biophysical and functional insights. *Curr. Opin. Virol.* 2, 128–133
68 Prelog, V. and Seiwerth, R. (1941) Über eine neue, ergiebigere Darstellung des Adamantans. *Ber. Dtsch. Chem. Ges. A/B* 74, 1769–1772
69 Kurtz, S. *et al.* (1995) Growth impairment resulting from expression of influenza virus M2 protein in *Saccharomyces cerevisiae*: identification of a novel inhibitor of influenza virus. *Antimicrob. Agents Chemother.* 39, 2204–2209
70 Bright, R.A. *et al.* (2006) High levels of adamantane resistance among influenza A (H3N2) viruses and interim guidelines for use of antiviral agents—United States, 2005–06 influenza season. *Morb. Mortal. Wkly. Rep.* 55, 44–46
71 Hu, W. *et al.* (2010) Identification of hits as Matrix-2 protein inhibitors through the focused screening of a small primary amine library. *J. Med. Chem.* 53, 3831–3834
72 Zhao, X. *et al.* (2011) Discovery of highly potent agents against influenza A virus. *Eur. J. Med. Chem.* 46, 52–57
73 Wang, J. *et al.* (2011) Exploring the requirements for the hydrophobic scaffold and polar amine in inhibitors of M2 from influenza A virus. *ACS Med. Chem. Lett.* 2, 307–312
74 Duque, M.D. *et al.* (2011) Exploring the size limit of templates for inhibitors of the M2 ion channel of influenza A virus. *J. Med. Chem.* 54, 2646–2657
75 Wang, J. *et al.* (2009) Discovery of spiro-piperidine inhibitors and their modulation of the dynamics of the M2 proton channel from influenza A virus. *J. Am. Chem. Soc.* 131, 8066–8076
76 Balannik, V. *et al.* (2009) Design and pharmacological characterization of inhibitors of amantadine-resistant mutants of the M2 ion channel of influenza A virus. *Biochemistry* 48, 11872–11882
77 Wang, J. *et al.* (2011) Molecular dynamics simulation directed rational design of inhibitors targeting drug-resistant mutants of influenza A virus M2. *J. Am. Chem. Soc.* 133, 12834–12841
78 Wang, J. *et al.* (2011) Exploring organosilane amines as potent inhibitors and structural probes of influenza A virus M2 proton channel. *J. Am. Chem. Soc.* 133, 13844–13847
79 Hu, J. *et al.* (2006) Histidines, heart of the hydrogen ion channel from influenza A virus: toward an understanding of conductance and proton selectivity. *Proc. Natl Acad. Sci. U. S. A.* 103, 6865–6870
80 Venkataraman, P. *et al.* (2005) Chemical rescue of histidine selectivity filter mutants of the M2 ion channel of influenza A virus. *J. Biol. Chem.* 280, 21463–21472
81 Tang, Y. *et al.* (2002) The gate of the influenza virus M2 proton channel is formed by a single tryptophan residue. *J. Biol. Chem.* 277, 39880–39886
82 Portela, A. and Digard, P. (2002) The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication. *J. Gen. Virol.* 83, 723–734
83 Ye, Q. *et al.* (2006) The mechanism by which influenza A virus nucleoprotein forms oligomers and binds RNA. *Nature* 444, 1078–1082
84 Ng, A.K-L. *et al.* (2008) Structure of the influenza virus A H5N1 nucleoprotein: implications for RNA binding, oligomerization, and vaccine design. *FASEB J.* 22, 3638–3647
85 Coloma, R. *et al.* (2009) The structure of a biologically active influenza virus ribonucleoprotein complex. *PLoS Pathog.* 5, e1000491
86 Boulo, S. *et al.* (2007) Nuclear traffic of influenza virus proteins and ribonucleoprotein complexes. *Virus Res.* 124, 12–21
87 Kao, R.Y. *et al.* (2010) Identification of influenza A nucleoprotein as an antiviral target. *Nat. Biotechnol.* 28, 600–605
88 Gerritz, S.W. *et al.* (2011) Inhibition of influenza virus replication via small molecules that induce the formation of higher-order nucleoprotein oligomers. *Proc. Natl Acad. Sci. U. S. A.* 108, 15366–15371
89 Su, C-Y. *et al.* (2010) High-throughput identification of compounds targeting influenza RNA-dependent RNA polymerase activity. *Proc. Natl Acad. Sci. U. S. A.* 107, 19151–19156
90 Chan, W.H. *et al.* (2010) Functional analysis of the influenza virus H5N1 nucleoprotein tail loop reveals amino acids that are crucial for oligomerization and ribonucleoprotein activities. *J. Virol.* 84, 7337–7345
91 Shen, Y-F. *et al.* (2011) E339. . .R416 salt bridge of nucleoprotein as a feasible target for influenza virus inhibitors. *Proc. Natl Acad. Sci. U. S. A.* 108, 16515–16520
92 Fedichev, P. *et al.* (2011) Structure-based drug design of a new chemical class of small molecules active against influenza A nucleoprotein *in vitro* and *in vivo*. *PLoS Curr.* RRN1253
93 Krug, R.M. *et al.* (1979) Are the 5′ ends of influenza viral mRNAs synthesized in vivo donated by host mRNAs? *Cell* 18, 329–334

94 Zhao, C. *et al.* (2009) Nucleoside monophosphate complex structures of the endonuclease domain from the influenza virus polymerase PA subunit reveal the substrate binding site inside the catalytic center. *J. Virol.* 83, 9024–9030

95 He, X. *et al.* (2008) Crystal structure of the polymerase PA(C)–PB1(N) complex from an avian influenza H5N1 virus. *Nature* 454, 1123–1126

96 Obayashi, E. *et al.* (2008) The structural basis for an essential subunit interaction in influenza virus RNA polymerase. *Nature* 454, 1127–1131

97 Sugiyama, K. *et al.* (2009) Structural insight into the essential PB1-PB2 subunit contact of the influenza virus RNA polymerase. *EMBO J.* 28, 1803–1811

98 Guilligay, D. *et al.* (2008) The structural basis for cap binding by influenza virus polymerase subunit PB2. *Nat. Struct. Mol. Biol.* 15, 500–506

99 Kuzuhara, T. *et al.* (2009) Structural basis of the influenza A virus RNA polymerase PB2 RNA-binding domain containing the pathogenicity-determinant lysine 627 residue. *J. Biol. Chem.* 284, 6855–6860

100 Tarendeau, F. *et al.* (2007) Structure and nuclear import function of the C-terminal domain of influenza virus polymerase PB2 subunit. *Nat. Struct. Mol. Biol.* 14, 229–233

101 Iwai, Y. *et al.* (2011) Anti-influenza activity of marchantins, macrocyclic bisbibenzyls contained in liverworts. *PLoS One* 6, e19825

102 Ghanem, A. *et al.* (2007) Peptide-mediated interference with influenza A virus polymerase. *J. Virol.* 81, 7801–7804

103 Martin, K. and Heleniust, A. (1991) Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. *Cell* 67, 117–130

104 Gómez-Puertas, P. *et al.* (2000) Influenza virus matrix protein is the major driving force in virus budding. *J. Virol.* 74, 11538–11547

105 Sha, B. and Luo, M. (1997) Structure of a bifunctional membrane-RNA binding protein, influenza virus matrix protein M1. *Nat. Struct. Biol.* 4, 239–244

106 Arzt, S. *et al.* (2001) Combined results from solution studies on intact influenza virus M1 protein and from a new crystal form of its N-terminal domain show that M1 is an elongated monomer. *Virology* 279, 439–446

107 Liu, T. and Ye, Z. (2002) Restriction of viral replication by mutation of the influenza virus matrix protein. *J. Virol.* 76, 13055–13061

108 Akarsu, H. *et al.* (2003) Crystal structure of the M1 protein-binding domain of the influenza A virus nuclear export protein (NEP/NS2). *EMBO J.* 22, 4646–4655

109 Hale, B.G. *et al.* (2008) The multifunctional NS1 protein of influenza A viruses. *J. Gen. Virol.* 89, 2359–2376

110 Cheng, A. *et al.* (2009) Structural basis for dsRNA recognition by NS1 protein of influenza A virus. *Cell Res.* 19, 187–195

111 Das, K. *et al.* (2008) Structural basis for suppression of a host antiviral response by influenza A virus. *Proc. Natl Acad. Sci. U. S. A.* 105, 13093–13098

112 Twu, K.Y. *et al.* (2006) The CPSF30 binding site on the NS1A protein of influenza A virus is a potential antiviral target. *J. Virol.* 80, 3957–3965

113 Basu, D. *et al.* (2009) Novel influenza virus NS1 antagonists block replication and restore innate immune function. *J. Virol.* 83, 1881–1891

114 Talon, J. *et al.* (2000) Influenza A and B viruses expressing altered NS1 proteins: a vaccine approach. *Proc. Natl Acad. Sci. U. S. A.* 97, 4309–4314

115 Akarsu, H. *et al.* (2011) Structure-based design of NS2 mutants for attenuated influenza A virus vaccines. *Virus Res.* 155, 240–248

116 McKimm-Breschkin, J.L. (2000) Resistance of influenza viruses to neuraminidase inhibitors—a review. *Antiviral. Res.* 47, 1–17