The Influenza Virus Polymerase Complex: An Update on Its Structure, Functions, and Significance for Antiviral Drug Design

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Abstract: Influenza viruses cause seasonal epidemics and pandemic outbreaks associated with significant morbidity and mortality, and a huge cost. Since resistance to the existing anti-influenza drugs is rising, innovative inhibitors with a different mode of action are urgently needed. The influenza polymerase complex is widely recognized as a key drug target, given its critical role in virus replication and high degree of conservation among influenza A (of human or zoonotic origin) and B viruses. We here review the major progress that has been made in recent years in unravelling the structure and functions of this protein complex, enabling structure-aided drug design toward the core regions of the PA endonuclease, PB1 polymerase, or cap-binding PB2 subunit. Alternatively, inhibitors may target a protein–protein interaction site, a cellular factor involved in viral RNA synthesis, the viral RNA itself, or the nucleoprotein component of the viral ribonucleoprotein. The latest advances made for these diverse pharmacological targets have yielded agents in advanced (i.e., favipiravir and VX-787) or early clinical testing, besides several experimental inhibitors in various stages of development, which are all covered here. Med. Res. Rev., 36, No. 6, 1127–1173, 2016

Key words: influenza virus; antiviral; polymerase; cap-snatching; nucleoside

1. INTRODUCTION

The influenza A, B, C, and D viruses belong to the Orthomyxoviridae, a family of enveloped viruses with a single-stranded negative-sense, and segmented RNA genome. Seasonal influenza A and B viruses affect each year approximately 5–10% of the adult and 20–30% of the pediatric population. In addition, there is permanent concern for sudden influenza pandemics, such as that of 2009 or the notorious "Spanish flu" of 1918.

A universal influenza vaccine that confers broad and long-term protection remains the "Holy Grail" in influenza research,[1] as vaccination is considered as the most effective way

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to prevent influenza virus infections. The existing tri- or quadrivalent vaccines contain the circulating A/H1N1, A/H3N2, and (one or two) B strains. They fail to provide broadly protective and long-lasting immunity, require annual updating, and are only partially protective in some target populations.[2,3]

For prevention or treatment of influenza virus infections, two classes of antiviral drugs are currently available. The M2-blockers, **amantadine** and **rimantadine**, inhibit influenza A virus replication by occluding the M2 proton channel,[4] but lack activity against influenza B virus.[5] In addition, central nervous system side effects[6] and the global spread of adamantane-resistant influenza viruses[7] undermine their clinical usefulness. The second group, the neuraminidase inhibitors (NAIs), prevents virus release from infected cells. **Zanamivir** requires administration by powder inhalation, while **oseltamivir** is administered orally as its prodrug oseltamivir phosphate. In the period 2007–2009, oseltamivir-resistant influenza H1N1 viruses were disseminated worldwide.[8] In contrast, resistance to zanamivir seems to be rare.[9,10] The search for novel M2-inhibitors and NAIs with improved resistance profile and higher potency is still ongoing, as is further optimization of original lead compounds.[11]

In conclusion, the armamentarium to prevent and treat influenza infections is currently limited, and new influenza virus inhibitors with an entirely different mode of action are urgently required. In this review, we focus on the influenza virus polymerase that is widely recognized as a superior antiviral drug target,[12] given its critical role in virus replication and high degree of sequence conservation in influenza A and B, particularly in the active sites for RNA binding, cleavage, or elongation. Before explaining the different antiviral strategies that are being explored, we provide relevant background on structure and functions of the viral polymerase. During recent years, major breakthroughs have been made in revealing the structure of this protein complex, its subunits, or the associated viral nucleoprotein (NP), creating unique opportunities for structure-aided drug design.

2. STRUCTURE AND FUNCTIONS OF THE INFLUENZA VIRUS POLYMERASE COMPLEX

To avoid overlap with other recent reviews on this topic,[13–16] this section is limited to provide an update on new findings with direct or indirect relevance for inhibitor design. The influenza virus polymerase complex (schematically depicted in Fig. 1) is composed of three subunits: PB1, PB2 (polymerase basic protein-1 and -2, respectively) and PA (polymerase acidic protein; referred to as P3 in the case of influenza C). The influenza A and B virions contain eight viral ribonucleoprotein (vRNP) segments, which have a double-helical hairpin structure and each carry one polymerase heterotrimer. These vRNPs contain vRNA that is coated by viral NP molecules. Viral RNA synthesis occurs in the cell nucleus in two stages (Fig. 1). (i) For primary transcription of vRNA to mRNA, primers are generated by the PA-dependent "cap-snatching" reaction on cellular capped RNAs. The PB2 subunit initially binds the capped RNAs, and actual RNA synthesis is performed by PB1. (ii) Replication of vRNA proceeds via complementary RNA (cRNA) intermediates and occurs in a primer-independent (de novo) manner. The nascent vRNA and cRNA replicates are immediately packaged into new vRNPs or cRNPs, respectively. The newly synthesized vRNP complexes are exported from the nucleus to the cytoplasm, for which different pathways have been proposed (reviewed in Eisfeld et al.[14]).

The influenza virus polymerase is a major determinant of virus pathogenicity and host adaptation.[26–28] One notable key factor is the PB2-627 signature residue, which is glutamic acid in avian viruses and lysine in human viruses, and was recently linked to species differences in the host nuclear protein ANP32A.[29]

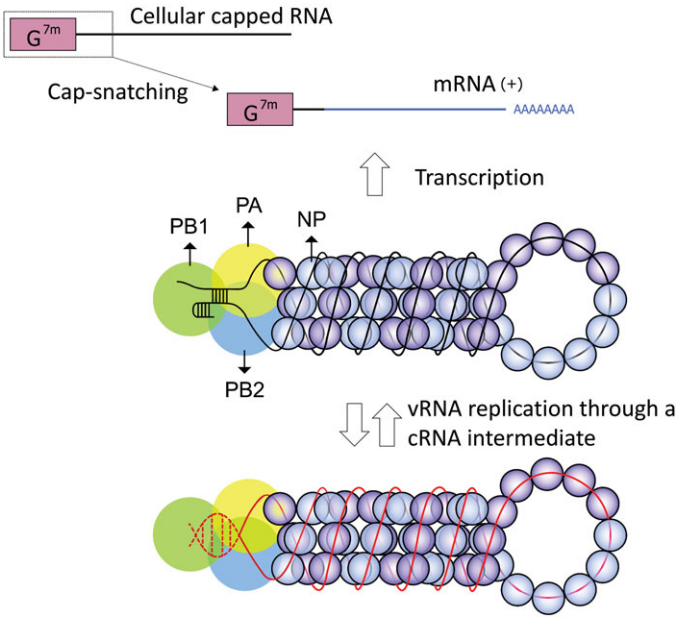

Figure 1. Role of the influenza virus polymerase complex in vRNA transcription and replication.
The influenza polymerase is a complex containing the PA, PB1, and PB2 proteins. One polymerase heterotrimer is attached to each vRNP segment inside the virion. These vRNPs have a double-helical hairpin structure consisting of two antiparallel vRNA strands that are coated by NP molecules.[17,18]
Transcription of vRNA to mRNA starts with the "cap-snatching" reaction, in which cellular capped RNAs are bound by PB2 and cleaved, by PA, at 10–15 nucleotides from the cap, to yield primers for viral mRNA synthesis.[19,20] Termination and polyadenylation occur at a stretch of five to seven U residues near the 5′ end of the vRNA.[21,22] Replication of vRNA proceeds through full-length complementary cRNAs, which are assembled as cRNP complexes. In both transcription and replication, RNA elongation is carried out by the PB1 subunit. The vRNA promoter is depicted as observed in the promoter-bound polymerase crystal structures, in which its 5′ end forms a "hook" structure.[23,24] The cRNA promoter is drawn in dashed lines, since its conformation is yet to be determined. (Adapted from Portela et al.[25])

A. The PB2 Cap-Binding Domain

The discovery that the cap-binding activity resides in the PB2 subunit was made in 1981.[30,31] In 2008, Guilligay et al.[32] reported the X-ray structure of the PB2 cap-binding domain (CBD; i.e., residues 318–483) of influenza A virus, complexed with the minimal cap analogue 7-methylguanosine 5′-triphosphate (m^7GTP; Fig. 2A). A similar approach for the influenza B PB2-CBD (Fig. 2B)[33] revealed that the binding mode of m^7GTP is similar in influenza A and B, indicating a conserved methylated cap recognition mechanism. Similar as in other cap-binding proteins (such as the eukaryotic initiation factor eIF4E) in which the 7-methylguanine moiety is sandwiched between two aromatic residues, a cation-π packing interaction occurs between Phe404 and His357 in the influenza A PB2-CBD, and Phe406 and Trp359 in the influenza B counterpart. However, the PB2-CBD has a completely different protein fold compared to other cap-binding proteins.[32] It also contains a remarkable cluster of phenylalanine residues with one noticeable difference (Phe323 vs. Gln325; compare Fig. 2A and B) between the influenza A and B proteins. In two other crystal structures, that is, the influenza B PB2-CBD in complex with GTP,[33] and a Q325F mutant form of this protein in complex with m^7GDP,[34] an inverted conformation for the guanine and ribose moieties was seen. These data indicate structural flexibility of the influenza B PB2-CBD, which explains its promiscuous cap recognition. In enzymatic assays with purified vRNPs[35] and binding assays with isolated PB2-CBD,[33] influenza A

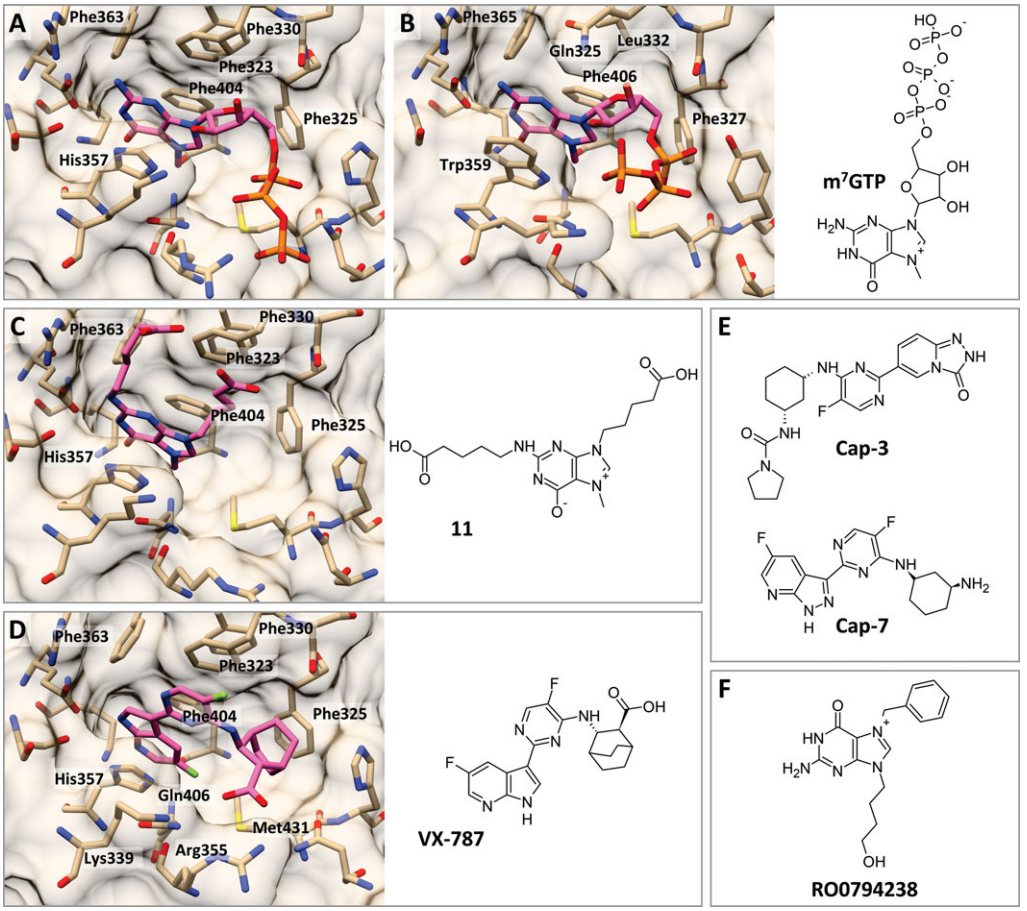

Figure 2. Chemical structures of the minimal cap analogue m^7GTP and reported inhibitors of cap-binding and X-ray structures of the PB2-CBD in complex with the corresponding ligands. (A–D) Comparison of the crystal structures of the PB2-CBD of influenza A[32] (A; PDB: 2VQZ) or influenza B[33] (B; PDB: 5EFA) PB2-CBD in complex with m^7GTP; and influenza A PB2-CBD with bound compound "11"[36] (C; PDB: 4CB6) or VX-787[37] (D; PDB: 4P1U). (E) Cap-binding inhibitors Cap-3 and Cap-7.[38] (F) For RO0794238,[39] direct binding to the PB2-CBD could not be demonstrated.

displays strict specificity for m^7G-capped RNA, while the influenza B protein recognizes various cap structures, including unmethylated GpppG-RNA.

B. The PA Endonuclease Domain

Until 2009, the endonuclease activity was thought to reside in PB1[40] or PB2.[41] A major leap forward was made when two groups independently revealed[42,43] that the endonuclease catalytic site resides in the N-terminal domain of PA (PA-Nter; residues 1 to ~195) and published the first crystal structures of the holo form of PA-Nter (Fig. 3A and B). Its structural core contains a characteristic (P)DX_N(D/E)XK motif (Fig. 3) conserved in all influenza viruses, which coordinates one, two, or three manganese or magnesium ions. It is still unresolved which and how many divalent metal ions are present in the native enzyme,[42–48] although this information is critical for efficient inhibitor design. The discrepancies in the crystal structures with regard to the identity of these metal ions (Fig. 3A–F) are possibly related to the crystallization conditions

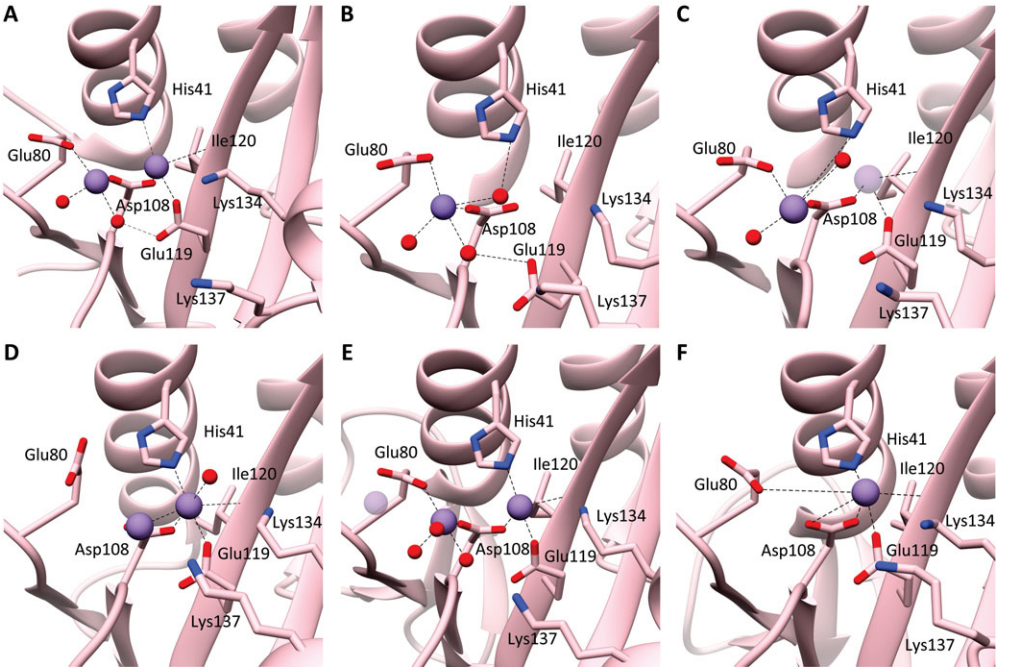

Figure 3. Comparison of PA-Nter active site structures as determined by crystallography. The metal coordinating and/or catalytic residues are labeled. The metal ions and water molecules are shown as purple and red spheres, respectively. (A) The PA-Nter crystal structure determined by Dias et al.[42] (PDB: 2W69) contains two metal ions (two Mn^{2+} ions or one Mn^{2+} and one Mg^{2+}). (B) Yuan et al.[43] (PDB: 3EBJ) obtained a PA-Nter crystal with one Mg^{2+} ion. (C) Zhao et al.[44] (PDB: 3HW6) discerned one (Mg^{2+}) metal (dark purple) or two (Mn^{2+}) metal ions, depending on the crystallization conditions. (D) Dubois et al.[45] (PDB: 4E5E) identified two Mn^{2+} ions. (E) Bauman et al.[50] (PDB: 4M5Q) described two Mn^{2+} ions (in dark purple) in the PA-Nter apo protein, and a third one (light purple) in the presence of a bound inhibitor (not shown; PDB: 4M5U). (F) In the crystal structure published by Tefsen et al.[48] (PDB: 4NFZ), one Mn^{2+} ion is accommodated in the PA-Nter active site.

(i.e., soaking *vs.* cocrystallization or different buffer composition, for instance related to pH or which metal ion was added). More conclusive evidence requires crystal structures obtained in the presence of an RNA substrate. As of today, only cocrystal structures with mononucleotides have been determined.[44,46,49]

In biochemical assays with isolated recombinant PA-Nter[51] or full-length PA,[52] the endonucleolytic activity is higher in the presence of manganese compared to magnesium. Experiments using isothermal titration calorimetry (ITC) showed a 500-fold higher affinity for Mn^{2+} ions than for Mg^{2+} ions.[51] This observation is at least partially explainable by the presence of a histidine residue in the PA-Nter active site, as Mn^{2+} ions have higher affinity for histidine than Mg^{2+}. However, these ITC measurements were performed in the absence of RNA substrate, which may be a different situation compared to the substrate-bound enzyme state. Since the intracellular concentration of free Mg^{2+} is at least 1000-fold higher than that of Mn^{2+}, magnesium may be more biologically relevant.[53,54] Also, in the context of the heterotrimeric polymerase, efficient cleavage is seen in the presence of the less reactive Mg^{2+},[55,56] which can be attributed to tight RNA binding by the nearby PB2-CBD.

In order to clarify this issue, Xiao and co-workers performed molecular dynamics (MD) simulations to construct structural models of PA-Nter in the presence of one or two magnesium ions and in complex with the RNA substrate.[57] In Figure 4, both active site conformations and

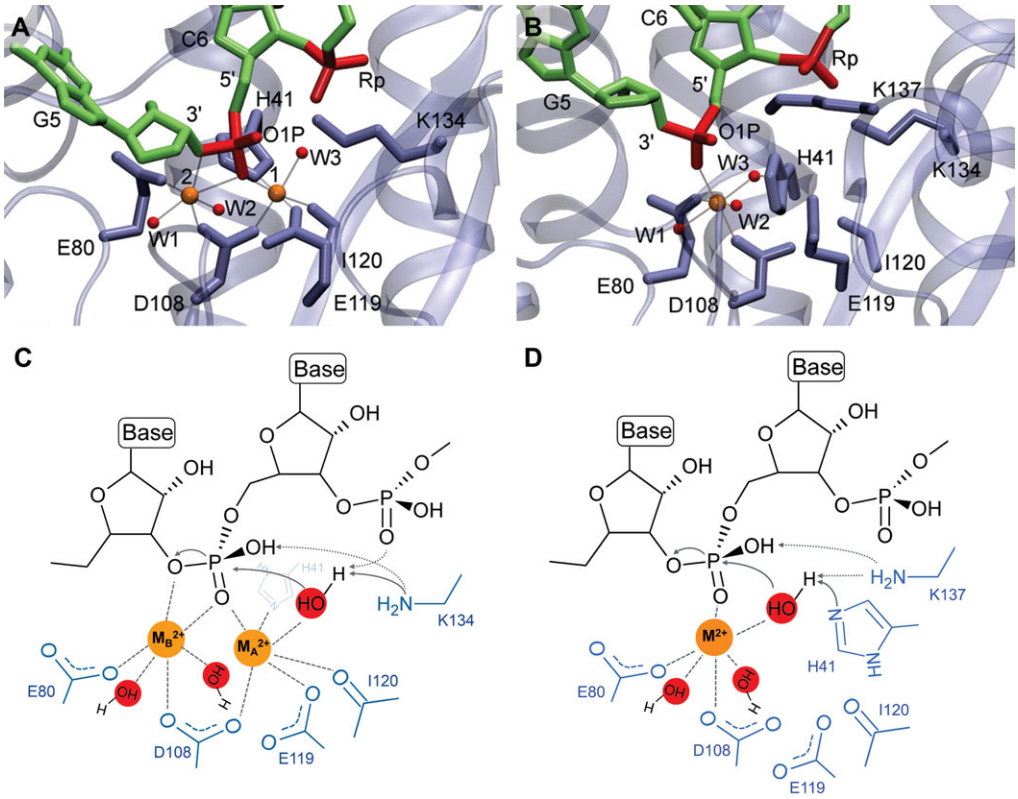

Figure 4. Comparison of the two- versus one-metal-ion models for the PA-Nter active site. The metal ions are depicted as orange spheres, while the red spheres represent the water molecules coordinated to the metal ions. (A and B) Structural models obtained by molecular dynamics (MD) simulations of the PA-Nter active site in complex with its RNA substrate, in the presence of two (A) or one Mg^{2+} ion(s) (B) [reprinted with permission from Xiao et al.[57] Copyright 2014 American Chemical Society]. (C and D) Possible reaction mechanism of the PA-Nter cleavage reaction, in the presence of two (C) or one (D) metal ion(s), based on the models of Xiao et al.[57] In each case, the metal ion has six coordinated ligands (indicated by dotted lines). (C) In the two-metal mechanism, both metal ions are coordinated to the scissile phosphodiester of the nucleic acid substrate, and the ribose 3′-O is coordinated apically to M_B^{2+}. M_A^{2+} would lower the pK_a of the attacking water molecule, together with the catalytic Lys134 and the adjacent 3′ phosphodiester, thus activating this water molecule as a nucleophile. M_A^{2+} might also assist the nucleophilic attack by moving toward M_B^{2+}, bringing the activated water molecule closer to the scissile phosphodiester. M_B^{2+} can stabilize the pentacovalent intermediate formed during the S_N2-type phosphodiester bond cleavage. (D) When the one-metal mechanism is operative, this metal ion is coordinated to the scissile phosphodiester. Its role would be to stabilize the transition state of the nucleophilic attack, like M_B^{2+} in the two-metal mechanism. Additionally, the single metal ion could decrease the pK_a of the nucleophilic water, together with the catalytic Lys137, while His41 would act as the general base that deprotonates the water molecule.

possible one- and two-metal-ion reaction mechanisms are compared and explained in full detail in the figure legend.

The two-metal-ion model is favored by the ITC data for Mn^{2+} binding to PA-Nter.[51] Doan et al.[55] found that the endonuclease activity in vRNP complexes isolated from virions depends on metal ion concentration in a cooperative manner, with Hill coefficients close to or larger than 2. Also, synergistic activation of the cleavage activity was observed with combinations of different metal ions. This suggests that PA-Nter requires two metal ions to perform RNA cleavage. The possibility was raised that binding of the second ion is stabilized when an RNA substrate or inhibitor is present.[51,57]

C. The Heterotrimeric Influenza Polymerase Complex

The crystal structure of the large (~260 kDa) polymerase complex was first resolved in 2014 by Cusack and co-workers,[23,24] who succeeded to achieve high resolution (2.7 Å) structures of the polymerase heterotrimer, in complex with the vRNA promoter and originating from bat influenza A (FluA; Fig. 5A) or human influenza B (FluB; Fig. 5B) virus. The crystal structures of the apo influenza C polymerase complex (FluC; without bound promoter; Fig. 5C),[58] and FluB polymerase in complex with a 5′ cRNA fragment (Fig. 5D),[56] were revealed more recently.

A first striking observation is the complex intertwining of the three subunits, which results in vastly more extensive intersubunit interactions than previously assumed (see other review articles[13,59] for the literature until 2013). Equally intriguing are some prominent conformational differences among the different crystal structures, which most likely represent functionally different states of the enzyme complex. Third, these crystallization studies are the first to reveal the architecture of the PB1 active site. These three insights undoubtedly create several new avenues for structure-aided drug design.

The polymerase body is formed by PB1, enclosed by the PA linker (which connects the N- and C-terminal domains of PA) on one side, and the N-terminal domain of PB2 (PB2-N) at the other side. Akin to other RNA-dependent RNA-polymerase (RdRp) enzymes, the overall structure of the catalytic core of PB1 resembles a right hand (Fig. 5E). It contains fingers, fingertips, palm, and thumb domains, formed mainly by conserved RdRp motifs designated "pre-A"/F, A, B, C, D, and E. By comparison with other polymerase structures, three conserved aspartic acids (Asp305, Asp444, and Asp445 in FluB) within the active site of PB1 were identified, which have a crucial role in catalysis[60] and together coordinate two divalent metal ions (Fig. 5, inset panel E).

The promoter-bound FluA and FluB polymerase complexes have a similar "open" conformation, a U-shaped structure (Fig. 5A and B), in which one arm is formed by PA-Nter and the other by the PB2-CBD. These domains face each other in the FluA structure, suggesting that this crystal represents the cap-snatching mode. In the FluB structure, the PB2-CBD is rotated by 70° toward the PB1 active site where primer elongation takes place (compare Fig. 5A and B, in orange). Hence, this FluB structure is thought to represent the cap-dependent priming step. In sharp contrast, in the crystal structures of the apo FluC polymerase (i.e., without bound promoter[58]; Fig. 5C) and the 5′ cRNA-bound FluB polymerase (Fig. 5D),[56] the polymerase adopts a "closed" conformation in which cap-snatching is disfavored; it is not yet clear whether these structures represent an inactive or "only replication-competent" state. Several interdomain interactions immobilize the PB2-CBD (Fig. 5C and D, in orange) and PA-Nter (which is named P3 in the case of influenza C) is repositioned and packed against the PB2-NLS-domain (Fig. 5C and D, in maroon). This pronounced difference between the open and closed forms of the polymerase complex is related to a radical in situ rearrangement of a large part of PB2. Hence, the regulatory role of the vRNA promoter, as evidenced by enzymatic studies,[61,62] may be related to triggering the transition from the closed preactivation state to the active transcription state. The crystallization studies further indicated that the influenza polymerase complex contains several hinge regions that provide high flexibility to enable its multiple roles in viral RNA synthesis.[56] Stabilization of one of its conformations by an inhibitor, such that a vital rearrangement cannot take place, would be an excellent approach to inhibit the polymerase complex.

D. Recent Structural Insights: A First Step Toward Solving Some Unknowns

The recent crystallographic analyses also help to interpret some issues that are already debated since many years.[13] A first issue with relevance for inhibitor design is related to the potential

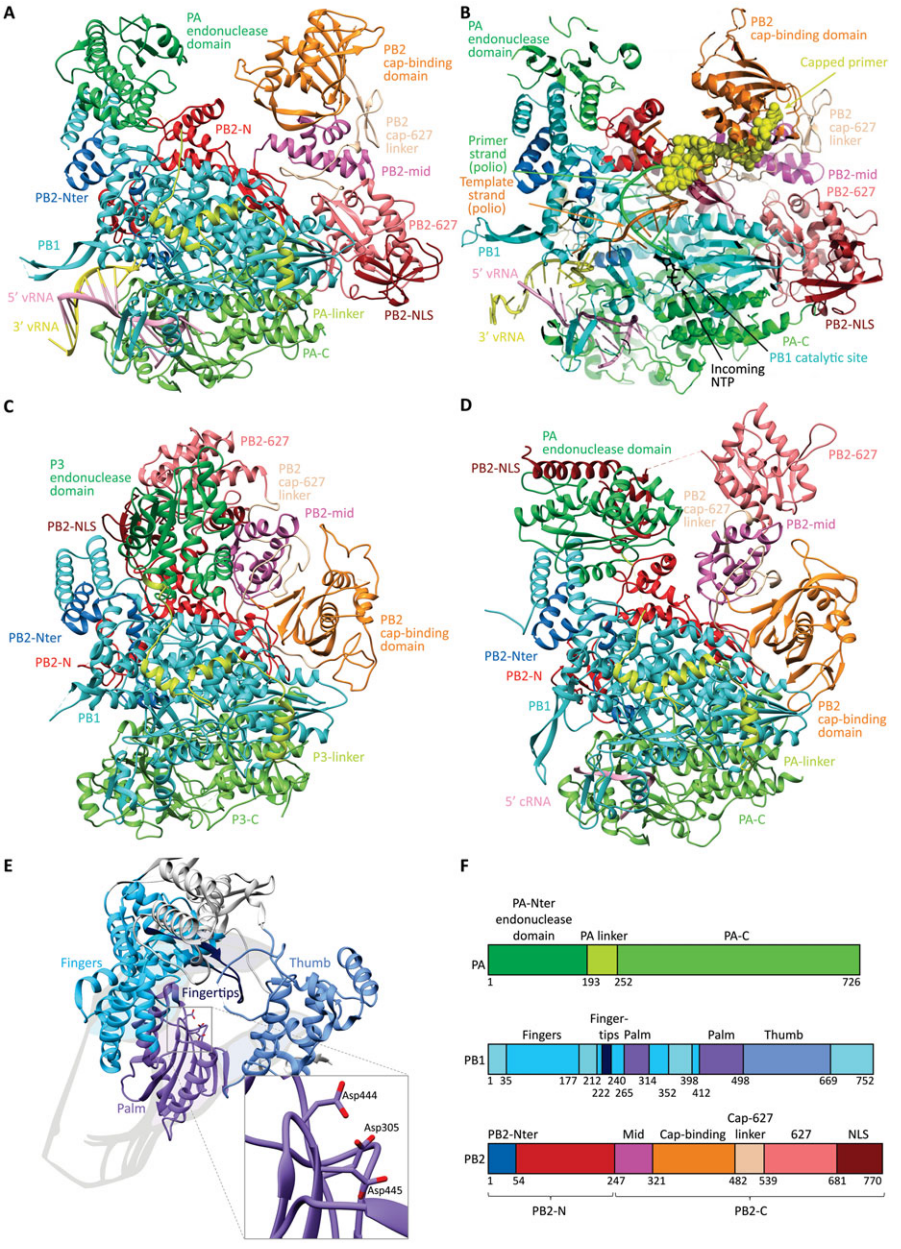

Figure 5. Comparison of the crystal structures of the heterotrimeric influenza polymerase complex containing full-length PA, PB1, and PB2. The models are shown in the same orientation, and the same coloring was applied for the different subdomains. (A) Bat FluA polymerase with bound vRNA promoter [PDB: 4WSB].[23] (B) Superposition model of the FluB polymerase crystal structure with a template–primer (orange–green) duplex and incoming NTP (black) (taken from a poliovirus polymerase crystal structure). The yellow spheres represent the capped primer bound to PB2, after cleavage by the PA endonuclease domain. This primer is now directed toward the PB1 catalytic cavity, where primer elongation occurs. (Adapted by permission from Macmillan Publishers Ltd: Nature, Reich et al.,[24] copyright 2014.) (C) Influenza C polymerase (PDB: 5D98) in apo form.[58] (D) FluB polymerase structure with bound cRNA 5′ end[56] (PDB: 5EPI). (E) Domain arrangement of FluB PB1, illustrating the right-hand-like polymerase fold. The inset shows a closeup of the PB1 catalytic residues, which coordinate two divalent metal ions (not shown). (F) Subdomain names and color scheme as applied in panels A–E, based on the FluB polymerase numbering. For clarity, the PB1 subunit is colored uniformly in cyan in panels A–D, while its different subdomains are differentiated in panel E.

substrate or sequence specificity of the cap-snatching reaction. The observation that the capped host RNAs is cleaved at a distance of 10–15 nucleotides from the 5′-cap can be explained by the 50 Å distance between the cap-binding and endonuclease domains in the "open" FluA structure.[23] Regarding the type of RNA substrate, the previous assumption that only host (pre-)mRNAs are targeted, is contradicted by recent data that noncoding RNAs are the primary source of capped primers.[63–65] The evidence for sequence preference is inconclusive,[19,20,42,52,62,66–68] but recent deep sequencing studies[63,69] indicate a preference for capped primers ending with a purine residue. The selection could be determined[20,70–72] (i) at the level of RNA cleavage and/or (ii) transcription initiation when requiring base-complementarity between the 3′ end of the vRNA template and a given cellular RNA. To gain insight into the first selection mechanism, a cocrystal of PA-Nter (or the entire polymerase complex) with an RNA substrate is required. This could elucidate substrate-dependent changes in the active site of PA-Nter, with relevance for design of optimized PA inhibitors. One indication in favor of such base preference comes from crystallization studies of PA-Nter in complex with a ribomononucleotide, which proved successful for uridine 5′-monophosphate (UMP)[44,46,49] (Fig. 6H–J) and adenosine 5′-monophosphate (AMP),[44] but not cytidine 5′-monophosphate (CMP) and guanosine 5′-monophosphate (GMP).[44,46]

Substrate selection at the level of initiation is corroborated by the polymerase crystal structures, more specifically the model that was proposed[24] (Fig. 5B) by superposition of the influenza B PB1 structure on the poliovirus polymerase primer–template complex.[80] This model suggests that the 3′ end of the vRNA would pass the PB1 active site by three nucleotides, creating a template overhang that could form three base pairs with the capped primer.

A second intriguing issue is how the different conformations captured in the crystal structures of the heterotrimeric polymerase complex, relate to the *cis/trans* RNA synthesis model[81] for which experimental support was provided by the cryo-electron microscopic analyses of vRNPs.[17,18] Namely, the branched arrangement of the RNP complexes in which a smaller nascent RNP seems to bud from a larger full-length RNP, indicate that genome replication is performed in *trans* by a second polymerase heterotrimer. On the other hand, vRNA transcription would be performed by the *cis*-acting polymerase that resides in the vRNP complexes. Given the high flexibility of the polymerase heterotrimer (see above), it is plausible that structural rearrangements occur in each of its functional states which, furthermore, might also be regulated by specific host cell factors.

3. STRATEGIES TO INTERFERE WITH THE INFLUENZA VIRUS POLYMERASE, THE NP, OR AN ASSOCIATED HOST CELL FACTOR

A. General Reflections

In the following section, we describe the diverse strategies that have been explored to block influenza virus RNA synthesis, from the stage of rational design or serendipitous screening, to unravelling the presumed mechanism of action (which includes analysis of the resistance profile), and evaluation in cell culture or mouse models, or, if appropriate, in clinical trials (see Table I for a concise overview). We added in this table some published antiviral activity data, in the awareness that these values may show large variations depending on assay conditions, for instance related to virus input, incubation time, or the parameters used to monitor inhibition of virus replication or cytotoxicity of the test compounds. In our own experience, in vitro evaluation based on cytopathic effect (CPE) reduction is more stringent compared to plaque reduction or virus yield assays. In the latter approach, a reduction in virus titer of at least two-$\log_{10}$ is required to conclude that a given compound has meaningful activity. For this reason,

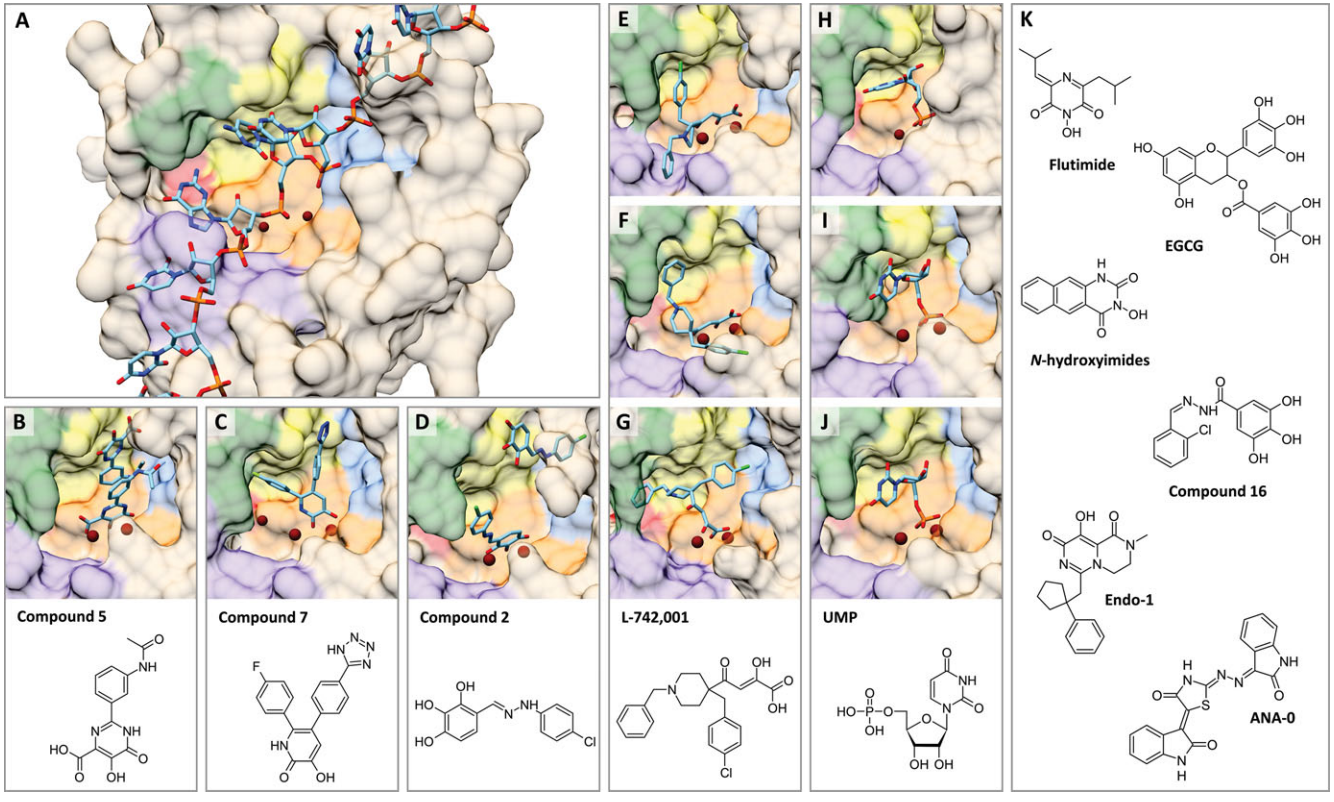

Figure 6. Comparison of the predicted orientation of RNA versus that of different ligands within the PA-Nter active site; and chemical structures of representative PA inhibitors. (A) Hypothetical disposition of the RNA substrate in PA-Nter with two bound Mg^{2+} ions, based on the molecular dynamics model (kindly provided by Dr. M. Alfonso-Prieto), as performed by Xiao et al.[57] The 3′ end of the mRNA is at the right-hand side, while its 5′ end is located at the left. (B–D) Crystal structures of PA-Nter in complex with (B) "compound 5"[45] (PDB: 4E5J); (C) "compound 7"[50] (PDB: 4M5U); or (D) "compound 2"[73] (PDB: 5I13).
(E–G) Position of L-742,001, as (E–F) determined by cocrystallization (E, PDB: 4E5H[45] and F, PDB: 5CGV[49]) or (G) predicted by docking.[74] (H–J) X-ray structures of PA-Nter with bound UMP, as determined by (H) Zhao et al.[44] (PDB: 3HW3), (I) Kowalinski et al.[46] (PDB: 4AWH), and (J) Song et al.[49] (PDB: 5CL0). In the latter two crystal structures, the UMP-binding mode fully overlaps. The binding regions in PA-Nter are colored—in orange: metal binding and catalytic residues (His41, Glu80, Asp108, Glu119, Ile120, and Lys134); in yellow: pocket P1 (Ala37, Ile38, Leu42, and Lys34); in blue: pocket P2 (Thr40, Val122, Arg124, Tyr130, and Phe150); in purple: pocket P3 (Arg84, Trp88, Phe105, and Leu106); in red: pocket P4 (Leu16 and Gly81); in green: pocket P5 (Ala20, Tyr24, and Glu26). The bound molecules (i.e., RNA-substrate or ligand) are shown in cyan, while the metal ions are colored dark red. (K) Chemical structures of flutimide,[75] EGCG,[76] *N*-hydroxyimides,[77] compound 16,[78] Endo-1,[38] and ANA-0.[79]

Table I. Overview of Published Approaches to Interfere with the Influenza Virus Polymerase, the Nucleoprotein or a Host Factor Involved in Viral RNA Synthesis

Compound name or code	Proposed mechanism of action[a]	Antiviral activity in cell culture[b]	Known spectrum of influenza virus activity[c]	Published resistance mutations	References
Inhibitors targeting PB1 (directly or indirectly)					
Nucleoside and nucleobase analogues					
T-705 (in Phase 3/approved in Japan)	Alternative substrate for viral polymerase; chain termination and lethal mutagenesis	0.1–10 μM[82–84][*]	A, B, and C	PB1: V43I[85]	82–85
Ribavirin	Alternative substrate for viral polymerase; lethal mutagenesis; IMPDH inhibitor	2.5–37 μM[86–88]; 2–3 $\log_{10}$ reductions in virus titer at 10 μM[89] [*]	A, B, and C	PB1: V43I[85] and D27N[90]	85–90
Prodrug 2	Ribavirin prodrug	0.5 μM[87]	A		87
Viramidine	Ribavirin prodrug	8.6–132 μM[88]	A and B		88
5-Azacytidine	Alternative substrate for viral polymerase; lethal mutagenesis	2–3 $\log_{10}$ reductions in virus titer at 10 μM[89]	A		89
5-Fluorouracil	Alternative substrate for viral polymerase; lethal mutagenesis	2–3 $\log_{10}$ reductions in virus titer at 80 μM[89]	A		89
EICAR	IMPDH inhibitor	1.5–8.6 μM[91]	A and B		91
ETCAR	IMPDH inhibitor	1.2–19 μM[92]	A and B		92
Selenazofurin	IMPDH inhibitor	0.7–1.2 μM[93]	A and B		93
Pyrazofurin	IMPDH inhibitor	0.24–0.88 μM[94]	A, B, and C		94
LY217896	IMPDH inhibitor	2.9–12 μM[95]	A and B		95

Continued

Table I. Continued

Compound name or code	Proposed mechanism of action[a]	Antiviral activity in cell culture[b]	Known spectrum of influenza virus activity[c]	Published resistance mutations	References
Compound 3c (analogue of T-705)	Alternative substrate for viral polymerase	1.9 μM[96]	A		96
Compound 8a (analogue of T-705)	?	5.6–7.4 μM[97]	A		97
2′-Deoxy-2′-fluoroguanosine	Alternative substrate for viral polymerase; nonobligate chain termination	2–22 μM[98,99] [*]	A and B	?[d]	98, 99
2′-Substituted carba-nucleoside analogues "6" and "10"	?	0.9–51 μM[100]	A and B		100
C-3′-modified analogues	?	3.7–65 μg/ml[102]	A and B		102
6-Methyl-7-substituted-7-deaza purine nucleoside analogues "5x" and "5z"	?	3.6–7.0 μM[103]	A		103
Protide 9j	2′-Deoxy-2′-fluoroguanosine prodrug	$EC_{99} = 12$ μM[104]	A		104
2′-Deoxy-2′-fluorocytidine	Alternative substrate for RNA synthesis?; immunomodulator?	0.05–8 μM[105]	A and B		105
2′-2′-Difluorodeoxycytidine	Interferes with pyrimidine nucleotide synthesis	<0.032–1.2 μM[105]	A and B		105
Protide 23a	2′-Deoxy-2′-fluorouridine prodrug	$EC_{99} = 49$ μM[106]	A		106
Compound A3	Interferes with pyrimidine nucleotide synthesis	0.04–1 μM[107]	A and B		107
N10169	Interferes with pyrimidine nucleotide synthesis	3 μM[108]	B		108
Carbodine	Interferes with pyrimidine nucleotide synthesis	0.6–36 μM[94,109,110] [*]	A, B, and C		94, 109, 110

Continued

Table I. Continued

Compound name or code	Proposed mechanism of action[a]	Antiviral activity in cell culture[b]	Known spectrum of influenza virus activity[c]	Published resistance mutations	References
Cyclopentenyl cytosine	Interferes with pyrimidine nucleotide synthesis	9.6–26 μM[94]	A, B, and C		94
3-Deazaguanine	IMPDH inhibitor	14–49 μM[94]	A, B, and C		94
Nonnucleoside analogues					
"367"	?	0.5 μM[111]	A	PB1: H456P[111]	111
ASN2	?	3-14 μM[112]	A and B	PB1: Y499H[112]	111
Inhibitors targeting PB2					
VX-787 (in Phase 2)	Inhibits cap-binding	0.32–2.8 nM[37]	A	PB2; cell culture: Q306H, S324I/N/R; F404Y and N510T[113]; patients: M431I[11]	11, 37, 113
Cap-3 and Cap-7	Inhibit cap-binding	1.17–9 μM[114]	A		114
Inhibitors targeting PA					
AL-794 (in Phase 1)	Endonuclease inhibitor	Undisclosed			
S-033188 (in Phase 2)	Endonuclease inhibitor	Undisclosed			
L-735,882 and analogues	Metal-chelating endonuclease inhibitor	0.18–1.6 μM[115,116]	A and B		115, 116
L-742,001	Metal-chelating endonuclease inhibitor	0.35[116] to 11[117] μM [*]	A and B	PA: T20A,[49,74,117] H41A,[74] L42T,[74] I79L,[49] G81F/T/V,[74] F105S,[49] E119D,[49] I120T,[74] V122T[74]	49, 74, 116, 117

Continued

Table I. Continued

Compound name or code	Proposed mechanism of action[a]	Antiviral activity in cell culture[b]	Known spectrum of influenza virus activity[c]	Published resistance mutations	References
Flutimide	Metal-chelating endonuclease inhibitor	5.9 μM[75]	A and B		75
Tetramic acid "36"	Metal-chelating endonuclease inhibitor	21 μM[118]	A		118
Phenethylphenylphthalimide analogs PPT-65 and PPT-66	Metal-chelating endonuclease inhibitor	26–48 μM[119]	A		119
Carboxamides "42," "44," "45"	Metal-chelating endonuclease inhibitor	13–19 μM[101]	A		101
Pyrimidinol "26"	Metal-chelating endonuclease inhibitor	13 μM[45]	A		45
Hydroxypyridinone "7"	Metal-chelating endonuclease inhibitor	11 μM[50]	A		50
Hexanetetrone "4" and benzohydrazide "16"	Metal-chelating endonuclease inhibitor	18–23 μM[78]	A		78
(Tri)hydroxyphenyls "1," "2," and "3"	Metal-chelating endonuclease inhibitor	11–14 μM[120]	A		120
Dihydroxyindoles "10" and "15"	Metal-chelating endonuclease inhibitor	EC_{99} = 5.7–12 μM[121]	A		121
THC19	?	31–45 μM[122]	A	PA: V44I and E165D[122]	122
Aptamer PAN-2	?	About 10 nM[123]	A		123
Pyridopiperazinediones Endo-1, Endo-8, and Endo-9	Metal-chelating endonuclease inhibitor	0.39–2 μM[114]	A		114
ANA-0	Endonuclease inhibitor	0.8–4 μM[79]	A		79

Continued

Table I. Continued

Compound name or code	Proposed mechanism of action[a]	Antiviral activity in cell culture[b]	Known spectrum of influenza virus activity[c]	Published resistance mutations	References
PPI inhibitors designed against the PA-PB1 interface					
Benzofuran derivatives	Inhibit PA-PB1 assembly	1–60 μM[124,125]	A		124,125
Benzbromarone and diclazuril	Inhibit PA-PB1 assembly	31–39 μM[126]	A		126
"1" and AL18	Inhibit PA-PB1 assembly	8.3–23 μM[127,128]	A and B		127,128
Cycloheptathiophene-3-carboxamides "6" and "19"	Inhibit PA-PB1 assembly	15–28 μM[129]	A and B		129
Thiophene-3-carboxamides "7," "10," "'18," and "19"	Inhibit PA-PB1 assembly	11–43 μM[130]	A and B		130
Triazolopyrimidines "16," "31," "'36," and "37"	Inhibit PA-PB1 assembly	5–51 μM[131]	A and B		131
4,6-Diphenyl pyridines "1," "11," "15"	Inhibit PA-PB1 assembly	7.3–26 μM[132]	A		132
ANA-1	Inhibit PA-PB1 assembly	0.09–1.2 μM[133]	A		133
Inhibitors targeting NP					
Nucleozin	Inhibits cytoplasmic trafficking of vRNPs	0.069–0.33 μM[134] [*]	A	NP: Y52H/C,[111,135] Y289H,[134,135] N309K/T,[134–136]	111,134–136
PPQ-581	Inhibits cytoplasmic trafficking of vRNPs	1 μM[137]	A	NP: S377G[137]	137
"3," "7," "'12," and "23"	Disrupts NP dimerization by targeting the E339 . . . R416-salt bridge	1.7–118 μM[138]	A		138
Naproxen	Blocks the RNA-binding groove of NP	11–25 μM[139]	A		139

Continued

Table I. Continued

Compound name or code	Proposed mechanism of action[a]	Antiviral activity in cell culture[b]	Known spectrum of influenza virus activity[c]	Published resistance mutations	References
F66	Blocks the RNA-binding groove of NP	~1–5 μM[140]	A		140
RK424	Inhibits nuclear export of NP; inhibits NP–RNA, and NP–NP interactions	0.40–0.63 μM[141]	A		141
Mycalamide analog "4"	Inhibits nuclear transport of NP?	59–97% reduction of plaque formation at 32 μM[142]	A		142
Ingavirin (approved in Russia)	Diverse effects on NP functioning?	Inconsistent[143–145]	A and B		143–145
Other					
AVI-7100 (in Phase 1)	Inhibition of M-gene translation by gene silencing	10–20 μM[146]	A		146
KPT-335 (in Phase 1)	CRM1 inhibition	0.01–0.42 μM[147]	A and B		147
BPR3P0128	Inhibitor of cap-binding	0.05–0.19 μM[148]	A and B		148
DPQ	Inhibition of RNA synthesis by binding to 5′ vRNA promoter	72–276 μM[149]	A and B		149
Geldanamycin, 17-AAG, and 3beta-acetoxydeoxodihydrogedunin	Hsp90 inhibition	1–2 $\log_{10}$ reductions in virus titer[150,151] [*]	A		150,151
U0126	MEK inhibition	1.2–141 μM[152,153] [*]	A		152,153

Continued

Table I. Continued

Compound name or code	Proposed mechanism of action[a]	Antiviral activity in cell culture[b]	Known spectrum of influenza virus activity[c]	Published resistance mutations	References
PD-0325901, AZD-6244, AZD-8330, and RDEA-119	MEK inhibition	0.005–0.75 μM[154]	A		154
WV970	Kinase inhibition	0.015–0.24 μM[155]	A and B		155
Acetylsalicylic acid, pyrrolidine dithiocarbamate, SC75741	NF-κB inhibition	1–4 $\log_{10}$ reductions in virus titer[156–158]	A		156–158
Bay 11–7082	NF-κB inhibition	40–85% reduction in virus titer at 15 μM[159]	A		159
NSC23766	Rac1 GTPase inhibition	22 μM[160]	A and B		160

[a]For compounds with broader antiviral activity, only the mechanism of action proposed for influenza virus is given. Note that for many of the experimental compounds, the antiviral mode of action in cell culture is still unproven (e.g., because no resistance data are thus far available).
[b]Unless stated otherwise, the EC50 value is given. The [*] annotation indicates that the antiviral activity was reported in two or more independent publications.
[c]The information in this column indicates only the clearly defined activity spectrum. For most compounds, only a limited number of influenza viruses was tested, and hence, the activity spectrum was not (yet) specified.
[d]Rollins et al.[99] described the selection of partially (fivefold) resistant influenza virus obtained by serial passaging; and Tisdale et al.[161] reported a partially (tenfold) less susceptible polymerase obtained from resistant virus; however, no sequence analysis of these viruses was reported.

we marked with an asterisk the published compounds for which the anti-influenza effect in cell culture has been confirmed in at least two independent studies. We also mentioned the published resistance mutations. Resistance studies help to reveal the precise interaction with the viral target and represent the gold standard to validate the proposed mechanism of action of any new class of antiviral molecules.

During compound testing, influenza B virus is sometimes ignored, although it accounts for about 25% of seasonal influenza cases. The protein sequences of PA, PB1, and PB2 are, overall, highly conserved among influenza A and B viruses, but even subtle amino acid differences in a binding pocket may render an inhibitor inactive against either of the two virus types. Several inhibitor classes described below were designed to interact with critical functional residues (for instance crucial for binding the capped RNA or NTP substrates). Still, high inhibitor binding affinity often requires involvement of adjacent and less conserved sites, which can give influenza A or B specific activity, or a reduced barrier for the virus to acquire resistance. In other words, although the viral polymerase complex is, generally speaking, an excellent target for developing broad influenza A and B inhibitors that do not readily select for resistance, this theoretical assumption requires biological verification for every single class of new inhibitors.

Besides application in seasonal influenza infections, antiviral drugs are essential to combat serious zoonotic infections (in particular, by highly pathogenic avian influenza A viruses). This seems less of a concern for inhibitors that directly interact with active domains in PA, PB1, or PB2, since these regions are highly conserved among human and avian influenza A viruses. On the other hand, there are species-dependent amino acid differences in some parts of the protein complex, supposedly related to a regulatory role for host cell factors such as ANP32A,[29,162] RNA polymerase II,[163] or nuclear import or export proteins.[14] The insights into the influenza virus host interactome are rapidly growing[164] and will hopefully rationalize the concept of host cell based antiviral therapeutics. As of today, some approaches (described in Section 3,H) have already been proposed, although their mechanistic details remain to be established.

B. Nucleoside and Nucleobase Analogue Inhibitors

1. Ribavirin and Structurally Related Carboxamide Analogues

Ribavirin [1-(β-D-ribofuranosyl)-1,2,4-triazole-3-carboxamide; Fig. 7A] was already discovered in 1972.[86] This nucleoside analogue inhibits many diverse RNA viruses including influenza virus. Ribavirin has been a first-line therapeutic for hepatitis C virus infections, but its clinical utility in the management of influenza infections seems limited. In clinical trials performed in the 1970s and 1980s, ribavirin was found to be ineffective against experimentally induced influenza,[165] though some benefit was provided at higher drug doses,[166] and in vivo data support its potential usefulness in combination therapy.[167] Although it has severe side effects (e.g., hemolytic anemia) and teratogenic properties,[168] ribavirin may be a last resort for clinicians encountering rare cases of multidrug-resistant influenza viruses.[169]

The mode of action of ribavirin is rather complex, since it appears to inhibit virus replication through a combination of different mechanisms. After phosphorylation by adenosine kinase[170] or cytosolic 5′-nucleotidase II,[171] ribavirin 5′-monophosphate inhibits the cellular enzyme inosine monophosphate dehydrogenase (IMPDH), resulting in a decrease in the intracellular GTP pool and, hence, indirect inhibition of viral RNA synthesis.[172–174] Other proposed mechanisms include direct inhibition of the viral polymerase by ribavirin 5′-triphosphate,[175,176] immunosuppression,[177] and lethal virus mutagenesis.[85] It was recently demonstrated that ribavirin induces mutagenesis in the influenza virus genome by acting as an ambiguous purine analogue and increasing G-to-A and C-to-U mutations.[85] Serial virus passaging in the presence of the compound gave rise to a mutant virus with twofold resistance to ribavirin and a lower

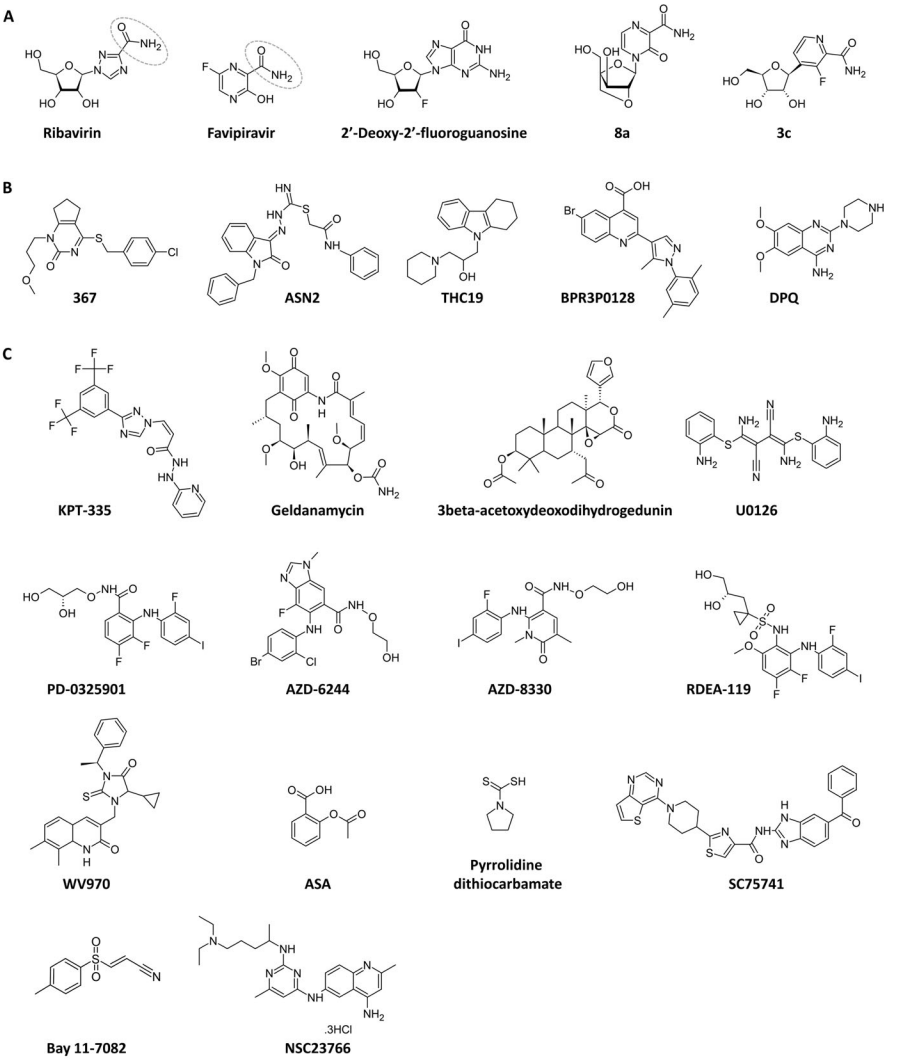

Figure 7. Chemical structures of proven or tentatively proposed polymerase inhibitors. (A) Nucleoside or nucleobase analogue inhibitors ribavirin,[86] favipiravir[82] (T-705), 2′-deoxy-2′-fluoroguanosine[98] (2′-FdG), "8a,"[97] and "3c."[96] The rotating carboxamide moiety (encircled) in ribavirin and favipiravir explains their ambiguous base pairing, since their base part mimics guanine as well as adenine. (B) Compounds for which the detailed mechanism of action at the level of the polymerase has not yet been revealed. (C) Compounds targeting a host cell factor that has been linked to viral polymerase function. See Table I for references on the individual compounds.

degree of resistance to favipiravir (described below). This mutant virus carried mutation V43I in PB1, which was associated with increased polymerase fidelity and reduced pathogenicity in mice.[85] This Val43 residue lies in close proximity of the putative NTP entrance channel, as predicted in the polymerase crystal structure of Pflug et al.,[23] which could explain how the V43I mutation may affect the activity of ribavirin, for instance by modifying the interaction of the polymerase with GTP or ribavirin 5′-triphosphate. The last analysis is difficult to reconcile with the observation that ribavirin 5′-triphosphate is a rather weak inhibitor of the viral polymerase in enzymatic assays, with reported IC_{50} values of 70 μM[173] or ~100 μM.[176] By using random mutagenesis, Binh et al.[90] identified PB1 mutation D27N, which conferred a 1.8-fold resistance to ribavirin. This mutation was also picked up by Pauly et al. after serial passaging of influenza

virus under ribavirin.[89] By inspecting the published crystal structure,[23] we noticed that Asp27 is located outside the catalytic site, in the vicinity of the 5′ vRNA promoter binding site. Pauly et al. further confirmed that ribavirin and two other nucleoside analogues, **5-azacytidine** and **5-fluorouracil**, are lethal mutagens for influenza virus in vitro.[89]

With the aim to improve the efficacy or safety, Dong et al. obtained an alkoxyalkylphosphodiester prodrug of ribavirin, prodrug "**2**," which still requires in vivo validation.[87] **Viramidine**, the 3-carboxamidine prodrug of ribavirin,[178] was found to be slightly less active against influenza virus in vitro and in vivo, but also showed less toxicity.[88] In addition to ribavirin, the following carboxamide-containing nucleoside analogues exhibit broad antiviral activity, presumably by inhibition of IMPDH: 5-fluoro-1-β-D-ribofuranosylimidazole-4-carboxamide (**FICAR**),[179] 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (**EICAR**),[91] 5-ethynyl-1-β-D-ribofuranosyl-1*H*-[1–3]triazole-4-carboxylic acid amide (**ETCAR**),[92] **selenazofurin**,[93] and **pyrazofurin**.[94] All these are cytostatic molecules mainly explored as anticancer agents.[180] The substituted thiadiazole compound **LY217896** was shown to display broad anti-influenza virus activity[95] via IMPDH inhibition,[181] but proved ineffective in a placebo-controlled clinical trial.[182]

2. Favipiravir

Favipiravir (6-fluoro-3-hydroxy-2-pyrazinecarboxamide; Fig. 7A), also known as **T-705**, was approved in Japan in March 2014 for restricted use in uncomplicated influenza virus infections, and is currently in Phase 3 clinical trials in the USA and Europe. Unpublished clinical data suggest that the antiviral effects of favipiravir are similar to those of oseltamivir.[183] In one reported Phase 2 study,[11] favipiravir significantly reduced the time to resolution of symptoms. No signs of drug resistance were seen in more than 700 samples tested.

Favipiravir has broad activity against influenza A, B, and C viruses,[82] including the 2009 pandemic A/H1N1 virus,[184] highly pathogenic avian influenza H5N1[185] and H7N9[186] viruses, and virus strains with resistance to M2 blockers or NAIs.[187] Among all nucleobase/nucleoside inhibitors of influenza virus reported thus far, favipiravir is absolutely unique in having a clearly superior therapeutic window (i.e., antiviral EC_{50} value in the range of 0.1–10 μM[82–84] and no cytotoxicity at 6400 μM[82]).

Mechanistically, favipiravir is a nucleobase mimetic that undergoes intracellular conversion to its ribofuranosyl 5′-triphosphate metabolite. The cellular hypoxanthine guanine phosphoribosyltransferase (HGPRT) converts favipiravir into its ribose-5′-monophosphate (RMP), which is further metabolized, by cellular kinases, to favipiravir-ribosyl-5′-triphosphate (favipiravir-RTP).[83] Its requirement for very high dosing (in the order of 1600–2400 mg per day in some clinical trials) may, at least partially, be related to the low efficiency of its metabolic activation.[83] This limitation could be solved by designing prodrug forms of favipiravir that bypass one or more of its activation steps.

Favipiravir-RTP is recognized by influenza virus RNA polymerase as an alternative for the natural substrates GTP and, to a lesser degree, ATP.[188–190] The precise mechanism of action of favipiravir remains to be fully explained, and two nonmutually exclusive hypotheses have been proposed. Similarly to what is described above for ribavirin, favipiravir can cause lethal virus mutagenesis by inducing a high rate of mutations and generating a nonviable viral phenotype. In virus grown under favipiravir, Baranovich et al.[84] observed a reduction in virus infectivity without a corresponding decrease in the number of viral RNA copies, together with a dose-dependent increase in mutation frequency in the influenza virus genome (primarily G-to-A and C-to-U). The biochemical basis for this mutagenic effect was revealed in the enzymatic studies by Jin et al.,[189] since the influenza polymerase was shown to efficiently incorporate favipiravir-RTP both opposite to C and U, meaning that favipiravir-RTP mimics GTP as well as ATP. This

ambiguous base-pairing behavior is related to the rotating carboxamide moiety (encircled in Fig. 7A). The second hypothesis, "non-obligate chain termination," is supported by the biochemical observation that incorporation of a single molecule of favipiravir-RMP (which carries a normal 3′-hydroxyl group) into a nascent influenza RNA strand causes inhibition of viral RNA extension.[190] However, another study did not confirm these results and concluded that at least two consecutive incorporation events of favipiravir-RMP are needed to terminate viral RNA elongation.[189] Hence, the RNA chain-terminating effect may prevail at higher favipiravir concentrations, while at lower compound concentrations, the mutagenic effect may become apparent. Interestingly, the compound appears to have an exceptionally high barrier for selecting resistance, since no favipiravir-resistant influenza virus was obtained in cell culture after up to 30 serial virus passages in the presence of favipiravir.[84,191] From a drug development perspective, this quality is of course clearly advantageous. On the other hand, it obstructs experiments aimed at providing conclusive evidence on favipiravir's mechanism of action.

Next to its activity against influenza virus,[82] favipiravir inhibits the replication of various other RNA viruses.[192] Until now, few structural analogues of favipiravir have been reported. The 6-fluoro substituent is not required for antiviral activity,[83,191] whereas the 3-hydroxyl group is indispensable.[83] An example of a base-modified analogue is "**3c**" (Fig. 7A), which displayed comparable antiviral activity as T-705 in cell culture and influenza polymerase enzymatic assays.[96] A recent example of a sugar-modified analogue is the 2′,4′-bridged analogue "**8a**" (Fig. 7A), which has anti-influenza virus activity comparable to that of favipiravir.[97] This compound has the 3′-hydroxyl of the pentose in the inverted xylo position. Evaluation of "8a" (or, rather, its 5′-triphosphate) at the level of the viral polymerase was not yet reported.

3. 2′-Deoxy-2′-Fluoroguanosine and Other Nucleoside Analogues

2′-Deoxy-2′-fluoroguanosine (2′-FdG; Fig. 7A) was reported years ago as a broad inhibitor of influenza A and B viruses in cell culture.[98] Its analogues **2′-deoxy-2′-fluoroadenosine** and **2′-deoxy-2′-fluoroinosine** were significantly less active.[99] In vivo, 2′-FdG was shown to reduce influenza virus titers in the respiratory tract of mice and ferrets.[193] Mechanistically, 2′-FdG-triphosphate was found to inhibit the influenza polymerase complex by nonobligate chain termination. This agrees with the observation that a virus selected for resistance to 2′-FdG, contained a polymerase with tenfold lower susceptibility to 2′-FdG-triphosphate in an enzymatic assay.[161] The identity of the amino acid changes in this mutant polymerase was not disclosed, which is unfortunate since this insight could be very helpful to explain the role of specific residues in the catalytic or other functional domain of PB1, as identified in the recent crystallographic studies.[23,24,56,58]

During more recent years, **2′-substituted carba-nucleoside analogues**,[100] **C-3′-modified analogues**,[102] and **6-methyl-7-substituted-7-deaza purine nucleoside analogues**[103] were reported to have anti-influenza activity comparable to 2′-FdG. The antiviral activity of 2′-FdG and analogues is dependent on intracellular conversion to the active nucleoside 5′-triphosphate form. ProTide prodrugs were employed to overcome the first (rate-limiting) phosphorylation step, and enable intracellular delivery of the nucleoside 5′-monophosphate species.[194] This **ProTide** concept was successfully applied to 2′-FdG and its uridine analogue 2′-FdU.[104,106]

The pyrimidine analogue **2′-deoxy-2′-fluorocytidine** (2′-FdC) seems more potent than 2′-FdG, with in vitro and in vivo activity against various strains of influenza A or B.[105] It remains to be demonstrated whether the 5′-triphosphate of 2′-FdC acts as an alternative substrate for the influenza polymerase complex, as is the case for 2′-FdG. Alternatively, since 2′-FdC was shown to be cytostatic in cells,[195] it could act as an immunomodulator in vivo, or inhibit influenza virus through inhibition of cellular enzymes which are involved in de novo pyrimidine biosynthesis. Similar to what is explained above for ribavirin and other inhibitors of IMPDH,

nucleoside analogues that interfere with purine or pyrimidine nucleotide synthesis can give strong inhibition of influenza virus replication in cell culture. Unfortunately, the therapeutic window of this approach is too narrow to allow broad application in influenza virus therapy. A few examples are **2′-2′-difluorodeoxycytidine**[196] (known as the anticancer drug gemcitabine), **compound** "**A3**,"[107] and **N10169**,[108] which lower the intracellular pyrimidine levels by inhibiting ribonucleotide reductase, dihydroorotate dehydrogenase, and orotidylate decarboxylase, respectively. Also, the anti-influenza activity of the carbocyclic nucleoside analogues **carbodine** and **cyclopentenyl cytosine** has been linked to pyrimidine depletion through CTP-synthetase inhibition.[94,197–199] For a few carbocyclic purine nucleoside analogues with borderline to modest activity against influenza virus, no mechanistic data are available.[200] For the broad antiviral agent **3-deazaguanine**, a mechanism involving IMPDH inhibition was proposed.[94,201]

4. Time to Revisit Nucleoside Inhibitors for Influenza?

That nucleoside analogues merit more attention in influenza drug development is nicely illustrated by the successful progression of T-705. The unprecedented bonuses of this agent are: high resistance barrier, low cytotoxicity, and broad coverage of diverse RNA viruses. As for 2′-FdG, the relevance of the 2′-fluoro modification to achieve nonobligate chain terminators of RNA virus polymerases[202] is underscored by the fact that this substitution is present in successful drugs for hepatitis C[203] or drug candidates for respiratory syncytial virus therapy.[204] An important aspect is the avoidance of inhibitory effects on cellular polymerases or enzymes of the purine or pyrimidine pathways, since this will de facto reduce the therapeutic index. Besides this relevance for drug development, nucleoside analogues (or their 5′-triphosphate forms) are unique tools to study the kinetics or fidelity of the influenza polymerase, and the role of specific PB1 residues.

C. Inhibitors of the Cap-Snatching Reaction

1. Inhibitors of Cap-Binding by PB2

As explained in Section 2,A, the first part of the cap-snatching reaction by the influenza virus polymerase involves cap-binding by PB2. Initially, the fact that PB2 recognizes capped RNA alike eukaryotic cap-binding proteins such as eIF4E,[205] raised doubts about the target drugability of the PB2-CBD and the possibility to design influenza virus-selective cap-binding inhibitors. However, Hooker et al.[39] used quantitative UV crosslinking to analyze the elements in cap analogues that contribute to their recognition by isolated vRNPs and eIF4E, and identified some notable differences. Based on this, compound **RO0794238** (Fig. 2F) was designed as a cap analogue lacking a negative charge and containing an acyclic moiety instead of a ribose, with the aim to achieve superior potency and selectivity for PB2. This molecule was indeed able to inhibit cap-binding by influenza virus vRNPs in a dose-dependent manner, with no effect on eIF4E.[39]

The revelation of the cocrystal structure of the PB2-CBD in complex with m^7GTP[32] (Fig. 2A and Section 2,A) finally enabled the rational design of selective inhibitors, given its unique protein fold and cluster of hydrophobic residues. Four compounds (e.g., compound "**11**"; Fig. 2C) displayed potent activity in a binding assay with the isolated PB2-CBD but, unfortunately, were devoid of antiviral activity in influenza virus-infected cells (possibly due to the presence of multiple negative charges, resulting in poor cellular uptake).[36] The previously identified compound RO0794238[39] however appeared inactive in this binding assay. The authors speculated that RO0794238 might inhibit cap-binding by PB2 in an indirect manner through its interaction with another part of the viral RNP complex. In addition, given its long substitution

at the *N*-7 position, it seems implausible that RO0794238 would be able to bind in the m^7GTP pocket of the PB2-CBD.

The validity of the PB2-CBD as a drug target is best demonstrated by the clinical candidate **VX-787** (JNJ-63623872 or JNJ-872; Fig. 2D). In a Phase 2a challenge study, VX-787 yielded a dose-dependent decrease in symptom scores and duration of symptoms.[11] Additional clinical trials with this promising compound are ongoing. Its discovery process was reported in 2014 by Clark et al.[37] VX-787 is an azaindole derivative that resulted from extensive iterative synthesis to develop PB2 inhibitors, which optimally occupy the m^7GTP binding pocket, as demonstrated by cocrystallizing VX-787 with the PB2-CBD of influenza A (Fig. 2D). In particular, optimal interactions with the hydrophobic as well as basic residues were achieved by introducing the bicyclooctane-carboxylate moiety. A further structure-activity relationship (SAR) exploration with isosteric replacements of the carboxylic group of VX-787 showed that the p*Ka* value and orientation of the negative charge significantly affect both anti-influenza potency and selectivity for unwanted protein kinase targets (the latter being related to compound binding to the ATP site of cellular kinases).[206] VX-787 possesses strong antiviral activity (i.e., EC_{50} values in the nanomolar range) in cellular assays with a broad range of influenza A virus strains, including NAI- and amantadine-resistant isolates, 2009 pandemic H1N1, and circulating avian H5N1 strains.[37,207] Its activity against influenza B virus is negligible, which can be attributed to amino acid differences in the PB2-CBD.[208] In particular, the π-stacking interaction between VX-787 and Phe323 in the influenza A PB2-CBD cannot be formed in the influenza B protein, which contains a glutamine (Q325) at the corresponding position (Fig. 2A and B). The apparent flexibility in the PB2-CBD of influenza B (see Section 2,A) may be another factor complicating the development of cap-binding inhibitors that cover both influenza A and B virus.

Resistance studies with VX-787 in cell culture delivered six resistance mutations in PB2 (Q306H, S324I, S324N, S324R, F404Y, and N510T), which yielded at least 60-fold reduction in VX-787 sensitivity.[207] In the Phase 2a clinical study, an M431I mutant virus was detected in a minority of the patients, which, in cell culture, displayed 57-fold lower sensitivity to VX-787, yet reduced viral fitness.[11] In mice, prophylactic use of VX-787 (which is orally bioavailable) provided 100% protection against the APR8 laboratory strain, a pandemic 2009 strain, or a highly pathogenic avian H5N1 virus. In addition, this inhibitor provided 100% survival when treatment was initiated up to 4 days after challenge with influenza virus.[207]

Recently, Roch et al.[38] reported two compounds, **Cap-3** and **Cap-7** (Fig. 2E), which bind to the PB2-CBD and inhibit transcription in an enzymatic assay with the polymerase complex. In addition, they inhibit virus replication in cell culture, albeit considerably less potently than VX-787 (i.e., EC_{50} values in the range of 1–9 μM). Hitherto, mechanistic validation was not yet reported.

2. Metal-Chelating Inhibitors of PA-Nter Endonuclease Activity

The conserved nature of several amino acid residues inside the catalytic site of PA-Nter implies that this domain is highly relevant to achieve inhibitors with broad activity against influenza A and B. The strategy explored thus far consists of metal-chelating scaffolds containing coplanar oxygens to bind the divalent metal ion(s), which resembles the action principle of approved HIV integrase inhibitors.[209,210] The challenge is to achieve inhibitors, which optimally occupy the PA-Nter catalytic site and surrounding regions, yet do not interact with metal-dependent proteins of the host cell. The concept seems now validated since the recent introduction of two PA inhibitors (**AL-794** and **S-033188**) into clinical trials.[211]

The literature contains diverse PA inhibitors with inhibitory activity in enzymatic assays with the viral polymerase or isolated PA-Nter. It is important to note that for many of these agents, cell culture data on antiviral activity and, in particular, mechanism of action are not

elaborated. Some compounds have a high anionic charge that impedes their cellular uptake. For example, we demonstrated that for **EGCG** (Fig. 6K), the activity in virus-infected cells is related to inhibition of virus entry.[74] To definitely prove that an assumed PA inhibitor acts upon PA in a virus/cell context, resistance studies should be carried out. As of today, this decisive mechanistic evidence is only available for **L-742,001** (Fig. 6E–G), since three laboratories including ours[49,74,117] demonstrated that specific mutations in PA confer moderate (3-) to high (>10-fold) viral resistance to this agent. The substitutions were present in the catalytic core of PA or surrounding hydrophobic pockets (colored purple, green, red, and orange in Fig. 6), confirming that these PA regions are critical for the antiviral mode of action of L-742,001. This molecule is the prototype of the first class of influenza endonuclease inhibitors, already discovered at Merck about two decades ago.[115,116] Among this series of 4-substituted-2,4-dioxobutanoic acids with a characteristic β-diketo acid motif, compound L-742,001 was identified as a particularly potent inhibitor of the influenza virus endonuclease reaction in an enzymatic assay, and of virus replication in cells.[49,74,116,117] In a mouse model, the compound provided up to 4-$\log_{10}$ reduction in virus lung titers.[116] Strong in vivo activity was also reported for the recently discovered inhibitor **ANA-0** proposed to act upon PA (Fig. 6K).[79] Other classes of reported endonuclease inhibitors (awaiting mechanistic validation in cell culture) include: **flutimide**[75] (Fig. 6K) and a series of more potent aromatic analogues,[212] ***N*-hydroxamic acid** and ***N*-hydroxyimide** (Fig. 6K) compounds,[77] **tetramic acid** derivatives,[118] **polyphenolic catechins**,[76] **trihydroxyphenyl**-bearing compounds[73,78,120] (Fig. 6D and K, compound 2[73] and compound 16[78]), **phenethylphenylphthalimide** analogues derived from thalidomide,[119] **macrocyclic bisbibenzyls**,[213] **fullerenes**,[214] **hydroxyquinolinones**,[215] **hydroxypyridinones**,[47,50] **hydroxypyridazinones**,[216] **hydroxypyrimidinones**,[216] **β-diketo acid (DKA)**, and DKA-bioisosteric compounds,[68] **bis-dihydroxyindolecarboxamides**,[121] **2-hydroxybenzamides**,[217] **thiosemicarbazones**,[218] **pyrimidinoles**,[45] **pyridopiperazinediones**[38] (Fig. 6K, Endo-1), and miscellaneous compounds bearing distinct pharmacophoric fragments.[45,78,101] All these different chemotypes have in common that they bear chelating motifs able to bind the bivalent metal ion(s) in the catalytic core of PA-Nter.[219]

As explained in Section 2,B, the different crystal structures for PA-Nter that have become available since 2009 converge on the overall structure of the active site, yet do not agree on whether the enzyme contains one or two (or possibly three) divalent metal ions in its catalytic site, nor on which metal ions (i.e., Mn^{2+} or Mg^{2+}) are present in the native enzyme.[42–47] Knowledge of the precise number and identity of these metal ions is critical to design optimized metal-chelating PA inhibitors. Similar to what was observed for HIV integrase,[220] we found that the potency of metal-chelating inhibitors in enzymatic assays with PA-Nter can show large variations depending on which bivalent metal ion (Mg^{2+} or Mn^{2+}) is used.[68] This phenomenon can be explained by the different ligand preferences of these two metal ions. For example, magnesium generally binds oxygen atoms rather than nitrogen, while manganese has slightly greater affinity for nitrogen.[221] Moreover, two tautomeric forms of an inhibitor can display a difference in metal preference. For DKA compounds, it was shown that Mn^{2+} preferentially binds to the diketo form, while Mg^{2+} predominantly recognizes the β-keto-enol form.[222] Dependent on which metal ion is physiologically relevant, the compounds' activity may increase by stabilization of the preferred tautomeric form, for instance by substituting the flexible β-diketo acid (DKA) moiety by a "locked" motif having optimal geometry for bidentate metal coordination.

Crystallographic analyses of PA-Nter in complex with diverse PA inhibitors have revealed that the catalytic center is surrounded by different hydrophobic pockets, which are amenable to inhibitor design.[45–47,49,50,120,215,216] Overall, the active site of PA-Nter is quite spacious and flexible, and in silico design of optimized PA inhibitors is further complicated by the fact that inhibitors bind via an induced-fit mechanism. Assuming that metal-chelating PA inhibitors act as substrate or product mimics, precise insight into the binding mode of the RNA substrate

or cleavage product is crucial to identify which of the hydrophobic pockets have the highest relevance for PA inhibitor design. A cocrystal structure for PA-Nter in complex with an RNA substrate is highly needed. Three X-ray structures are available of PA-Nter in complex with UMP (Fig. 6H–J), which can be regarded as a mimic of one of the two cleavage products.[44,46,49] Two distinct UMP-binding poses were observed, particularly related to the position of the ribose and base, which underlines the impact of crystallization procedures (i.e., cocrystallization vs. soaking; addition of metal ions or not). In the context of PA, cocrystallization seems more reliable since it enables the binding pocket to adapt to the ligand. One particularly flexible region is the helix containing residue Tyr24, which, in two cocrystal structures, stacks with the uracil base[46,49] of UMP (Fig. 6I and J, in green) and also shows hydrophobic interactions with PA inhibitors like those shown in Figures 6C and E.[45,46,49,50] To accommodate these ligands, this Tyr24 moves or rotates compared to its pose in the apo form. Since, upon crystal soaking, this conformational change is unlikely to occur, the ligand may be forced into an alternative and biologically less relevant binding pose.

Another interaction site that seems of high relevance is Arg124. A role for this residue in RNA substrate binding was suggested by Yuan et al.[43] This was corroborated by Xiao et al.,[57] whose MD simulations (Fig. 6A) pointed to Arg124, Arg125, and Arg192 as the residues responsible for interaction with the 3′ end of the RNA substrate. Likewise, the relevance of this Arg124 pocket (in blue in Fig. 6) was suggested by our cell-based antiviral studies in which we introduced mutations at different sites within or around the PA-Nter active center, and analyzed the resistance of the mutant viruses to the prototype PA inhibitor L-742,001.[74] The data agreed to a docking model[74] for L-742,001 in PA-Nter (Fig. 6G), but only partially agreed to the cocrystallization results[45,49] (Fig. 6E and F). In addition, an analogous orientation as for the docked L-742,001 molecule was seen in the X-ray structures of PA-Nter in complex with the dihydropyrimidine-based "compound 5"[45] (Fig. 6B), and the particularly potent hydroxypyridinone-based PA inhibitor "compound 7"[50] (Fig. 6C). For both these compounds, the authors reported a role for Arg124 in binding of the inhibitor.

Consequently, we speculate that a PA inhibitor with a binding orientation that allows interactions with these RNA-binding residues, mimics the 3′ end of the capped RNA substrate and is able to efficiently compete with the substrate. Presumably, the interactions with the 3′ end are essential for correct positioning and cleavage of the substrate. In contrast, the interactions between the 5′ end of the capped RNA and PA-Nter (i.e., at the pocket colored in purple in Fig. 6) may be weaker, since the cap-binding site in the PB2 subunit already ensures strong binding of the 5′ end. As a result, the pocket in PA-Nter, which binds the 5′ end could possibly be more flexible. An additional argument is the fact that, once cleavage has occurred, the PA active site has to release the capped 5′ end in order to allow its relocation into the PB1 catalytic site for primer elongation and transcription of the viral mRNA. Hence, tight binding affinity of PA to the 5′ end of the capped RNA substrate would substantially decrease the rate of product release, which would be detrimental to the overall process of transcription.

3. PA Targeting Aptamers

Recently, a DNA aptamer library screening performed by Yuan et al.[123] yielded a number of aptamers with binding affinity to either entire PA or PA-Nter. Four aptamers were able to inhibit the endonuclease reaction and one of them showed broad influenza A inhibition when transfected into virus-infected cells. At present, aptamers still require significant improvements in terms of pharmacokinetics and—dynamics before having broad clinical applicability. Nevertheless, this study demonstrates that aptamers can be excellent tools to identify new relevant binding pockets for inhibitors of the PA endonuclease or other viral target proteins.

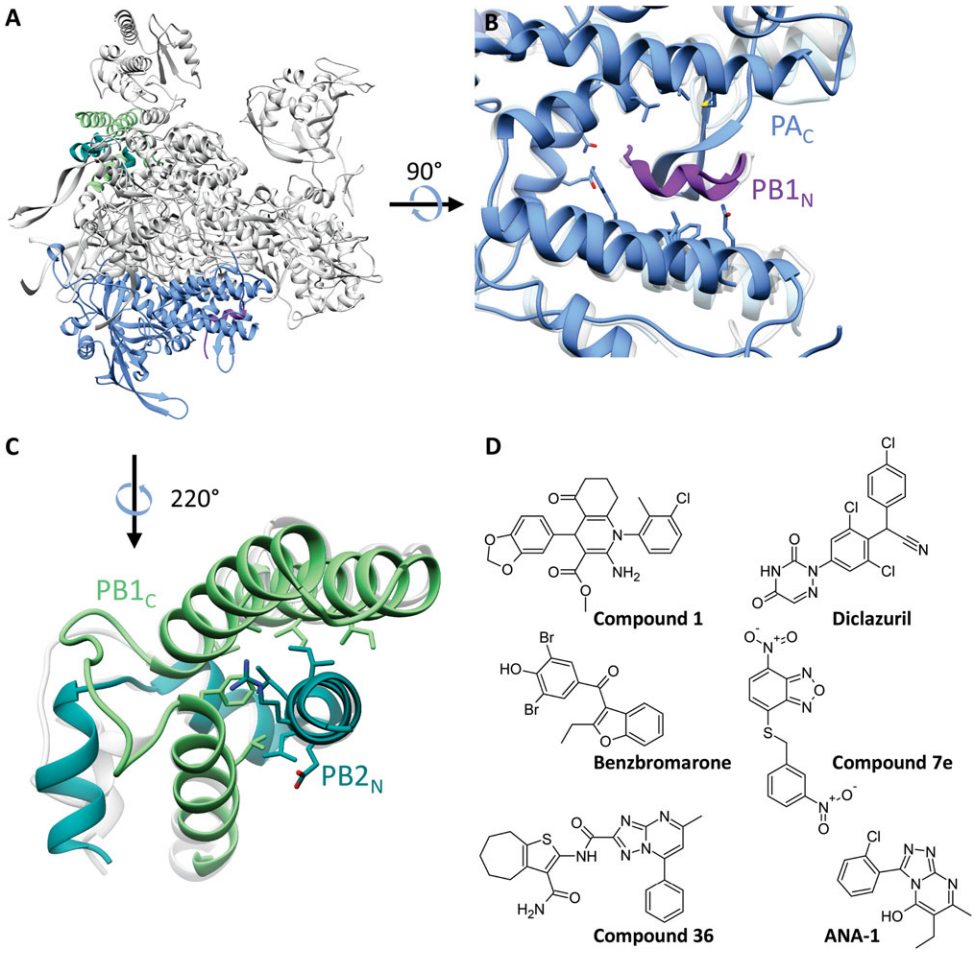

Figure 8. The two thus far explored protein–protein interaction (PPI) domains and chemical structures of PA$_C$-PB1$_N$ PPI inhibitors. (A) Location in the FluA polymerase crystal structure[23] (PDB: 4WSB) of the two PPI domains, which have been targeted by peptides or small molecules. (B) Closeup showing a superposition of the crystal structures of the PA$_C$-PB1$_N$ interface[223] (PDB: 3CM8) on that of the FluA polymerase (light gray) and the apo form of PA$_C$[224] (light blue; PDB: 4IUJ). (C) Closeup showing a superposition of the crystal structure of the PB1$_C$-PB2$_N$ interface[225] (PDB: 3A1G) on the FluA polymerase complex, in light gray. (D) Chemical structures of representative PA$_C$-PB1$_N$-PPI inhibitors "compound 1,"[127] diclazuril,[126] benzbromarone,[126] "compound 7e,"[124] "compound 36,"[131] and ANA-1.[133]

D. Protein–Protein Interaction Inhibitors of the Influenza Polymerase Complex

The assembly of the three subunits (PB1, PB2, and PA) into a functional viral polymerase complex is essential for influenza RNA synthesis and virus replication. Thus, interference with its correct assembly through inhibition of a crucial protein–protein interaction (PPI) is currently explored as an innovative antiviral strategy. Until 2014, tailored design of assembly inhibitors was based on at that time available crystal structures for two specific interfaces in the polymerase heterotrimer (Fig. 8). The revelation of the full polymerase structure now creates the possibility to explore other inter- or intrasubunit interfaces, yet two important issues are: (i) the size of a specific interface, to allow fitting of small-molecule inhibitors; and (ii) the conservation of its residues when envisaging both influenza A and B.

The structural details for one important PA-PB1 interaction domain were revealed in 2008, based on cocrystallization of the PA C-terminal domain (PA_C, residues 239 or 256–716) with a short N-terminal PB1 peptide ($PB1_N$).[223,226] This PA_C domain resembles a dragon's head that clamps the $PB1_N$ peptide. As shown in Figure 8B, the base of this pocket overlaps between the crystal structures of the $PB1_N$-bound PA_C domain[223] (in dark blue), the full polymerase heterotrimer[23] (in gray), and the apo PA_C domain[224] (in light blue). In contrast, there is less overlap at the periphery, meaning that the binding groove is slightly more narrow in the structure of the $PB1_N$-bound PA_C compared to the two other crystal structures. The less mobile base of the pocket appears relevant as the initial interaction point for PPI inhibitors; to these scaffolds, structural elements should be added to target also the more flexible protein parts on the periphery of the interface.

Three features explain the drugability of this PA_C-$PB1_N$ interface: several residues are conserved (among influenza A, B, and C), and the interface is hydrophobic and relatively small, implying that it can be targeted by small molecules.[227] Initially, the concept to disrupt the PA_C-$PB1_N$ interface was explored with PB1-derived peptides.[228–230] Among a first series of small-molecule inhibitors identified by in silico screening followed by a PA_C-$PB1_N$ biochemical interaction assay, "**compound 1**" emerged as particularly relevant given its broad anti-influenza A and B virus activity in cell culture (Fig. 8D).[127] Other early lead molecules with anti-influenza A activity in cell culture are **benzbromarone** and **diclazuril**[126] and "**compound 7e**"[124] (Fig. 8D). This was followed by rational development of diverse lead compounds,[125,129–132,231] some of which were used to generate a pharmacophore model for PA_C-$PB1_N$ interaction inhibitors.[130] For the recently identified inhibitor **ANA-1** (Fig. 8D), the anti-influenza activity was confirmed in a mouse model.[133] On the basis of docking, the binding site of ANA-1 in PA_C was predicted to lie in an allosteric site adjacent to the PB1 interacting domain. Yet, for all the PA_C-$PB1_N$ interaction inhibitors reported thus far, mechanistic studies using cocrystallization or resistance selection remain to be performed to verify their antiviral mode of action and precise binding mode. This could also aid to design PA_C-$PB1_N$ assembly inhibitors, which establish hydrophilic besides hydrophobic interactions, thereby leading to better solubility and potentially higher antiviral potency than the current lead compounds.[232]

Another protein–protein interface that was validated with peptide inhibitors[233,234] but is as yet unexplored with small molecules, is the interaction domain between $PB1_C$ (residues 678–757; Fig. 8C, in light green) and $PB2_N$ (residues 1–37; in dark green). The crystal structure of this isolated domain was published in 2009[225] and nicely overlaps with that in the full polymerase complex[23] (in gray). Despite the fact that this $PB1_C$-$PB2_N$ interface carries conserved amino acids, the inhibition by a $PB2_N$-derived peptide seemed prone to strain dependency.[235] One proposed hypothesis relates this observation to a conformational change in PB1, when it is bound in the PB1-PA dimer intermediate, thereby rendering the $PB2_N$-binding part in PB1 inaccessible. Hence, this PB1-PB2 interface appears a more challenging target for development of broad influenza virus inhibitors.[234,235]

E. Gene Silencing Approaches

Pharmaceutical development of oligonucleotide inhibitors faces some common obstacles, particularly related to their low in vivo stability and inefficient cellular delivery. Antisense oligonucleotides function as a single strand and block mRNA processing or translation by binding to the mRNA to which they are complementary. One influenza virus inhibitor currently in Phase 1 clinical trials,[236] is the antisense oligonucleotide AVI-7100 (Radavirsen), a phosphorodiamidate morpholino oligomer (PMO). AVI-7100 contains nonionic morpholino rings (instead of ribose rings) and three phosphorodiamidate intersubunit linkages, and was designed to interfere with translation and splicing of mRNA derived from the M-gene. In preclinical studies, AVI-7100

proved effective against influenza A virus infection in mice and ferrets, even when administered after virus challenge.[146]

The possible application of RNA interference (RNAi) for influenza therapy has been the subject of a series of studies.[237] This includes siRNAs, small RNA duplexes, which trigger the destruction of specific mRNAs by associating with the RNA-induced silencing complex. This concept can be valuable for PA, PB1, PB2, and NP since some regions in their nucleotide sequences are conserved among influenza A subtypes. The siRNAs for NP and PA were shown to prevent accumulation of their mRNAs, and reduce the mRNA, vRNA, and cRNA levels for other viral genes.[238] siRNAs against NP, PA, and PB1 were shown to have antiviral activity in cell culture and mice.[238,239] 5′-Triphosphate modification of siRNA (for NP) provided a dual effect by combining gene silencing with RIG-I activation.[240] Alternatively, to circumvent the poor delivery of siRNA, a lentiviral[241] or *Escherichia coli* vector[242] was explored to express NP- and PB1-targeting, or NP- and PA-targeting RNA interfering sequences, respectively.

F. Small-Molecules Targeting the Viral NP

The viral NP directly interacts with the viral polymerase complex to support viral RNA synthesis.[243–245] However, it primarily has a structural function since it forms the protein scaffold of the vRNP complexes through its homo-oligomerizing[246–248] and RNA-binding properties.[246,249] The NP protein is crescent shaped with head, body, and tail domains[246,247] and a putative RNA-binding groove (Fig. 9A and B).[246] NP–NP oligomerization is the result of one tail loop inserting into the body of a neighboring monomer. This insertion is stabilized by intermolecular β-sheets, hydrophobic interactions, and salt bridges.[246]

The NP protein contains different sites that are amenable to inhibitor design and are briefly described below (Fig. 9C–E).[136] A first series are NP-aggregating agents such as the aryl piperazine amide compound called **nucleozin** (Fig. 9D).[111,134,135,251] This molecule forms bridges between NP subunits to give higher order NP oligomers.[135] Depending on the assay, nucleozin exerts its activity prior to nuclear import of the vRNPs; at the stage of viral RNA synthesis; or during cytoplasmic trafficking of progeny vRNPs.[252] It exhibits robust activity against influenza A virus in vitro and in vivo, yet is inactive against influenza B. Viral resistance to nucleozin emerged after only five cell culture passages.[111,134,135] This agent is inherently inactive against some influenza virus strains (such as the 2009 pandemic H1N1 virus) that already carry one of its resistance mutations.[111,134] Another NP-aggregating compound, **PPQ-581**, shows no cross-resistance to nucleozin and selects for a resistance mutation adjacent to the nucleozin-binding site (Fig. 9D).[137]

A second type of NP inhibitors target the highly conserved Glu339-Arg416 salt bridge among NP neighbor monomers.[246] Shen et al.[138] identified four small-molecule influenza inhibitors, which are able to disrupt NP oligomerization, with compound "**3**" (Fig. 9E) being the most potent one in cell culture.

A third possibility is to target the RNA-binding groove in NP. I*n silico* screening[139] led to the identification of **naproxen** (Fig. 9C), which was found to inhibit RNA binding by NP in biochemical assays, and display anti-influenza virus properties in cell culture and mouse models. The latter activity is likely potentiated by naproxen's well-known anti-inflammatory effect. The analogue **naproxen C0**,[250] which carries a 1,3-dicarboxylated phenyl group instead of the propionic acid in naproxen, has higher NP binding affinity in vitro, but its activity in cell culture was not yet reported. A different virtual screening hit encoded **F66** showed antiviral activity in cell culture and mouse models,[140] yet its RNA-NP disrupting activity and binding mode in NP remain to be established.

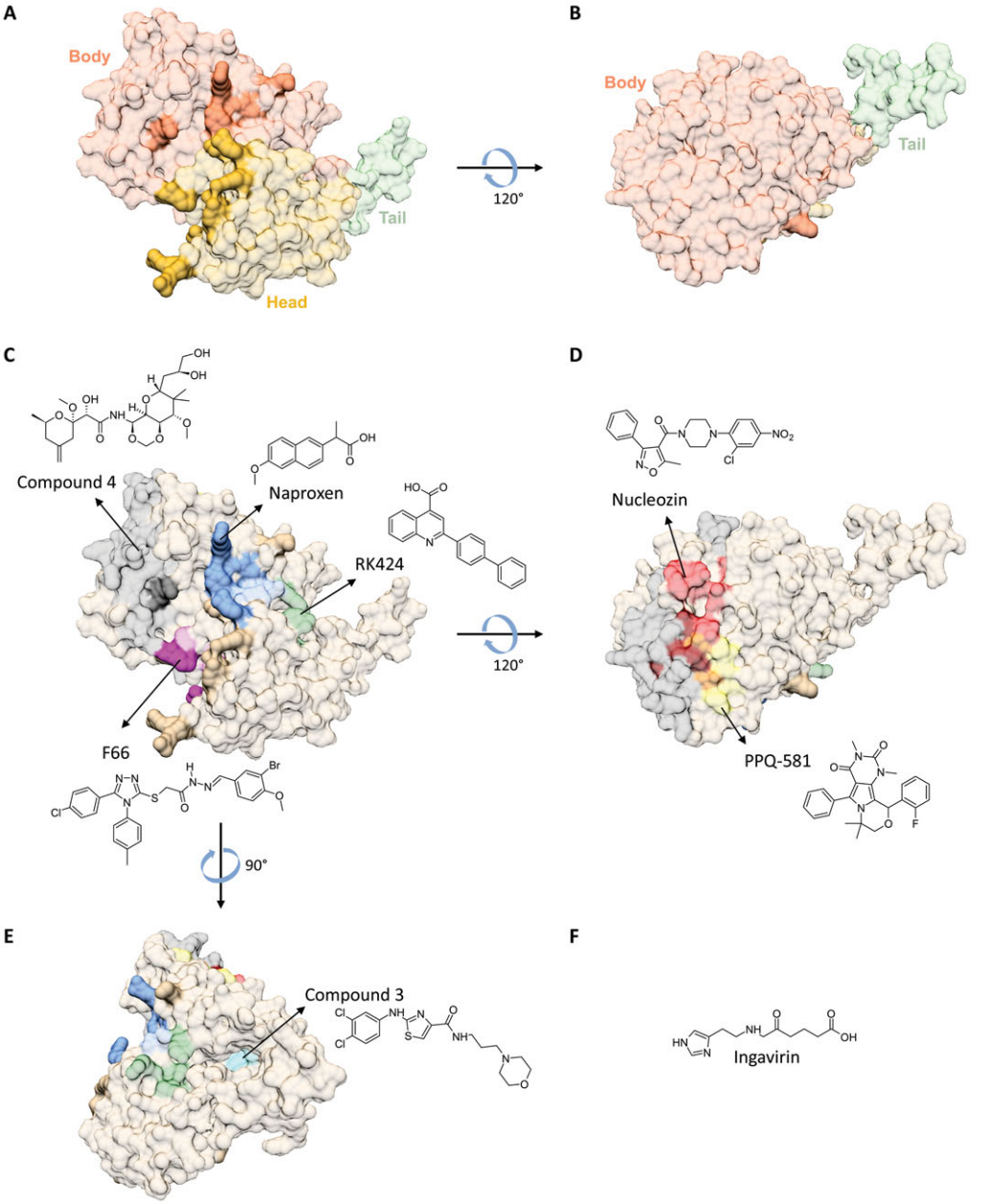

Figure 9. Structure of the viral nucleoprotein and chemical structures of proposed or proven NP inhibitors. The NP structures are based on the H1N1 NP X-ray structure[246] (PDB: 2IQH). The residues lining the RNA-binding groove are shown in darker colors. (A) Head, body, and tail domains in NP. (B) As in panel A, but rotated 120° around the *x*-axis. (C) Predicted binding sites for compound 4,[142] naproxen[139] and naproxen C0,[250] RK424,[141] and F66.[140] (D) Binding site for nucleozin[111,134,135] (proven by cocrystallization) and that predicted for PPQ-581.[137] The overlap of the nucleozin-binding site with that of compound 4 or PPQ-581 is colored in maroon and orange, respectively. (E) The model in (C) is rotated 90° around the *y*-axis to show the internal tail loop binding cavity (in cyan), which is the predicted binding pocket for compound 3.[138] (F) Chemical structure of Ingavirin®, which was reported to impair biogenesis and oligomerization of NP in vitro.[144]

Compound **RK424**[141] was reported to inhibit influenza A virus in cell culture and mouse models, and interfere with RNA binding to NP, the NP–NP interaction, and nuclear export of NP. Although this complex mode of action remains to be verified, it seems to agree with the docking analysis for RK424, which predicted its binding in an NP pocket surrounded by the RNA-binding groove, NP dimer interface, and nuclear export signal.[141] Other proposed NP inhibitors are compound "**4**" (Fig. 9C) and **Ingavirin®** (Fig. 9F), a controversial[143] influenza inhibitor that is commercially available in Russia. This agent was reported to impair biogenesis and oligomerization of NP in vitro[144] and suppress influenza A and B infections in animal models.[145,253] However, since its broad antiviral activity encompasses unrelated respiratory viruses,[145] Ingavirin® most likely targets a cellular rather than a virus-specific component.

Taken together, besides nucleozin, several lead molecules to target NP have been proposed during recent years. Among these, nucleozin is the only inhibitor for which the mechanism has been firmly proven by cocrystallization and resistance analysis.

G. Less Defined Approaches to Inhibit the Influenza Polymerase Complex

For a number of compounds suggested to act on influenza virus polymerase activity in cell culture, the detailed mechanism of action has not yet been revealed. Compound "**367**"[111] (Fig. 7B) was proposed to target PB1 since mutation H456P in PB1 rendered the virus resistant to "367" and its close analogue "**715**." Compound "367" inhibited viral RNA synthesis in the vRNP reconstitution (minigenome) assay, an effect that possibly contributes to its antiviral activity in influenza virus-infected cell cultures. Another small molecule that may target PB1 is "**ASN2**" (Fig. 7B). This compound inhibits influenza A virus polymerase function while simultaneously activating the innate immune system by inducing type I interferon expression.[112] ASN2 inhibited viral transcription but not all genes were equally affected. It induced preferential downregulation of mRNAs encoded by the smallest genome segments, that is M1, M2, NS1, and nuclear-export protein (NEP). Loss of NS1 expression resulted in induction of interferon-I, which may prove beneficial in an in vivo setting. Resistance selection revealed that the antiviral activity of ASN2 is linked to the serine or tyrosine residue at position 499 in PB1.

Before the three-dimensional structure of PB1 was revealed, it was impossible to analyze whether the identified resistance mutations for ASN2 and "367" (at position 499 and 456 of PB1, respectively) are located close to each other. Using the crystal structures published by Pflug et al.,[23] we were now able to establish that both residues are located at the outside of PB1, close to the PB2-627 domain (Fig. 5, in maroon) and at a distance of 15–20 Å from each other. Both molecules appear relevant not only in terms of drug development, but also as tools to unravel as yet unknown functions of particular regions in PB1.

Compound **THC19** (Fig. 7B) was reported to inhibit influenza RNA synthesis and virus replication by targeting the PA subunit, yet its mode of action remains to be established.[122] The quinoline-based compound **BPR3P0128** (Fig. 7B) was shown to strongly inhibit viral mRNA synthesis.[148] This molecule inhibited binding and cleavage of capped RNA fragments by influenza polymerase complexes in nuclear extracts, yet did not compete with cap structures in an in vitro cap-binding assay using isolated PB2. Based on this, it was hypothesized[148] that BPR3P0128 might target the interaction between PB2 and a host factor, such as the heat shock proteins Hsp70 or Hsp90. Based on NMR-based fragment screening, Lee et al.[149] identified the small molecule **DPQ** (Fig. 7B) that appears to interact with the influenza vRNA promoter. This molecule was found to inhibit virus replication in a plaque reduction assay.[149]

H. Strategies Directed Toward Associated Host Cell Factors

The concept of targeting a host factor to indirectly inhibit influenza virus RNA synthesis is still in its infancy. Like all other viral pathogens, influenza viruses strictly rely on a variety of host factors to support their replication.[254] Thus, targeting such a cellular component represents an alternative to traditional drugs that are directed toward a viral factor.[255–257] This approach could diminish the emergence of resistance since the mutation rate of the host cells is intrinsically lower compared to that of the virus, and the virus most probably does not readily adapt itself to use an alternative cellular factor. Obviously, the inherent challenge of all host-directed drug discovery campaigns is to ensure that inhibition of the cellular protein is not detrimental to the host. Hence, the pharmaceutical industry has been rather hesitant when considering host cell targets to treat virus infections.

As explained below, miscellaneous host targeting approaches for influenza have been published, which, at least conceptually, may be linked to indirect inhibition of the viral polymerase complex. The interpretation is complicated by the fact that many of these molecules have multiple pharmacological (such as anti-inflammatory) effects. Hence, their therapeutic activity in mouse influenza models may be related to immune-related besides antiviral effects. Although this deviates from the traditional view on antiviral therapy, it fits with an ongoing call for broader acting or immunomodulatory antiviral agents.[258]

One explored host-targeting strategy is to interfere with nuclear transport. All details on the mechanisms for vRNP nuclear import and export can be found in a recent review.[14] Inhibition of the cellular CRM1 protein by the cytotoxic agent leptomycin B was already shown several years ago to give nuclear retention of the vRNPs, presumably by disrupting the interaction between NP and the CRM1 nuclear export receptor.[259] In 2014, the more selective CRM1-antagonist **KPT-335** (Verdinexor; Fig. 7C), which is currently in Phase 1 clinical trials,[260] was proven to increase survival in influenza virus-infected mice.[147]

An alternative approach relates to the heat shock protein Hsp90, which is involved in assembly and nuclear transport of the viral RNA polymerase, possibly as a molecular chaperone for its protein subunits prior to the formation of a mature ternary polymerase complex.[261] It was demonstrated that Hsp90 inhibitors (Fig. 7C) such as **geldanamycin**,[150] its analogue 17-allylamino-17-demethoxygeldanamycin[150] (**17-AAG**), and **3beta-acetoxy-deoxodihydrogedunin**,[151] impair influenza viral growth by reducing the levels of ribonucleoprotein complexes. Recently, Swale et al.[262] revealed how the PA-PB1 heterodimer assembles with host RanBP5 into a complex, probably being the PA-PB1 import complex. Targeting the assembly or disintegration of this complex with inhibitors, could be a valuable antiviral strategy.

Another pharmacological class is protein kinase inhibitors to inhibit phosphorylation of a viral protein or crucial cellular factor. The MEK (mitogen-activated protein kinase kinase) inhibitor **U0126** (Fig. 7C) was shown to cause nuclear retention of viral RNPs, impaired function of the NEP, and concomitant inhibition of virus production.[263] U0126 proved able to suppress propagation of influenza viruses, including highly pathogenic avian influenza viruses, and was reported to have low cytotoxicity in vitro and in vivo.[152,153] Four other MEK inhibitors (Fig. 7C), **PD-0325901**, **AZD-6244**, **AZD-8330**, and **RDEA-119** (which are all orally available and at least in Phase 1 clinical trials for cancer therapy) demonstrated antiviral activity in vitro as single agents or in combination with oseltamivir.[154] Also, the multiple kinase inhibitor **WV970** (Fig. 7C) was reported to suppress influenza virus in cell culture through vRNP inhibition.[155]

The NF-κB signaling pathway is critical for efficient replication of influenza virus, and its inhibition results in reduced virus titers. Mazur et al.[156] reported that **acetylsalicylic acid** (aspirin; Fig. 7C) can efficiently diminish influenza virus replication in vitro and in vivo through NF-κB inhibition, which blocks caspase-mediated nuclear export of VRNPs. Three

other NF-κB inhibitors, **pyrrolidine dithiocarbamate**, **SC75741**, and **Bay 11–7082** (Fig. 7C), were reported to significantly decrease influenza virus-induced disease in mice.[157–159,264]

Another host cell factor with diverse regulatory functions, the Rac1 GTPase, was demonstrated to have both virus-supportive as antiviral effects. The Rac1 GTPase inhibitor **NSC23766** (Fig. 7C) was found to possess anti-influenza virus properties by affecting viral polymerase activity in a cell-based (mini-genome) assay. NSC23766 was able to reduce virus replication in mice and prolong the survival rate of infected mice.[160]

4. CONCLUSION AND PERSPECTIVES

For many viruses such as HIV, hepatitis B virus, hepatitis C virus, and herpesviruses, inhibitors of viral DNA or RNA polymerases or hereto related activities are the cornerstone of current antiviral interventions. For influenza virus, the development of viral polymerase inhibitors has been lagging behind, but presently seems to be at a turning point. During the past years, major progress was made in the structural elucidation of different influenza virus polymerase subdomains, and very recently of the entire heterotrimeric complex. In parallel, significant advances were achieved in unravelling the reaction mechanisms and functioning of the influenza replication machinery. These accomplishments fuel the ongoing search for unique influenza polymerase inhibitors, which has evolved from serendipitous discovery to rational structure-aided drug design. It can be anticipated that, in the near future, some of these inhibitors will enter the clinic to fundamentally reshape the field of influenza therapy and prevention.

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