Anti-Influenza Drug Discovery and Development: Targeting the Virus and Its Host by All Possible Means

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Abstract

Infections by influenza virus constitute a major and recurrent threat for human health. Together with vaccines, antiviral drugs play a key role in the prevention and treatment of influenza virus infection and disease. Today, the number of antiviral molecules approved for the treatment of influenza is relatively limited, and their use is threatened by the emergence of viral strains with resistance mutations. There is therefore a real need to expand the prophylactic and therapeutic arsenal. This chapter summarizes the state of the art in drug discovery and development for the treatment of influenza virus infections, with a focus on both virus-targeting and host cell-targeting strategies. Novel antiviral strategies targeting other viral proteins or targeting the host cell, some of which are based on drug repurposing, may be used in combination to strengthen our therapeutic arsenal against this major pathogen.

Keywords

Antiviral · Drug repurposing · Replication · Entry · Immune modulator

Abbreviations

CoV	Coronavirus
COX	Cyclooxygenase
HA	Hemagglutinin
IAV	Influenza A virus
IFN	Interferon
M2	Matrix 2
NA	Neuraminidase
NOX	NADPH oxidase
NP	Nucleoprotein
p09	H1N1 2009-pandemic strain
PA	Polymerase acidic subunit
PB1	Polymerase basic subunit 1
PB2	Polymerase basic subunit 2
PPI	Protein-protein interaction
RdRP	RNA-dependent ribonucleoprotein complex
RIG-I	Retinoic acid-inducible gene-I
TNF-α	Tumor necrosis factor-α
vRNP	Viral ribonucleoproteins

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8.1 Introduction

Infections by influenza virus constitute a major and recurrent threat for human health. Influenza viruses are the causative agents of seasonal flu epidemics, associated with up to 1 billion infections and 300,000–650,000 deaths worldwide and consequently with a large economic price including hospitalization costs and missing working days [1, 2]. In addition, influenza A viruses (IAV) have been the cause of several pandemics in recent human history, from the Spanish flu H1N1 in 1918 to the more recent H1N1 2009 pandemic [3].

Together with vaccines, antiviral drugs play a vital part in the prevention and treatment of influenza virus infection and disease. During a normal influenza season, antiviral drugs are mainly used to treat critically ill patients, such as those hospitalized in intensive care. In a pandemic context, pending the availability of a vaccine, antiviral drugs are essential both to treat patients who have been infected and to prevent infection in those exposed, including healthcare workers. Today, the number of antiviral molecules approved for the treatment of influenza, based on the targeting of viral proteins, is relatively reduced and threatened by the emergence of strains with resistance mutations. There is therefore a real need to expand the prophylactic and reinforce the current therapeutic arsenal. This chapter summarizes the state of the art in drug discovery and development for the treatment of influenza virus infections, with a focus on both virus-targeting and host cell-targeting strategies (Fig. 8.1). Novel antiviral strategies targeting other viral proteins or targeting the host cell, some of which are based on drug repurposing, may be used in combination to strengthen our therapeutic arsenal against this major pathogen.

8.2 From Existing Classic Antiviral Drugs to New Pre-Clinical Candidates

8.2.1 M2 Ion Channel Blockers (Amantadine/Rimantadine)

Influenza A M2 is a multifunctional viral homotetramer protein [4]. Its transmembrane (TM) domain forms a proton channel. This channel is required for the acidification of the viral endosome formed after fusion and endocytosis of the virus within the host cell. This process allows viral ribonucleoproteins (vRNPs) to dissociate from the matrix 1 (M1) protein. The proton conductance mechanism relies on the conserved H37XXXW41 sequence which is responsible for selectively gating H^+ ions [5–8]. Channel blockers interfere with the proton conductance mechanism by binding to the transmembrane pore [9] (Fig. 8.2). When proton conductance through M2 is blocked by the adamantane drug, this dissociation is prevented, and the virus is no longer able to replicate. In recent years, adamantane drug-resistant mutants have become prevalent in circulating viruses. The most prevalent drug-resistant mutations are S31N, L26F, and V27A, all of which are located in the transmembrane region of M2 [11]. Figure 8.2a shows the strong interaction of amantadine with V27 in the upper part of the pore. Upon drug resistance V27A mutation, this interaction is lost. Recently developed spiro-amantadyl amine effectively binds to A27 of the pore (Fig. 8.2b) [10]. Recently, new amantadine derivatives effective against double mutants M2-S31N/L26I and M2-S31N/V27A viral strains have been developed by Musharrafieh et al. [12]. The antiviral efficacy of such compounds is summarized in Table 8.1. As a consequence of resistance mutations that

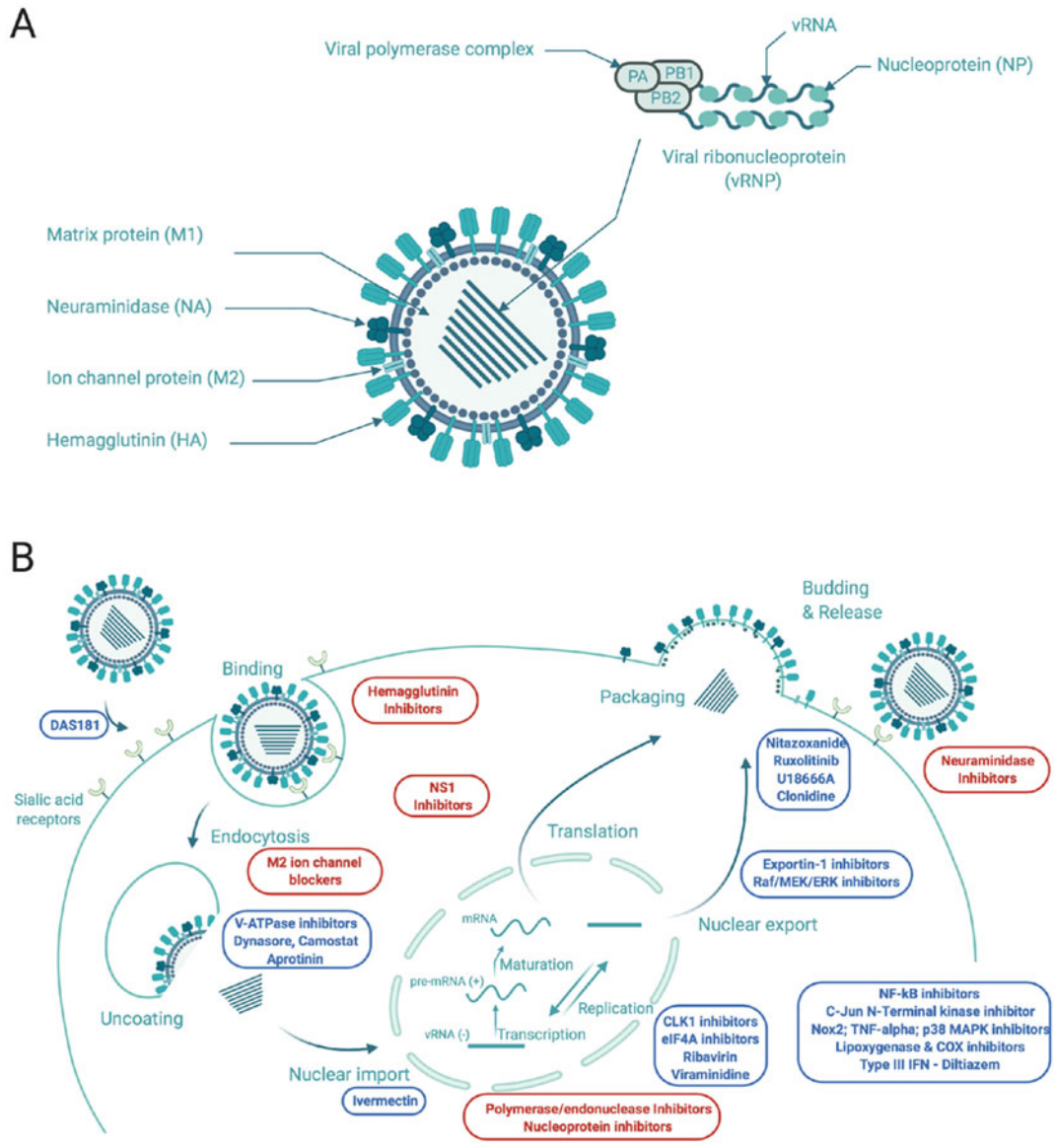

Fig. 8.1 Influenza viral particle and viral cycle; current state of anti-influenza drug discovery and development. (**a**) Influenza A virus (IAV) particle. The IAV genome is composed of eight ribonucleoprotein complexes (vRNPs), composed of single-stranded negative-sense viral RNA (vRNA) encapsidated by viral nucleoprotein (NP) and a viral polymerase complex (PA, PB1, and PB2) positioned at the extremity of the vRNA segment. Three viral proteins, hemagglutinin (HA), neuraminidase (NA), and ion channel protein (M2), are embedded within the viral membrane. Matrix protein 1 (M1) holds the vRNPs inside the virion. (**b**) The viral particle binds to sialic acid receptors and enters the cell via receptor-mediated endocytosis. Acidification of the endocytic vesicles leads to virus uncoating mediated by the M2 ion channel. vRNPs are then released into the cytoplasm and transported into the nucleus. There, the viral RNA-dependent RNA polymerase complex snatches the host mRNA caps to initiate the negative vRNA transcription. Transcribed vRNAs then undergo an mRNA maturation phase, before export to the cytoplasm to be translated. vRNAs are also replicated in the nucleus to generate new vRNPs in association with neosynthesized viral proteins. Progeny vRNPs are transported toward the cytoplasmic membrane with viral components to be packaged into new infectious particles which are formed by cellular envelope budding. Classic virus-targeting strategies are highlighted in red and virus-host-targeted strategies in blue. Figure created by BioRender.com

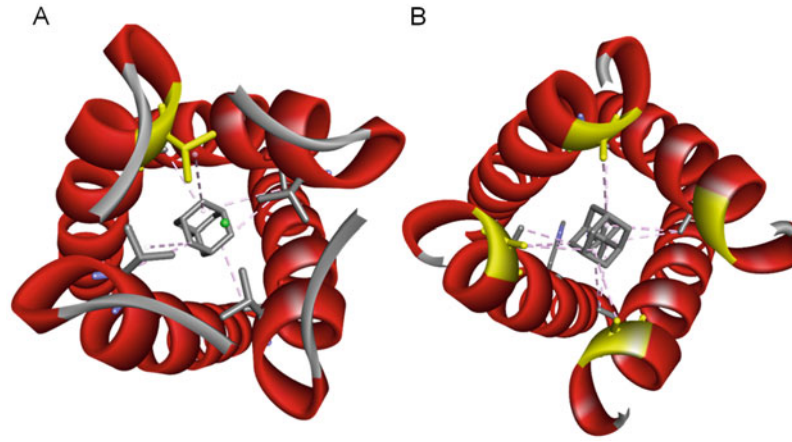

Fig. 8.2 Looking down the M2 channel in the presence of inhibitors: Structure of M2 WT and VA27 mutant in complex with amantadine and spiroamantadine. View down the pore channel in (**a**) WT-amantadine (V27 is colored in yellow, PDB ID 6BKK [9]) and (**b**) V27A-spiroamantadine complexes (A27 is colored in yellow, PDB ID 6NV1 [10])

Table 8.1 Summary of the activity and structures of the main antiviral compounds bound to their target, the proton channel M2 of influenza A or the neuraminidase NA of influenza A and B

Target	Compound	IC_{50}	PDB ID	Stage year approval)	References
M2	Amantadine	100μM (H1N1 WT) > 500μM (S31N) 15.7μM (WT channel [a,]) [13]	6BKK	Approved (1976)	Thomaston et al. [9], Cady et al. [14]
	Rimantadine	0.1μM (H1N1 WT) > 200μM (S31N)	2RLF	Approved (1994)	Schnell and Chou [15]
	Spiro-adamantyl amine	18.7μM (WT channel[a]) 0.2μM (V27A [a])	6BMZ 6NV1 6OUG	Pre-clinical	Thomaston et al. [9, 10]
NA	Oseltamivir (Tamiflu)	0.8 nM (N5 NA)	2HT7	Approved (1999)	Russell et al. [16]
	Peramivir	3.4 nM	2HTU	Approved (2014)	Russell et al. [16]
	Zanamivir	0.6 nM (N5 NA)	3CKZ	Approved (1999)	Collins et al. [17]
	Chebulinic acid Chebulagic acid	1.36 ± 0.36μM (H1N1 PR8) (Oseltamivir-resistant and H1N1 pdm09 viruses) CC_{50} > 100μM		Pre-clinical	Li et al. [18]
	Oseltamivir derivatives	0.66μM (H5N1)	Docking 150/430 cavity	Pre-clinical	Ai et al. [19], Jia et al. [20]; Zhang et al. [21]
	Triazole oseltamivir derivatives C1-modified oseltamivir derivatives	0.05–0.15μM (H5N1, H5N2, and H5N6) 0.1μM (H5N1, H5N6) 0.7μM (oseltamivir-resistant virus)	Docking 430 cavity	Pre-clinical	Ju et al. [22]

[a]Patch clamp assays [10]

appeared in M2 in H1N1/H3N2 circulating strains, both amantadine and rimantadine were removed from the WHO list of recommended anti-influenza agents for clinical use in 2009 [23].

8.2.2 Neuraminidase (NA) and Hemagglutinin (HA) Inhibitors

8.2.2.1 NA Inhibitors

NA inhibitors competitively inhibit terminal sialic acid residue removal from glycoproteins and carbohydrates found at the surface of host (mammalian) cells and influenza virus particles. Binding of virions to intact (uncleaved) sialic acid inhibits virion release. Among these NA inhibitors, peramivir, zanamivir, and oseltamivir carboxylate are the most frequently prescribed drugs and considered standard of care for influenza management (Table 8.1 and Fig. 8.3). Resistance to oseltamivir can be observed experimentally in a few cell passages and also found in the clinic. Typically, resistance originates from substitutions in the viral NA protein such as H274Y and I223R (predominant in H1N1 and H5N1 viruses) and E119V, R292K, or N294S (predominant in H3N2 viruses). Oseltamivir, peramivir and zanamivir are three NA inhibitors currently approved worldwide for the treatment of influenza A and B infections, oseltamivir being the most widely used. There is still a lot of debate about the effectiveness and real impact of inhibitors on the prevention and treatment of influenza. New oseltamivir derivatives, targeting either multiple sites or different NA cavities (as the "430" or the "150" cavity), have been recently developed. Some of these derivatives are very potent against multiple IAV and IBV strains, including oseltamivir-resistant ones (Table 8.1).

8.2.2.2 Hemagglutinin Inhibitors

The surface glycoprotein HA is associated with viral entry into host cells. HA binding to cell-surface, sialic-acid-containing glycans further enables fusion between the viral and host membranes in endosomal compartments. HA is composed of head (HA1) and stem (HA2/HA1) domains. As the regions on HA involved in binding and fusion are highly conserved, they are attractive sites for the design of new antivirals (Table 8.2). The broad-spectrum antiviral drug arbidol shows efficacy against influenza viruses by targeting the hemagglutinin (HA) stem region [24]. This molecule is currently licensed in Russia and China for the treatment of influenza and other infections [35]. A challenging strategy aiming at mimicking antibodies binding sites was successfully developed by Wilson et al., targeting the conserved stem region and more recently at the interface of the trimeric head region [13, 27, 36] (Fig. 8.4a). The binding sites of the binding sites for CBS1117 and JNJ4796 were both found in the stem region close to the fusion peptide, highlighting the possibility of further structure-based designed compounds [29]. De novo design of high-affinity trimeric proteins called "HA mini-binders" that bind influenza A hemagglutinin trimer at a conserved region binding site (Fig. 8.4b) [33]. These molecules were developed as alternative to antibodies. These and other compounds are summarized in Table 8.2.

8.2.3 Polymerase/Nucleoprotein/RNA inhibitors

8.2.3.1 Polymerase/Endonuclease Inhibitor (Favipiravir, Baloxavir Marboxil)

Influenza viruses transcribe and replicate their genome in the nucleus of infected cells by the means of a hetero-trimeric polymerase, PA, PB1, and PB2. The polymerase complex function requires the nucleoprotein NP, a protein associated with and protecting the segmented genomic RNA. Therefore, all four proteins are essential for replication. Whereas replication requires the generation of complementary positive polarity RNA intermediates (cRNA) that are then copied into progeny negative polarity segments (vRNPs), viral message is directly synthesized from vRNPs. Since the influenza virus polymerase is unable to form 5′ mRNA cap structures, its subunit PA is necessary for the generation of viral mRNAs via its endonuclease activity, transferring host mRNAs 5′-capped RNA primers in a cap-snatching mechanism. The endonuclease active site of PA-N terminal comprises a histidine and a cluster of three strictly

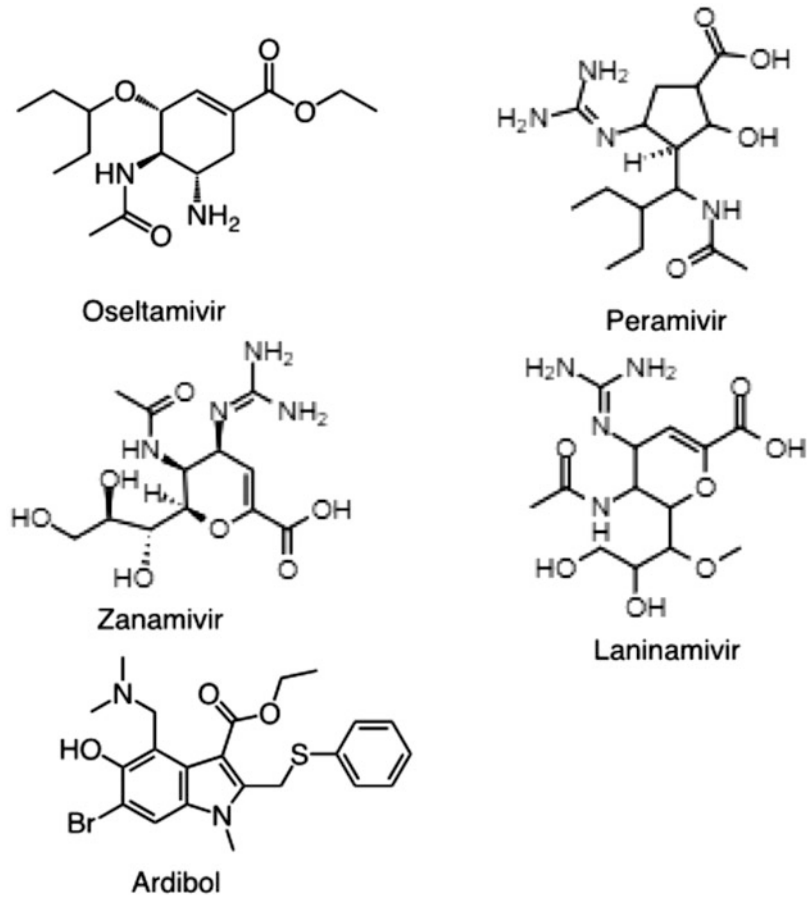

Fig. 8.3 Structures of the approved NA inhibitors

Table 8.2 Recent antiviral candidates targeting HA, their activity, and structures of their complexes with HA

Target	Compound/binding site	IC_{50}/CC_{50}	PDB ID	Stage	References
HA	Arbidol/stem region	4–12μM CC_{50} = 59μM	5T6S, 5T6N	Pre-clinical and clinical NCT03787459	Kadam and Wilson [24], Wang et al. [25], Wright et al. [26]
	F0045(S)/stem region	0.5–2μM (H1 HA)	6WCR	Pre-clinical	Yao et al. [13]
	JNJ4795/stem region	0.01–0.07μM (H1 HA)	6CF7	Pre-clinical	Van Dongen et al. [27]
	IY7640/stem region	0.5–7μM (H1 HA) CC_{50} > 800μM	Docking studies	Pre-clinical	Kim et al. [28]
	CBS1117/stem region	3μM For H5 HA	6VMZ	Pre-clinical	Antanasijevic et al. [29]; [30]); Hussein et al. [31]
	MB2746/stem region	0.3μM (H1 HA) CC_{50} > 100μM	Docking studies	Pre-clinical	Basu et al. [32]
	De novo design of "mini-binder" proteins	0.15–0.19 nM (H3 and H1 HA)	6KUY		Strauch et al. [33]
	penindolone		HA1 and HA2	Pre-clinical	Wu et al. [34]

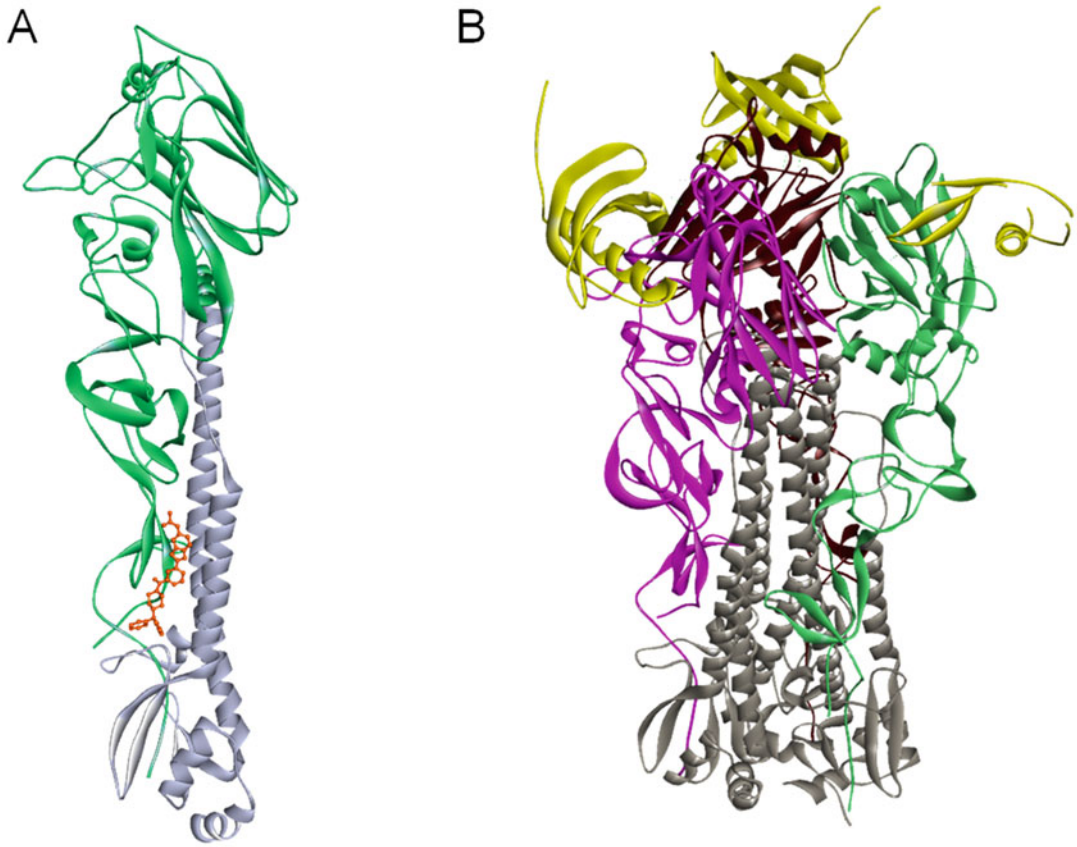

Fig. 8.4 Structure of some of the pre-clinical candidates targeting HA: (**a**) Structure of HA in complex with JNJ4796 shown in orange (PDB ID 6CF7) [27]. (**b**) Structure of trimeric HA in complex with mini-binder highlighted in yellow (PDB ID 6KUY) [33]

conserved acidic residues (Glu80, Asp108, Glu119), which coordinate (together with Ile120) one or two manganese or magnesium ions [37] (Fig. 8.5a). PB2 binds capped primers, the enzymatic activity for phosphodiester bond formation being associated with the PB1 subunit.

Several classes of inhibitors are in the clinics (Fig. 8.6): baloxavir (PA), favipiravir (PB1), and pimodivir (PB2, Fig. 8.5b).

8.2.3.2 Pre-clinical Compounds Targeteinf the Polymerase PA, PB1 and PA subunits, Escape Mutations and Resistance

Pre-clinical candidates, some of them being listed in Tables 8.3 and 8.4, are in development, benefiting from the recent insight provided by the structures of PA-PB1, PB1-PB2, and whole polymerase complex with or without RNA by X-ray crystallography [71–77] and cryo-electron microscopy [78–82]. The error-prone nature of influenza viral replication can rapidly generate point mutants for the selection of resistance that have seriously compromised the efficacy of influenza therapeutics. Escape mutations were identified under the pressure selection of PA inhibitors: the hotspot mutation for escape from baloxavir marboxil is located at PA residue 38, including several substitutions (PA I38T/M/F) [41]. Similarly, escape mutations from L-742.001 [42] and RO-7 [44] treatments were also characterized although in laboratory

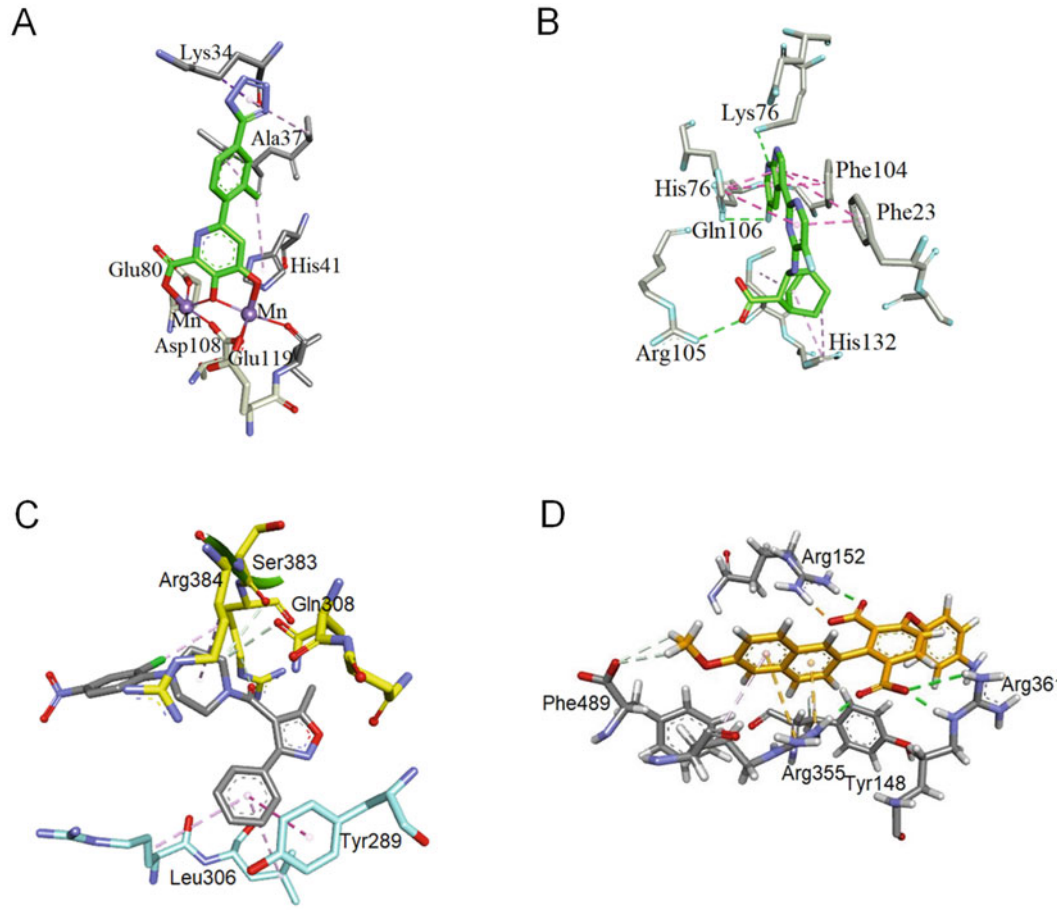

Fig. 8.5 Structure of some of the pre-clinical candidates targeting the polymerase. (**a**) Active-site PA N-terminal inhibitor compound 22 [38]; (**b**) PB2 inhibitor pimodivir [39] (the numbering is associated with this structure corresponding to the full-length PB2); (**c**) nucleozin-NP oligomeric complex PDB ID 5B7B; monomers A and B are in cyan and yellow, respectively; (**d**): naproxen F1-NP monomeric complex from docking studies [40]

resistance assays, escape mutants were not detected after multiple passages for L-742.001. While very tight affinities have been achieved by designing metal binding inhibitors to block the active site of the endonuclease activity in PA N-terminal (Table 8.2), the appearance of escape mutants often rapidly decreases their efficacy. Several recent reviews focus on the development of PA and polymerase inhibitors [83–86].

Different strategies have been undertaken to attempt overcoming induced resistance. Interfering with its proper assembly of the RdRP polymerase to inhibit function is pursued using protein-protein interaction (PPI) inhibitors. The advantage of such an approach is the relatively large interacting surface between the two proteins as compared to the binding site of an active-site ligand. Indeed, inducing simultaneous mutation of at least one residue on both proteins while maintaining their interaction is less likely to develop resistance and suggests that PPI inhibitors could be less prone to drug resistance than inhibitors of enzyme active sites. The recent identification of a single- domain antibody (nanobody) allowing to disrupt dimerization of FluA polymerase is among these lines [79]. PPI inhibitors have been developed based on the structural insight given by PA-PB1 crystal

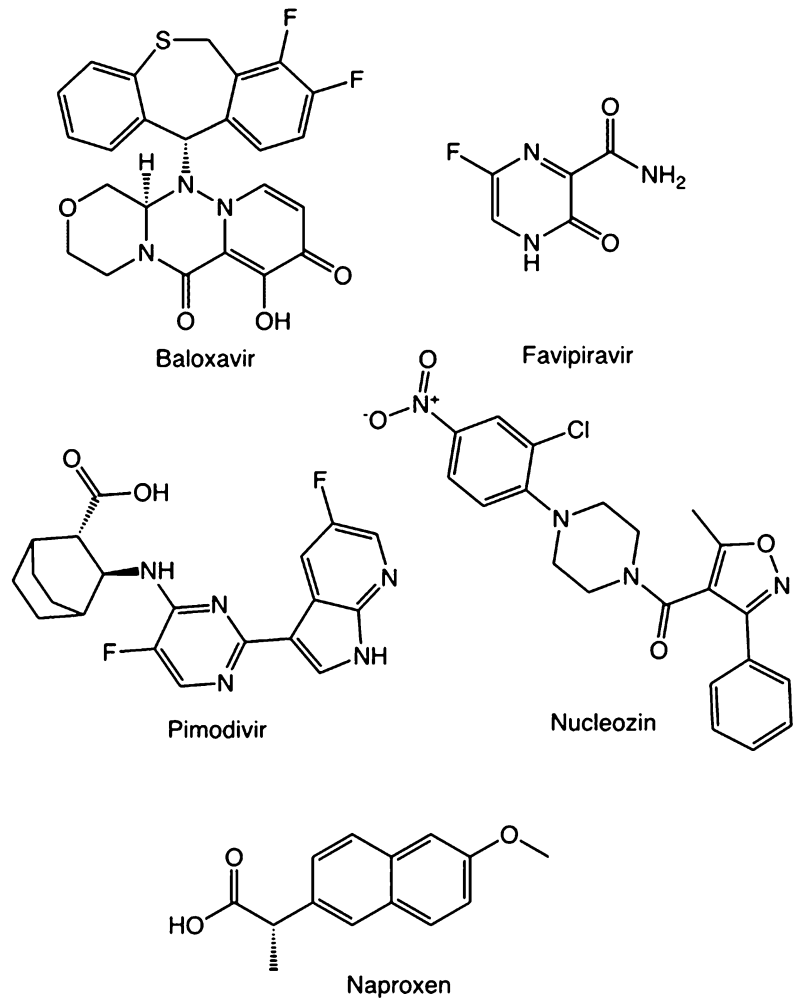

Fig. 8.6 Structures of the approved polymerase inhibitors and some pre-clinical candidates

structures in 2012 [87]. The inhibition of the polymerase PA-PB1 subunit interface has become an active field of research with the goal of remaining active against resistant strains to amantadine and to oseltamivir (Table 8.3). Recently, compound 12 was identified by structure-based screening of compounds targeting the PA-PB1 structure. No resistant virus was selected in vitro under drug selection pressure of compound 12a [48]. Moreover, derivatives of cyclothiophene and R151785 were found active against multiple strains of influenza A and B [50–52].

Based on the ability of PA-PB1 to bind viral RNA, it is likely that novel types of inhibitors could be developed by structure-based design [88]. Additionally, inhibitors targeting PA C-terminal [47] and its interactions with vRNA or with PolII could be effective targets, based on the accumulating wealth of structural data [74, 75, 79, 82] and deeper insight in the multi-protein assembly required during replication/transcription.

8.2.3.3 Broad-Spectrum Inhibitors

Favipiravir inhibits RNA viruses of the arenavirus, bunyavirus, flavivirus, alphavirus, norovirus, picornavirus, paramyxovirus, and rhabdovirus families, in addition to influenza viruses;

Table 8.3 Inhibitors of PA, PA-PB1 interactions, and PB1

Target	Compound	IC_{50}/CC_{50}	PDB ID	Stage	References
PA	Baloxavir marboxil	0.3–1μM (H1N1/H3N2)	6FS6 6FS9	Approved (2019) NCT02954354 NCT0294901	Omoto et al. [41]
	L-742,001	3μM (WT H1N1) 24μM (WT H1N1 pdm09) 236μM (H1N1 pdm09 PA F105S)	5CGV 5D9J	Clinical trial NCT01526785	Song et al. [42]
	RO7	16 nM (WT H1N1) 3 nM (H1N1 pdm09)	5VPX	Pre-clinical	Jones et al. [43]; Kowalinski et al. [44]
	Ana-0	0.8μM	Docking	Pre-clinical	Yuan et al. [45]
	Compound 22	110 pM	6E6W	Pre-clinical	Credille et al. [38]
	N-Acylhydrazone derivatives	11μM	5EGA	Pre-clinical	Carcelli et al. [46]
	"312"	37μM (H1N1, H2N2, and H3N2)	PA–C-terminal	Pre-clinical	Lo et al. [47]
PA-PB1	Compound 12a	0.9–2.7μM (FluA amantadine- and oseltamivir-resistant, FluB)	Docking	Pre-clinical	Zhang et al. [48]
	Amino-acids adducts of diphenyl-pyridine derivatives	39 ± 2μM (H1N1)	Docking	Pre-clinical	D'agostino et al. [49]
	Cycloheptathiophene-3-carboxamide	0.2μM–0.7μM H1N1 pdm09, H1N1 oseltamivir-resistant, H3N2, influenza B	Docking	Pre-clinical	Desantis et al. [50]; Nannetti et al. [51]
	R151785	2.5, 5.0μM p09, H1N1 oseltamivir- and amantadine-resistant influenza B	Docking	Pre-clinical	Zhang et al. [52]
PB1	Favipiravir	Broad-spectrum		Approved (2014)	Yoon et al. [53]
	β-d-N4-Hydroxycytidine/ EIDD-2801	Broad-spectrum influenza, SARS-CoV2		Clinical trial NCT04405739	Sheahan et al. [54]; Toots et al. [55]

therefore, it is considered as a broad-spectrum drug [53]. This drug is incorporated into newly synthesized RNA by the viral polymerase in place of purines but not pyrimidines, resulting in increased frequencies of C-to-U and G-to-A transition mutations. Although the barrier for resistance is relatively high, this drug seems to present toxicity issues. N4-Hydroxycytidine (NHC) inhibits RSV and both highly pathogenic avian and seasonal influenza viruses as well as SARS-CoV-2 virus, thus being also a broad-spectrum antiviral candidate with oral efficacy [55].

8.2.3.4 Pre-Clinical Compounds Targeting the Polymerase PB2 Subunit

Crystal structure of the PB2 cap-binding domain has been exploited to develop different 7-methylguanine derivatives [59]. Pimodivir (VX-787) is an inhibitor targeting the polymerase PB2 subunit at the m^7 GTP-binding site, forming extensive stacking interactions with several aromatic residues His (Figs. 8.5b and 8.6). It inhibits influenza virus replication and reduced viral load in animal infection models of H3N2 and H1N1

Table 8.4 Inhibitors of PB2 cap-binding, PB1-PB2, NP, and NS1

Target	Compound/binding site	IC_{50}/CC_{50}	PDB ID	Stage	References
PB2	Pimodivir (VX787)	2.6 nM	4P1U	Approved (2017)	Byrn et al. [56]; Clark et al. [39]
	5,7-Difluoroindole derivative of pimodivir	11 nM	6S5V	Pre-clinical	Mcgowan et al. [57]
	D 715–2441	3.6–4.4μM (H1N1, H3N2, H5N1, H7N9)	Docking	Pre-clinical	Liu et al. [58]
	Cap analogs	7.5μM H3N2	4CB5	Pre-clinical	Pautus et al. [59]
PB1-PB2	PP7	1.4–9.5μM (strain-specific)	Docking	Pre-clinical	Yuan et al. [60]
NP	Nucleozin	0.07μM (H1N1) 0.16μM (H3N2) 0.33μM (H5N1Y287H)	5B7B	Pre-clinical	Kao et al. [61]; Pang et al. [62]
	Compound 3	0.1 μM (H1N1 and H5N1)	3RO5	Pre-clinical	Gerritz et al. [63]
	2-(4-Chloro-3,5-difluorophenylamino)thiazole-4-carboxamide derivatives	0.11μM	Docking	Pre-clinical	Shen et al. [64]; Woodring et al. [65]
	Naproxen Naproxen C0 (naproxen derivative 2) Naproxen F1 (naproxen derivative 4)	Broad-spectrum FluA and Sars-CoV2 16 ± 5μM (H1N) 2.9 ± 0.3μM (H1N1) 1.8μM (H1N1 pdm09) 1.3 ± 0.2μM (H1N1) 0.7μM (H1N1 pdm09, H3N2, resistant to oseltamivir)	Docking	Pre-clinical	Dilly et al. [40]; Lejal et al. [66]; Tarus et al. [67]
	Hydroquinolinone derivatives (NUD)	1.8–7.0μM (H1N1)	Docking	Pre-clinical	Makau et al. [68]
NS1	A22	≅ 1μM (H1N1 PR8)	Docking	Pre-clinical	Kleinpeter et al. [69]
	ML303	0.7–17μM (H1N1 pdm09, H3N2)	HTS	Pre-clinical	Patnaik et al. [70]

viruses, although potency was highest against H1N1 strains [39, 56]. Phase II clinical studies indicated that this drug is well-tolerated, reduced viral load, and resulted in slightly faster resolve of clinical signs. Further derivatives of pimodivir have been designed [57]. Targeting the PB1-PB2 interface by PPI inhibitors has been challenging: although PP7 exhibited antiviral activities against influenza virus subtypes A pandemic H1N1, H7N9, and H9N2, resistances have been unexpectedly detected in laboratory assays [60].

8.2.3.5 Pre-Clinical Compounds Targeting the Nucleoprotein or the Nucleoprotein-RNA Interactions

The nucleoprotein associated with viral RNA and the polymerase complex is essential for transcription and replication [77, 89, 90]. The assembly of NP-RNA oligomers into RNP has been determined by cryo-electron microscopy studies [77, 78, 89, 91]. In the X-ray structures of the NP [92], the protein adopts a trimeric structure. NP self-association to achieve trimer formation is

mediated by a flexible tail loop that protrudes into a pocket of the adjacent subunit, via the formation of a critical interaction between R416 of one subunit and E339 of the adjacent subunit. The R416A mutant lacking this interaction adopts a monomeric structure [93]. The native protein can also be purified in a monomeric form at low salt and concentration conditions [93–95]. The ability to modify the oligomeric state of NP is the structural basis of most NP inhibitors presently developed. Nucleozin was the first NP inhibitor developed as a molecule impeding nuclear accumulation. Nucleozin enhanced higher-order structures [61, 63]. Figure 8.5c shows the interactions of one of the nucleozin ligands found in the X-ray structure (PDB ID 5B7B) stabilizing the interface between two NP subunits [62]. Escape mutants to nucleozin have been identified in laboratory assays. The opposite approach to impede nucleoprotein self-association has also been pursued by disrupting the important salt bridge R416-E339 mediating NP oligomerization [64]. Recently, new compounds with high affinity for NP were designed stabilizing monomeric NP [65]. Impeding NP binding to viral RNA has been achieved by naproxen drug repurposing, naproxen being a known inhibitor of cyclooxygenase (COX) [66]. As NP oligomerization is enhanced by the presence of RNA, naproxen binding to NP reduced NP oligomers and favored monomeric NP. Docking and single mutation studies identified Tyr148, the only aromatic residue within the RNA binding groove, and residues of the C-terminal part of NP R355, R361 and Phe489 being involved in the interaction of naproxen with NP. Laboratory assays showed no resistance after eight cell passages infected with influenza A. Naproxen exhibited antiviral effects in mice models of influenza A infection [40, 66] as well as influenza B virus [96]. Further structure-based design yielded new naproxen derivatives with improved antiviral effects and selectivity for NP without COX inhibition (Figs. 8.5d and 8.6) [40, 67] (Table 8.4). Some of these derivatives were found inhibiting NP-PA interactions [40, 97]. Naproxen derivatives also present antiviral properties against oseltamivir-resistant strains [40]. Additional compounds with some similarity of their hydroxyquinoline scaffold to the methoxynaphthalene scaffold of naproxen called NUD were designed and were also found to be resistant in escape mutation laboratory assays [68].

8.2.4 Drugs Targeting the Non-structural Protein-1 (NS1)

NS1 has a plethora of strategies to inhibit the host immune response due to its ability to establish multiple protein-protein and protein-RNA interactions. NS1 hampers different pathways both in the cytoplasm and in the nucleus of infected cells. NS1 antagonizes interferon-mediated antiviral host response by binding to double-stranded (ds) viral RNA, thus protecting it from cellular factors, by blocking retinoic acid-inducible gene-I (RIG-I) and NF-kB activation. One pathway by which NS1 increases virulence is through the activation of phosphoinositide 3-kinase (PI3K) by binding to its p85β subunit [98]. NS1 has two structural domains – RNA-binding domain (RBD) and the effector domain (ED) – connected by a short linker (LR) and a disordered C-terminal tail. New drugs binding to NS1 effector domain have been designed with low micromolar antiviral efficacy [69] (Table 8.4).

8.3 Host-Targeting and Drug Repurposing Approaches for the Treatment of Influenza

Considerable progress has been made in understanding the interactions between influenza viruses and the host cell in recent years. In this context, and in light of the emerging problem of resistance to available classical antivirals, many studies have focused on targeting host factors to limit virus replication, but also to modulate host immune response. The targeting of host factors and/or signaling pathways makes sense in the context of virally induced hypercytokinemia (also known as “cytokine storm”), which is directly correlated with tissue injury and an

unfavorable prognosis of severe influenza [99]. Indeed, approaches to control or attenuate this disproportionate immune response are of particular interest and are the subject of numerous pre-clinical and clinical studies. As with all viruses, influenza viruses depend on cellular machinery for their replication and propagation. Many cellular factors essential for the replication of influenza viruses have been uncovered through genome-wide RNA interference approaches [100–103] but also more broadly through different integrated cell biology approaches using interactome and transcriptome data, for example [104, 105]. In order to list the different host-targeting strategies developed, a distinction can be made between molecules with a mode of action associated with a relatively well-defined stage of the viral cycle and molecules associated with the modulation of signaling pathways. It is these two main classes that will be described in the following sections.

8.3.1 Drugs Targeting Host Cell Component at Different Stages of Influenza Replication Cycle

The replication cycle of influenza viruses consists in distinct successive phases, 1) entry, 2) nuclear import of viral genome (viral ribonucleoprotein; vRNPs), 3) genome replication and protein synthesis, 4) nuclear-cytoplasmic export of vRNPs, and 5) plasma membrane transport and budding of neo-virions (Fig. 8.1). A number of molecules targeting host factors in these different steps, at different pre-clinical/clinical development stages, are known today.

Viral entry is a target of great interest, as it is likely to allow prophylactic approaches, by blocking the infection in its early stages. One of the most advanced strategies consists to target the viral receptor. DAS181 (Table 8.5) (Fludase, Ansun BioPharma) is a sialidase fusion protein that cleaves both the Neu5Ac α(2,3)- and Neu5Ac α(2,6)-Gal linkages of sialic acid on host cells. DAS181 is administered as an inhalable dry powder to deliver sialidase to the pulmonary epithelium for cleavage of sialic acids, which renders the cells inaccessible to infection by virus [131]. DAS181 was demonstrated to have broad-spectrum activity, given the conserved nature of influenza and parainfluenza viruses binding to respiratory epithelium. Pre-clinical in vitro and in vivo studies demonstrated that DAS181 has activity against a number of seasonal influenza strains including those containing the H274Y mutation (conferring resistance to oseltamivir), highly pathogenic avian influenza strains (H5N1), and pandemic 2009 influenza A (H1N1). This compound was assessed in different Phase I and Phase II clinical trials (NCT00527865, NCT01651494, NCT01037205) with results indicating a significant reduction of viral load in treated influenza patients [106] but with identification of respiratory adverse events and rapid clearance of the drug being consistent with the induction of antibodies against DAS-181 – this could be a limitation in the duration and dosages of such treatment [107]. Other approaches targeting viral entry have also been described (Table 8.5), e.g., targeting the endosome acidification step by inhibition of V-ATPase (e.g., bafilomycin A1, concanamycin) or inhibition of the internalization (e.g., Dynasore) or cleavage steps of hemagglutinin (e.g., camostat). Most of these strategies were primarily evaluated at the pre-clinical stage and have not been further evaluated as their efficacy was either limited or accompanied by cytotoxicity. One exception is the protease inhibitor aprotinin, which was approved as anti-influenza drug in Russia [112].

The step of **nuclear import of vRNPs** is a crucial one, for which there are today very few molecules with antiviral potential described in literature. Interestingly, it has been shown in vitro that ivermectin (Table 8.5), a well-known anti-parasite drug, was able to inhibit viral replication via inhibition of importins (IMPα/β) and therefore the nuclear import of vRNPs [116].

Targeting the **replication** stage of the virus is one of the earliest host-targeting strategies, with pioneer works on the antiviral efficacy of ribavirin in the 1970s [119]. However, this nucleoside analogue and its prodrug, less toxic, do not appear

Table 8.5 Drugs targeting host cell component at different levels of viral cycle stages

Viral cycle stage	Drug name	Mode of action	Research phase	References
Viral entry	DAS181	Sialidase – Removes sialic acid receptors	Phase I/II	Moss et al. [106], Zenilman et al. [107]
	Bafilomycin A1	V-ATPase inhibitors – Inhibits endosomal acidification	Pre-clinical	Yeganeh et al. [108]
	Concanamycin			Müller et al. [109]
	Diphyllin			Chen et al. [110]
	Saliphenylhalamide			Bimbo et al. [111]
	Aprotinin	Protease inhibitors – Inhibit HA0 cleavage	Approved (2011)	Zhirnov et al. [112]
	Camostat		Pre-clinical	Yamaya et al. [113]
	Dynasore	Inhibition of internalization		de Vries et al. [114]
	EIPA			
	Fattiviracin			Harada et al. [115]
Nuclear import of vRNP	Ivermectin	Inhibits importin-α/β		Gotz et al. [116]
Genomic replication and protein synthesis	TG003	CLK1 inhibitors – Regulation of splicing – Decrease in M2 mRNA expression		Karlas et al. [100]
	Clypearin			Zu et al. [117]
	Corilagin			
	Silvestrol	eIF4A inhibitors – Inhibit viral protein synthesis		Slaine et al. [118]
	Pateamine			
	Ribavirin	Nucleoside analogue	Approved (1986)	Durr et al. [119]
	Viramidine (ribavirin prodrug)		Phase III (HCV)	Sidwell et al. [120]
	Cyclosporin A	Inhibits host RNA polymerase II	Pre-clinical	Liu et al. [58]
vRNP nuclear export		Inhibits nuclear export of vRNPs		
	Verdinexor	Exportin 1 inhibitors		Perwitasari et al. [121]
	DP2392-E10			Chutiwitoonchai et al. [122]
	CI-1040	MEK inhibitor – Nuclear retention of VRNP complex		Haasbach et al. [123]
	UO126			Pleschka et al. [124]
	PBP10/BOC2	Formyl peptide receptor 2 antagonists – Raf/MEK/ERK inhibition		Courtin et al. [125]
	Trametinib	MEK1/2 inhibitor – Inhibition of vRNP export	Approved (cancer)	Schräder et al. [126]
	Dapivirine	Reverse transcriptase inhibitor – Inhibition of vRNP export	Phase III (HIV)	Hu et al. [127]

(continued)

Table 8.5 (continued)

Viral cycle stage	Drug name	Mode of action	Research phase	References
Apical transport and budding	Nitazoxanide	Anti-parasitic – Inhibition of HA maturation and transport	Phase III	Rossignol et al. [128]
	Ruxolitinib	Virion formation and vRNA incorporation inhibition	Approved (myelofibrosis)	Watanabe et al. [105]
	U18666A	Hydrophobic polyamine – Reduces plasma membrane cholesterol level and decreases virion egress	Pre-clinical	Musiol et al. [129]
	Clonidine	Alpha2-adrenergic receptors inhibitor – Inhibits transport of HA transport to plasma membrane		Matsui et al. [130]

to be options being considered for the treatment of influenza virus infections of influenza viruses, despite interesting preliminary in vitro and in vivo results [120] (Table 8.5). Other, more recent strategies propose to target **mRNA splicing**. Influenza viruses are known to hijack cellular splicing machinery to their benefit, making them extremely dependent on it [132, 133]. Several studies show that the inhibition of Cdc2-like kinase 1 (CLK1), involved in the alternative splicing of M2 gene of influenza, appears to be an interesting antiviral option, with several molecules available (TG003, clypearin, corilagin, Table 8.5). Of all its molecules, clypearin has relatively low EC50s and very low toxicity, making it an attractive potential antiviral candidate [100, 117].

While strategies to prevent the nuclear import of vRNPs are relatively uncommon, paradoxically there are many more therapeutic approaches to block the **nuclear-cytoplasmic transport of vRNPs**. Indeed, in contrast to the inhibition of importins, the inhibition of exportin 1 (XPO1) by verdinexor (XPO1 antagonist KPT-335) allows to significantly reduce viral production in vitro and in vivo [121]. Another compound, DP2392-E10, inhibits nuclear export of both viral NP and nuclear export protein (NEP). More specifically, in vitro pull-down assays revealed that DP2392-E10 directly binds cellular CRM1, which mediates nuclear export of NP and NEP – highlighting CRM1 as a target of interest [122]. With the same objective, other strategies consist to target the Raf/MEK/ERK signaling pathway, known to be involved in the export of vRNPs [134]. Several MEK inhibitor molecules have been studied for their ability to inhibit the replication of influenza viruses, such as CI-1040 or U0126 [124, 125]. Interestingly, Schräder and colleagues have demonstrated that trametinib (GSK-1120212), a licensed MEK inhibitor used for the treatment of malignant melanoma, efficiently blocks influenza viral replication of different subtypes in vitro and in vivo [126] (Table 8.5).

Apical transport and budding, the last part of the last major step of the replication cycle, is also the object of several antiviral strategies, notably by blocking the transport of viral proteins to the plasma membrane (e.g., clonidine; [130]) or the cholesterol pathway, which would reduce virion egress (U18666A; [129]). One of the most advanced strategies is nitazoxanide, which was first approved for parasite infections' treatment. Its antiviral properties against influenza virus were first reported by Rossignol et al. [128]. Interestingly, the proposed mode of action of nitazoxanide against influenza clearly differs from its anti-parasitic effects, acting at the post-translational level by selectively blocking the maturation of the viral glycoprotein HA. Consecutively, it impacted on intracellular trafficking and insertion into the host plasma membrane [135]. This drug is a potent antiviral against a large panel of circulating strains [136]. A Phase IIb/III trial showed the efficacy of nitazoxanide in treating patients with non-complicated influenza [137], with a further, currently assessed, Phase III clinical trial (NCT01610245).

Table 8.6 Drugs targeting host cell signaling pathway and host responses that are crucial for influenza replication cycle

Host signaling pathway/response	Drug name	Mode of action	Research phase	References
NF-kB pathway	Acetylsalicylic acid	Immune dysregulation Inhibition of caspase/vRNP export inhibition	Approved	Mazur et al. [138]
	Pyrrolidine dithiocarbamate		Pre-clinical	Wiesener et al. [139]
	SC75741		Pre-clinical	Ehrhardt et al. [140] Haasbach et al. [123]
	LASAG		Phase II	Droebner et al. [141] Scheuch et al. [142]
C-Jun-N-terminal-kinase	SP600125	C-Jun N-terminal kinase inhibitor – Immune dysregulation	Pre-clinical	Nacken et al. [143]
p38 MAPK	NJK14047	Immune dysregulation	Pre-clinical	Choi et al. [144]
HMG-CoA	Statins	Immunomodulation	Phase II	Fedson [145], Mehrbod et al. [146]
TNF-alpha	Etanercept	Anti-inflammatory drug – Prevents TNF-mediated lung injury and edema	Pre-clinical	Shi et al. [147]
Nox2	Apocynin	ROS scavenger, inhibits Nox2 activity	Pre-clinical	Ye et al. [148] Oostwoud et al. [149]
	Ebselen	ROS scavenger and glutathione peroxidase mimetic, inhibits Nox2	Pre-clinical	
Lipoxygenase and COX pathways	Celecoxib	Immune dysregulation	Phase III	
	Mesalazine	Immune dysregulation	Pre-clinical	Davidson et al. [150] Carey et al. [151] Zheng et al. [152]
Type III IFN response	Type III IFN	Induction of type III IFN response	Pre-clinical	Davidson et al. [153] Kim et al. [154]
	Diltiazem		Phase II	Pizzorno et al. [155, 156]

8.3.2 Drugs Targeting Host Cell Signaling Pathways and Host Response that Are Crucial for Influenza Replication Cycle

Our increased knowledge of signaling pathways that are crucial in the response to infection and/or those hijacked by the virus has allowed many research teams to explore complementary antiviral strategies that can be described here (Table 8.6). The targeting of the ref./MEK/ERK channel, mentioned above, could of course also have been listed here. At the crossroads of the regulatory pathways of the immune response and the stress response, the **NF-kB pathway** was one of the first to be studied (Table 8.6). In the context of cell biology approaches, it was initially shown that the anti-inflammatory drug acetylsalicylic acid (ASA) had interesting antiviral effect against influenza viruses in vitro and in vivo, via inhibition of the NF-kB activating IkkB kinase [138, 157, 158]. Several drugs targeting the NF-kB pathway have been evaluated since then, such as pyrrolidine dithiocarbamate or SC7574, with encouraging in vivo results [123, 139, 140]. BAY81–8781/LASAG (D,L-lysine

acetylsalicylate-glycine) (Table 8.6), a modified version of ASA, demonstrates in vitro antiviral activity against several human and avian influenza viruses. In a mouse infection model, inhalation of LASAG reduced lung viral titers and protected mice from lethal infection [141]. More recently, a Phase II proof-of-concept trial compared LASAG versus placebo in patients with severe influenza. Aerosolized LASAG was demonstrated improving the time to symptom alleviation compared to placebo, although the reduction of viral load in LASAG-treated group was not statistically significant [142].

Based on clinical observations, 3-hydroxy-3-methylglutaryl coenzyme A (**HMG-CoA**) reductase inhibitors such as statins (Table 8.6), approved for indication of cholesterol metabolism regulators, have demonstrated pleiotropic anti-inflammatory and immunomodulatory properties, which could increase survival of patients with severe influenza [145, 146]. However, most in vivo studies reported so far failed to clearly demonstrate such a beneficial effect for influenza patients [159–161]. Nevertheless, an association between statin treatment with a reduction of mortality in patients hospitalized with laboratory-confirmed seasonal influenza was highlighted in observational studies [162, 163]. A randomized placebo-controlled Phase II clinical trial (NCT02056340) to evaluate the potential beneficial effect of atorvastatin in improving the status severely-ill influenza-infected patients is currently undergoing. The combination of naproxen with clarithromycin and oseltamivir twice daily reduced the both 30- and 90-day mortality and length of hospital stay of patients hospitalized for A(H3N2) influenza [164]. Other approaches, at the pre-clinical validation stages, propose to target the **TNF-alpha** (etanercept) or **NOX2** (apocynin/ebselen) or lipoxygenase/COX pathway (celecoxib/mesalazine) pathways [147–152, 165]. A Phase III clinical trial is currently investigating the benefit of celecoxib for the treatment of severe influenza (NCT02108366). These molecules could be of interest to better control the inflammatory response, which is a very important aspect of the pathology.

Modulation of immune and inflammatory responses is a therapeutic avenue that has been much explored, but which may present risks given the ambivalent aspect of these pathways in relation to viral replication and the evolution of the pathology. Indeed, such treatment should stimulate induction of antiviral genes to control IAV spread, without driving immunopathology. In this context, **IFN-lambda** (Table 8.6) appears as a potent anti-influenza therapeutic, without the inflammatory side effects of IFN-alpha treatment [153]. Intranasal administration of IFN-λ2/3 was shown to significantly suppress infection of various influenza strains, including WS/33 (H1N1), PR (H1N1), and H5N1 in the mouse lung, and was accompanied by greater upregulation of ISGs [154]. More recently, using a transcriptome-based screening approach, we identified and validated diltiazem, a calcium channel blocker used as an anti-hypertensive drug, as a very promising host-targeted inhibitor of influenza infection. Interestingly, the study of the mode of action revealed that diltiazem was a strong induced or type III IFN [156]. An ongoing French multicenter randomized clinical trial is investigating the effect of diltiazem oseltamivir bi-therapy compared with standard oseltamivir monotherapy for the treatment of severe influenza infections in intensive care units (FLUNEXT trial NCT03212716).

8.4 Perspectives and Concluding Remarks

Among all the molecules listed in this chapter, some are already available on the market for other therapeutic indications and fall within the scope of drug repurposing. This is the case for naproxen, diltiazem, LASAG, or nitazoxanide, for example. Drug repurposing bypasses the long, risky, and expensive pre-clinical studies, an early clinical evaluation stage conventionally used for de novo drug development. It takes advantage of available resources, as extensive human clinical, pharmacokinetics, and safety data, as the starting point for the development [155] All these aspects make the repositioning of drugs a very interesting approach, in particular

to enable a rapid response to the need for new antiviral strategies in the context of the emergence of a virus with pandemic potential.

Another very interesting perspective is the interest in combining different antiviral approaches with each other, including classical approaches targeting the virus with those targeting the host cell. The concept of combining therapies has already been used successfully, notably in the design of antiretroviral treatments [166]. Combination therapy can have several objectives, such as reducing the risk of the emergence of resistance by simultaneously targeting several viral proteins and/or key host factors, but also increasing the effectiveness of the treatments by obtaining additive or synergistic effects.

While there is relatively little convincing evidence to support the use of conventional virus-targeting antivirals in combination [167, 168], there are interestingly a growing number of examples of combinations of oseltamivir with host-targeted approaches. For example, we have shown that the combination of diltiazem and oseltamivir provides a much greater reduction in viral titers in a reconstructed human epithelium model compared to single treatments [156]. More recently, Schloer and colleagues have shown that a combination treatment of an antifungal molecule, itraconazole, with oseltamivir achieves much greater antiviral activity compared to monotherapy, making it possible to consider reducing the concentrations of drugs used and thus possibly reducing the problems of adverse effects and emergence of resistance mutations [169]. These results open up interesting prospects for the development of future therapeutic strategies, particularly for the treatment of severe forms of influenza. The potential arsenal for fighting influenza virus infections is potentially very extensive, in particular thanks to the combination of new molecules targeting the virus, resulting from docking and structure-based design strategies, with approaches targeting cellular factors and signaling pathways. In this context, the quality and relevance of the pre-clinical models, as well as the quality of the tools for evaluating combinations of molecules, are important critical elements.

Beyond influenza viruses, many of the antiviral molecules described in this chapter have the potential for broader-spectrum use. Indeed, some virus-targeted strategies can target viral determinants with very strong similarities between different viruses. This is particularly the case with naproxen for which we have previously demonstrated antiviral activity against both influenza viruses and SARS-CoV-2 [66, 170]. This property is explained by the fact that the nucleoproteins N of enveloped, positive-sense, single-stranded viruses coronavirus (CoV) share with negative-sense single-stranded viruses such as influenza A virus the ability to bind to and protect genomic viral RNA without sequence specificity and to form self-associated oligomers. Despite their differences, viruses induce and divert many common cellular pathways. As a result, host-targeted approaches can identify molecules with a broad spectrum of antiviral activity. An example is diltiazem, for which we have shown antiviral activity against influenza viruses [156], but which has been shown to be effective against other respiratory viruses, such as SARS-CoV-2 [171, 172], due to its mode of action involving the type III interferon response. Efforts to identify anti-influenza molecules therefore open up very interesting prospects for the broader development of antivirals. In many ways, antiviral research on influenza viruses is pioneering in this area and provides a starting point for the study of other emerging viruses.

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