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Searching for effective antiviral small molecules against influenza A virus: A patent review

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Abstract

Introduction: Despite the current interest caused by SARS-Cov-2, influenza continues to be one of the most serious health concerns, with an estimated 1 billion cases of influenza across the globe, including 3-5 million severe cases and 290,000-650,000 deaths worldwide.

Areas covered: This manuscript reviews the efforts made in the development of small molecules for the treatment of influenza virus, primarily focused on patent applications in the last five years. Attention is paid to compounds targeting key functional viral proteins, such as the M2 channel, neuraminidase, and hemagglutinin, highlighting the evolution toward novel ligands and scaffolds motivated by the emergence of resistant strains. Finally, the discovery of compounds against novel viral targets, such as the RNA-dependent RNA polymerase, is discussed.

Expert opinion: The therapeutic potential of antiviral agents is limited by the increasing presence of resistant strains. This should encourage research on novel strategies for therapeutic intervention. In this context, the discovery of arbidol and JNJ7918 against hemagglutinin, and current efforts on RNA-dependent RNA polymerase have disclosed novel opportunities for therapeutic treatment. Future studies should attempt to expand the therapeutic arsenal of anti-flu agents, often in combined therapies, which might be relevant to prevent future health challenges caused by influenza virus.

Keywords: influenza, antivirals, M2 proton channel, neuraminidase, hemagglutinin, RNA polymerase, druggable targets, drug discovery

Article highlights

● Influenza represents a severe health challenge that requires novel strategies to protect people from this threatening disease.

● Besides the M2 proton channel and neuraminidase, structural and molecular biology studies have expanded the number of druggable targets and understand the resistance mechanisms.

●The flip-flop binding mode of adamantylamino-derivatives offers novel opportunities to target the wild-type M2 channel and amantadine-resistant mutated variants.

● The druggability of hemagglutinin is confirmed by arbidol and JNJ7918, which represents antiviral compounds endowed with different mechanisms of action.

● The design of compounds that target the protein-protein interaction between domains in RNA-dependent RNA polymerase opens avenues for anti-flu agents.

● The potential therapeutic interest of these novel antiviral compounds used in conjunction with other drugs could lead to synergistic combined therapies.

1. Introduction

The 1918 influenza pandemic, also known as 'Spanish flu', had a dramatic impact on the public health worldwide, infecting around one-third of the population. More than 100 years later, influenza continues to be one of the most serious global health threats, as recognized by the estimated 1 billion cases of influenza across the globe, including 3-5 million severe cases and 290,000-650,000 deaths in seasonal outbreaks worldwide [1]. Continued efforts in developing better diagnostic tools and promoting the discovery of effective influenza treatments, including vaccines and antiviral drugs, were challenged by pandemics in 1957, 1968 and 2009 [2-4]. These outbreaks underscore the latent risk of facing a new pandemic in the future, as has been exemplified with the severe acute respiratory syndrome (SARS-CoV-2) in 2019 following the first SARS-CoV-1 (commonly referred as SARS-CoV) infection in 2002 and the Middle East respiratory syndrome (MERS) in 2012 [5,6]. This threatening scenario motivated the Global Influenza Strategy initiative launched by the World Health Organization in 2019 [7].

Seasonal flu vaccination has a major role in preventing influenza A and B virus infections. However, the limited ability to generate new vaccines and the fluctuating therapeutic efficacy of vaccines from year to year make it advisable to improve the therapeutic arsenal with small antiviral molecules. [8,9]. Historically, the first drugs available for the treatment of influenza were amantadine and rimantadine [10,11], which contain the adamantane motif and inhibit viral replication by blocking the M2 proton channel in influenza A virus. Their use, however, is no longer recommended due to the emergence of drug-resistant viral strains, the lack of activity against influenza B, and the detection of adverse effects in the gastrointestinal tract and the central nervous system [12]. Currently the only licensed class of antiviral agents target neuraminidase, also called sialidase, which is a glycoside hydrolase enzyme that catalyses the

hydrolysis of sialic acid residues from the host cell receptor and from the newly formed virions [13]. Zanamivir and oseltamivir are potent influenza virus NA inhibitors [14,15] and are licensed worldwide. Two other NA inhibitors, peramivir [16] and laninamivir [17,18], are also available in some countries, the former being approved in China, Japan, South Korea and the United States, and the latter in Japan [19]. The efficacy of these drugs is also challenged by the occurrence of drug-resistant strains, as noticed for the oseltamivir-resistant virus associated to the NA H275Y mutation in 2008 [20]. Nevertheless, the resistance to NA inhibitors in circulating strains is still low, supporting the use of these drugs for the prophylaxis of influenza virus infections [21-23].

In this context, this review aims to discuss the efforts invested in the discovery of antivirals compounds targeting key proteins of the influenza virus, often directed at novel targets, with the goal of increasing potency and mitigating the emergence of resistance to current treatments. For the sake of brevity, the material related with combined therapies, antibodies and immunomodulatory agents will not be explored here, and we limit ourselves to the discovery of small compounds. To this end, particular attention has been paid to the development of antiviral molecules against the major targets –the M2 proton channel, neuraminidase and hemagglutinin–, with special emphasis to their mode of action, and finalizes with recent work focused on the identification of alternative strategies that exploit novel targets.

2. Life cycle of the influenza A virus

Influenza viruses belong to the Orthomyxoviridae family, and are categorized in four distinct types (A, B, C, and D) [24]. The influenza A virus is covered by a lipid-protein envelope that contains 8 single-stranded RNA segments that encode the genetic

information for viral proteins (**Figure 1**) [25-27]. They include the matrix protein M1, the transmembrane M2 proton channel, the membrane-bound hemagglutinin (HA) and neuraminidase (NA), the non-structural proteins (NS1, NS2/NEP), the heterotrimeric RNA-dependent RNA polymerase (RdRp), formed by three domains (PA, PB1 and PB2), and nucleoprotein (NP) components.

The three largest RNA segments encode the PA, PB1 and PB2 subunits of RdRp, which participate in RNA synthesis and replication in infected cells. Two RNA segments encode HA, which mediates binding to sialic acid-containing receptors and viral entry, and NA, which is implicated in the release and spread of new virions. The remaining RNA segments encode M1, which is thought to sustain the structure of the virion and, together with NEP, regulates the trafficking of the viral RNA in the cell, M2, which is a proton channel required for acidification and fusion of viral and host cell membranes in the endosome, NS1, which is a virulence factor that inhibits host antiviral responses, and NP, which is wrapped around the viral RNA. Additional accessory viral proteins can also be expressed in infected cells, such as PB1–F2 and PA-x (influenza A), that participate in preventing host innate antiviral responses together with the NS1 protein [26].

The infection is triggered upon binding of HA to the sialic acid residues in glycoconjugates attached to the host cell membrane (**Figure 1**). This process is assisted by the sialidase activity of NA, which permits to suppress nonproductive and scan the cell surface for productive binding with sialylated receptors [28]. HA-mediated binding to the receptor triggers endocytosis of the virion. In the interior of the endosome, the low pH causes a large-scale conformational in HA that involves multiple steps, including cleavage of HA by host cell proteases into two subunits, HA1 and HA2. This process releases the fusion peptide and ultimately promotes the fusion of both viral and

host cell membranes. On the other hand, the M2 channel transports protons to the interior of the virus, which facilitates the disassembly of the packaged vRNPs from M1, thus enabling their transfer to the host cytoplasm following HA-mediated fusion.

Trafficking of the viral RNA to the nucleus is mediated by the importin-α–importin-β nuclear import pathway [30, 31]. While entry and fusion processes occur rather quickly (~10 min), nuclear import may require *ca*. 1 h, revealing an efficient recruitment of the vRNP by the host nuclear import factors. Inside the nucleus, RdRp carries out the transcription and replication of the viral RNAs, as well as to mRNA transcription from the viral RNA templates in a primed process named cap-snatching performed upon association of the PA domain of RdRp with the cellular RNA polymerase II [32]. Synthesis of the viral proteins exploits the translation machinery of the host cell, making use of endoplasmic reticulum-associated ribosomes for HA, NA and M2, and cytosolic ribosomes for the other protein components [33]. NS2/NPE and M1 are assumed to participate in the export of the vRNPs from the nucleus to the cytoplasm [34], where the assembly and maturation of the novel virions occurs. Among others, these processes include the translocation of HA, NA and M2 into the lumen of the endoplasmic reticulum, oligomerization of HA and NA and addition of glycans to these proteins, trafficking through the Golgi to the plasma membrane, co-localization of the replicated vRNPs in appropriate budding regions in the plasma membrane, remodeling of the plasma membrane to induce bud formation and subsequent scission of the viral envelope from the plasma membrane, and release of the novel virions assisted by the hydrolysis of the glycosidic linkages by NA [26, 27, 35].

The preceding comments highlight the complexity of the multiple processes implicated in the generation of new virions in the host cell, taking advantage of a combination of viral and cellular mechanisms to coordinate cell entry, replication, intracellular

trafficking, virion assembly and release. In this scenario, the search for effective antiviral treatments has been inspired by the identification of druggable viral targets with a relevant role in the life cycle of virus infection. The discovery of small-molecule antivirals against these targets, which will be discussed in the next sections, have benefited from the progresses made in disclosing the fine structural details of these proteins, the molecular mechanisms that underlie their biological function, and the characterization of the mode of action of inhibitory compounds.

3. M2 proton channel

Located in the viral envelope of influenza A virus, the M2 proton channel is a selective, pH-regulated channel that regulates the acidification of the interior of the virion. This process ultimately leads to dissociation of the viral RNA from its bound matrix proteins and release of the viral genetic material for replication [36]. From a structural point of view, the M2 channel is a homotetramer, each monomer containing an unstructured extracellular N-terminal region, a transmembrane domain, an amphiphilic membrane-anchored helix, and a C-terminal cytoplasmic tail. The transmembrane region mediates proton conductance and contains the binding site for M2 inhibitors in the interior of the pore, filling a pocket located between the tetrads formed by residues Val27, Ser31, Gly34 and His37 [37-39], thus blocking the passage of protons (**Figure 2**).

Figure 2 here

Amantadine and rimantadine are cyclic amines used in the treatment of flu infection. Amantadine was patented in 1961 and approved for flu prophylaxis by the FDA in 1966 [40]. Its ethyl analog, rimantadine, was patented in 1963 [41], and the methylated

derivative of rimantidine, adapromine, in 1977 [42]. Nowadays their prescription is no longer recommended due to the limited effectiveness against influenza B virus, unwanted side effects, and the emergence of adamantane-resistant strains in circulating influenza A viruses, primarily associated to single mutated variants V27A, L26F and S31N, which modify the physicochemical properties of the binding pocket, thus preventing an effective inhibition of the proton conductance by these compounds [43-45]. Indeed, the thermodynamics and kinetics of amantadine binding to the M2 channel, which has been proposed to follow a sequential flipping mechanism, is very sensitive to the V27A mutation, providing a quantitative rationale to the drastic decrease in inhibitory potency against the V27A variant [46].

Different strategies have been developed to expand the chemical diversity of adamantane-related compounds in order to circumvent the resistance to these compounds [47, 48]. An example is the identification of BL-1743 [49, 50], a spirene-containing imidazole compound. The inhibitory activity was determined using electrophysiological measurements of whole-cell current with a two-electrode voltage clamp (TEVC) using the M2 ion channel expressed in oocytes of *Xenopus laevis*. BL-1743 has an apparent inhibition constant (K_i) of 4.7 μM, which was found to be slightly larger than the value determined for amantadine (K_i of 0.3 μM), but the majority of amantadine-resistant influenza viruses were also resistant to BL-1743. Subsequent efforts led to the spiropiperidine **1** and spiranamine **2**, which inhibit the wild-type channel in TEVC electrophysiological assays with IC_{50} values of 0.9 and 12.6 μM, the latter being also an inhibitor of the L26F and V27A variants (IC_{50} of 32.4 and 46.6 μM, respectively) [51]. Expansion of the hydrophobic cage in the spiro derivative **3** and the polycyclic pyrrolidine **4** further improved the inhibitory potency against the V27A channel (IC_{50} of 0.3 and 0.7 μM), while retaining a similar potency against the wild-

type channel [52, 53]. Finally, it is also worth mentioning the development of compounds targeting specifically the S31N variant, such as the adamantylamino-containing isoxazole and thiophene compounds **5** and **6** [54, 55]. This latter compound was found to have EC_{50} values of 1.8 and 4.6 μM in assays performed using MDCK cells infected with M2-S31N-expressing A/WSN/33 and wild-type M2-expressing A/Udorn/72 viruses. Remarkably, a flip-flop binding mode was proposed for compound **6**, adopting distinct orientations upon binding to the pore of the wild type and S31N channels [55].

4. Neuraminidase

Neuraminidase (NA) is a surface glycoprotein that mediates the release of newly formed viral particles from host cells. With its sialidase activity, it cleaves sialic acid (SA) from both cellular receptors and from synthesized HA and NA on new virions during viral budding. While SA recognition is necessary for virus adhesion and endocytosis, cleavage of SA in NA prevents virions to aggregate on the dying cell, thus facilitating the release to the extracellular media and the diffusion toward new cells [56]. Although its main activity influences late stages of virus infection, it has been pointed out that the NA sialidase activity could be crucial for virus entry [57, 58]. A total of 10 subtypes of NA have been detected up to date as a consequence of the antigenic drift [59].

NA assembles as a homotetramer, each monomer consisting of four structural domains: the cytoplasmic tail, the transmembrane region, the stalk and the catalytic (sialidase) domain (**Figure 3**). Arg118, Asp151, Arg152, Arg224, Glu276, Arg292, Arg371 and Tyr406 shape the catalytic binding site. The FDA-approved NA inhibitors zanamivir and oseltamivir block sialidase activity by targeting the SA binding site in the catalytic

head domain of the protein (**Figure 3**). Mutations near this site are quite frequent and can affect the activity of NA inhibitors. In this regard, of great importance are the H274Y and the E119G mutations, which induce resistance to oseltamivir and zanamivir, respectively [60, 61]. Detailed reports about influenza resistance to NA inhibitors can be found elsewhere [62, 63]. Here, attention will be focused on the most relevant NA inhibitors developed in the last years, especially regarding the chemical modifications introduced in the reference compounds, zanamivir and oseltamivir.

Figure 3 here

4.1 Zanamivir and its derivatives

Zanamivir was the first commercially available NA inhibitor and was patented by Biota Scient Management in 1993 [64]. Endowed with an K_i of 10^{-11} M, the binding mode involves several hydrogen bonds (**Figure 3**): the carboxylate unit at position 2 interacts with Arg292, Arg371 and Arg118, the guanidine moiety at position 4 is hydrogen-bonded to Glu119, the amide group at position 5 contacts Arg152, and the poly-hydroxylated aliphatic chain at position 6 contacts Glu276 [65]. The use of acylguanidine prodrugs seemed to ameliorate the pharmacokinetic profile by improving cell permeability and bioavailability, leading to an effective oral administration [65, 67]. The zanamivir derivative, laninamivir, has been patented in 2010 [68]. The invention provided a therapeutic or prophylactic solution to H5N1 influenza. It basically differs from zanamivir for a methoxy group at position 7. Following an enzymatic assay of NA activity [69], the compound showed an IC_{50} of 0.33 nM, in line with the inhibitory potency of zanamivir (IC_{50} of 0.96 nM).

4.2 Oseltamivir and its derivatives

Oseltamivir was patented by Roche in 2009 [70], while the lead compound was originally discovered by Gilead (GS4071) in 1998 [71] and approved by the FDA in 2000. According to a recent report [72], A/H1N1 and A/H3N2 and influenza B viruses are susceptible to oseltamivir. In particular, IC_{50} values of 130 and 150 nM were observed for two A(H1N1)pdm09 isolates using a fluorescence-based neuraminidase inhibition assay [17]. The crystallographic structure of oseltamivir bound to NA (H5N1; PDB: 2HU4) [73] reveals that the carboxylate moiety is stabilized by electrostatic interactions with Arg119, Arg294, and Arg372. Additional polar contacts are formed between the amide unit at position 4 and Arg153 and Glu229, and finally between the 5-amino substituent and Glu120 (**Figure 3**). The effect of N-alkyl, -aryl and -aryl substituents at positions 3 and 5 has been explored in several patents [74-78]. Interestingly, the modified compounds retained the inhibitory activity on NA mutants (compounds **7-11** in **Figure 3**).

4.3 Peramivir

Peramivir was developed by BioCryst Pharmaceuticals [79] and received a regular FDA approval in 2014, after demonstrating to be a “life-saving” treatment during the 2009 swine flu. The compound is a unique cyclopentane derivative and its binding mode to NA (H8; PDB: 2HTU) closely resembles that of oseltamivir and zanamivir [73]. Thus, the carboxyl group at position 1 forms polar interactions with Arg118, Arg294 and Arg376, the 2-hydroxy substituent is hydrogen-bonded to Asp151, and the amide moiety present in the substituent at position 3 contacts Arg152. Finally, the 4-guanidine moiety interacts with Glu229, the backbone oxygen of Trp180, and Asp151 (see **Figure**

3 for a comparative analysis of the binding modes for zanamivir, oseltamivir and peramivir).

4.4 Drug design on mutated NAs

Although zanamivir and laninamivir are still active on the H273Y NA mutant, several efforts have been made to overcome drug resistance, especially for oseltamivir. A promising strategy was associated to the 150-cavity, and several oseltamivir derivatives, bearing large substituents at position 3 and 4, have been proposed to target this site [80]. However, since the 150-loop stably marks only the SA cavity of group-1 influenza A virus (N1, N4, N5, N8), it is unclear whether this approach would lead to broad spectrum NA inhibitors [81].

Another strategy has exploited the flexible 430-loop, which marks an accessory cavity close to the SA binding site of both group-1 and group-2 NAs. In this regard, Feng et al. [82] have recently proposed a series of zanamivir analogues, which would be able to reach the 430-loop due to the presence of a large substituent at position 1. The most potent compound had an inhibitory potency in the nanomolar range (IC_{50} values of 1.0, 1.4 and 12 nM against H1N1, H3N2 and H5N1, respectively, as determined from a fluorescent-based assay of the NA enzymatic activity for A/Guangdong/376/2001 (H1N1), A/Sydney/5/97 (H3N2) strains, and recombinant virus containing HA and NA genes from A/Indonesia/5/2005 (H5N1) [83]). These data are encouraging and suggest that structure-based design may be valuable for finding new, powerful NA inhibitors.

5. Hemagglutinin

Hemagglutinin (HA) is a spike-like glycoprotein primarily involved in early stages of viral infection. It is located at the virus envelope, together with the M2 proton channel

and NA and mediates host-virus recognition and membrane fusion. The fusion event starts when HA recognizes the SA exposed by glycans, promoting the virus particle to be wrapped by the host cell. Subsequent reduction of the endosomal pH, facilitated by M2 proton channel, triggers a conformational rearrangement in HA, which allows the viral and host membranes to fuse, and eventually enables translocation of the viral genetic material into the cell [84].

HA is characterized by a homotrimeric ensemble and each monomeric unit is formed by head (HA_1) and stem (HA_2) functional domains. The HA_1 unit contains the receptor-binding domain (RBD) involved in host-cell recognition, and the HA_2 subunit includes the fusion peptide (residues 1-24). Specific mutations can dramatically affect the virus avidity for the host cell, producing changes in viral antigenicity, transmissibility and pathogenicity [85, 86], making HA to be a promising target for therapeutic intervention. Eighteen HA subtypes (H1 to H18) have been identified as a consequence of this "antigenic drift", although only H1 and H3 subtypes are associated to seasonal flu and circulate among people [4], while H5, H7 and H9 subtypes pertain to high pathogenic avian influenza viruses with low human infectivity [87, 88].

Up to date anti-influenza compound targeting viral adhesion (acting at the SA binding site; Site 1 in **Figure 4**) and fusion inhibitors (acting at the HA_2 stem region; Sites 2 and 3 in **Figure 4**) are the most effective approaches. In this regard, a vast list of vaccines and human broadly neutralizing antibodies, SA-peptide mimetics and conjugates have been examined, although we limit ourselves to discuss small inhibitors targeting the fusion process [89]. Fusion inhibitors prevent the conformational changes necessary for membrane fusion by targeting and stabilizing the stem region of HA, near the fusion peptide. Accordingly, "influenza A HA inhibitors" and "arbidol" were used as search criteria to interrogate the "Espacenet" database [90], leading to the identification of

about 500 results specifically related to HA, which include 11 patents related to small HA fusion inhibitors (see **Figure 4**).

Figure 4 here

5.1 Tert-butyl hydroquinone (TBHQ) and its derivatives

The compound tert-butyl hydroquinone (TBHQ) and a list of substituted hydroquinone derivatives were patented in 1994 [91]. Crystallographic data revealed that TBHQ is bound to a hydrophobic pocket located at the central stem region of HA formed by residues Arg54D, Lys58D, Phe294C, Leu99D, Leu98B, Tyr94B, Ile29A, and Glu97B [92]. Binding of TBHQ and related compounds, such as **12** (**Figure 4**), would impede the conformational changes associated to membrane fusion by stabilizing a region proximal to the fusion peptide. No improvements in HA fusion inhibition were observed by rising the concentration of these compounds. In fact, using scintillation proximity assays involving binding of HA to specific antibodies [91], a maximum of 66% of inhibition was observed, which was attributed to the formation of weak interactions with HA or to the involvement of "off-target", mechanistically ineffective interactions with other regions of HA. TBHQ was able to inhibit haemolysis, syncytia formation and infectivity with an IC_{50} of respectively 0.1 mM, 0.01-0.001 mM, and 0.1-0.001 mM.

5.2 Arbidol and its derivatives

The fusion inhibitor arbidol was patented in 1993 and is approved for antiviral treatment in Russia [93]. The chemical structure of arbidol and other 5-hydroxy-bromoindole derivatives are shown in **Figure 4**. Arbidol has a broad-spectrum activity against both

influenza A and B with a mean binding affinity (K_d) of 40-100 μM [94], though improvement in binding affinity up to 90-500 nM has been reported for some analogues (general chemical structure **13**, with X = O, S; **Figure 4**) [95] using biolayer interferometry assays to determine the ratio between association and dissociation rate constant of the inhibitor to HA loaded onto streptavidin-coated biosensors (patent published by the Scripps Research Institute in 2018 [96]). The best activity profile was obtained for compound **14**, which contains a 2-methylthiophenol moiety, enhancing the binding affinity by 100- and 1000-fold against H1 and H3 strains compared to arbidol.

Arbidol and TBHQ bind to the same cavity of TBHQ [97]. Besides a hydrogen-bond formed between the carboxylate moiety of arbidol and $Lys307^{A}$ (note that in this notation the superindex denotes the chain in the heterotrimeric HA), a hydrophobic pocket is filled by substituents at positions 1, 5 and 6, whose nature may lead to marked changes in potency relative to the parent compound. The amine group in position 4 occupies a region surrounded by $Asp90^{D}$, $Lys310^{C}$, $Trp92^{B}$ and $Asn60^{B}$, and a slight improvement in potency was observed upon replacement by hydrogen. The 2-methylthiophenol moiety of arbidol is stably accommodated in a hydrophobic region formed by $Leu98^{D}$, $Ile29^{C}$, $Ala101^{D}$, and $Val55^{B}$. Replacement of a structural water that mediates the intramolecular interaction between the backbone oxygen of $Ile29^{C}$ and $Glu103^{B}$ in this cavity by the hydroxyl group of the *m*-hydroxy derivative, **14**, results in enhanced binding affinity. Interestingly, no binding was observed for the *o*- and *p*-hydroxy derivatives. Finally, introduction of a *m*-methoxy group abolished the inhibitory activity, thus confirming the critical role exerted by the hydroxy group in **14**.

A novel series of antiviral compounds having both immunostimulatory and anti-influenza A/B activity involves indole derivatives with different alkyl substituents on the 4-aminomethyl group (R_1 and R_2 in compound **15**; **Figure 4**) and at position 2 (R_3 in

compound **15**; **Figure 4**) [98]. The 5-hydroxy-4-dimethylaminomethyl-2-methyl-1-cyclohexyl-3-ethoxycarbonylindole derivative resulted to be active at a dose (15 mg/kg/day), 4 times lower than that of arbidol (60 mg/kg/day) in a model of influenza infection in mice (A/Aichi virus/2/69).

A series of 5-methoxy-6-bromo-indol-3-carboxylic acid derivatives (general chemical structure **16**, with phenyl or alkyl groups at R_1, H, -S/O-phenyl, -S/O-alkyl groups at R_2, and H, alkyl- or aryl-esters at R_3; **Figure 4**) were proposed in 2016 [99]. The antiviral effect was tested on the influenza virus infection in culture cells of MDSC, and with a model of influenza pneumonia in mice infected with A/Aichi/2/69 (H3N3) virus. The effectiveness of these compounds was similar to arbidol. Further efforts explored modifications at position 2 of the generic compound **17** (**Figure 4**), with Y = C, N, O, S and Ar being aryl, substituted aryl, heteroaryl or substituted heteroaryl groups [100], complementing studies on 5-hydroxy-3-carboxylic acid esters steroid derivatives (**18** in **Figure 4**) [101].

Finally, a new series of piperazine derivatives exhibiting high affinity to the HA stem region have been patented by Janssen Vaccines & Prevision BV in 2020 [102]. A set of 500,000 small molecules was screened using an AlphaLISA (Amplified Luminiscent Proximity Homogeneous Assay)-based high-throughput screening protocol related to competition for displacing a small protein (HB80.4) bound to HA. Representative scaffolds for the most interesting piperazine derivatives are compounds JNJ7918 (**19** in **Figure 4**) and JNJ4796 [103]. JNJ7918 exhibited an IC_{50} of 1.39 and 13.06 µM against A/California/07/2009 (H1N1) and A/Vietnam/1203/2004 (H5N1) HAs, respectively. On the other hand, JNJ4796 lead to an inhibitory activity (EC_{50}) of 0.012-0.066 µM in virus neutralization assays performed using MDCK cells and a variety of viral strains (H1N1, H3N1, H5N1 and H7N7). Structural details (PDB: 6CF7 and 6CFG for H1N1

and H5N1, respectively) about the mode of action for JNJ7918 revealed binding to the stem region in a site typically targeted by broadly neutralizing antibodies. The complex with group-1 H1/SI06 HA shows that the compound occupies a conserved, hydrophobic region formed by His18, His38, Val40, Leu42 and Thr318 of HA_1, Ile48, Thr49, Val52, Asn53 and Ile56 of helix-A, and Trp21 of HA_2. In particular, a direct hydrogen-bonding interaction was observed between the hydroxy group of the ligand and Thr318 of HA.

6. Searching for novel targets

Efforts have been conducted in the search of novel opportunities for therapeutic intervention beyond the three main targets discussed above. This is exemplified by the RNA-dependent RNA polymerase (RdRp; **Figure 5**), which is responsible for replication and transcription of the viral genome in the nucleus of infected cells [104]. RdRp consists of three subunits, which are denoted polymerase acidic (PA), and polymerase basic 1 (PB1) and 2 (PB2). RdRp is highly conserved among different strains and the presence of multiple sites may lead to the disclosure of new drugs, which may affect RNA synthesis, cap-snatching, and protein-protein interactions between the distinct domains.

Efforts targeting the RNA synthesis have primarily involved nucleoside analogs, such as favipiravir, which acts as a purine mimic that inhibits the polymerase activity in a broad range of influenza virus, including strains resistant to amantadine and oseltamivir [105]. In the cap-snatching process, the viral polymerase uses its PB2 cap-binding domain to capture the 5′-cap of nascent host capped RNAs, the PA endonuclease domain cleaves the capped RNA 8–14 nucleotides downstream of the cap structure, and the capped RNA fragments are then used as primers to initiate transcription of viral mRNAs. Drug discovery studies led to the development of pimodivir (VX-787), which

blocks the PB2 activity (K_d of 24 nM) of the viral polymerase, being active against a diverse panel of influenza A virus strains, including H1N1pdm09 and H5N1 strains [106]. On the other hand, baloxavir marboxil (**Figure 5**) inhibits the endonuclease activity within the polymerase PA subunit (IC_{50} of 2.5 nM in an enzymatic assay of the endonuclease activity) [107]. Baloxavir acid potently and selectively inhibits the PA domain of influenza A and B viruses, without affecting the RdRp activity of PB1 or cap binding activity of PB2. Nevertheless, treatment resistance have been associated to the occurrence of mutation I38T (and at less extent I38M and I38F) that reduces the susceptibility to the drug [108].

Inhibition of the protein-protein interaction between RdRp domains has also been the focus of recent studies [109], leading to the development of thiophene-3-carboxamides, such as compound **20** [110], and 4,6-diphenylpyridines, such as compound **21** [111], which are able to inhibit viral growth in the low micromolar range by targeting the interaction between PA and PB1 domains (**Figure 5**). More recent studies have led to the rational design of amino acid derivatives that contain a 3-cyano-4.6-diphenylpyridine scaffold [112]. In particular, hybridization with isoleucine led to a compound (**22**) endowed with an IC_{50} value of 36±3 μM in an ELISA PA-PB1 interaction assay. Moreover, using an in vitro split luciferase complementation-based assay, high-throughput screening of 10,000 compounds (MyriaScreen Diversity collection) led to the identification of two inhibitors of the PA-PB1 interaction, R151785 and R160792, which showed a broad-spectrum antiviral activity against multiple drug-resistant strains of influenza A and B viruses (IC_{50} values in the range 0.9-7.5 μM) [113].

Figure 5 here

Nitazoxanide is an FDA-approved thiazolide initially licensed for the treatment of parasitic infections with minimal adverse side effects but patented as anti-influenza agent [114] (**Figure 6**). The compound is a prodrug that is rapidly hydrolyzed to the active form, tizoxanide, and has been reported to inhibit a broad array of viruses in both tissue culture and animal models. This compound blocks the maturation of the viral glycoprotein HA, altering its intracellular trafficking and insertion into the host plasma membrane, but the net effect is reinforced by a broad amplification of the innate immune response [115].

Dapivirine, originally designed as an HIV nonnucleoside reverse transcriptase inhibitor, has been identified as a broad-spectrum antiviral agent with low micromolar efficacy (**Figure 6**). This compound inhibits the nuclear entry of viral ribonucleoproteins at the early stage of viral replication, thus preventing the synthesis of viral protein and RNA, while has a high in vitro genetic barrier to drug resistance [116]. Along this line, trametinib, an allosteric inhibitor of MEK kinase used against malignant melanoma (**Figure 6**), efficiently blocks replication of different influenza A viral subtypes by interfering with the export of progeny viral nucleoproteins from the nucleus [117]. Similarly, ruxolitinib, an inhibitor of the JAK kinase for the treatment of myelofibrosis (**Figure 6**), has also been patented for the inhibitory activity against the uptake of viral RNA or nucleoprotein in a host cell [118]. Finally, nucleozin has been described as a 'molecular staple' due to the stabilizing effect exerted on the interactions between monomers of the viral nucleoprotein (**Figure 6**), leading to nonfunctional aggregates [119]. Interestingly, it has been remarked that at early stages of the viral infection nucleozin inhibits the synthesis of viral proteins and RNA, and that at late stages it

blocks the production of viral particles affecting the trafficking of viral ribonucleoproteins.

Figure 6 here

7. Expert opinion

Data collected in this report has been compiled with the aim to illustrate the efforts made by the scientific community in an attempt to guarantee a continued updating of the antiviral arsenal available for the treatment of influenza infection. Particular emphasis has been given to selected studies taken from both the scientific literature and patents, focused on the identification of small molecules endowed with antiviral activity, primarily but not exclusively on the last five years (the reader is addressed to specific reviews for further details [120-123]). Most of these efforts have targeted proteins with a key role in viral life cycle, such as the M2 proton channel and NA, and more recently HA and RdRp. However, the limited efficacy and the susceptibility to the emergence of resistance strains have encouraged the search for alternative treatment strategies. At this point, the dramatic scenario produced by the unexpected SARS-CoV-2 outbreak has inevitably raised concerns about the risk of facing a new influenza pandemic in the future and the need to enlarge the therapeutic efficacy with novel antiviral compounds.

In case of flu, M2 ion blockers in the class of adamantanes and NA inhibitors as zanamivir and oseltamivir were developed to treat flu, but this therapeutic reservoir has become obsolete by the advent of resistant influenza strains. In particular, up to 100% of seasonal H1N1 and H3N2 circulating strains are now resistant to adamantanes, and the NA inhibitor oseltamivir is no longer effective in treating the pandemic H1N1

subtypes [124]. As a result, there is an urgent need of new active compounds and most importantly, of new effective therapeutic strategies. In this regard, different approaches could be applied.

A short-term strategy to temporarily to potentiate the therapeutic efficacy of anti-flu treatments and overcome drug resistance could consist on the application of antivirals in combinatorial therapy. In fact, combination of two or three antivirals with different mechanism of action would enhance the antiviral potency, possibly limiting the rate of inactivating mutations [125]. Although interesting for the treatment of mutated strains associated to virulence, the real impact of this approach is not completely explored and thus its effectiveness is yet a matter of intense debate.

An alternative approach is the search of novel antiviral compounds extracted from natural sources. As noted by Zhang et al., 37% of the 98 antiviral drugs registered from Januanry 1981 to September 2019 pertain to natural product botanical medicines, synthetic but natural product mimics, or a combination of these two options [126] (see also [127]). A representative example is oseltamivir, which can be synthesized using quinic and shikimic acids, i.e., two natural products, as starting materials. Pharmacological studies have also disclosed other anti□influenza agents that include a variety of chemical scaffolds, such as terpenoids, quinones, diarylheptanoids, phenylpropanoids, polyphenols, flavonoids, and alkaloids, isolated from traditional herbs [126, 128]. Therefore, they can be an inspiration source for accelerating the development of effective anti-influenza drugs.

A long-term, more demanding strategy consists on the identification of new synthetic inhibitors targeting the already established viral targets (M2, NA) and especially other emerging viral components, such as HA, RNA-polymerase, and nucleoprotein, as well as the blockade of virus-cell interactions that mediate processes such as trafficking of

viral ribonucleoproteins and release of viral particles and modulation of the host immune response. However, the need of finding new antiviral targets and strategies will require a better comprehension of the molecular biology of these processes. Significant progresses have been made in understanding the functional role of the different domains of the viral RdRp, not only targeting the polymerase activity, but also exploiting the druggability of protein-protein interfaces, as noted above. However, current knowledge about other processes, such as the export of ribonucleoprotein complexes to the cytoplasm, which involve the role of nucleoproteins and M1 protein, remain still poorly understood [129].

On the other hand, a deeper characterization of the molecular mechanisms implicated in the activity of the viral proteins may be of outmost relevance for unveiling novel drug discovery strategies. This is illustrated by the efforts performed to take advantage of the presence of subpockets in the binding site of NA and exploit the flexibility of specific loops for the design of antiviral compounds [73, 80]. Another example is provided by the drastic conformational changes that are triggered upon pH acidification in the structure of HA, leading to a complete remodelling of the protein fold that is required for the fusion of viral and host membranes [130, 131]. Besides the binding sites already known for arbidol and JNJ7918, the complex structure of HA may offer opportunities for disclosing novel binding cavities that may prevent the release of the fusion peptide [132]. At this point, it is worth noting a recent study where, following an antibody-guided strategy, several small molecules were identified at fusion inhibitors upon binding to the membrane proximal external region of the HIV□1 envelope spike [133]. Furthermore, understanding the complex structural rearrangements that take place in HA may be valuable not only to identify novel mechanisms for inactivating the fusion

process, but also to explore the impact of resistance mutations on the drug effectiveness against this protein target.

Overall, we hope that this review will be valuable to foster the discussion on the design and implementation of novel approaches to enrich our current anti-influenza therapeutic arsenal. The development of antiviral strategies that combine treatment effectiveness and low susceptibility to resistance is a challenge that requires an urgent response.

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Declaration of interests

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Abbreviations: AlphaLISA: amplified luminiscent proximity homogeneous assay; HA: hemagglutinin; NA: neuraminidase; RBD: receptor binding domain; RdRp: RNA-dependent RNA polymerase; SA: sialic Acid; TBHQ: tert-butyl hydroquinone; TEVC: two-electrode voltage clamp.

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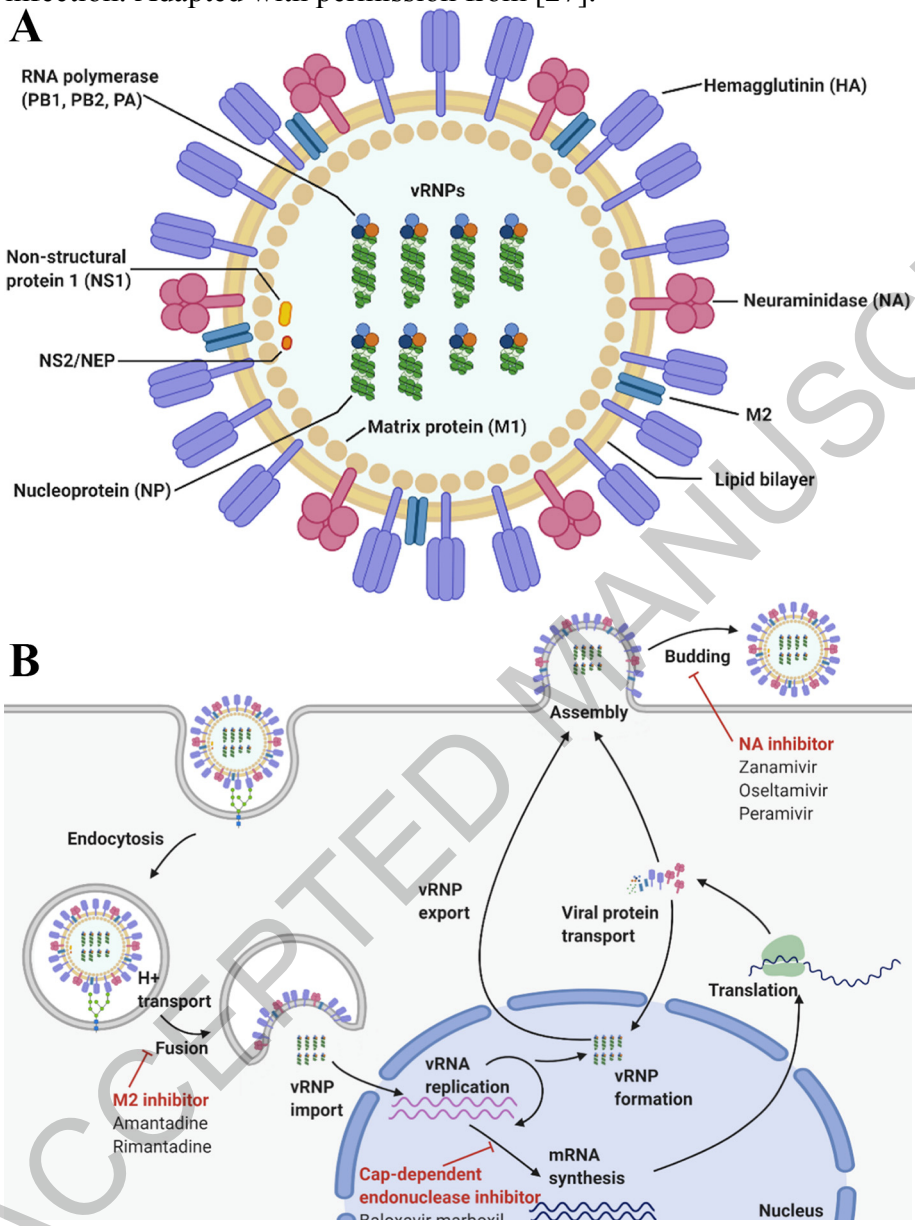
Figure 1. (A) Schematic representation of the influenza A virus, showing the matrix protein M1, the transmembrane M2 proton channel, the membrane-bound hemagglutinin (HA) and neuraminidase (NA), the non-structural proteins, and the 8 segments of the viral ribonucleoprotein (vRNP). The 5'- and 3'-segments of the viral RNA are bound to the heterotrimeric RNA-dependent RNA polymerase, formed by three domains (PA, PB1 and PB2). (B) Representation of the life cycle of viral infection. Adapted with permission from [27].



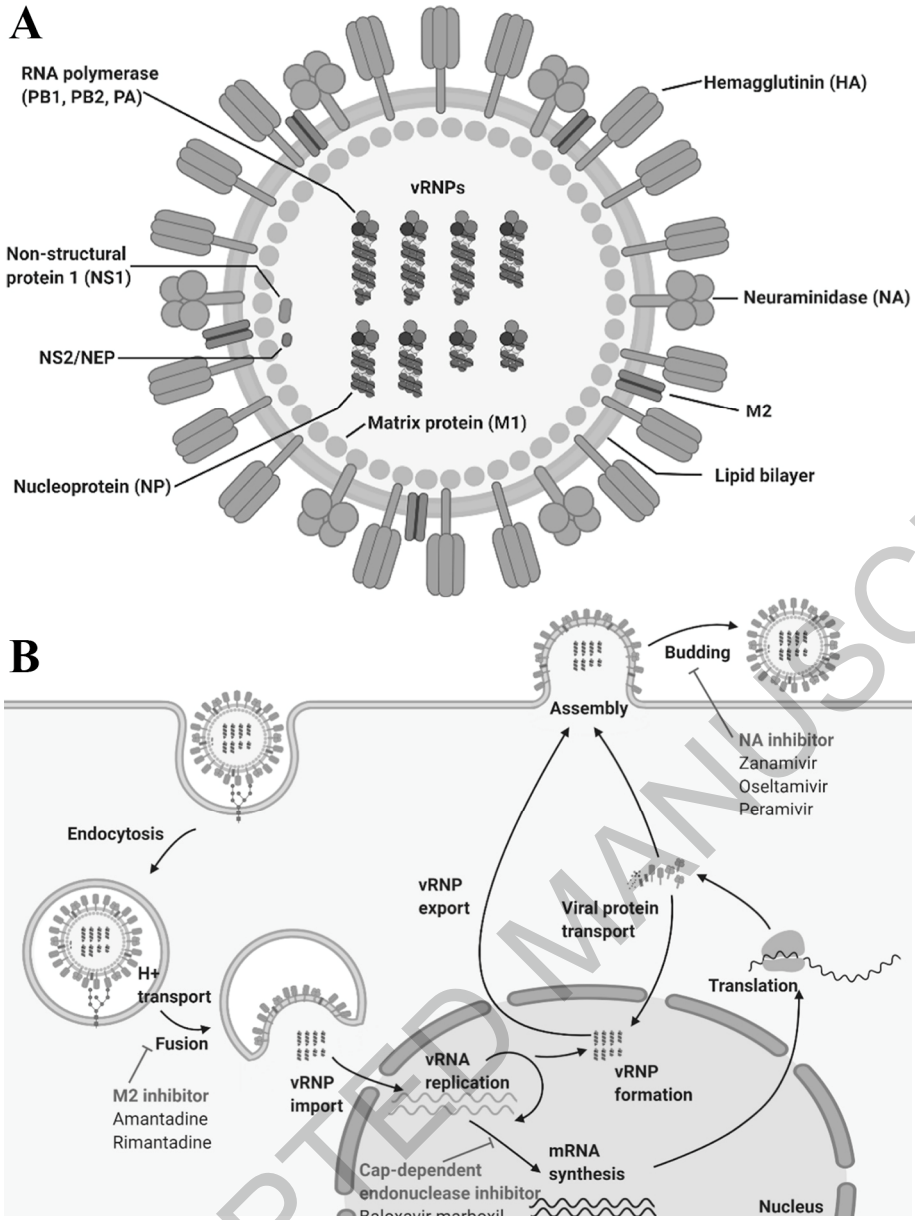
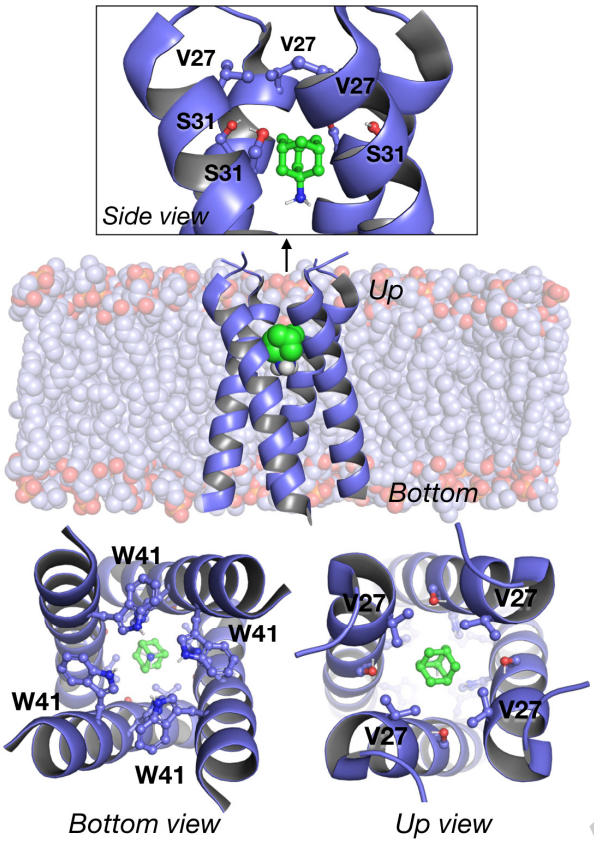
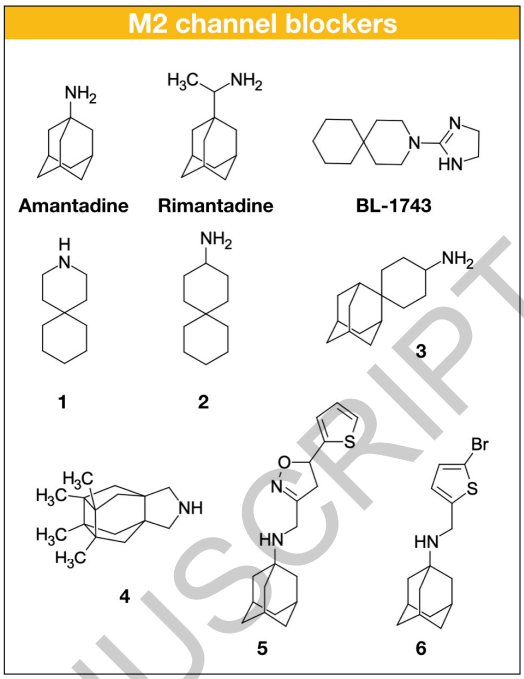


Figure 2. Representation of the crystallographic structure of the M2 proton channel (PDB: 2KQT), binding mode of amantadine in the interior of the pore, and chemical structures of selected compounds targeting wild-type and mutated variants of the proton channel.





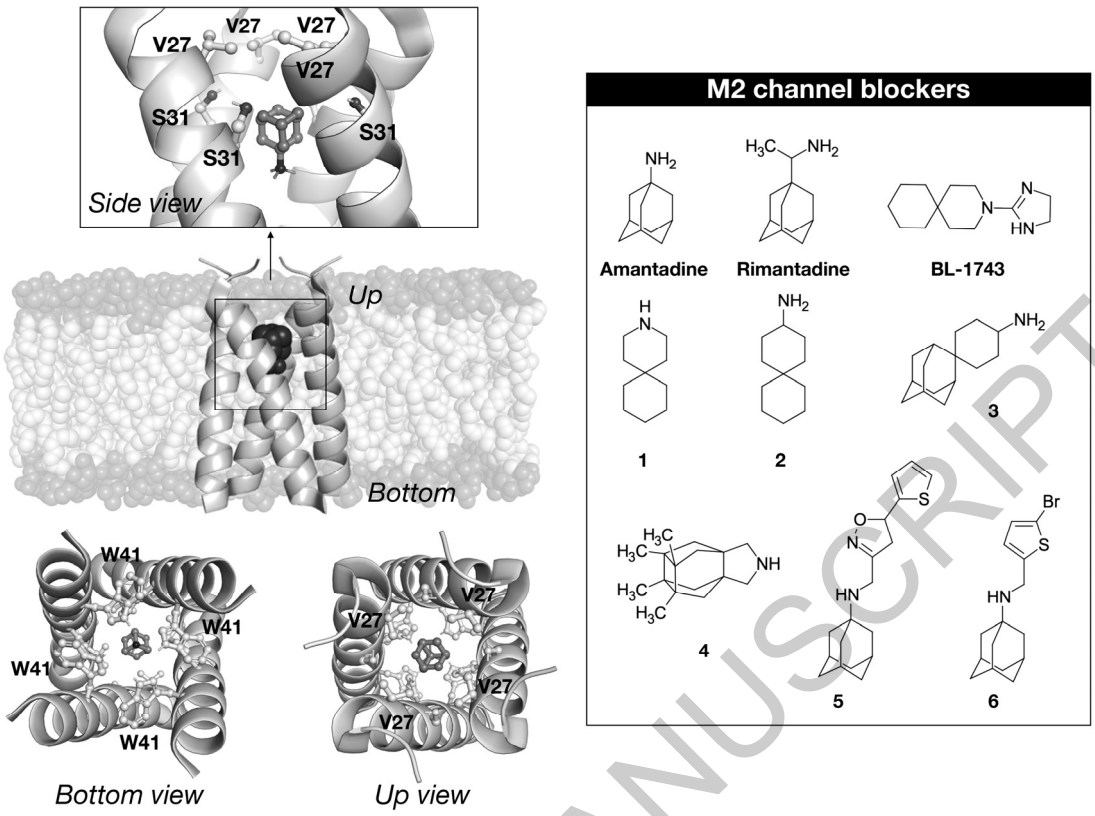
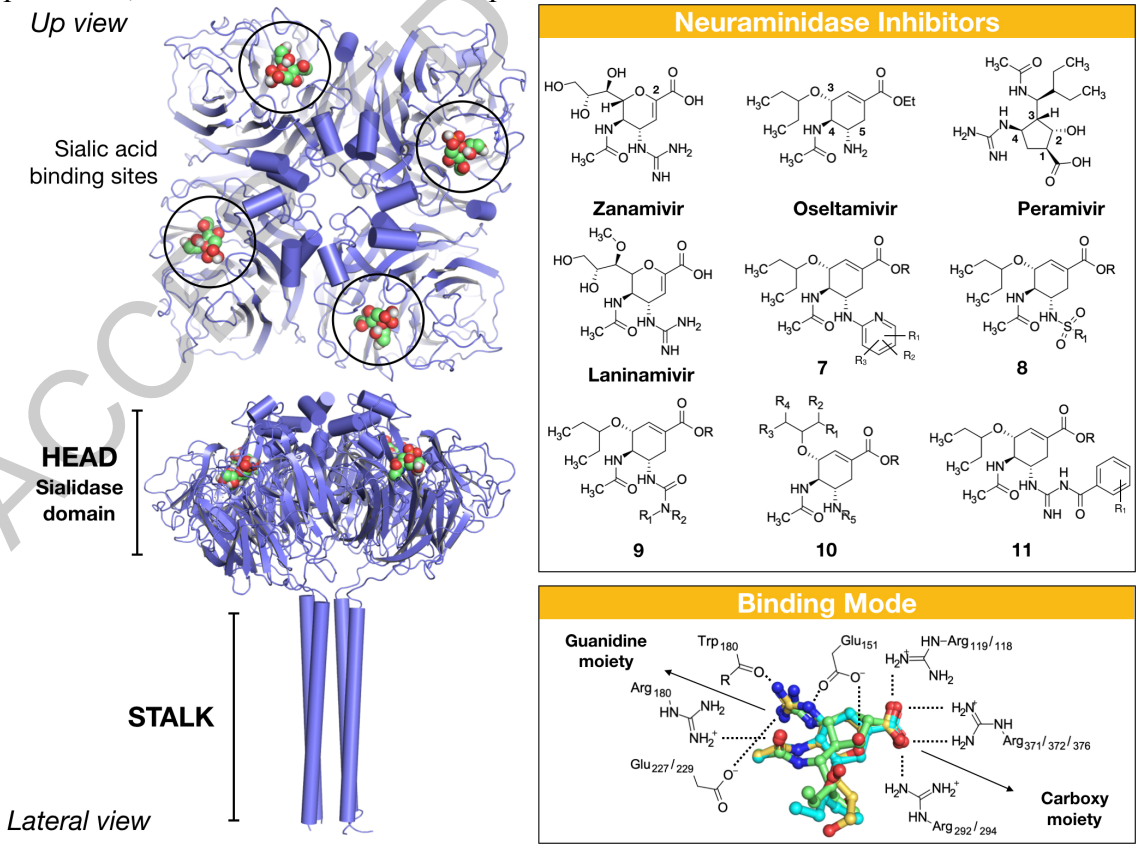


Figure 3. Representation of the crystallographic structure of NA protein (PDB: 6CRD, from recombinant H1N9 strain), binding modes for zanamivir, oseltamivir and peramivir, and chemical structures of patented NA inhibitors.



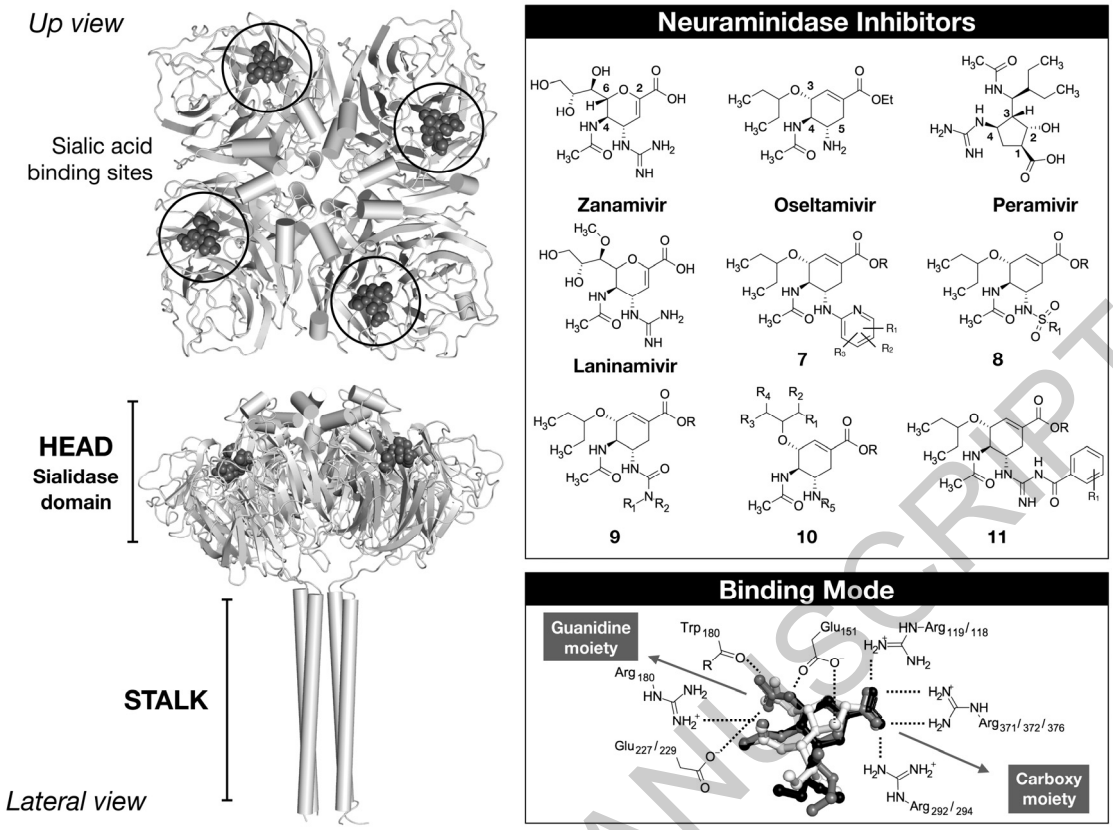


Figure 4. Representation of the crystallographic structure of the pre-fusion HA protein (PDB: 3EYM for complex with TBHQ; PDB: 5T6N for complex with arbidol; PDB: 6CFG for complex with JNJ7918) and chemical structures for patented HA fusion inhibitors.

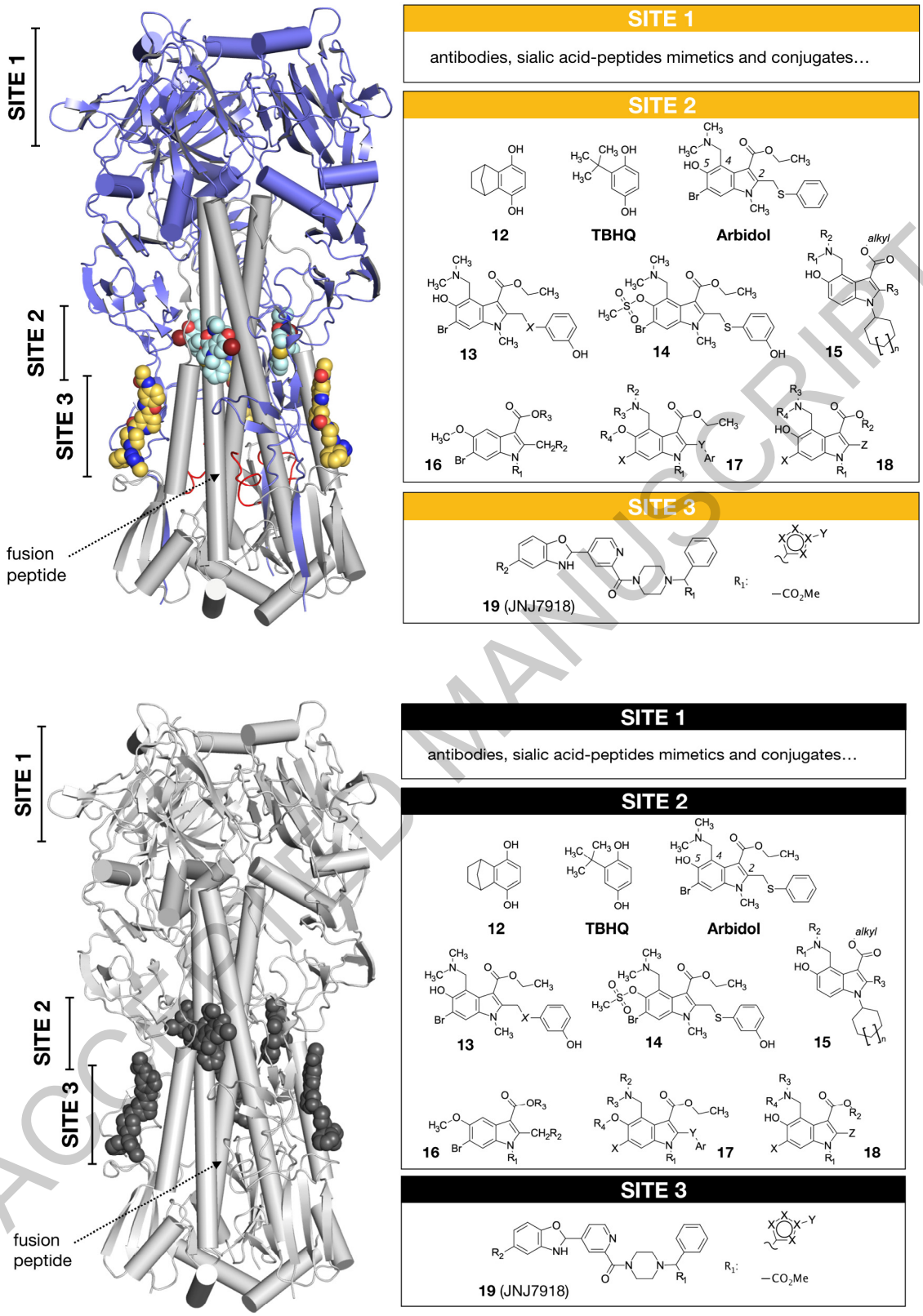
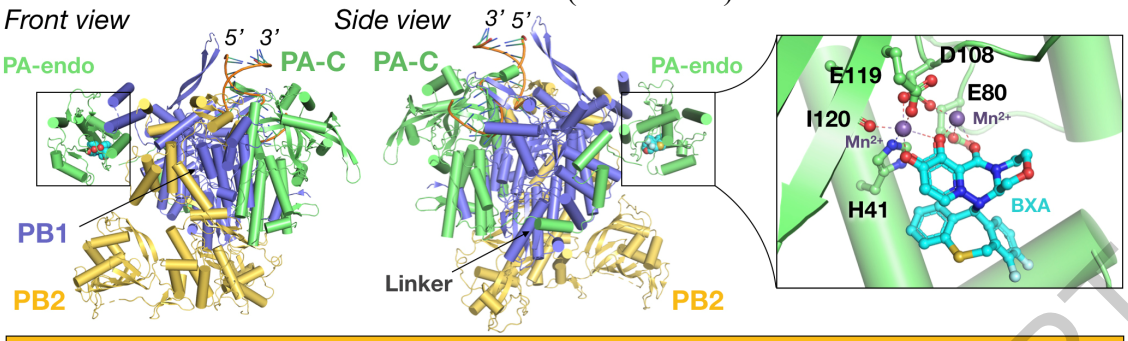
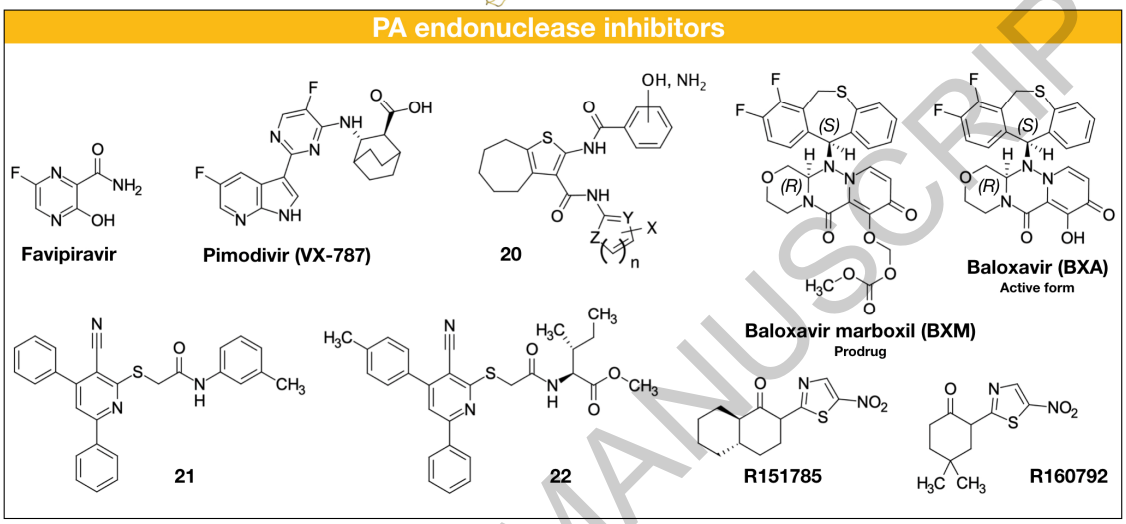
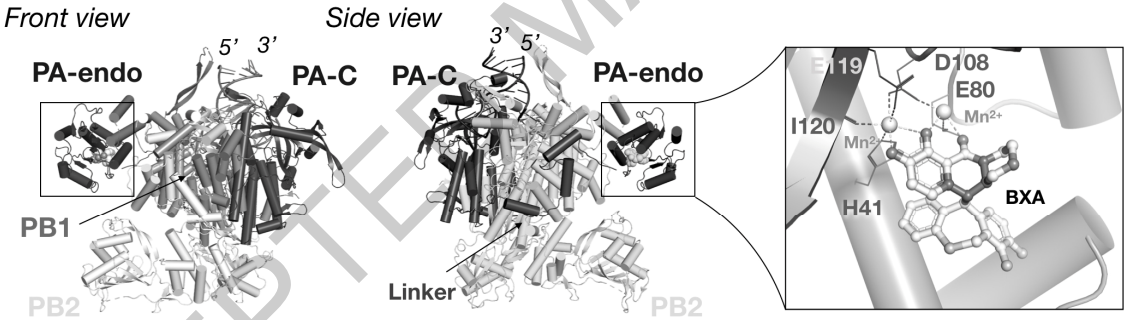


Figure 5. Representation of the three-dimensional structure of the heterotrimeric unit of Influenza A RNA polymerase (PDB: 4WSB). PB1, PB2 and PA domains are

highlighted. Chemical structure of some representative inhibitors and binding mode of baloxavir into the PA endonuclease domain (PDB: 6FS6).







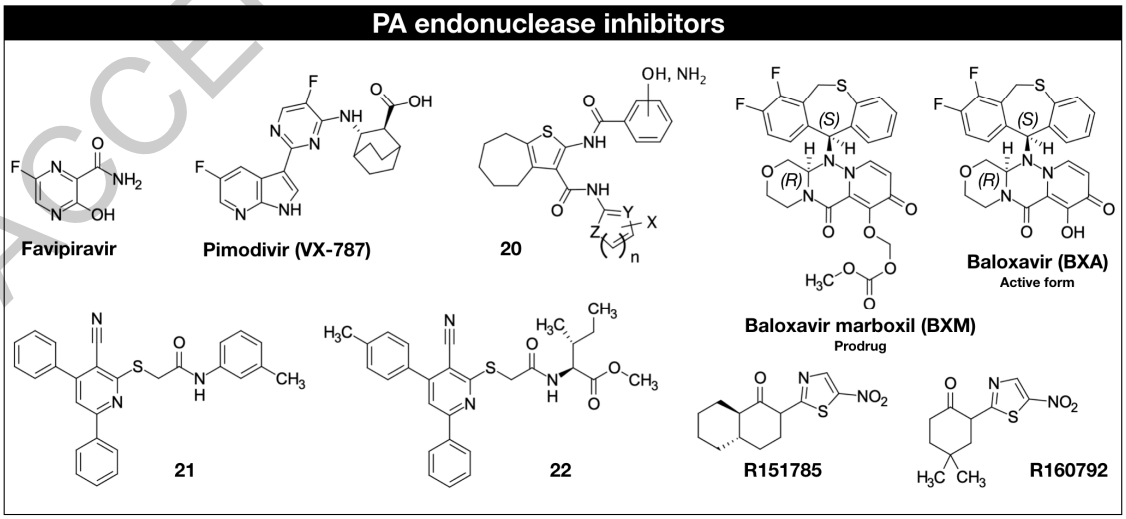


Figure 6. Representation of the chemical structures of selected compounds developed as antiviral inhibitors of the influenza infection.