

## REVIEW

# Compounds with anti-influenza activity: present and future of strategies for the optimal treatment and management of influenza.

## Part I: influenza life-cycle and currently available drugs

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### Key words

Influenza • Influenza life cycle • Antiviral • Licensed drugs • Amantadine • Rimantadine • Oseltamivir • Zanamivir • Peramivir • Laninamivir • Ribavirin • Arbidol

### Summary

*Influenza is a contagious respiratory acute viral disease characterized by a short incubation period, high fever and respiratory and systemic symptoms.*

*The burden of influenza is very heavy. Indeed, the World Health Organization (WHO) estimates that annual epidemics affect 5-15% of the world's population, causing up to 4-5 million severe cases and from 250,000 to 500,000 deaths.*

*In order to design anti-influenza molecules and compounds, it is important to understand the complex replication cycle of the influenza virus. Replication is achieved through various stages. First, the virus must engage the sialic acid receptors present on the free surface of the cells of the respiratory tract. The virus can then enter the cells by different routes (clathrin-mediated endocytosis or CME, caveolae-dependent endocytosis or CDE, clathrin-caveolae-independent endocytosis, or macropinocytosis). CME is the most usual pathway; the virus is internalized into an endosomal compartment, from which it must emerge in*

*order to release its nucleic acid into the cytosol. The ribonucleoprotein must then reach the nucleus in order to begin the process of translation of its genes and to transcribe and replicate its nucleic acid. Subsequently, the RNA segments, surrounded by the nucleoproteins, must migrate to the cell membrane in order to enable viral assembly. Finally, the virus must be freed to invade other cells of the respiratory tract. All this is achieved through a synchronized action of molecules that perform multiple enzymatic and catalytic reactions, currently known only in part, and for which many inhibitory or competitive molecules have been studied. Some of these studies have led to the development of drugs that have been approved, such as Amantadine, Rimantadine, Oseltamivir, Zanamivir, Peramivir, Laninamivir, Ribavirin and Arbidol. This review focuses on the influenza life-cycle and on the currently available drugs, while potential antiviral compounds for the prevention and treatment of influenza are considered in the subsequent review.*

### Introduction

Influenza is a contagious acute respiratory viral disease characterized by a short incubation period, high fever, respiratory (e.g. runny and stuffy nose) and systemic symptoms (e.g. muscle or body aches) [1]. Most of the people affected by influenza recover in a few days or, at most, in 2 weeks. However, some patients may develop complications that may be very serious. The most common complications are bronchitis, pneumonia, ear infections, etc. People with underlying diseases, such as asthma, subjects with Chronic Obstructive Pulmonary Disease (COPD) or individuals with heart disease are at high risk of complications [2]. Related complications, such as myositis, acute encephalopathy or Reye's syndrome, are rare [3]. Reye's syndrome is classically characterized by rashes, vomiting and liver damage. It can typically occur during viral illness in children who have been taking aspirin for a long period [4].

Pneumonia can be caused by bacterial superinfection also called secondary pneumonia or viral pneumonitis [5, 6]. This is characterized by the complex interactions of host-co-infecting pathogens [7], and, in particularly frail and debilitated subjects, can result in the impairment of physical capabilities, dysregulation of immune responses and a delayed return to homeostasis [5, 6].

The burden of influenza is very heavy. Indeed, the World Health Organization (WHO) estimates that annual epidemics affect 5-15% of the world's population, causing up to 4-5 million severe cases and from 250,000 to 500,000 deaths [7]. The European Centre for Disease Prevention and Control (ECDC) estimates that approximately 10% of Europeans are infected each year [8]. Furthermore, the US government estimates that 5-20% of US residents catch influenza each year [9].

Influenza viruses belong to the *Orthomyxoviridae* family [10], with the other two genera being *Isavirus* and *Thogotovirus*, and have the ability to change their surface antigens relatively frequently [11]. When a major

variation occurs, if the virus adapts to humans during zoonotic spill-over, widespread diffusion of the virus is possible, resulting in a pandemic [12]. The most severe influenza pandemic was that of 1918, which caused 500 million cases and from 50 to 100 million deaths [13, 14]. During that devastating pandemic, the treatment of patients suffering from influenza was empirical and symptomatic, and was intended primarily to relieve fever and pain (e.g. aspirin administration), while epinephrine was used to treat forms of secondary pneumonia [15]. Only in the 1960s did the first antiviral drug against influenza, namely Amantadine, become available in the US [16, 17], while in 1993 another drug, Rimantadine, was authorized [18]. Later, in 1999, the anti-neuraminidase (NA) medications Zanamivir and Oseltamivir were both licensed in the US [19].

Since 1999, much knowledge concerning viral replication has been acquired, and new experimental hypotheses have been advanced for the development of new flu drugs and new protocols for both prevention and treatment. Anti-influenza drugs are an important complement to vaccination, which is the most efficacious weapon against the disease. In this review, it therefore seemed useful to deal with the issue of new/potential antiviral medications against influenza infections, especially in the light of the most recent scientific advances.

## Biology of influenza viruses

Regarding the antigenic characteristics of the core proteins (nucleoproteins [NP] and Matrix proteins [M proteins]), three influenza virus types have been identified: A, B and C. Given the relevance of Influenza A Virus (IAV) to human pathology, we will provide a brief overview of its biology and life-cycle and underline the main differences among the three virus types in terms of structural and molecular biology.

The IAV particle varies in the range of 80-120 nm and is pleomorphic, being usually spherical, though cord-like forms with a diameter of 40-100 nm and a length in the range of 300 nm-20  $\mu$ m can occur [11, 20, 21]. Transition from spherical to tubular form is not well understood: what is known so far is that M1 [22, 23], M2 [23, 24] and NP [25] could play a role in determining and modulating this process. Besides genetic traits, also the phenotype of the host cell, in terms of shape and polarization, seems to influence the viral form [21, 26]. Influenza B has a similar shape, being structurally indistinguishable from IAV [11], while Influenza C virus is usually filamentous and 500 nm long [11]. The IAV viral particle is an envelope made up of lipid rafts and spikes of two main types of glycoproteins: hemagglutinin (HA) accounts for about 80% (about 500 molecules) and NA for about 17% (about 100 molecules); M2 is the least abundant protein, with only 16-20 copies per virion [11, 27].

The particle of influenza B virus contains four proteins, namely HA, NA, NB, and BM2 [11, 28], while the particle of influenza C virus is made up of a major

surface glycoprotein (HEF, hemagglutinin-esterase-fusion protein) and a minor surface glycoprotein (CM2). These surface glycoproteins form ordered hexagonal arrays [11, 29-32].

Underneath the viral membrane, M1 tightly binds the vRNPs, which consist of RNA strands (usually single strands but in certain cases double strands) [11] wrapped around NP and NEP, with a terminal polymerase ternary complex (PA, PB1, PB2). The genome is small (about 13-16 kilobases) and contains seven or eight pieces of segmented negative-sense RNA (eight segments for IAV and influenza B, seven for influenza C), each piece of RNA containing either one or two genes of 890 to 2,341 nucleotides each [11]. The genome codes for at least 11 proteins: hemagglutinin (HA) of about 76-77 kDa, NA of about 60 kDa, NP of about 60 kDa, M1 of about 28 kDa, M2 of about 15 kDa, non-structural protein type 1 (NS1) of about 26 kDa, non-structural protein type 2 (NS2) (also known as NEP: nuclear export protein) of about 11 kDa, PA of about 85 kDa, PB1 (polymerase basic type 1) of about 88 kDa, PB1-F2 of about 80 kDa [33-35] and PB2 (polymerase basic type 2) of about 91 kDa [36].

In particular, segments 1-3 code for the ternary polymerase complex (PB1, PB2 and PA, respectively), segment 4 for HA, segment 5 for NA, segment 6 for NP, segment 7 for the matrix proteins, and segment 8 for the non-structural proteins [11]. On the basis of the gene structures, segments can be divided into three classes: intronless, intron-containing and unspliced, and intron-containing and spliced. On the basis of the kinetics of the gene expression, they can be classified into "early" (segments 1-3, 5 and the unspliced segment 8 transcript) and "late" (segments 4, 6, 7 and spliced segment 8) classes. Usually, structural and functional/kinetic classifications do not correspond [37, 38].

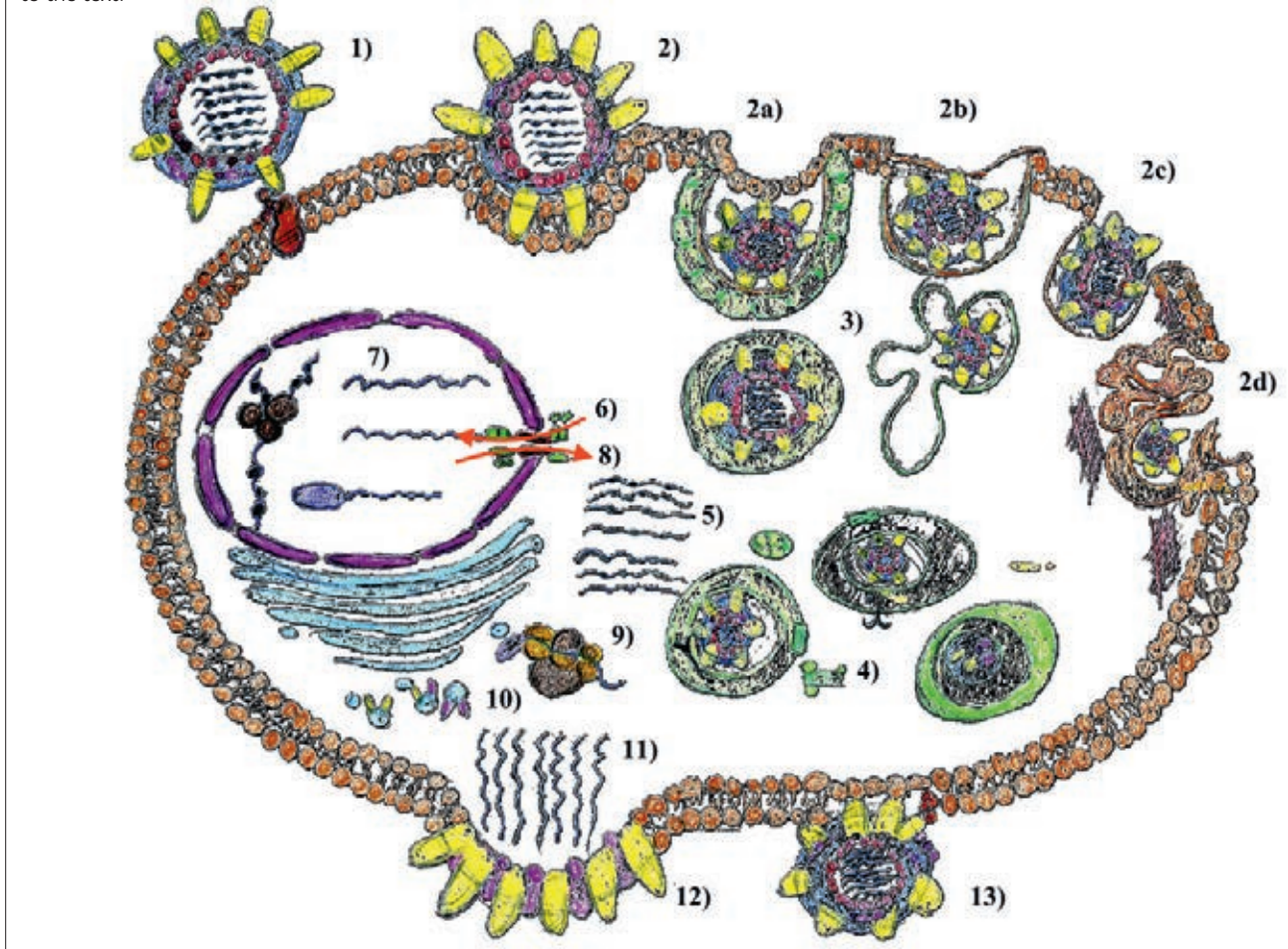
Moreover, the presence of overlapping genes and different splicing mechanisms may give rise to further accessory proteins, such as PB1-N40 [39], PA-X [39-40], PA-N155 [41], PA-N182 [42], M42 [43], and NS3 [43], which have been discovered and characterized only recently. Another accessory protein, NEG8 ORF, has been predicted [44].

The viral proteome thus reveals unexpected dynamism and complexity [43-44]; Matsuoka and coll. have designed a comprehensive map of influenza interactions, termed FluMap, which contains 960 factors and 456 reactions as of April 2012 [47].

## Replication cycle of influenza viruses

The replication cycle of the virus (Fig. 1) is a complex, highly dynamic, biological process which consists of the following steps: 1) attachment of the virion to target cells and receptor binding (virus adsorption); 2) internalization into cellular regions by means of clathrin-mediated endocytosis (CME), caveolae-dependent endocytosis (CDE), clathrin-caveolae-independent endocytosis, and macropinocytosis; 3) endosomal trafficking via

**Fig. 1.** Schematic representation of the replication cycle of the influenza: 1) attachment of the virion to target cells and receptor binding (virus adsorption); 2) internalization into cellular regions by means of clathrin-mediated endocytosis (CME), caveolae-dependent endocytosis (CDE), clathrin-caveolae-independent endocytosis, and macropinocytosis; 3) endosomal trafficking via endosomes / caveosome / macropinosome / lysosomes to the perinuclear compartment; 4) pH-dependent fusion of viral and endosomal / organellar membranes; 5) uncoating; 6) nuclear importation; 7) transcription and replication; 8) nuclear exportation; 9) protein synthesis; 10) post-translational processing and trafficking; 11) viral progeny assembly and packaging; 12) budding; and 13) release. For further details, the reader is referred to the text.



endosomes / caveosome / macropinosome / lysosomes to the perinuclear compartment; 4) pH-dependent fusion of viral and endosomal / organellar membranes; 5) uncoating; 6) nuclear importation; 7) transcription and replication; 8) nuclear exportation; 9) protein synthesis; 10) post-translational processing and trafficking; 11) viral progeny assembly and packaging; 12) budding; and 13) release (modified from [48]).

The cells infected by the influenza virus are: alveolar and bronchial epithelial tissue (BET) cells, alveolar macrophages (AM), lung epithelial tissue (LET) cells and, in particular, type II pneumocytes, plasmacytoid dendritic cells (pDCs) and natural killer cells (NKs) [49, 50].

Influenza virus is able to activate Endoplasmic Reticulum (ER) stress, caspase pathway [51] or to finely tune host secreted molecules, such as lung mucins [52], in order to avoid being trapped and subsequently eliminated. Moreover, it recruits host factors and misuses them [53]. Although the mechanisms of influenza virus replication are not fully understood, scientific projects for new

drugs against influenza cannot ignore the biological cycle of this virus.

#### INFLUENZA VIRUS ENTRY

The first important event during infection in humans is the attachment of influenza virions to the apical cell surface (event known also as virus adsorption). Indeed, the entry of the Influenza virus into target cells is an essential process whereby viral genomes are delivered from extracellular virions to sites of transcription/replication in the cell nucleus [54]. During this phase, thanks to the surface glycoprotein HA, the virus interacts with (-2,3)- or (-2,6)-linked sialic acid receptors [55]. The physico-chemical conformation of these receptors is not identical in different species of animals – humans, seals, birds, pigs, horses, etc., which are the natural reservoir of the virus. The vast majority of human receptors are located in the upper respiratory tract, but man also possesses receptors typical of birds, which are located deep in the respiratory tract [56].

HA is a homotrimeric integral type 1 membrane cylinder-like glycoprotein, approximately 13.5 nanometres long. HA monomers are synthesized as precursors (HA0) containing a hydrophobic signal sequence. After being translated, they are glycosylated and cleaved into two smaller subunits: namely, HA1 of 50 kDa and HA2 of 27 kDa, which are linked by a disulfide bridge. Each subunit is characterized by a long, central,  $\alpha$ -helix connected to the membrane by HA2 and surmounted by HA1, a spherical head containing the sialic acid binding sites (receptor binding sites or RBSs, also known as receptor binding pocket or RBP) [57]. The apolar domain of HA2, near the cleavage site, is known as the “fusion peptide” or HAfp23 [58, 59], since it is characterized by a domain of highly conserved N-terminal 23 residues. HAfp23 has a helical-hairpin structure consisting of two tightly-packed helices, which are fundamental to inducing the negative curvature (“fusogenic conformation” of HA) [60].

There are at least eighteen HA subtypes [61]. These are further subdivided into two groups: group 1 comprises H1, H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18 (from waterfowl, the last two having been recently isolated from bats in Guatemala and Peru) [61, 62]; group 2 comprises H3, H4, H7, H10, H14, and H15 [62]. However, a recent experiment has proved that the virus can also enter into cells whose surface has been completely depleted of sialic acid-based glycoproteins and glycolipids [63]. This seems to suggest alternative entry routes. Indeed, H17 and H18 do not bind to sialic acid and their receptor is still unknown [64].

Entry is essentially through receptor-mediated endocytosis [65], though an alternative uptake pathway, namely macropinocytosis, has quite recently been discovered [66-68]. Receptor-mediated endocytosis can be CME or mediated by lipid rafts: CDE or non-clathrin non-caveolae endocytosis [69-71].

CME is the most common pathway through which the virion is internalized. The clathrin triskelion is made up of three heavy chains, which constitute the backbone of the polyhedral structure, and of three light chains, which finely tune the assembly / disassembly of the triskelion [72]. Adaptor proteins, such as AP-2 [73], epsin-1 [74], epsin-15, recognize specific internalization signals located on cargo receptors, and take part in the formation of clathrin-coated pits (CCPs).

Caveolae are small flask-like infoldings of the membrane, with high levels of cholesterol and glycosphingolipids and with caveolin as the integral membrane scaffolding structure. The remaining entry routes have been investigated less. In clathrin caveolae-independent endocytosis, the Ras-phosphoinositide 3-kinase (PI3K) signalling pathway may play a major role [75].

The PI3K inactive complex (PI3K p110-p85 heterodimer) moves to the plasma membrane, where the SH2 (Src Homology 2) domains of p85 engage the phosphotyrosine residues present in receptor-associated proteins, causing a molecular rearrangement of the complex, in such a way that p110 is now enzymatically active and can recruit / produce intracellular second messenger,

such as PtdIns(3,4,5)P3 (phosphatidylinositol-(3,4,5)-trisphosphate), from PtdIns(4,5)P2 (phosphatidylinositol-(4,5)-bisphosphate). Subsequently, several effector proteins with pleckstrin homology (PH) domains mediate an array of different signalling cascades: namely, activation of Akt, mTOR (mammalian Target Of Rapamycin), PKC (Protein Kinase C), PTEN (Phosphatase and tensin homolog) pathways [76].

Macropinocytosis can be activated by cell growth factors such as the Epidermal Growth Factor (EGF) or Nerve Growth Factor (NGF) or can be induced by phorbol esters. Macropinocytosis is Rac1-, PAK1- (p21-activated kinase type 1), cholesterol- and actin-dependent, since it extensively reorganizes the plasma membrane and implies energy mechanisms, such as Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) activity [77, 78]. Also other pathogens such as Ebola virus [79, 80], arenavirus [81], adenovirus [82, 83], HPV (Human Papillomavirus) [84], HIV (Human Immunodeficiency Virus) [85], *Escherichia coli* [86], *Trypanosoma cruzi* [87], vaccinia virus [88], African Swine Fever virus [89], RSV (Respiratory Syncytial Virus) [90] and HCV (Hepatitis C Virus) [91], among the others, use macropinocytosis as additional entry pathways.

These different routes (CME, CDE, non-clathrin non-caveolae endocytosis and macropinocytosis) result in different internalization yields and may be related to the different degrees of virulence and pathogenicity of influenza strains [92].

## ENDOSOMAL TRAFFICKING

After attaching to the respiratory cell surface, the influenza virus, as already mentioned, can penetrate into the cell through more than one mechanism, and the final result is that the virions are internalized in an endosomal compartment [93]. According to the previous routes, virions can be packaged into CCPs and clathrin-coated vesicles (CCVs) or into caveosomes / macropinosomes. Subsequently, they are further processed into early endosomes (EEs) and into late endosomes (LEs) [94].

Endosomal trafficking usually follows influenza virus uptake. Virions are taken up into Rab-5/Vps4 and Rab-7/LAMP (lysosomal-associated membrane protein) positive endosomes. Rab-5/Vps4 and Rab-7 are Rab guanosine triphosphatases (GTPases) that are responsible for the processes of trafficking and fusion for EEs and LEs, respectively [95-97].

LAMPs are a series of proteins – 3 isoforms are currently known, namely, LAMP1 (known also as CD107a), LAMP2, and LAMP3 (DC-LAMP, TSC403 or CD208) – that are involved in endosomal maturation [98]. Moreover, the expression of LAMP3 has been found to be predictive of the host's response to influenza [99].

Cullin-3 (Cul3) belongs to the Cullin-RING-ligase family (CRL). It functions as a scaffolding protein in the Bric-a-brac, Tramtrack, Broad-complex (BTB)-Cul3-Rbx1 ubiquitin E3 ligase complex and is involved in LE maturation and cargo trafficking [100, 101].

Endosomal trafficking is a complex multifaceted process that can be divided into three stages: stage I, which

is actin-dependent, stage II, which is dynein- and microtubule-dependent, and stage III, which is microtubule-dependent [102].

### INFLUENZA ENVELOPE FUSION

In order for the virus to emerge from the endosome and for ribonucleoprotein (RNP) to be released into the cytosol, the envelope must fuse with the endosome membrane. This part of the life-cycle of the virus requires the acidity of the lumen of the endosome to decrease to a pH value of about 5 [103, 104]. In this process, a crucial role is played by M2, which not only acts on ion channels, thereby allowing acidification of the interior of the virus [105], but also alters its conformation, resulting in changes of the curvature of the viral envelope [106, 107]. This leads to two important events, namely: the dissociation of the M1 protein from RNP and a dramatic change in the conformation of HA [108], which can expose its fusion peptide. This peptide may allow fusion of the viral envelope and the endosome membrane and the release of RNP into the cytoplasm. The cleavage of HA occurs through a proteasic enzymatic action [109]. Proteases that can cause the cleavage of HA are widely distributed throughout the human body, and the precise role that each plays in cutting the HA is not exactly known. Nevertheless, proteases are known to belong to two main classes, namely trypsin-like enzymes or furin-like serine enzymes [109]. These enzymes are produced by the cells, particularly those of the respiratory system, in the presence of inflammation (proinflammatory cytokines/chemokines, neutrophils, etc.) [110].

The release of RNP into the cytosol is also enabled by the host's aggresome processing machinery (made up of dynein, dynactin and myosin II) [111]. Further molecules and pathways could take part in this process.

M2 is more than just a simple ion channel. Indeed, it plays a multifaceted role in the entire viral life-cycle, as the mechanism of proton permeation, conductance and acidification is crucial to each different step and activity of the virus. After endocytosis, the low endosomal pH (in the range 5-6) activates the M2 channel prior to HA-mediated fusion, and favours dissociation of the M1 protein from the vRNPs, thereby facilitating the entry of RNPs into the nucleus. M2 is fundamental to genome unpacking and the release of the viral RNA during viral uncoating [112]. Moreover, M2 enables the viral RNA to package and facilitates virion scission and budding during viral maturation and assembly, replication and infection: during transport to the cell surface, in the trans-Golgi network (TGN) membrane, M2 acts as a proton-leak channel and prevents the activation of *de novo* synthesized HA by regulating the pH of the Golgi apparatus of the host cell and equilibrating its pH with the pH of the viral interior. Indeed, if it were not elevated in this way, the low pH would cause a conformational rearrangement of HA, whose intracellular cleavage would prematurely inactivate the new influenza viral progeny [113, 114]. M2, together with other proteins such as NS1 and HA, could play an additional role of fine-tuning the apoptosis of infected cells, thus favouring viral replication [115].

NS1 is involved in several biological tasks, such as mRNA splicing and translation, cell survival, and immune defence. In particular, as far as the type I interferon (IFN-I)-mediated response is concerned, it interacts with PACT/PRKRA (Protein activator of the interferon-induced protein kinase / Protein kinase, interferon-inducible double stranded RNA dependent activator), which is an important cofactor for the IFN-I response elicited by the viral RNA-sensor RIG-I (Retinoic acid-Inducible Gene I). Therefore, it blocks PACT/RIG-I-mediated activation of IFN-I [116, 117]. Moreover, it binds latent protein kinase PKR (Protein Kinase R, also known as Protein kinase RNA-activated or interferon-induced, double-stranded RNA-activated protein kinase, or eukaryotic translation initiation factor 2-alpha kinase 2 – EIF2AK2), whose activation would inhibit viral protein translation and synthesis [118], and also TRIM25 (tripartite motif-containing protein 2) [119, 120]. Recently, it has been shown to interact with an array of host proteins, such as interleukin-6 receptor (IL-6R), MHC class I HLA-B, cathepsin B, ubiquitin, and adenosine deaminase acting on RNA (ADAR1) [121].

With regard to M2 ion channel activity, the heart of this mechanism is the HxxxW motif of the inner transmembrane (TM) residues [122-125]. In this HxxxW motif, Histidine 37 putatively acts as the pH sensor and, when the pH is low, the protonation of the imidazolic ring destabilizes TM packing because of electrostatic repulsion. Tryptophan 41, which acts as a primary gate, rotates, becomes unlocked from Aspartic acid 44 (“the channel lock”) and, being now parallel to the axis of the pore, makes the protons flow. By contrast, Valine 27 acts as a secondary gate (the so-called “Valine 27 valve”); its importance has been confirmed only recently by the multi-scale simulation carried out by Liang and coll. [126]. On the basis of the exact role of the Histidine 37 tetrad, two models have been proposed: the shutter model, in which the biprotonated charge of Histidine 37 does not change during the proton flux (proton diffusion is coupled with water wire via the Grotthuss mechanism), and the shuttle model, in which the protonation status of Histidine 37 is subject to changes during excess proton transfer [127]. However, the exact mechanism of highly selective transport of protons is not known.

Acidification is enhanced by the viral activation of the PI3K cascade [76].

### NUCLEAR IMPORTATION

The next event in replication is the importation of RNP into the nucleus. The trafficking of RNP into the cytoplasm is achieved extremely rapidly, by means of a mechanism of diffusion, without the intervention of microtubules, intermediate filaments or actin filaments, through the nuclear pore complex (NPC). Some important components of the NPC are the nucleoporins Nup37, Nup43, Nup45 [128], Nup50 [129], Nup54 [130], Nup58 [128], Nup62 [131], Nup75, Nup88, Nup93, Nup98, Nup107, Nup133 [128], Nup153 [132-135], Nup160 [128], Nup214 [130], NuTF2 (Nuclear Transport Factor 2) and Nup358/RanBP2 [128].

Nup37, Nup88, Nup96, Nup107, Nup133 and Nup160 belong to the so-called Nup107 subcomplex [136], while NupL1 (Nup45/Nup58), Nup54, and Nup62 belong to the Nup62 subcomplex [137]. Other components are Magoh, ALADIN, Tpr (Translocated promoter region), EJC (exon junction complex), NLP1/CG1 (Nucleoporin-Like Protein type 1), Seh1, Rae1/Gle2 and POM121 (nuclear envelope pore membrane protein type 121) [128].

When RNPs reach the nuclear membrane, nuclear importation is mediated by the binding of the nucleoprotein with the alpha importins, which then bind importin- $\beta$  [129]. Simultaneously, the importins must interact with PA, PB1 and PB2 [130], and this affects the interaction of the RNP with the same importins [131]. Specifically, subunits PB1 and PA are imported by Ran-BP5 or karyopherin beta3 (also known as importin beta3, or importin 5), whilst subunit PB2 is imported by importin alpha-3 or importin alpha-7. NPs are imported by importin alpha-1 [132-141]. Other molecules that take part in nuclear importation are Hsp70 and Hsp90 [142-144]. In the nucleus, the importins detach from RNP. Although it is not clear, nor is the mechanism known at the molecular level, dissociation of the RNP should occur after separation from the importins. Thus, after the spreading of RNP in the nucleoplasm, transcription and replication can initiate.

### TRANSCRIPTION AND REPLICATION

The transcription and replication of viral nucleic acid are not fully understood. However, the phenomena involve coordinated and differentiated RNA, NP and RdRp activities. The package that consists of the genomic segments (sRNA), the unit of trimeric polymerase and the nucleoprotein is the elementary replication unit of the influenza virus (vRNP). Therefore, in the nucleoplasm, vRNP needs to trigger the first round of its replication cycle, i.e. copying its genomic information onto mRNAs. Subsequently, mRNA exportation occurs in the cytoplasm.

The influenza polymerase is a heterotrimeric ~250 kD complex. It plays central roles in the viral life-cycle and is directly responsible for RNA synthesis for both viral replication and transcription. Moreover, it recruits host factors such as DnaJA1/Hsp40 [145].

The PA subunit interacts with host factors such as the mini-chromosome maintenance complex (MCM, a putative DNA helicase) and hCLE/CGI-99 [146, 147]. The PB2 subunit binds to the host RNA cap (7-methylguanosine triphosphate (m(7)GTP)) and supports the endonuclease activity of PA in order to “snatch” the cap from host pre-mRNAs [148, 149]. Moreover, PB2 interacts with the acetyl-CoA found in eukaryotic histone acetyltransferases (HATs) [150].

Viral mRNA synthesis is initiated by a *cis*-acting viral RNA polymerase, which is part of the vRNP structure and is bound to the vRNA promoter. However, mRNAs are not able to translate the genetic message efficiently; indeed, they need to be capped. Specifically, the virus must use the pre-mRNA of the cell and, for this purpose,

a coordinated process mediated by PB2, PA and the cellular Polymerase II (Pol II) is necessary. Very briefly, PB2 binds mRNAs with cellular Pol II and PA, which, by means of an endonuclease mechanism, generates capped-mRNAs, which are translatable at the ribosomal level.

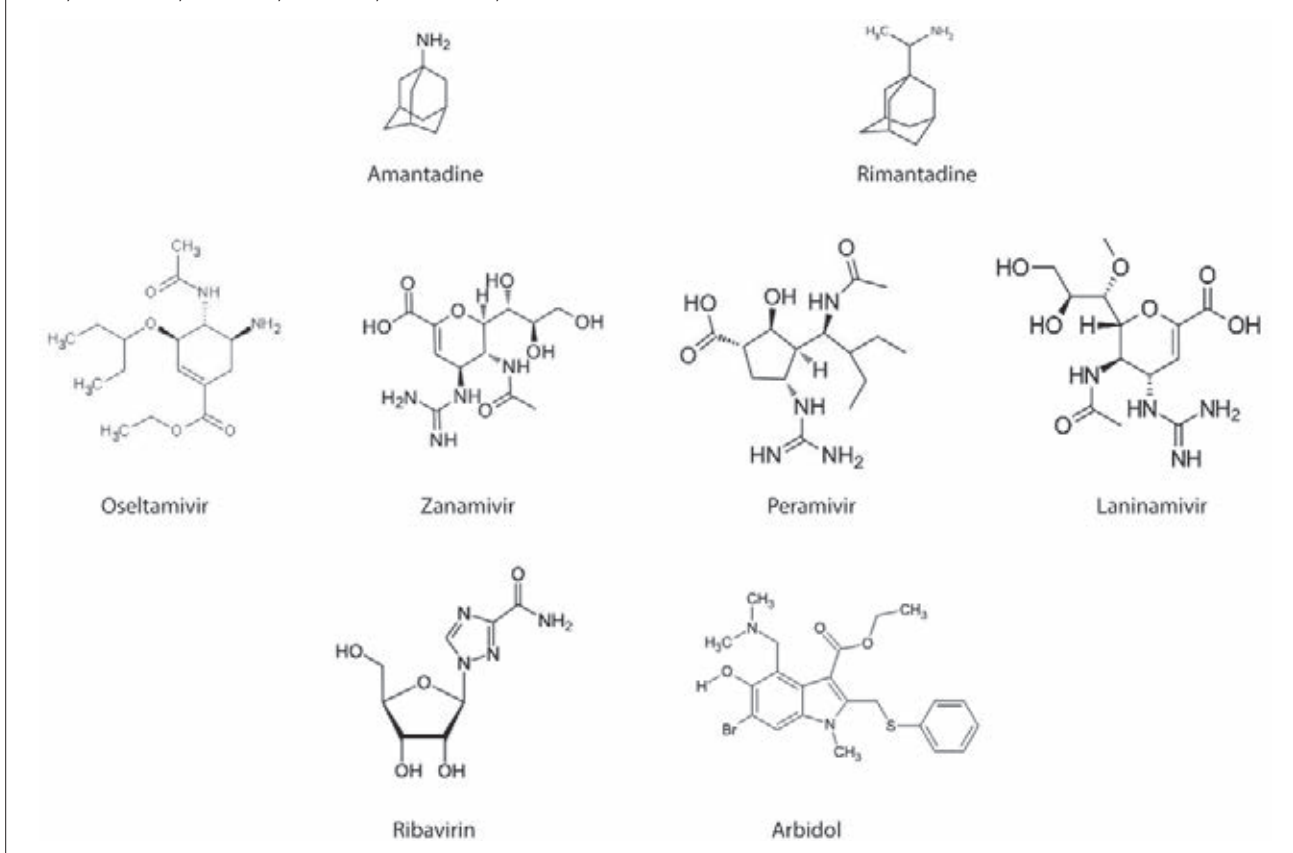
According to a model proposed by Fodor [151], a *trans*-acting polymerase activity is necessary for vRNA replication. Because of the negative polarity of vRNA, positive RNA (cRNA) must be synthesized from the mRNA templates. The replication of vRNA could then be performed by a *trans*-acting RNA polymerase, which may be distinct from the polymerase in the packages of RNPs. Various hypotheses have been formulated regarding the proteins, for instance NPs, that trigger *trans*-acting polymerase [152, 153]. Although this assumption is contradicted by other studies, which have shown viral replication in the absence of NP [154], the presence of newly synthesised NPs appears to be important in stabilizing the length of the segments of viral RNA. Furthermore, other studies have suggested that NEP could regulate viral RNA synthesis [155]. The importation of newly synthesised nucleoproteins and polymerases is also very important in order to assemble nucleoprotein with the negative-sense viral RNA. This event is not completely understood and requires coordinated interaction between Nuclear Localisation Signals (NLSs), PA, PB1, PB2, NP and importins [156-158]. In addition, as recent research has demonstrated [159, 160], NP plays a major role in the assembly of vRNP by interacting with polymerase subunits and is involved in the nuclear and cytoplasmic transportation of vRNP. Specifically, NPs are oligomerized in ring structures, which interact with viral RNA segments and also with polymerase trimers. This latter interaction allows the viral RNA to twist into a double helix with the polymerase complex at one termination, and with a loop of RNA at another termination [161-164].

To progress, the infection requires a coordinated two-way flow between proteins synthesized in the cytoplasm (for instance M1 protein and NP) and the viral RNA that has been replicated in the nucleus. In this way, the RNP, which is full of vRNA, NP, M1, PA, PB1, PB2, and the non-structural proteins can reconstitute in the nucleus.

The transcription factory is made up of components such as SFPQ/PSF (splicing factor proline-glutamine rich [164], though its entire molecular anatomy is not known. As already mentioned, splicing mechanisms are crucial to increasing the dynamism and complexity of the influenza proteome. Specifically, the RED-SMU1 spliceosomal complex interacts with PB1 and PB2 and is responsible for the splicing of NS1. Other components of the spliceosomal complex are: EJC, SR (serine/arginine-rich proteins, such as SRp40, SRp55 and pre-mRNA Splicing Factor type 2 / Alternative Splicing Factor – SF2/ASF), NS1-BP and hnRNP K (heterogeneous nuclear ribonucleoproteins K) [165-168].

Transcription and replication are enhanced by activation of the PI3K cascade via NS1 [76].

**Fig. 2.** Chemical structure of the available licensed anti-influenza drugs: M2 blockers (Amantadine, Rimantadine), Neuraminidase inhibitors (NAIs; Oseltamivir, Zanamivir, Peramivir, Laninamivir), Ribavirin and Arbidol.



### NUCLEAR EXPORTATION

Subsequently, through more than one mechanism, such as that of small proteins capable of passing through the nuclear pores easily, NS1 and NEP/NS2, the RNP complexes can be exported into the cytoplasm.

The export machinery is a factory made up of several components: E1B/AP5, Rae1/mRNP41 [132], NXF1-TAP (Nuclear RNA export factor type 1) [133], CBC (nuclear cap-binding complex, in particular the 80 kDa subunit), REF/Aly, TREX, P15-INXT, YB-1 (Y-box-binding protein type 1), CRM1/XPO1 (exportin type 1) [169], Rcc1 (Regulator of chromosome condensation type 1), CHD3 (chromodomain helicase DNA-binding protein type 3), RMB15B [170], RanBP3 [171], DDX19B, an ATP-dependent RNA helicase [172], and Hsc70 (heat shock cognate protein 70) [173] among others.

Briefly, two main export routes can be described: the NXF1-dependent and the CRM1-dependent pathways. RNPs are exported via CRM1, whilst HA, NA are transported by NXF1.

CRM1 pathway includes: Nup88, Nup214, Rcc1, RanBP3, CHD3, Hsc70, NS2, among others [174, 175].

### POST-TRANSLATIONAL PROCESSING AND TRAFFICKING

After being translated, proteins are transported to the Golgi network, where they are modified. Modifications,

such as glycosylation of HA and NA, palmitoylation/S-acylation of HA and M2, phosphorylation of NS1, and SUMOylation of M1, NS1, NP, PB1 and NEP by SUMO-1/2/3 (Small Ubiquitin-like Modifier type 1/2/3) are essential steps in the production of functionally active viral proteins [176]. These tasks are performed by kinases, such as Cyclin-Dependent Kinases (CDKs) and extracellular signal-regulated kinases (ERKs) [177].

Proteins are retrogradely transported by COPI and Rab8 from the *trans*-Golgi apparatus to the *cis*-Golgi and ER [178].

The quality of protein folding and the correctness of post-translational processing are checked by proteins such as malexin, calnexin, calreticulin and ERp57 [179-183].

### APOPTOSIS PATHWAY

The exportation of RNPs is favoured as the infection progresses, which results in activation of the mechanism of spontaneous cell death (apoptosis) through the activation of caspase 3 [184]. By altering the nuclear membrane, the activation of caspase 3 increases perviousness through the nuclear pore [185, 186]. Indeed, caspase signalling pathways play an important role in the activation, replication, propagation and pathogenicity of the influenza virus, and are therefore related to the severity of influenza symptoms and its clinical burden [187]. The virus finely tunes and modulates the host cellular pro-

teins involved in the processes of regulation and control of the induction of apoptosis [188].

The units of RNP released from the nucleus are concentrated in the perinuclear cytoplasm [189], particularly in the region of the centre for organizing microtubules (MTOC) [190] and, subsequently, in the area of recycling endosomes (REs) [191]. Interaction with the REs allows the RNPs to interact better with the network of microtubules, and thus to orient themselves and to travel towards the cell membrane [192]. The exportation of RNPs is a complicated mechanism that requires well-synchronized timing, and results in an accumulation of RNPs on the apical surface of the cell in the late stages of viral multiplication.

In the late stages of viral replication, the accumulation of HA molecules on the cell membrane, probably by activating the mitogen-activated protein kinase (MAPK) [193], increases the exportation of RNPs, which, through a still unknown mechanism, is oriented towards the apical surface of the cell.

Subsequently, as each unit of RNP contains only one of the eight segments of the viral genome, it is particularly important that the different segments be assembled properly. The viral RNAs themselves mediate this process through a “hierarchical assembly” signalling mechanism [194].

#### **BUDDING AND RELEASE**

As the infection progresses, the apical membrane becomes rich in viral proteins, which together initiate the budding of the virus around the complex of RNPs, at the regions of the membrane where the extruded HA, the NA, and M1 and M2 proteins are concentrated [195]. Viral proteins are then delivered to the plasma membrane and assembled. Here, Rab11a [196] and HRB (HIV Rev-binding protein) [197] play an important role. HA is able to initiate the process of budding, but not to complete it. This requires the mediation of NA, M1 and M2 proteins [195]. It is also important to consider that, during the formation of the positive curvature of the cell membrane, the suitably assembled units of RNPs move toward the distal part of the viral bud, so that they can be properly wrapped by the viral envelope. As the budding process progresses, a stalk is generated which holds the virion to the cell. The viral envelope must then detach itself from the cell membrane. The M2 protein appears to be crucial to this process. Indeed, it is capable of generating a positive curvature of the membrane, which is necessary in order to enable the spherical virions to split off [195]. However, the virus is not yet free; it is bound to the cell by the binding of HA molecules with the units of sialic acid of the membrane surface. NA molecules must therefore detach the sialic acid from the cell surface in order to accomplish viral budding.

The enzymatic mechanism of influenza virus sialidase has been studied by Taylor, who showed that the enzyme catalysis process is particularly complex and consists of four steps. During the first step the  $\alpha$ -sialoside is distorted from a chair conformation to a pseudoboat conformation when the sialoside binds to the sialidase. The

second step leads to sialosyl cation, an oxocarbenium ion intermediate. The third step is the formation of Neu5Ac, as  $\alpha$ -anomer. The fourth step involves its mutarotation and the subsequent release of the thermodynamically-stable  $\beta$ -Neu5Ac [198]. Finally, these steps lead to sialic acid hydrolysis.

Currently, 11 isoforms of NA are known; NA10 and NA11 have recently been isolated from bats and are not able to cleave sialic acid. Their precise role and mechanism are still unknown [54].

NA is further classified into two groups: group 1 (N1, N4, N5 and N8) and group 2 (N2, N3, N6, N7 and N9), based upon primary sequence [199]. Group 1 NAs contain a 150-cavity (formed by amino acids 147–151 of the 150-loop), an exposed pocket near the active catalytic site, whereas group 2 NAs lack this cavity [200].

Budding occurs via a VPS4 and VPS28 independent pathway [201, 202].

### **Currently available drugs**

#### **M2 INHIBITORS**

Amantadine and Rimantadine (Figs. 2, 3) were the first generation of influenza antiviral agents [203]. Amantadine (1-aminoadamantane) is a derivative of the hydrocarbon tricyclo[3.3.1.1.3,7]decane. Amantadine can be administered either as a hydrochloride derivative (Symmetrel), as 100 mg tablets or syrup, or as its effective derivative Rimantadine ( $\alpha$ -methyl-1-adamantanemethylamine hydrochloride, Flumadine). At high concentrations, Amantadine and Rimantadine non-specifically raise the pH within cellular endosomes, thus inhibiting or retarding the acid-induced conformational change in viral HA. At low concentrations, Amantadine and Rimantadine specifically inhibit the ion-channel activity of the M2 protein [204].

Crossing the brain-blood barrier and being present in the cerebrospinal fluid (CSF) with a concentration around 75% of serum level, Amantadine can also be used to treat Parkinson's disease [205], depression and obsessive-compulsive disorder (OCD) [206], Huntington's disease [207], attention deficit hyperactivity disorder (ADHD) and other neuropsychiatric diseases [208], cocaine abuse and dependence [209], HCV [210], Creutzfeldt-Jakob's disease [211], Borna's disease [212], herpes and post herpes zoster neuralgia (PHN) [213].

This variety of uses seems to suggest that Amantadine, besides blocking the M2 channel, acts on an array of receptors, from the dopaminergic receptors to noradrenergic, serotonergic, cholinergic, and N-Methyl-D-aspartate (NMDA) receptors [214, 215].

After being rapidly adsorbed, with an excellent bioavailability profile (usually in the range 86-94%) [216], the drug reaches peak plasma levels within 4 hours [216]. The plasma elimination half-life is about 11–15 h in patients with normal renal function. It has a plasma protein binding of about 67% [216].



The drug, after being poorly metabolized and being widely distributed, is almost completely excreted via glomerular filtration and tubular secretion: this implies a dose adjustment when administered to patients with renal failure or to the elderly, such as reducing the daily dose of 100 mg, instead of 200 mg.

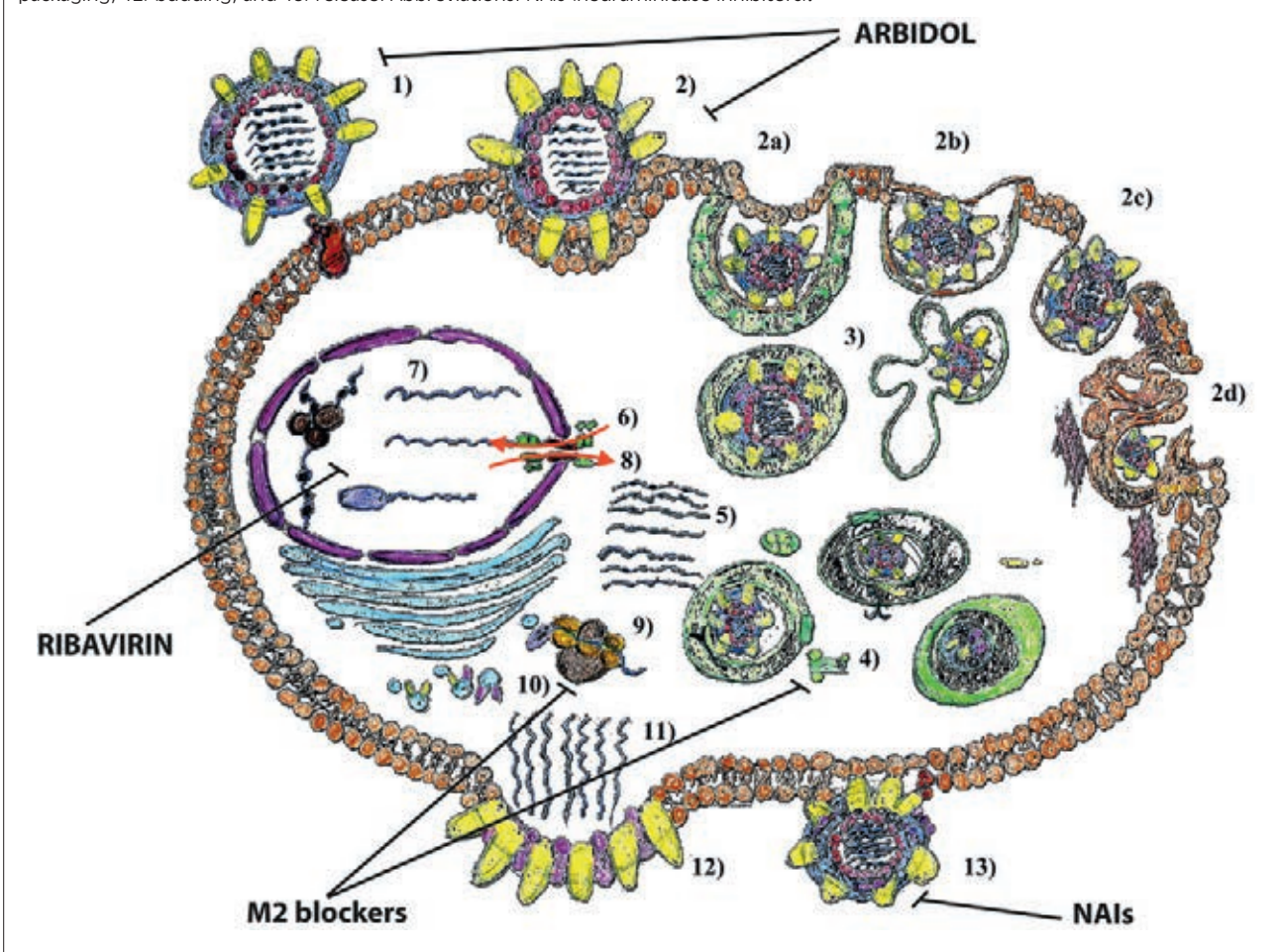
Acting on muscarinic receptors, some patients may experience anti-muscarinic adverse effects such as orthostatic hypotension, gastrointestinal discomfort (nausea, vomiting, anorexia), congestive heart failure [216]. Moreover, because Amantadine has some Central Nervous System (CNS) stimulatory properties, adults may complain of confusion, disorientation, jitteriness, anxiety, mood disorders, slurred speech, insomnia, ataxia, tremors, and, rarely, nightmares, oculogyric episodes. These symptoms are usually more common (up to 15-30%) when the drug is used for different weeks for prophylactic purpose. When instead used for treatment (less than a week), it is better tolerated. In rare cases, seizures, hallucinosis/hallucinations, coma, acute psychosis and cardiac arrhythmia may occur, usually in patients with

underlying psychiatric comorbidities [216, 217]. Adult Respiratory Distress Syndrome (ARDS) has been rarely and anecdotally reported [218].

Crossing the placenta and being present in breast milk, it is teratogenic at least in animals, even though safety has not been established in pregnant women: for precaution sake, it belongs to class C. Pregnancy is recognized as one of the risk factors for catching influenza and untoward outcomes (higher morbidity, hospitalization and mortality rates), but cannot benefit from adamantanes. Also in children and in the elders, the effectiveness in preventing, treating and shortening the duration of influenza A appears to be limited, according to a recent systematic review [219].

Rimantadine can be administered as 100 mg tablets. It reaches peak plasma concentration after 3-6 hours. The plasma half-life is long (24-36 hours). Rimantadine is more metabolized than Amantadine: only 25% is secreted unchanged [216]. It has a plasma protein binding of about 40%.

**Fig. 3.** Schematic representation of the sites of action of anti-influenza licensed drugs. The steps of the replication cycle of the influenza virus are the following: 1) attachment of the virion to target cells and receptor binding (virus adsorption); 2) internalization into cellular regions by means of clathrin-mediated endocytosis (CME), caveolae-dependent endocytosis (CDE), clathrin-caveolae-independent endocytosis, and macropinocytosis; 3) endosomal trafficking via endosomes / caveosome / macropinosome / lysosomes to the perinuclear compartment; 4) pH-dependent fusion of viral and endosomal / organellar membranes; 5) uncoating; 6) nuclear importation; 7) transcription and replication; 8) nuclear exportation; 9) protein synthesis; 10) post-translational processing and trafficking; 11) viral progeny assembly and packaging; 12) budding; and 13) release. Abbreviations: NAIs (neuraminidase inhibitors).



Rimantadine is characterized by lower rates of ADRs [220]: compared to Amantadine, it is better tolerated by children and the elderly.

Unfortunately, the use of M2 inhibitors has been limited by the emergence of drug-resistant strains of influenza viruses [221], such as the mutations of pore-facing residues (V27A, A30T, S31N, G43E), mutations of close interhelical residues located at the N-terminal half of the channel (L26F), and mutations of far interhelical residues far located at the C-terminal half of the channel (L38F) [221-222].

## NA INHIBITORS

In recent times, the most widely used antivirals against influenza have been the inhibitors of Neuroaminidase: namely Oseltamivir and Zanamivir (Figs. 2, 3). From a chemical point of view, NA inhibitors can be classified into: sialic acid derivatives (or 5,6-dihydro-4H-pyran derivatives), benzoic acid derivatives, cyclohexene derivatives, cyclopentane derivatives, pyrrolidine derivatives and natural products. The first NA inhibitors were representatives of the first chemical class, being unsaturated sialic acid analogs, such as DANA and FANA, which were initially described by Meindl and Tuppy in 1969 [223].

NA is a homotetrameric enzyme of about 220-240 kDa that is essential to the reproduction of the influenza virus. Indeed, it exerts at least three crucial actions. First, neuraminidase frees the virus from the respiratory mucus and allows it to reach the cells of the respiratory mucosa more easily. The coordinated action of HA, NA, M1 and M2 is required during the phase of viral budding. Finally, NA is required in order to release the virus from the cell surface by cutting the molecules of sialic acid that still anchor the virus to the cell surface by means of HA after completion of the replication cycle. This action also facilitates separation of the self-aggregated virions of the viral progeny. Burnet and coll. [224] first had the idea that an inhibitor of NA could be an effective antiviral agent, but only when the crystal structure of NA and its complex with neuroaminic acid were defined by Coleman in 1993 was it possible for von Itzstein [225] to synthesize a neuroaminic acid derivative with an enhanced affinity for influenza NA. This compound is Zanamivir (4-guanidino-Neu5Ac2en, or 4-GU-DANA). Its mechanism of action, which is identical to that of Oseltamivir, is characterized by the fact that the molecule mimics sialic acid; thus, it enters into competition with the acid and reversibly binds the molecules of viral NA. Zanamivir was designed to concentrate locally in the respiratory tract, while Oseltamivir (GS4104) was designed to have a high bioavailability (80%) concentration after oral administration. Indeed, Oseltamivir is very well absorbed from the gastrointestinal tract and is rapidly metabolised to active Oseltamivir carboxylate (GS4071) ([3R,4R,5S]-4-acetamido-5-amino-3-(1-ethylpropoxy)-1-cyclohexene-1-carboxylate phosphate) in the liver by hepatic carboxyl-esterase 1. The active metabolite is distributed throughout the body, including the upper and lower respiratory tract, middle ear, tracheal lining,

nasal mucosa and lungs [226]. Plasma half-life varies from 1-2 to 3-4 hours, being 6-10 hours for the carboxylate form [226]. Oseltamivir is extensively metabolized (more than 95%).

Oseltamivir and Zanamivir result comparable in terms of clinical profile and superior to the adamantanes [227, 228]. Regarding the clinical effectiveness of the two drugs, a recent meta-analysis by Jefferson et al. [229] revealed that both drugs had a modest therapeutic effect in healthy adults and that prophylactic treatment with either Zanamivir or Oseltamivir was effective in preventing the disease.

Zanamivir is supplied in "Rotadisks" with four blisters containing 5 mg of powder each. Five Rotadisks are packaged with a Diskhaler inhalation device. Oseltamivir is available as 75 mg capsules or as an oral suspension containing 12 mg/mL (Oseltamivir phosphate) [217].

ADRs are usually nausea, vomiting. Recently, CNS toxicity in infants younger than one year of age has been reported [217].

While adjustments are necessary in subjects suffering from renal failure, no adjustments are needed in patients with hepatic failure or mild obesity. Oseltamivir can be administered to pregnant women [230].

While the majority of currently circulating influenza viral strains are resistant to amantadanes, the resistance of both H1N1 and H3N2 strains and of type B viruses to Oseltamivir and Zanamivir is very low. For this reason, the US CDC and the WHO recommend their use for the treatment and prevention of seasonal and pandemic influenza [231, 232].

## Other licensed drugs

### OTHER NAIS

Two analogues of Zanamivir and Oseltamivir, which are currently licensed in Japan and other Asian countries, are Peramivir and Laninamivir octanoate hydrate (CS-8958) (Figs. 2, 3).

The former is only administered intravenously because of its very poor bioavailability [233]. It has been licensed in Japan and in South Korea, but was only temporarily approved for emergency use in the USA during the H1N1 pandemic [234].

The latter drug is very promising because of its long-acting inhibitory effect [235]. Inavir was launched in Japan in October 2010 as a 20-mg dry powder inhaler. It is a prodrug that is converted in the airway to laninamivir (R-125489), the active NAI and is retained at high concentrations for at least 10 days after a single inhalation of 40 mg. Only 15% of the drug is orally bioavailable [223]. Commonly reported ADRs were psychiatric, gastrointestinal and CNS disorders [223]. A single inhalation dose makes Inavir a quite convenient drug, even though children and young adolescents could not inhale it properly [232].

### RIBAVIRIN

Considering the compounds targeted against the transcription and replication of vRNA, one of the first de-

veloped drug is Ribavirin (Figs. 2, 3). Ribavirin, also known as the trade name of Virazole, is the guanosine nucleoside analog: 1 $\beta$ -D-ribofuranosyl-1,2,4,-triazole-3-carboxamide. Its mechanism of action is not completely known. However, Inosine 5'-monophosphate dehydrogenase (IMPDH) appears to be the principal target of the molecule [234]. This inhibition diminishes the intracellular concentration of GTP (Guanosine-5'-triphosphate), and this would stop viral protein synthesis and limit the vRNA replication. Crotty et al also demonstrated that Ribavirin is a vRNA lethal mutagen, resembling guanosine or adenosine and causing mutations in RNA replication [235]. However, the need of high doses of the drug to have good clinical results have limited the use of Ribavirin as anti-influenza drug, and a recent revision of the literature by Chan-Tack et al. suggests that there are not conclusive results about the beneficial use of Virazole for treatment of influenza [236]. Ribavirin can be administered orally, by aerosolization, rarely by intravenous route [237]. ADR is dependent on the administration route, being extravascular haemolytic anemia if the drug is delivered intravenously, a bronchospasm if aerosolized [216].

#### ARBIDOL

There are several potentially effective drugs, which act as HA inhibitors. However, only one medication, the small indole derivative Arbidol (ARB) or Umifenovir (Figs. 2, 3), or ethyl-6-bromo-4-[(dimethylamino)methyl]-5-hydroxy-1-methyl-2[(phenylthio)methyl]-indole-3-carboxylate hydrochloride monohydrate, has been licensed [232-248].

ARB was created by the Center for Drug Chemistry in Moscow [246], has been licensed in Russia 20 years ago and since 2006 has been used in China for the prophylaxis and treatment of pneumonia caused by influenza viruses A and B [234]. ARB probably exerts a multiple antiviral action: a direct virucidal effect, a block of the virus at the level of cell-entry (attachment and internalization), and impairment of viral replication, because of its ability to bind with proteins and lipids [232-248]. Several studies have demonstrated that ARB is also effective against other enveloped and non-enveloped viruses, such as Hepatitis B Virus (HBV), HCV, RSV, some Picornavirus (such as rhinovirus 14), Poliovirus 1, Coxsackievirus B5), parainfluenza type 3 (PIV3), as well as the avian coronavirus, infectious bronchitis virus, Chikungunya virus, Reovirus, Hantaan virus, Vesicular stomatitis virus (VSV) and Marek disease virus, an avian oncogenic herpesvirus [234].

It is metabolized in the liver and redistributed in the body tissues.

The principal biotransformation pathways include sulfoxidation, dimethylamine N-demethylation, glucuronidation, and sulfate conjugation. The major metabolite is sulfinylarbidol, followed by unmetabolized arbidol, N-demethylsulfinylarbidol, and sulfonylarbidol. CYP3A4 is the major isoform involved in ARB metabolism, whereas the other P450s and flavin-containing monooxygenases (FMOs) play minor roles. Plasma half-life is long (up to 25 hours) [243].

## Conclusions

In recent decades, few antiviral drugs against influenza virus infections have been available. This has limited their use in human and animal outbreaks. Indeed, antiviral drugs used during seasonal and pandemic outbreaks have usually been administered as mono-therapy and, sometimes, in an uncontrolled manner in farm animals. This has led to the emergence of viral strains that are resistant, especially to the compounds of the amantadane family. For this reason, it is particularly important to develop new antiviral drugs against influenza viruses. Indeed, although vaccination is currently the most effective means of mitigating the effects of influenza epidemics and can delay the spread of new pandemic viruses, as maintained by the Advisory Committee on Immunization Practice (ACIP), antiviral drugs can be very useful in allowing manufacturers to prepare large quantities of pandemic vaccines. In addition, antiviral drugs are particularly valuable in complicated cases of influenza, particularly in hospitalized patients and in individuals at risk, such as the elderly or patients with chronic respiratory diseases. In such cases, it would be particularly desirable to have more antivirals and to administer them in an appropriate manner [249, 250].

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## Abbreviations

A: Alanine; ACIP: Advisory Committee on Immunization Practice; ADARI: adenosine deaminase acting on RNA type 1; ADHD: Attention Deficit and Hyperactivity Disorder; ADR: Adverse Drug Reactions; AM: alveolar macrophage; AP-2: Adaptor Protein 2; ARB: Arbidol; ARDS: Adult Respiratory Distress Syndrome; ASF: Alternative Splicing Factor; BET: Bronchial Epithelial Tissue; BM2: Type B Influenza Virus Matrix Protein 2; BTB: Bric-a-brac, Tramtrack, Broad-complex; CBC: cap-binding complex; CCP: Clathrin-Coated Pit; CCV: Clathrin-Coated Vesicle; CD: Cluster of Differentiation; CDC: Center for Disease Control and Prevention; CDE: Caveole-Dependent Endocytosis; CDK: Cyclin-Dependent Kinase; CHD3: chromodomain-helicase-DNA-binding protein type 3; CME: Clathrin-Mediated Endocytosis; CNS: Central Nervous System; COPD: Chronic Obstructive Pulmonary Disease; CRL: Cullin-RING-Ligases; CRM1: Chromosome Region Maintenance type 1; cRNA: complementary RNA (positive RNA necessary as template for viral RNA replication, or template RNA); CSF: Cerebrospinal Fluid; DANA: 2,3-didehydro-2-deoxy-N-acetylneuraminic acid; E: Glutamic acid; ECDC: European Centre for Disease Prevention and Control; EE: Early Endosome; EGF: Epidermal Growth Factor; EJC: Exon Junction Complex; ER: Endoplasmic Reticulum; ERK: extracellular signal-regulated kinase; F: phenylalanine; FANA: 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid; FMO: flavin-containing monooxygenase; G: Glycine; GTP: Guanosine-5'-triphosphate; GTPase: guanosine triphosphatase; HA: Hemagglutinin; HAfp23: Hemagglutinin fusion peptide 23; HAT: histone acetyltransferase; HBV: Hepatitis B Virus; HCV: Hepatitis C Virus; HEF: Hemagglutinin-Esterase-Fusion protein; HIV: Human Immunodeficiency Virus; hnRNP K: heterogeneous nuclear ribonucleoproteins K; HPV: Human Papillomavirus; HRB: HIV Rev-binding protein; Hsc: Heat shock cognate protein; HSP: Heat Shock Protein; IAV: Influenza A Virus; IFN-1: type I interferon; IL: interleukin; IL6R: interleukin 6 receptor; IMPDH: Inosine 5'-monophosphate dehydrogenase; kD: kilodalton; L: Leucine; LAMP: lysosomal-associated membrane protein; LE: Late Endosome; LET: Lung Epithelial Tissue; M protein: Matrix protein; M1: Matrix protein 1; M2: Matrix Protein 2; m(7)GTP: RNA cap 7-methylguanosine triphosphate; MAPK: Mitogen-Activated Protein Kinase; MCM: mini-chromosome maintenance complex; MHC: Major Histocompatibility Complex; mRNA: messenger RNA; mRNAs: messenger RNA segment; mRNP: messenger ribonucleoprotein; MTOC: MicroTubules-Organizing Centre; mTOR: mammalian Target Of Rapamycin; N: Asparagine; NA: Neuroaminidase; NAIs: Neuroaminidase inhibitors; NEP: Nuclear Export Protein; NES: Nuclear Export Signal; NGF: Nerve Growth Factor; NHE: Na<sup>+</sup>/H<sup>+</sup> exchanger; NK: Natural Killer cell; NLP1: Nucleoporin-Like Protein type 1; NLSs: Nuclear Localization Signals; NMDA: N-Methyl-D-aspartate; NP: Nucleoprotein; NPC: Nuclear Pore Complex; NS1: Non-Structural protein type 1; NS1-BP: NS1 Binding Protein; NS2: Non-Structural protein type 2; NS3: Non-Structural protein type 3; Nup: Nucleoporin; NuTF2: Nuclear Transport Factor 2; NXF1: Nuclear RNA export factor type 1; OCD: Obsessive-Compulsive Disorder; ORF: Open Reading Frame; PA: Acidic Polymerase; PACT: Protein ACTivator of the interferon-induced protein kinase; PAK: p21-activated Kinase; PB1: Basic Polymerase 1; PB2: Basic Polymerase 2; pDC: plasmacytoid dendritic cell; PH: pleckstrin homology; PHN: Post-Herpetic Neuralgia; PI3K: phosphoinositide 3-kinase; PIV: Para-Influenza Virus; PKC: Protein Kinase C; PKR: Protein Kinase R (also known as Protein kinase RNA-activated, or interferon-induced, double-stranded RNA-activated protein kinase, or eukaryotic translation initiation factor 2-α kinase 2 – EIF2AK2); Pol II: Polymerase II; POM121: nuclear envelope pore membrane protein type 121; PRKRA: Protein kinase, interferon-inducible double stranded RNA dependent activator; PtdIns(3,4,5)P3: phosphatidylinositol-(3,4,5)-trisphosphate; PtdIns(4,5)P2: phosphatidylinositol-(4,5)-bisphosphate; PTEN: Phosphatase and tensin homolog; qPCR: quantitative Polymerase Chain Reaction; RanBP: Ran Binding Protein; RBP: Receptor Binding Pocket; RBS: Receptor Binding Site; Rcc1: Regulator of chromosome condensation type 1; RdRp: RNA-dependent RNA polymerase complex; REs: Recycling Endosomes.; RIG-I: Retinoic acid-Inducible Gene 1; RNA: Ribonucleic Acid; RNP: Ribonucleoprotein; RSV: Respiratory Syncytial Virus; S: Serine; SF: pre-mRNA Splicing Factor; SFPQ/PSF: splicing factor proline-glutamine rich; SFRS: Serine/arginine-rich splicing factor; SH2: Src Homology 2; siRNA: short interfering RNA; SR: Serine/arginine-Rich protein; sRNA: genomic segments of RNA; SUMO: Small Ubiquitin-like Modifier; T: Threonine; TGN: Trans-Golgi Network; TM: transmembrane; Tpr: Translocated promoter region; TRIM: tripartite motif-containing protein; US: United States of America; V: Valine; Vps: Vacuolar protein sorting; vRNP: viral Ribonucleoprotein; VSV: Vesicular stomatitis virus; WHO: World Health Organization; XPO1: exportin 1; YB-1: Y-box-binding protein type 1.

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