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New drug-strategies to tackle viral-host interactions for the treatment of influenza virus infections

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ABSTRACT

The influenza virus (IV) is a highly contagious virus causing seasonal global outbreaks affecting annually up to 20% of the world's population and leading to 250,000–500,000 deaths worldwide. Current vaccines have variable effectiveness, and, in particular during a pandemic outbreak, they are probably not available in the amounts needed to protect the world population. Therefore we need effective small molecule drugs to combat an IV infection and that can be produced, in case of pandemic, rapidly and in large quantities. Unfortunately, natural occurring IV becomes more and more resistant to current anti-IV drugs. And thus, there is an urgent need for development of alternative agents with new mechanisms of action. This review provides an overview of the pharmacology and effectiveness of new anti-IV agents, focusing on inhibition mechanisms directed against virus-host interactions.

1. Introduction

Influenza virus (IV) is an highly contagious virus with global outbreaks. IV belongs to the Orthomyxovirus family and is classified in three antigen types: A, B and C (Hampson and Mackenzie, 2006; Martín-Benito and Ortín, 2013). Only IV A and B subtypes cause large outbreaks and serious illness. The seasonal IV flu affects up to 20% of the world population and leads to an excess of 250,000–500,000 deaths each year (Król et al., 2014). In this review, the focus lies on IV A. IV A can be further subdivided on the basis of the antigenic nature of the major surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). There are 18 different HA- and 10 different NA classes (Hamilton et al., 2012). The human IV A pandemic in 1918, with 50–100 million deaths, was caused by the H1N1 strain (Hamilton et al., 2012). Currently, highly pathogenic H5N1 cause large numbers of sporadic infections with little to no continuing transfer between humans. However, when such viruses become easily transferable in humans, this may lead to a new pandemic (Reperant et al., 2014). The continuous threat of highly pathogenic IV viruses emphasizes the continuing threat to public health and the need to develop new anti-IV treatments (Lee et al., 2014).

The viral proteins constantly mutate due to the unreliability of RNA-polymerases. Because of genetic drift and genetic shift, new IV viruses arise continuously, making previously acquired immunity no longer opportune, and antiviral drugs potentially less effective (Hampson and Mackenzie, 2006; Lofgren et al., 2007; Hutchinson and Fodor, 2013). Due to the high mutation rate of IV, the efficacy of existing vaccines are variable and annually development of new vaccines is required.

Currently there are two classes of anti-IV drugs approved by the FDA for clinical use: the M2 protein inhibitors amantadine and rimantadine, and the NA inhibitors oseltamivir and zanamivir (Król et al., 2014). More and more resistant strains arise against these drugs and their use is often associated with adverse side effects (Loregian et al., 2014). Therefore, there is urgent need for development of alternative anti-IV agents with new mechanisms of action to combat IV. The majority of the novel antiviral strategies focus on conserved domains of the viral proteins. Many cellular factors also play a crucial role in an IV infection and are also attractive as target for the development of new antiviral agents. When host proteins are used as target, toxicity and disruption of the regular cellular function is a problem. On the other hand, there will be less drug resistance. In addition, such drugs may have antiviral broad-spectrum effects, because many other virus species use similar uptake routes (Król et al., 2014; Edinger et al., 2014).

The focus of this review lies on inhibitory mechanisms directed against virus-host interactions, but also compounds that attach to the IV will be described. The anti-IV agents are described on the basis of a detailed description of the cell biology of IV, wherein different druggable targets of the host cells are mentioned. Many antiviral

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candidates described in this review are still in early phases of drug development.

2. Structure of the influenza virus

IV is an enveloped virus with a single-stranded negative-sense RNA genome with a negative polarity (Rossman and Lamb, 2011). Its genome consists of eight RNA segments containing ten genes (Garcia-Robles et al., 2005; Martín-Benito and Ortín, 2013). The RNA segments are present as viral ribonucleoprotein particles (vRNPs) that contain besides RNA, a RNA-polymerase subunit and several nucleoproteins (NP) (Neumann et al., 2004; Martín-Benito and Ortín, 2013). NP is a multifunctional protein important for, besides packaging RNA, transcription and replication (Martín-Benito and Ortín, 2013; Gerritz et al., 2011). The IV RNA-polymerase is a heterotrimer consisting of a PB1-, PB2-, and PA-fragment (Kranzusch and Whelan, 2012), which is important for viral transcription and replication (Poch et al., 1989). The viral membrane contains two antigenic glycoproteins, HA and NA, and two matrix proteins, matrix protein 1 (M1) and matrix protein 2 (M2). HA is a glycosylated homotrimer and provides the viral uptake into host cells via receptor-binding and viral-endosome fusion activity (Rossman and Lamb, 2011). Glycosylation differs among different HA subtypes. HA consist of a globular head domain and a stem domain (Reperant et al., 2014; Lee et al., 2014). As result of the high mutation rate, the globular head domain varies widely between different IV strains. Both the receptor binding domain and the stem domain of HA are very conserved. Hence, they may act as an interesting druggable target for universal anti-IV therapies (Liu et al., 2013; Edinger et al., 2014). NA, a tetrameric enzyme, plays a role in the late stage of the infection by causing enzymatic splicing of HA from sialic acid and allows release of newly synthesized viruses from the host cells (Rossman and Lamb, 2011; Monod et al., 2015). The third integral membrane protein, M2, is a selective ion channel consisting of four parallel transmembrane α-helices. This protein is important for viral absorption and budding (Rossman and Lamb, 2011; Monod et al., 2015). M1 is important for the structure of the virus by interacting with the viral lipid membrane and vRNP (Rossman and Lamb, 2011).

3. Cell attachment and internalization

3.1. Hemagglutinine-sialic acid interaction

HA binds to sialylated receptors on host cells (Hamilton et al., 2012; Edinger et al., 2014). Sialic acid is the distal residue in oligosaccharide chains of glycoproteins and glycolipids on the cell surface. Sialic acid is linked to underlying galactose by α2,3- or α2,6 bonds. Human-adapted HA subtypes bind to α2,6-sialic acid, while avian HA subtypes bind to α2,3-sialic acid. This difference is an important factor regarding host tropism. A mutation in only one amino acid in the receptor binding domain of HA may affect the receptor-specificity considerably (Hamilton et al., 2012; Edinger et al., 2014). It is assumed that the interaction between HA and sialic acid has a low affinity (Edinger et al., 2014). In order to increase the overall strength of the interaction, several HA molecules on the surface of the virion bind to various glycoproteins. Thus, the viral HA protein is an attractive target for the development of anti-IV drugs.

Soluble synthetic sialylated receptors and peptide mimetics, which block the very conserved receptor binding domain of HA, compete with the naturally occurring sialylated receptors on host cells, and can potentially be used to block the absorption of IV (Król et al., 2014; Edinger et al., 2014). Gangliosides such as sialylparagloboside, pentadecapeptides, and liposomes with glycan sialylneolacto-N-tetraose c are examples of such compounds which block the interaction of IV with host cells (Król et al., 2014). Matsubara et al. showed in an in vitro study that sialic acid peptide mimetics can block an infection with H1N1 and H3N2 viruses (Matsubara et al., 2010). In addition, in the study by Hendricks et al. (2013). antiviral effects were observed with liposomes, which were coated with sialic acid analogues.

Blocking viral entry has also been achieved using various synthetic peptides (for review see Skalickova et al., 2015). For instance, Nicol et al. (2012) described a family of peptides that interact with a variety of HA subtypes (H1, H3, and H5) and were active in an *in vitro* assay in nanomolar concentrations. A minimal sequence of 6 amino acids was needed to block infection. One of the peptides was also tested successfully in a mouse model when given at the time of viral administration. Also peptides have been described that disrupt the viral envelope (Skalickova et al., 2015).

Another interesting strategy to combat the virus uptake is by use of sialidases, which remove sialic acid from the epithelial cell surface and in this way prohibit the virus uptake in target cells. DAS181 (Fludase) is a bacterial sialidase and can effectively remove α2,6- and α2,3-linked sialic acid. DAS181 shows anti-IV activity in *in vitro* and *in vivo* mouse models against a wide range of IV A and B subtypes, including H5N1, H1N1 and H7N9 and oseltamivir resistant strains (Triana-Baltzer et al., 2009; Liu et al., 2013; Nicholls et al., 2013; Król et al., 2014). In a phase II study, DAS181 significantly reduced the viral load and viral shedding of IV, as compared to placebo, but had no effect on the severity of the clinical symptoms (Moss et al., 2012). Treatment of IV in humans for three days with DAS181 reduced the viral spread. Unfortunately, administration of DAS181 longer than seven days was associated with adverse respiratory events. Furthermore, anti-DAS181 neutralizing IgG Abs have been observed in a number of treated patients (Zenilman et al., 2015). Overall DAS181 does not seem to be a very promising agent for the treatment of an IV infection.

3.2. Intracellular transport

Like many other viruses, IV enters the host cells by receptor-mediated endocytosis (Rossman and Lamb, 2011). Drugs that block viral endocytosis may be of great clinical importance. IV utilize two endocytosis systems: clathrin-mediated endocytosis and macropinocytosis (Edinger et al., 2014). To initiate these endocytosis mechanisms, several co-receptors are involved: annexin V (Huang et al., 1996), 6-sulfo sialyl Lewis X receptors (Gambaryan et al., 2008), C-type lectin receptors (Londrigan et al., 2011), receptor tyrosine kinases (RTKs), epidermal growth factor receptor and c-Met kinase (Eierhoff et al., 2010; De Vries et al., 2011; De Vries et al., 2012). All these proteins are potential targets to block an IV infection. According to the study of De Vries et al. (2012) sialylated N-glycans are important for viral uptake through macropinocytosis, while clathrin-mediated endocytosis is not affected by the absence of N-glycosylation (De Vries et al., 2012). The adapter protein Epsin-1 is required for the formation of clathrin-coated pits. Knockdown of Epsin-1 inhibits the clathrin-mediated endocytosis of IV (Hutchinson and Fodor, 2013; Edinger et al., 2014). During clathrin-mediated endocytosis, virions are transferred to the endosomal compartments by a dynamin-dependent route. In the case of macropinosomes, the virions are transferred *via* an unknown, dynamin-independent route (Hutchinson and Fodor, 2013). The clathrin-mediated endocytosis of IV can be efficiently blocked by the dynamin inhibitor dynasore. A complete blockade of the internalization can be achieved by treatment with dynasore in combination with ethylisopropylamiloride (EIPA). EIPA is a Na^+/H^+-transporter inhibitor that blocks the macropinocytosis through prevention of a cytosolic pH increase. As a result, activation of GTPases, which are required for the actin remodelling, does not take place. In tissue culture experiments, dynasore and EIPA are cytotoxic at higher concentrations and prolonged exposure and are therefore unsuitable for clinical use (De Vries et al., 2011, Edinger et al., 2014). Virus endocytosis may also be inhibited by membrane fluidity modulators, such as the glycolipids fattiviracin and glycyrrhizin, which limit the movement of membrane molecules on the virus (Harada et al., 2007). These agents have a broad

activity against various enveloped viruses. Glycyrrhizin also has immunomodulatory effects. It interferes with activation of the H5N1 induced signalling events NFκB, p38 MAPK and C-Jun-N-terminal kinase (Michaelis et al., 2011) (see also chapter 6). The aryl methylidene rhodamine derivative LJ001 is another interesting compound, that inhibits membrane fluidity in both viral and cellular membranes (Wolf et al., 2010). LJ001 can affect the membrane fluidity and curvature through O_2-mediated lipid oxidation (Vigant et al., 2013; Edinger et al., 2014).

The endosomal route can be divided in three steps. First, the virion is transported from the cell surface into the periphery of the cell by an actin-dependent process. The second phase is characterized by a rapid dynein-directed movement with early endosomes. In the third phase, the virions move along microtubules to the perinuclear region (Martin and Helenius, 1991; Edinger et al., 2014). Early endosomes are transported to the perinuclear region *via* motorproteins kinesin-1 and dynein along microtubules. Rab5, endosomal autoantigen 1 and PI3K are important regulators of the maturation process and are used as marker proteins for early endosomes. Rab proteins are cellular GTPases that play an important role in the regulation of the endosomal traffic. During the microtubule-dependent transport into the perinuclear region, late endosomes are formed from early endosomes by vesicle exchange with lysosomes or other late endosomes. The switch of Rab5 to Rab7 indicates the progression of early endosomes to late endosomes. Both Rab5 as Rab7 are essential for IV trafficking (Sieczkarski and Whittaker, 2003). The pH drops in the vesicles from 6.8 to 5.9 in early endosomes to 6.0-4.8 in late endosomes (Edinger et al., 2014). At an optimal pH of 4.9 (Krumbiegel et al., 1994), HA-mediated fusion of the viral membrane with the late endosomes membrane occurs and the virus content is released into the cytosol. A summary of the endocytosis process is shown in Fig. 1.

PKCβII is another important protein in the endosomal movement of IV. As described by Sieczkarski et al. (2003) overexpression of inactive PKCβII leads to accumulation of virions in late endosomes because the fusion process does not occur (Sieczkarski et al., 2003). The broad-spectrum PKC inhibitor, bisindolymaleimide I, prevents IV entry and the subsequent infection. The drawback of PKCβII inhibition is that this enzyme is also important for trafficking and degradation of many other proteins including epidermal growth factors and thus PKCβII inhibitors may have severe side effects . Histone deacetylases (HDACs) are other important proteins in LE-mediated transport. HDACs have different functions, they are generally associated with nuclear epigenetic regulation of gene expression. While HDAC1 inhibits the acidification and penetration, HDAC3 and HDAC8 enhance endocytosis, acidification and penetration of IV. HDAC3 and HDAC8 regulate the properties of the microtubule system, and have an influence on the motility, distribution and maturation of late endosomes and lysosomes. In the study of Yamauchi et al. (2011) with a siRNA silencing approach against HDAC3 and 8, inhibition of HDAC3 and HDAC8 *in vitro* appears to reduce the risk of an IV infection to respectively 28% and 36% (Yamauchi et al., 2011). The effects have been associated with major changes in the organization of the microtubule-system and showed reduced nuclear import of vRNP. Depletion of HDAC1 reduced splitting of centrosomes, and enhanced the IV infection. HDAC6 and class III HDACs also appear to have anti-IV characteristics (Husain and Cheung, 2014; Nagesh and Husain, 2016). Taken together, HDACs may be attractive targets for the treatment of an IV infection. In this context, it is important to mention that many of the naturally occurring cyclotetrapeptides (primarily obtained from fungi) are HDAC inhibitors (Abdalla, 2016). These cyclic peptides have

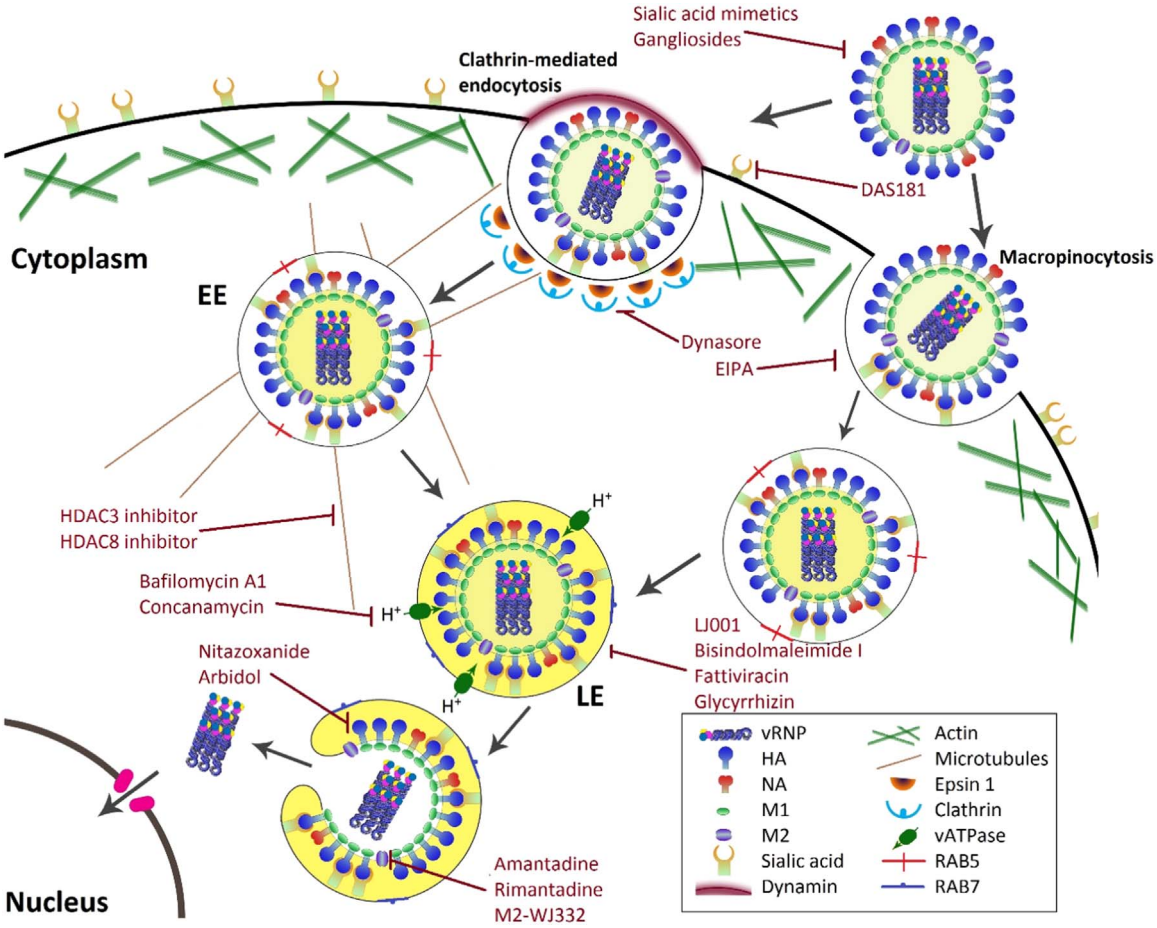

Fig. 1. A schematic representation of the absorption process of the IV.

a much more favourable bioavailability compared to linear peptides because they are not recognized by exoproteases.

3.3. Membrane fusion

Before fusion can occur between the viral and endosomal membrane, the HA protein must first undergo post-translational splicing. HA is synthesized as a fusion-inactive precursor (HA0). To start the fusion process HA0 has to be spliced by host proteases into two functional subunits HA1 and HA2. HA1 includes the receptor binding domain, which initiates endocytosis. Once in the endosome, HA2 controls the fusion between the viral envelope and the endosomal membrane to transport vRNPs to the cytosol of the host cell (Hampson and Mackenzie, 2006; Hamilton et al., 2012). Vascular ATPases (vATPases), are proton pumps and cause acidification of the endosomal lumen of the late endosomes (Edinger et al., 2014). A decrease in the pH to about 5.0, is necessary for the activation of HA-mediated fusion between the viral- and endosomal membrane (Rossman and Lamb, 2011; Edinger et al., 2014). Low pH alters the conformation of HA2, which leads to exposure of the N-terminal fusion peptide that subsequently anchors in the endosomal membrane by a strong hydrophobic interaction with the lipid acyl chains (Hamilton et al., 2012; Worch, 2014; Greber, 2016). The C-terminal transmembrane fragment of HA2 is embedded in the viral envelope of IV, and thus the two ends of HA2 are embedded in two different membranes (Worch, 2014). Further conformational changes induce a hairpin-like folding of the protein, forcing the membranes of the endosome to fuse and release the viral content into the cytoplasm (see Fig. 2) (Hutchinson and Fodor, 2013; Worch, 2014).

Inhibition of viral fusion can be achieved by inhibition of the acidification of endosomes (Król et al., 2014; Edinger et al., 2014). For instance, the acidification can be prevented by blocking the vATPases. One of the most potent membrane fusion inhibitors is the macrolide antibiotic bafilomycin A1 (Edinger et al., 2014). Bafilomycin A1 inhibits the activity of vATPases, which prevents the nuclear uptake of vRNPs. Another macrolide antibiotic, concanamycin, is also a competent inhibitor of vATPase (Król et al., 2014). Because of the inhibition of the acidification, these agents can prevent the influx of IV A and B in host cells. Similar antiviral effects have been demonstrated for the vATPase inhibitors diphyllin and saliphenylhalamide (Edinger et al., 2014). Another class of fusion inhibitors, *tert*-butylhydroquinone and t-butyl benzene-1,4-diol, bind to a conserved stem domain on HA and with this inhibit the conformational changes of HA0 that are necessary for fusion. However, further research on these agents has been stopped because of their high strain-specific activity as well as the fact that the particular IV strains became quickly resistant (Leneva et al., 2009; Król et al., 2014; Edinger et al., 2014; Monod et al., 2015). Another drug that selectively blocks the maturation of HA is nitazoxanide and is currently in phase 3 clinical trial. It inhibits replication of various IVs and no drug resistance occurred. Nitazoxanide acts synergistically with oseltamivir or zanamivir to reduce the severity of the infection. Further studies in seriously ill patients are needed to determine the efficacy of nitazoxanide (Haffizulla et al., 2014; Belardo et al., 2015). Arbidol is like nitazoxanide an inhibitor of HA-mediated membrane fusion by increasing the stability of HA at low pH. The advantage of arbidol is that it has a broad-spectrum activity by its binding to the conserved regions and works not only against IV A and B (H5N1, H9N2, H2N2 and H6N1 (Leneva et al., 2005)), but also against

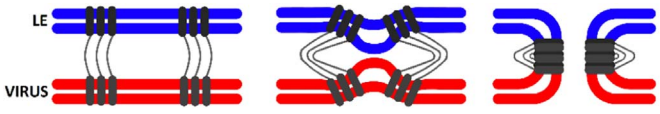

Fig. 2. The HA2 subunit is embedded in the viral envelope with the transmembrane domain and in the endosomal membrane with the fusion peptide. Following a pH drop in the LEs, a pore is formed whereby vRNP can be released into the cytosol of the host cell.

various other viruses (Król et al., 2014; Monod et al., 2015). Another advantage of arbidol is, that little virus resistance has been observed against it. In addition, arbidol has an immunomodulatory activity that may contribute to the broad antiviral effects (Boriskin et al., 2008). Arbidol is currently licensed in Russia and China for treatment and prophylaxis of IV A and B (Sun et al., 2013). Furthermore, in an orally administration, arbidol is broadly distributed within the body, which is important when the IV infection spreads throughout the body (*e.g.* with an H5N1 infection) (Leneva et al., 2009).

At a low pH, the M2-protein provides proton transfer to the viral lumen. As a result of increasing acidity in the viral lumen, a conformational change in M1 is induced which causes dissociation of vRNPs from M1 (Edinger et al., 2014; Hutchinson and Fodor, 2013; Greber, 2016). As soon as the viral- and endosomal membrane are fused, vRNP and viral proteins are released into the cytosol of the host cell. vRNP is subsequently transported to the cell nucleus where the viral replication starts (Rossman and Lamb, 2011; Hamilton et al., 2012).

Amantadine and rimantadine are inhibiters of the viral M2 protein and belong to the first generation of anti-IV drugs (Król et al., 2014). It has been shown that these agents shorten the duration of disease and counter the infection symptoms (Liu et al., 2013). These drugs inhibit IV infection by blocking the proton influx by occlusion of the pores in the M2 protein (Król et al., 2014). Unfortunately, resistance to these adamantanes has spread since the beginning of the 21st century, and the current treatment guidelines no longer recommend the use of adamantanes (Bright et al., 2005). Resistance can occur as a result of only one single amino acid mutation, which has little effect on the action of M2 itself. The most common substitution S31N is found in more than 95% of resistant viruses (Król et al., 2014; Edinger et al., 2014). Currently, there is much research going on into new M2 inhibitors that impair amantadine-resistant viruses. Di-, tri-, and tetrazole derivatives of adamantane have been found with a high anti-IV activity against rimantadine-resistant IV strains. In addition, several derivatives of imidazole and guanazole pinanamine have been synthesized. Unfortunately, these molecules have just a slight inhibitory effect on the amantadine-resistant M2 mutant S31N (Król et al., 2014). Recently, there has been shown that M2-WJ332 can inhibit the M2 S31N variant stronger than amantadine could inhibit wild type M2 (Król et al., 2014; Shen et al. (2015)). Some benzyl-substituted amantadine derivatives appear to have an antiviral activity against both S31N and wild-type viruses (Król et al., 2014). Despite several promising compounds, no universal M2 inhibitor which is active against both the wild-type and all circulating amantadine-resistant strains is yet developed (Król et al., 2014).

4. Transcription and replication

4.1. Import

Once out of the LE, transport of vRNPs to the nuclear membrane takes place through diffusion (Hutchinson and Fodor, 2013). vRNP passage through the nuclear membrane takes place *via* nuclear pore complexes and depends on nuclear transport receptors, so-called karyopherins. These karyopherins move in both directions through nuclear pore complexes under the influence of a concentration gradient of Ran-GTP. In order to gain access to the nucleus, viral proteins must contain nuclear localization signals (NLS) to bind to karyopherins (Hutchinson and Fodor, 2013). Although all proteins of vRNP contain NLSs, NP is the main contributor for vRNP import (Resa-Infante et al., 2011; Zheng and Tao, 2013). NP proteins first bind to α-importins. Next α-importins bind to β-importins to start nuclear import. vRNPs are released in the nucleus by the binding of Ran-GTP to importins. vRNPs distribute through the nucleus by diffusion (Boulo et al., 2007; Resa-Infante et al., 2011; Hutchinson and Fodor, 2013).

Each vRNP works as an independent functional unit during

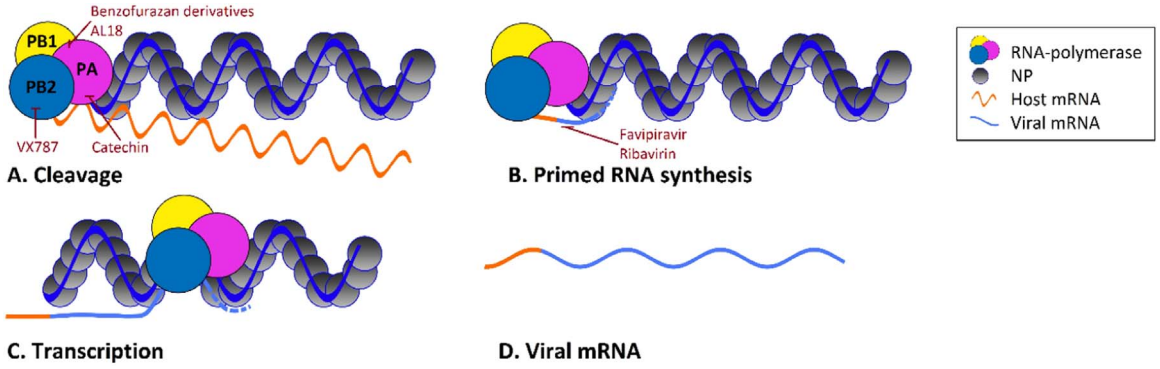

Fig. 3. The mechanism of cap-snatching. **A:** PB2 binds to an oligonucleotide primer of the pre-mRNA of the host, and the oligonucleotide primer is spliced from the cellular pre-mRNA by the endonuclease activity of the PA subunit. **B-C:** RNA-polymerase transcribes ns-vRNA in viral ps-mRNA and the beginning end of the viral ps-mRNA is linked to the oligonucleotide primer of the host. **D:** In the end viral ps-mRNA is formed that starts with an oligonucleotide primer which is obtained from the host.

transcription and replication and is dependent on the nuclear functions of the host (Martín-Benito and Ortín, 2013). Proteins from the vRNP are attractive targets, since they are more conserved and thus less vulnerable to mutations than other IV proteins (Monod et al., 2015). So far, most of these agents are still at the preclinical research stage. NP has a head- and a body domain separated by a groove with many basic residues, which are involved in RNA-binding. Several approaches to inhibit NP-RNA interactions are under development (Ng et al., 2008, Monod et al., 2015). In the first approach, the interface between the NP monomers is stabilized by nucleozine derivatives, causing the formation of non-functional oligomers (Król et al., 2014; Monod et al., 2015). Nucleozine derivatives thereby disrupt viral RNA- and protein synthesis and the production of new virions by the inhibition of NP traffic across the nuclear membrane. Nucleozine inhibits effectively the growth of IV in cell cultures, suggesting that NP is a valid target for anti-IV therapy (Gerritz et al., 2011). Nucleozine promotes the formation of large deviant aggregates of perinuclear vRNP along with the cellular protein Rab11 (Gerritz et al., 2011; Cheng et al., 2012; Król et al., 2014). The nucleozine analogue pyrimido-pyrrolo-quinoxaline-dione (PPQ) has similar anti-IV effects as nucleozine and is effective against nucleozine resistant viruses. Conversely nucleozine has an effect on a PPQ resistant strain which suggests that both nucleozine analogues have different binding sites (Lin et al., 2015). In the second approach, the oligomer formation is prevented by blocking the oligomerization loop of NP (Król et al., 2014; Monod et al., 2015; Cheng et al., 2012). The peptides ingavirin and compound 3 (Shen et al., 2011) inhibit the NP oligomerization and subsequent uptake in the nucleus of newly synthesized NP. Finally, there is a third approach in which naproxen competes with RNA for binding to the NP binding groove (Monod et al., 2015). Naproxen and its derivatives stabilize NP monomers by altering the groove in which the oligomerization loop interacts with the RNA. Naproxen has in addition to the anti-IV activity, by its cyclooxygenase (COX) inhibiting actions, a beneficial anti-inflammatory effect. Another compound, RK424, binds to a highly conserved NP pocket and has shown potent anti-IV activity against many different strains in vitro and a lethal H1N1 infection in a mouse in vivo model (Kakisaka et al., 2015). RK424 inhibits both the NP-RNA and NP-NP interactions, and inhibits nuclear export of NP.

In the beginning of the infection primarily transcription takes place, while in a later stage replication occurs (Resa-Infante et al., 2011). Recent studies suggest that transcription and replication is mediated by different sources of RNA-polymerase (Hutchinson and Fodor, 2013). Only the RNA-polymerase that is part of the vRNP-complex can initiate the transcription. For the replication, newly synthesized unbound RNA-polymerases are required (Jorba et al., 2009). In the primary transcription, viral negative-sense RNA (ns-vRNA) in the vRNP serves as an active template for the synthesis of viral positive sense messenger RNA (ps-mRNA) and viral complementary RNP (cRNP) (Martín-Benito and Ortín, 2013; Zheng and Tao, 2013). The cRNP is a replication intermediary that does not occur in high concentrations and does not leave the cell nucleus (Hutchinson and Fodor, 2013).

4.2. Transcription

In order to form ps-mRNA during transcription, a process called ‘cap-snatching’ is required. Cap-snatching results in the degradation of host transcripts and destruction of host RNA-polymerase II, leading to elimination of the host transcription (Hutchinson and Fodor, 2013). Cap-snatching is mediated by RNA-polymerase. Cap-snatching is initiated by the combined action of the PB2 subunit of RNA-polymerase, which interact with the capped RNA, and the PA subunit with endonuclease activity for splicing of the host pre-mRNA to obtain 5′ capped mRNA oligonucleotide primers of 10–13 nucleotides (Martín-Benito and Ortín, 2013; Garcia-Robles et al., 2005). These oligonucleotide primers are required for further processing of the viral ps-mRNA (Garcia-Robles et al., 2005; Zheng and Tao, 2013). The capped oligonucleotide primers are coupled in front of the newly synthesized viral ps-mRNA by the PB1 subunit (see Fig. 3) (Poch et al., 1989). The created ps-mRNA can be exported from the nucleus, where the oligonucleotide primers can initiate the translation using ribosomes to form viral proteins. Newly synthesized NP, PB1, PB2 and PA are imported back into the nucleus to form new vRNPs and to promote the replication (Garcia-Robles et al., 2005; Martín-Benito and Ortín, 2013).

Because IV RNA-polymerase is highly conserved among different IV strains, it is an attractive target for the development of anti-IV agents. The cap-snatching processes can be inhibited by blocking the PB2 cap-binding domain, or the PA endonuclease domain (Król et al., 2014; Monod et al., 2015; Zheng and Tao, 2013). The cap-binding domain of PB2 is bound to 7-methyl guanosine triphosphate (m7GTP), a modified purine nucleoside (Monod et al., 2015; Pautus et al., 2013). The promising azaindole pyrimidine derivative, VX787, binds PB2 on the m7GTP binding domain. This compound has a strong efficacy against many IV strains in in vitro and in vivo models, including H1N1 and H5N1 (Monod et al., 2015; Byrn et al., 2015). The PA domain is also an important target to block the cap-snatching activity. This domain is similar between different IV A, B and C subtypes. Several compounds directed at the cap-snatching endonuclease activity of the RNA-polymerase complex, such as catechin, have been discovered in recent years. Catechin has residues at the endonuclease active site of PA as target. Further research is necessary to demonstrate the exact efficacy (Król et al., 2014; Monod et al., 2015). There is also evidence that catechin derivatives can bind to NA. The binding to a conserved groove on the NA molecule is different from current NA inhibitors, oseltamivir and zanamivir, which makes this drug effective against the current-resistant viruses. Through this binding, the catechin derivatives can

reduce the binding of NA to sialic acid and thus inhibit the virus release. Catechin derivatives have great potential as a therapeutic treatment against circulating strains of the IV (Müller and Downard, 2015). Finally, the PA-PB1 interaction is an interesting target for new drugs since it is essential for the replication and pathogenicity of IV, and the sequence of these domains are highly conserved in many IV strains (Monod et al., 2015). Benzofurazan derivatives appear to disrupt the viral RNA-polymerase complex. AL18 is also a compound capable of blocking the interaction between PA and PB1 and may inhibit both replication of IV A and B (Król et al., 2014; Monod et al., 2015).

Peptides derived from the PB1 or PB2 subunits have been successfully used in *in-vitro* systems to study the biological functions of these subunits (Skalickova et al., 2015). Unfortunately, there are serious hurdles, in particular at the level of intracellular targeting and potential immunogenicity that prevent their current use as antiviral drug in human patients.

A very different approach makes use of the ribonucleotide analogue favipiravir, which acts as a purine analogue and inhibits the formation of viral RNA (Król et al., 2014). Favipiravir has recently been approved for human use in Japan and a phase III study currently takes place in the United States. Favipiravir contains a strong anti-IV activity and is effective against a variety of IV subtypes (A, B, and C), including the amantadine and oseltamivir resistant strains, both in vitro and in vivo (Liu et al., 2013; Furuta et al., 2013; Monod et al., 2015; Gowen et al., 2015). In addition to the anti-IV activity, favipiravir blocks the replication of nine other RNA virus families (Furuta et al., 2013; Gowen et al., 2015) and viral resistance against favipiravir is (so far) very low (Król et al., 2014). Ribavirin is another nucleoside analogue with anti-IV activity. The target of ribavirin is the enzyme inosine-5′-monophosphate dehydrogenase, which is involved in viral RNA synthesis and cellular biosynthesis of GTP. Ribavirin is active against many IV strains, including H1N1, H3N2, H5N1 and IV B *in vitro* and *in vivo* (Sidwell et al., 2005; Liu et al., 2013; Król et al., 2014). Resistance to this agent has been reported rarely. One of the disadvantages of the therapy with ribavirin is the development of hemolytic anemia, but this is reversible after discontinuation of treatment (Król et al., 2014; Liu et al., 2013). This together, with the fact that the clinical efficacy of ribavirin for IV is less effective than adamantanes or NA inhibitors makes ribavirin not a very attractive drug for the treatment of IV infections (Liu et al., 2013).

4.3. Replication

The production of new vRNP takes place in two separate steps. First, cRNP is formed from the original vRNP. And second, the cRNP serve as template for the synthesis of new vRNP. With the aid of RNA-polymerase, large amounts of new ns-vRNA are synthesized which are encapsulated into new vRNP structures (Resa-Infante et al., 2011; Martín-Benito and Ortín, 2013; Zheng and Tao, 2013). In Fig. 4, an overview is shown of the transcription and replication of IV and side of actions of potential inhibitors.

4.4. Export of vRNP

Newly synthesized vRNPs are exported from the cell nucleus to the plasma membrane, where they are incorporated into new virions. This process is initiated by HA and nuclear export-protein (NEP). In the late infection process HA on the cell membrane activates the cellular mitogen-activated protein kinase (MAPK) signalling pathway, which stimulates vRNP export. This can be seen as an auto-regulating mechanism that coordinates the timing of vRNP output during virus budding (Zheng and Tao, 2013; Hutchinson and Fodor, 2013). NEP also regulates the timing of vRNP export by the slow accumulation of NEP during an infection. The mRNA of NEP is generated by splicing at a weak 5′ splice site, resulting in a low rate of NEP synthesis in comparison with the production of other viral proteins. The NEP levels correlate with the output of vRNP, this indicates the slow accumulation of NEP results in a 'molecular timer' that stimulates vRNP export in a later stage of the infection (Hutchinson and Fodor, 2013; Chua et al., 2013). As the infection persists, other viral proteins with a NLS arrive in the nucleus. M1 is able to bind in the nucleus with newly formed vRNP by an interaction with NP (Yu et al., 2012). The binding probably masks the NLSs of vRNPs (Zheng and Tao, 2013). Subsequently, NEP can bind to M1 in the nucleus. NEP has two nuclear export signals that both are recognized by the exportin chromosome region maintenance 1 (CRM1) that mediates the export of the vRNP-M1-NEP complex out of the nucleus (Yu et al., 2012; Zheng and Tao, 2013). CRM1 is activated by binding to Ran-GTP. The proteins along with Ran-GTP are subsequently excreted into the cytoplasm (Hutchinson and Fodor, 2013). Verdinexor is a selective inhibitor of exportin and blocks nuclear export of vRNP. It effectively inhibits the replication of various IV strains *in vitro*, including H1N1, H5N1 and H7N9. *In vivo*, verdinexor protected mice against H1N1 and H3N2 infection, as well as reduced IV titers and cytokine expression, while having negligible cytotoxicity (Perwitasari et al., 2014).

Once in the perinuclear cytoplasm, vRNP interacts with the microtubule organizing centre (Hutchinson and Fodor, 2013). This process is mediated by the Y-box binding protein 1 (YB-1) that binds in the nucleus to vRNP and can interact with the microtubules. The cellular human immunodeficiency virus Rev binding protein assists in the separation from vRNP of the CRM1-Ran-GTP complex and makes further transport of vRNPs possible. In order to take part in vesicular transport, vRNPs are able to interact with recycling endosomes *via* Rab11 in the vicinity of the microtubule organizing centre. The viral PB2 subunit binds the active GTP-bound form of Rab11 that is bound to the recycling endosome. The vRNP complex will be transported across the cytoplasm along the microtubule network to the plasma membrane. However, this vesicular transport system does not appear to be the only method in which vRNP can be transported through the cytoplasm. A number of vRNPs, turn out to migrate away from the perinuclear region through diffusion, while other vRNP can move over short distances along actin filaments. Apart of the transport of vRNPs to the plasma membrane, recycling endosomes serve as a platform for vRNPs to interact with other vRNPs. Because each vRNP only contains one segment of the viral genome, the chance of a successful infection is greatly increased when vRNP of different segments associate with each other before they are packaged into new virions. Eventually vRNP arrives in an area that is adjacent to the plasma membrane. vRNP comes loose from Rab11 as a result of hydrolysis of GTP, thereby Rab11 is converted to its inactive GDP-bound form. From this area vRNP migrates to the apical plasma membrane (Hutchinson and Fodor, 2013). Fig. 5 shows an overview of the transport of the viral complexes in an infected cell. So far, these processes as far as we know, are not being investigated as drug targets.

5. Cell budding

IV use lipid raft domains on the plasma membrane of infected cells for virus assembly and budding. Lipid rafts are regions which are rich in cholesterol and sphingolipids. HA and NA are intrinsically linked to lipid raft domains, while the M2 protein accumulates at the border of the lipid raft domains (Rossman and Lamb, 2011; Hutchinson and Fodor, 2013). HA and NA proteins curve the membrane. This creates bud formation, which subsequently initiates the budding process (see Fig. 6A). The cytoplasmic tails of HA and NA serve as docking sites for M1 (Rossman and Lamb, 2011). The M1 proteins form a spiral-like web below the viral membrane and are responsible for the structure of the new virions. In addition, M1 recruits the vRNP-complexes through an interaction with NP, in order to complete the budding process (Zhang et al., 2000; Rossman and Lamb, 2011). vRNP are selectively packaged into new virions (Zheng and Tao, 2013; Hutchinson and

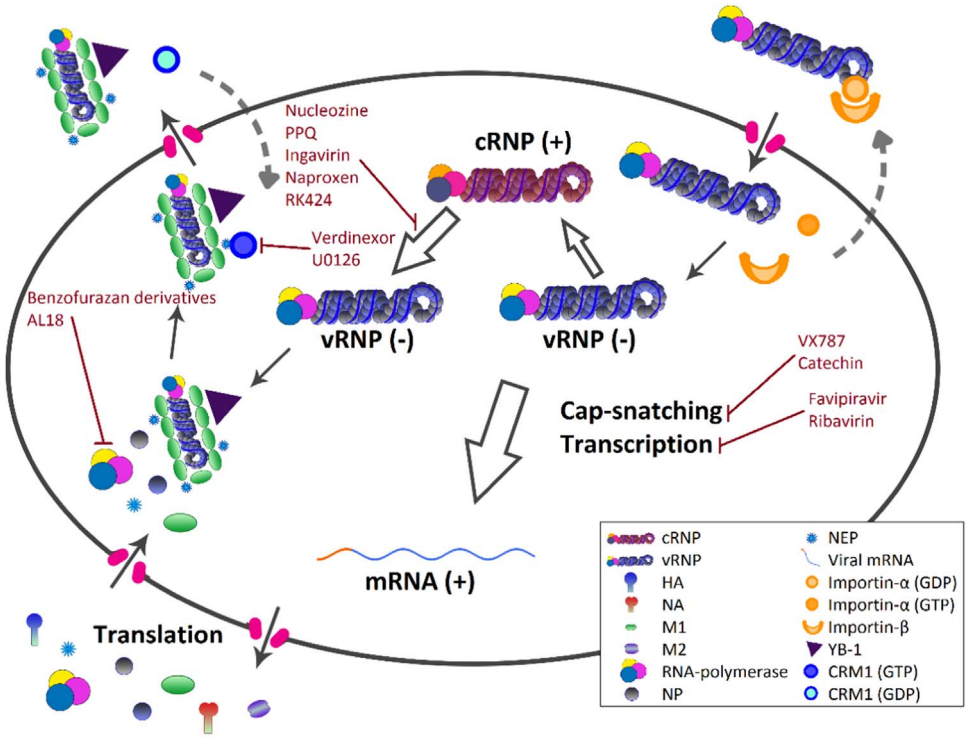

Fig. 4. The viral transcription-replication cycle. The figure shows the active transport of initially vRNP in the nucleus, the transcription process, the translation process, the two-step method vRNP replication and active export of new vRNP to the cytoplasm.

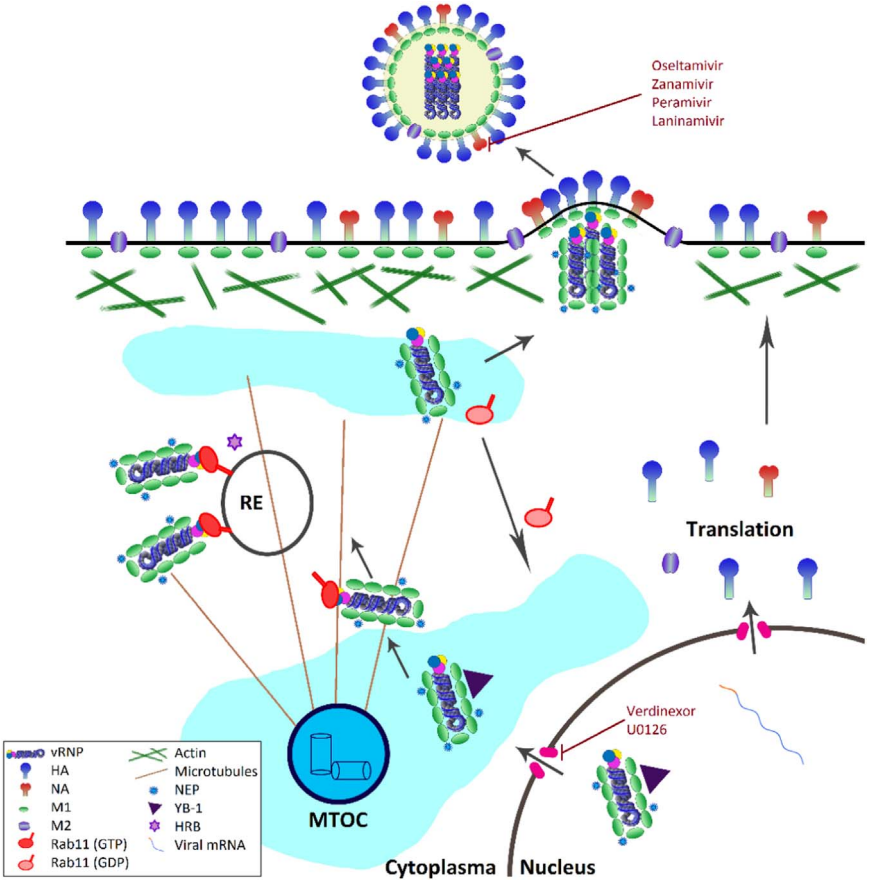

Fig. 5. Export of vRNPs and viral proteins in an infected host cell.

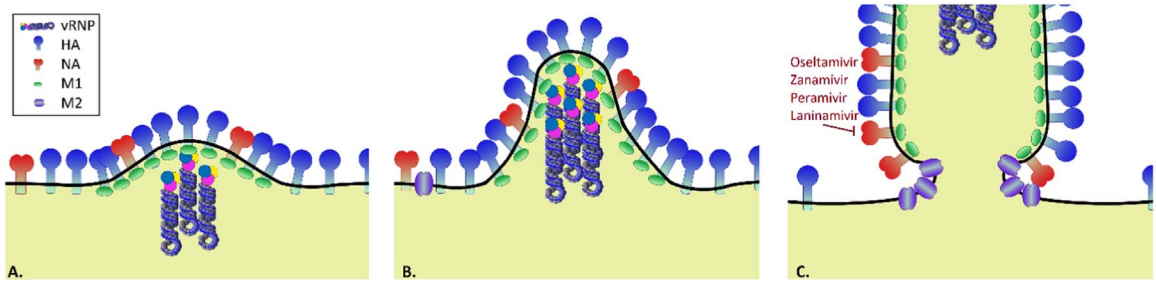

Fig. 6. IV budding. **A:** Clustering of HA and NA in lipid raft domains introduce virus budding. M1 binds to the cytoplasmic tail of HA and NA, and serves as a docking site for vRNPs. **B:** Extension of the budding virion is induced by polymerization of M1. **C:** M2 is recruited to the periphery of the budding virus *via* interaction with M1. M2 causes membrane splicing, which results in secretion of a new virion.

Fodor, 2013) and presumably induce the starting signal for the initiation of budding by M1 polymerization, which provides extension of the virion (Rossman and Lamb, 2011). Binding of M1 in the spiral-like web to HA and NA, curve the membrane in such a way that budding takes place (see Fig. 6B).

For the last step in the release of new virions, M2 is critical. By means of the delayed expression of M2, only mature virions are able to split off from the membrane (Rossman and Lamb, 2011). The amphipathic helix of the M2 protein creates a rotation in the membrane, which causes a tension between the two lipid phases and finally separation occurs between the budding virion and the cell membrane. In this process, M2 depends on cholesterol which is bound to the cytoplasmic tail of M2 (Rossman and Lamb, 2011). Studies with mutations in the M2 protein indicate that M2 only plays a role in the completion of virus budding (Rossman and Lamb, 2011; Chen et al., 2007). After completion of membrane splicing, the virion may still be tethered to the cell membrane *via* interactions of HA with sialic acid residues on the cell surface. NA can subsequently remove sialic acid from the cell surface releasing the virus (Rossman and Lamb, 2011). Currently, two NA inhibitors, oseltamivir and zanamivir, are approved in the United States and Europe. Oseltamivir and zanamivir are both synthetic analogues of sialic acid which in vivo competitively block the active site of NA and thus inhibit the enzymatic activity of NA. This prevents NA to split off releasing virions that are bound to sialic acid in the late phase of the infection (Król et al., 2014; Monod et al., 2015). NA is a promising drug target mainly due to the highly conserved structure of the active site among IV A and B strains. Nevertheless, resistant variants with an H275Y mutation have emerged against oseltamivir (Król et al., 2014). Also virus strains H7N9 and H1N1 with a R292K substitution are described with resistance against oseltamivir and partially to zanamivir (Sheu et al., 2011; Liu et al., 2013; Hai et al., 2013). New generation of NA inhibitors are currently being evaluated to improve the effectiveness of the current compounds. The modifications include multimeric derivatives of zanamivir, which are significantly more active than the monomeric form, and importantly, they exhibit a long-term protective effect in mice experiments. Diverse dimeric derivatives of zanamivir conjugates have demonstrated, both *in vitro* and *in vivo*, to be 100-fold more potent and polymeric conjugates appear to have even a 1000 to 10,000-fold higher antiviral activity *in vivo*, and have a longer-lasting effect than the monomeric form of zanamivir (Król et al., 2014; Monod et al., 2015). Furthermore, there are two other promising NA inhibitors, peramivir, which is already marketed in Japan, and laninamivir, which is tested in phase III clinical trial (Monod et al., 2015; Liu et al., 2013). Peramivir is a cyclopentane based compound with strong NA inhibition characteristics. It is active against various zanamivir- and oseltamivir resistant IV A and B strains, however peramivir is not effective against virus strains with the R292K substitution (Hai et al., 2013; Król et al., 2014; Monod et al., 2015). Laninamivir, which is structurally similar with zanamivir, inhibits the NA activity of various IV A and B strains, including the oseltamivir-resistant strains. Furthermore, laninamivir appears to be particularly well effective against the pandemic H1N1 strain of 2009 (Król et al., 2014; Monod et al., 2015). Various other cyclopentane-based compounds, such as A-192558 and A-315675, have been synthesized with improved NA-inhibiting properties. Both compounds effectively inhibit different strains of IV A and B (Król et al., 2014).

6. Involvement of the immune system

6.1. Infection process

An IV infection can result in multiple complications for the patient resulting in multi-organ failure, which leads to a high pathogenicity and mortality (Yuan, 2013). Usually, IV A infects the upper respiratory tract and causes mild respiratory symptoms. In serious cases, the infection spreads to the lower respiratory tract with viral pneumonia as result. This occurs mainly in patients with a weakened immune system, including the elderly. Pneumonia may develop into acute lung injury, pulmonary oedema, hypoxia and ultimately respiratory failure. Unfortunately, current treatments have limited effectiveness when administered in the late phase of the disease and this highlights the need for additional therapies (Armstrong et al., 2013). A large part of the damage to the respiratory tract is attributed to necrosis of the infected cells. Cytolysis of infected endothelial cells is caused by the suppression of gene expression and protein synthesis (Armstrong et al., 2013; Janke, 2014). Cytolytic mortality occurs within 20–40 h after infection of the host cell. In addition to the damage that is caused by necrosis of infected cells, IV raises a strong inflammatory response (Janke, 2014). The amount of transcription factor NFκB increases in endothelial cells and stimulates the production of pro-inflammatory cytokines and chemokines, which subsequently attract leukocytes. These leukocytes extend the duration of the inflammatory response and contribute to further tissue damage (Liu et al., 2013; Armstrong et al., 2013; Short et al., 2014). Increased concentrations of interleukin-6 (IL-6), and IL-1β cause vascular hyperpermeability. The increase in endothelial permeability due to remodelling of endothelial cell junctions leads to endothelial apoptosis, and to pulmonary oedema (Armstrong et al., 2013). Although leukocytes influx into the lungs is crucial for recovery against an IV infection, massive infiltration of neutrophils is a major contributor to the development of acute lung damage (Narasaraju et al., 2011; Armstrong et al., 2013). An IV infection can be further linked to thrombogenicity. Produced cytokines and chemokines, such as IL-1, IL-6, platelet activation factor, intercellular adhesion molecule 1, p-selectin and the von Willebrandfactor, increase the expression of platelet binding receptors on the endothelium. In addition, platelets become activated by binding to the exposed extracellular matrix of damaged endothelial cells (Armstrong et al., 2013).

6.2. Hyper immune reaction

Some strains of IV induce a very strong immune reaction, with high release of pro-inflammatory cytokines (Mehrbod et al., 2014). Especially the H5N1 strain as well as some H1N1 strains cause pro-inflammatory cytokine dysregulation (Walsh et al., 2011; Lee et al. (2012)). The stronger the immune system acts after an H5N1 infection, the more severe the disease symptoms. This makes healthy young people most vulnerable to an H5N1 infection. Immunosuppressive agents are very important to combat the hyper immune response induced by various IV strains (Yuan, 2013).

The study of Zheng et al. (Zheng et al., 2008) shows that combinations of the immuno-modulating agents celecoxib and mesalazine, in combination with anti-IV agents, substantially reduce the mortality of H5N1-infected mice. Celecoxib inhibits COX-2 and mesalazine inhibits lipoxygenase and COX pathways. These drugs do not cause an overall immunosuppression, but suppress the induced cytokine production by H5N1 and H1N1 strains (Yuan, 2013; Zheng et al., 2008). Statins may likewise be an alternative therapy against IV infections. Statins are hydroxy-methylglutaryl coenzyme A reductase (HMG-CoA) inhibitors, and inhibit cholesterol synthesis. Cholesterol plays an important role in an IV infection (Mehrbod et al., 2014). In addition, statins have also immunomodulatory effects and can reduce sepsis. Statins, for instance, inhibit the expression of MHC-II, which leads to suppression of CD4 T lymphocytes (Lee et al., 2012;). In vitro studies have shown effects of atorvastatin, simvastatin and pravastatin on several cellular pathways to constrain the virus-host interactions (Mehrbod et al., 2014). These actions of statins restrict cell destruction and add to conventional IV therapies. Several retrospective observational studies have identified an association between statin use and reduced mortality in IV-infected patients and demonstrate the therapeutic potential of statins (Frost et al., 2007). Nevertheless, these studies have some potential biases and there are also studies that do not show effects of statins on the mortality in IV-infected patients (Fleming et al., 2010, Brassard et al., 2016). However, the articles of Fleming et al. (2010) and Brassard et al. (2016) do not only focus on the IV strains that cause a hyper immune response like H5N1. Despite conflicting reports, statins have on basis of all current information some potential and further research should be carried out for specific IV strains, in order to identify a possible beneficial effect of statins. Corticosteroids are another group of immunomodulatory drugs that have been tested in IV infections. The available data suggest corticosteroid therapy against an IV infection might be associated with increased mortality, however there is a lot of criticism about these trials as mentioned in the systematic review by Rodrigo et al. (2016). Similar results are found in the studies of Nedel et al. (2016) and Cao et al. (2016), which indicate, next to increased mortality, also a longer viral shedding (Nedel et al., 2016; Cao et al., 2016). Furthermore, dexamethasone was not effective to prevent the development of acute respiratory distress syndrome in H5N1 infected mice (Xu et al., 2009).

Another factor that can modulate the immune response is sphingosine-1-phosphate (S1P). S1P is present in plasma and is the ligand for five different S1P receptors. These receptors are expressed on many cell types. Activation of S1P receptors regulates various cellular activities and plays an important role in inflammation and immunity (Lee et al., 2012). The sphingosine analogue, AAL-R, is a broad-spectrum agonist of S1P receptor 1, 3, 4, and 5. In mice, AAL-R suppresses dendritic cell maturation, inhibits IV specific T-cell responses and reduces release of cytokines and chemokines including interferon (IFN) responses in H1N1 infections (Marsolais et al., 2009; Walsh et al., 2011). It decreases infiltration of macrophages and granulocytes in the lung leading to less pulmonary tissue injury during an IV infection. Importantly, AAL-R does not affect antibody responses and virus titers in the lung do not increase. Combined administration of both AAL-R and an antiviral compound may seem the best approach for treatment. This combination will inhibit IV directly as well as suppress the cytokine storm to reduce lung injury, while maintaining the benefits of the protective immune response (Walsh et al., 2011; Lee et al. (2012)). Although S1P receptors appear to be a promising therapeutic target, development of agonists against specific S1P receptors is needed in order not to disrupt normal cellular activities of these receptors. CYM-5442 is an example of a specific agonist of the $S1P_1$ receptor. *In vivo*, CYM-5442 significantly reduced cytokine and chemokine responses associated with IV induced lung injury. However, unlike AAL-R, it has no effect on dendritic cells and T-cell responses (Teijaro et al., 2011; Lee et al. (2012)).

The IKK/NFκB pathway and the Raf/MEK/ERK cascade are two signalling pathways required for an efficient virus replication and are of crucial importance for cytokine synthesis during an IV infection (Ludwig, 2009). These cascades may be suitable targets for anti-IV strategies. In this way, inhibitors of these cascades inhibit not only the replication of the virus, but can also moderate the systemic inflammation. This hypothesis has been confirmed by several studies both in vitro and in vivo, although further studies are needed to define the activity in humans (Liu et al., 2013; Mazur et al., 2007).

The NFκB pathway is responsible for diverse cellular processes, including apoptosis. As a result of the production of various antiviral and proinflammatory cytokines, an IV infection activates the NFκB pathway by activation of IKK2 (Julkunen et al., 2001). NFκB is an important regulator of cytokine expression, especially for IFN-β to mediate antiviral activities (Lee et al., 2012). On the other hand, IV can also control the activity of NFκB with its non-structural protein NS1 (Lee et al., 2012). NS1 is a highly conserved multifunctional protein which protects IV against antiviral IFN responses of the host (Rossman and Lamb, 2011; Monod et al., 2015). NS1 binds to double-stranded viral RNA and prevents binding of cellular factors to the vRNA. In addition it blocks retinoic acid inducible gene-I, which is involved in immune regulation and viral RNA recognition (Monod et al., 2015). Hereby, NS1 inhibits the production of IFN-α/β and the antiviral effects of IFN-induced proteins such as NFκB (Liu et al., 2013). The viral NS1 protein is a good target for antiviral drugs. Small molecules such as polyphenol and quinoxaline derivatives are potential inhibitors of NS1 (Monod et al., 2015). Another compound, JJ3297, inhibits NS1-induced inhibition of the mRNA production of IFN in an RNase L-dependent manner causing suppression of replication and spread of IV (Liu et al., 2013). The C-Jun-N-terminal kinase inhibitor SP600125 reduces the replication of IV in vitro and in vivo by indirect inhibition of NS1-mediated functions in the early stages of infection (Zhang et al., 2016).

Furthermore, IV mediates the IKK/NF-κB pathway to express pro-apoptotic factors, such as TNF-related apoptosis-inducing ligand (TRAIL) and FasL/CD95L (Wurzer et al., 2004). NFκB is a novel therapeutic target to reduce the cytokine storm. Current research suggests that a high dose of acetylsalicylic acid, an inhibitor of IKK2, can block the IV replication effectively both in vitro and in vivo (Mazur et al., 2007; Lee et al., 2012). Another inhibitor of the NFκB pathway, the antioxidant pyrrolidine dithiocarbamate, was shown to increase survival up to 80% in H1N1-infected mice by immediate administration of the drug (Wiesener et al., 2011). NFκB inhibitor SC75741 significantly protects mice against a lethal infection with highly pathogenic IV strains H5N1 and H7N7. SC75741 reduces expression of cytokines, chemokines, and pro-apoptotic factors and blocks nuclear export of vRNPs in lung of infected mice. By inhibition of the cytokine storm it might protect against highly pathogenic strains. Furthermore, SC75741 has broad activity against resistant virus variants and no adverse effects were shown during treatment with SC75741 in the required concentrations to protect mice against IV. More studies are needed to determine the potential of SC75741 (Ehrhardt et al., 2013; Haasbach et al., 2013b).

The Raf/MEK/ERK signalling pathway belongs to the MAPK cascade. This pathway controls cell proliferation, differentiation and survival. The MAPK cascade can be activated by IV.

Table 1
An overview of host factors and their inhibitors in different research phases.

Targeted host factor	Infection process	Inhibitors	Research phase	Potential	Reference
Sialic acid	Attachment	DAS181	Phase II	+/-	Moss et al. (2012)
Dynamin	Internalization	Dynasore	Preclinical	–	de Vries et al. (2011)
Na^+/H^+-transporter	Internalization	EIPA	Preclinical	–	de Vries et al. (2011)
Membrane fluidity	Internalization,	Fattiviracin	Preclinical	+/-	Harada et al. (2007)
	Immune dystegulation	Glycyrrhizin	Preclinical	+	Harada et al. (2007), Michaelis et al. (2011)
		LJ001	Preclinical	+/-	Wolf et al. (2010)
PKC	Internalization	Bisindolymaleimide I	Preclinical	–	Root et al. (2000)
vATPase	Fusion	Bafilomycin A1	Preclinical	+/-	Yeganeh et al. (2015)
		Concanamycin	Preclinical	+/-	Müller et al. (2011)
		Diphyllin	Preclinical	+/-	Chen et al. (2013)
		Saliphenylhalamide	Preclinical	+/-	Bimbo et al. (2013)
Inosine-5′-monophosphate dehydrogenase	Transcription, replication	Ribavirin	Approved[a]	+	Marcellin et al. (2010)
		Viramidine	Phase III	+	Marcellin et al. (2010)
Exportin	Nuclear export	Verdinexor	Preclinical	+/-	Perwitasari et al. (2014)
COX-2	Immune dysregulation	Celecoxib	Approved[a]	+/-	Zheng et al. (2008)
Lipoxygenase and COX pathways	Immune dysregulation	Mesalazine	Approved[a]	+/-	Zheng et al. (2008)
HMG-CoA	Immune dysregulation	Statins	Approved[a]	+/-	Mehrbod et al. (2014), Frost et al. (2007)
Multiple targets	Immune dysregulation	Corticosteroids	Approved[a]	–	Rodrigo et al. (2016)
S1PRs	Immune dysregulation	AAL-R	Preclinical	+/-	Marsolais et al. (2009), Walsh et al. (2011)
		CYM-5442	Preclinical	+/-	Teijaro et al. (2011)
C-Jun-N-terminal kinase	Immune dysregulation	SP600125	Preclinical	+/-	Zhang et al. (2016)
IKK2	Immune dysregulation	Acetylsalicylic acid	Approved[a]	+/-	Mazur et al. (2007)
NFκB pathway	Immune dysregulation	Pyrrolidine dithiocarbamate	Preclinical	+	Wiesener et al. (2011)
NFκB	Immune dysregulation	SC75741	Preclinical	+	Ehrhardt et al. (2013), Haasbach et al. (2013b)
MEK	Nuclear export	U0126	Preclinical	+	Pleschka et al. (2001), Droebner et al. (2011)
p38 MAPK	Immune dysregulation	NJK14047	Preclinical	+/-	Choi et al. (2016)

[a] Approved for another indication.

to initiate the nuclear export of vRNP. The anti-IV potential of some MAPK/ERK kinase (MEK) inhibitors have been investigated (Lee et al., 2012; McKimm-Breschkin et al., 2016). U0126 is a specific MEK inhibitor which blocks the MAPK cascade, to supress the viral production by blocking nuclear export of vRNPs (Pleschka et al., 2001). U0126 is a promising drug that shows a decrease in IV virus titers in infected mice, and protection of IV infected mice against a 100x lethal viral challenge (Droebner et al., 2011). U0126 has high anti-IV activity and low cytotoxicity in vitro and in vivo. The novel compound NJK14047 inhibits IV mediated p38 MAPK activation selectively in epithelial cells and can suppress the viral replication as well (Choi et al., 2016). Other MEK inhibitors have demonstrated to increase the anti-IV activity of oseltamivir synergistically at suboptimal concentrations (Haasbach et al., 2013a).

Interferon-inducible transmembrane proteins (IFITMs) are other important host factors which combat IV (Everitt et al., 2012; Blyth et al., 2015). In response to a viral infection IFITMs stimulate type I IFN production to induce an antiviral condition in neighbouring cells. Especially IFITM3, localized to late endosomes and lysosomes, can restrict replication of various viruses including IV strains H1N1, H6N2, and H11N9 by restriction of the endocytic pathway. Overexpression of IFITM3 reduces the percentage of IV-infected cells *in vitro* and *in vivo* in a strain-independent manner. This demonstrates that IFITM3 is a significant factor to the innate immune system against IV and IFITM3 related drug developing might be interesting.

In Table 1 an overview is shown of the described inhibitors against host factors.

7. Combination therapy

Single treatment with one antiviral drug might promote the development of drug resistance by the virus, especially in immunocompromised hosts and patients The usage of combination therapies of anti-IV agents with different mechanisms of action can optimize antiviral therapies. At present, there are already antiviral combination therapies used to control HIV. A potential advantage is that in combination lower dosage of the drugs can be used than when given alone, which may result in fewer side effects, and reduces the development of resistance (Król et al., 2014; Monod et al., 2015). At present, combination therapies of current therapies with anti-IV NA inhibitors did not reduce resistance (Dunning et al., 2014). However, combining NA inhibitors in combination with antiviral agents with other mechanism of action maybe more advantageous. Several *in vitro* and *in vivo* studies have demonstrated an increased activity of anti-IV agents when these agents are used in combination. However, the clinical relevance has to be determined for most combinations. A double-blind, randomized, placebo-controlled study of a treatment with zanamivir in combination with rimantadine which has been compared to rimantadine alone, has been carried out for the treatment of IV in hospitalized adults (Ison et al., 2003). This clinical study has confirmed a higher efficacy of the combination of zanamivir and rimantadine. In another clinical study performed in Korea by Kim et al. (2011) the comparison was made between a triple-combination with amantadine, ribavirin and oseltamivir *versus* oseltamivir monotherapy in patients with the severe H1N1 (2009) IV infection. This study showed that the mortality rate was significantly lower in patients treated with the combination therapy compared with patients treated with only oseltamivir after 14 days (17% *versus* 35%), but not any more after 90 days. In the study of Seo et al. (2013). the pharmacokinetics and safety of this triple anti-IV therapy was investigated in humans. Similar pharmacokinetics for the combination therapy were seen in comparison with amantadine, oseltamivir or ribavirin when given in a single dose. In addition they showed that the triple therapy can be administered safely in immunocompromised patients. A combination of the two cap-snatching inhibitors favipiravir and ribavirin exhibited significant synergistic effects against the R292K mutant virus in cell-

based assays (Zhang et al., 2014). This combination should be tested further in animal models and ultimately in human patients to confirm the therapeutic benefit. Furthermore, additive and synergistic effects are observed in tissue culture and IV A infected mice for many more combinations (rimantadine or amantadine with ribavirin (Wilson et al., 1980), oseltamivir, peramivir, or zanamivir with rimantadine (Govorkova et al., 2004), oseltamivir with amantadine Ilyushina et al. (2007)), oseltamivir with ribavirin (Ilyushina et al., 2008), and oseltamivir, peramivir or zanamivir with favipiravir (Furuta et al., 2013; Tarbet et al., 2014).

8. Summary

The constant threat of an IV pandemic and the increasing appearance of multi-resistant IV strains, emphasize the need for development of improved antiviral agents. Increased knowledge about the structure of IV and the underlying host mechanisms involved in the viral life cycle may enable the development of novel anti-IV agents. Because current vaccines do not provide universal protection and need several month of development and production time, the use of small molecule antiviral agents is important as a first line of defence against new IV strains. In this review, various potential druggable targets and novel antiviral agents in various stages of preclinical to advanced clinical development were described. Drug strategies against host proteins involved in a IV infection are promising. No viral resistance can occur against these antiviral agents and agents with immunomodulatory effects can decrease inflammation and tissue damage. However, drug therapies against host proteins that perturbing the cellular environment may be toxic for normal cellular functions and could cause adverse side effects. This may hamper their use and should be carefully evaluated. Local administration of these drugs in the respiratory tracts may reduce systemic toxic side effects. Combining drugs with different modes of action is an important point of interest to optimize antiviral therapies. Simultaneous use of multiple anti-IV agents can lead to a greater clinical efficacy. Anti-IV combination preparations can be developed which target both viral proteins as cellular host factors. Currently, several combination therapies based on approved and/or new drugs are under investigation and demonstrate increased activity of combined anti-IV agents. Although several combinations are promising, clinical relevance has to be determined in most combination therapies.

In summary, in addition to viral factors many host factors are required for an IV infection. These include among others immune regulators, transport proteins, G-proteins, kinase activity, vATPases activity and host proteins involved with the replication of the IV. Increasing the understanding of these processes can be used to develop novel anti-IV agents with less risk of resistance and toxicity. Besides direct inhibition of the replication of IV, development of antiviral agents with immunosuppressive characteristics is very important to combat pandemic IV strains. Both conserved viral proteins as host proteins may apply as target in order to obtain a broad antiviral activity. Novel approaches that have both immunomodulatory and antiviral effects therefore deserve special attention. Future research is needed to determine which approaches and combination therapies are most suitable as broad-spectrum anti-IV therapy that offer a long lasting protection.

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