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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