Current trends of hemagglutinin and M2 protein inhibition for the treatment of influenza using antivirals

Abstract

Influenza is an annually returning illness with a great physical and economic burden. Currently, the most commonly used medication for influenza is vaccination. However, every year, a new vaccine has to be created to combat a newly formed influenza strain as a result of antigenic drift and shift. Moreover, creating new vaccines to combat new influenza strains costs money and time. Another way of treating influenza is via the use of antivirals. Production of these drugs is faster and they are not strain-specific. Currently used antivirals focus on targeting neuraminidase and cap-dependent nuclease proteins of the exit and replication phase of influenza virus, respectively. This review investigates ongoing and future research in antivirals targeting hemagglutinin and matrix protein 2 of the entry phase of influenza. The most promising upcoming antivirals are hemagglutinin inhibiting drugs, such as Arbidol, Nitazoxanide, and Urumin. More research in matrix protein 2 inhibition is still necessary, with a low number of viable candidates and low barrier of drug-resistance being the main problems.

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Introduction

Influenza virus is a RNA virus belonging to the *Orthomyxoviridae* family (Bouvier & Palese, 2008). Four types of the virus have been identified: A, B, C, and D, of which only D has not shown to be infectious to humans (CDC, 2017b). Influenza A and B can cause acute respiratory diseases and can spread rapidly via infectious droplets distributed via sneezing or coughing by those infected. Symptoms commonly associated with influenza virus infection are fever, coughing, and pain in joints and muscles. For most people the disease lasts around a week, however, severe illness or death can occur, especially for young children, the elderly, and immunosuppressed patients. Influenza A and B can cause annual epidemics (Paules & Subbarao, 2017) that result in around 4 million cases of severe illness and 500.000 deaths (WHO, 2018).

Influenza A virus (IAV) can be classified into subtypes according to the hemagglutinin (HA) and neuraminidase (NA) proteins present on the surface of the virus: H1 through H18 and N1 through N11 respectively. A well-known example is the H1N1 subtype that caused the Spanish flu pandemic in 1918 and the more recent swine flu pandemic in 2009 (CDC, 2017b) (Garenne & Noymer, n.d.) (Paules & Subbarao, 2017).

Currently, vaccination is paramount for influenza control (WHO, 2018). Current vaccines produce antibodies focusing on the HA proteins present on the virus surface. However, due to antigenic drift, in which mutations in the influenza genome cause slight

changes in antibody binding sites, annual vaccination is necessary. Vaccinations effective against current strains are not effective against influenza epidemics the following year (CDC, 2017a). Antigenic shift is the cause for the emergence of antigenically novel viruses. Here, two or more influenza virus strains combine to form a new subtype with new antigens present on the surface (CDC, 2017a). This makes development of vaccines expensive and time-consuming (Houser & Subbarao, 2017) (Duwe, 2017).

Antivirals are drugs capable of targeting and disabling (parts of) viral proteins common across a variety of strains. Their use is second in the line of action against influenza, following vaccines. As a result, they are not strain-specific and not affected by antigenic drift and shift. These antivirals can interfere at any level of the virus life cycle and can even target proteins of the host that ease virus infection (Król, 2014). These drugs pose a great alternative for when vaccination is not (yet) possible and therefore, extensive research is going on in the field of antivirals.

The European Centre for Disease Prevention and Control (ECDC) currently recommends two antiviral drugs for the treatment of influenza. These are Oseltamivir (Tamiflu) and Zanamivir (Relenza). These drugs belong to a class of neuraminidase inhibitors and therefore inhibit budding from the host cell after replication (McKimm-Breschkin, 2013) (ECDC, n.d.). The American Centers for Disease control and Prevention (CDC) and the Food and Drug Administration (FDA) recommend Oseltamivir and Zanamivir, as well as Peramivir (Rapivab) and Baloxavir Marboxil (Xofluza). The latter two inhibit neuraminidase and cap-dependent endonucleases respectively (CDC, 2018). This means that currently, antiviral treatments against influenza virus only target the replication phase inside the host cell nucleus and the release from the host cell. During the entry phase, however, other influenza proteins play a role that might pose as a great target for novel influenza antivirals (Shen et al., 2013).

In this review, two targets of antiviral treatment belonging to virus entry into host cells are investigated: hemagglutinin and matrix protein 2 (M2) ion channel inhibitors. Current available drugs are discussed as well as new, emerging drugs targeting these proteins.

Entry phase of the influenza life cycle

In order for viruses to replicate, they must deliver their genome into a host cell. For this, the virus first needs to enter the cell. IAV uses specialized proteins present on the surface: hemagglutinin (Lakadamyali, 2004). This protein can recognize N-acetylneuraminic acid (sialic acid) bound to the carbon-3 or carbon-6 of galactose to form α-2,3 or α-2,6-linkages present on the host cell surface. These linkages are present predominately in the human tracheal and respiratory epithelial cells (Bouvier & Palese, 2008). Hemagglutinin is a trimer consisting of two distinct regions: a stem and a head. The stalk region connects the HA to the envelope of the virus and is glycosylated, possibly for the stability of the molecule. The stalk region is a conserved epitope. The head region of the virus holds the receptor-binding pocket and is also highly conserved among different HA subtypes (Edinger et al., 2014) (Smrt & Lorieau, 2017) (Luo, 2012). Serine proteases cleave the HA protein into HA1 and HA2 during virus replication. The HA1 portion of the protein contains the actual receptor-binding pocket, whereas HA2 helps with fusion of the virus envelope and cell membrane after endocytosis (Bouvier & Palese, 2008). Since the binding of HA with sialic acid is of low affinity, multiple HA-sialic acid connections must be formed to strengthen the virus-host cell interaction (Edinger et al., 2014).

Once sufficient binding has taken place, virus internalization commences. IAV internalization happens via several pathways. Clathrin-mediated endocytosis is most common, however, uncoated vesicles containing IAV

have been observed, suggesting IAV does not require Clathrin-dependent pathways (Smrt & Lorieau, 2017) (Edinger et al., 2014). Internalization via the caveolin-independent pathway and micropinocytosis is also possible (Lakadamyali et al., 2004).

After internalization, IAV localizes to early endosomes. These early endosomes are trafficked away from the host cell periphery via actin-dependent transport and later towards the nucleus via microtubule-mediated transport. During this process, maturation of early endosomes into late endosomes takes place as the pH of the endosomal interior drops from around 6.5 to 5.0 (Edinger et al., 2014).

IAV needs to be present in the nucleus of the host cell for replication to take place (Amorim & Digard, 2006). This is mediated via nuclear transport of influenza viral ribonucleoproteins (vRNPs) present in the cytosol. However, these vRNPs, present in the virus need to be released into the cytoplasm first. Fusion between the viral and endosomal membranes and subsequent viral uncoating is necessary for this step.

Fusion starts with a conformational change of the HA protein as a result of lowering pH to expose a fusion peptide present on the stalk of HA (Amorim & Digard, 2006) (Edinger et al., 2014). The fusion peptide is positioned towards the endosomal membrane. HA trimers at the fusion site tilt away from the endosomal membrane (Edinger et al., 2014) and the virus membrane and endosomal membrane interact in a hemi-fusion stage. The fusion peptide inserts itself into the endosomal membrane, finalizing complete fusion of viral and endosomal membranes (Samji, 2009).

In the virus, vRNPs are connected to the virus membrane via the M1 protein and have to be released using the M2 protein (Samji, 2009). This protein is normally present in the virus membrane and forms tetramers that possess ion-channel activity. Its ion channeling properties start when the pH in the virus particle drops during endocytosis. Upon acidification of the endosome during maturation, activity of the ion-channel increases, allowing larger proton influx into the virus particle and mediating detachment of M1 protein from vRNPs (Edinger et al., 2014) (Pielak & Chou, 2011). The now freely present vRNPs can finally enter the cytoplasm.

M2 also functions as a buffer by preventing lowering pH in the ER of the host cell after translation of the viral RNA. This prevents premature conformational rearrangement of HA (Pielak & Chou, 2011).

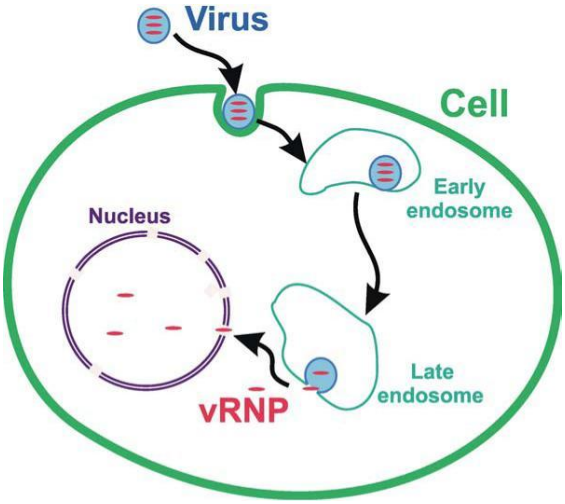

Figure 1: schematic overview of influenza virus entry. After endocytosis using virus HA and host sialic acid, conformational changes due to dropping pH in maturing endosomes lead to membrane fusion of virus and host cell, releasing vRNPs into host cell cytoplasm (Lakadamyali et al., 2004).

Hemagglutinin

Currently, no antiviral treatments targeting HA are in use. However, ongoing research has produced promising new candidates. Hemagglutinin inhibiting antivirals can be subdivided into different types of inhibitors: small molecules, proteins and peptides, and monoclonal antibodies (mAbs).

Small molecules targeting HA

Small molecule drugs are compounds low in molecular weight. Once inside a virion or cell,

they can affect other molecules and alter their functioning (Gurevich & Gurevich, 2015). These compounds could act as a drug targeting HA on the virus surface.

Arbidol (Umifenovir), a drug developed by the Center for Drug Chemistry in Russia, has been in use in Russia for over 20 years (Zeng et al., 2017). By binding to the HA trimer in a hydrophobic cavity, it can alter its conformation. Salt bridges are created, making Arbidol act as a glue. This prevents necessary conformational changes of HA when pH drops during endosome maturation, thereby inhibiting fusion (Kadam & Wilson, 2017) (Boriskin et al., 2008). Recent research has used x-ray structures of Arbidol to assess binding of this compound to the binding pocket of HA. They were able to make modifications to the compound resulting in increased interactions between it and the binding pocket (Wright et al., 2017). To test the cytotoxicity and efficacy of Arbidol, Madin-Darby Canine Kidney (MDCK) cells were infected with an H1N1 influenza strain and virus titer was observed at different intervals. *In vitro* IAV inhibition by Arbidol was observed. Mice were then infected with the same IAV strain and administered Arbidol at different concentrations. 12 mice were observed for mortality and weight. A control group did not receive Arbidol. This research found that mice treated with 180.0 and 90.0 mg * kg^{-1} * $dose^{-1}$ Arbidol showed less weight loss and higher survival rates than the control group, suggesting alleviation of clinical signs of IAV by Arbidol. Moreover, the study observed a downregulation of cytokines commonly associated with infection (IL-1β, IL-6, IL-12p40, TNF-α, and IFN-β), suggesting regulation of IAV infection by modulating the expression of inflammatory cytokines (Liu et al., 2013). This drug is not approved by de FDA for the treatment of influenza and is therefore only used in Russia.

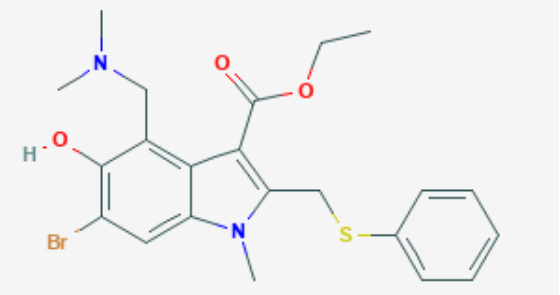

Figure 2: structural formula of Arbidol. Retrieved from: https://pubchem.ncbi.nlm.nih.gov/compound/Arbidol

Nitazoxanide is a compound currently in the phase three clinical trial as of 2017 (Koszalka et al. 2017). Developed in the 1970s in the United States, its first commercial application was as an antiprotozoal agent, but later research identified its broad-spectrum antiviral capabilities. Nitazoxanide is converted in the body to its circulating metabolite tizoxanide. Influenza virus has been shown to be sensitive to tizoxanide (IC_{50}: 0.2 – 1.5 µg/mL, with a MOI of 5 PFU/cell to 0.001 PFU/cell. Its antiviral effectiveness did not change when using different cell cultures, and a high resistance barrier was observed. The mechanism of action of Nitazoxanide depends on interference HA maturation after translation. NA and M2 protein are unaffected by Nitazoxanide. In a phase 2b/3 double-blind, randomized, placebo-controlled clinical trial, subjects receiving 600 mg Nitazoxanide experienced shorter times of symptom alleviation ($p = 0.008$), compared to subjects receiving placebo. A phase 3 clinical trial of 2000 subjects experiencing influenza symptoms has been conducted for treatment of acute uncomplicated influenza by Romark Laboratories. Their results showed a reduction of the symptom duration when treated twice daily with 600 mg Nitazoxanide. However, further research is necessary to investigate drug efficacy in combination with existing drugs and in complicated influenza infections (Haffizulla et al., 2014) (Rossignol, 2014) (McMahon & Martin-Loeches, 2017).

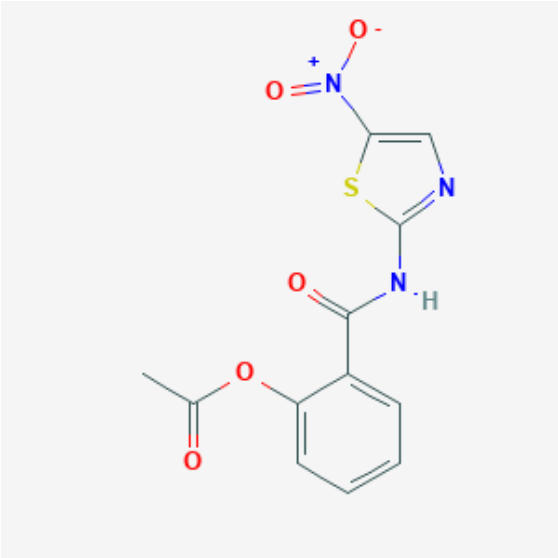

Figure 3: structural formula of Nitazoxanide. Retrieved from: https://pubchem.ncbi.nlm.nih.gov/compound/41684#section=2D-Structure

Two compounds, MBX2329 and MBX2546, were identified in the United States in 2014 as HA-mediated viral entry inhibitors using pseudotype virus-based high-throughput screens of over 100.000 small molecules. These compounds were able to inhibit different influenza A H1N1 strains with an IC_{50} ranging from 0.29 – 0.53 µM, and 0.3 – 5.8 µM for MBX2329 and MBX2546 respectively. Inhibitory activity against influenza A H3N2 strains was significantly lower, or non-existent. Both MBX2329 and MBX2546 were found to bind to the conserved epitope in the HA stem region, thereby inhibiting HA-mediated fusion by preventing pH-dependent conformational changes. Strong synergic properties were observed when these compound were administered in combination with oseltamivir, a NA inhibitor (Basu et al. 2014). Currently, no clinical trials are conducted to test treatment applications for these compounds.

Peptides targeting HA

For almost a century, peptides have played a major role in medicine, with notable therapeutic peptides being insulin and vasopressin. (Lau & Dunn, 2018) Being constantly under development, therapeutic applications increase continually, with newer peptides created being capable of targeting multiple targets simultaneously (Lau & Dunn, 2018). Determining peptides' therapeutic properties against influenza virus is still being investigated.

Examining pre-existing host innate defense mechanisms of other species, a research group from the United States found a host defense peptide able to target and inhibit HA of influenza viruses. Based on pre-existing studies showing that amphibians are capable of excreting large amounts of peptides to fight microbial infections, the research group collected skin secretions of the south Indian frog *Hydrophylax bahuvistara* and identified its constituents. 32 host defense peptides were identified and after plating with MDCK epithelium cells and counting virus plaques three days later, 4 peptides showed a viral titer reduction of more than 50%. After checking for toxicity against mammalian cells, one compound, named Urumin, remained. The IC_{50} of Urumin was 3.8 µM with a toxic dose against mammalian cells of 2450 µM. Using eight different influenza A H1N1 strains and four influenza A H3N2 strains, subtype specificity of Urumin was determined. Virus titer of all H1N1 strains was decreased by at least 60%, however, virus titer of H3N2 strains were reduced by less than 50%. Determining virus inhibition after adding Urumin to H1N1 strains that have circulated for over half a century, it was reasoned that Urumin binds to a conserved part of influenza A. Inhibition of a chimeric H9N3 virus (H9 head region, PR8 H1 stalk) but not non-chimeric H9N3 virus (H9 head region and stalk) confirmed this and showed the binding point to be H1 HA. Electron microscopy showed that Urumin-treated influenza virions were actively being destroyed. *In vivo* studies of PR8 virus infected mice showed that intranasal admission of Urumin was able to inhibit influenza induced disease (Holthausen et al., 2017). At this

moment, no clinical trial is set up to investigate Urumin's treatment possibilities.

Based on recent breakthroughs in identification of human broadly neutralizing antibodies (bnAbs) targeting specific parts of the conserved HA stalk region, researchers from the United States developed a cyclic HA fusion inhibitor peptide. Using the bnAb FI6v3, a starting point for a peptide based on its looping HA-binding epitope was created. By continually optimizing the cyclic peptide by swapping proteins of the peptide and determining its affinity for HA of different influenza strains, an effective peptidic inhibitor was created. Since the peptide is based on bnAbs capable of stabilizing HA at low pH, thereby preventing conformational changes necessary for fusion, the researchers expected the peptide to have similar mechanisms. Crystallization of the peptide-HA complexes under low pH conditions showed H1 HA structures to be identical to prefusion H1 HA states. To test whether the cyclic peptide would be stable *in vitro*, *in vitro* and *in vivo* pharmacokinetic studies were performed using human and mouse plasma. No proteolytic degradation was observed, nor cytotoxic effects in human lung derived Calu-3 cells. *In vivo* studies demonstrated a half life of 2.7 hours and plasma clearance of 24 hours (Kadam et al., 2017).

Monoclonal antibodies targeting HA

Creating monoclonal antibodies against HA is a challenging feat. It is possible to find mAbs that target the globular head region of HA, however, due to great variability of the head between different strains, these mAbs are not broadly inhibiting (Corti, 2017). A better, though more challenging target is the conserved stalk region of HA.

One such compound that targets the stalk of HA is MHAA4549A. Developed the United States, it is in its second phase of clinical trial as of 2018. By binding to the conserved HA stalk region and blocking fusion, MHAA4549A is able to inhibit all seasonal influenza A strains. Moreover, it induces immune cells to lyse influenza-infected cells by binding to HA on their surface (Deng et al., 2018). Two phase 1 studies have been performed, demonstrating the safety and tolerability of 1.5, 5, 15 or 45 mg/kg MHAA4549A admissions (Lim et al., 2016). For a phase IIa clinical trial, the pharmacokinetics of MHAA4549A were studied. MHAA4549A isolated from a human plasma blast cell from an influenza-vaccinated donor. A group of 100 healthy subjects was administered live influenza A virus (H3N2) intranasally. After a day, 32 subjects received placebo infusion and 60 received an infusion of either 400, 1200 or 3600 mg MHAA4549A. Eight subjects received oseltamivir as a positive control group. Viral load was collected three times daily for 8 days and treatment-emergent adverse events (TEAEs) were recorded. A pharmacokinetic assay showed a quick distribution phase, followed by a slower elimination phase of the antibody. No significant TEAEs were recorded, and one percent of subjects showed anti-drug antibodies (Deng et al., 2018). Another study has showed that a 3600 mg dose of MHAA4549A leads to a reduction in viral burden, peak viral load, viral shedding duration, and infection characteristics. No reduction was found in subjects administered 1200 mg doses (McBride et al., 2017).

VIS410 is a IgG1 monoclonal antibody developed in the United States for the possible treatment of H7N9 and N3N2 strains of influenza. These strains can cause pneumonia with acute respiratory distress syndrome. However, VIS410 was observed also capable of binding the stalk region of H1, H2, H3, H5, H6, H7, and H9 subtypes, implying broader antiviral capabilities (Tharakaraman et al., 2015). Mice infected with an H7N9 strain of influenza showed a reduction in lung viral load after treatment with 50 mg/kg VIS410, demonstrating virus replication and spread inhibition capabilities of VIS410 (Baranovich et

al., 2016). A phase one clinical trial consisting of 41 subjects aimed to determine the safety (compared to placebo) and pharmacokinetics of VIS410. Safety was determined by measuring and examining a variety of physiological aspects, ranging from vital signs to urinalysis. Blood samples were taken for pharmacokinetic analysis and anti-drug antibodies. This study showed that VIS410 is generally safe, with diarrhea being the most common side-effect. Anti-drug antibodies were only observed at low levels in a few subjects (Wollacott et al., 2016). A phase two clinical trial was set up to determine the safety and efficacy of VIS410 in adults with uncomplicated influenza A infections. 148 subjects from different countries positively tested for influenza were administered a single infusion of 2000 or 4000 mg VIS410 and compared to placebo. Physiological assessments (among which a FLU-PRO symptom score), nasopharyngeal viral load, pharmacokinetics were determined. Based on FLU-PRO symptom scores, a reduction of baseline in mean total symptom scores was significantly larger in the VIS410-treated group compared to placebo up until day 6. After this, reductions in symptom scores were similar. 39.2% of subjects reported adverse events. Subjects that had received 4000 mg VIS410 reported adverse effects significantly more than those receiving placebo ($p = 0.014$), with diarrhea being the most common adverse effect. A reduction in nasopharyngeal viral load was observed in subjects receiving VIS410. In 23% of VIS410 recipients, measurable amounts of anti-drug antibodies were observed. However, this had close to no impact on VIS410 pharmacokinetics in the month following treatment (Hershberger et al., 2019).

Matrix protein 2

Discovered in 1964, amantadine was the first discovered inhibitor of M2 protein. As a derivative of adamantane, its workings depend on inserting itself into the M2 protein ion channel, thereby blocking proton flow into the virus particle. This stop dissociation of M1 protein from vRNPs, inhibiting viral entry. Rimantadine is another compound derived from adamantane with M2 protein inhibiting properties (Król et al., 2014). Currently, the CDC and FDA allow the use of adamantanes for treatment of influenza A, however, their use is not recommended due to widespread resistance of circulating H1N1 and H3N2 subtypes (CDC, 2018). A mutation of S31N and V27A of the M2 protein allows for normal proton conductance while disrupting the adamantane binding pocket, preventing its antiviral machinery (Pielak et al., 2009). New drugs are necessary to stop viral entry via inhibition of the M2 protein.

WJ379 and BC035 are two compounds created for resistant influenza virus strains. These drugs are capable of inhibiting influenza A strains with S31N-mutated M2 protons, resulting in effective channel blockage and antiviral effects. To test therapeutic potential, the effectiveness of these drugs was tested on currently circulating IAV strains. Moreover, resistance development against these drugs was tested. First, MDCK cells were used to determine the cytotoxicity and selectivity of the compounds for further testing. The highest drug concentrations found to cause minimal cytotoxicity was observed to be 5 and 15 μM for WJ379 and BC035 respectively. Plaque assays against multidrug-resistant IAV for both compounds were then set up with concentrations of WJ379 and BC035 ranging from 3 nM to 3 μM and 30 nM to 30 μM for WJ379 and BC035 respectively. It was observed that rising concentrations of drugs lead to lower plaque numbers, indicating an antiviral effect against multidrug-resistant IAVs. To test the genetic barrier to drug resistance, MDCK infected with a resistant IAV were serially infused with WJ379 or BC035. After four passages, resistant strains were selected and after eleven passages, complete resistance to WJ379 and BC035 was observed. The resistant strains were found to have L26I and I32T

mutations for treatment with WJ379 and BC035 respectively. Using a H1N1 influenza strain with a N31S mutation, emergence of resistance to WJ379 and BC035 compared to amantadines was tested. This strain was observed resistant to WJ379 and BC035 after four passages, however, resistance to amantadines was observed after just one passage (Ma et al., 2016).

To find M2 protein inhibitors not derived from an amantadine scaffold, one research group performed a high-throughput screening assay using over 100.000 compounds on *Saccharomyces cerevisiae*. This yeast expressed WT M2 protein, which causes slow growth of the organism, unless acted upon by a M2 protein inhibitor. Of the over 100.000 compounds, one compound showed an inhibitory potency equal to amantadine. This spirothiazamenthane compound was able to cause normal growth of the yeast with an EC_{50} of 0.03 μM, but weak growth restoration in amantadine-resistant (V27A) yeast strains (EC_{50} = 100 μM), and no growth restoration against S31N-mutated strains. To test whether this compound could inhibit live influenza virus replication, MDCK cells infected with an amantadine-sensitive H3N2 IAV strain was treated with the novel compound and amantadine. Both compounds could inhibit cell deaths in similar concentration (3 μM) ranges and neither was toxic to the cells. This compound was further modified to find derivatives capable of inhibiting amantadine-resistant M2 protein strains. Recombinant influenza A strains bearing S31N and V27A substitutions were created and MDCK cells were infected. Using a 96-well plate miniplaque assay, multiple spirothiazamenthane derivatives were tested for inhibitory properties. It was found that some derivatives were able to inhibit the S31N and V27A strains, but at a cost of lower potency towards the WT strain. However, the same compounds were able to inhibit WT M2 protein, but not S31N mutants in the yeast assay, possibly due to inhibition of a protein other than M2 (Arns et al., 2016).

Discussion

Every year, many people suffer from the consequences of an influenza infection. To combat this virus, yearly vaccination programs are set up and antiviral therapeutics are used. The economic burden for the United States of seasonal influenza infection was estimated at 11.2 billion dollars (Putri et al., 2018), not to mention the physical burden of those infected. Current antiviral treatment options rely on neuraminidase and cap-dependent nuclease inhibition. However, resistance against neuraminidase inhibitors Zanamivir and Oseltamivir can occur fast (Trebbien et al., 2017). New compounds capable of treating influenza infections are necessary to be able to treat normal and drug-resistant influenza subtypes. The current review highlights several ways researchers try to tackle this problem, with some way showing greater potential than others.

The monoclonal antibody MHAA4549A has shown potential for treatment of IAV infection, however the efficacy of the mAb is difficult to determine due to great variability between preexisting neuraminidase antibodies and $CD4^+$ T cells interference of subjects. (McBride et al., 2017). The role of MHAA4549A on influenza infection is therefore more difficult to determine. Thus, future research is necessary to further optimize ways of MHAA4549A inhibition quantification. For VIS410, adverse effects of medication were reported in roughly a third of subjects. Though, these effects could be mitigated using a single dose of ibuprofen or aspirin. To test the efficacy of VIS410, nasopharyngeal swabs were taken to determine virus titer, however, viral titers of the lungs would show more relevant results since lung involvement of IAV infection correlates with higher infection severity. Because VIS410 targets patients with lower

respiratory tract infections, further research will be necessary to accurately quantify the virus titer in the lungs of IAV infected patients (Herschberger, 2019). Development of monoclonal antibodies against HA is an upcoming research field with promising therapeutic agents. However, as shown, many mAbs are still in the early states of development and more time will be necessary before these can accurately be used as antiviral options.

Currently, Arbidol, Nitazoxanide, and Urumin have shown great potential for future antiviral IAV treatment. Though it has been in use in Russia for over two decades, Arbidol remains prohibited by the FDA for use against influenza infections, despite its clinical significance (Liu et al., 2017). Nitazoxanide is currently in third phase clinical trials (Koszalka et al., 2017) and has been demonstrated to reduce symptom duration after influenza infection. Future research is still necessary to study its efficacy in combination with other medicine and in the treatment of complicated influenza infections (Haffizulla et al., 2014) (Rossignol, 2014) (McMahon & Martin-Loeches, 2017). It is important to take into account that timely administration of antivirals to combat disease is paramount to their effectiveness. Administration of antivirals should preferably be within 48 hours after a patient becomes ill (Koonin & Patel, 2018). In their study, Haffizulla et al. only admitted patients whose symptoms (cough, nasal discharge or congestion, sneezing, or sore throat) appeared in 48 hours or less. Since timely application of influenza antivirals is often made difficult by the fact that the disease has very general symptoms, only taking into account patients whose general influenza symptoms appeared less than 48 hours ago makes the result of this study more credible. Finally, Urumin, an amphibian derived peptide has been demonstrated able to combat IAVs via HA inhibition in phase 2 clinical trials. Their virucidal activity and binding site, however, remains yet to be discovered. Mice used in this study were administered 20 μg Urumin 5 minutes before being infected with IAV, fulfilling the early administration requirement for effective antiviral treatment, and solidifying the study's results (Holthausen et al., 2017). In my opinion, despite still in need of further research, Nitazoxanide could be a strong contender for IAV inhibition via the HA pathway. It has shown great potential in the treatment of uncomplicated influenza with a high barrier of emergence of resistance. Treatment of patients with severe acute respiratory illness (SARI) with Nitazoxanide did not result in a reduction of hospital stay (Gamiño-Arroyo et al., 2019), suggesting that the use of this drug might be restricted to uncomplicated influenza cases.

Inhibitors for matrix protein 2 already exist, however, due to resistance they are not recommended. Because of this, antiviral compounds targeting M2 need to be effective against the wild-type strains, as well as the mutated strains. This has shown to be a difficult feat. Spirothiazamenthane was the only compound observed capable of inhibiting M2 protein of a collection of over 100.000 compounds, demonstrating the rarity of such compounds. Moreover, this compound was not able to inhibit amantadine-resistant influenza strains, while its derivatives were capable of inhibiting amantadine-resistant strains, but not wild-type. Other compounds, like WJ379 and BC035 were created after careful crafting and compound modification (Wang et al., 2013) (Wu et al., 2014) and used in further research (Ma et al., 2016) to test their antiviral properties. Though looking promising, these compounds are still in an early state of development. This, combined with the small number of effective M2 protein inhibitors capable of inhibiting both resistant and non-resistant IAV strains already discovered, suggests that novel, clinically applicable antiviral targets are more likely to develop in other fields first. Current research is still conducted to study the function and possible inhibition of M2 protein. Using metadynamics

simulations binding trajectories of M2 inhibitors are determined (Sakai et al., 2018). Furthermore, recent development Proton Flux (pHlux) Assays can be used to study the inhibition of M2 protein by high-throughput screening (Santner et al., 2018).

The influenza life cycle is dependent on many factors, from virus entry and replication to virion assembly and exocytosis. Many of the factors that play a role in this cycle could be an antiviral target and research towards existing and new antivirals should always continue. The current trend of development of antivirals targeting conserved parts of HA show promising results. However, M2 protein inhibition still mainly relies on blockage of the ion channel, thereby preventing proton flow. Amantadine and Rimantadine both worked in this fashion, however, a single mutation made these antivirals ineffective. To prevent resistance of future M2 protein inhibitors, development against conserved parts of the protein is important.

For future research, antiviral targets of HA should be investigated more. Inhibition of this protein can happen via several ways, as shown in this paper. Newer routes involve targeting HA using host cell mimetics, thereby inhibiting HA-sialic acid binding (Chen & Guo, 2016), or might involve the host factors that aid in virus replication (Watanabe & Kawaoka, 2015). The latter has the added benefit of being less prone to resistance emergence, an important factor for future antivirals.

Acknowledgements

I would like to thank dr. Yoshita Bhide and prof. dr. Anke Huckriede of the Faculty of Science and Engineering of the UMCG, Groningen, The Netherlands for their help and input during the writing of this review.

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