Review Article

Antiviral Drugs for the Control of Pandemic Influenza Virus

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Abstract

In the advent of an influenza virus pandemic it is likely that the administration of antiviral drugs will be an important first line of defence against the virus. The drugs currently in use are effective against seasonal influenza virus infection, and some cases have been used in the treatment of patients infected with the avian H5N1 influenza virus. However, it is becoming clear that the emergence of drug-resistant viruses will potentially be a major problem in the future efforts to control influenza virus infection. In addition, during a new pandemic, sufficient quantities of these agents will need to be distributed to many different parts of the world, possibly at short notice. In this review we provide an overview of some of the drugs that are currently available for the treatment and prevention of influenza virus infection. In addition, basic research on influenza virus is providing a much better understanding of the biology of the virus, which is offering the possibility of new anti-influenza virus drugs. We therefore also review some new antiviral strategies that are being reported in the scientific literature, which may form the basis of the next generation of antiviral strategies during a future influenza virus pandemic.

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Introduction

Over the past 100 years there have been 3 major influenza virus pandemics, which have among them claimed millions of lives. In 1918 the first of these pandemics occurred suddenly, and without warning. The source of this pandemic is likely to have been Kansas in the USA, but by the end of 1918 the virus had spread across the globe. Two subsequent major pandemics followed in 1957 and 1968, but these were less severe than that experienced in 1918. However, it is worth noting that during these latter pandemics there was no adequate time to prepare suitable quantities of vaccine against the respective pandemic virus strains. There is currently an influenza virus pandemic among birds, predominantly involving the highly pathogenic avian influenza (HPAI) H5N1 virus. It is now known that this virus can be directly transmitted to humans from birds, resulting in a high mortality rate in infected individuals. However, several other avian influenza virus strains have been reported to infect humans (e.g. H7N7, H7N3, H7N2, H9N2[1]), and although the most recent cases of transmission have involved H5N1, it is by no means certain that a new pandemic will be caused by H5N1. It is difficult to predict with certainty what vaccine will be effective in a future pandemic, and a similar scenario to that faced by people in previous pandemics may arise. The use of drugs against influenza virus could therefore represent a first line defence against a new pandemic, allowing the control of the infection until sufficient quantities of a suitable vaccine can be produced. In this article we will discuss some of the current drugs that are available to prevent influenza virus infection, and describe more recent developments that could make the transition from "bench to bedside" in the near future.

The Current Anti-influenza Drugs

Amantadine is effective against all influenza A virus types,[2,3] and was originally approved in 1976 for the treatment of influenza A virus infection. Several formulations of amantadine are currently available on prescription (e.g. Symmetrel®). It should be administered as soon as possible after the onset of symptoms, and continued for at least 2 days after the disappearance of symptoms. A number of reports have suggested that amantadine treatment is associated with several side effects, including dizziness, insomnia, nervousness and nausea.[4]

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Rimantadine (Flumadine®) is also available on prescription, and although it is closely related to amantadine, it exhibits fewer side effects in treated individuals.

In the final stage in the virus entry process, the virus genomic segments must be released from the incoming virus particle, to allow transport of the virus genome to the cell nucleus,[5] and the virus encoded matrix-2 (M2) protein plays an important role in this process. It exhibits an essential ion channel activity that allows the transport of hydrogen ions (H^+) across the virus envelope into the internalised virus particle.[6,7] This increases acidity within the virus particle causing the dissociation of the virus gene segments from the internalised virus particle.[8] Amantadine binds to the transmembrane region of the M2 protein and blocks the membrane pore,[9-11] thus preventing the uncoating process.[12] In HPAI viruses (e.g. H5 and H7), amantadine also acts by inhibiting the final stages of influenza virus maturation. In all influenza A viruses, the HA protein is initially synthesised as an inactive precursor (HA0) that is activated by proteolytic cleavage into 2 smaller protein subunits, called HA1 and HA2.[13] In HPAI viruses, the HA protein undergoes intracellular cleavage in the trans-Golgi urea, and this activated intracellular form of the HA protein can prematurely change into its fusogenic form in the more acidic post-Golgi compartments. In these viruses, the M2 protein protects the HA protein by maintaining the pH in these cell compartments close to neutral.[14]

Early studies demonstrated that drug-resistant viruses were readily generated in tissue culture in the presence of amantadine, and that these mutant viruses appeared to grow as well as wild type viruses.[15] These mutations gave rise to specific amino acid changes in the transmembrane region of the M2 protein that prevented amantadine binding.[9,10] Several early studies showed that administering amantadine to people resulted in the production of drug-resistant viruses,[16-19] and more recent epidemiological studies have demonstrated that amantadine-resistant influenza virus strains are currently circulating in several countries.[20] Furthermore, in addition to human influenza virus isolates, the emergence of amantadine resistance has also been observed in avian influenza viruses.[21] Since drug-resistant mutants are readily generated in the presence of amantadine, the drug is not recommended as a prophylactic for seasonal influenza, suggesting that amantadine may be of limited use in a future pandemic.

At the same time as the development of amantadine, the efficacy of other drugs that were able to inhibit influenza virus replication was examined. Ribavirin is a nucleoside analogue that interferes with the duplication of either DNA or RNA.[22-24] Early studies showed that, in tissue culture, ribavirin was effective against influenza A and B,[25,26] and some studies have suggested that it may be effective in the treatment of influenza virus. However, carefully controlled clinical studies to study the efficacy of ribavirin in patients infected with influenza virus have given inconsistent results.[27] Although ribavirin is recommended for the treatment of several virus infections, including respiratory syncytial virus and hepatitis C virus,[28] it is currently not approved by the Food and Drug Administration (USA) or Health Protection Agency (UK) for the treatment of influenza virus infection.

Partly as a result of the emergence of amantadine-resistant influenza viruses, alternative antiviral strategies have been developed. The sialidase activity in the virus neuramindase (NA) protein plays a critical role in the influenza virus replication cycle, and the design of NA protein inhibitors is currently one of the most common approaches in the development of anti-influenza virus drugs. The NA protein was the first virus glycoprotein for which a high resolution molecular structure was obtained.[29] Subsequent functional and structural studies of the NA protein have allowed several pharmaceutical companies to produce NA inhibitors using a structure-based inhibitor design. Zanamivir and oseltamivir are the 2 virus NA inhibitors that are currently available for the prevention of virus infection (see review[30]). These drugs target the active site of the NA protein, thus inhibiting its sialidase activity that is essential for virus release (see review[31]). They are effective both *in vitro* and *in vivo*, and are effective against both influenza A and B viruses. Zanamivir was the first NA inhibitor available, and it is currently marketed by GlaxoSmithKline under the market name of Relenza®. Oseltamivir, initially developed by Gilead Sciences, is currently produced by Hoffman-La Roche under the market name of Tamiflu®. These drugs, in particular oseltamivir, are currently the primary drugs available for the prevention of influenza virus infection. An injectable form of NA inhibitor called peramivir has been developed by BioCryst Pharmaceuticals Inc, and it is currently undergoing phase II clinical trials in the USA.

Although zanamivir and oseltamivir are similar in their mode of action, the drugs have different biochemical properties, which influence how they are administered. Due to the poor bioavailability of zanamivir it must be administered by inhalation,[32] and there have been several reports of respiratory complications following the inhalation of Relenza®.[33,34] The route of administration has been a major reason for the limited use of zanamivir by the public. In contrast, oseltamivir is administered orally as a pro-drug ester, usually as oseltamivir carboxylate. Once administered, the pro-drug is processed by human carboxyesterase into its active form.[35] It is currently the drug of choice for the prevention of influenza virus infection, and as a consequence it is being stockpiled by organisations in many countries in

anticipation of a pandemic.[36] Although it has general acceptance by the public, there have been a series of reports regarding serious side effects that are associated with the drug, which have included nausea and vomiting.[37] In rare cases, neurological side-effects have been reported that have led to several deaths among teenagers,[38,39] and in some countries warnings have been issued about administering oseltamivir to this age group.[40]

Early studies on the effect of oseltamivir and zaminivir on influenza virus replication in tissue culture showed a variation in the degree of susceptibility of several virus isolates,[41] and the degree of drug susceptibility for each virus correlated with the affinity of its HA protein for sialic acid.[42] The prolonged passage of influenza virus in tissue culture in the presence of these drugs led to the emergence of drug-resistant mutants, which exhibited amino acid changes in both the HA and NA proteins.[43-46] The changes in the HA protein reduced the affinity of the virus for its sialic acid receptor, and presumably reduced its dependency on the NA protein. There is no evidence of the emergence of drug-resistant viruses in patients treated with zanamivir, which is presumably a reflection of its low usage. However, drug-resistant H3N2 and H1N1 viruses were isolated from oseltamivir-treated children,[36,47] and the emergence of drug resistance in patients infected with H5N1 has been reported in Vietnam.[48]

Anti-influenza Drugs, the Future Perspective

The available evidence suggests that the current drugs used to prevent influenza virus infection eventually give rise to drug-resistant viruses. Furthermore, a pandemic will potentially involve a greater part of the world's population, requiring the capacity to mass produce effective drugs on a global scale. These are 2 major factors that are driving the search for new antiviral strategies that are both efficacious in preventing influenza virus infection, and that will be cost-effective. There are many potential anti-influenza virus strategies that are currently being described in the literature that may become effective in the clinic, and here we will examine some of these different approaches.

Although the virus polymerase is an obvious therapeutic target, there is currently no high-resolution structural data for the proteins that form the virus polymerase complex. This has hampered the development of polymerase inhibitors using a structure-based approach. Although most of the current focus is on the development of drugs which target the activity of the NA protein, agents that target the HA are also effective in the laboratory setting. These agents have a great potential to be developed into antiviral drugs for the treatment and prevention of influenza virus infection, and the availability of several high-resolution HA structures should facilitate their further development.[49]

There is a long history of the use of passive immunisation to control virus infections (see review[50]), and humanised antibodies that exhibit neutralising activity are currently being developed for the treatment and prevention of several viruses. In at least 1 case, a humanised monoclonal antibody against the respiratory syncytial virus (RSV) fusion protein (e.g. Synagis® from MedImmune) has been approved for clinical use, and has been used for several years in the prevention of RSV infection in hospitalised high-risk patients.[51] Similarly, antibodies against the HA protein that can neutralise virus infection (e.g. by blocking cell attachment[52]) can be potentially developed into an effective influenza virus prophylactic. In mice challenged with influenza A virus, neutralising antibodies to the HA glycoprotein was shown to be effective both as a prophylactic and a therapeutic.[53] Several candidate antibodies against H5N1 have been identified, and have found to be effective in neutralising the virus infectivity in tissue culture and in experimental animals. Recent studies found that equine hyperimmune globulin F(ab')2,[54] humanised mouse monoclonal antibodies[55] and human monoclonal antibodies generated from the memory cells of recovered patients,[56] protected mice infected with H5N1. Although antibody escape mutants represent a serious drawback,[57] it is envisaged that several different humanised antibodies given in combination may facilitate their longer term effectiveness. Although immunoprophylaxis using humanised antibodies could be an option during a pandemic, the probable high cost to mass produce them is an obstacle. An even more serious obstacle in the advent of a sudden pandemic will be the lag-time that may be required to produce sufficient quantities of the humanised antibodies, particularly in the advent of a new virus subtype for which neutralising antibodies are not yet available.

Short interfering (si)RNAs are double-stranded RNA duplexes that are able to inhibit the expression of specific genes by inducing sequence-specific degradation of target mRNA through the RNA interference (RNAi) pathway (see review[58]). RNAi as a generic antiviral strategy is efficacious, both *in vitro* and in animal models, against several viruses, including influenza A virus (see reviews[59,60]). siRNAs designed against conserved sequences in the influenza A virus nucleoprotein, acidic polymerase and matrix genes, are able to suppress virus replication in tissue culture, and significantly reduced virus yields in tissue culture, and in the lungs of infected mice.[61-64] The antiviral effect was shown to be due to specific degradation of virus mRNA and was effective against a broad spectrum of different human and avian influenza subtypes.[63] There are currently several biotechnology companies developing RNAi-based drugs for clinical use. Alnylam Pharmaceuticals (see http://www.alnylam.com for further

details) have successfully completed phase I clinical trials for siRNAs against the human RSV in mid-2007, showing that administration of the siRNAs was well-tolerated and safe. A Phase II clinical trial is currently in progress to determine the efficacy of the siRNA drug in volunteer patients, and initial reports indicate that the drug demonstrated "statistically significant antiviral activity".[65] Alnylam, in collaboration with Novartis, is also conducting preclinical trials for siRNAs against influenza virus. One of the major advantages of RNAi-based therapeutics in a pandemic influenza situation is that the design of specific siRNAs only requires knowledge of the gene sequence, and the siRNAs synthesis can be achieved within a short period of time and at relatively low cost. However, there have been reports that some antiviral siRNA molecules can exhibit off-target effects that inhibit the expression of some host genes.[66,67] Therefore, before a siRNA approach can be used in the clinical scenario, it will have to be carefully evaluated to examine its specificity in silencing virus gene expression.

One of the key challenges in RNAi-based therapeutics is the issue of delivery to specific cells or tissues, especially in the case of a systemic disease. Selective non-viral methods of delivery include siRNAs coupled to nanoparticles coated with receptor-targeting ligands, antibody-fragments or aptamers.[68-70] For long-term delivery of RNAi-drugs, viral delivery methods using lentiviruses and adenoviruses have been examined.[71,72] Reduced lung titres and pulmonary pathology were observed in mice infected with RSV when "naked" siRNAs or siRNAs coupled to nanoparticles were administered intranasally.[73,74] Likewise for influenza, siRNAs delivered intranasally could be effective both as prophylactic and therapeutic agents.

Many RNA viruses replicate their virus genome via a double-stranded RNA intermediate, referred to as a replicative intermediate (RI). Such molecules are not produced in the host cell, and their presence in mammalian cells stimulates an antiviral response. This is mediated by a Toll-like receptor 3 (TLR3) which is able to recognise the RI, resulting in the production of alpha/beta interferon (IFN-α/β).[75] Molecules have been synthesised that mimic the structure of RIs, and these are being evaluated as antiviral drugs. Studies using synthetic dsRNA molecules composed of polyriboinosinic polyribocytidylic acid (poly[I:C]) have been effective in countering influenza virus infection.[76,77] It is envisaged that a similar method to deliver these molecules as that described for siRNA could be used. Although the data obtained in the laboratory looks promising, it remains to be established if this approach will be effective in preventing influenza virus infection in human patients.

Over the past 20 years there has been an increasing knowledge on how the influenza virus is able to interact with the host cell during virus replication, and in particular the role that host cell factors play in the virus replication cycle.[78,79] Although many current antiviral strategies focus on drugs that inhibit activities associated with specific virus proteins, it is perhaps worth mentioning drugs whose antiviral activities may arise because they modify a host cell activity that is essential for virus replication, which may be beneficial in the treatment of acute virus infections. The activity of these drugs will be more generic in nature, and should potentially have a broad-spectrum antiviral activity, and many of these potential drugs are currently available for the treatment of other medical conditions. In the final part of this review, 2 of these approaches will be briefly discussed.

Chloroquine has been used for several years in the treatment or prevention of malaria caused by *Plasmodium falciparum*.[80] Although the appearance of drug-resistant parasites has started to limit its use in malaria control, chloroquine also inhibits bacterial and fungal growth (see review[81]), and its efficacy as an antiviral is currently being evaluated. Treatment of cells with chloroquine elevates the endosomal pH, and previous studies have demonstrated its inhibitory effects on influenza virus replication.[82,83] More recent studies have re-examined its anti-influenza activity in tissue culture, and the available data is encouraging,[84,85] but it will need to be further evaluated to determine its efficacy as an anti-influenza virus drug in humans. Furthermore, chloroquine treatment can cause side-effects in humans,[86,87] and the effects of taking this drug on a long-term basis will have to be evaluated.

Work in several laboratories have shown that during influenza virus replication specialised host-cell membranes called lipid-rafts play an important role in the virus maturation process.[88-90] Lipid-rafts are characterised by the enrichment of certain classes of lipid, including cholesterol, that are important for their stability. Drugs that are collectively referred to as statins are currently available for the treatment of individuals with abnormally high cholesterol levels in their blood. These drugs are also able to disrupt lipid-raft membranes by removing membrane-bound cholesterol, and in so doing are able to inhibit the assembly process of influenza virus in tissue culture.[90] The role that lipid-rafts play during RSV replication has been established[91,92] and recent studies have shown that lovastatin-treated mice exposed to RSV did not show the symptoms associated with RSV infection e.g. weight loss.[93] This has suggested that statins may be an effective drug in treating virus infection, including influenza virus. In 2 recent studies, it was observed that people taking statins were less likely to develop severe complications that are associated with

influenza virus infection,[94,95] and the reason for this is currently unclear. Although lipid-rafts play an important role in the virus maturation process, they are also pivotal to many cellular processes, including cell signalling pathways. Some cytokine signalling pathways are associated with lipid-rafts, and it is possible that statins may counter the detrimental effects of virus infection that arise by excessive cytokine production i.e. preventing a cytokine storm. Although side-effects have been reported in individuals receiving these drugs, the extent and type of side effect reported is dependent on the type of statin involved. However, given the reported immunomodulatory effects of statins, the consequences of their long-term use during an influenza pandemic will have to be carefully evaluated.

Conclusion

Since the last influenza virus pandemic, our knowledge of how influenza virus interacts with its host has greatly increased. This increase in knowledge has not only facilitated our understanding of the biology of influenza virus, but has opened up new avenues in the search for antiviral strategies. It is clear that the development of new antiviral strategies will not be a major limitation in countering a new pandemic. However, it is likely that during a pandemic people that live in many parts of the world will not be able to afford the cost of drugs that are expensive to produce. One of the major challenges in a new pandemic will be the availability of anti-influenza virus drugs that can be easily mass produced, and distributed to all parts of the world. This challenge will require a partnership between researchers, government bodies and the pharmaceutical industry.

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