EXISTING INFLUENZA ANTIVIRALS: THEIR MECHANISMS OF ACTION AND POTENTIAL IN THE FACE OF AVIAN INFLUENZA*

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INTRODUCTION

The outbreaks of avian influenza A (H5N1) in Southeast Asia² (with several clusters recently identified in Indonesia³), the expanding geographic distribution of this epizootic virus (with well-documented cases in Eastern Turkey in 2006⁴), and the ability of influenza A to transfer to humans and cause severe infection have aroused serious concerns on the control measures that should be undertaken if a pandemic with influenza A, whether avian or human, would strike. In the wake of such pandemic, several preventive and therapeutic strategies have been formulated, among which are the stockpiling of antiviral drugs^{2,5} and in particular the neuraminidase inhibitors oseltamivir (Tamiflu™) and zanamivir (Relenza™).

*This chapter represents an adjusted and updated version of De Clercq, E. Antiviral agents active against influenza viruses, *Nature Rev. Drug Discovery* **5**:1015–1025 (2006).¹

Combating the Threat of Pandemic Influenza: Drug Discovery Approaches,
Edited by Paul F. Torrence
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Many governments are now stockpiling large quantities of oseltamivir, providing an expanding and durable market for the drug.⁶ Public and media interest in avian influenza has sparked demand for oseltamivir in the private sector, leading to increases in prescriptions amid fears of a shortage.⁶ Roche has announced plans to dramatically increase production by as much as 8- to 10-fold to meet these demands. Other companies are now considering to resurrect their antiviral programs to take advantage of the increased interest generated by the possibility of a pandemic.⁶ Given the considerable challenges to the rapid development of an effective vaccine against influenza A (H5N1) antiviral agents will play an important role as a first-line defense if a new pandemic strikes.⁷ However, the large-scale use of drugs for chemoprophylaxis and chemotherapy imposes new challenges—that is, those of the selection and ensuing transmission of drug-resistant virus strains.⁷

There are, in principle, two mechanisms by which pandemic influenza may originate (i) by direct transmission (of a mutated virus?) from animal (bird) to man, as happened in 1918 with the “Spanish influenza” (H1N1), “the mother of all pandemics,”⁸ or (ii) through reassortment of an avian with a human influenza virus, as occurred in 1957 with the “Asian influenza” (H2N2) and, again, in 1968 with the “Hong Kong influenza” (H3N2)⁹ (Fig. 1.1).¹⁰ Whether a new influenza pandemic may arise through (i) antigenic “drift” from an avian influenza or (ii) antigenic “shift” by recombination of an avian and human influenza virus can only be speculated upon. Whereas this question is of crucial importance for future vaccine development, it basically should have little bearing on antiviral drug design, because the antiviral drug targets, as depicted in Fig. 1.2, should be applicable to all influenza A virus variants.¹¹

M2 ION-CHANNEL INHIBITORS: AMANTADINE, RIMANTADINE, AND NEW ADAMANTANAMINE DERIVATIVES

The first synthetic compound ever shown to inhibit influenza virus replication was amantadine.¹² As indicated in Fig. 1.2 amantadine blocks, within the endosomes, the migration of H⁺ ions (protons) into the interior of the virus particles (virions), a process that is needed for the uncoating to occur. The H⁺ ions are imported through the M2 (matrix 2) channels.¹³ The transmembrane domain of the M2 protein, with the amino acid residues facing the ion-conducting pore,¹⁴ is shown in Fig. 1.3. Amantadine has been postulated to plug up the interior

Fig. 1.1. The two mechanisms whereby pandemic influenza originates. In 1918, the "Spanish influenza" H1N1 virus closely related to an avian virus adapted to replicate efficiently in humans. In 1957 and 1968, reassortment events led to, respectively, the "Asian influenza" H2N2 virus and the "Hong Kong influenza" H3N2 virus. The "Asian influenza" H2N2 virus acquired three genetic segments from an avian species [a hemagglutinin, a neuraminidase, and a polymerase (PB1) gene]. The "Hong Kong influenza" H3N2 virus acquired two genetic segments from an avian species (hemagglutinin and PB1). Future pandemic strains could arise through either mechanism.¹⁰ (Taken from Belshe.¹⁰) See color insert.

channel within the tetrameric M2 helix bundle.¹⁵ The adamantan(amin)e derivatives amantadine and rimantadine (Fig. 1.4) have for a considerable time been available for both the prophylaxis and therapy of influenza A virus infections, but their use has been limited essentially because of the rapid emergence of virus-drug resistance, the ready transmissibility of the drug-resistant viruses.

In addition to amantadine and rimantadine, a variety of new adamantanamine derivatives have been accredited with marked activity against influenza A (H2N2 and/or H3N2): spiro[cyclopropane-1,2'-adamantan]-2-amine,¹⁶ spiro[pyrrolidine-2,2'-adamantane],¹⁶ spiro[piperidine-2,2'-adamantane],¹⁷ 2-(2-adamantyl)piperidine,¹⁸ 3-(2-adamantyl)pyrrolidine,¹⁹ rimantadine 2-isomers,²⁰ 2-(1-adamantyl)piperidine,²¹ 2-(1-adamantyl)pyrrolidine,²¹ and 2-(1-adamantyl)-2-methyl-pyrrolidine²² (Fig. 1.4). Whether any of these new adamantyl derivatives may offer any advantage—in terms of potency, selectivity, safety, or resistance

Fig. 1.2. Inhibition of influenza virus replication cycle by antivirals. After binding to sialic acid receptors, influenza virions are internalized by receptor-mediated endocytosis. The low pH in the endosome triggers the fusion of viral and endosomal membranes, and the influx of H^+ ions through the M2 channel releases the viral RNA genes in the cytoplasm. Adamantamine derivatives block this uncoating step. RNA replication/transcription occurs in the nucleus. This process can be blocked by inhibitors of IMP dehydrogenase (a cellular enzyme) or viral RNA polymerase. The stability of the viral mRNA and its translation to viral protein may be prevented by siRNAs. Packaging and budding of virions occur at the cytoplasmic membrane. Neuraminidase inhibitors block the release of the newly formed virions from the infected cells. (Taken from Palese,¹¹ with modifications.¹) See color insert.

profile—over the parent compounds amantadine and rimantadine needs to be further explored.

RESISTANCE TO THE M2 ION-CHANNEL INHIBITORS AMANTADINE AND RIMANTADINE

Resistance to amantadine and rimantadine develops rapidly as a result of single amino acid substitutions 26, 27, 30, 31, or 34 within the trans-membrane domain of the M2 protein.²³ In particular, the Ser → Asn mutation at position 31 (S31N) engenders high-level resistance to

Fig. 1.3. Model of the proposed transmembrane domain of the M2 protein, showing top view as seen from the extracellular side and a cross section in the plane of the lipid layer. Residues that were identified as facing the ion-conducting aqueous pore are indicated. (Taken from Shuck et al.¹⁴) See color insert.

adamantan(amin)es (Fig. 1.5).²⁴ The incidence of adamantan(amin)e (M2-inhibitor) resistance among human influenza A (H3N2) virus in the United States has increased from less than 2% until 2004 to 14.5% for the period October 2004–March 2005 to 92.3% for the period October–December 2005.²⁴ The incidence of adamantane resistance among influenza A (H3N2) viruses isolated in the United States²⁵ and worldwide²⁶ has been a cause for concern. More than 98% of the adamantane-resistant isolates identified worldwide between 1995 and 2005 contain the same S31N substitution.²⁶

The rate of adamant(amin)e resistance began to increase in Asia in the 1997–1998 influenza season and increased markedly in China to 57.5% in 2002–2003 and 73.8% in 2003–2004.²⁶ Misuse of the adamant(amin)es most likely contributed to this rapid increase in resistance. In China, Russia, and some other countries, amantadine and

Fig. 1.4. The adamantane (or adamantanamine) derivatives amantadine and rimantadine and various new adamantanamine derivatives. Amantadine, rimantadine, and adamantanamine derivatives share a number of common structural features, which relate to their eventual mode of action—that is, blockage of the M2 channel—responsible for transporting H^+ ions (protons) into the interior of the virions and initiating the viral uncoating process (see legend to Fig. 1.2). Several new adamantanamine derivatives were found to be more active than amantadine: i.e. 230-fold (spiro[pyrrolidine-2,2'-adamantane]), 101-fold (spiro[cyclopropane-1,2'-adamantan]-2-amine), and 4.3-fold (3-[2-adamantyl]pyrrolidine).²²

Fig. 1.5. Mechanism of action of and development of resistance to M2 inhibitors. In the absence of amantadine, the proton channel mediates an influx of H^+ ions into the infecting virion early in the viral replication cycle, which facilitates the dissociation of the ribonucleoproteins from the virion interior and allows them to be released into the cytoplasm and transported into the cell nucleus. In highly pathogenic avian viruses (H5 and H7), the M2-proton channel protects the hemagglutinin from acid-induced inactivation in the trans-Golgi network during transport to the cell surface. In the presence of amantadine, the channel is blocked and replication is inhibited. The serine at position 31 lies partially in the protein–protein interface and partially in the channel (see inset). Replacement of serine by a larger asparagine leads to the loss of amantadine binding and the restoration of channel function. Depending on the particular amino acid, other mutations at position 26, 27, 30, or 34 may inhibit amantadine binding or allow binding without the loss of ion-channel function. [Taken from Hayden.²⁴ Inset courtesy of Rupert Russell, Phillip Spearpoint, and Alan Hay (National Institute for Medical Research, London).] See color insert.

rimantadine are both available without a prescription and are included in over-the-counter “antiflu” and “cold” preparations at a range of doses.²⁷ In North America, the increase in resistance began about 5 years after the initial increase in Asia to reach the current 92%.²⁵ In January 2006, the Centers for Disease Control (CDC) issued a Health Alert and recommended that neither amantadine nor rimantadine should be used for the treatment or prophylaxis of influenza A infections in the United States for the remainder of the 2005–2006 influenza season.²⁷

The distribution of amantadine-resistant avian influenza H5N1 in Asia has been examined.²⁸ More than 95% of the H5N1 viruses isolated in Vietnam and Thailand contained resistance mutations, as compared to 6.3% in Indonesia and 8.9% in China. The dual mutation Leu26Ile and Ser31Asn was found almost exclusively in all resistant isolates from Vietnam, Thailand, and Cambodia.²⁸

The startling increase in the incidence of adamantan(amin)e resistance in the United States²⁵ has obviously been looking for an explanation, one (still hypothetical) being the wide-scale use of the amantadine derivative memantine (3,5-dimethyl-L-adamantanamine). Memantine, which interacts with the *N*-methyl-D-aspartate (NMDA) receptor,²⁹ has been launched in March 2004 for the treatment of Alzheimer’s disease, accounting for 26% of all prescriptions for this disease by March 31, 2005. The introduction of memantine may, according to this provocative hypothesis,²⁹ have inadvertently led to the inability of amantadine to be used in the prophylaxis or therapy of influenza A.

NEURAMINIDASE INHIBITORS: ZANAMIVIR AND OSELTAMIVIR

Whereas the viral hemagglutinin (H) is needed for the virus to interact with the receptor bearing the *N*-acetylneuraminic acid (NANA, sialic acid), the viral neuraminidase (N) that cleaves off NANA enables the progeny virions to leave the infected cells and to spread to other host cells. By blocking the release of these newly formed virus particles, neuraminidase inhibitors should prevent further spread of the virus^{30,31} (Fig. 1.6). The neuraminidase may also play a role early in influenza infection of the human airway epithelium.³² The viral neuraminidase cleaves NANA (sialic acid or SA) from the cell surface glycoprotein at a specific bond [SA α 2,3Gal (sialic acid linked to galactose by an α -2,3 linkage) or SA α 2,6Gal (sialic acid linked to galactose by an α -2,6 linkage)] (Fig. 1.7).

Fig. 1.6. Mechanism of action of neuraminidase inhibitors. Neuraminidase inhibitors, such as zanamivir and oseltamivir (see Fig. 1.8), interfere with the release of progeny influenza virions from the surface of infected host cells. In doing so, the neuraminidase inhibitors prevent virus infection of new host cells and thereby halt the spread of infection in the respiratory tract. The neuraminidase cleaves off sialic acid (*N*-acetylneuraminic acid) from the cell receptor for influenza virus (see Fig. 1.7), so that the newly formed virus particles can be released from the cells. Neuraminidase inhibitors prevent this process. (Taken from Moscona.³¹) See color insert.

Fig. 1.7. Sialic acid (SA) [also known as *N*-acetylneuraminic acid (NANA)] linked to galactose (Gal) by an α 2-3 linkage (SA α 2-3Gal) or α 2-6 linkage (SA α 2-6Gal). Galactose is linked to *N*-acetylglucosamine (GlcNAc) through a β 1-4 linkage).

Avian (H5N1) influenza and human (H3N2, H1N1) influenza viruses seem to target different receptors of the human respiratory tract: Whereas human-derived viruses preferentially recognize SA α 2,6Gal located on epithelial cells of the nasal mucosa, paranasal sinuses, pharynx, trachea, and bronchi, avian viruses would preferentially recognize SA α 2,3Gal located more deeply in the respiratory tract, at the alveolar cell wall and junction between the respiratory bronchiole and alveolus.³³ The avian influenza H5N1 virus may cause severe lower respiratory tract (LRT) disease in humans because it attaches predominantly to type II pneumocytes, alveolar macrophages, and nonciliated bronchiolar cells of the human LRT.³⁴ In terms of the effectiveness of neuraminidase inhibitors, it would not, in theory, matter whether NANA is bound via an α -2,3 or α -2,6 linkage, because the neuraminidase inhibitors act as transition state analogues³⁵ of NANA, irrespective on how it is bound to the penultimate galactose unit.

The first neuraminidase inhibitors designed according to the “transition state analogue” principle were DANA and FANA. They served as the lead compounds for the development of the neuraminidase inhibitors that were eventually marketed for the treatment (and prophylaxis) of influenza A and B virus infections: zanamivir (Relenza[®], 4-guanidino-Neu5Ac2en, GG167)³⁶ and oseltamivir (Tamiflu[®], GS4071 ethyl ester, GS4104, Ro64-0796)³⁷ (Fig. 1.8). Both compounds have been found to be highly potent inhibitors ($IC_{50} \leq 1$ ng/ml) of the influenza neuraminidase, to inhibit influenza A and B virus replication *in vitro* and *in vivo* (mice, ferrets), to be well-tolerated, and to be both prophylactically (significant reduction in number of ill subjects) and therapeutically (significant reduction in duration of illness) effective against influenza A/B virus infection in humans. A crucial difference between zanamivir and oseltamivir, however, is that zanamivir has to be administered by inhalation (10 mg bid), whereas oseltamivir can be administered orally (75 or 150 mg b.i.d.).

The benefits to be expected from the neuraminidase inhibitors are that they may be expected to reduce illness duration by 1–3 days, to reduce the risk of virus transmission to household or health-care contacts, to reduce the number and severity of complications (sinusitis, bronchitis), to reduce the use of antibiotics and to prevent seasonal influenza virus infection. As shown in particular for oseltamivir, the earlier the administration of oseltamivir, the shorter the duration of fever, the greater the alleviation of symptoms and the faster the return to baseline activity and health scores.³⁸ Oseltamivir treatment of influenza illness reduces lower respiratory tract complications (LRTCs), particularly bronchitis and pneumonia, concomitantly with a reduction

DANA

FANA

Zanamivir
Relenza® Osetamivir
Tamiflu® Peramivir
RWJ-270201 Cyclopentane
derivative Cyclopentane
derivative Cyclopentane amide
derivative Pyrrolidine
A-192558 Pyrrolidine
A-315675

Fig. 1.8. DANA, FANA, eanamivir (Relenza®, 4-guanidino-Neu5Ac2en, GG167), osetamivir (Tamiflu®, GS4071 ethyl ester, GS4104, Ro64-0796), peramivir (RWJ-270201), and cyclopentane and pyrrolidine derivatives.

in antibiotic use and need for hospitalization.³⁹ Also, post-exposure prophylaxis with oseltamivir, 75 mg once daily for 7 days, was found to protect close contacts of influenza-infected persons against influenza illness and prevented spread within households.⁴⁰ Post-exposure prophylaxis with oseltamivir can be considered an effective option for preventing the transmission of influenza within households.⁴¹ It should be recognized, however, that oseltamivir is less effective against influenza B than against influenza A—that is, with regard to duration of fever and virus persistence.⁴²

The neuraminidase inhibitors (i.e., GS4071) have been positioned in the active center of the neuraminidase (Fig. 1.9).^{37,43} The structure of

Fig. 1.9. GS4071 within the active site of the influenza A viral neuraminidase. Locations of oseltamivir-resistance mutations (i.e., H274Y) showing that the tyrosine at position 252 is involved in a network of hydrogen bonds in group-1 (H5N1 and H1N1) neuraminidases.⁴⁴ (Figure 1.9A was taken from Kim et al.³⁷ and De Clercq,⁴³ and Fig. 1.9B was taken from Russell et al.⁴⁴) See color insert.

the influenza A virus neuraminidase has recently been resolved in two groups (group 1 contains the subtypes N1 (as in H5N1), N4, N5, and N8, and group 2 contains the subtypes N2 (as in H3N2), N3, N6, N7, and N9).⁴⁴ The crystal structures of the N1, N4, and N8 neuraminidases reported by Russell et al.,⁴⁴ surprisingly reveal that the active site of these group 1 enzymes have a different three-dimensional structure from that of group-2 enzymes.⁴⁵ The differences lie in a loop of amino acids known as the 150-loop. Group-1 neuraminidases contain a cavity adjacent to their active site that closes on ligand binding (Fig. 1.10).⁴⁴ When an inhibitor binds to group-1 subtypes, the 150-loop adopts a conformation similar to that of group-2 neuraminidases.⁴⁵ The cavity near the active site that is exposed by the open conformation of the 150-loop might be exploited in further drug design.⁴⁵

Fig. 1.10. Molecular surfaces of group-1 (N1) and group-2 (N9) neuraminidases with bound oseltamivir showing the 150-cavity in the group-1 (N1) structure that arises because of the distinct conformation of the 150-loop. (Taken from Russell et al.⁴⁴) See color insert.

RESISTANCE TO NEURAMINIDASE INHIBITORS ZANAMIVIR AND OSELTAMIVIR

The neuraminidase inhibitors zanamivir and oseltamivir make contact, through their carboxylic acid group, with the neuraminidase amino acid residue arginine in position 292 and, through their basic amine (oseltamivir) or guanidinium (zanamivir) group, with the neuraminidase amino acid residue glutamic acid in position 119. Hence, it is not surprising that at these positions (R292K, E119G), mutations may arise that engender resistance to both zanamivir and oseltamivir.⁴⁶ The R292K mutation causes high-level resistance to oseltamivir but only low-level (5- to 30-fold) resistance to zanamivir.

In a comprehensive study of over 1000 clinical influenza isolates recovered from 1996 to 1999, there was no evidence of naturally occurring resistance to either oseltamivir or zanamivir in any of the isolates.⁴⁷ During the subsequent 3 years (1999–2002) the frequency of variants with decreased sensitivity to the neuraminidase inhibitors did not increase significantly (the percent variants with a >10-fold decrease in susceptibility to oseltamivir was 0.41% in 2002, as compared to 0.33% in 2000).⁴⁸ However, in children treated for influenza with oseltamivir, Kiso et al.⁴⁹ found neuraminidase mutations in viruses from nine patients (18%), six of whom had mutations at position 292 (R292K) and two at position 119 (E119V). It has been postulated that zanamivir-resistant influenza H3N2 viruses may not readily arise *in vivo* due to their poor viability (reduced fitness).⁵⁰

Recombinant viruses containing either the wild-type neuraminidase or a single amino acid change at residue 119 (E119V) or 292 (R292K) were generated in the influenza A (H3N2) influenza virus background by reverse genetics: Both mutants showed decreased sensitivity to oseltamivir, and the R292K virus showed cross-resistance to zanamivir. The R292K mutation was associated with compromised viral growth and transmissibility (in accordance with earlier studies^{51,52}), whereas the growth and transmissibility of the E119V virus was comparable to those of wild-type virus.⁵³

Of note, influenza virus A (H3N2) carrying the R292K mutation in the neuraminidase gene did not transmit to ferrets under conditions the wild-type virus was readily transmitted.⁵¹ However, other mutant viruses of influenza A (H3N2) (i.e., E119V and H274Y, both engendering resistance to oseltamivir) were found to be readily transmissible in ferrets, although the H274Y mutant required a 100-fold higher dose for infection and was transmitted more slowly than the wild type.⁵²

It has been hypothesized that neuraminidase inhibitors could, in theory, inhibit the 1918 pandemic virus.⁵⁴ In fact, recombinant viruses

possessing the 1918 neuraminidase, or both the 1918 neuraminidase and 1918 hemagglutinin, were shown to be effectively inhibited, both *in vitro* and *in vivo* (mice) by the neuraminidase inhibitors zanamivir and oseltamivir; and a recombinant virus possessing the 1918 M2 ion channel could be effectively inhibited by amantadine and rimantadine. This means that current antiviral strategies would be effective in curbing a reemerging 1918 or 1918-like influenza (H1N1) virus.⁵⁵

Particular vigilance is warranted for drug-resistant influenza virus in immunocompromised patients, which may harbor, and shed, multi-drug-resistant influenza A (H3N2) for a prolonged time (1 year), as has been demonstrated for H3N2 influenza A H3N2 carrying the neuraminidase E119V mutation.^{56,57}

The conserved amino acid residues that interact with neuraminidase inhibitors are under selective pressure, but only a few have been linked to resistance. In the A/Wuhan/359 (H3N2) recombinant virus background, seven charged neuraminidase residues (R118, R371, E227, R152, R224, E276, and D151) were characterized that directly interact with the neuraminidase inhibitors but have not been reported to confer resistance to neuraminidase inhibitors.⁵⁸ Of the mutations that may arise at these positions, only the E276D mutation was predicted to likely emerge under selective pressure.⁵⁸ Other mutations, in addition to those that are currently associated with influenza resistance to zanamivir and oseltamivir (E119G, R152K, H274Y, and R292K), are so-called “outlier” mutations (i.e., A18S, L23F, C42F, R143V, E199K, S332F, and K431N): these “outlier” mutations have not been associated with resistance to neuraminidase inhibitors.⁵⁹

Resistance of influenza H5N1 to oseltamivir due to the H274Y mutation in the N gene has been described:⁶⁰ The patient from whom the oseltamivir-resistant H5N1 strain was isolated recovered from the disease, and the virus was found to be less pathogenic in ferrets than the parent strain and did not show cross-resistance to zanamivir. Although the H274Y mutation in influenza A H1N1 neuraminidase had been previously reported,^{61,62} its occurrence in influenza A (H5N1) infection raised concern because it was associated with death in two of the eight influenza A (H5N1) virus-infected patients.⁶³ Whether there was a causal relationship between the emergence of the H274Y mutation and the lethal outcome could not be ascertained, however.⁶³

The efficacy of oseltamivir in the treatment of H5N1 infection in humans could, because of its anecdotal use, not been unequivocally demonstrated. Yet, it should be pointed out that oseltamivir has been shown to protect ferrets against lethal influenza H5N1 infection: Treatment with oseltamivir at 5 mg/kg/day for 5 days twice daily (orally) resulted in complete inhibition of virus replication in the lungs

and small intestine on day 5 p.i. and, consequently, prevented mortality.⁶⁴ Similarly, oseltamivir has proven efficacious in the treatment of mice infected with the highly pathogenic H5N1 A/Vietnam/1203/04 influenza virus strain, although prolonged and higher-dose oseltamivir regimens were required for achieving the most beneficial antiviral effect.⁶⁵

Recent data have demonstrated that the sensitivities of the neuraminidase of H5N1 viruses isolated in 2004 and 2005 to oseltamivir are about 10-fold higher than those of earlier H5N1 viruses.⁶⁶ Although the clinical relevance of a 10-fold increase in sensitivity of neuraminidase to oseltamivir needs to be investigated further, the possibility that sensitivity to neuraminidase inhibitors could increase (or possibly decrease) significantly, even in the absence of treatment, underscores the need for continuous evaluation especially for influenza viruses with pandemic potential.⁶⁶

NEURAMINIDASE INHIBITORS: PERAMIVIR AND OTHER CYCLOPENTANE OR PYRROLIDINE DERIVATIVES AND DIMERIC ZANAMIVIR DERIVATIVES

Whereas oseltamivir can be described as a cyclohexenyl derivative, there are a number of cyclopentane and pyrrolidine derivatives that have been described as neuraminidase inhibitors: peramivir (RWJ-270201, BCX-1182)^{67–69} and other cyclopentane^{69,70} and cyclopentane amide⁷¹ derivatives as well as a variety of pyrrolidine derivatives, including A-192558,⁷² A-315675,^{73–75} and other pyrrolidines⁷⁶ (Fig. 1.8). Also, 2,3-disubstituted tetrahydrofuran-5-carboxylic acid derivatives have been described as influenza neuraminidase inhibitors, albeit with reduced inhibitory potency as compared to the corresponding pyrrolidine analogues.⁷⁷

Peramivir (RWJ-270201) and A-315675 represent novel neuraminidase inhibitors that were shown to retain activity against various zanamivir- and oseltamivir-resistant influenza A and B viruses:⁷⁸ Specifically, a new oseltamivir-resistant influenza B variant carrying the D198N substitution at the viral neuraminidase was found to retain susceptibility to peramivir and A-315675. Also, the neuraminidase E119V mutant displaying 6000-fold lower susceptibility to oseltamivir and 175-fold lower susceptibility to zanamivir than did wild-type virus still retained full susceptibility to A-315675.⁷⁹

Taken together the different studies performed with influenza A (H3N2) virus mutants,^{49–53,78,79} it appears that neuraminidase inhibitors

may select for mutations at a number of positions (E119V, R152K, D198N, H274Y, and R292K) that do only partially overlap,⁸⁰ that is, only partially engender cross-resistance.

In vivo, peramivir (BCX-1812, RWJ-270201) was found to strongly suppress influenza A (H1N1) infection in mice upon a single intramuscular injection (10 mg/kg). This was ascribed to a tight binding of peramivir to the neuraminidase.⁸¹ Complete protection against lethality was afforded by peramivir given once daily for five days after influenza A virus infection in a murine model.⁸² Similarly, a single intramuscular/intravenous injection of peramivir, 1 hour pre-virus exposure, offered protection against influenza A (H5N1) in mice.⁸³ When given orally to humans, however, peramivir did not offer robust protection against human influenza A virus infection, which was attributed to the very low oral bioavailability (<5%) of peramivir;⁸⁴ further studies with parenteral formulations of peramivir are, therefore, warranted.

Dimeric derivatives of zanamivir with linking groups of 14–18 atoms in length were found to be 100-fold more potent inhibitors of influenza virus replication *in vitro* and *in vivo* than zanamivir.^{85,86} These compounds exhibited long-lasting antiviral activity due to extremely long resistance times in the lungs, thus allowing a once-weekly dosing regimen. This raises the prospect for a new type of anti-influenza drug that could be administered as a single dose in the treatment of influenza, or just once a week in the prevention of infection.⁸⁶

IMP DEHYDROGENASE INHIBITORS: RIBAVIRIN AND VIRAMIDINE

Ribavirin has long been recognized as a broad-spectrum antiviral agent with particularly distinct activity against orthomyxo (i.e., influenza) and paramyxo (i.e., measles, respiratory syncytial) viruses.⁸⁷ Respiratory syncytial virus (RSV) infection is the only (–)RNA virus infection for which aerosolized ribavirin has been formally approved. Oral ribavirin is also used, in combination with parenteral pegylated α -interferon, in the treatment of chronic hepatitis C virus (HCV) infection. The intravenous form of ribavirin has been registered for the treatment of hemorrhagic fever with renal syndrome (HFRS). In addition to ribavirin, viramidine, which can actually be considered as the amidine prodrug of ribavirin (Fig. 1.11), has been accredited with marked potential as an anti-influenza drug.⁸⁸ Of interest, ribavirin has not been shown to generate virus-drug resistance, and resistance of influenza virus replication to ribavirin has not been reported to date. Obviously, this lack of drug

Fig. 1.11. Ribavirin and viramidine, with IMP dehydrogenase being the target enzyme for ribavirin 5'-monophosphate. Viramidine acts as a prodrug (precursor) of ribavirin, which is converted intracellularly to its 5'-monophosphate derivative, ribavirin-MP. The latter inhibits IMP dehydrogenase, a crucial enzyme in the biosynthesis of RNA, including viral RNA. The IMP dehydrogenase is responsible for the conversion of IMP (inosine 5'-monophosphate) to XMP (xanthosine 5'-monophosphate), which, in turn, is further converted to GMP (guanosine 5'-monophosphate), GDP (guanosine 5'-diphosphate), and GTP (guanosine 5'-triphosphate). The latter serves as substrate, together with ATP, UTP, and CTP, in the synthesis of RNA.

resistance development is due to the fact that ribavirin's main target of antiviral action (as demonstrated for paramyxo- and flaviviruses⁸⁹) is a cellular enzyme, the inosine 5'-monophosphate (IMP) dehydrogenase (responsible for the conversion of IMP to XMP), a key enzyme involved in the biosynthesis of GTP and viral RNA synthesis (Fig. 1.11).

Ribavirin is active against both human and avian (H5N1) influenza viruses within the 50% effective concentration (EC_{50}) range of 6–22 μ M.⁸⁸ Of the three routes (oral, aerosolized, intravenous) by which ribavirin could be administered in the treatment of avian/human influenza, the intravenous should be the preferred route when it comes to therapy of an acute influenza virus infection. Oral ribavirin did not offer the expected clinical or virological efficacy in earlier studies with influenza A (H1N1).⁹⁰ Ribavirin aerosol has been used successfully (based on reduction of virus shedding and clinical symptoms) in the treatment of influenza virus infections in college students.⁹¹ Intravenous ribavirin (producing mean plasma concentration at 20–60 μ M) was associated with symptomatic improvements and elimination of influenza virus from nasopharyngeal swabbings and tracheal aspirates.⁹²

Intravenous ribavirin has been further investigated, with success, in the treatment of Lassa fever⁹³ and HFRS.⁹⁴ Both studies demonstrated significant benefits of ribavirin in terms of survival and reduction of disease severity. The dosing regimen for intravenous ribavirin consists of a loading dose of 2 g of ribavirin followed by 1 g every 6 hr for 4 days. During the next 5 days, a maintenance dose of 0.5 g should be administered every 8 hr. This should generate the effective concentrations needed for achieving suppression of (human and avian) influenza virus replication. Dose-limiting toxicity would be hemolytic anemia, which should be reversible upon cessation of therapy.

SHORT INTERFERING RNAs AND PHOSPHOROTHIOATE OLIGONUCLEOTIDES

Short interfering RNAs (siRNAs) specific for conserved regions of influenza virus genes were found to reduce virus production in the lungs of infected mice, when the siRNAs were given intravenously (i.v.) in complexes with a polycation carrier either before or after initiating virus infection.⁹⁵ Delivery of siRNAs specific for highly conserved regions of the nucleoprotein or acidic polymerase significantly reduced lung virus titers in influenza A virus-infected mice and protected the animals from lethal challenge. This protection was specific and not

mediated by an antiviral interferon response. The influenza-specific siRNA treatment was broadly effective and protected animals against lethal challenge with highly pathogenic avian influenza A viruses of the H5 and H7 subtypes.⁹⁶ That specific siRNA would be effective against influenza could be readily predicted from equally effective results obtained with other specific siRNAs—that is, against the SARS (severe acute respiratory syndrome) coronavirus in comparable situations.⁹⁷

Phosphorothioate oligonucleotides (PS-ONs) (i.e., REP, a 40-mer PS-ON) offer potential, when administered as aerosol in the prophylaxis and therapy of influenza infection.⁹⁸ Similarly, antisense phosphorodiamidate morpholino oligomers (ARP-PMOs) could be further pursued for their potential in the treatment of H5N1 influenza A virus infections.⁹⁹ Similarly, peptide-conjugated phosphorodiamidate morpholino oligomers (P-PMO), designed to base-pair with influenza viral RNA sequences that are highly conserved across viral subtypes, proved highly efficacious in reducing the viral titer in a dose-responsive and sequence-specific manner in influenza A virus-infected cells.¹⁰⁰

INFLUENZA VIRUS RNA POLYMERASE INHIBITORS

The influenza viral RNA polymerase consists of a complex of three virus-encoded polypeptides (PB1, PB2, and PA), which, in addition to the RNA replicative activity, also contains an endonuclease activity so as to ensure “cap snatching” for initiating the transcription and subsequent translation process.¹⁰¹ The polymerase complex genes¹⁰² contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. This observation underscores the importance of novel antivirals targeting the polymerase for further development for the therapy and prophylaxis of human and avian influenza virus infections.

Few compounds have been reported to be operating at either the RNA replicase (RNA polymerase) or endonuclease level. In analogy with the inhibitors that have been identified to inhibit the reverse transcriptase (RNA-dependent DNA polymerase) of HIV or RNA replicase (RNA-dependent RNA polymerase) of HCV, influenza RNA replicase inhibitors could be divided into two classes: nucleoside and non-nucleoside type of inhibitors. Examples of the nucleoside type of inhibitors are 2'-deoxy-2'-fluoroguanosine (FdG)^{103,104} and T-705^{105–107} (Fig. 1.12).

T-705 is a substituted pyrazine that has been found to exhibit potent anti-influenza virus activity *in vitro* and *in vivo*. According to a com-

2'-Deoxy-2'-fluoroguanosine
2'-Fluoro-dGuo
FdG

Flutimide

Thiadiazolo[2,3-a]pyrimidine

Pyrimidinyl acylthiourea

Fig. 1.12. FdG, Flutimide, thiadiazolo[2,3-*a*]pyrimidine, pyrimidinyl acylthiourea, and T-705, with the postulated mode of action of T-705, according to Furuta et al.¹⁰⁷

parative study, T-705 would even be more potent than oseltamivir when increasing the multiplicity of infection (*in vitro*) or using a higher virus challenge dose (*in vivo*).¹⁰⁶ It has been postulated that T-705 would be converted intracellularly to its ribonucleotide, T-705 4-ribofuranosyl-5'-monophosphate (T-705 RMP), through a phosphoribosyl transfer-

ase, and, upon further phosphorylation to its 5'-triphosphate (Fig. 1.12), T-705 RTP would then inhibit influenza virus RNA polymerase in a GTP-competitive manner.¹⁰⁷ Unlike ribavirin 5'-monophosphate, T-705 RMP did not significantly inhibit IMP dehydrogenase, indicating that it owes its anti-influenza virus activity mainly, if not exclusively, to an inhibition of the influenza virus RNA polymerase.

In addition to the RNA polymerase, the “cap snatching” or “cap scavenging” endonuclease activity associated with the PB1-PB2-PA complex could be considered as an attractive target for influenza virus inhibitors: It can be inhibited by 4-substituted 2,4-dioxobutanoic acid derivatives¹⁰⁸ and *N*-hydroxamic acid/*N*-hydroxy-imide derivatives.¹⁰⁹ Likewise, flutimide, a 2,6-diketopiperazine (Fig. 1.12), identified in extracts of the fungal species *Delitschia confertaspera*, has been demonstrated to specifically inhibit the cap-dependent endonuclease activity associated with influenza viral RNA polymerase and to inhibit the replication of influenza A and B virus in cell culture.¹¹⁰ Both the viral RNA polymerase and endonuclease should be further explored as targets for the development of anti-influenza agents.

Recently, a new class of potent influenza virus inhibitors (EC_{50} for virus replication: 0.08–0.09 μ M), as represented by thiadiazolo[2,3-*a*]pyrimidine and pyrimidinyl acylthiourea (Fig. 1.12), has been reported.¹¹¹ Although the mechanism of action of this highly potent and selective inhibitors of influenza virus remains to be established, they represent a highly interesting lead worth pursuing. A series of novel bisheterocycle tandem derivatives consisting of methyloxazole and thiazole may also serve as leads for further optimization, although the lead compounds exhibited only modest activity against influenza A virus.¹¹²

INTERFERON (INDUCERS)

Interferon was originally discovered, exactly 50 years ago, with influenza virus as inducer.¹¹³ In some earlier studies, interferon, instilled by the intranasal route, did not offer much protection in the prophylaxis of influenza A virus infections.^{114,115} Meanwhile, interferon has come a long way, and pegylated α -interferon (injected parenterally), in combination with (oral) ribavirin, has become the standard therapy for chronic hepatitis C virus (HCV) infections. This means that with this combination, extensive experience has been accumulated,¹¹⁶ which could be readily implemented in the prophylaxis and therapy of human and avian influenza virus infections in humans. In the prophylaxis/therapy of influenza virus infections, the duration of treatment would

be much shorter than that for hepatitis C, which would obviously reflect on the convenience (cost/benefit) and side effects that are inherently linked to the use of interferon and ribavirin.

In addition to interferon, interferon inducers such as poly(I)·poly(C), discovered some 40 years ago,¹¹⁷ may also play a role in the control of influenza virus infections. Prophylaxis using liposome-encapsulated double-stranded RNA [poly(I)·poly(C)] provided complete and long-lasting protection against influenza A virus infection.¹¹⁸ Furthermore, poly(I)·poly(C), when combined with (intranasal) vaccination, conferred complete protection against influenza virus infection, which may have been mediated by an upregulated expression of Toll-like receptor 3 and α/β interferons as well as Th1- and Th2-related cytokines.¹¹⁹ It is unclear whether the use of exogenous interferon, or the induction of endogenous interferon by poly(I)·poly(C) or other double-stranded RNAs, may help in the prophylaxis or therapy of avian or human influenza virus infections, but in view of the “renaissance” of interferon, as witnessed in the treatment of HCV infection, the potential of interferon in control measures against influenza may well deserve to be revisited.

ANTIVIRAL DRUG COMBINATIONS

From the drug combination regimens utilized in the treatment of *Mycobacterium tuberculosis* and human immunodeficiency virus (HIV) infections (AIDS), we have learned that such drug combinations (i) achieve greater benefit than each compound given individually, (ii) reduce the likelihood of drug resistance development, and (iii) may allow us to decrease the individual drug doses, thereby diminishing adverse side effects. The concept of using two or more antivirals for influenza to enhance antiviral efficacy and possibly reduce resistance emergence is several decades old,^{120–123} and it was first demonstrated for the combination of interferon with amantadine.¹²¹ Many more drug combinations are possible, even if limited only to those compounds that are available (marketed) today: neuraminidase inhibitors (oseltamivir and zanamivir), adamantanamines (amantadine and rimantadine), ribavirin, and (pegylated) interferon (Fig. 1.13).

In the therapy (or prophylaxis) of influenza virus infections, the combination of (pegylated) interferon and ribavirin could be further complemented with amantadine (or rimantadine). This triple-drug combination has shown efficacy in the treatment of chronic HCV infection,¹²⁴ and it may also be worth pursuing in the treatment (or

Fig. 1.13. Drug combination possibilities among neuraminidase inhibitors (oseltamivir and zanamivir), adamantanamines (amantadine or rimantadine), ribavirin (or viramidine), and interferon (or interferon inducers).

prophylaxis) of influenza. The three drugs are, individually, all active against influenza virus replication *in vitro* and act through different mechanisms, which implies that, when combined, they may achieve an additive or even synergistic action while reducing the risk of emergence of drug-resistant virus variants. As early as 1984, Hayden et al.¹²⁵ pointed to the additive synergistic action between interferon- $\alpha 2$, rimantadine, and ribavirin.

Also combinations of (pegylated) interferon with neuraminidase inhibitors (zanamivir, oseltamivir, peramivir) may be considered, and thus might be combinations of ribavirin (or viramidine) with the neuraminidase inhibitors, although in a recent study with a lethal influenza A (H1N1) infection model in mice, Smee et al.¹²⁶ found that the combination of oseltamivir with ribavirin did not score better than ribavirin alone. On the other hand, combination of oseltamivir with amantadine appeared to effect a significantly greater antiviral activity against influenza A (H1N1, H3N2, and H5N1) while reducing the emergence of drug-resistant influenza A variants.¹²⁷

Combinations of the adamantan(amin)es (i.e., amantadine or rimantadine) with the neuraminidase inhibitors (zanamivir or oseltamivir) should, therefore, receive due attention. *In vitro*, rimantadine was found to act synergistically with zanamivir, oseltamivir, or peramivir in reducing the extracellular yield of influenza A (H3N2) virus.¹²⁸ *In vivo*, oseltamivir at 10 mg/kg/day and amantadine at 15 mg/kg/day provided similar protection against influenza A (H5N1)-associated death risk in mice; but when both were combined, they provided an incremental protection against lethality as combined to both compounds given as single-agent chemotherapy.¹²⁹ The only controlled study in humans was

a comparison of rimantadine alone versus rimantadine plus inhaled zanamivir in hospitalized (adult) patients with serious influenza,¹³⁰ although preliminary, this study pointed to a higher clinical benefit for the combination of zanamivir with rimantadine.

RECOMMENDATIONS

Among the antivirals, the neuraminidase inhibitors oseltamivir and zanamivir are the most likely to be considered for use against an avian influenza H5N1 pandemic, with oseltamivir being the preferable option of the two neuraminidase inhibitors because it is less expensive and administered orally (whereas zanamivir is administered by inhalation).¹³¹ The stockpiling of an appropriate antiviral agent such as oseltamivir is currently the most crucial single defense to be utilized against influenza H5N1.¹³²

Zanamivir remains an attractive antiviral drug for combination with oseltamivir because of a non-overlapping resistance pattern, but then the route of administration will need to be reconsidered, since, if administered by inhalation, it does not reach the sites (lower respiratory tract and extrapulmonary) where avian influenza H5N1 replicates.¹³³

Some authors have questioned the effectiveness of neuraminidase inhibitors in interrupting viral spread and argued that their use in a serious epidemic or pandemic should not be considered without concomitant public-health measures such as barriers, distance, and personal hygiene.¹³⁴ The same authors also argued against the use of amantadine and rimantadine.¹³⁴ Yet, in severe influenza A virus infections, amantadine may be lifesaving,¹³⁵ due to the fact that, independently of its antiviral properties (and thus unaffected by antiviral resistance), it may increase distal airway function and thus improve oxygenation.¹³⁶

Finally, when considering the use of antivirals in the prevention and/or treatment of influenza, particular attention should be paid to high-risk groups: children, pregnant women, immunocompromised hosts, and nursing home residents.¹³⁷

CONCLUSIONS

Several drugs are available that could be used, either alone or in combination, in the treatment (or prophylaxis) of a pandemic influenza virus infection, whether avian or human. These include adamantan(amine)

derivatives (i.e., amantadine, rimantadine), neuraminidase inhibitors (i.e., zanamivir, oseltamivir), ribavirin, and interferon. In the meantime, attempts should be intensified to further design and develop new antivirals whether based on known molecular targets, such as the neuraminidase and M2 ion channel, or on yet relatively unexplored targets such as the viral RNA polymerase. The latter could, in principle, be targeted by both nucleoside and non-nucleoside inhibitor types, an approach that has proven most successful in the cases of the HIV reverse transcriptase and HCV RNA polymerase.

ACKNOWLEDGMENTS

I thank Christiane Callebaut for her invaluable editorial assistance.

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