COMBATING THE THREAT OF PANDEMIC INFLUENZA

Drug Discovery Approaches

Edited by

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PREFACE

It seems impossible not to see or hear of the threat of avian influenza in the printed media or the nightly news. The avian influenza A (H5N1) epizootic that is occurring in Asia and in parts of Europe, the Near East, and Africa will not likely diminish soon. Thus far, there is no evidence to suggest genetic recombination between avian influenza A virus and human genes. Nonetheless, since there is little preexisting natural immunity to H5N1 infection in human populations, if H5N1 viruses become able to maintain efficient and sustained transmission among humans, an influenza pandemic could result. High rates of morbidity and mortality could result along with staggering economic and societal disruption.[1–4]

While vaccines will likely be the most effective at reducing morbidity and mortality, they may be available in the beginning of a pandemic because of production capacity.[5] Therefore, antivirals, such as neuraminidase inhibitors, would be expected to provide pharmaceutical intervention until sufficient vaccine can be produced.[6–8] This places a huge onus on antiviral chemotherapy, especially when the phenomenon of emerging resistance is considered.[9] Of great significance is the finding that H5N1 viruses from humans in Thailand and Vietnam show resistance to amantadine and rimantadine, Therefore, novel approaches of all kinds to effective therapeutics for influenza should be sought. The contributions in this volume provide some insight into such possibilities.

In Chapter 1, De Clercq provides an overview of the current state of antiviral therapeutics for influenza. Covered are: M2 ion-channel

inhibitors such as amantadine, rimantadine, and new adamantanamine congeners; interferon and interferon inducers; neuraminidase inhibitors such as the well-known zanamivir and oseltamivir as well as more recent compounds such as peramivir and other cyclopentane or pyrrolidine derivatives and dimeric zanamivir derivatives; the IMP dehydrogenase inhibitors ribavirin and viramidine; RNA polymerase inhibitors such as 2′-deoxy-2′-fluoroguanosine and the newer thiadiazolo[2,3-a]pyrimidine and pyrimidinyl acylthiourea. In addition, the key issues of drug combinations and resisitance devlopment are treated.

The vital matter of high-throughput screening (HTS) to aid in the identification of new leads for influenza antivirals is dealt with by Noah et al. in Chapter 2. Described is the potential of each influenza component as an assay target, along with the current state of influenza assays that are adaptable or have already been adapted to HTS formats for diagnostic strain characterization, vaccine evaluation, and identification of potential antivirals.

Interferon and interferon inducers have been around for some time and may prove to be of use as interferon antivirals, but it may well be the targets revealed through mechanisms of their antiviral activity that will provide clues for alternative therapeutics. In Chapter 3, Ezelle and Hassel review the ever-expanding knowledge base of interferon actions.

In Chapter 4, Shigeta describes his laboratory's experiences in mass-screening trial of potential anti-myxovirus agents including both synthetic and natural substances, Several broad-spectrum anti-myxovirus agents have been found in these studies, and some of these compounds exceeded ribavirin in potency and selectivity *in vitro* and some displayed activity *in vivo*.

Wong and colleagues look at nucleic acid-based agents (with the exception of RNAi interference) as antiviral agents in Chapter 5, whereas Haasnoot and Berkhout review the activity and potential of RNA interference against influenza and other respiratory viruses in Chapter 6.

Hayashi and co-workers review their research on fucoidan, a sulfated polysaccharide isolated from an edible alga *Undaria pinnatifida*, as an inhibitor of influenza A virus replication in Chapter 7. This fucoidan shows *in vitro* antiviral activity and synergistic antiviral action in combination with oseltamivir.

Carbohydrate chains of mammalian host cells are known to be receptors for influenza virus and many other viruses and bacteria. In Chapter 8, Bovin and Gambaryan presented the rational design of an anti-adhesion drug for influenza. This includes self-assembling glycopeptides and symmetric star-like molecules.

Verma and Hansch apply quantitative structure–activity relationships (QSAR) in the search for new neuraminidase inhibitors as anti-influenza drugs in Chapter 9. From data gathered on known specific influenza A and B neuraminidase inhibitors, the authors have developed two QSAR models that may be used to narrow the synthetic challenges required to develop new inhibitors.

In Chapter 10, Gao relates recent progress in the development of anti-virus fusion peptides by addressing the important hepted repeat (HR) polypeptides in some notorious viruses, such as HIV, Newcastle disease virus (NDV), and influenza virus, which are all Class I enveloped viruses.

Finally, in Chapter 11, Aschenbrenner and colleagues describe several new approaches under development at NexBio, Inc. These include a chemical entity that inactivates the virus receptor and another that inhibits the viral RNA polymerase.

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1

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 Asia[2] (with several clusters recently identified in Indonesia[3]), the expanding geographic distribution of this epizootic virus (with well-documented cases in Eastern Turkey in 2006[4]), 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

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

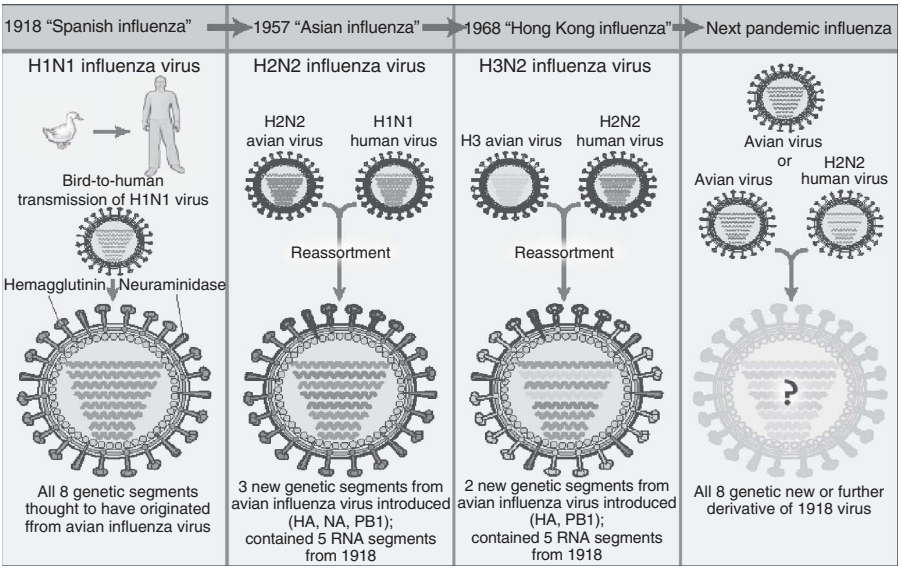


Fig. 1.1. The two mechanisms whereby pandemic influenza originates. In 1918, the "Spanish influenza" H1N1 virus closely related to an avian virus adapted to replicate efficiently in humans. In 1957 and 1968, reassortment events led to, respectively, the "Asian influenza" H2N2 virus and the "Hong Kong 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-pyrrolidine[22] (Fig. 1.4). Whether any of these new adamantyl derivatives may offer any advantage—in terms of potency, selectivity, safety, or resistance

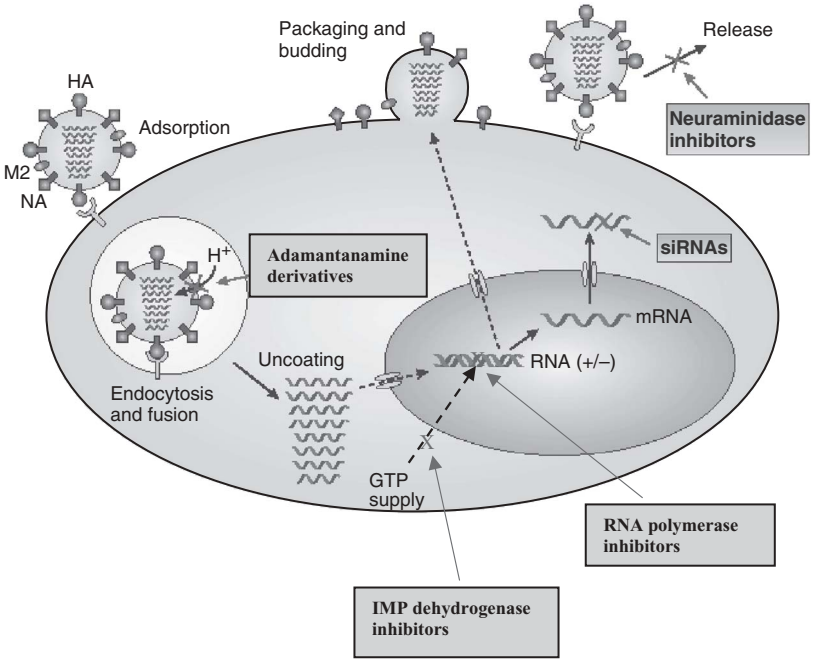


Fig. 1.2. Inhibition of influenza virus replication cycle by antivirals. After binding to sialic acid receptors, influenza virions are internalized by receptor-mediated endocytosis. The low pH in the endosome triggers the fusion of viral and endosomal membranes, and the influx of H^+ ions through the M2 channel releases the viral RNA genes in the cytoplasm. Adamantanamine 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 transmembrane domain of the M2 protein.[23] In particular, the Ser → Asn mutation at position 31 (S31N) engenders high-level resistance to

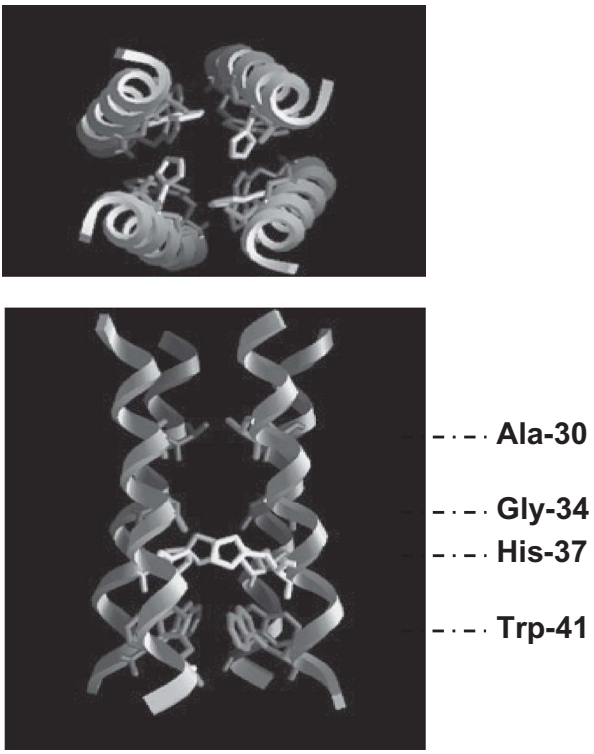
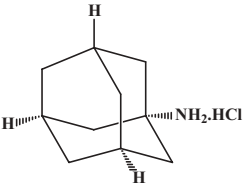


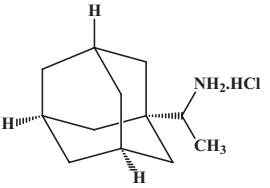
Fig. 1.3. Model of the proposed transmembrane domain of the M2 protein, showing top view as seen from the extracellular side and a cross section in the plane of the lipid layer. Residues that were identified as facing the ion-conducting aqueous pore are indicated. (Taken from Shuck et al.[14]) See color 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 States[25] and worldwide[26] 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



Amantadine
Symmetrel®, Mantadix®



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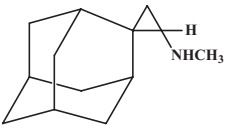
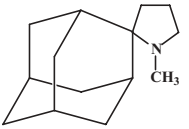
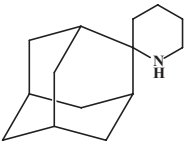


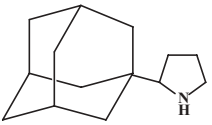
Spiro[cyclopropane-1,2′-adamantan]-2-amine



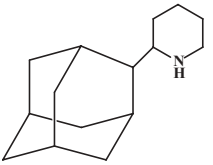
Spiro[pyrrolidine-2,2′-adamantane]



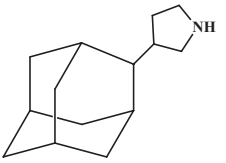
Spiro[piperidine-2,2′-adamantane]



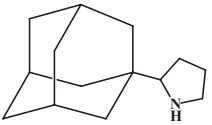
2-(1-Adamantanyl)pyrrolidine



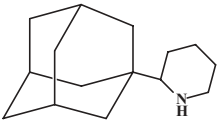
2-(2-Adamantyl)piperidine



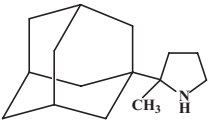
3-(2-Adamantyl)pyrrolidine



2-(1-Adamantyl)pyrrolidine



2-(1-Adamantyl)piperidine



2-(1-Adamantyl)-2-methyl-pyrrolidine

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 adamantamine 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]

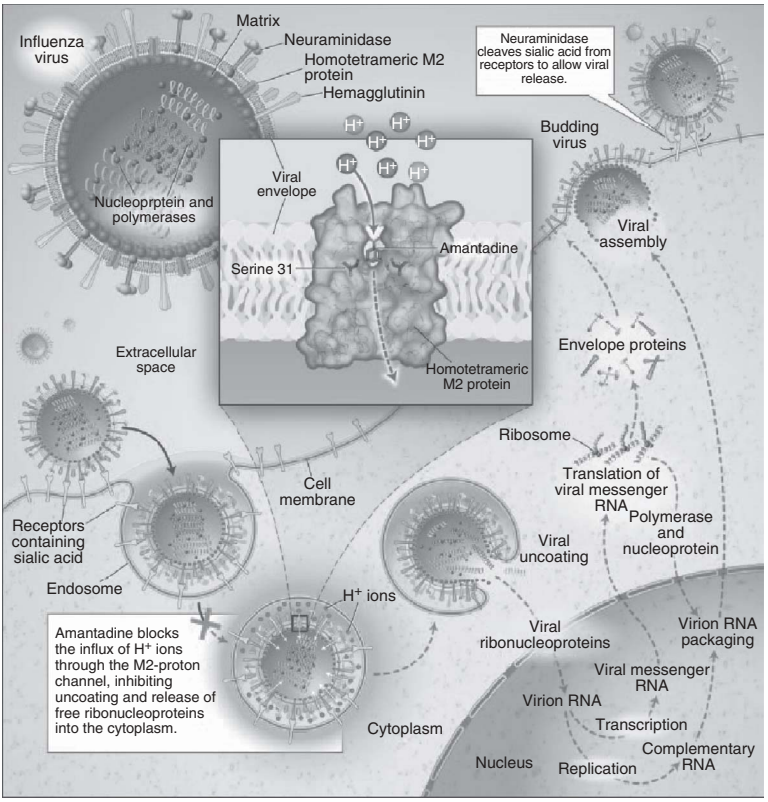


Fig. 1.5. Mechanism of action of and development of resistance to M2 inhibitors. In the absence of amantadine, the proton channel mediates an influex of H^+ ions into the infecting virion early in the viral replication cycle, which facilitates the dissociation of the ribonucleoproteins from the virion 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 States[25] 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 [SAα2,3Gal (sialic acid linked to galactose by an α-2,3 linkage) or SAα2,6Gal (sialic acid linked to galactose by an α-2,6 linkage)] (Fig. 1.7).

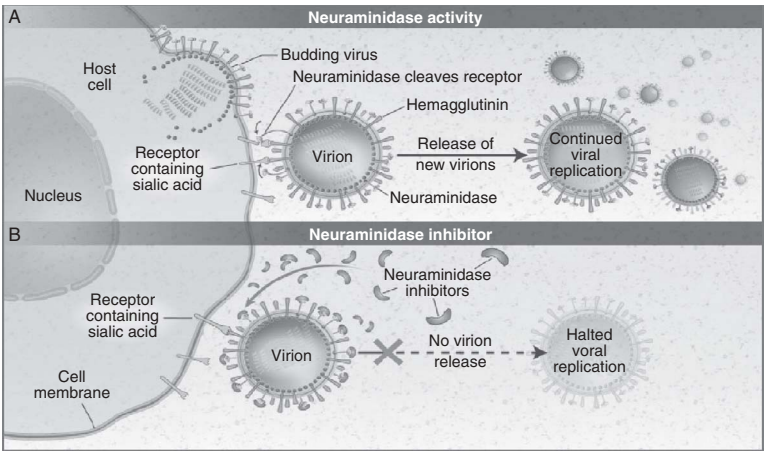


Fig. 1.6. Mechanism of action of neuraminidase inhibitors. Neuraminidase inhibitors, such as zanamivir and oseltamivir (see Fig. 1.8), interfere with the release of progeny influenza virions from the surface of infected host cells. In doing so, the neuraminidase inhibitors prevent virus infection of 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.

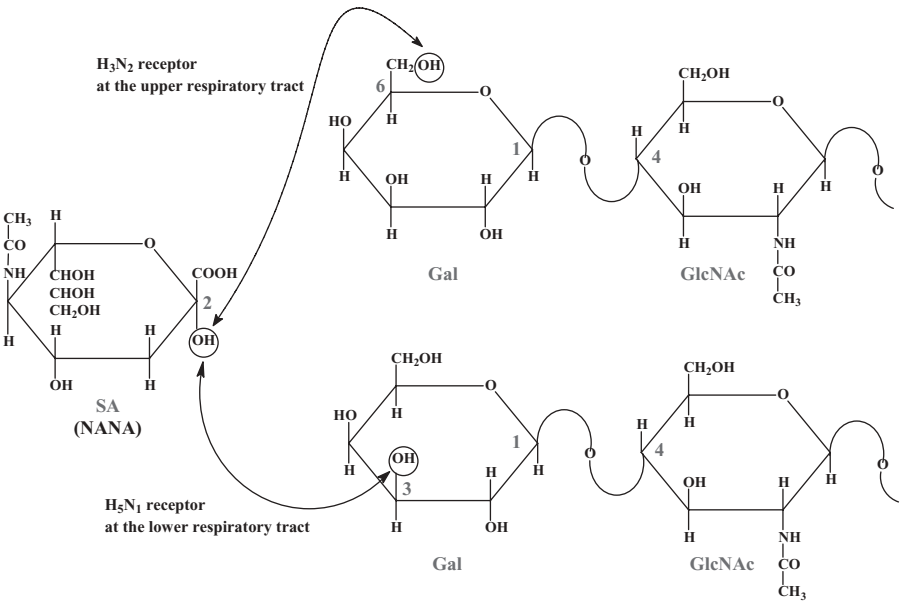
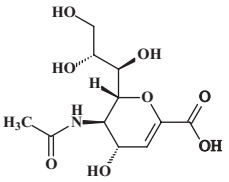


Fig. 1.7. Sialic acid (SA) [also known as *N*-acetylneuraminic acid (NANA)] linked to galactose (Gal) by an α2–3 linkage (SAα2–3Gal) or α2–6 linkage (SAα2–6Gal). Galactose is linked to *N*-acetylglucosamine (GlcNAc through a β1–4 linkage).

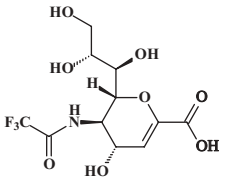
Avian (H5N1) influenza and human (H3N2, H1N1) influenza viruses seem to target different receptors of the human respiratory tract: Whereas human-derived viruses preferentially recognize SAα2,6Gal located on epithelial cells of the nasal mucosa, paranasal sinuses, pharynx, trachea, and bronchi, avian viruses would preferentially recognize SAα2,3Gal 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 analogues[35] 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.).

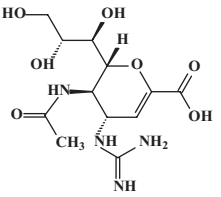
The benefits to be expected from the neuraminidase inhibitors are that they may be expected to reduce illness duration by 1–3 days, to reduce the risk of virus transmission to household or health-care contacts, to reduce the number and severity of complications (sinusitis, bronchitis), to reduce the 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



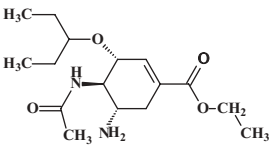
DANA



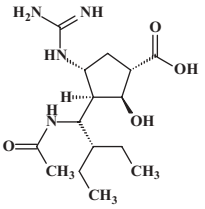
FANA



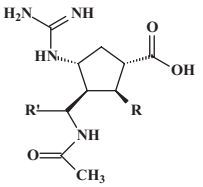
Zanamvir
Relenza®



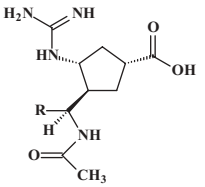
Oseltamivir
Tamiflu®



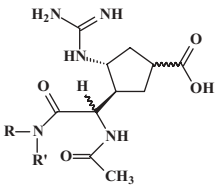
Peramivir
RWJ-270201



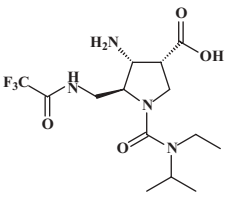
Cyclopentane
derivative



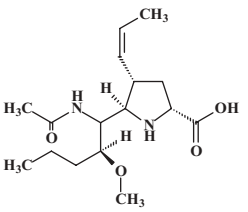
Cyclopentane
derivative



Cyclopentane amide
derivative



Pyrrolidine
A-192558



Pyrrolidine
A-315675

Fig. 1.8. DANA, FANA, eanamivir (Relenza®, 4-guanidino-Neu5Ac2en, GG167), oseltamivir (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

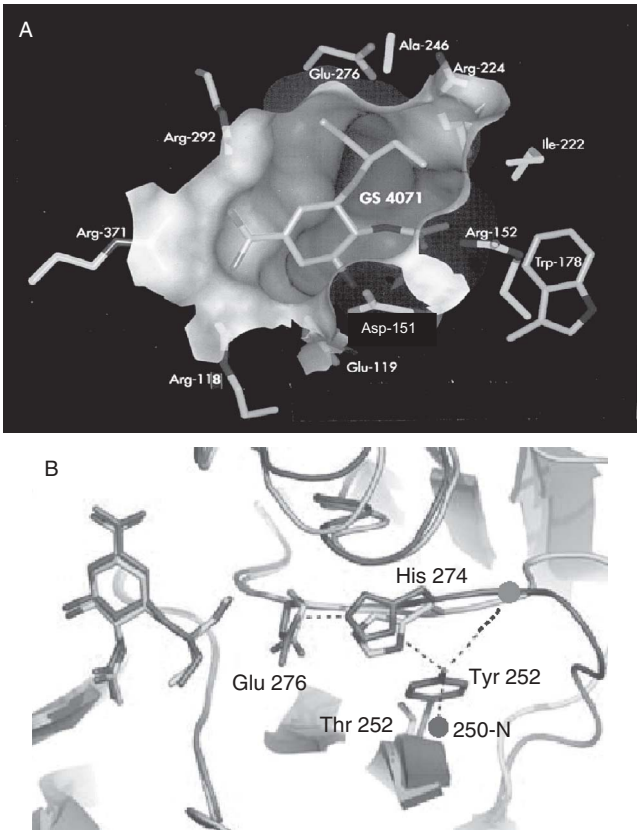


Fig. 1.9. GS4071 within the active site of the influenza A viral neuraminidase. Locations of oseltamivir-resistance mutations (i.e., H274Y) showing that the tyrosine at position 252 is involved in a network of hydrogen bonds in group-1 (H5N1 and H1N1) neuraminidases.[44] (Figure 1.9A was taken from Kim 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]

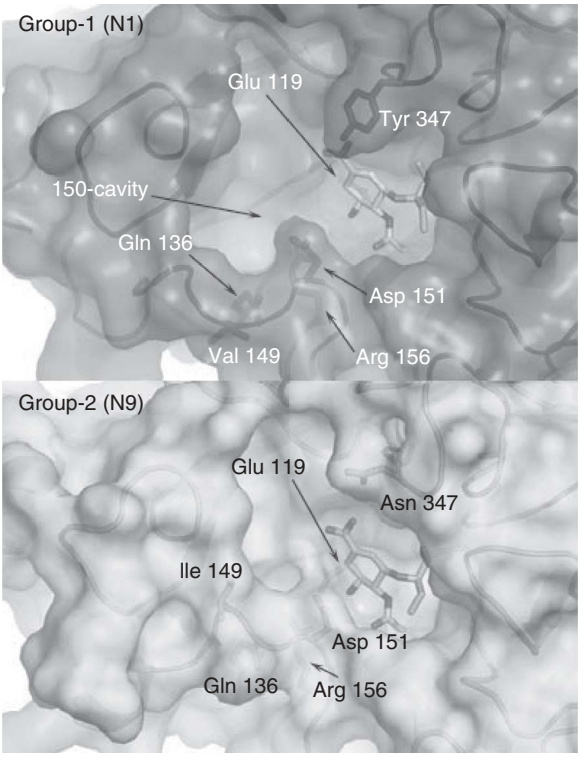


Fig. 1.10. Molecular surfaces of group-1 (N1) and group-2 (N9) neuraminidases with bound oseltamivir showing the 150-cavity in the group-1 (N1) structure that arises because of the distinct conformation of the 150-loop. (Taken from Russell et al.[44]) See color insert.

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 zanamvir-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