

Antiviral agents active against influenza A viruses

Erik De Clercq

Abstract | The recent outbreaks of avian influenza A (H5N1) virus, its expanding geographic distribution and its ability to transfer to humans and cause severe infection have raised serious concerns about the measures available to control an avian or human pandemic of influenza A. In anticipation of such a pandemic, several preventive and therapeutic strategies have been proposed, including the stockpiling of antiviral drugs, in particular the neuraminidase inhibitors oseltamivir (Tamiflu; Roche) and zanamivir (Relenza; GlaxoSmithKline). This article reviews agents that have been shown to have activity against influenza A viruses and discusses their therapeutic potential, and also describes emerging strategies for targeting these viruses.

HXNY

In the naming system for virus strains, H refers to haemagglutinin and N to neuraminidase.

In the face of the persistent threat of human influenza A (H3N2, H1N1) and B infections, the outbreaks of avian influenza (H5N1) in Southeast Asia, and the potential of a new human or avian influenza A variant to unleash a pandemic, there is much concern about the shortage in both the number and supply of effective anti-influenza-virus agents^{1–4}. There are, in principle, two mechanisms by which pandemic influenza could originate: first, by direct transmission (of a mutated virus perhaps) from animal (bird) to humans, as happened in 1918 with the ‘Spanish influenza’ (H1N1)⁵; or second, through reassortment of an avian influenza virus with a human influenza virus, as occurred in 1957 with the ‘Asian influenza’ (H2N2) and, again, in 1968 with the ‘Hong Kong influenza’ (H3N2)^{6,7} (FIG. 1).

Whether a new influenza pandemic could arise through antigenic ‘drift’ from an avian influenza virus or antigenic ‘shift’ through recombination of an avian and human influenza virus can only be speculated on. However, although this question is of crucial importance for future vaccine development, it has much less bearing on antiviral-drug design, as the antiviral drug targets shown in FIG. 2, and others which will be discussed here, should be relevant to all variants of influenza A virus⁸. In this article, I focus on agents that have been shown to have activity against influenza A viruses, and consider their therapeutic potential.

Adamantan(amin)e derivatives

The first synthetic compound shown to inhibit influenza-virus replication was amantadine⁹. As indicated in FIG. 2, amantadine blocks the migration of H⁺ ions

into the interior of the virus particles (virions) within endosomes, a process that is needed for the uncoating to occur. The H⁺ ions are imported through the M2 (matrix 2) channels¹⁰; the transmembrane domain of the M2 protein, with the amino-acid residues facing the ion-conducting pore, is shown in FIG. 3a (REF. 11). Amantadine has been postulated to block the interior channel within the tetrameric M2 helix bundle¹².

The adamantan(amin)e derivatives amantadine and rimantadine (FIG. 3b) have long been available for both the prophylaxis and therapy of influenza A virus infections, but their use has been limited because of the rapid emergence of drug resistance, the ready transmissibility of drug-resistant viruses, and, particularly for amantadine, the occurrence of central nervous system (CNS) side effects. These drawbacks compromise the potential usefulness of amantadine or rimantadine if used as single agents in the treatment of avian or human influenza A virus infections.

In particular, the incidence of adamantane resistance among influenza A (H3N2) viruses isolated in the United States¹³ and worldwide¹⁴ has been a cause for concern. More than 98% of the adamantane-resistant isolates identified worldwide between 1995 and 2005 contain the same S31N substitution¹⁴. The global circulation of adamantane-resistant H3N2 viruses is unprecedented and does not seem to be mediated by continued selective drug pressure. It could be argued that if resistance exists in a relatively homogeneous strain of H3N2, and if antiviral use would be curtailed, susceptible strains might (re-)emerge and adamantanes might regain their utility against both epidemic and pandemic influenza¹⁵.

Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium.
e-mail: erik.declercq@rega.kuleuven.be
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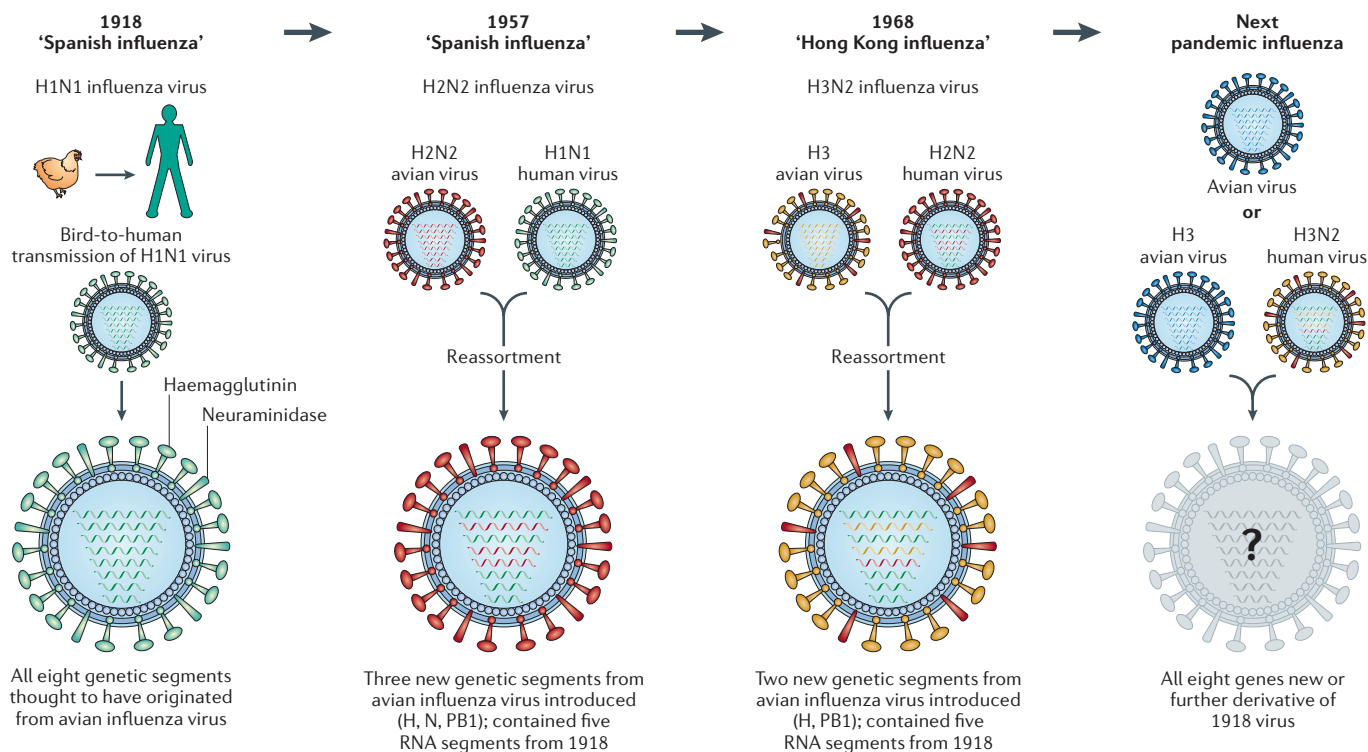


Figure 1 | The two mechanisms by which pandemic influenza originates. In 1918, the ‘Spanish influenza’ H1N1 virus, closely related to an avian virus, adapted to replicate efficiently in humans. In 1957 and 1968, reassortment events led to, respectively, the ‘Asian influenza’ H2N2 virus and the ‘Hong Kong influenza’ H3N2 virus. The ‘Asian influenza’ H2N2 virus acquired three genetic segments from an avian species (a haemagglutinin (H), a neuraminidase (N) and a polymerase (PB1) gene). The ‘Hong Kong influenza’ H3N2 virus acquired two genetic segments from an avian species (H and PB1). Future pandemic strains could arise through either mechanism. Figure adapted, with permission, from REF. 7 © (2005) Massachusetts Medical Society.

In addition to amantadine and rimantadine, various new adamantan(amin)e derivatives have shown marked activity against influenza A (H2N2 and/or H3N2)^{16–22} (FIG. 3b). Whether any of these new derivatives might offer any advantage in terms of potency, selectivity, safety or resistance profile over the parent compounds amantadine and rimantadine needs to be further explored. Also, further investigation of the antiviral potential of other ‘cage-like’ compounds structurally related to the adamantyl entity, such as bananin²³, might be worthwhile. At present, it can only be speculated whether any of the new adamantan(amin)e derivatives might be active against amantadine-resistant variants and efficacious *in vivo* in humans or relevant animal models.

Neuraminidase inhibitors

Viral haemagglutinin (H) is needed for the virus to interact with the receptor bearing *N*-acetylneuraminic acid (NANA, sialic acid). The viral neuraminidase (N) then cleaves off NANA 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. 4)). This enables the progeny virions to leave the infected cells and to spread to other host cells. So, by blocking the release of these newly formed virus particles, neuraminidase inhibitors should prevent further spread of the virus^{24,25} (FIG. 4). The

neuraminidase might also have a role early in influenza infection of the human airway epithelium²⁶.

Avian (H5N1) influenza viruses 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 seem to 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 alveolus²⁷. The avian influenza (H5N1) virus might cause severe lower respiratory tract (LRT) disease in humans because it attaches predominantly to type II pneumocytes, alveolar macrophages and non-ciliated bronchiolar cells of the human LRT²⁸. In terms of the effectiveness of neuraminidase inhibitors, it would not, in theory, matter whether NANA is bound through an α -2,3 or α -2,6 linkage, as the neuraminidase inhibitors act as transition state analogues²⁹ of NANA, irrespective of how it is bound to the penultimate galactose unit.

Oseltamivir and zanamivir. The first neuraminidase inhibitors designed according to the ‘transition state analogue’ principle²⁹ were DANA (2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid) and FANA (2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid). They

Alveoli
Lung structures responsible for gas (O₂ ↔ CO₂) exchange.

Transition state analogue
A structural mimic of the intermediary state between reactant(s) and product(s) in a given reaction.

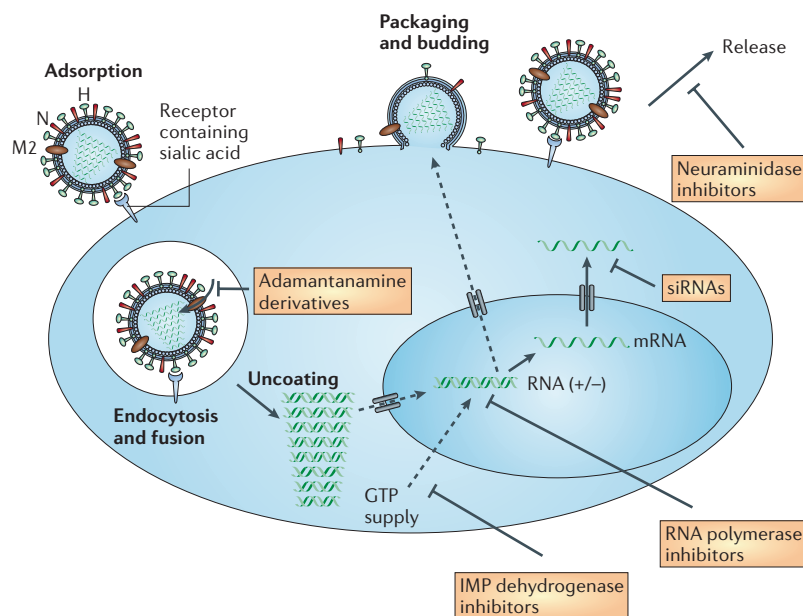


Figure 2 | Inhibition of the influenza-virus replication cycle by antiviral agents.

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. Adamantan(amin)e derivatives block this uncoating step. RNA replication and transcription occur in the nucleus. This process can be blocked by inhibitors of inosine 5'-monophosphate (IMP) dehydrogenase (a cellular enzyme) or viral RNA polymerase. The stability of the viral mRNA and its translation to viral protein might be prevented by small interfering RNAs (siRNAs). Packaging and budding of virions occur at the cytoplasmic membrane. Neuraminidase (N) inhibitors block the release of the newly formed virions from the infected cells. Figure adapted with permission from REF. 8 © (2004) Macmillan Magazines Ltd. H, haemagglutinin.

served as the lead compounds for the development of the neuraminidase inhibitors that are marketed at present for the treatment (and prophylaxis) of influenza A and B virus infections: zanamivir (Relenza, 4-guanidino-Neu5Ac2en, GG167)³⁰ and oseltamivir (Tamiflu, GS4071 ethyl ester, GS4104, Ro64-0796)³¹ (FIG. 5a). Both compounds have been found to be highly potent inhibitors ($IC_{50} \leq 1 \text{ ng ml}^{-1}$) 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 and B virus infection in humans. A crucial difference between zanamivir and oseltamivir, however, is that zanamivir has to be administered by inhalation (10 mg twice daily), whereas oseltamivir can be administered orally (75 or 150 mg twice daily).

Neuraminidase inhibitors are anticipated to reduce illness duration by 1–3 days, to reduce the risk of virus transmission to household or healthcare 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, the shorter the duration of fever, the greater the alleviation of symptoms and the faster the return to baseline

activity and health scores³². Oseltamivir treatment of influenza illness reduces lower respiratory tract complications, particularly bronchitis and pneumonia, concomitantly with a reduction in antibiotic use and need for hospitalization³³. Also, post-exposure prophylaxis with oseltamivir, 75 mg once daily for seven days, was found to protect close contacts of influenza-infected persons against influenza illness and to prevent spread within households³⁴. Post-exposure prophylaxis with oseltamivir can be considered an effective option to prevent the transmission of influenza within households³⁵. It should be recognized, however, that oseltamivir is less effective against influenza B than against influenza A with regard to duration of fever and virus persistence³⁶.

The neuraminidase inhibitor GS4071 has been found to be positioned in the active centre of the neuraminidase^{31,37} (FIG. 5b). Two structures of the influenza A virus neuraminidase have now been solved: the first containing the N1 neuraminidase of H5N1, and the second containing the N2 neuraminidase as in H3N2³⁸ (FIG. 5c). The neuraminidase inhibitors make contact with arginine in position 292 through their carboxylic acid group, and with glutamic acid in position 119 through their basic amine (in the case of oseltamivir) or guanidinium (in the case of zanamivir) group. So, it is not surprising that at these positions mutations (R292K, E119G) can arise that engender resistance to both zanamivir and oseltamivir³⁹. The R292K mutation causes high-level resistance to oseltamivir but only low-level (5–30-fold) resistance to zanamivir. In a comprehensive study of more than 1,000 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 isolates⁴⁰. However, in children treated for influenza with oseltamivir, Kiso *et al.*⁴¹ found neuraminidase mutations in viruses from nine patients (18%), six of whom had mutations at position 292 (R292K) and two at position 119 (E119V). Zanamivir-resistant influenza H3N2 viruses might not readily arise *in vivo* owing to their poor viability (reduced fitness)⁴².

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) virus 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^{43,44}), whereas the growth and transmissibility of the E119V virus were comparable to those of wild-type virus⁴⁵.

Of note, influenza A (H3N2) virus carrying the R292K mutation in the neuraminidase gene did not transmit to ferrets under conditions in which the wild-type virus was readily transmitted⁴³. However, other mutant viruses of influenza A (H3N2) — that is, 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 type⁴⁴.

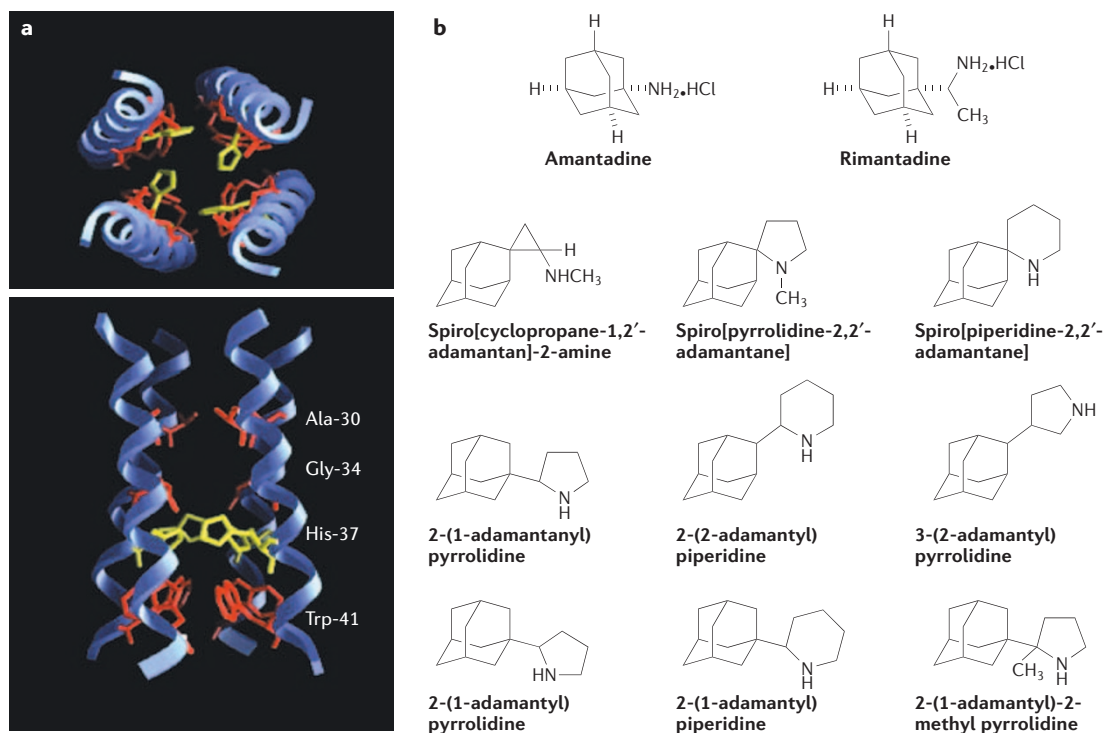


Figure 3 | Adamantan(amine) derivatives as antiviral drugs. **a** | Amantadine, rimantadine and adamantanamine derivatives share several common structural features which relate to their mode of action: blockade of the M2 channel, which is responsible for transporting H^+ ions (protons) into the interior of the virions and initiating the viral uncoating process (FIG. 2). The figure shows a model of the proposed transmembrane domain of the M2 protein with a top view as seen from the extracellular side and a cross-section in the plane of the lipid layer. Residues which were identified as facing the ion-conducting aqueous pore are indicated. **b** | Structures of the adamantan(amine) derivatives amantadine and rimantadine, and various new adamantanamine derivatives: spiro[cyclopropane-1,2'-adamantan]-2-amine¹⁶, spiro[pyrrolidine-2,2'-adamantane]¹⁶, spiro[piperidine-2,2'-adamantane]¹⁷, 2-(2-adamantyl)piperidine¹⁸, 3-(2-adamantyl)pyrrolidine¹⁹, rimantadine 2-isomers²⁰, 2-(1-adamantyl)piperidine²¹, 2-(1-adamantyl)pyrrolidine²¹ and 2-(1-adamantyl)-2-methyl-pyrrolidine²². Panel **a** reproduced with permission from REF. 11 © (2000) American Society for Microbiology.

The use of neuraminidase inhibitors in the therapy and prophylaxis of influenza A and B virus infections has been considered a new 'millennium conundrum', and the stockpiling of zanamivir and/or oseltamivir has been proposed in preparation of an influenza pandemic⁴⁶. It was also hypothesized that these compounds could, in theory, also inhibit the virus that caused the 1918 pandemic⁴⁶. In fact, recombinant viruses possessing the 1918 neuraminidase or both the 1918 neuraminidase and 1918 haemagglutinin were shown to be effectively inhibited, both *in vitro* and *in vivo* (mice), by zanamivir and oseltamivir, and a recombinant virus possessing the 1918 M2 ion channel could be effectively inhibited by amantadine and rimantadine. This indicates that current antiviral strategies could be effective in curbing a re-emerging 1918 or 1918-like influenza (H1N1) virus⁴⁷.

Recently, resistance of influenza A (H5N1) virus to oseltamivir owing to the H274Y mutation in the neuraminidase gene was described⁴⁸: 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^{49,50}, its occurrence in influenza A (H5N1) infection raised concern as it was associated with death in two of the eight influenza A (H5N1)-infected patients⁵¹. However, whether there was a causal relationship between the emergence of the H274Y mutation and the lethal outcome was not established⁵¹.

The efficacy of oseltamivir in the treatment of H5N1 infection in humans could, because of its anecdotal use, not be unequivocally shown. However, it should be noted that oseltamivir has been shown to protect ferrets against lethal influenza H5N1 infection: treatment with oseltamivir at 5 mg kg⁻¹ per day for 5 days twice daily (orally) resulted in complete inhibition of virus replication in the lungs and small intestine on day 5 post-infection and, consequently, prevented mortality⁵². Similarly, oseltamivir has proven efficacious in the treatment of mice infected with the highly pathogenic H5N1 A/Vietnam/1203/04 influenza strain, although prolonged and higher-dose oseltamivir regimens were required for achieving the most beneficial antiviral effect⁵³.

Peramivir and other cyclopentane or pyrrolidine derivatives. Whereas oseltamivir can be described as a cyclohexenyl derivative, there are several cyclopentane and pyrrolidine derivatives that have been described as neuraminidase inhibitors: peramivir (RWJ-270201, BCX-1182)^{54–56} and other cyclopentane^{56,57} and cyclopentane amide⁵⁸ derivatives, as well as various pyrrolidine derivatives, including A-192558 (REF. 59), A-315675 (REFS 60–62) and other pyrrolidines⁶³ (FIG. 5a). In addition, 2,3-disubstituted tetrahydrofuran-5-carboxylic acid derivatives have been described as influenza neuraminidase inhibitors, albeit with reduced inhibitory potency compared with the corresponding pyrrolidine analogues⁶⁴.

Peramivir and A-315675 retain activity against various zanamivir- and oseltamivir-resistant influenza A and B viruses⁶⁵. Specifically, a new oseltamivir-resistant influenza B variant that carries the D198N substitution in the viral neuraminidase was found to retain

susceptibility to peramivir and A-315675. In addition, the neuraminidase E119V mutant, which shows 6,000-fold lower susceptibility to oseltamivir and 175-fold lower susceptibility to zanamivir than wild-type virus, retained full susceptibility to A-315675 (REF. 66). Taken together, the studies performed with influenza A (H3N2) virus mutants^{41–45,65,66} indicate that neuraminidase inhibitors might select for mutations at several positions (E119V, R152K, D198N, H274Y and R292K) that only partially overlap⁶⁷ — that is, only partially engender cross-resistance.

In vivo, peramivir was found to strongly suppress influenza A (H1N1) infection in mice following a single intramuscular injection (10 mg kg⁻¹). This was ascribed to tight binding of peramivir to neuraminidase⁶⁸. Similarly, a single intramuscular/intravenous injection of peramivir one hour before virus exposure offered protection against influenza A (H5N1) in mice⁶⁹. When given orally to humans, however, peramivir did not offer robust protection

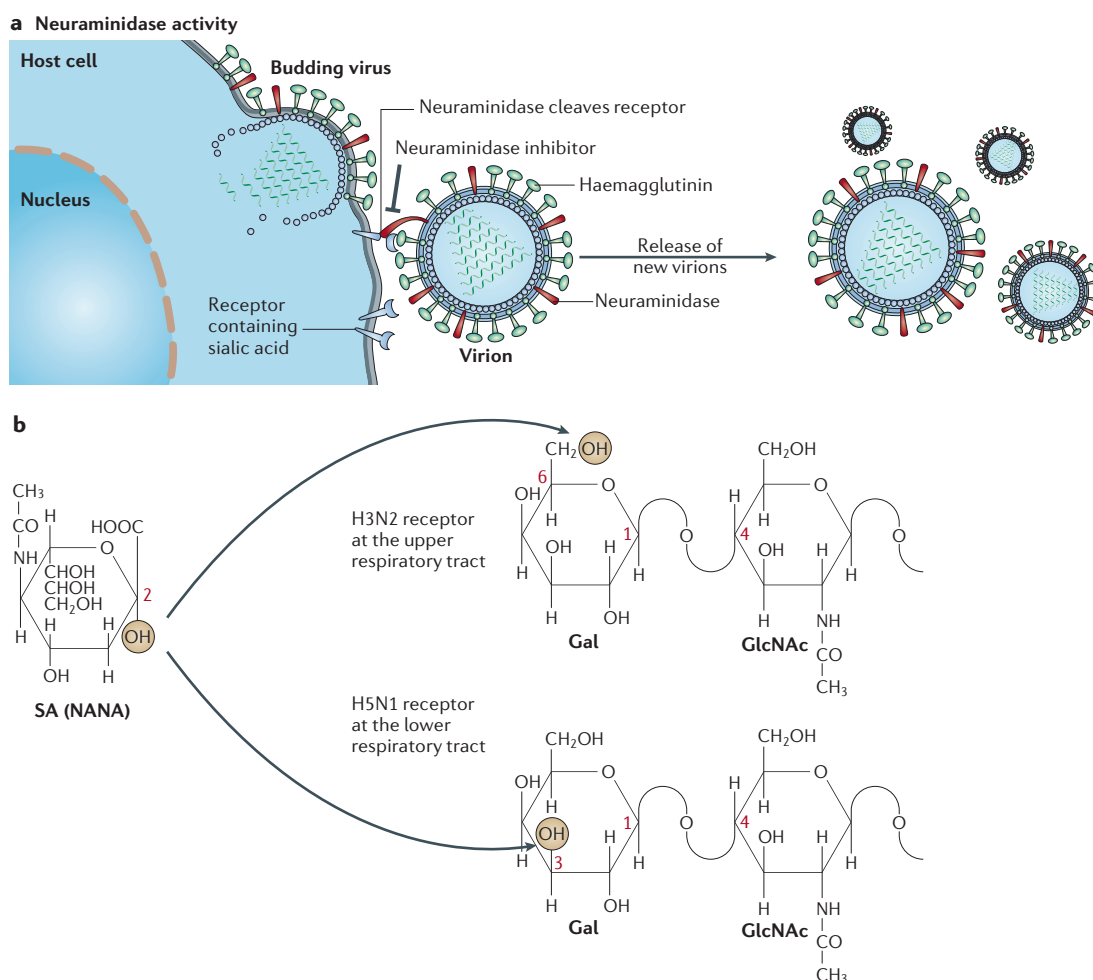


Figure 4 | Viral neuraminidase inhibition. a | The neuraminidase cleaves off sialic acid (SA, also known as *N*-acetylneuraminic acid or NANA) from the cell receptor for influenza virus (**b**), so that the newly formed virus particles can be released from the cells. Neuraminidase inhibitors, such as zanamivir and oseltamivir (FIG. 5), 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. **b** | SA 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. Panel **a** adapted with permission from REF. 25 © (2005) Massachusetts Medical Society.

against human influenza A infection, which was attributed to the very low oral bioavailability (<5%) of peramivir⁷⁰; further studies with parenteral formulations of peramivir are therefore warranted.

Dimeric neuraminidase inhibitors. Starting from zanamivir, 7-alkyl ether derivatives and bicyclic ether derivatives were synthesized which showed improved influenza A virus plaque-reduction activity *in vitro*⁷¹, and improved oral efficacy *in vivo* (mice)⁷², respectively, compared with the parent compound, zanamivir. 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^{73,74}. These compounds showed long-lasting antiviral activity owing to extremely long persistence times in the lungs, allowing a once-weekly

dosing regimen. This raises the prospect of 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 infection⁷⁴.

Ribavirin and viremide

Ribavirin has long been recognized as a broad-spectrum antiviral agent with particularly distinct activity against orthomyxoviruses (that is, influenza) and paramyxoviruses (that is, measles and respiratory syncytial virus (RSV))⁷⁵. 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 interferon- α , in the treatment of chronic hepatitis C virus (HCV) infection. The intravenous form of ribavirin has been registered for the treatment of haemorrhagic fever with renal

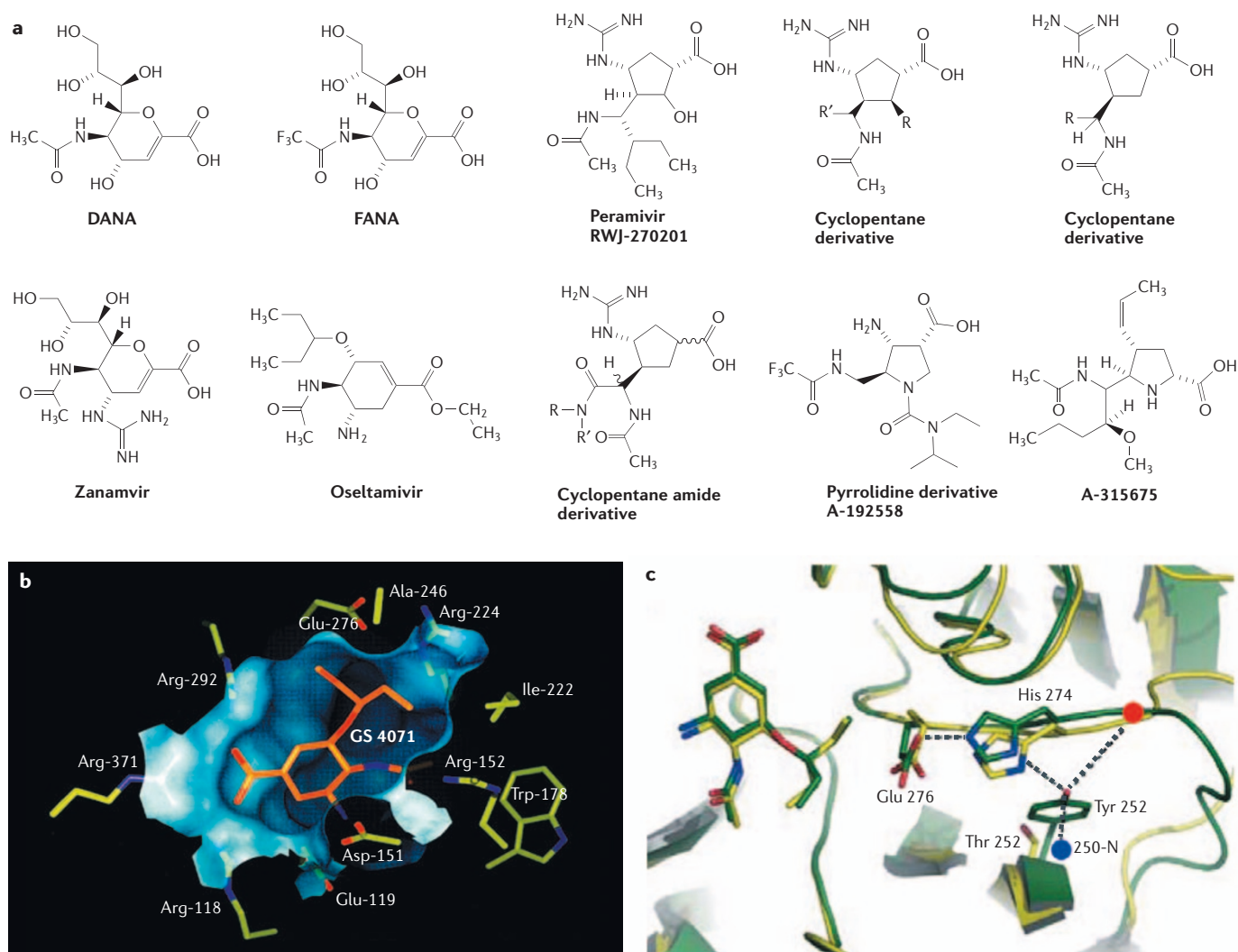


Figure 5 | **Neuraminidase inhibitors.** **a** | Structures of DANA, FANA, zanamivir (4-guanidino-Neu5Ac2en, GG167), oseltamivir (GS4071 ethyl ester, GS4104, Ro64-0796), peramivir (RWJ-270201), cyclopentane derivatives, a cyclopentane amide derivative and pyrrolidine derivatives (A-192558 and A-315675). **b** | GS4071 within the active site of the influenza A viral neuraminidase. **c** | Locations of oseltamivir-resistance mutations (H274Y) showing that the tyrosine at position 252 is involved in a network of hydrogen bonds in group-1 (H5N1 and H1N1) neuraminidases³⁸. Panel **b** reproduced with permission from REF. 31 © (1997) American Chemical Society and REF. 37 © (2002) Macmillan Magazines Ltd. Panel **c** reproduced with permission from REF. 38 © (2006) Macmillan Magazines Ltd.

syndrome (HFRS). In addition to ribavirin, viramidine — which can be considered to be the amidine prodrug of ribavirin (FIG. 6) — has been accredited with marked potential as anti-influenza drug⁷⁶. Ribavirin is notable for not generating drug resistance, and resistance of influenza-virus replication to ribavirin has, to the best of my knowledge, not been reported so far. Obviously, the lack of drug-resistance is due to the fact that ribavirin's main target of antiviral action is a cellular enzyme: inosine 5'-monophosphate (IMP) dehydrogenase (responsible for the conversion of IMP to xanthosine 5'-monophosphate), a key enzyme involved in the biosynthesis of GTP and viral RNA synthesis (FIG. 6).

Ribavirin is active against both human and avian (H5N1) influenza viruses within the 50% effective concentration (EC_{50}) range of 6–22 μM ⁷⁶. Of the three routes (oral, aerosolized and intravenous) by which ribavirin could be administered in the treatment of influenza, the intravenous route is preferred for therapy of acute influenza-virus infection. Oral ribavirin did not offer the expected clinical or virological efficacy in earlier studies with influenza A (H1N1)⁷⁷. Ribavirin aerosol has been used successfully (based on reduction of virus shedding and clinical symptoms) in the treatment of influenza-virus infections in college students⁷⁸. Intravenous ribavirin (producing a mean plasma concentration of 20–60 μM) was associated with symptomatic improvements and elimination of influenza virus from nasopharyngeal swabbings and tracheal aspirates⁷⁹.

Intravenous ribavirin has been further investigated, with success, in the treatment of Lassa fever⁸⁰ and HFRS⁸¹. Both studies showed 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 grams of ribavirin followed by 1 gram every 6 hours for 4 days. During the following 5 days, administering a maintenance dose of 0.5 gram every 8 hours should generate the concentrations needed to achieve suppression of (human and avian) influenza-virus replication. The dose-limiting toxicity would be haemolytic anaemia, which should be reversible on cessation of therapy.

Sialidase fusion protein and sialylglycopolymers

Recently, a recombinant fusion protein composed of the sialidase (neuraminidase) catalytic domain derived from *Actinomyces viscosus* fused with a cell-surface-anchoring sequence was reported as a novel broad-spectrum inhibitor of influenza-virus infection⁸². The sialidase fusion protein is to be applied topically as an inhalant to remove the influenza-virus receptors — sialic acids — from the airway epithelium. A sialidase fusion construct, DAS181 (Fludase), was shown to effectively cleave sialic-acid receptors used by both human and avian influenza viruses. DAS181 showed potent antiviral and cell-protective efficacies against a panel of laboratory strains and clinical isolates of influenza A and influenza B, with virus-replication inhibition EC_{50} values in the range of 0.04–0.9 nM. Significant *in vivo* efficacy of the sialidase fusion construct was noted in both prophylactic and therapeutic approaches⁸².

The sialic-acid receptors could also be targeted by sialic-acid polyacrylamide conjugates, also termed sialylglycopolymers^{83,84}. Sialylglycopolymers inhibit influenza-virus attachment to the cells. *In vivo*, when administered in aerosol form within 24–110 hours of infection, they were found to completely prevent mortality in mice infected with mouse-adapted influenza A strains (H3N2, H1N1). These sialylglycopolymers target the receptor determinant SA α 2-6Gal β 1-4GlcNAc, recognized by human influenza A and B viruses. This would make them potentially valuable for protection against any newly emerging (human) influenza virus strains.

siRNAs and phosphorothioate oligonucleotides

Small 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 in complexes with a polycation carrier either before or after initiating virus infection⁸⁵. Delivery of siRNAs specific for highly conserved regions of the nucleoprotein or acidic polymerase significantly reduced lung virus titres in mice infected with influenza A virus 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 subtypes⁸⁶. It could be predicted that specific siRNAs would be effective against influenza from equally effective results obtained with other specific siRNAs against the SARS (severe acute respiratory syndrome) coronavirus, in comparable situations⁸⁷.

Phosphorothioate oligonucleotides (PS-ONs) (that is, REP 9, a 40-mer PS-ON) offer potential, when administered as aerosol in the prophylaxis and therapy of influenza infection⁸⁸. Similarly, antisense phosphorodiamidate morpholino oligomers (ARP-PMOs) could be further pursued for their potential in the treatment of H5N1 influenza A virus infections⁸⁹.

Influenza-virus RNA-polymerase inhibitors

The influenza-virus 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' to initiate the transcription and subsequent translation process⁹⁰. The polymerase-complex genes contribute to the high virulence of the human H5N1 influenza-virus isolate A/Vietnam/1203/04 (REF. 91). This observation highlights the importance of novel antivirals that target the polymerase for further development of 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. Like the inhibitors that have been found to inhibit the reverse transcriptase (RNA-dependent DNA polymerase) of HIV or RNA replicase (RNA-dependent RNA polymerase) of HCV, influenza RNA-replicase inhibitors can be divided into two classes:

PEGylation

Addition of poly(ethylene glycol) (PEG) groups to proteins can increase their resistance to proteolytic degradation, improve their water solubility and reduce their antigenicity.

Prodrug

A pharmacologically inactive compound that is converted to the active form of the drug by endogenous enzymes or metabolism. It is generally designed to overcome problems associated with stability, toxicity, lack of specificity or limited (oral) bioavailability.

Small interfering RNAs

(siRNAs). Small (~ 20 nucleotide) RNA constructs that interfere with RNA translation.

Phosphorothioate oligonucleotides

Antisense oligonucleotides in which phosphate groups are replaced by thiophosphate groups.

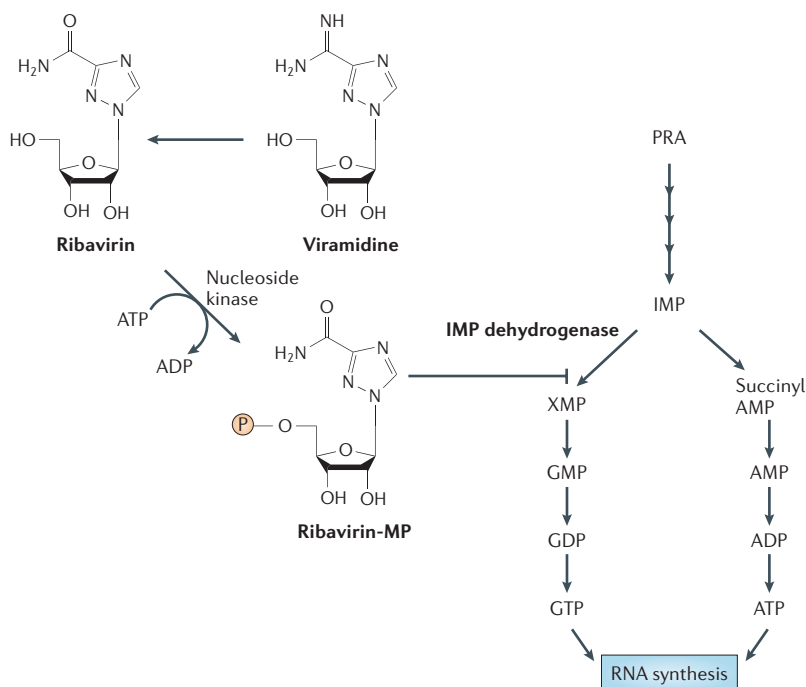


Figure 6 | IMP dehydrogenase inhibition. Viramidine acts as a prodrug (precursor) of ribavirin, which is converted intracellularly to its 5'-monophosphate derivative, ribavirin-MP. The latter inhibits inosine 5'-monophosphate (IMP) dehydrogenase, a crucial enzyme in the biosynthesis of RNA, including viral RNA. IMP dehydrogenase is responsible for the conversion of IMP into xanthosine 5'-monophosphate (XMP) 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.

nucleosides and non-nucleosides. Examples of the nucleoside type of inhibitors are 2'-deoxy-2'-fluoroguanosine (FdG)^{92,93} and T-705⁹⁴⁻⁹⁶ (FIG. 7).

T-705 is a substituted pyrazine with potent anti-influenza-virus activity *in vitro* and *in vivo*. According to a comparative 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*)⁹⁵. It has been postulated that T-705 is converted intracellularly to the ribonucleotide T-705-4-ribofuranosyl-5'-monophosphate (T-705 RMP) by a phosphoribosyl transferase and, on further phosphorylation to its 5'-triphosphate (FIG. 7), T-705 RTP would then inhibit influenza-virus RNA polymerase in a GTP-competitive manner⁹⁶. 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 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 an attractive target for influenza-virus inhibitors: it can be inhibited by 4-substituted 2,4-dioxobutanoic acid derivatives⁹⁷ and *N*-hydroxamic acid/*N*-hydroxy-imide derivatives⁹⁸. Likewise, flutimide, a 2,6-diketopiperazine (FIG. 7) identified in extracts of the fungal species *Delitschia confertasporea*, has been shown 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 culture⁹⁹. 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 (EC₅₀ for virus replication: 0.08–0.09 μM) has been reported¹⁰⁰, represented by thiadiazolo[2,3-*a*]pyrimidine and pyrimidinyl acylthiourea (FIG. 7). Although the mechanism of action of this class of 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 might also serve as leads for further optimization, although the lead compounds showed only modest activity against influenza A virus¹⁰¹.

Interferon (inducers)

Interferon was originally discovered almost 50 years ago, with influenza virus as its inducer¹⁰². In fact, Baron and Isaacs alluded to the absence of interferon in lungs from fatal cases of influenza¹⁰³. In some earlier studies, interferon instilled by the intranasal route did not offer significant protection in the prophylaxis of influenza-A-virus infections^{104,105}.

Since then, PEGylated interferon-α (injected parenterally), in combination with (oral) ribavirin has become the standard therapy for chronic HCV infections. So, extensive experience with this combination has been accumulated¹⁰⁶, which could be readily implemented in the prophylaxis and therapy of human as well as avian influenza-virus infections in humans. In the prophylaxis and therapy of influenza-virus infections, the duration of treatment would be much shorter than in the treatment of hepatitis C, which would obviously affect 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 ~40 years ago¹⁰⁷, might also have 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 infection¹⁰⁸. Furthermore, when combined with (intranasal) vaccination, poly(I)-poly(C) conferred complete protection against influenza-virus infection, which might have been mediated by an upregulated expression of Toll-like receptor 3 and interferon-α/β as well as T_H1- and T_H2-related cytokines¹⁰⁹. 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 might help in the prophylaxis or therapy of avian or human influenza-virus infections, but in view of the 'renaissance' of interferon in the treatment of HCV infection, the potential of interferon for influenza might well deserve to be revisited.

Signal-transduction inhibitors

Signal-transduction inhibitors, such as those targeted at either ErbB tyrosine kinase¹¹⁰ or the Abl kinase family

(that is, imatimib¹¹¹), have recently been shown to suppress the *in vitro* replication and *in vivo* dissemination of poxviruses. Likewise, the signalling cascade could be considered an attractive opportunity for future strategies to block influenza-virus production. The export of the influenza-virus ribonucleoprotein (RNP) from the nucleus depends on the cellular Raf/MEK/ERK kinase (MAPK) signalling cascade¹¹², and this PKC α -mediated activation of ERK signalling is specifically triggered by the accumulation of influenza-A-virus haemagglutinin

at the cell membrane¹¹³. The PKC α -mediated ERK activation could therefore be considered a potential target for intervention with influenza-virus propagation.

Other potential targets

Recently, the CPSF30-binding site on the NS1A protein of influenza A virus was proposed as a potential target for the development of antivirals directed against influenza A virus¹¹⁴. The NS1A protein inhibits the 3'-end processing of cellular pre-mRNAs by binding to the 30-kDa subunit of cleavage and polyadenylation specificity factor (CPSF30). This binding site is also required for efficient virus replication. A fragment of CPSF30, termed F2F3 because it spans the second and third zinc-finger domains, was found to specifically bind to the CPSF30 binding site: it inhibited influenza-A-virus replication but did not inhibit the 3'-end processing of cellular pre-mRNAs. This might indicate that the CPSF30-binding site of NS1A could possibly be targeted by low-molecular-mass inhibitors of HIV replication¹¹⁴.

Combination therapy

Drug-combination regimens used in the treatment of *Mycobacterium tuberculosis* and HIV infections achieve greater benefit than each compound given individually, reduce the likelihood of drug-resistance development and might allow the individual drug doses to be lowered, thereby diminishing adverse effects. 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 infection¹¹⁵. Against influenza, such a triple-drug regimen might theoretically be expected to yield a beneficial outcome. The three drugs are, individually, all active against influenza-virus replication *in vitro* and act through different mechanisms, which implies that, when combined, they might 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.*¹¹⁶ pointed to the additive synergistic action between interferon- α 2 and rimantadine or ribavirin. Similarly, combinations of (PEGylated) interferon with neuraminidase inhibitors (zanamivir, oseltamivir or peramivir) could also be considered, and so might combinations of ribavirin (or viremide) with the neuraminidase inhibitors.

Combinations of the adamantan(amin)es (amantadine or rimantadine) with the neuraminidase inhibitors (zanamivir or oseltamivir) should also receive attention. *In vitro*, rimantadine was found to act synergistically with zanamivir, oseltamivir or peramivir in reducing the extracellular yield of influenza A (H3N2) virus¹¹⁷. *In vivo*, oseltamivir at 10 mg kg⁻¹ per day and amantadine at 15 mg kg⁻¹ per 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 compared with both compounds given as single-agent chemotherapy¹¹⁸. The only controlled

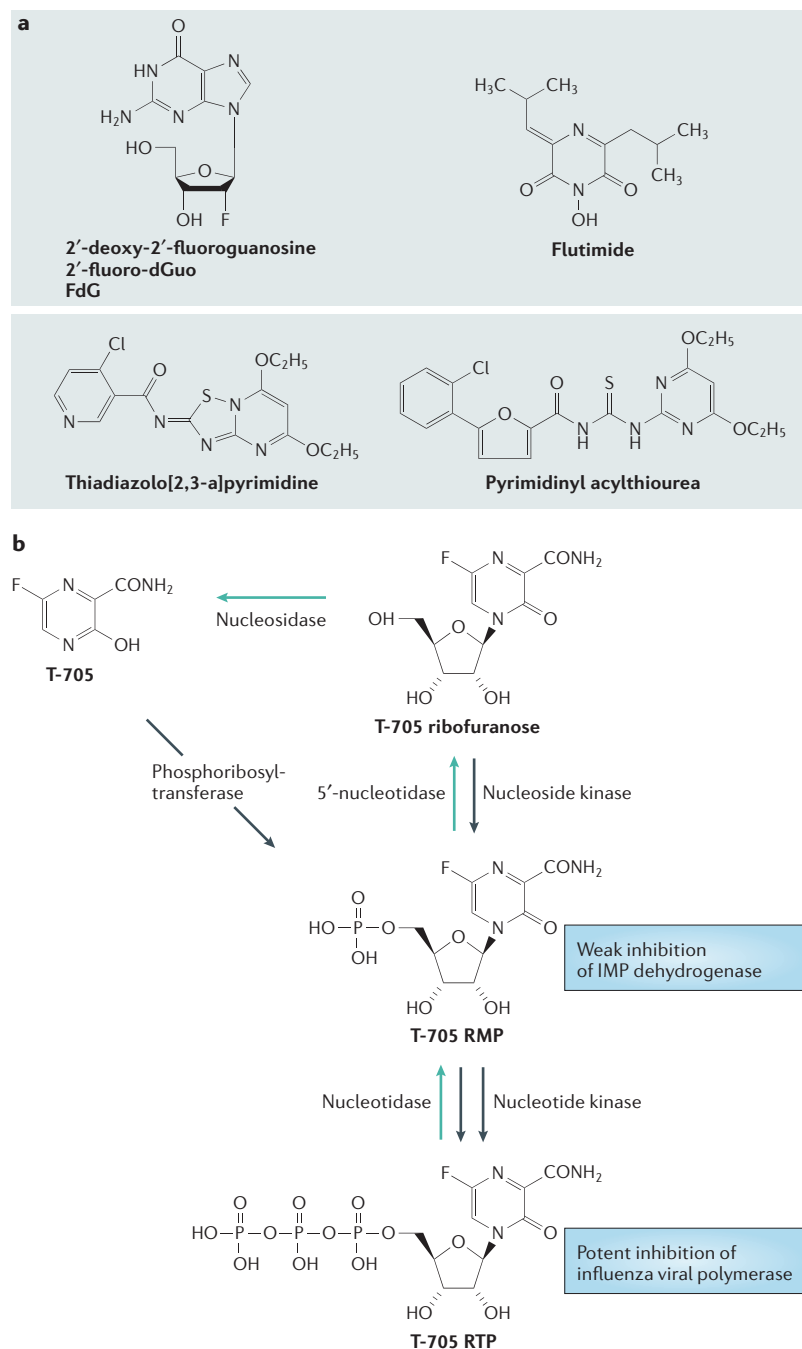


Figure 7 | **Influenza-virus RNA-polymerase inhibitors.** **a** | FdG, Flutimide, thiadiazolo [2,3-a]pyrimidine and pyrimidinyl acylthiourea. **b** | The postulated mode of action of T-705, according to Furuta and colleagues⁹⁶.

study in humans was a comparison of rimantadine alone versus rimantadine plus inhaled zanamivir in hospitalized (adult) patients with serious influenza¹¹⁹. Although preliminary, this study pointed to a higher clinical benefit for the combination of zanamivir with rimantadine.

Conclusions

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

derivatives (amantadine), neuraminidase inhibitors (zanamivir and 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 or viral uncoating process, or on as-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 which has proven most successful in the cases of the HIV reverse transcriptase and HCV RNA polymerase.

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Competing interests statement

The author declares no competing financial interests.

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