Avian influenza A (H5N1) infection: targets and strategies for chemotherapeutic intervention

Erik De Clercq and Johan Neyts

Rega Institute for Medical Research, Minderbroedersstraat 10, B-3000 Leuven, Belgium

In an avian flu pandemic, which drugs could be used to treat or prevent infection with influenza A (H5N1) virus? Foremost are the viral neuraminidase inhibitors oseltamivir and zanamivir, which have already been used to treat human influenza A (H1N1 and H3N2) and B virus infections. The use of the M2 ion channel blockers amantadine and rimantadine is compounded by the rapid development of drug resistance. Although formally approved for other indications (i.e. treatment of hepatitis C), ribavirin and pegylated interferon might also be useful for controlling avian flu. Combined use of the currently available drugs should be taken into account and attempts should be made to develop new strategies directed at unexplored targets such as the viral proteins hemagglutinin, the viral polymerase (and endonuclease) and the non-structural protein NS1. As has been shown for other viral infections, RNA interference could be a powerful means with which to suppress the replication of avian H5N1.

Introduction

The outbreaks of avian influenza A (H5N1) in Southeast Asia, the increasing geographic distribution of this epizootic virus and its ability to transfer to humans and cause severe infection (i.e. pneumonia) have aroused serious concerns regarding the control measures that should be undertaken to curb a potential pandemic of the disease [1–3]. In the wake of such a pandemic, several preventive and therapeutic strategies have been formulated [1], the most prominent being the stockpiling of neuraminidase inhibitors – in particular, oseltamivir (Tamiflu®).

The current avian H5N1 originated in 1997 in Hong Kong and has spread (through birds) to Southeast Asia and other countries, with occasional transmission to humans (∼200 human cases, more than half of which were fatal). Whether the current avian H5N1 will evolve further to cause a pandemic through either mutation of the current H5N1 virus ('antigenic drift') or reassortment of an avian influenza virus with a human (or other non-avian) influenza virus ('antigenic shift') [4] is unpredictable at present.

We have recently reviewed the antiviral agents that are active against influenza viruses and that could be used, either therapeutically and/or prophylactically, in an influenza virus pandemic, whether it be human, avian, equine,

porcine or other [1]. Here, we address which strategies could be undertaken and which targets could be envisaged to combat avian H5N1 infections specifically.

The influenza virus replicative cycle

The replicative cycles of human (H1N1, H2N2 and H3N2) and avian (H5N1) influenza viruses [1,5] follow a similar 'scenario' [1] (Figure 1). After binding to sialic acid receptors, influenza virions are internalized by receptor-mediated endocytosis. The low pH in the endosomes triggers the fusion of the viral and endosomal membranes, and the influx of H^+ through the M2 channel releases the viral RNA genes in the cytoplasm ('uncoating'). Adamantane derivatives block this uncoating step. The RNA replication and transcription steps [which require repeated cycles of $(-)RNA \leftrightarrow (+)RNA$ polymerization reactions] occur in the nucleus and can be blocked indirectly by inosine 5′ monophosphate (IMP) dehydrogenase inhibitors (e.g. ribavirin), which suppress the biosynthesis of GTP, or directly by RNA polymerase inhibitors (e.g. T705). The translation of viral mRNA to proteins could be prevented by interferon and small interfering (si)RNAs. Packaging and budding of virions occur at the cytoplasmic membrane, and neuraminidase inhibitors block the release of newly formed virions from the infected cells [6].

Anti-influenza-virus compounds

The anti-influenza-virus compounds – both those that are currently available as antiviral drugs and those that are still being developed – can be divided into different classes according to their molecular target of interaction:
 (i) neuraminidase inhibitors (e.g. zanamivir, oseltamivir and peramivir);
 (ii) M2 ion channel blockers (e.g. amantadine and rimantadine);
(iii) IMP dehydrogenase inhibitors (e.g. ribavirin and viramidine);
(iv) interferon and siRNAs;
 (v) RNA polymerase (or endonuclease) inhibitors (e.g. T705 and flutimide).
 Representative congeners belonging to classes (i), (ii), (iii) and (v) are shown in Figure 2.

Neuraminidase inhibitors

Whereas viral hemagglutinin (HA) is needed for the influenza virus to interact with its receptor bearing
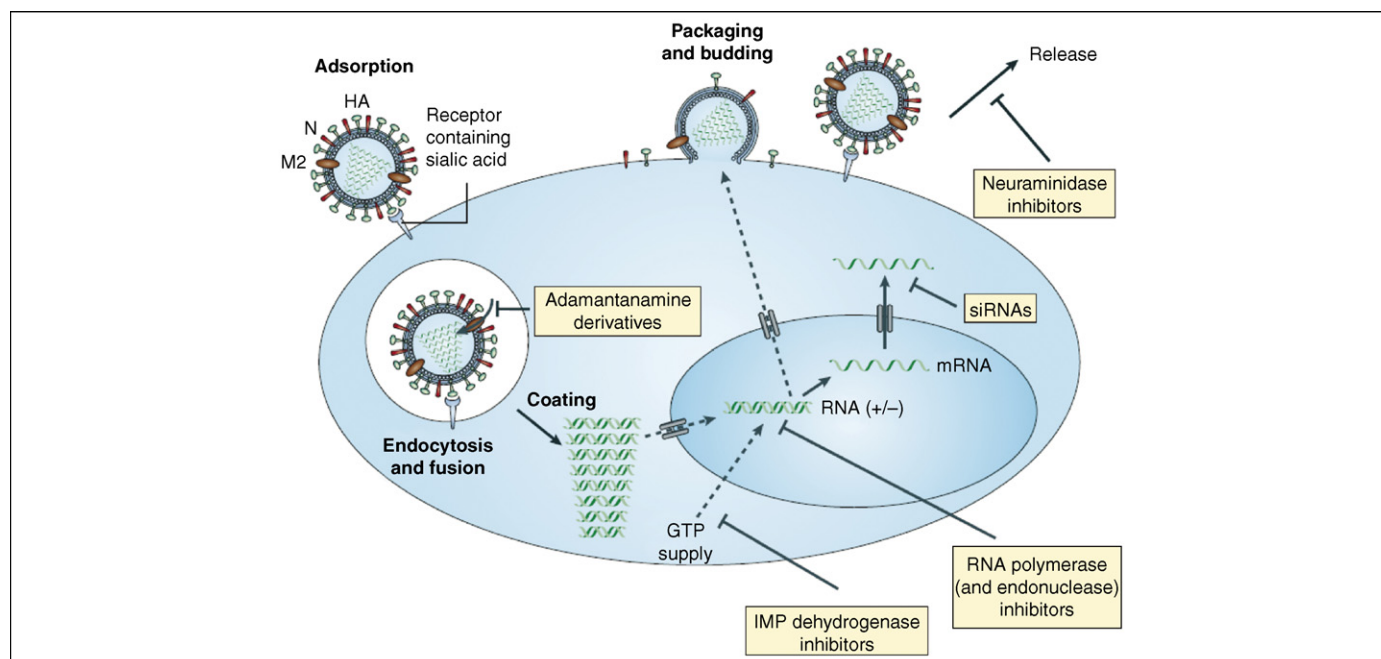

Figure 1. The life cycle of the influenza virus. Shown are the targets of chemotherapeutic intervention. Anti-influenza-virus compounds fall into different classes according to their molecular target of interaction: neuraminidase inhibitors, M2 ion channel blockers (adamantanamine derivatives), IMP dehydrogenase inhibitors, interferon and siRNAs, and RNA polymerase (and endonuclease) inhibitors. Abbreviation: N, neuraminidase. Reproduced, with permission, from Ref. [1].

N-acetylneuraminic acid (NANA, sialic acid), viral neuraminidase, which cleaves NANA, enables the progeny virions to leave the infected cells and spread to other host cells. By blocking the release of virus particles, neuraminidase inhibitors prevent this spread of virus [6,7].

Of the neuraminidase inhibitors, oseltamivir has received most attention as 'the' drug that should be stockpiled for therapeutic use in humans infected with avian flu. Given its ease of administration (orally, 75 mg twice daily for five days) and systemic availability, oseltamivir can certainly be recommended as the drug of choice to be used against H5N1. It should not be ignored, however, that the use of oseltamivir can lead to the development of resistance. Resistance to oseltamivir in clinical isolates of human influenza A has been associated with mutations at positions 119 (E119V), 152 (R152K), 198 (D198N), 274 (H274Y) and 292 (R292K) of the neuraminidase. In particular, the R292K mutation has been associated with resistance of the (human) influenza A virus to oseltamivir [8].

Recently, resistance of H5N1 to oseltamivir was shown to be caused by the H274Y mutation. 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 [9]. However, two other patients from whom the H274Y mutant H5N1 virus was isolated during oseltamivir treatment died from the infection [10], indicating a possible association of this mutation with death.

Interestingly, probenecid was found to prevent the renal secretion of the parent compound of oseltamivir (an oral prodrug form), thus markedly increasing the systemic exposure of oseltamivir [11]. Combining oseltamivir with probenecid might be an important therapeutic option in severely ill patients and might enable the use of the

standard dose (75 mg twice daily) of oseltamivir, as currently recommended. From studies of the highly pathogenic A/Vietnam/1203/04 strain of H5N1 in mice [12], it could be inferred that a prolonged oseltamivir regimen (i.e. eight- or ten-day, rather than five-day, treatment) and a higher dose (150 mg, instead of 75 mg, twice daily) might be required for the most beneficial antiviral effect. Alternatively, intravenous administration of oseltamivir might be considered a therapeutic option, particularly in severely ill patients.

The other currently available neuraminidase inhibitor is zanamivir, which must be administered through an inhalation device. There is low systemic availability of zanamivir following administration, including low drug levels in the lower respiratory tract (where most of the replication of current H5N1 viruses seems to take place) [13]. However, zanamivir is active against the oseltamivir-resistant H5N1 H274Y variant. Although not yet demonstrated for avian influenza, neuraminidase-based resistance is more likely to develop against oseltamivir than against zanamivir [14]. Combination of the two drugs might be considered if it is further corroborated that the resistance patterns of zanamivir and oseltamivir do not overlap. If needed urgently (e.g. in severely ill patients), zanamivir and oseltamivir should be administered parenterally (i.e. intramuscularly or intravenously) and, hence, the adequate formulations should be made available for this purpose.

Several other neuraminidase inhibitors such as peramivir (RWJ270201) and A315675 have been described [15,16]. Interestingly, both peramivir and A315675 have proven to be effective against a panel of five zanamivir-resistant and six oseltamivir-resistant influenza A and B viruses [17]; again, this indicates that resistance to neuraminidase inhibitors might not overlap. Also, the structure of the influenza A (H5N1) neuraminidase has recently been
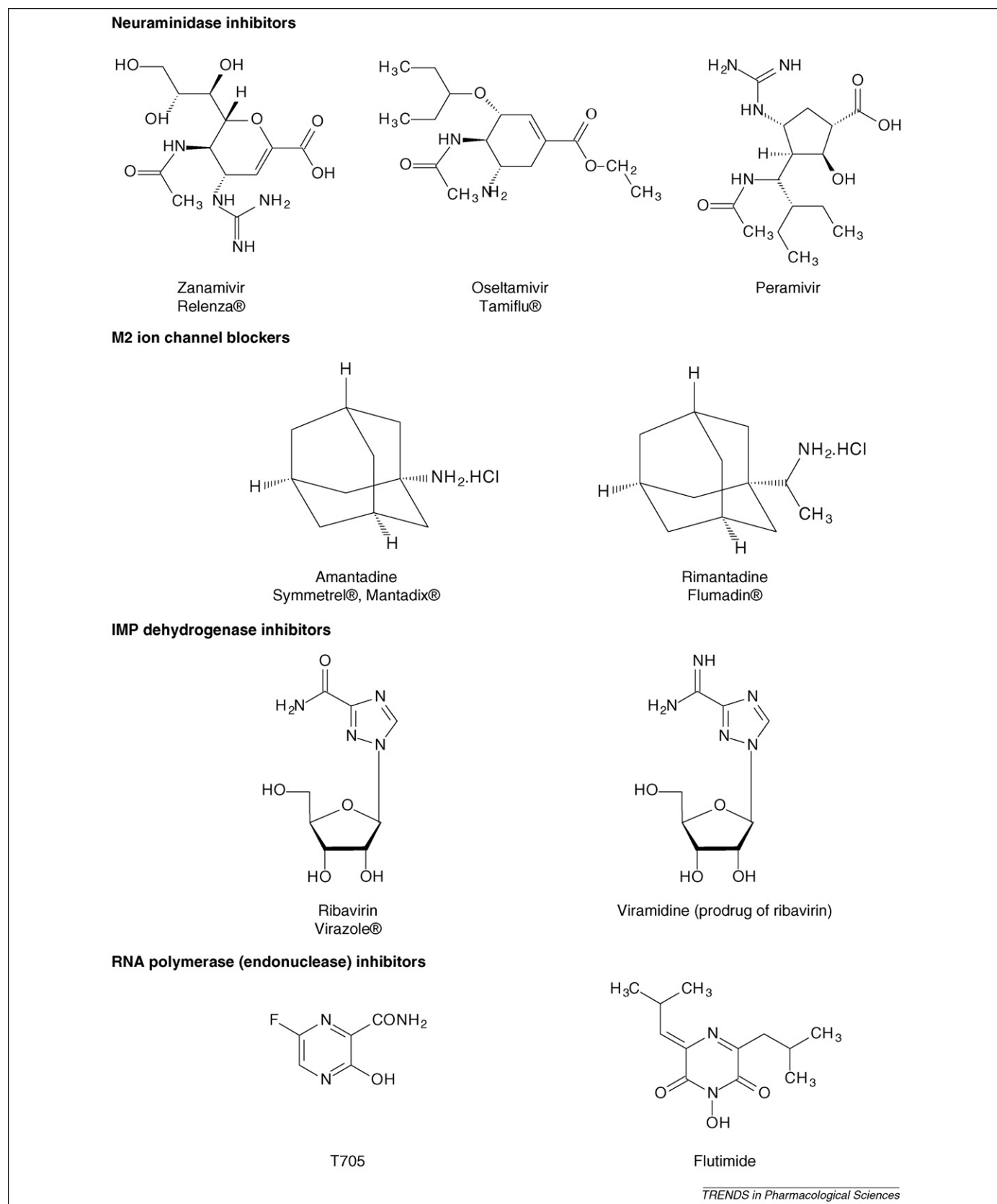
Neuraminidase inhibitors

Zanamivir
Relenza®

Oseltamivir
Tamiflu®

Peramivir

M2 ion channel blockers

Amantadine
Symmetrel®, Mantadix®

Rimantadine
Flumadin®

IMP dehydrogenase inhibitors

Ribavirin
Virazole®

Viramidine (prodrug of ribavirin)

RNA polymerase (endonuclease) inhibitors

T705

Flutimide

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Figure 2. Major classes of anti-influenza-virus compounds. Shown are typical examples of compounds for the different classes.

resolved [18], which should help the rational design of inhibitors.

In initial clinical studies, oral administration of peramivir did not offer robust protection against infection with human influenza A virus [19]; further studies with parenteral formulations of peramivir are warranted. Meanwhile, long-acting dimeric inhibitors of influenza virus neuraminidase have been developed, which offer

the prospect of a new type of anti-influenza drug that could be administered as a once-weekly dose to prevent infection [20].

M2 ion channel blockers

Do any antiviral agents other than neuraminidase inhibitors have potential in the control (prevention or therapy) of influenza A (H5N1) infections? The adamantane derivatives amantadine and rimantadine are specifically active against influenza A. They interfere with the viral uncoating process through a direct interaction with the matrix (M2) protein, which functions as a channel for hydrogen ions (protons). Amantadine and rimantadine, however, are notorious for rapidly leading to drug resistance, which compromises their potential usefulness, if used as single agents, in the treatment of H5N1 infections. This concern has been highlighted by recent data about adamantane resistance among influenza A (H3N2) viruses isolated in the USA [21] and worldwide [22].

Of concern with regard to the potential use of the adamantane derivatives against influenza A (H5N1) is that, of the two clades of currently circulating H5N1 viruses [clade 1 (Vietnam, Thailand and Cambodia) and clade 2 (Indonesia)], the whole of clade 1 is amantadine resistant, and drug resistance has also been noted in strains from clade 2 [23,24].

Several new adamantane derivatives that are effective against influenza A (H3N2), such as 2-(1-adamantyl)-2-methylpyrrolidine [25], have been synthesized; however, these compounds have not yet been evaluated for their activity against H5N1 viruses.

IMP dehydrogenase inhibitors

The broad-spectrum antiviral agent ribavirin has been used as an inhibitor of influenza A and B virus infections for >30 years [26]. Ribavirin is particularly active against (−)RNA viruses, including the orthomyxoviruses and paramyxoviruses. The paramyxovirus respiratory syncytial virus (RSV) is the only (−)RNA virus infection for which ribavirin has been formally approved (as an aerosol). Although oral ribavirin has not been successful in the treatment of influenza A (H1N1) infection [27], it is used, in combination with (injectable) pegylated α-interferon, to treat hepatitis C virus (HCV) infection. Recently, viramidine – the carboxamidine analogue of ribavirin – was shown to have similar efficacy to ribavirin against influenza virus infections and, considering its lower toxicity, viramidine might warrant further evaluation as a possible therapy for influenza A virus, including H5N1, infections [28].

Interferon and siRNAs

Ironically, interferon was discovered, almost 50 years ago, with influenza virus as the inducer [29]. In fact, Baron and Isaacs described the absence of interferon from the lungs in fatal cases of influenza [30]. Since then, interferon and its use have come a long way, and pegylated α-interferon, in combination with ribavirin, has become the standard therapy for HCV infection. Therefore, extensive experience has been accumulated with this combination that could be readily implemented in the therapy of avian flu, for which the duration of treatment would be much shorter than for HCV. When using interferon for the prophylaxis or treatment of influenza, one should, however, take into account the fact that interferon alone might cause flu-like symptoms.

Based on the principle of RNA interference, siRNAs that are specific for highly conserved regions of the viral nucleoprotein or acidic polymerase have been found to protect mice against a lethal influenza virus (i.e. H5N1) challenge *in vivo* [31]. Delivery systems compatible with human use have demonstrated the potential use of siRNAs for prophylaxis and therapy of influenza virus infections in humans [32]. Similarly, siRNAs have proven to be a powerful new means with which to combat other respiratory virus infections such as those involving RSV and severe acute respiratory syndrome (SARS) [33,34].

RNA polymerase (or endonuclease) inhibitors

More than ten years ago, 2′-deoxy-2′-fluoroguanosine was described as an inhibitor of influenza virus transcription, although it has not been studied further in this capacity [35]. Also, a substituted pyrazine (T705) was reported that has potent *in vitro* activity against influenza A, B and C, and is more effective than oseltamivir at protecting mice that have been experimentally infected with influenza A/PR/8/34. T705 has not yet been evaluated against avian H5N1 but, considering its target of action, it can be assumed to be effective. This pyrazine is apparently recognized by cellular enzymes as a nucleobase and converted to the ribofuranosyl-5′-triphosphate metabolite, which – in turn – inhibits the viral polymerase [36]. A polymerase inhibitor can be expected to have a resistance profile that does not overlap with those of the other known influenza virus inhibitors.

Another possibility for selectively inhibiting influenza virus replication is to target the cap-dependent endonuclease activity of the polymerase complex. In the mid-1990s, several inhibitors of influenza endonuclease (e.g. flutimide) were reported as selective inhibitors of influenza virus A and B replication, but they have not been developed further [37]. In view of the need for additional anti-H5N1 drugs, it might seem mandatory to explore further the viral polymerase (and/or endonuclease) as an antiviral target.

Recently, new compounds such as thiadiazolo[2,3-a]pyrimidine and pyrimidinyl acylthiourea were reported to inhibit influenza A (H1N1) virus replication at a low (<0.1 μM) concentration [38]. However, the mechanism and target of action of these compounds have not been elucidated. In addition, a sialidase fusion protein [DAS181 (fludase)] has been reported that, at subnanomolar concentrations, inhibits both human and avian influenza virus replication [39]. The potential of fludase in the (topical) treatment of influenza A virus infections remains to be established.

Drug combinations

A plausible, but not yet proven, option for the prophylaxis or treatment of influenza A (H5N1) virus infection is the combination of pegylated interferon and ribavirin, which could be complemented with amantadine (or rimantadine). This triple-drug combination has shown efficacy in the
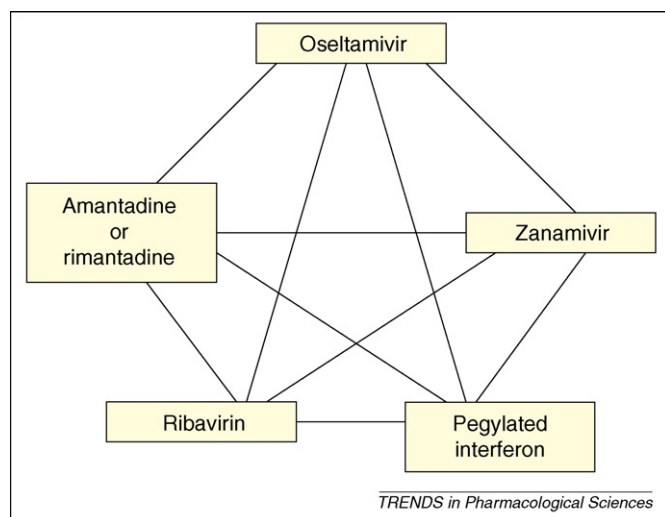
Figure 3. Potential double-, triple- and quadruple-drug combinations of currently available anti-influenza-virus compounds. Drug combination regimens are aimed at synergizing antiviral action, diminishing (toxic) side effects and/or reducing the risk of developing drug resistance.

treatment of HCV infection [40] and might be worth exploring further for the treatment and/or prevention of influenza virus infections. Even if based only on the currently available drugs, there are several double-, triple- and quadruple-drug combinations that could be envisaged for the prevention and treatment of avian H5N1 (Figure 3) – including the combination of oseltamivir and zanamivir (because their resistance profiles overlap only partially), the combination of these neuraminidase inhibitors with M2 ion channel blockers, and further extension of these combinations to include pegylated interferon and ribavirin.

The compounds shown in Figure 3 are active against influenza through different mechanisms and might, when combined, synergize their antiviral action while reducing the risk of the emergence of drug-resistant virus variants.

New targets and approaches
In addition to viral RNA polymerase and/or endonuclease, mentioned earlier as potential targets for new anti-influenza-virus agents, there are some other clues regarding the virulence of H5N1 viruses in humans [41] that could be considered as points of attack for chemotherapeutic intervention. First, the amino acid at position 627 in the viral polymerase protein PB2 is mutated from glutamic acid to lysine in H5N1 viruses, and this might represent an adaptation of H5N1 viruses for efficient replication in mammalian cells [42]. Second, the HA of H5N1 viruses contains an unusual stretch of basic amino acids (RRRKKRG) that is cleaved by ubiquitous intracellular proteases (including furin). Recombinant H5N1 viruses that lack these basic amino acids are not virulent in mice [40,43]. Third, the C-terminal domain of the non-structural protein NS1 of avian H5N1 viruses contains a sequence motif (ESEV/EPEV) that can be considered a virulence factor because it binds to human host proteins and disrupts their morphology and functioning [44]. In ferrets, however, the C-terminal sequence is not required for the virulence of H5N1 viruses [45]. Therefore, the role of the ESEV/EPEV motif and other

molecular determinants of the virulence of H5N1 viruses must be explored further [41].

Concluding remarks
Several drugs are available that could be used, either alone or in combination, for the prophylaxis and treatment of an influenza A (H5N1) pandemic. This includes compounds that are already used against influenza A virus infections – such as amantadine, rimantadine, zanamivir and oseltamivir – in addition to compounds that are used for infections with other viruses, such as ribavirin and pegylated interferon, which are used to treat HCV. Attempts to design and develop further new antiviral drugs should be intensified, whether based on known molecular targets such as neuraminidase and/or the viral uncoating process or on relatively unexplored targets such as the viral endonuclease or the viral RNA polymerase, which, in principle, could be targeted by both nucleoside and non-nucleoside inhibitors. The latter approach can be thought of as analogous to the nucleoside and non-nucleoside inhibitors of the HIV reverse transcriptase and of the HCV RNA polymerase.

References
1 De Clercq, E. (2006) Antiviral agents active against influenza A viruses. *Nat. Rev. Drug Discov.* 5, 1015–1025
2 Kaye, D. and Pringle, C.R. (2005) Avian influenza viruses and their implication for human health. *Clin. Infect. Dis.* 40, 108–112
3 Beigel, J.H. *et al.* (2005) Avian influenza A (H5N1) infection in humans. *N. Engl. J. Med.* 353, 1374–1385
4 Belshe, R.B. (2005) The origins of pandemic influenza – lessons from the 1918 virus. *N. Engl. J. Med.* 353, 2209–2211
5 Lamb, R.A. and Krug, R.M. (2001) Orthomyxoviridae: the viruses and their replication. In *Fields Virology* (4th edn) (Knipe, D.M. and Howley, P.M., eds), pp. 1487–1531, Lippincott Williams & Wilkins
6 De Clercq, E. (2004) Antivirals and antiviral strategies. *Nat. Rev. Microbiol.* 2, 704–720
7 Moscona, A. (2005) Neuraminidase inhibitors for influenza. *N. Engl. J. Med.* 353, 1363–1373
8 Kiso, M. *et al.* (2004) Resistant influenza A viruses in children treated with oseltamivir: descriptive study. *Lancet* 364, 759–765
9 Le, Q.M. *et al.* (2005) Avian flu: isolation of drug-resistant H5N1 virus. *Nature* 437, 1108
10 de Jong, M.D. *et al.* (2005) Oseltamivir resistance during treatment of influenza A (H5N1) infection. *N. Engl. J. Med.* 353, 2667–2672
11 Hill, G. *et al.* (2002) The anti-influenza drug oseltamivir exhibits low potential to induce pharmacokinetic drug interactions via renal secretion – correlation of *in vivo* and *in vitro* studies. *Drug Metab. Dispos.* 30, 13–19
12 Yen, H.L. *et al.* (2005) Virulence may determine the necessary duration and dosage of oseltamivir treatment for highly pathogenic A/Vietnam/1203/04 influenza virus in mice. *J. Infect. Dis.* 192, 665–672
13 Shinya, K. *et al.* (2006) Avian flu: influenza virus receptors in the human airway. *Nature* 440, 435–436
14 Varghese, J.N. *et al.* (1998) Drug design against a shifting target: a structural basis for resistance to inhibitors in a variant of influenza virus neuraminidase. *Structure* 6, 735–746
15 Sidwell, R.W. *et al.* (2001) Influence of virus strain, challenge dose, and time of therapy initiation on the *in vivo* influenza inhibitory effects of RWJ-270201. *Antiviral Res.* 51, 179–187
16 Kati, W.M. *et al.* (2002) *In vitro* characterization of A-315675, a highly potent inhibitor of A and B strain influenza virus neuraminidases and

influenza virus replication. *Antimicrob. Agents Chemother.* 46, 1014–1021

17 Mishin, V.P. *et al.* (2005) Susceptibilities of antiviral-resistant influenza viruses to novel neuraminidase inhibitors. *Antimicrob. Agents Chemother.* 49, 4515–4520

18 Russell, R.J. *et al.* (2006) The structure of H5N1 avian influenza neuraminidase suggests new opportunities for drug design. *Nature* 443, 45–49

19 Barroso, L. *et al.* (2005) Efficacy and tolerability of the oral neuraminidase inhibitor peramivir in experimental human influenza: randomized, controlled trials for prophylaxis and treatment. *Antivir Ther.* 10, 901–910

20 Macdonald, S.J.F. *et al.* (2004) Potent and long-acting dimeric inhibitors of influenza virus neuraminidase are effective at a once-weekly dosing regimen. *Antimicrob. Agents Chemother.* 48, 4542–4549

21 Bright, R.A. *et al.* (2006) Adamantane resistance among influenza A viruses isolated early during the 2005-2006 influenza season in the United States. *JAMA* 295, 891–894

22 Bright, R.A. *et al.* (2005) Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. *Lancet* 366, 1175–1181

23 World Health Organization Global Influenza Program Surveillance Network (2005) Evolution of H5N1 avian influenza viruses in Asia. *Emerg. Infect. Dis.* 11, 1515–1521

24 Smith, G.J.D. *et al.* (2006) Evolution and adaptation of H5N1 influenza virus in avian and human hosts in Indonesia and Vietnam. *Virology* 350, 258–268

25 Zoidis, G. *et al.* (2006) Heterocyclic rimantadine analogues with antiviral activity. *Bioorg. Med. Chem.* 14, 3341–3348

26 Sidwell, R.W. *et al.* (1972) Broad-spectrum antiviral activity of virazole: 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* 177, 705–706

27 Smith, C.B. *et al.* (1980) Lack of effect of oral ribavirin in naturally occurring influenza A virus (H1N1) infection. *J. Infect. Dis.* 141, 548–554

28 Sidwell, R.W. *et al.* (2005) *In vitro* and *in vivo* influenza virus-inhibitory effects of viramidine. *Antiviral Res.* 68, 10–17

29 Isaacs, A. and Lindenmann, J. (1957) Virus interference. I. Interferon. Proc. Royal Soc. London 147, 258–267

30 Baron, S. and Isaacs, A. (1962) Absence of interferon in lungs from fatal cases of influenza. *Br. Med. J.* 5270, 18–20

31 Tompkins, S.M. *et al.* (2004) Protection against lethal influenza virus challenge by RNA interference *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* 101, 8682–8686

32 Ge, Q. *et al.* (2004) Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl. Acad. Sci. U. S. A.* 101, 8676–8681

33 Bitko, V. *et al.* (2005) Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* 11, 50–55

34 Li, B.J. *et al.* (2005) Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in rhesus macaque. *Nat. Med.* 11, 944–951

35 Tisdale, M. *et al.* (1995) Inhibition of influenza virus transcription by 2′-deoxy-2′-fluoroguanosine. *Antimicrob. Agents Chemother.* 39, 2454–2458

36 Furuta, Y. *et al.* (2005) Mechanism of action of T-705 against influenza virus. *Antimicrob. Agents Chemother.* 49, 981–986

37 Tomassini, J.E. *et al.* (2005) A novel antiviral agent which inhibits the endonuclease of influenza viruses. *Antimicrob. Agents Chemother.* 40, 1189–1193

38 Sun, C. *et al.* (2006) A novel class of potent influenza virus inhibitors: polysubstituted acylthiourea and its fused heterocycle derivatives. *Bioorg. Med. Chem. Lett.* 16, 162–166

39 Malakhov, M.P. *et al.* (2006) Sialidase fusion protein as a novel broad-spectrum inhibitor of influenza virus infection. *Antimicrob. Agents Chemother.* 50, 1470–1479

40 Bizollon, T. *et al.* (2005) Triple antiviral therapy with amantadine for IFN–ribavirin nonresponders with recurrent posttransplantation hepatitis C. *Transplantation* 79, 325–329

41 Krug, R.M. (2006) Clues to the virulence of H5N1 viruses in humans. *Science* 311, 1562–1563

42 Shinya, K. *et al.* (2004) PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. *Virology* 320, 258–266

43 Hatta, M. *et al.* (2001) Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 293, 1840–1842

44 Obenauer, J.C. *et al.* (2006) Large-scale sequence analysis of avian influenza isolates. *Science* 311, 1576–1580

45 Govorkova, E.A. *et al.* (2005) Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. *J. Virol.* 79, 2191–2198