THERAPEUTIC TARGETS FOR INFLUENZA – PERSPECTIVES IN DRUG DEVELOPMENT

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Since new and dangerous influenza virus strains, such as H5N1 "avian flu" and more recently the swine-origin H1N1 "swine flu", are constantly evolving, the need for effective anti-influenza drugs is pressing. It is becoming clear that the emergence of drug-resistant viruses will be a major potential problem in future efforts to control influenza virus infection. Moreover, development of vaccines against new influenza strains takes several months, and their production capacity is limited. Thus, new classes of anti-influenza drugs are highly sought after. This review focuses mainly on novel strategies, including targeting viral entry into host cells, inhibition of viral transcription and genome replication, and targeting of the NS1 influenza protein. Another approach involves viral RNA silencing by siRNAs or by

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antisense oligonucleotides. Inhibitors of viral neuraminidase have been the most successful approach in influenza virus breakdown to date. Viral maturation can also be blocked by inhibition of hemagglutinin-processing cellular proteinases. Compounds modifying the host cell immune response have also been reported. Design of specific compounds universally active against all viral variants with a reduced potential for the emergence of drug-resistant mutants is the main challenge in anti-influenza drug development, and the goals in this field are discussed here. A review with 140 references.
Keywords: Influenza; Drug research; Protein structure; Oligonucleotides; Nucleosides; Antivirals; RNA viruses; Antiviral therapy; Neuramidinase inhibitors.

1. INTRODUCTION

Seasonal influenza is an epidemic disease. The most frequent symptoms are fever and cough; in serious cases the disease can sometimes lead to fatal complications, particularly in older persons. In temperate climates, influenza infections peak during the cold months. In tropical and subtropical areas, infections occur throughout the year, usually with one or two bursts of increased activity. At irregular intervals, severe worldwide pandemics have broken out. These outbreaks are less constrained by season, and they usually have a different age-susceptibility profile. Historical evidence suggests that pandemics have occurred at 10–40-year intervals since the 16th century, originating mainly in Asia[1]. During the past 100 years there have been three major influenza pandemics. The largest of these began slowly in July 1918, and the second wave of infection hit in the autumn[2]. The virus was profoundly virulent, and by the end of the pandemic in June 1920 it had killed an estimated 35–100 million people worldwide, which corresponds to 1.9–5.5% of the world population at the time[3]. In 1957 the Asian flu pandemic killed 100,000 people, while the Hong Kong flu pandemic of 1968 claimed 700,000 victims[4].

Influenza viruses are classified as members of the family *Orthomyxoviridae* and are grouped into types A, B, and C according to the antigenic properties of their matrix and nucleocapsid proteins[5]. Only the influenza A viruses have pandemic potential. The virus is lipid-enveloped, with a genome composed of eight strands of negative-sense RNA that encodes ten viral proteins. Further classification of influenza A viruses is based on the antigenic properties of its surface glycoproteins, namely hemagglutinin (H) and neuraminidase (N) – more precisely named sialidase. These two surface antigens are essential for viral infection of host cells, and they are also major targets of the host immune response[6]. Genetic changes in hemagglutinin and neuraminidase allow the viruses to overcome host immunity acquired

through a previous infection or vaccination and can lead to a new epidemic or pandemic viral strain. The first type of genetic changes, known as "antigenic drift", restores the epidemic potential of influenza viruses by accumulation of mutations resulting from error-prone replication of the single-stranded RNA. The second type of genetic changes, "antigenic shift", is more extensive. After coinfection of one host cell by two viral subtypes, the viruses can undergo a process known as reassortment, in which hybrid viral progeny are assembled by the mixing of gene segments from the two ancestral viruses. The host species in which the reassortment events take place is not known. Wild waterfowl and shorebirds are often infected asymptomatically (harmless gut infections) and provide a vast natural reservoir from which influenza viruses emerge to cause disease in domestic poultry, horses, pigs, and humans. Since avian viruses generally do not replicate well in humans, and *vice versa*, pigs have been proposed as a potential "mixing vessel" for reassortment, as both avian and human strains can replicate in these hosts[7]. In addition to changes in antigenic properties, changes in pathogenicity and in the ability of new viral variants to cross the species barrier are important in evolution of novel pandemic strains. These changes involve mutations effecting hemagglutinin receptor specificity or hemagglutinin processing in host cells (polybasic cleavage site in highly pathogenic avian strains). Production of viral progeny is influenced by mutations in the polymerase complex (mutation of residue 627 of the PB2 protein to lysine in mammalian viruses or glutamic acid in avian viruses). A multibasic hemagglutinin cleavage site and PB2-Lys627 seem to be universal markers of viral pathogenicity, whereas the effects of mutations in other proteins are strain dependent. The viral nuclear export protein (NEP, formerly called NS2), an interferon antagonist, suppresses the antiviral effects of host interferon and influences production of pro-inflammatory cytokines, high levels of which can lead to life threatening "cytokine storms" (hypercytokinemia). The viral protein PB1-F2 induces apoptosis in host cells. It could also increase production of viral progeny, although this claim has recently been challenged[8]. For details regarding virulence factors, see review[9].

Two hypotheses about the origin of the causative agent of Spanish influenza in 1918 – virus type A(H1N1) – exist. Initially, it was supposed that the virus evolved entirely from an avian influenza virus that underwent adaptive mutations[10]. However, recent works favor the opinion that the virus evolved by reassortment/recombination of avian and mammalian viruses[11,12]. The Asian (H2N2) and Hong Kong (H3N2) pandemics were results of reassortment of human influenza subtypes and avian influenza

viruses[13]. Since 1977 influenza A viruses (H1N1 and H3N2) have been circulating in human populations, causing seasonal flu. Seasonal influenza A kills more than 250,000 people worldwide every year[14]. Influenza A and B strains are included in each year's influenza vaccine[15]. In recent years, the ability of avian influenza viruses to infect humans after direct contact with infected poultry has drawn attention. Human infection with these viruses has ranged from low (H9N2 and H7N2) or mild (H7N3, H7N7) to severe and fatal (H7N7, H5N1)[16]. Analysis of the human and avian isolates of H5N1 over the history of the avian flu outbreaks in Hong Kong from 1997 to the present showed that the virus underwent extensive genetic changes, both by repeated reassortment with avian viruses and by mutations that increased its pathogenic potential and enabled it to overcome the species barrier. Fortunately, although the H5N1 virus shows a very high-mortality rate in humans, it has a very low potential for human-to-human transmission[17]. In contrast, the swine-origin H1N1 virus has a good potential for human to human transmission but usually only a mild pathogenicity[18]. Its genome contains a unique combination of gene segments from both North American and Eurasian swine lineages. Its low genetic diversity suggests that the introduction into humans was either a single event or multiple events involving similar viruses. Isolates of swine-origin H1N1 viruses are antigenically homogeneous and are similar to North American swine A(H1N1) viruses but distinct from seasonal human A(H1N1)[19].

The genetic repertoire of influenza A viruses is abundant, and it is difficult to predict which mosaic could be pieced together in the near future. One of the most fearful visions is the possibility of a viral variant with a high mortality rate (like H5N1) and a high human to human transmission potential (like the swine-origin H1N1 virus). However, at present, "There is nothing more predictable about flu than its unpredictability" [20].

Vaccines are an important tool for control of influenza outbreaks. At present, an inactivated vaccine (containing killed virus or viral components) and the nasal-spray live vaccine, containing attenuated viruses, are available to prevent seasonal flu. The viruses incorporated into the vaccine change each year on the basis of international surveillance about which types and strains of influenza viruses will circulate in a given year. A novel vaccine against swine-origin H1N1 influenza has recently been developed. In general, the first doses of influenza vaccine could be available within five to six months after identification of a new viral strain[21]. However, safety aspects have to be taken into account. As has been pointed out, "Memories are still vivid of the 1976 flu-vaccine fiasco. That year, a new swine flu emerged at an army barracks in New Jersey, killing one person but failing to

spread further. A mass vaccination campaign ordered by president Gerald Ford caused neurological side effects in some people, and killed 25" [22]. Another problem is the efficacy of the vaccine, which depends on the overlap between the influenza strains selected for vaccine production and the types of influenza in circulation that year. The classical trivalent inactivated vaccine against seasonal flu can reduce the probability of getting the flu by 70–90% in healthy adults, assuming complete antigenic agreement of circulating strains and strains used to produce the vaccine. The vaccine is less effective in elderly persons and very young children. However, in some years the vaccine was not successful as a preventive tool for any age group; e.g., during the 1996–1997 seasonal flu outbreak, the vaccine seemed to be nearly ineffective[23].

In this context, the need for effective antiviral drugs is obvious. At present, several anti-influenza drugs (the M2 ion channel blockers amantadine and rimantadine and the neuraminidase inhibitors oseltamivir and zanamivir) are licensed (Fig. 1). Ribavirin has also been used to a limited extent to treat influenza. However, it is becoming clear that the emergence of drug-resistant viruses will potentially be a major problem in future efforts to control influenza infection. Advances in understanding the mechanisms in-

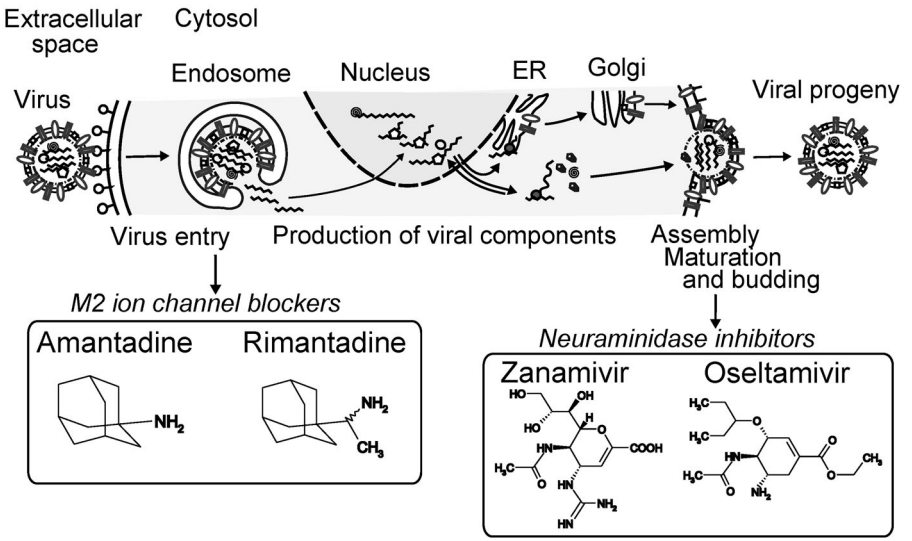


FIG. 1
Schematic view of the basic stages in the influenza virus life cycle, with clinically approved anti-influenza drugs indicated

volved in influenza virus replication have revealed a number of potential targets that might be exploited in the development of new antiviral agents. This review focuses mainly on these novel strategies. As in all cases, basic ideas and synthetic possibilities are limiting factors for novel drug development, but in this case, drug development is also compromised by the limited availability of simple assays to quickly and inexpensively test potential inhibitors. The gold standards in cell-based systems are inhibition of viral plaque formation, evaluation of viral cytopathic effects, and inhibition of viral yield. Animal models used for the study of potential influenza virus inhibitors include the ferret, the laboratory mouse, and the chicken. A variety of parameters are used to assess the severity of the infection and the effect of the therapy[24]. However, simplified cell-based assays, *in vitro* assays exploiting isolated viral proteins, and molecular modeling are useful tools for drug development. Such approaches pose fewer requirements for safety precautions, laboratory equipment, and personnel proficiency. Some of these approaches will be discussed here together with a review of the basic steps of the influenza life cycle and a description of potential drugs directed against novel targets.

2. VIRUS ENTRY INTO CELLS

2.1. Hemagglutinin Targeting

The initial step of infection is specific binding of viral hemagglutinin to a sialic acid-galactose structure attached to a cell-surface glycoprotein or glycolipid (α-2,6-linked sialylated glycan receptors predominate in human airways, α-2,3-linked receptors predominate in birds, and both are prevalent in pigs). The virion is then taken up into endocytic vesicles, where acidification enables hemagglutinin to trigger the fusion of the viral envelope and the endosomal membrane[6]. It seems that the key determinant of hemagglutinin adaptation to human receptors is not the interaction with the α-2,6 linkage itself but the ability of the viral hemagglutinin to recognize long α-2,6 glycans (which adopt a characteristic umbrella-like shape)[25].

Heterologous low-molecular-weight ligands of hemagglutinin that can retard or block viral entry have been reported (Fig. 2), including sialic acid analogues (e.g. **1**)[26,27], *tert*-butylhydroquinone[28], 4a,5,8,8a-tetrahydro-5,8-methano-1,4-naphthoquinone[29], diiodofluorescein[30], thiobenzamide fusion inhibitors[31], and quinolizidine salicylamide derivatives (e.g. **2**)[32], as well as a hemagglutinin inhibiting peptide with the sequence NDFRSKT [33].

An easy, noninfectious, and nonradioactive assay exists to test compounds of interest. The assay employs purified biotinylated hemagglutinin and relies on the observation that following treatment at low pH, the exposed fusion peptide is sensitive to thermolysin digestion, resulting in a decrease in the apparent molecular weight of a hemagglutinin subunit from ca. 20 to 18 kDa [29].

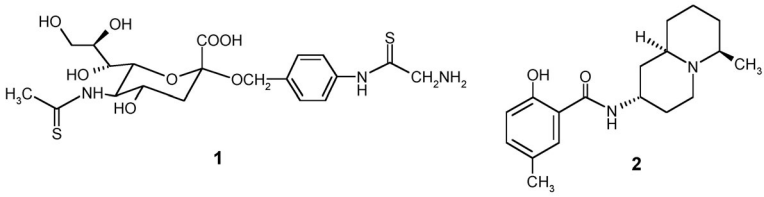


FIG. 2
An example of a sialoside inhibitor of hemagglutinin (**1**) and a quinolizidine hemagglutinin antagonist (**2**)

Attachment of the virus to the host cell can be blocked by multivalent macromolecules that mimic cellular receptors for viral entry, such as sialoglycopolymers with a chitosan backbone[34], glycopolymers carrying lactosamine[35], sialyloligosaccharides with a poly(γ-glutamic) acid backbone[36], derivatives of *N*-thioacetylneuraminic acid attached to a polymeric carrier[37], and spacer-*N*-linked sialoglycopeptides[38]. Sialoglycopeptides have been tested in a hemagglutinin inhibition assay[39]. Alternatively, since the recognition motifs in human influenza virus hemagglutinin are very similar to *Sambucus sieboldiana* lectin, the lectin is a possible model for study of the specific carbohydrate–carbohydrate interactions that take place[40]. The disadvantage of using macromolecular sialoglycans in interaction assays is that the preference for sugar structures could differ among viral strains. Preparation of polymer mixtures carrying sialyl groups that differ in their sugar positions could be a solution.

Entry of influenza virus into host cells was also successfully blocked by the lectin cyanovirin – an 11 kDa protein originally obtained from the cyanobacterium *Nostoc ellipsosporum*[41].

Viral hemagglutinin is also the main target of host defense antibodies. Using phage display libraries for antibody V-region production, unique neutralizing antibodies have been selected, revealing a common mechanism of preventing the large structural reorganizations that are required for hemagglutinin-mediated membrane fusion[42].

Moreover, regions of hemagglutinin that are highly conserved among different viral strains have been identified[43]. These regions could become

a shared target for development of a universal vaccine against diverse influenza subtypes. Furthermore, these findings, together with structural data[28,42,43] and molecular modeling, could help with rational design of universal inhibitors of influenza virus entry.

Since such compounds act extracellularly, they need not (or even should not) pass across the cell membrane and could be administered topically. For these reasons, they may show low toxicity. Targeting the initial step of viral infection makes such compounds ideal for prophylaxis. The fact that conserved residues exist in different viral hemagglutinins gives the prospect of low viability of emerging viral mutants resistant to drugs interacting with these residues.

Another interesting strategy to disable viral entry is removal of cell receptors from the airway epithelium. A recombinant fusion protein composed of a sialidase catalytic domain derived from *Actinomyces viscosus* fused with a cell surface-anchoring sequence was constructed for this purpose. This fusion protein, DAS181, is intended for topical use as an inhalant to remove sialic acids from receptors in the airway epithelium. In trials, DAS181 showed a long-term effect and no cytotoxicity[44]. However, proteins in general could trigger an allergic reaction in humans, so the possible allergenic potential of DAS181 should be taken into account.

2.2. M2 Channel Blockers

After endocytosis of attached virions by a host cell, acidification enables the flow of protons through the M2 channel into the interior of the endocytosed virions, causing dissociation of the virion components and releasing viral ribonucleoproteins and matrix protein into the cytoplasm. Amantadine, a blocker of M2 H^+ ion channels, was the first synthetic anti-influenza drug[45]. The adamantane[46] derivatives amantadine and rimantadine have been approved as drugs for the prophylaxis and therapy of influenza A infection; however, viral strains resistant to these compounds are common[47]. Several new inhibitors of M2 proton channels have been synthesized as potential anti-influenza drugs, such as aminoadamantane derivatives[48], ring-contracted amantadine analogues[49], and 1,2-fused adamantane piperidines[50] or heterocyclic rimantadine analogues[51,52]. Recently, spiro-piperidine derived compounds have been studied, and 3-azaspiro[5,5]undecane hydrochloride was identified as a potent M2 channel inhibitor[53]. Since a variety of adamantane-derived compounds have been synthesized and tested, it should be relatively easy to derive general structural requirements for inhibitors of influenza M2 channels[54,55]. Moreover,

a short fragment, possessing only 24 amino acid residues of the M2 channel, has been identified to be sufficient for folding, amantadine binding, and proton translocation[56]. This finding has the potential to simplify future studies, including drug design and testing of potential M2 channel inhibitors. However, it is not yet clear whether or not any of these novel compounds will hold an advantage over the existing drugs (such as activity against amantadine-resistant viral variants, better pharmacokinetics, or fewer adverse effects).

Of drugs in clinical use, chloroquine (an antimalarial) has been reported to aggravate influenza virus release into the cytosol by increasing endosomal pH [57].

3. REPLICATION OF THE VIRAL GENOME AND VIRAL PROTEIN EXPRESSION

3.1. Targeting the Polymerase Complex

After the release of virion components into the cytoplasm, ribonucleoproteins are transported into the cell nucleus, where a viral polymerase complex transcribes and replicates the viral genome.

The influenza virus polymerase is a heterotrimer composed of three subunits (PB1, PB2, and PA). The proper synthesis of viral mRNA (the template for the synthesis of viral proteins) starts with a unique "cap-snatching" mechanism. In the first step, the polymerase PB2 subunit specifically binds the 5′-cap of host pre-mRNAs [58], which is subsequently cleaved after 10–13 nucleotides by the viral endonuclease PA subunit. The crystal structure of the PA domain shows a structural core that closely resembles resolvases and type II restriction endonucleases[59]. Subsequent RNA strand lengthening is mediated by the PB1 subunit[60]. The PB1 gene of most viral strains also encodes a PB1-F2 protein in the +1 reading frame. This protein induces apoptosis and is also an important virulence factor[61].

Viral mRNA synthesis is the target of several classes of antivirals. The effect of RNA synthesis inhibitors can be easily studied *in vitro* with purified influenza RNA polymerase[62] or in tissue cultures using cells transiently transfected with vectors carrying the RNA polymerase complex[63].

For cap-binding by the PB2 protein, the importance of the mesoionic properties of the N7-methylguanine (N(7m)G) component of the mRNA cap has been reported. Compounds analogous to the mesoionic N(7m)G component of mRNA cap structures comprise a large class of potential inhibitors of the influenza virus polymerase. Although the design of biologi-

cally active analogues has been unsuccessful so far, further investigation of compounds with this mechanism of action is warranted[64].

The endonuclease activity of the PA subunit of the influenza RNA polymerase is inhibited by 2,4-dioxo-4-phenylbutanoic acid[65], by its analogue L-742,001 [66], by tetramic[67] or hydroxamic[68] acid derivatives, and by flutimide (**3**) and analogous fully substituted pyrazine-2,6-diones[69] (Fig. 3).

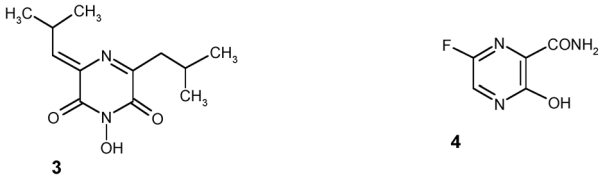


FIG. 3

Drugs under development targeting the influenza virus polymerase complex: an endonuclease inhibitor, flutimide (**3**), and T-705 (favipiravir, **4**), which competes with GTP in the chain lengthening phase. T-705 has entered phase III clinical trials for use against seasonal influenza[9]. T-705 also acts against highly pathogenic H5N1 avian influenza, and it shows synergy with neuraminidase inhibitors in mice[140]

Inhibition of influenza RNA synthesis in the chain-lengthening step is inhibited by T-705 (favipiravir, **4**)[70] and by 2′-deoxy-2′-fluoroguanosine[71] (Fig. 3). Both these compounds are converted to ribofuranosyl triphosphate by the host cells. After that, they can compete with GTP to inhibit virus RNA polymerase. The activity of these compounds against cellular polymerases is low or undetectable[72]. Depletion of GTP is also inflicted by the clinically available drug ribavirin (administered as an inhalation aerosol) and its amidine prodrug viramidine, which both act on a cellular enzyme, inosine 5′-monophosphate dehydrogenase[73]. Ribavirin, together with inhibitors of viral RNA polymerase, could act synergistically against viral RNA synthesis, so these compounds seem to be very promising.

Development of peptides that inhibit polymerase complex assembly *via* interactions with the protein–protein binding sites is also a strategy for anti-influenza drug development. A 25-amino-acid peptide derived from the PA-binding domain of PB1 polymerase subunit has been shown to block the polymerase activity[73].

3.2. Antagonists of Viral Non-Structural Proteins NS2/NEP and NS1

Some viral transcripts require splicing, and to accomplish this, the virus exploits the host splicing machinery[74]. However, these processes are influ-

enced by viral factors such as the viral nonstructural protein NS1 [75] and the nuclear export protein NS2/NEP [76]. NS2/NEP mediates nucleocytoplasmic export of viral ribonucleoproteins[77] and may also play a role in regulating transcription and replication of the viral genome[76].

NS1 is a multifunction protein. It binds and sequesters dsRNA, interferes with host mRNA processing, controls viral RNA replication, and facilitates preferential viral mRNA translation. NS1 disables the host immune response primarily *via* interactions with interferon production and action and also by inhibition of activation of sentinel dendritic cells. For details see a recent review[78].

Some NS1 variants are associated with high mortality rates in humans (H5N1), whereas others are connected with low-pathogenicity (swine-origin H1N1)[79].

Targeting NS1 seems to be a promising novel strategy for influenza therapy since it offers the possibility of inhibiting the disease progression on several levels with only one compound. NS1 antagonists could prevent disabling of the host immune defense, as well as decrease production of viral progeny.

It has been suggested that the NS1 protein could be effectively targeted with a divalent ligand that interferes with double-stranded RNA binding[80]. Additionally, compounds such as NSC95676 (**5**) and NSC125044 (**6**) have been reported as suitable NS1 inhibitors (Fig. 4). The inhibition potency of these compounds was evaluated using an NS1 – expressing yeast strain with a slow-growth phenotype[81]. Also, a filter-binding assay involving recombinant His-NS1 protein and a radiolabeled model viral RNA has been applied to the study of interactions between viral RNA and NS1 influenza A protein[82]. This assay is applicable to characterization of NS1 variants as well as to evaluation of the potency of NS1 inhibitors.

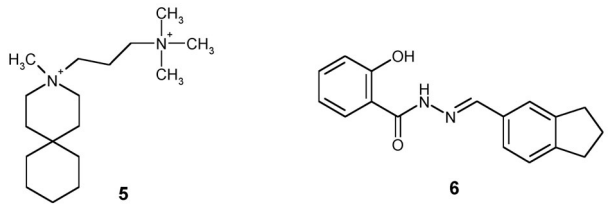


FIG. 4
"Two in one": Antagonists of influenza multifunctional non-structural protein NS1 could block infection on at least two levels: examples of the NS1 inhibitors NSC95676 (**5**) and NSC125044 (**6**)

3.3. Silencing of Viral RNA

Viral RNA can be silenced by exogenously delivered short interfering RNAs (siRNA)[83–89] or by antisense oligonucleotides like nuclease-resistant and water-soluble peptide-conjugated phosphorodiamidate morpholino oligomers. These molecules enter cells and form stable duplexes with complementary RNA [90,91].

Delivery to the cells and stability of anionic DNA or RNA molecules can also be enhanced using phosphorothioate oligonucleotides[92], by covalent modification of siRNA by a signal peptide for transmembrane transport of bacterial protein toxins[93], by 2′-*O*-methyl and 2′-deoxy-2′-fluoro substitutions in the ribose moiety[94], or by linkage with cholesterol[95]. Delivery systems utilizing polyethyleneimine-based siRNA [96], liposomes[97], or complexing to cationic lipids[98] have been also reported.

4. TRAFFICKING OF VIRAL COMPONENTS

Translated viral proteins – the nucleoprotein and the polymerase subunits – are transported from the cytoplasm into the nucleus, where they form ribonucleoproteins together with the viral RNA [99]. These processes are very complex, and detailed studies could reveal novel therapeutic targets in the future. Of this group of potential drugs, only the antiviral activity of LY294002, an inhibitor of the host signalling enzyme phosphatidylinositol 3-kinase, has been evaluated. LY294002 reduces production of viral progeny, inhibits viral RNA and protein synthesis, and affects nuclear export of viral ribonucleoproteins[100].

The newly synthesized hemagglutinin, neuraminidase, and membrane-ion-channel M2 molecules undergo glycosylation in the Golgi apparatus and then must be brought together with other virion components (such as ribonucleoprotein and the matrix protein M1) to the budding site on the host cell membrane, where the process of morphogenesis and budding occurs. Inhibition of protein glycosylation by 2-deoxy-2-fluoro-D-mannose has been demonstrated to have an antiviral effect[101].

5. MORPHOGENESIS OF VIRAL PARTICLES

The eight segments of viral RNA that occur in budding virions are present in an equimolar ratio. This is ensured by packaging signals in each of these RNAs that occur at two separate regions at the 3′ and 5′ termini[102]. Phosphorothioate oligonucleotides derived from the packaging signals have been designed. These phosphorothioate oligonucleotides were associated

with liposomes and tested for activity against influenza A and B viruses *in vitro*; the oligos were markedly inhibitory[103]. Morphogenesis of the viral particles has been disrupted by the calmodulin antagonist trifluoroperazine[104], by a peptide derived from hemagglutinin[105], or by binding of the matrix protein M1 to viral RNA with a fragment of a monoclonal antibody with the potential to act against different viral strains[106]. For details regarding the assembly and budding of the influenza virus see a comprehensive review[107]. Better understanding of the morphogenic process leading to identification of novel therapeutic targets is desirable, and this field remains open to further investigation.

6. MATURATION OF VIRAL PROGENY

Cleavage of hemagglutinin into two disulfide-linked subunits is essential for the maturation of viral progeny. In mammalian and most avian viruses, hemagglutinin is cleaved by tissue- or organ-specific trypsin-like extracellular proteinases following its glycosylation and exposition to the outer side of the plasma membrane. In these cases, the production of complete viral progeny is limited to organs or tissues in which appropriate trypsin-like proteinases are expressed. However, the H5 and H7 hemagglutinins from highly pathogenic avian strains undergo intracellular processing in the Golgi apparatus by ubiquitous subtilisin-like proteinases (furin, PC6), enabling systemic infection[7]. Inhibition of hemagglutinin-processing cellular proteinases could be a possible antiviral strategy. Since the host protein is targeted, the influence of such compounds on the host organism must be addressed.

Furin inhibitors – dicoumarols – have been reported as potential drugs[108]. It has also been demonstrated that ambroxol, an over-the-counter muclolytic agent, up-regulates the levels of endogenous protease inhibitors in lung[109].

7. BUDDING OF INFECTIOUS INFLUENZA VIRUSES

7.1. Neuraminidase – an Established Anti-Influenza Target

Newly synthesized viral proteins – the matrix protein M1, the ion channel M2 (blocked by adamantane-derived drugs, see above), and neuraminidase – play important roles in the budding of viral progeny from the membranes of infected cells. During the budding process as well as during movement of the infectious viral particles across the airway, the viral hemagglutinin binds to sialylated cell-surface receptors. The release of viral particles requires

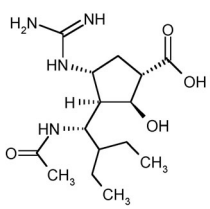
the scission of *N*-acetylneuraminic acid by neuraminidase, a glycosylated enzyme anchored in the viral envelope. Preferences for the type of scissile bond determine the cell tropism and are an important factor for pathogenicity. Human strains preferentially cleave off 2,6-α-linked sialic acid. These types of receptors occur in the upper respiratory tract, whereas avian influenza viruses bear neuraminidases preferring cleavage of 2,3-α-linked sialic acid. Such receptors predominate on the surfaces of cells occurring in human alveoli, causing severe infections of the lower respiratory tract.

At present, the most potent anti-influenza drugs in clinical use are inhibitors of viral neuraminidase – zanamivir (Relenza), an inhalant, and the orally bioavailable drug oseltamivir (Tamiflu). Both drugs are the result of structure-based inhibitor design[5]; they mimic the transition state of the *N*-acetylneuraminic acid cleavage, independent of the type of linkage to the rest of saccharide structure. Thus, these compounds are active against both human and avian viral strains[72].

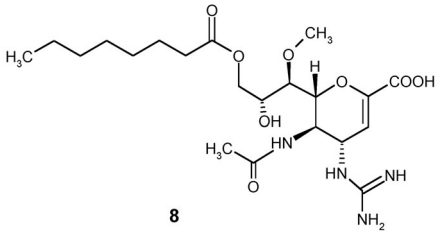
Oseltamivir and zanamivir reduce the duration of symptoms by an average of 1 day and reduce the number of complications and the mortality rate. Prophylactic long-term administration of oseltamivir (6 weeks) significantly reduces the incidence of influenza[110]. However, drug resistance has developed. During the 2008/2009 influenza season, oseltamivir resistance developed mainly in the H1N1 strains of seasonal influenza, whereas influenza H3N2 strains, which are resistant to adamantanes, retained susceptibility to both neuraminidase inhibitors[111]. As for the swine-origin H1N1 strains, all the viruses tested to date are resistant to amantadine and rimantadine, but most of them are susceptible to oseltamivir and zanamivir[112]. Infectious influenza A strains resistant to zanamivir have not yet been reported. A possible explanation is that zanamivir is closely related to the natural substrate *N*-acetylneuraminic acid. Thus, mutants with decreased affinity to the drug also have low affinity for their natural substrates, leading to limited infectivity of zanamivir-resistant strains. A more straightforward reason could be that this drug is not used frequently[6].

Novel neuraminidase inhibitors are under development (Fig. 5): peramivir (RWJ-270201, BCX-1182) (**7**) and other cyclopentane and cyclopentane amide derivatives, pyrrolidine derivatives like A-192558 and A-315675, and disubstituted tetrahydrofuran-5-carboxylic acid derivatives. A major focus has been placed on development of dimeric neuraminidase inhibitors. Dimeric derivatives of zanamivir showed an extremely long half-life in lungs, allowing once-weekly administration[71]. Also, the esterified form CS-8958 (**8**; Fig. 5) of the zanamivir derivative R-125489 has been shown to have an antiviral effect in mice even when it was administered

intranasally in a single dose 7 days before infection. In this case, the prolonged half-life of CS-8958 compared to that of R-125489 was the result of increased hydrophobicity due to the esterification. The optimal acyl chain length has been determined to be octanoyl[113].



7



8

FIG. 5
Neuraminidase inhibitors in phase II clinical trials against seasonal influenza[9]: Peramivir RWJ-270201 (**7**) and CS-8958 (**8**)

Recently, other neuraminidase inhibitors have been reported: NSC89853, which was obtained by computational molecular docking[114], aurintricarboxylic acid[115], and benzyl sulfonate inhibitors and their derivatives[116]. Natural sources have also been used to obtain novel neuraminidase inhibitors: xanthones from *Cudrania tricuspidata*[117], and pterocarpans and flavanones from *Sophora flavescens*[118].

Several cell based assays exist for evaluation of the susceptibility of neuraminidase variants to inhibitors[119]. Enzyme inhibition assays typically exploit artificial substrates, such as an assay using the fluorogen 2′-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA)[120] or a more sensitive chemiluminescence assay with the 1,2-dioxetane derivative of sialic acid (NA-STAR) as the substrate[121]. More recently, an assay mimicking viral particle release has been reported; pseudotyped viral particles bearing retroviral gag-pol polyprotein, influenza hemagglutinin and neuraminidase, and luciferase genes as a quantification marker were constructed[122].

Since neuraminidase displays a tetrameric structure on the virus surface, carbosilane dendrimers carrying thiosialoside residues on their termini have been synthesized as promising multivalent-type therapeutic agents. This thioglycosidic linkage is not hydrolyzed by neuraminidases in general. These compounds block neuraminidase activity in H1N1 as well as in H2N3 viral strains[123]. Computational approaches for the design of neuraminidase inhibitors have also been reported[124,125].

8. DRUGS TARGETING THE HOST IMMUNE RESPONSE

Antiviral treatment sometimes has limited clinical efficacy because host reactions leading to induction of apoptosis in respiratory epithelial cells and production of pro-inflammatory cytokines by virus-infected cells are also important factors of pathogenicity[126]. In clinics, corticosteroids are used to treat the "cytokine storm" [127].

Several pre-clinical approaches focus on virus-induced intracellular signalling by targeting cell-signalling pathways that are essential for virus replication, such as the classical mitogen-activated protein kinase cascade Raf/MEK/ERK and the IKK/NF-κB module[128].

U0126 – a MAPK/ERK kinase (MEK) inhibitor – reduces accumulation of viral ribonucleoproteins in the cell nucleus and impairs the nuclear-export mediated by the viral protein NEP/NS2. These actions result in decreased production of viral progeny[129]. Moreover, since the Raf/MEK/ERK cascade is also a regulator of pro-inflammatory cytokines, inhibition of the pathway could prevent the "cytokine burst" sometimes induced by highly pathogenic viral strains.

The transcription factor NF-κB triggers expression of antiviral cytokines; however, it also induces pro-apoptotic factors. Inhibition of NF-κB by Bay11-7082 and ammonium pyrrolidinedithiocarbamate resulted in decreased production of viral genomic RNA [130]. Findings such as these show that targeting cell signalling pathways can be a promising antiviral strategy. Since not viral but host factors are involved, development of drug-resistance is less probable.

Acetylsalicylic acid (aspirin, 2-acetoxybenzoic acid) inhibits the NF-κB-activating kinase IKK2, and indeed, in cell-culture and mouse model experiments acetylsalicylic acid showed antiviral activity[128]. However, this finding should be applied to human medicine with maximal caution, as administration of acetylsalicylic acid during viral illnesses increases the incidence of life threatening Reye's syndrome in children[131].

Both processes – induction of apoptosis and pro-inflammatory cytokine gene expression – lead to activation of macrophages, which produce toxic superoxide[132]. Scavengers of superoxide radicals such as ammonium pyrrolidinedithiocarbamate[133] and nordihydroguaiaretic acid[134] have been reported as potential anti-influenza drugs. The mechanism of action of ammonium pyrrolidine dithiocarbamate seems to be more complex; it has also been shown to influence the inhibition of viral gene replication and transcription[135].

Existing generic drugs have also been evaluated for possible use in influenza treatment: statins, which show cardioprotective, anti-inflammatory, and immunomodulatory effects[136], and the fibrate gemfibrozil, a peroxisome proliferator-activated α-receptor agonist[57].

9. CONCLUSIONS

A universal anti-influenza vaccine has not yet been developed, and its development is not imminent. Vaccines against individual viral strains take time to prepare, and usually, the strains included in the annual vaccine overlap only partially with the strains actually in circulation that year. Furthermore, the evolution of influenza viruses is difficult to predict, as is their ability to escape previously acquired human immunity, their pathogenic potential, and the potential for drug resistance development. Thus, novel anti-influenza drugs are desired.

Many influenza isolates have been sequenced[137], and several 3D structures of different influenza proteins are available[138]. This opens new gateways for rational drug design. The viral proteins could be targeted by low-molecular-weight ligands as well as by biomacromolecules and their derivatives, thus opening possibilities for applications of various chemical and biological approaches. These include nucleotide and oligonucleotide chemistry (inhibitors of viral polymerase or endonuclease, antagonists of host cell nucleotide biosynthesis, siRNA, antisense oligonucleotides and their derivatives); carbohydrate chemistry (inhibitors of neuraminidase and hemagglutinin by modified sugars, glycopeptides, or glycodendrimers); peptides, proteins, and their derivatives (inhibitors of oligomerization of viral oligomeric proteins – hemagglutinin, neuraminidase, polymerase complex, nucleoprotein, matrix protein, and non-structural protein NS1); as well as a heterologous group of low-molecular-weight organic compounds targeted against all the viral components.

The major challenges in anti-influenza drug design are the identification of compounds universally active against all viral variants and the minimization of drug resistance development. Identification of residues and structural motifs conserved among all viral subtypes would help the search for universal viral protein antagonists.

The search for compounds capable of inhibiting more than a single influenza protein seems to be a challenging strategy. The first attempt in this direction is dual targeting of both neuraminidase and hemagglutinin by NCI0353858, which was selected by virtual screening and docking[139].

To avoid drug resistance development, it seems that compounds that are as structurally similar to the natural substrates as possible are advantageous, since the decreased affinity of viral targets for these antiviral drugs could also lead to decreased affinity for the natural interaction partners, thus decreasing the viral fitness of such drug-resistant mutants.

Improving the pharmacokinetic properties (usually by increasing their hydrophobicity and stability) of novel as well as existing drugs is desirable, enabling different administration routes or longer dosage intervals. Also, novel simple and low-cost synthetic routes applicable to large-scale drug production are needed.

All of the anti-influenza compounds approved to date are intended only for short-term administration; many of them can be administered topically by inhalation. For these reasons, the adverse effects associated with the potential toxicity of these compounds are not a major issue.

Combination therapy gives hope for preventing drug resistance development as well as for antiviral treatment with increased potency. For this reason, the identification and characterization of as many potentially useful compounds as possible is solicited, and a wide range of mechanisms of action is exploited.

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