Antiviral agents targeting the influenza virus: a review and publication analysis

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ABSTRACT: Influenza is a serious infectious disease, which is life-threatening especially in children, seniors and immunocompromised patients. In addition to vaccination, the development of new anti-influenza agents represents a crucial defence strategy to combat seasonal and pandemic influenza strains. At present most attention is paid to the development of inhibitors of influenza neuraminidase, which has been established as a key drug target for the prophylaxis and treatment of influenza infections. However, the emergence of drug-resistant influenza variants highlights the need of continuously innovative strategies for the development of new drugs with improved antiviral effects, higher safety and increased tolerability. In this review article, an analysis of publications describing anti-influenza agents indexed in the Web of Science® database has been carried out. The most important publications are presented in tables and are characterised by several key words, abstracts and references. The presented publications have been sorted according to five basic criteria: (i) review articles, (ii) design, synthesis and evaluation of new anti-influenza drugs, (iii) major classes of anti-influenza drugs, (iv) combination therapy of influenza infections and (v) influenza drug resistance. The design of this review article allows us to offer a complex overview of known antiviral agents targeting influenza viruses, facilitates easy and rapid orientation in numerous publications written on this subject, and aids the gathering of required data.

Keywords: influenza; virus; antiviral; agents; drug; resistance; therapy; structure-based drug design

Contents

Supported by the Ministry of Education, Youth and Sports, Czech Republic (AdmireVet; Grant No. CZ 1.05/2.1.00/01.0006-ED 0006/01/01) and the Ministry of Agriculture of the Czech Republic (Grant No. MZe 0002716202).

1. INTRODUCTION

Influenza is considered to be one of the life-threatening infectious diseases. In some countries seasonal influenza affects annually up to 40% of the population and 500 million people die from it worldwide every year. New highly-virulent influenza strains can arise unexpectedly to cause world-wide pandemics with markedly increased morbidity and mortality, such as the "avian flu" in 1997 and "swine flu" in 2009. At present, the development of antiviral drugs represents a crucial strategy in the control and prevention of seasonal and pandemic influenza infections. Antiviral drugs can overcome the limitations of vaccination strategies, such as the time-consuming vaccine design, insufficient protection for immunocompromised patients and the unpredictable antigenic changes in influenza strains which render vaccination ineffective. In general, the anti-influenza agents can be divided into two basic groups, i.e., synthetic analogues of biomolecules required during virus infection and substances derived from natural plant extracts. With regard to the considerable genetic and antigenic variability of the influenza virus, research has been predominantly focused on broad-spectrum antiviral drugs, which are effective against a large variety of influenza strains. The development of antivirals targeting host-cell proteins, which play an important role in viral replication, has also gathered pace recently. Moreover, combination therapy based on the application of two or more different antivirals represents a promising approach to combat influenza infections.

The aim of this review is to offer an overview of known antiviral agents targeting influenza viruses and to discuss their characteristic properties, modes of action and advantages or limitations of their therapeutic use. Publications which contain key information concerning the issues of anti-influenza agents are in this review presented in tables and characterised with descriptive words, full or shortened abstracts and relevant references. The text in the tables contains several format imperfections, which exist in the Web of Science® database and are caused by transmission and copying of data between various information sources. The loss of cursive typeface in Latin names in titles of references and in abstracts is such an example. The presented publications have been classified according to five basic criteria: (i) review articles, (ii) design, synthesis and evaluation of new anti-influenza drugs, (iii) major classes of anti-influenza drugs, (iv) combination therapy of influenza infections and (v) influenza drug resistance. This review article should facilitate orientation in numerous publications dealing with anti-influenza drugs and enable rapid and easy search for specific data, information and protocols. The previous reviews in this format have been well received (Vass et al. 2008; Eyer and Hruska 2012; Hruska and Kaevska 2012; Hruska and Franek 2012).

1.1. Database used as the source of information

The publications were retrieved from the Web of Science® database using the general search profile:

Table 1.1. Analysis of publications: search profiles and numbers of results retrieved

Timespan	All years	2010–2012
General search profile		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*)		
Results	14 458	4 673
Review articles cited in Table 2	17	
Design, synthesis and evaluation of new anti-influenza drugs		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND (design OR synthesis OR evaluation)		
Results	3083	1048
Cited in Table 3	19	
Haemagglutinin inhibitors		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND (hemagglutinin OR haemagglutinin)		
Results	2616	742
Cited in Table 4.1	18	
M2 ion channel blockers		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND (adamantane* OR amantadine OR rimantadine)		
Results	897	249
Cited in Table 4.2	15	
Inhibitors of viral RNA polymerase		
Topic= (influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND polymerase*		
Results	824	344
Cited in Table 4.3	22	
Inhibitors of neuraminidase		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND neuraminidase*		
Results	2658	930
Cited in Table 4.4	21	
Host cell factor targeting		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND ("host factor*" OR "host protein*")		
Results	43	22
Cited in Table 4.5	14	
Other anti-influenza agents		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND (nucleoprotein* OR "non-structural protein" OR siRNA* OR plant* OR herbal* OR antibod*)		
Results	3899	1382
Cited in Table 4.6	16	
Combination therapy of influenza infections		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND "combination therapy"		
Results	102	38
Cited in Table 5	11	
Influenza drug resistance		
Topic=(influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*) AND resistance		
Results	1757	700
Cited in Table 6	19	

Topic = (influenza OR flu) AND (antivir* OR virostatic* OR drug* OR "anti-influenza" OR "anti-flu" OR inhibit*), Timespan = all years. A total of 14 458 publications were obtained, demonstrating the huge number of papers on the subject published during the period from 1945 to 2012. To ease orientation through the numerous publications, the results were subsequently refined using more specific search profiles as described in Table 1.1. A selection of the most important publications has been based on reading abstracts and hundreds of available full papers.

1.2. Basic analysis of publications on antiviral agents targeting the influenza virus

The Web of Science® utilities were employed for search result analysis. Regarding the high number of retrieved publications, attention has been predominantly paid to papers published in the period 2010 to 2012; however, some older key papers are also included in the tables. As is evident from Figure 1, publication activity on the topic has continuously increased since the early 1990s. In the last three years, 4673 papers were published, with 1529 papers in 2012. The oldest papers were published in the late 1940s and describe the research on the first influenza haemagglutinin inhibitors in pigs. In 1960s, the antiviral effects of the first ion channel blockers were reported. The latest publications are focused on computer-aided structure-based design of new neuraminidase and viral polymerase inhibitors to combat pandemic influenza strains. Of all the published papers, original research articles prevail (82.7%). Our analysis shows that 2938 institutions from 112 countries are concerned with the

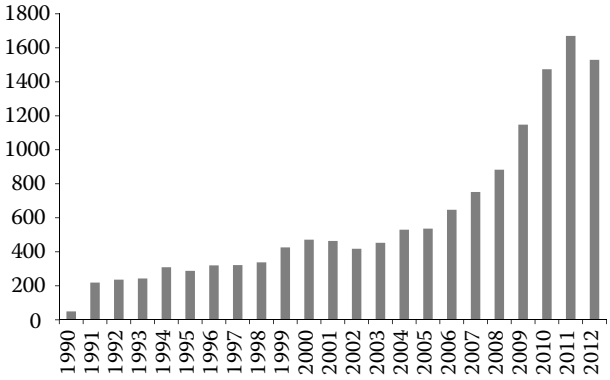

Figure 1. The number of publications on anti-influenza agents during the period from 1990 to 2012

Table 1.2. Analysis of publications: TopTen authors, institutions and countries according to the number of published papers (Web of Science®, publications in total 14 458)

Item	Number of publications
Authors (12 596 in total)	
Garcia-Sastre A	156
Webster RG	112
Suzuki Y	103
Hayden FG	95
Kawaoka Y	85
Gubareva LV	74
Suzuki T	66
Palese P	62
Kochs G	61
Li Y	61
Institutions (2938 in total)	
Centers for Disease Control and Prevention	307
Harvard University	197
Chinese Academy of Sciences	175
Icahn School of Medicine at Mount Sinai, New York	165
National Institute of Allergy and Infectious Diseases	164
University of Virginia	155
University of Wisconsin	150
University of Washington	149
University of Oxford	133
Emory University	125
Countries (112 in total)	
USA	5695
Japan	1344
England	1169
People's Republic of China	1097
Germany	963
Canada	710
France	653
Australia	637
Italy	561
Netherlands	484

subject of antiviral agents targeting the influenza virus. The authors, institutions and countries which show the highest publication activities (TopTen) are listed in Table 1.2.

2. Review articles

Review articles represent 9.2% of all publications retrieved from the Web of Science® database using the above mentioned search profile. In Table 2, seventeen key review articles are presented and characterised with a few descriptive words (left column), abstract (in the middle), and source reference (right column). These papers offer overall reviews on all important topics, especially regarding structure-based drug design techniques (Du et al. 2012), chemical synthesis of antiviral drugs (Gong and Xu 2008; Marra et al. 2008), biochemical and cell-based antiviral screen assays (Tisdale 2000; Atkins et al. 2012), animal models for study of influenza virus inhibitors (Sidwell and Smee 2000), structure, chemical properties and modes of action of the major groups of anti-influenza drugs (Gong et al. 2009; Chintakrindi et al. 2012; Grienke et al. 2012), influenza virus-neutralising antibodies (Martinez et al. 2009), natural products with antiviral activity (Grienke et al. 2012), combination therapy of influenza infections (Kaihatsu and Barnard 2012) and mechanisms of influenza drug resistance (Pizzorno et al. 2011). All the topics are also discussed in detail in the original papers presented in Tables 3 to 6.

3. Design, synthesis and evaluation of new anti-influenza drugs

The first anti-influenza drugs were usually identified using large-scale screening methods or by chance and their chemical structure and modes of action were not completely understood (Davies et al. 1964). In contrast, the current development of new antivirals is based on detailed knowledge of the X-ray crystallography-derived structure of influenza proteins as drug targets. Such an inventive process of finding new drugs, which is termed structure-based drug design, involves the development of organic molecules or macromolecular scaffolds that are complementary in shape and charge to potential ligand-binding pockets of the viral target protein (Kim et al. 1997; Lv et al. 2011).

The designed structures that are predicted to show high affinity to the viral target are then synthesised using various chemical procedures (Shie et al. 2011; Rawat et al. 2012) and their anti-influenza effects are evaluated using standardised *in vitro* screening methods. These methods include biochemical assays (Hung et al. 2012) and cell-based antiviral screens (Schmidtke et al. 2001), such as the plaque reduction assay to monitor viral replication efficiency, dye-uptake assays for measuring virus cytopathic effect, and yield-reduction assays for quantification of specific virus antigens. Through the combination of bioinformatic approaches, applicative robotics and miniaturisation strategies, most of these techniques can be adapted to high-throughput screen formats, which are applicable for testing large drug libraries containing millions of unique chemical structures (Dai et al. 2012). As an alternative to experimental screening methods, virtual (computational) screening can be used to select the desired chemical structure from large molecular databases (Yamada et al. 2012).

Besides *in vitro* tests, potential influenza virus inhibitors are further studied using animal models, such as the ferret, laboratory mouse, and chicken (Yoshimoto et al. 2000; Sauerbrei et al. 2006). Such *in vivo* studies play a crucial role in the preclinical evaluation of new drugs. The final step in the development of new medications is represented by clinical trials, which focus particularly on clinical pharmacokinetic studies and evaluation of antiviral efficacy, safety and tolerability in human volunteers (He et al. 1999; Cao et al. 2012). For key publications describing the design, synthesis and evaluation of anti-influenza drugs see Table 3.

4. Major classes of anti-influenza drugs

The anti-influenza drugs are usually classified according to their target in the viral life-cycle, which is schematically depicted in Figure 2. Such antiviral molecules are particularly used as inhibitors of the following processes: attachment of the virus to host cell receptors, endocytosis and fusion of viral and cell membranes, replication and transcription of the viral genome, synthesis of viral proteins, assembly of the viral progeny and release of the new virions into the outside environment. The following paragraphs are focused on the description of basic classes of influenza virus inhibitors.

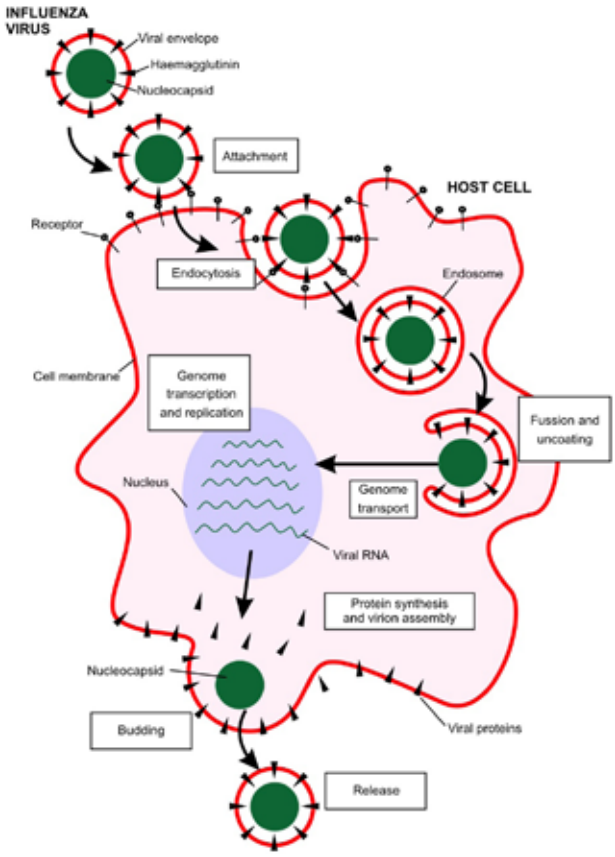

Figure 2. Life-cycle of the influenza virus (according to Beigel and Bray 2008)

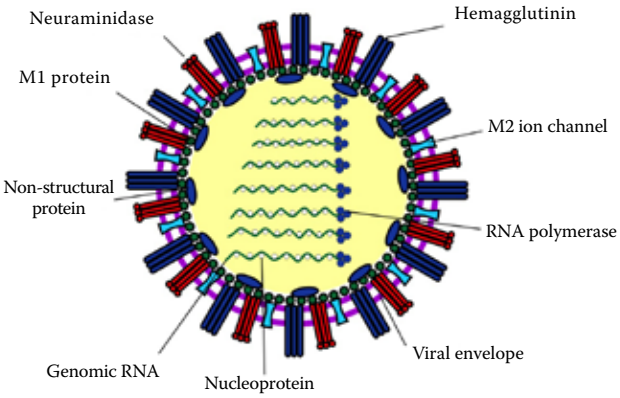

Figure 3. Structure of the influenza virus (according to Ludwig et al. 2003)

4.1. Inhibitors of haemagglutinin

Haemagglutinin is a trimeric rod-shaped glycoprotein located on the surface of influenza virions (Figure 3). During the initial step of infection, haemagglutinin molecules bind specifically to host cell sialic acid receptors and enable entry of the virus into the cell cytoplasm by fusion of viral and cell membranes (Skehel and Wiley 2000). The binding interaction of haemagglutinin with cellular receptors can be efficiently inhibited by synthetic macromolecules composed of multiple sialic acid residues conjugated to glycan, glycopeptide or polyacrylamide backbones (Sigal et al. 1996; Feng et al. 2010; Narla and Sun 2012). Such therapeutic constructs have been observed to exhibit higher antiviral effects than monovalent sialic acid analogues. Haemagglutinin-mediated membrane fusion has been successfully blocked by a large variety of small organic compounds, e.g. quinones (Larson et al. 2012), the antibiotic stachyflin produced by *Stachybotrys* sp. (Yoshimoto et al. 1999) and derivatives of benzamide and podocarpic acid (Luo et al. 1997; Staschke et al. 1998). Although these structures show significant anti-influenza potency *in vitro*, most of them are characterised by strong strain specificity and apparent cytotoxicity.

The sialidase fusion protein DAS181 is an entirely novel broad-spectrum haemagglutinin inhibitor that enzymatically removes sialic acid receptors from respiratory epithelium cells, preventing virus attachment (Malakhov et al. 2006). This new antiviral is active against both A and B influenza strains at nanomolar concentrations, causing minimal cytopathic effects. This substance is at present being tested in phase II human trials (Moss et al. 2012). Recently, research has also focused on fusion-inhibitory peptides (Lee et al. 2011), and anti-haemagglutinin RNA aptamers (Park et al. 2011). Eighteen key publications concerning influenza haemagglutinin inhibitors are presented in Table 4.1.

4.2. M2 ion channel blockers

M2 ion channel is a transmembrane viral protein (Figure 3) that mediates the selective transport of protons into the interior of the influenza virion. Conductance of protons acidifies the internal space of the viral particle and facilitates the haemagglutinin-mediated membrane fusion which in turn results in the uncoating of the influenza nucleocapsid and import of the viral genome into the nucleus (Schnell and Chou 2008). Adamantanes are potent M2 channel blockers, which are known as the first synthetic anti-influenza drugs described in the mid-1960s (Davies et al. 1964). Two adamantane derivatives, amantadine and rimantadine

(Figure 4), have been licensed for influenza control and are commercially available under the trademarks Symmetrel® and Flumadine®, respectively (Galvao et al. 2012). The adamantanes are relatively cheap, highly stable in storage and show strong anti-influenza activity at micromolar concentrations. At present, the application of adamantanes for prevention and treatment of influenza infections is, however, not recommended because of the rapid emergence of drug-resistant virus variants that retain full virulence and transmissibility (Bright et al. 2005; Barr et al. 2008). Moreover, serious gastrointestinal and neurological side effects were observed in patients undergoing adamantane therapy (Galvao et al. 2012). Another disadvantage of adamantanes is their strong specificity against influenza A strains only (Rosenberg and Casarotto 2010). Selected publications describing the structure, chemical properties and application of adamantanes, adamantane-derivatives and other M2 ion channel blockers are presented in Table 4.2.

4.3. Inhibitors of viral RNA polymerase

Transcription and replication of the influenza virus genome is carried out by the influenza RNA polymerase holoenzyme, which is characterised by two catalytic activities. Polymerase activity is needed for the elongation of nascent RNA chains, whereas endonuclease activity is essential for cleavage of the 5'-capped primer sequence of the host mRNA. The cap is the terminal 7-methylguanosin bound through a triphosphate group to the host mRNA. This "cap snatching" process is needed for the initiation of viral RNA transcription (Lv et al. 2011). Influenza RNA polymerase is an extremely suitable target for the development of new broad-specific antivirals because of its highly conserved structure among influenza strains. It is thought that the influenza polymerase plays a crucial role in virus adaptation to human-to-human transmission and, consequently, in the formation of pandemic influenza variants (Miotto et al. 2008; Boivin et al. 2010; Aggarwal et al. 2011; Ping et al. 2011).

Two basic classes of RNA polymerase inhibitors have been described based on different mechanisms of action. The first group is represented by nucleoside analogues for the blocking of viral RNA chain elongation (Tisdale et al. 1995). A typical member of this group is favipiravir (T-705, Figure 4), which is an inhibitor of influenza A, B and C strains, including variants resistant to amantadine or oseltamivir. This compound is currently in the stage of clinical testing (Furuta et al. 2005). Other nucleoside analogs with anti-influenza activity include ribavirin (Virazole®)

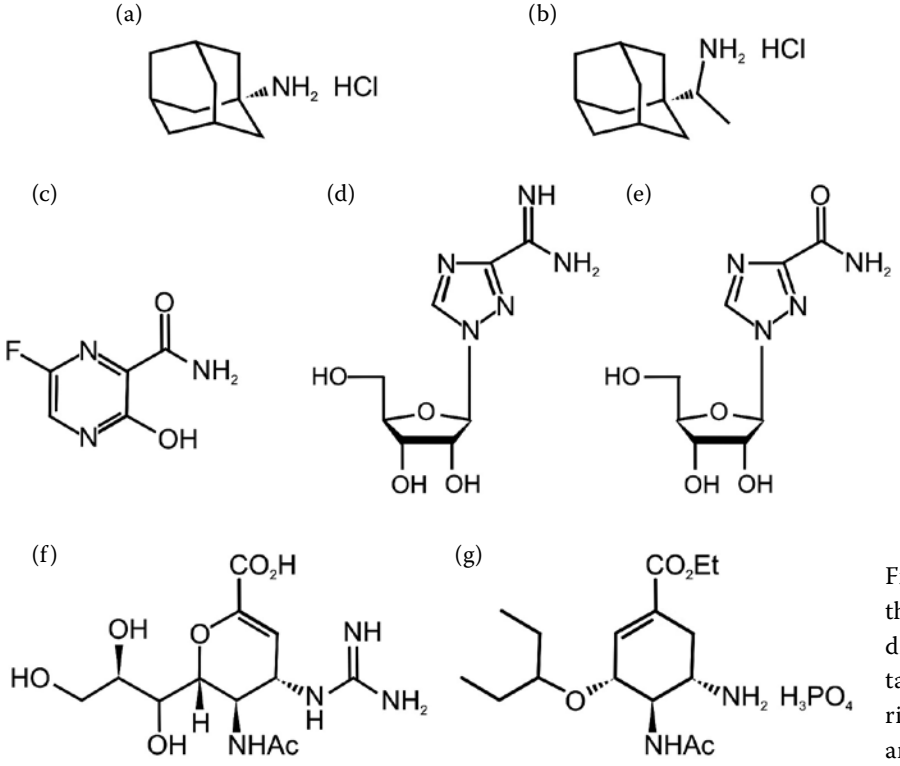

Figure 4. Chemical structures of the most important anti-influenza drugs; (a) amantadine, (b) rimantadine, (c) favipiravir (T-705), (d) ribavirin, (e) viramidine, (f) zanamivir, (g) oseltamivir

and its derivative viramidine, originaly licensed for treatment of hepatitis C infections (Figure 4). Their application is, however, sometimes connected with the development of haemolytic anaemia (Sidwell et al. 2005). The second class of antiviral molecules targeting the influenza polymerase is represented by compounds which block the endonuclease and cap-binding domains of the polymerase holoenzyme. These antivirals include cap analogues (Lv et al. 2011), short capped oligonucleotides (Tado et al. 2001), and small organic compounds, such as 4-substitued 2,4-phenylbutanoic acid (Hastings et al. 1996) and flutimide isolated from the fungus *Delitschia confertaspora* (Tomassini et al. 1996). For the representative publications concerning viral RNA polymerase inhibitors, see Table 4.3.

4.4. Inhibitors of neuraminidase

Neuraminidase, also referred to as sialidase, is an antigenic glycoprotein anchored in the surface envelope of the influenza virions, which hydrolytically cleaves the terminal sialic acid from the host cell receptors (Figure 3). Thus, it plays a crucial role in the release of viral progeny from the membranes of infected cells, prevents self-aggregation of virions and facilitates the movement of the infectious viral particles in the mucus of the respiratory epithelia (Matrosovich et al. 2004; Suzuki et al. 2005). Influenza neuraminidase has been established as a key drug target for the prophylaxis and treatment of influenza infections, predominantly for the following reasons: Firstly, the structure of the influenza neuraminidase active site is highly conserved between influenza A and B strains, making neuraminidase an attractive target for the development of broad-spectrum inhibitors (Yen et al. 2006). Secondly, resistance to neuraminidase inhibitors develops less commonly than to other anti-influenza drugs. Nevertheless, the intensive application of neuraminidase inhibitors for influenza treatment results in a permanently increasing number of drug-resistant strains (Garcia et al. 2009). Thirdly, in contrast to adamantanes, neuraminidase inhibitors are mostly well tolerated in patients under therapy (Cao et al. 2012). Finally, neuraminidase protein is a freely accessible target for antiviral molecules with an extracellular mode of action.

The development of neuraminidase inhibitors started in the middle 1970s, when the first structural analogues of sialic acid were described and denoted as DANA (2-deoxy-2,3-didehydro-N-acetyl neuraminic acid) and its trifluoroacetyl derivative FANA (Schulman and Palese 1975). At present, several licensed anti-influenza medications are available on the market, most notably the inhalant zanamivir with the trademark Releza®, and the orally administered oseltamivir (Tamiflu®) having excellent bioavailability and relatively long half-life *in vivo* (He et al. 1999; Greengard et al. 2000) (Figure 4). In response to the emergence of some oseltamivir-resistant influenza strains, peramivir and laninamivir have been recently developed (Bantia et al. 2006; Kubo et al. 2010). New-generation neuraminidase inhibitors are currently under investigation, e.g., multimeric forms of zanamivir (Watson et al. 2004), dual-targeted bifunctional antivirals (Liu et al. 2012), and several herbal remedies, such as flavonols, alkaloids and saponins (Jeong et al. 2009). The key publications on the structure, synthesis and therapeutic application of neuraminidase inhibitors are presented in Table 4.4.

4.5. Host cell factor targeting

Many human host cell molecules play a crucial role in influenza virus propagation and, therefore, represent promising targets for the design of new generation inhibitors of the virus-cell interaction. Muller et al. (2012) describes in his review 35 cellular factors essential for influenza virus infection for which 57 inhibitors with apparent anti-influenza activity are available. The most intensively studied are the compounds which effectively inhibit intracellular signalling cascades with a resulting negative influence on the establishment of viral infection (Nacken et al. 2012). Studies have also focused on inhibitors of vacuolar proton-ATPase which render the viral M2 ion channels inactive (Guinea and Carrasco 1994), inhibitors of cellular proteases which block the proteolytic activation of haemagglutinin (Zhirnov et al. 2011), and blockers of the cellular ubiquitin-proteasome system (Dudek et al. 2010). Although the development of host factor inhibitors is a promising research strategy to limit the emergence of drug-resistant mutants, their possible toxic side-effects *in vivo* need to be carefully studied. Selected papers on the topic are presented in Table 4.5.

4.6. Other anti-influenza agents

During the last decades, a large variety of chemical compounds with anti-influenza activity have been investigated. Several examples of such novel drugs are the inhibitors of viral nucleoprotein (Hung et al. 2012), blockers of influenza non-structural proteins (Basu et al. 2009) and short interfering oligonucleotides (siRNAs) used for viral RNA silencing (Stewart et al. 2011). Using large-scale screening techniques, new antiviral molecules which show significant anti-influenza effects have been identified; however, their chemical structure and mechanism of action remains unknown. These include, for instance, natural substances isolated from plants in chemical and pharmaceutical studies (He et al. 2012; Jiao et al. 2012). Another important group of prospective therapeutics are monoclonal antibodies and recombinant antibody fragments with high virus-neutralising activities (Hanson et al. 2006; Wei et al. 2011). Sixteen publications describing alternative anti-influenza agents are presented in Table 4.6.

5. Combination therapy of influenza infections

Recent *in vitro* and *in vivo* studies have demonstrated that the simultaneous application of two or more anti-influenza drugs with different modes of action, e.g. oseltamivir and amantadine, results in increased virus inhibition and enhanced therapeutic efficiency (Masihi et al. 2007). Similar findings were made with the combination of influenza virus inhibitors and immunomodulatory agents, especially corticosteroids (Zheng et al. 2008; Quispe-Laime et al. 2010). These observations are in accordance with the hypothesis that the applied drugs exert additive or synergistic anti-influenza effects in the infected cells. Such a combination regimen enables a reduction in the concentration of the individual inhibitory drugs, resulting in decreased drug toxicity and a reduced risk of antiviral resistance emergence in seasonal and pandemic influenza viruses (Govorkova et al. 2004; Smee et al. 2010; Nguyen et al. 2012). The principals of the combination therapy can in the future become a crucial strategy not only in the treatment of influenza infections, but also in the therapy of other serious viral, bacterial and parasitic diseases. Selected publications, which discuss combination antiviral therapy, are presented in Table 5.

6. Influenza drug resistance

As with all antimicrobials, propagation of viruses in the presence of antiviral drugs increases the selection pressure for mutations in the viral target proteins, which results in the induction of virus drug resistance. As an example, adamantane-resistant strains are typically characterised by a single substitution in the transmembrane region of the M2 ion channel (Saito et al. 2003; Shiraishi et al. 2003). On the other hand, resistance to neuraminidase inhibitors can result from mutations in the neuraminidase active cavity, but also from amino acid substitutions on the molecular surface of the neuraminidase protein (Yen et al. 2006; Du et al. 2010). It is noteworthy that resistance to adamantanes is acquired rapidly and by a high number of virus strains (Bright et al. 2005). In contrast to adamantane resistance, neuraminidase inhibitor resistance has developed over a longer time period and occurs with a relatively lower frequency (Garcia et al. 2009). This may be due to the fact that some mutations significantly affect viral infectivity and ability to replicate in the host cell. While amantadine-resistant strains do not show growth or virulence impairment (Sweet et al. 1991), influenza variants resistant to oseltamivir exhibit reduced neuraminidase activity and viral fitness *in vitro* (Yen et al. 2006), and decreased transmissibility in ferret models (Herlocher et al. 2004). The increasing emergence of drug-resistant influenza strains highlights the need to search continuously for innovative strategies for the development of new drugs with improved antiviral effects, higher safety and better tolerability. For key publications describing the mechanisms of viral drug resistance, as well as prevalence and clinical impact of drug resistant influenza strains see Table 6.

7. Acknowledgement

We wish to thank A. Durisova (Veterinary Research Institute, Brno) for her excellent graphical service.

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Received: 2013–03–25
Accepted after corrections: 2013–04–05

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Table 2. Review articles

Structure-based drug design Neuraminidase inhibitors Drug resistance	Recent emergence of influenza A H5N1 and H1N1 strains has heightened concern, especially as a result of their drug resistance. The life cycle of influenza viruses has been well studied and nearly all the viral proteins are becoming potential therapeutic targets. In this review, we present an overview of recent progress in structure-based anti-influenza drug design, paying close attention to the increasing role of computation and strategies for overcoming drug resistance.	Du et al. 2012
Structure-based drug design Haemagglutinin inhibitor Neuraminidase inhibitor M2 inhibitor Polymerase inhibitor Kinase inhibitor	In this review, we will discuss drug design based on proven and potential anti-influenza drug targets including viral hemagglutinin (HA), neuraminidase (NA), M2 ion channel, 3P polymerase complex, and host factors such as kinases. We have summarized influenza inhibitors based on their mode of actions. For instance, included are descriptions of (1) inhibitors of HA cleavage, such is nafamostat, camostat, gabexate, epsilon-aminocapronic acid and aprotinin, (2) inhibitors of fusion and entry, such as benzoquinones and hydroquiriones, CL 385319, BMY-27709, stachyflin, and their analogues, (3) inhibitors of viral RNPs/polymerase/endonuclease, such as T-705, L-735,822, flutimide and their analogues, (4) inhibitors of MEK, such as PD 0325901, CI-1040 and ARRY-142886, and (5) inhibitors of NA such as DANA, FANA, zanamivir, and oseltamivir, etc. Although amantadine and rimantadine are not recommended for treating influenza virus infections because of drug resistance problem, these viral M2 ion channel blockers established a proof-of-concept that the endocytosis of virion into host cells can be a valid drug target because M2 protein is involved in the endocytosis process. The influenza polymerase complex not only catalyzes RNA polymerization but also encodes the "cap snatching" activity. After being exported from the nucleus to the cytoplasm, the newly synthesized vRNPs are assembled into virions at the plasma membrane. The progeny virions will then leave the host cells through the action of NA. The strategies for discovery of small molecule inhibitors of influenza virus replication based on each particular mechanism will be discussed. Finally, the lessons learned from the design of NA inhibitors (NAI) are also included. Many exciting opportunities await the cadre of virologists, medicinal chemists, and pharmacologists to design novel influenza drugs with favorable pharmacological and pharmacokinetic properties to combat this threatening infectious disease.	Hsieh and Hsu 2007
High-throughput screen techniques	Introduction: Influenza antiviral high-throughput screens have been extensive, and yet no approved influenza antivirals have been identified through high-throughput screening. This underscores the idea that development of successful screens should focus on the exploitation of the underrepresented viral targets and novel, therapeutic host targets. Areas covered: The authors review conventional screening applications and emerging technologies with the potential to enhance influenza antiviral discovery. Real-world examples from the authors' work in biocontained environments are also provided. Future innovations are discussed, including the use of targeted libraries, multiplexed assays, proximity-based endpoint methods, non-laboratory-adapted virus strains, and primary cells, for immediate physiological relevance and translational applications. Expert opinion: The lack of successful anti-influenza drug discovery using high-throughput screening should not deter future efforts. Increased understanding of the functions of viral targets and host-pathogen interactions has broadened the target reservoir. Future screening efforts should focus on identifying new drugs against unexploited viral and host targets using currently developed assays, and on the development of novel, innovative assays to discover new drugs with novel mechanisms. Innovative screens must be designed to identify compounds that specifically inhibit protein-protein or protein-RNA interactions or other virus/host factor interactions that are crucial for viral replication. Finally, the use of recent viral isolates, increased biocontainment (for highly-pathogenic strains), primary cell lines, and targeted compound libraries must converge in efficient high-throughput primary screens to generate high-content, physiologically-relevant data on compounds with robust antiviral activity.	Atkins et al. 2012

Susceptibility monitoring Plaque-reduction assay Yield-reduction assay Dye-uptake assay Neuraminidase Haemagglutinin	With the clinical development of anti-viral agents, monitoring for the continued susceptibility of wild-type strains has become important in disease management. Various methods have been used to monitor viral susceptibility; the advantages and disadvantages of which depend on the virus, the target and the scale of the research being undertaken. The plaque-reduction assay is valuable for measuring susceptibility of most viruses but is not ideal for large-scale monitoring. Yield-reduction, measuring specific virus antigens, and dye-uptake assays, measuring virus cytopathic effects, are more suitable for high-throughput requirements, but the IC50 value (the concentration that inhibits 50% of virus) varies with the viral inoculum. Surveillance of influenza susceptibility to rimantadine/amantadine in the clinic has predominantly used EIA-based assays, since plaquing of influenza clinical isolates is variable. With development of the influenza NA inhibitors it became apparent that current cell-based assays were unsuitable for monitoring susceptibility to this new class of drugs. Variability may result from virus spread directly from cell to cell in culture by-passing the NA function. Furthermore, mutations selected in the HA, while not apparently contributing to phenotypic resistance in vivo, may result in cell-culture based resistance, and may mask NA resistance in cell culture by modifying receptor-binding specificity. One important distinction between NA inhibitors and other antiviral enzyme inhibitors is that both target enzyme and inhibitor work extracellularly. NA assays are therefore most representative of the in vivo situation for monitoring susceptibility, supported by HA sequencing. As the clinical use of NA inhibitors escalates, a major change will be required in approaches used to monitor susceptibility of influenza isolates in virology laboratories world-wide.	Tisdale 2000
Synthetic strategies Oseltamivir phosphate	Oseltamivir phosphate (Tamiflu) is the only orally active anti-influenza drug that potently inhibit neuraminidase. The recent emergence of avian flu, especially the H5N1 type, makes the situation of Tamiflu supply and demand increasingly serious. Further optimization of the current commercial approach and exploration of new synthetic routes are urgent. Here, different synthetic strategies of oseltamivir phosphate are reviewed, including discovery and improved synthetic route from (–)-quinic acid or (–)-shikimic acid, new asymmetric synthesis via catalytic desymmetrization of a mesoaziridine (CDMA), Diels-Alder Reaction and from other available materials.	Gong and Xu 2008
Synthetic strategies Tetra- and octavalent sialoside clusters	Tetra- and octavalent sialoside clusters were prepared in good yields exploiting for the first time the multiple copper-catalyzed cycloaddition of a propargyl thiosialoside with calix[4]arene polyazides. The cycloadducts featured the hydrolytically stable carbon-sulfur bond at the anomeric position and the 1,4-disubstituted triazole ring as the spacer between the sialic acid moieties and the platform. It was demonstrated that these unnatural motifs did not hamper the desired biological activity of the sialoclusters. In fact, they were able to inhibit, at submillimolar concentrations, the hemagglutination and the viral infectivity mediated both by BK and influenza A viruses.	Marra et al. 2008
Antiviral activity evaluation In vitro antiviral assays Animal models	Evaluation of potential influenza virus inhibitors may utilize multiple steps. First would be to determine if the viral target (e.g. influenza virus neuraminidase) being focused upon will be inhibited in the appropriate assay. Standard in vitro antiviral assays, used next in antiviral evaluations, may utilize inhibition of viral plaques, viral cytopathic effect (CPE), and viral hemagglutinin or other protein, with inhibition of viral yield used in follow-up evaluations. The CPE can be determined visually and by dye uptake, Animal models used for study of potential influenza virus inhibitors include the ferret, the laboratory mouse, and the chicken, with a variety of parameters used to indicate the severity of the infection and its inhibition by therapy. Multiple parameters are recommended in any in vivo antiviral evaluation. The ferret and the mouse infection models have been useful in studying the development of drug resistance and the relative virulence of drug-resistant viruses. The influenza mouse model has also been of value for the evaluation of immunomodulating effects of test compounds and for the study of the utility of antiviral drugs for use against influenza virus infections in the immunocompromised host. In considering the use of any animal model, species differences in drug pharmacology and metabolism must be taken into account. This review has described the systems which have been used most frequently by antiviral investigators, using, as examples, recent studies with the clinically approved influenza virus neuraminidase inhibitors oseltamivir and zanamivir.	Sidwell and Smee 2000

Anti-influenza agents	Influenza is a disease for deeply affecting millions of people every year. Recently, there has been considerable concern regarding the highly pathogenic H5N1 avian influenza virus, and its human pandemic potential. With developments in viral biology, there are more novel antiviral strategies targeting these viruses. In this review, we will discuss several proven and potential anti-influenza targets, including viral factors (such as hemagglutinin (HA), M2 ion channel protein, RNA-dependent RNA polymerase (RdRp), nucleoprotein (NP), non-structural protein (NS) and neuraminidase (NA)) and host factors (such as v-ATPase, protease, inosine monophosphate dehydrogenase (IMPDH) and intracellular signalling cascades), and their relevant inhibitors.	Gong et al. 2009
Neuraminidase Natural products Secondary plant metabolites	Influenza neuraminidase (NA), a key enzyme in viral replication, spread, and pathogenesis, is considered to be one of the most promising targets for combating influenza. Despite the substantial medical potential of NA inhibitors (NAIs), only three of these drugs are currently on the market (zanamivir, oseltamivir, and peramivir). Moreover, sudden changes in NAI susceptibility revealed the urgent need in the discovery/identification of novel inhibitors. Nature offers an abundance of biosynthesized compounds comprising chemical scaffolds of high diversity, which present an infinite pool of chemical entities for target-oriented drug discovery in the battle against this highly contagious pathogen. This review illuminates the increasing research efforts of the past decade (2000–2011), focusing on the structure, function and druggability of influenza NA, as well as its inhibition by natural products. Following a critical discussion of publications describing some 150 secondary plant metabolites tested for their inhibitory potential against influenza NA, the impact of three different strategies to identify and develop novel NAIs is presented: (i) bioactivity screening of herbal extracts, (ii) exploitation of empirical knowledge, and (iii) computational approaches. This work addresses the latest developments in theoretical and experimental research on properties of NA that are and will be driving anti-influenza drug development now and in the near future.	Grienke et al. 2012
Neuraminidase inhibitors Neuraminidase crystal structure 150- and 430-loop region	Neuraminidase inhibitors such as oseltamivir and zanamivir have been widely used in the treatment and have gained remarkable success. Although, they are effective in prevention of influenza; the concern for drug resistance still remains a question. Recently, the availability of crystal structures of the enzyme gave a new trend to the structure based drug designing of neuraminidase inhibitors. The article reviews a detailed understanding of the structural features within neuraminidase enzyme which turnouts to be crucial for future drug development. In depth analysis for the newly proposed spots within the 150 and 430-loop regions in N1 makes it distinguishable among the subtypes. Further we have discussed the various computational studies carried out in optimizing the designing of neuraminidase inhibitors thereby providing new clues to modify the currently available drugs.	Chintakrindi et al. 2012
Cellular drug targets	This review summarizes current knowledge on influenza A virus replication with a focus on emerging cellular drug targets. Interestingly, for many of these targets, compounds for which safety testing has been carried out in humans are available. It is possible that some of these compounds, such as inhibitors of heat shock protein 90, proteasome, importin 0/5 or protein kinase C, will be used for treatment of IAV infections after careful evaluation in human primary cells and severely ill flu patients.	Muller et al. 2012
Influenza neutralizing antibodies	The human antibody response to influenza virus infection plays a protective role against re-infection, yet little molecular detail is available regarding how human antibodies, when characterized at the monoclonal level, neutralize this important human pathogen. Recent studies, using a diverse array of strategies, have isolated and characterized human anti-virus neutralizing antibodies and shed light not only on the specificity and origin of these antibodies but on their potential for therapeutic use against influenza virus infection.	Martinez et al. 2009

Traditional Chinese medicine drugs Swine flu	After new human transmissible H1N1 (swine flu) viruses were reported in Mexico and the United States in April 2009, the World Health Organization (WHO) announced the emergence of a novel influenza A virus. Most governments in the world have been alerted and are monitoring the situation closely. As one of the official responses to the H1N1 pandemic, the Chinese government has released three editions of a document entitled "Recommended Schemes for Pandemic Influenza A Diagnoses and Treatments". The third edition recommended the use of not only two targeted anti-flu drugs, oseltamivir and zanamivir, but also four anti-flu TCM (Traditional Chinese Medicine) prescriptions. Since then, TCM has played a significant role in fighting the pandemic. TCM drugs comprise multiple compounds regulating multiple targets for a given class of medical indications, and are tunable to the symptoms of the individual. This review summarizes anti-influenza agents from TCM, including compounds, herbs, and TCM prescriptions, and suggests that, by further investigating TCM theory and mining TCM databases, a better drug discovery paradigm may arise – one that can be beneficial to both TCM and modern medicine.	Ge et al. 2010
Combination therapy	The pandemic potential of influenza viruses has engaged a large portion of the antiviral drug discovery research community in the development of numerous antiviral agents, with the ultimate goal to supplement effective immunization when new strains arise, especially after an antigenic shift. Antiviral agents against influenza A targets different replication steps of the virus life cycles. Some of the agents are analogues of biomolecules required during virus infection and others are inspired from natural plant extracts. In this review, we summarize their mechanisms of action during the influenza life cycle in vitro and the efficacies of combinational therapies with these agents against the influenza virus infections in vivo.	Kaihatsu and Barnard 2012
Combination therapy Currently available agents	The emergence of pandemic H1N1 influenza viruses in April 2009 and the continuous evolution of highly pathogenic H5N1 influenza viruses underscore the urgency of novel approaches to chemotherapy for human influenza infection. Anti-influenza drugs are currently limited to the neuraminidase inhibitors (oseltamivir and zanamivir) and to M2 ion channel blockers (amantadine and rimantadine), although resistance to the latter class develops rapidly. Potential targets for the development of new anti-influenza agents include the viral polymerase (and endonuclease), the hemagglutinin, and the non-structural protein NS1. The limitations of monotherapy and the emergence of drug-resistant variants make combination chemotherapy the logical therapeutic option. Here we review the experimental data on combination chemotherapy with currently available agents and the development of new agents and therapy targets.	Govorkova and Webster 2010
Protease inhibitors Ambroxol Clarithromycin Neuraminidase inhibitors Combination therapy	Influenza A virus (IAV) is one of the most common infectious pathogens in humans. Since IVA genome does not have the processing protease for the viral membrane fusion glycoprotein precursors, entry of' this virus into cells is determined primarily by host cellular, trypsin-type, processing proteases that proteolytically activate the fusion glycoprotein precursors of IAV. At least five different processing proteases have been identified in the airways of animals and humans. These proteases determine the infectious organ tropism of IAV infection as well as the efficiency of viral multiplication in the airway, and sometimes in the brain. Proteases in the upper respiratory tract are suppressed by secretory leukoprotease inhibitor, and those in the lower respiratory tract are suppressed by pulmonary surfactant which, by adsorption, inhibits the interaction between the proteases and viral membrane proteins. Since protease activities predominate over those of endogenous inhibitory compounds under normal airway conditions, administration of protease inhibitors in the early-stage of infection significantly suppresses viral entry and viral multiplication. Several viral neuraminidase inhibitors are used clinically as anti-influenza virus agents, based on their inhibitory action oil viral release from infected cells. Furthermore, protease inhibitors of viral entry could be potentially useful against influenza virus as well as neuraminiclase inhibitor-resistant viruses. We also found that ambroxol, a mucolytic and anti-oxidant agent, up-regulates the levels of endogenous protease inhibitory compounds in the airway fluids in early-phase infection, and that clarithromycin, a macrotide antibiotic, increases IgA levels and mucosal immunity through augmentation of interleukin-12 levels in the airway. The combination of neuraminidase inhibitors and protease inhibitors, clarithromycin or ambroxol, could be potentially used as a potent anti-influenza therapy to minimize the emergence of drug-resistant mutant viruses.	Kido et al. 2007

Drug resistance Adamantanes and neuraminidase inhibitors	Influenza viruses are major human pathogens with a global distribution, accounting for more than 500 000 annual deaths worldwide and with considerable impact on the quality of life and productivity of the society. Due to the limited efficacy of vaccination, antiviral drugs constitute a complementary approach in the control and prevention of influenza infections and thus play an important role in the management of influenza outbreaks and pandemics. Currently, adamantanes and neuraminidase inhibitors (NAIs) are the only two classes of anti-influenza agents approved for clinical use. However, the worldwide emergence and high prevalence of adamantane-resistant virus variants has discouraged the use of the former drugs. NAIs have proved to be very effective against influenza A and B viruses. Nevertheless, oseltamivir-resistant strains have also been reported quite frequently, as in the case of seasonal H1N1 viruses that circulated between 2007 and 2009. Indeed, the emergence of drug-resistant virus variants is always a matter of concern because it could significantly compromise the usefulness of such intervention. This highlights the need for continuous monitoring of resistance markers, as well as the development of new anti-influenza drugs and combination therapies.	Pizzorno et al. 2011

Table 3. Design, synthesis and evaluation of new anti-influenza drugs

Drug design and antiviral activity evaluation Drug database of 4000 compounds Docking simulation Viability assay Plaque assay Benzbromarone Diclazuril RNA polymerase	We performed a docking simulation targeting the interface of PA interacting with PB1 using a drug database including similar to 4000 compounds. We then conducted cell viability assay and plaque assay using IAV/WSN/33. Finally we examined their anti-IAV mechanism by surface plasmon resonance and IAV replicon assay. Results: We found that benzbromarone, diclazuril, and trenbolone acetate had strong anti-IAV activities. We confirmed that benzbromarone and diclazuril bound with PA subunit, and decreased the transcriptional activity of the viral RNA polymerase. Conclusions: Benzbromarone and diclazuril had strong anti-IAV activities with novel action mechanism, i.e. inhibition of viral RNA polymerase. General significance: Since benzbromarone and diclazuril are already used in public as medicines, these could be the candidates for alternatives in case of an outbreak of IAV which is resistant to pre-existing anti-IAV drugs.	Fukuoka et al. 2012
Structure-based drug design Computational methodologies Virtual screening Docking Pharmacophore modeling 200 new designed ligands	The M2 channel protein on the influenza A virus membrane has become the main target of the anti-flu drugs amantadine and rimantadine. The structure of the M2 channel proteins of the H3N2 (PDB code 2RLF) and 2009-H1N1 (Genbank accession number GQ385383) viruses may help researchers to solve the drug-resistant problem of these two adamantane-based drugs and develop more powerful new drugs against influenza A virus. In the present study, we searched for new M2 channel inhibitors through a combination of different computational methodologies, including virtual screening with docking and pharmacophore modeling. Virtual screening was performed to calculate the free energies of binding between receptor M2 channel proteins and 200 new designed ligands. After that, pharmacophore analysis was used to identify the important M2 protein-inhibitor interactions and common features of top binding compounds with M2 channel proteins. Finally, the two most potential compounds were determined as novel leads to inhibit M2 channel proteins in both H3N2 and 2009-H1N1 influenza A virus.	Tran et al. 2011

Interaction of adamantane with M2 protein Pore docking model	Interaction of aminoadamantanes with the influenza A virus M2 proton channel was analyzed by docking simulations of a series of synthetic aminoadamantane derivatives, of differing binding affinity, into the crystal structure of the transmembrane (M2TM) pore. The pore blocking model tested in the 'gas phase' describes qualitatively the changes on the relative binding affinities of the compounds (although a series of highly hydrophobic ligands which seem to have little capacity for different specific interactions with their receptor). The docking calculations predicted poses in which the adamantane ring is surrounded mainly by the alkyl side chains of Val27 or Ala30 and the ligand's amino group is generally hydrogen bonded with hydroxyls of Ser31 or carbonyls of Val27 or carbonyls of Ala30, the former (Ser31) being the most stable and most frequently observed. The binding of the ligand is a compromise between hydrogen bonding ability, which is elevated by a primary amino group, and apolar interactions, which are increased by the ability of the lipophilic moiety to adequately fill a hydrophobic pocket within the M2TM pore. A delicate balance of these hydrophobic contributions is required for optimal interaction.	Eleftheratos et al. 2010
Structure-based drug design Polymerase basic protein 2 Polymerase inhibitors	Influenza viruses cause a significant level of morbidity and mortality in the population every year. Their resistance to current anti-influenza drugs increases the difficulty of flu treatment. Thus, development of new anti-influenza drugs is necessary in regards of prevent the tragedy of influenza pandemic. The Polymerase basic protein 2 (PB2) subunit of influenza virus RNA polymerase is one of potential targets for new drugs because the binding of PB2 with the 5' cap of the host pre-mRNAs is the initial step of the virus' protein synthesis. In this study, we compared the binding potency of PB2 cap binding domain with two small molecules, i.e., RO and PPT28, with that of PB2 with cap analog m7GTP. The calculated binding energies showed that RO and PPT28 had higher binding affinity with PB2. Further interaction analysis showed that the important parts for binding were the five-and six-member heterocyclic rings (the 6/5-member rings) of small molecules, as well as the hydrophobic parts of RO and PPT28 which had good interactions with the hydrophobic residues in the binding cavity. Thus, RO and PPT28 are two potential anti-influenza drugs targeted PB2, which may inhibit the growth of influenza virus by competitively binding with the cap structure binding domain of PB2.	Lv et al. 2011
Design, synthesis, in vitro evaluation Transition-state-based neuraminidase inhibitors X-ray crystallographic structure Hydrophobic pocket	The design, synthesis, and in vitro evaluation of the novel carbocycles as transition-state-based inhibitors of influenza neuraminidase (NA) are described. The double bond position in the carbocyclic analogues plays an important role in NA inhibition as demonstrated by the antiviral activity of 8 (IC50 = 6.3 μM) vs 9 (IC50 > 200 μM). Structure-activity studies of a series of carbocyclic analogues 6a-i identified the 3-pentyloxy moiety as an apparent optimal group at the C-3 position with an IC50 value of 1 nM for NA inhibition. The X-ray crystallographic structure of 6h bound to NA revealed the presence of a large hydrophobic pocket in the region corresponding to the glycerol subsite of sialic acid. The high antiviral potency observed for 6h appears to be attributed to a highly favorable hydrophobic interaction in this pocket. The practical synthesis of 6 starting from (–)-quinic acid is also described.	Kim et al. 1997
Enantioselective synthesis (–)-oseltamivir free base (–)-methyl 3-epi-shikimate	A new enantioselective synthesis of the anti-influenza agent (–)-oseltamivir free base (7.1% overall yield; 98% ee) and (–)-methyl 3-epi-shikimate (16% overall yield; 98% ee) has been described from readily available raw materials. Sharpless asymmetric epoxidation and diastereoselective Barbier allylation of an aldehyde are the key reactions employed in the incorporation of chirality, while the cyclohexene carboxylic ester core was constructed through a ring closing metathesis reaction.	Rawat et al. 2012

Synthetic strategies 4-amino-1-phosphono-DANA Phosphono-zanamivir	Two phosphonate compounds la (4-amino-1-phosphono-DANA) and 1b (phosphono-zanamivir) are synthesized and shown more potent than zanamivir against the neuraminidases of avian and human influenza viruses, including the oseltamivir-resistant strains. For the first time, the practical synthesis of these phosphonate compounds is realized by conversion of sialic acid to peracetylated phosphono-DANA diethyl ester (5) as a key intermediate in three steps by a novel approach. In comparison with zanamivir, the high affinity of la and 1b can be partly attributable to the strong electrostatic interactions of their phosphonate, group's with the three arginine residues (Arg118, Arg292, and Arg371) in the active site of neuraminidases. These phosphonates are nontoxic to the human 293T cells; they protect cells from influenza: virus infection with EC50 values. in low-nanomolar range; including the wild-type WSN (H1N1), the 2009 pandemic (H1N1), the oseltamivir-resistant H274Y (H1N1), RG14 (H5N1); and Udorn (H3N2) influenza strains.	Shie et al. 2011
Synthetic strategies (+)-stachyflin	A novel and potent hemagglutinin inhibitor, (+)-stachyflin, was efficiently synthesized in an enantioselective manner starting from the (+)-5-methyl-Wieland-Miescher ketone. The synthetic method features a BF(3) center dot Et(2) O-induced cascade epoxide-opening/rearrangement/cyclization reaction to stereoselectively construct the requisite pentacyclic ring system in one step. In order to rationalize the mechanism of the cascade reaction, quantum chemical calculations of the possible intermediary carbocations and transition states in the model synthesis were carried out. An alternative approach to synthesize (+)-stachyflin by employing a similar cascade reaction was also described.	Sakurai et al. 2011
Nucleoprotein screening assay Tryptophan fluorescence quenching	Recent studies have shown that NP (nucleoprotein), which possesses multiple functions in the viral life cycle, is a new potential anti-influenza drug target. NP inhibitors reliably induce conformational changes in NPs, and these changes may confer inhibition of the influenza virus. The six conserved tryptophan residues in NP can be used as an intrinsic probe to monitor the change in fluorescence of the tryptophan residues in the protein upon binding to an NP inhibitor. In the present study, we found that the fluorescence of recombinant NP proteins was quenched following the binding of available NP inhibitors (such as nucleozin) in a concentration- and time-dependent manner, which suggests that the inhibitor induced conformational changes in the NPs. The minimal fluorescence-quenching effect and weak binding constant of nucleozin to the swine-origin influenza virus H1N1pdm09 (SOIV) NP revealed that the SOIV is resistant to nucleozin. We have used the fluorescence-quenching property of tryptophans in NPs that were bound to ligands in a 96-well-plate-based drug screen to assess the ability of promising small molecules to interact with NPs and have identified one new anti-influenza drug, CSV0C001018, with a high SI value. This convenient method for drug screening may facilitate the development of antiviral drugs that target viruses other than the influenza virus, such as HIV and HBV.	Hung et al. 2012
Neuraminidase enzymatic assay Recombinant neuraminidase expression Recombinant baculovirus	Context: Development of inexpensive and safe enzymatic assays to screen for putative neuraminidase inhibitors. Objective: Validate the use of recombinant neuraminidase expressed in baculovirus located on the viral surface capsule to develop a neuraminidase inhibitor screening assay. Materials and methods: Recombinant baculovirus particles displaying neuraminidase N1 and N3 were used as enzyme sources. The assay set-up required the use of 2'-(4-methylumbelliferyl)-alpha-D-acetyl neuraminic acid as substrate and oseltamivir carboxylate as benchmark inhibitor. Results: The assay was set up in a standard 96-well plate. The within- and between-assay coefficients of variation were, on average, less than 10%. The 50% inhibitory concentration values of the inhibitor were in good agreement with those determined by independent kinetic experiments. Discussion and conclusions: The assay showed satisfactory within- and between-assay repeatability. The obtained results suggest that recombinant baculovirus expressing neuraminidase located on the virus membrane capsule can be used to set up affordable and reliable neuraminidase inhibitors screening assays.	Kongkamnerd et al. 2012

Fluorescence polarisation assay Endonuclease inhibitors	The endonuclease activity of the influenza virus PA(N) protein is essential for virus replication and is a promising target for novel anti-influenza drugs. To facilitate the discovery of endonuclease inhibitors, we have developed a high-tluoughput fluorescence polarization (FP) assay, utilizing a novel fluorescein-labeled compound (K–d = 0.378 mu M) and a PA(N) construct, to identify small molecules that bind to the PA(N) endonuclease active site. Several known 4-substituted 2,4-dioxobutanoic acid inhibitors with high and low affinities have been evaluated in this FP-based, competitive binding assay, and there was a general correlation between binding and the reported inhibition of endonuclease activity. Additionally, we have demonstrated the utility of this assay for identifying endonuclease inhibitors in a small diverse targeted fragment library. These fragment hits were used to build a follow-up library that that led to new active compounds that demonstrate FP binding and anti-influenza activities in plaque inhibition assays. The assay offers significant advantages. over previously reported assays and is suitable for high-throughput and fragment-based screening studies. Additionally the demonstration of the applicability of a mechanism-based "targeted fragment" library supports the general potential of this novel approach for other enzyme targets. These results serve as a sound foundation for the development of new therapeutic leads targeting influenza endonuclease.	Baughman et al. 2012
High-throughput screening Cytopathic effect inhibitory assay Crystal violet uptake assay Influenza virus A Coxsackie virus B3 Herpes simplex virus-1	In order to identify new potential antiviral drugs, small amounts of extracts or compounds have to be examined for cytotoxicity and antiviral activity in primary screening using a rapid, easy, inexpensive, and highly standardised test system. In this study, high-throughput cytopathic effect (CPE) inhibitory assays were established for coxsackie virus B3 on HeLa Ohio cells, influenza virus A on Madin-Darby canine kidney cells, and herpes simplex virus type (HSV-1) on green monkey kidney cells that meet these requirements. The cytotoxic and the antiviral effects were quantified using a crystal violet uptake assay allowing automated handling of large numbers of candidate agents. To ensure comparable results with plaque reduction assays. the 50 and 90% plaque inhibitory concentrations of guanidine, amantadine, and phosphonoformic acid were used to standardise the anti-coxsackie virus B3, anti-influenza virus A, and anti-HSV-l tests, respectively. The strong correlation between the antiviral activity determined by CPE-inhibitory assays and plaque reduction assay was further proved for other antivirals. In summary, low amounts of large numbers of compounds may be tested inexpensively and standardised within 24 h (coxsackie virus B3 and influenza virus A) or 48 h (herpes simplex virus type 1) post-infection using CPE inhibitory assays.	Schmidtke et al. 2001
High-throughput screening Plaque inhibition assay Time-of-addition experiment Traditional Chinese medicine Procyanidin	In this research, we have established a high-throughput screening (HTS) platform based on the influenza A virus (IAV) vRNA promoter. Using this HTS platform, we selected 35 medicinal plants out of 83 examples of traditional Chinese medicine and found that 7 examples had not been reported. After examining many previous reports, we found that Vaccinium angustifolium Ait., Vitis vinifera L, and Cinnamomum cassia Presl had a common active compound, procyanidin, and then determined the anti-IAV effect of procyanidin and explored its mechanism of action. With a plaque inhibition assay and a time-of-addition experiment, we found that procyanidin could inhibit the IAV replication at several stages of the life cycle. In the Western blot and EGFP-LC3 localization assays, we found that procyanidin could inhibit the accumulation of LC3II and the dot-like aggregation of EGFP-LC3. In the RT-PCR and Western blot assays, we found procyanidin could inhibit the expression of Atg7, Atg5, and Atg12. Finally, by the bimolecular fluorescence complementation-fluorescence resonance energy transfer and co-immunoprecipitation assays, we found that procyanidin could inhibit the formation of the Atg5-Atg12/Atg16 heterotrimer and the dissociation of the beclin1/bcl2 heterodimer. In conclusion, we have established an HTS platform and identified procyanidin as a novel and promising anti-IAV agent.	Dai et al. 2012

Computational/ experimental screening Flow cytometry analysis Caspase-3 assay Mini-genome assay Time-of-addition experiment Ribonucleoprotein complex	In this study, computational and experimental screening of an extensive compound library identified THC19, which was able to suppress influenza virus replication. This compound had no cytotoxic effects and did not disrupt cell cycle progression or induce apoptosis in MDCK cells as confirmed by WST-1 assays, flow cytometry analysis, and caspase-3 assays. Time-of-addition experiments showed that THC19 acts at a relatively early stage of the viral life-cycle. Subsequent mini-genome assays revealed that THC19 inhibited viral genome replication and/or transcription, suggesting that it interferes with one or more of the viral components that form the ribonucleoprotein complexes, namely polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), nucleoprotein (NP) and viral RNA. Finally, mini-genome assays where PB2, PB1, PA or NP from A/WSN/33 (H1N1) virus were replaced with those from A/Udorn/307/1972 (H3N2) virus effectively demonstrated that THC19 inhibited viral multiplication in a manner dependent upon the PA subunit. Taken together, these results suggest that influenza virus PA protein is a potential target for, and may aid the development of, novel compounds that inhibit influenza A virus replication.	Yamada et al. 2012
Ferret/mouse model Intranasal administration Stachyflin derivatives	The in-vivo anti-influenza-virus activity of Stachyflin derivatives (III and its phosphate ester, III-Phos), a new class of haemagglutinin fusion inhibitor, and the improvement of their absorption after oral or intranasal administration were studied in mice, rats, and ferrets. The absorption of III in PEG 4000 and III-Phos aqueous solution increased about three and four fold in AUC after oral administration to uninfected mice compared with that of 0.5% HPMC (hydroxy-propyl-methylcellulose) suspension. Using a mouse influenza virus infection model, significant anti-influenza-virus activity was observed in infected mice treated orally with these compounds dissolved in PEG 4000 or distilled water, respectively, but not in mice treated with 0.5% HPMC. The in-vivo anti-influenza-virus activity in ferrets, a good model for influenza virus infection in man, was also studied. Although the concentration of III in plasma was above the IC50 against the influenza virus strain used for 6h after the oral administration of III in PEG 400 to uninfected ferrets, no in-vivo antiinfluenza-virus activity was observed at the same dosage given 4 times daily for 3 days. The intranasal administration of III-Phos, which was expected to have a more notable in-vivo anti-influenza-virus activity, was examined. III-Phos, whose intranasal absorption had been improved by the modification of III with phosphate ester in rats, inhibited viral replication in the nasal cavity and suppressed influenza-virus-induced fever when administered intranasally to infected ferrets. This study demonstrates that intranasally administered compounds with anti-influenza-virus activity must permeate the nasal membranes to produce their anti-influenza-virus effect.	Yoshimoto et al. 2000
Chicken egg model Comparative study Amantadine Rimantadine Oseltamivir Zanamivir	Previous studies have shown that embryonated egg provides a convenient and easy to use system for in vivo screening of anti-influenza virus inhibitors. However, it is not known whether this model is suitable for testing neuraminidase (NA) inhibitors, too. Therefore, the present study describes the evaluation of the ion-channel blockers amantadine and rimantadine in comparison with the NA inhibitors oseltamivir and zanamivir by using the influenza A virus hen's egg model. The treatment was started immediately before or after the challenge dose was placed on the chorioallantoic membrane (CAM). Differences between the survival rate of treated and untreated chick embryos infected with influenza A virus were analyzed statistically. As result, the survival rate of chick embryos could be significantly increased when the treatment with amantadine, rimantadine, oseltamivir, or zanamivir was started before the CAM was inoculated with one egg infective dose 50% (EID_{50}) influenza A virus. When the drugs were administered shortly after viral inoculation, significant antiviral efficacy was shown for rimantadine, oseltamivir, and zanamivir. Antiviral efficacy could be demonstrated exclusively for both oseltamivir and zanamivir after the embryos were infected with higher challenge doses of 10^2 EID_{50} influenza A virus. In conclusion, the NA inhibitors oseltamivir and zanamivir have a significantly better antiviral activity against influenza A virus than amantadine and rimantadine tested in embryonated hen's eggs. Therefore, this model can be a valuable alternative approach for in vivo pre-testing anti-influenza virus activity of NA inhibitors.	Sauerbrei et al. 2006

Pharmacokinetic studies Oseltamivir Bioavailability Renal clearance Steady-state plasma concentration	Oseltamivir is an ethyl ester prodrug of Po 64-0802, a selective inhibitor of influenza virus neuraminidase. Oral administration of oseltamivir delivers the active antiviral Po 64-0802 to the bloodstream, and thus all sites of influenza infection (lung, nasal mucosa, middle ear) are accessible. The pharmacokinetic profile of oseltamivir is simple and predictable, and twice daily treatment results in effective antiviral plasma concentrations over the entire administration interval. After oral administration, oseltamivir is readily absorbed from the gastrointestinal tract and extensively converted to the active metabolite. The absolute bioavailability of the active metabolite from orally administered oseltamivir is 80%. The active metabolite is detectable in plasma within 30 minutes and reaches maximal concentrations after 3 to 4 hours. After peak plasma concentrations are attained, the concentration of the active metabolite declines with an apparent half-life of 6 to 10 hours. Oseltamivir is eliminated primarily by conversion to and renal excretion of the active metabolite. Renal clearance of both compounds exceeds glomerular filtration rate, indicating that renal tubular secretion contributes to their elimination via the anionic pathway. Neither compound interacts with cytochrome P450 mixed-function oxidases or glucuronosyltransferases. The pharmacokinetic profile of the active metabolite is linear and dose-proportional, with less than 2-fold accumulation over a dosage range of oseltamivir 50 to 500mg twice daily. Steady-state plasma concentrations are achieved within 3 days of twice daily administration, and at a dosage of 75mg twice daily the steady-state plasma trough concentrations of active metabolite remain above the minimum inhibitory concentration for all influenza. strains tested. Exposure to the active metabolite at steady state is approximately 25% higher in elderly compared with young individuals; however, no dosage adjustment is necessary. In patients with renal impairment, metabolite clearance decreases linearly with creatinine clearance. A dosage reduction to 75mg once daily is recommended fbr patients with creatinine clearance <30 ml/min (1.8 l/h). The pharmacokinetics in patients with influenza are qualitatively similar to those in healthy young adults. In vitro and in vivo studies indicate no clinically significant drug interactions. Neither paracetamol (acetaminophen) nor cimetidine altered the pharmacokinetics of Ro 64-0802. Coadministration of probenecid resulted in a 2.5-fold increase in exposure to Ro 64-0802, however, this competition is unlikely to result in clinically relevant effects. These properties make oseltamivir a. suitable candidate for use in the prevention and treatment of influenza.	He et al. 1999
Multicenter clinical study Zanamivir Antiviral activity Safety Tolerability China 2010–2011 Adolescents/adults	Background It is the first multicenter clinical study in China to investigate zanamivir use among Chinese adolescents and adults with influenza-like illness (ILI) since 2009, when inhaled zanamivir (RELENZA (R)) was marketed in China. Methods An uncontrolled open-label, multicentre study to evaluate the antiviral activity, and safety of inhaled zanamivir (as Rotadisk via Diskhaler device); 10 mg administered twice daily for 5 days in subjects ≥ 12 years old with ILI. Patients were enrolled within 48 hours of onset and followed for eight days. Patients were defined as being influenza-positive if the real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) test had positive results. Results A total of 400 patients ≥ 12 years old were screened from 11 centers in seven provinces from March 2010 to January 2011. Three hundred and ninety-two patients who took at least one dose of zanamivir were entered into the safety analysis. The mean age was 33.8 years and 50% were male. Cardiovascular diseases and diabetes were the most common comorbidities. All the reported adverse events, such as rash, nasal ache, muscle ache, nausea, diarrhea, headache, occurred in less than 1% of subjects. Mild sinus bradycadia or arrhythmia occurred in four subjects (1%). Most of the adverse events were mild and did not require any change of treatment. No severe adverse events (SAE) or fatal cases were reported. Bronchospasm was found in a 38 years old woman whose symptoms disappeared after stopping zanamivir and without additional treatment. All the 61 influenza virus isolates (43 before enrollment, 18 during treatment) proved to be sensitive to zanamivir. Conclusions Zanamivir is well tolerated by Chinese adolescents and adults with ILIs. There is no evidence for the emergence of drug-resistant isolates during treatment with zanamivir.	Cao et al. 2012

Tolerability/ pharmacokinetics Neuraminidase inhibitors Adult/elderly volunteers Steady-state plasma concentrations	The tolerability and pharmacokinetics of Ro 64-0802, a potent, selective inhibitor of influenza neuraminidase, and ifs oral prodrug oseltamivir were investigated in three double-blind, placebo-controlled studies. Two studies involved healthy adult volunteers (18–55 years) (n = 48) who received single (20–1000 mg) or bid doses (50–500 mg) (n = 32) of oseltamivir or placebo for 7 days. Healthy elderly volunteers (greater than or equal to 65 years) (n = 24) received oseltamivir 100 to 200 mg bid or placebo for 7 days in a third study. Measurable plasma concentrations of the active metabolite appeared rapidly in plasma and were significantly higher and longer lasting than those of oseltamivir. Pharmacokinetics of both compounds were linear. Multiple-dose exposure was predictable from single-dose data, and steady-state plasma concentrations were achieved within 3 days of bid drug administration. Oseltamivir was well tolerated at single doses of up to 1000 mg and twice-daily doses of up to 500 mg. Adverse events were mild in intensity. Exposure to both prodrug and active metabolite was increased in elderly patients by approximately 25%. However, due to the wide safety margin of both compounds, no dose adjustment is necessary for elderly patients.	Massarella et al. 2000

Table 4.1. Inhibitors of haemagglutinin

Haemagglutinin X-ray crystalography Receptor binding	Hemagglutinin (HA) is the receptor-binding and membrane fusion glycoprotein of influenza virus and the target for infectivity-neutralizing antibodies. The structures of three conformations of the ectodomain of the 1968 Hong Kong influenza virus HA have been determined by X-ray crystallography: the single-chain precursor, HA0; the metastable neutral-pH conformation found on virus, and the fusion pH-induced conformation. These structures provide a framework for designing and interpreting the results of experiments on the activity of HA in receptor binding, the generation of emerging and reemerging epidemics, and membrane fusion during viral entry.	Skehel and Wiley 2000
Sialylglycans Molecular dynamics simulation Haemagglutinin-receptor interaction	Recognition of cell-surface sialyldisaccharides by influenza A hemagglutinin (HA) triggers the infection process of influenza. The changes in glycosidic torsional linkage and the receptor conformations may alter the binding specificity of HAs to the sialylglycans. In this study, 10-ns molecular dynamics simulations were carried out to examine the structural and dynamic behavior of the HAs bound with sialyldisaccharides Neu5Ac alpha(2-3) Gal (N23G) and Neu5Ac alpha(2-6) Gal (N26G). The analysis of the glycosidic torsional angles and the pair interaction energy between the receptor and the interacting residues of the binding site reveal that N23G has two binding modes for H1 and H5 and a single binding mode for H3 and H9. For N26G, H1 and H3 has two binding modes, and H5 and H9 has a single binding mode. The direct and water-mediated hydrogen bonding interactions between the receptors and HAs play dominant roles in the structural stabilization of the complexes. It is concluded from pair interaction energy and Molecular Mechanic-Poisson-Boltz-mann Surface Area calculations that N26G is a better receptor for H1 when compared with N23G. N23G is a better receptor for H5 when compared with N26G. However, H3 and H9 can recognize N23G and N26G in equal binding specificity due to the marginal energy difference (approximate to 2.5 kcal/mol). The order of binding specificity of N23G is H3 > H5 > H9 > H1 and N26G is H1 > H3 > H5 > H9, respectively. The proposed conformational models will be helpful in designing inhibitors for influenza virus.	Priyadarzini et al. 2012

Sialyllactose-containing glycopolymers Chemoenzymatic synthesis Glyco-biosensor Glycoarray	We report a chemoenzymatic synthesis of chain-end functionalized sialyllactose-containing glycopolymers with different linkages and their oriented immobilization for glycoarray and SPR-based glyco-biosensor applications. Specifically, O-cyanate chain-end functionalized sialyllactose-containing glycopolymers were synthesized by enzymatic a2,3- and alpha 2,6-sialylation of a lactose-containing glycopolymer that was synthesized by cyanoxyl-mediated free radical polymerization. H-1 NMR showed almost quantitative alpha 2,3- and alpha 2,6-sialylation. The O-cyanate chain-end functionalized sialyllactose-containing glycopolymers were printed onto amine-functionalized glass slides via isourea bond formation for glycoarray formation. Specific protein binding activity of the arrays was confirmed with alpha 2,3- and alpha 2,6-sialyl specific binding lectins together with inhibition assays. Further, immobilizing O-cyanate chain-end functionalized sialyllactose-containing glycopolymers onto amine-modified SPR chip via isourea bond formation afforded SPR-based glyco-biosensor, which showed specific binding activity for lectins and influenza viral hemagglutinins (HA). These sialyloligo-macroligand derived glycoarray and SPR-based glyco-biosensor are closely to mimic 3D nature presentation of sialyloligosaccharides and will provide important high-throughput tools for virus diagnosis and potential antiviral drug candidates screening applications.	Narla and Sun 2012
Sialyllactose Structural analysis Computer simulation	We designed and synthesized novel trivalent anti-influenza reagents. Sialyllactose was located at the terminal of each valence which aimed to block each receptor-binding site of the hemagglutinin (HA) trimer on the surface of the virus. Structural analyses were carried out with a model which was constructed with a computer simulation. A previously reported cyclic glycopeptide blocker bound to the HA in the model. The analyses suggest that the glutamine residue in the cyclic peptide bearing Neu5A alpha 2,3Gal beta 1,4Glc trisaccharide via a linker interacts with the Gln189 in HA through hydrogen bonding. The present anti-influenza reagents likely interact with a glutamine residue included in the vicinity of Gln189. A plague reduction assay of the influenza virus, A/PR/8/1934 (H1N1), was performed in MDCK cells to evaluate for the synthesized compounds to inhibit viral replication. One of the compounds showed approximately 85% inhibition at the concentration of 400 mu M at 4 degrees °C.	Feng et al. 2010
Sialic acid analogues C2-, C4- and C5-substitutions Molecular docking AutoDock3.05	A molecular docking tool of AutoDock3.05 was evaluated for its ability to reproduce experimentally determined affinities of various sialic acid analogues toward hemagglutinin of influenza A virus. With the exception of those with a C6-modified glycerol side chain, the experimental binding affinities of most sialic acid analogues (C2, C4 and C5-substituted) determined by viral hemadsorption inhibition assay, hemagglutination inhibition assay and nuclear magnetic resonance correlated well with the computationally estimated free energy of binding. Sialic acid analogues with modified glycerol side chains showed only poor correlation between the experimentally determined hemagglutinin inhibitor affinities and AutoDock3.05 scores, suggesting high mobility of the glutamic acid side chain at the glycerol binding pocket, which is difficult to simulate using a flexi-rigid molecular docking approach. In conclusion, except for some glycerol-substituted sialic acid analogues, the results showed the effectiveness of AutoDock3.05 searching and scoring functions in estimating affinities of sialic acid analogues toward influenza A hemagglutinin, making it a reliable tool for screening a database of virtually designed sialic acid analogues for hemagglutinin inhibitors.	Al-qattan and Mordi 2010a

Sialic acid analogues C2-, C5- and C6-substitutions Fragment-based drug design	In this study fragment-based drug design is combined with molecular docking simulation technique, to design databases of virtual sialic acid (SA) analogues with new substitutions at C2, C5 and C6 positions of SA scaffold. Using spaces occupied by C2, C5 and C6 natural moieties of SA when bound to hemagglutinin (HA) crystallographic structure, new fragments that are commercially available were docked independently in all the pockets. The oriented fragments were then connected to the SA scaffold with or without incorporation of linker molecules. The completed analogues were docked to the whole SA binding site to estimate their binding conformations and affinities, generating three databases of HA-bound SA analogues. Selected new analogues showed higher estimated affinities than the natural SA when tested against H3N2, H5N1 and H1N1 subtypes of influenza A. An improvement in the binding energies indicates that fragment-based drug design when combined with molecular docking simulation is capable to produce virtual analogues that can become lead compound candidates for anti-flu drug discovery program.	Al-qattan and Mordi 2010b
Sialic acid analogues Polyacrylamide conjugate Polyvalent inhibitor	An ELISA assay is described for measuring the binding of influenza virus A-X31 to alpha-sialoside groups that are linked to biotin-labeled polyacrylamides. The efficacy of these polymers in inhibiting the adhesion of influenza virus to erythrocytes (as measured by a hemagglutination assay) was shown to be directly related to the binding affinity of the polymers for the viral surface: the differences in inhibitory efficacy among the polymeric inhibitors and monomeric alpha-methyl sialoside, among fractions of a polymeric, polyvalent inhibitor with narrow molecular weight ranges, and among polymeric inhibitors prepared by copolymerization or modification of a preformed polymer chain, all correlated with differences in the affinity of the inhibitors for the surface of the virus, The polymeric inhibitors studied had affinities for the viral surface that ranged between 10^3 and $> 10^6$ greater than alpha-methyl sialoside, on the basis of total sialic acid groups in solution, The role of steric stabilization in the mechanism by which these polymers inhibit hemagglutination was investigated, The ability of the polymeric, polyvalent inhibitors to inhibit the binding of a polyclonal antibody to the viral surface suggests that steric stabilization may also be an important effect in this system.	Sigal et al. 1996
Quinone molecules Bicyclic quinones Polymeric inhibitors Influenza A virus	Using the plaque reduction assay, relatively simple bicyclic quinone molecules, as well as multiple copies thereof covalently attached to a long polyglutamate-based polymeric chain, were examined as new inhibitors of various naturally occurring strains of influenza A virus. The polymer-conjugated inhibitors were found to have a far greater potency (for some as high as two orders of magnitude when a long spacer arm was employed) than their corresponding parent molecules against the human Wuhan influenza strain. However, such polymeric inhibitors failed to exhibit higher potency compared with their small molecule predecessors against the human Puerto Rico and avian turkey influenza strains. These observations, further explored by means of molecular modeling, reveal the previously unrecognized unpredictability of the benefits of multivalency, possibly because of poor accessibility of the viral targets to polymeric agents.	Larson et al. 2012
Benzoquinones, hydroquinones Conformational change of haemagglutinin Virus-cell membrane fusion	Past efforts to employ a structure-based approach to design an inhibitor of the fusion-inducing conformational change in the influenza virus hemagglutinin (HA) yielded a family of small benzoquinones and hydroquinones, The most potent of these, tert-butyl hydroquinone (TBHQ), inhibits both the conformational change in HA from strain X:31 influenza virus and viral infectivity in tissue culture cells with 50% inhibitory concentrations in the micromolar range. A new structure-based inhibitor design search was begun which involved the recently refined crystal structure (2.1-Angstrom resolution) of the HA ectodomain, new insights into the conformational change, and improvements in the molecular docking program, DOCK. As a result, we identified new inhibitors of HA-mediated membrane fusion. Like TBHQ, most of these molecules inhibit the conformational change, One of the new compounds, however, facilitates rather than inhibits the HA conformational change, Nonetheless, the facilitator, diiodofluorescein, inhibits HA-mediated membrane fusion and, irreversibly, infectivity. We further characterized the effects of inhibitors from both searches on the conformational change and membrane fusion activity of HA as well as on viral infectivity. We also isolated and characterized several mutants resistant to each class of inhibitor. The implications of our results for HA-mediated membrane fusion, anti-influenza virus therapy, and structure-based inhibitor design are discussed.	Hoffman et al. 1997

New haemagglutinin inhibitors	We reported previously that a small molecule named CL-385319 could inhibit H5N1 influenza virus infection by targeting hemagglutinin, the envelope protein mediating virus entry. In the present study, a novel series of derivatives focused on the structural variation of CL-385319 were synthesized as specific inhibitors against the H5 subtype of influenza A viruses. These small molecules inhibited the low pH-induced conformational change of hemagglutinin, thereby blocking viral entry into host cells. Compound 11 was the most active inhibitor in this series with an IC50 of 0.22 μM. The structure activity relationships analysis of these compounds showed that the 3-fluoro-5-(trifluoromethyl)benzamide moiety was very important for activity, and the -F group was a better substituent group than -CF3 group in the phenyl ring. The inhibitory activity was sensitive to the benzamide because the oxygen and hydrogen of the amide served as H-bond acceptor and donor, respectively.	Zhu et al. 2012
Stachyflin Conformational change of haemagglutinin	Stachyflin is a novel compound having H1 and H2 subtype-specific anti-influenza A virus activity. Stachyflin has no inhibition on H3 subtype influenza A or influenza B viruses. The susceptibility of the reassortant viruses between H1 and H3 subtype influenza A viruses to Stachyflin indicated that its target was virus-encoded hemagglutinin (HA). The results of the timing of Stachyflin addition against in vitro virus infection and virus-mediated hemolysis assay suggested that the drug inhibited the HA-mediated virus-cell fusion process. More directly, Stachyflin interfered with HA conformational change induced by low pH or heat treatment. The effect of Stachyflin could not be eliminated by washing of the Stachyflin-treated virus, which caused very specific virucidal effect. This is a remarkable property among small molecules which inhibit low-pH induced HA conformational change. From these findings, we concluded that the mechanism of Stachyflin action is to inhibit HA conformational change which is necessary for virus-cell membrane fusion. Stachyflin may be used as a tool for a study of molecular mechanism of low-pH induced HA conformational change, and offers potential as a pharmaceutical agent.	Yoshimoto et al. 1999
(+)-stachyflin Enantioselective synthesis	A novel and potent hemagglutinin inhibitor, (+)-stachyflin, was efficiently synthesized in an enantioselective manner starting from the (+)-5-methyl-Wieland-Miescher ketone. The synthetic method features a BF(3)center dot Et(2) O-induced cascade epoxide-opening/rearrangement/cyclization reaction to stereoselectively construct the requisite pentacyclic ring system in one step. In order to rationalize the mechanism of the cascade reaction, quantum chemical calculations of the possible intermediary carbocations and transition states in the model synthesis were carried out. An alternative approach to synthesize (+)-stachyflin by employing a similar cascade reaction was also described.	Sakurai et al. 2011
Benzamid derivatives Virus-cell membrane fusion Sequence analyses Haemagglutinin gene Drug resistance	In the initial stages of influenza virus infection, the hemagglutinin (HA) protein of influenza virus mediates both adsorption and penetration of the virus into the host cell. Recently, we identified and characterized BMY-27709 as an inhibitor of the H1 and H2 subtypes of influenza A virus that specifically inhibits the HA function necessary for virus-cell membrane fusion. Studies presented herein show that the inhibition is mediated through specific interaction with the HA protein. This binding represses the low-pH-induced conformational change of the HA protein which is a prerequisite for membrane fusion. In an attempt to define the binding pocket within the HA molecule, a number of drug-resistant viruses have been isolated and characterized. Sequence analyses of the HA gene of these drug-resistant viruses mapped amino acid changes responsible for drug resistance to a region located near the amino terminus of HA2. In addition, we have identified inactive analogs of BRY-27709 which are able to compete out the inhibitory activity of BMY-27709. This finding suggests that inhibition of the HA-mediated membrane fusion by this class of compounds is not solely the result of binding within the HA molecule but requires specific interactions.	Luo et al. 1997

Podocarpic acid derivatives Sequence analyses Haemagglutinin gene Drug resistance	Entry of influenza virus into the host cell is dependent on the fusion of the viral envelope with the endosomal membrane and is mediated by a low-pH-induced change of the viral hemagglutinin (HA) to a conformation that is fusogenic. A compound related to podocarpic acid (180299] was identified that inhibits multicycle replication of influenza A/Kawasaki/86 (H1N1) Virus in culture. Treatment of Madin-Darby canine kidney (MDCK) cells with 180299 at 1 h before infection resulted in the inhibition of viral protein synthesis. Addition of 20 μg of 180299/ml at 1 h p.i. had no effect, indicating that 180299 affects an early step of the influenza viral replication cycle. Genetic analysis of reassortants between sensitive and resistant viruses demonstrated that hemagglutinin (HA) conferred the 180299-resistant (180299(r)) phenotype. Twelve independent isolates of influenza A/Kawasaki/86 were selected for resistance to 180299, and sequence analysis revealed that each of these viruses contained amino acid substitutions in the HA. These mutations are dispersed throughout the HA primary amino acid sequence and cluster in one of two regions: the interface between HA(1) and HA(2) and in a region near the fusion domain of HA(2). When compared with the parent virus, the pH-of-inactivation of the resistant mutants was increased by 0.3 to 0.6 pH unit, suggesting that the mutant HAs undergo the conformational change at an elevated pH. Fusion of human erythrocytes to MDCK cells infected with parent influenza A/Kawasaki/86 was inhibited by 180299 (0.1–10 μg/ml) in a concentration-dependent: manner, whereas fusion of erythrocytes to MDCK cells infected with 180299(r) mutants was not: affected. These results suggest that 180299 interacts with the neutral pH conformation of influenza A HA and prevents the low-pH-induced change of HA to its fusogenic conformation.	Staschke et al. 1998
DAS181 Sialidase fusion protein Influenza A and B Mouse/ferret model	Influenza is a highly infectious disease characterized by recurrent annual epidemics and unpredictable major worldwide pandemics. Rapid spread of the highly pathogenic avian H5N1 strain and escalating human infections by the virus have set off the alarm for a global pandemic. To provide an urgently needed alternative treatment modality for influenza, we have generated a recombinant fusion protein composed of a sialidase catalytic domain derived from Actinomyces viscosus fused with a cell surface-anchoring sequence. The sialidase fusion protein is to be applied topically as an inhalant to remove the influenza viral receptors, sialic acids, from the airway epithelium. We demonstrate that a sialidase fusion construct, DAS181, effectively cleaves sialic acid receptors used by both human and avian influenza viruses. The treatment provides long-lasting effect and is nontoxic to the cells. DAS181 demonstrated potent antiviral and cell protective efficacies against a panel of laboratory strains and clinical isolates of IFV A and IFV B, with virus replication inhibition 50% effective concentrations in the range of 0.04 to 0.9 nM. Mouse and ferret studies confirmed significant in vivo efficacy of the sialidase fusion in both prophylactic and treatment modes.	Malakhov et al. 2006
DAS181 Phase II clinical trial	Background. DAS181, a novel host-directed antiviral in development for influenza treatment, was assessed in this phase II clinical trial. Methods. This study was a double-blind, placebo-controlled phase II clinical trial assessing influenza viral load and patient safety in otherwise healthy influenza-infected participants. Participants were randomized to a single-dose, multiple-dose, or placebo group and were followed for safety and virologic outcomes. Results. A total of 177 laboratory-confirmed influenza-infected participants were enrolled in the trial, which encompassed 3 influenza seasons from 2009–2011 in both the Northern and Southern Hemispheres. Thirty-seven percent of participants had confirmed infection with influenza B, 33% with seasonal H3N2, 29% with pandemic 2009 H1N1, and 1 participant was positive for both influenza B and pandemic 2009 H1N1. Significant effects were observed in regard to decreased change from baseline viral load and viral shedding in the multiple-dose group compared with placebo as measured by quantitative polymerase chain reaction ($P < 0.05$). No instances of H274Y were observed among viral isolates from this trial. Overall, the drug was generally well tolerated. Conclusions. DAS181 significantly reduced viral load in participants infected with influenza, thus warranting future clinical development of this novel host-directed therapy.	Moss et al. 2012

Fusion-inhibitory peptides Cholesterol-conjugated compounds	We previously described fusion-inhibitory peptides that are targeted to the cell membrane by cholesterol conjugation and potently inhibit enveloped viruses that fuse at the cell surface, including HIV, parainfluenza, and henipaviruses. However, for viruses that fuse inside of intracellular compartments, fusion-inhibitory peptides have exhibited very low antiviral activity. We propose that for these viruses, too, membrane targeting via cholesterol conjugation may yield potent compounds. Here we compare the activity of fusion-inhibitory peptides derived from the influenza hemagglutinin (HA) and show that although the unconjugated peptides are inactive, the cholesterol-conjugated compounds are effective inhibitors of infectivity and membrane fusion. We hypothesize that the cholesterol moiety, by localizing the peptides to the target cell membrane, allows the peptides to follow the virus to the intracellular site of fusion. The cholesterol-conjugated peptides trap HA in a transient intermediate state after fusion is triggered but before completion of the refolding steps that drive the merging of the viral and cellular membranes. These results provide proof of concept for an antiviral strategy that is applicable to intracellularly fusing viruses, including known and emerging viral pathogens.	Lee et al. 2011
RNA aptamers SELEX technology Haemagglutination inhibition assay	In this study, we investigated the efficacy of using hemagglutinin (HA) as a target for antiviral therapy through nucleic acid aptamers. After purification of the receptor binding domain (HA1) of HA protein, activity of recombinant HA1 was confirmed by using hemagglutination assay. We selected RNA aptamer candidates after 15 rounds of iterative Systematic Evolution of Ligands by EXponential enrichment (SELEX) targeting the biologically active HA protein. The selected RNA aptamer HAS15-5, which specifically binds to HA1, exhibited significant antiviral efficacy according to the results of a hemagglutination inhibition assay using egg allantoic fluids harboring the virus. Thus, the RNA aptamer HAS15-5, which acts by blocking and inhibiting the receptor-binding domain of viral HA, can be developed as a novel antiviral agent against type H5 avian influenza virus.	Park et al. 2011

Table 4.2. M2 ion channel blockers

Structure and function of M2 protein Conductance of protons Haemagglutinin-mediated membrane fusion M2-rimantadin complex	The integral membrane protein M2 of influenza virus forms pH-gated proton channels in the viral lipid envelope (1). The low pH of an endosome activates the M2 channel before haemagglutinin-mediated fusion. Conductance of protons acidifies the viral interior and thereby facilitates dissociation of the matrix protein from the viral nucleoproteins - a required process for unpacking of the viral genome (2). In addition to its role in release of viral nucleoproteins, M2 in the trans- Golgi network (TGN) membrane prevents premature conformational rearrangement of newly synthesized haemagglutinin during transport to the cell surface by equilibrating the pH of the TGN with that of the host cell cytoplasm (3). Inhibiting the proton conductance of M2 using the anti- viral drug amantadine or rimantadine inhibits viral replication(4–7). Here we present the structure of the tetrameric M2 channel in complex with rimantadine, determined by NMR. In the closed state, four tightly packed transmembrane helices define a narrow channel, in which a 'tryptophan gate' is locked by intermolecular interactions with aspartic acid. A carboxy-terminal, amphipathic helix oriented nearly perpendicular to the transmembrane helix forms an inward-facing base. Lowering the pH destabilizes the transmembrane helical packing and unlocks the gate, admitting water to conduct protons, whereas the C-terminal base remains intact, preventing dissociation of the tetramer. Rimantadine binds at four equivalent sites near the gate on the lipid- facing side of the channel and stabilizes the closed conformation of the pore. Drug-resistance mutations are predicted to counter the effect of drug binding by either increasing the hydrophilicity of the pore or weakening helix – helix packing, thus facilitating channel opening.	Schnell and Chou 2008
Structure of M2 protein Chimeric M2 channel Drug-binding sites Drug resistance	The M2 channel of influenza A is a target of the adamantane family antiviral drugs. Two different drug-binding sites have been reported: one inside the pore, and the other is a lipid-facing pocket. A previous study showed that a chimera of M2 variants from influenza A and B that contains only the pore-binding site is sensitive to amantadine inhibition, suggesting that the primary site of inhibition is inside the pore. To obtain atomic details of channel-drug interaction, we determined the structures of the chimeric channel with and without rimantadine. Inside the channel and near the N-terminal end, methyl groups of Val27 and Ala30 from four subunits form a hydrophobic pocket around the adamantane, and the drug amino group appears to be in polar contact with the backbone oxygen of Ala30. The structures also reveal differences between the drug-bound and -unbound states of the channel that can explain drug resistance.	Pielak et al. 2011
Affinity study Amantadine/rimantadine binding to M2 Surface plasmon resonance Liposomes	The influenza A virus contains a proton-selective ion channel (M2) that is the target of the adamantane family of drug inhibitors. Two recently published studies relating to adamantane binding of the M2 ion channel using X-ray crystallography and solution NMR have re-ignited interest in the potential use of adamantanes in combating the spread of influenza A. However, these two studies propose different binding sites for the adamantane drugs with the X-ray M2/amantadine structure favoring an ion channel pore-binding model and the solution NMR M2/rimantadine structure suggesting the existence of a lipid-facing binding pocket. We conducted a series of surface plasmon resonance (SPR) experiments designed to accurately measure the affinity of amantadine and rimantadine to M2 ion channels embedded in 1,2-dimyristoyl-sn-glycero-phosphocholine (DMPC) liposomes. We find that this class of drug is capable of binding M2 with two different affinities in the order of 10^{-4} and 10^{-7} M, suggesting that both proposed binding sites are feasible. Furthermore, by examining drug binding to M2 mutant constructs (V27A, S31N, and D44A), it was possible to probe the location of the two binding sites. We show that a high-affinity binding site corresponds to the M2 ion channel pore whereas the secondary, low-affinity binding site can be attributed to the lipid face of the pore. These SPR results are in excellent agreement with the most recent solid-state NMR study of amantadine-bound M2 in lipid bilayers and provide independent support that the ion channel pore-binding site is responsible for the pharmacological activity elicited by the adamantane drugs.	Rosenberg and Casarotto 2010

M2 ion channel inhibition Rimantadine Molecular dynamics simulations	In order to understand how rimantadine (RMT) inhibits the proton conductance in the influenza A M2 channel via the recently proposed "allosteric mechanism", molecular dynamics simulations were applied to the M2-tetrameric protein with four RMTs bound outside the channel at the three protonation states: the 0H-closed, 1H-intermediate and 3H-open situations. In the 0H-closed state, a narrow channel with the RMT-Asp44-Trp41 H-bond network was formed, therefore the water penetration through the channel was completely blocked. The Trp41-Asp44 interaction was absent in the 1H-intermediate state, whilst the binding of RMT to Asp44 remained, which resulted in a weakened helix-helix packing, therefore the channel was partially prevented. In the 3H-open state it was found that the electrostatic repulsion from the three charged His37 residues allowed the Trp41 gate to open, permitting water to penetrate through the channel. This agreed well with the potential of the means force which is in the following order: 0H > 1H > 3H.	Intharathep et al. 2011
Discovery of amantadine Evaluation of virus inhibition	I-Adamantanamine (amantadine) causes a selective, reproducible, dose-related inhibition of influenza infections in tissue culture, chick embryos, and mice. The compound is not virucidal and appears to act by interfering with the penetration of the host cell by the virus. In influenza infections of mice, greatest efficacy occurs with treatment at the time of infection; however, there is significant antiviral activity with treatment delayed up to 72 hours after infection. Virus inhibition is not complete and survivors are immune to a challenge infection with the original infecting virus.	Davies et al. 1964
Adamantane derivatives 3-(2-adamantyl)pyrrolidines Synthesis and evaluation	The 3-(2-adamantyl)pyrrolidines 8a-g. 14 were synthesized and evaluated for activity against influenza A virus. The parent N-H compound 14 was several times more active than amantadine against H2N2 and H3N2 influenza A virus. The combined use of NMR spectroscopy and computational chemistry showed that the conformation around the pyrrolidine-adamantyl carbon-carbon bond is trans and the pyrrolidine heterocycle has an envelope conformation with C-2 out of the plane of the other ring atoms. N-Dialkylaminoethyl substitution of compound 14 resulted in the potent diamine analogues 8e,f,g. Interestingly, their lactam amine precursors were also active. Compounds 8e,f,g are the first adamantane derivatives, bearing two amine groups, reported to be active against influenza A virus.	Stamatiou et al. 2001
Adamantane derivatives Pyrrolidine Azetidines Aziridines	E2-(1-adamantyl)-2-methyl-pyrrolidines 3 and 4, 2-(1-adamatityl)-2-methyl-azetidines 5 and 6, and 2-(1-adamantyl)-2-methyl-aziridines 7 and 8 were synthesized and tested for their antiviral activity against influenza A. Parent molecules 3, 5, and 7 contain the alpha-methyl-1-adimantan-methanamine 2 pharmacophoric moiety (rimantadine). The ring size effect on anti-influenza A activity was investigated. Pyrrolidine 3 was the most potent anti-influenza virus A compound, 9-fold more potent than rimantadine 2, 27-fold more potent than amantadine 1, and 22-fold more potent than ribavirin. Azetidines 5 and 6 were both markedly active against influenza A H2N2 virus, 10- to 20-fold more potent than amantadine. Aziridine 7 was almost devoid of any activity against H2N2 virus but exhibited borderline activity against H3N2 influenza A strain. Thus, it appears that changing the five-, to four- to a three-membered ring results in a drop of activity against influenza A virus.	Zoidis et al. 2006
Adamantane derivatives Azolo-adamantanes Rimantadine-resistant strains	Chemotherapy and chemoprophylaxis of influenza is one of the most important directions of health protection activity. Due to the high rate of drug-resistant strains of influenza virus, there is a need for the search and further development of new potent antivirals against influenza with a broad spectrum of activity. In the present study, a set of di-, tri- and tetrazole derivatives of adamantane was efficiently prepared and their anti-influenza activities evaluated against rimantadine-resistant strain A/Puerto Rico/8/34. In general, derivatives of tetrazole possessed the highest virus-inhibiting activity. We demonstrated that several compounds of this set exhibited much higher activity than the currently used antiviral rimantadine, a compound of related structure. Moreover, we showed that these azolo- were significantly less toxic. This study demonstrates that influenza viruses can be inhibited by adamantylazoles and thus have potential for developing antiviral agents with an alternate mechanism of action.	Zarubaev et al. 2010

New M2 ion channel inhibitors	The A/M2 proton channel of influenza A virus is a target for the anti-influenza drugs amantadine and rimantadine, whose effectiveness was diminished by the appearance of naturally occurring point mutants in the A/M2 channel pore, among which the most common are S31N, V27A, and L26F. We have synthesized and characterized the properties of a series of compounds, originally derived from the A/M2 inhibitor BL-1743. A lead compound emerging from these investigations, spiro[5.5]undecan-3-amine, is an effective inhibitor of wild-type A/M2 channels and L26F and V27A mutant ion channels in vitro and also inhibits replication of recombinant mutant viruses bearing these mutations in plaque reduction assays. Differences in the inhibition kinetics between BL-1743, known to bind inside the A/M2 channel pore, and amantadine were exploited to demonstrate competition between these compounds, consistent with the conclusion that amantadine binds inside the channel pore. Inhibition by all of these compounds was shown to be voltage-independent, suggesting that their charged groups are within the N-terminal half of the pore, prior to the selectivity filter that defines the region over which the transmembrane potential occurs. These findings not only help to define the location and mechanism of binding of M2 channel-blocking drugs but also demonstrate the feasibility of discovering new inhibitors that target this binding site in a number of amantadine-resistant mutants.	Balannik et al. 2009
Synthetic strategies Microwave-assisted three-component one-pot cyclocondensation method Adamantyl moiety	A microwave-assisted three-component one-pot cyclocondensation method was applied for the synthesis of novel N-(1-thia-4-azaspiro[4.5] decan-4-yl)carboxamide compounds carrying an adamantyl moiety. The structures of the compounds were confirmed by spectral and elemental analysis. All compounds were evaluated for antiviral activity against influenza A (H1N1 and H3N2) and influenza B virus in MDCK cell cultures. The compounds displayed a confined structure-activity relationship. The N-(2,8-dimethyl-3-oxo-1-thia-4-azaspiro[4.5] dec-4-yl)adamantane-1-carboxamide 3b was the most potent inhibitor [antiviral EC50: 1.4 μM against influenza A/H3N2 virus]. Its strong inhibitory effect in a virus hemolysis assay supports that 3b acts as an influenza virus fusion inhibitor by preventing the conformational change of the influenza virus hemagglutinin at low pH.	Goktas et al. 2012
Encapsulation of adamantanes in liposomes	The aim of the present study was to encapsulate mannosylated 1-aminoadamantane and mannosylated adamantyltripeptides, namely [(2R)-N-(adamant-1-yl)-3-(alpha,beta-D-mannopyranosyloxy)-2-methylpropanamide and (2R)-N[3-(alpha-D-mannopyranosyloxy)-2-methylpropanoyl]-D,L-(adamant-2-yl)glycyl-L-alanyl-D-isoglutamine] in liposomes. The characterization of liposomes, size and surface morphology was performed using dynamic light scattering (DLS) and atomic force microscopy (AFM). The results have revealed that the encapsulation of examined compounds changes the size and surface of liposomes. After the concanavalin A (ConA) was added to the liposome preparation, increase in liposome size and their aggregation has been observed. The enlargement of Liposomes was ascribed to the specific binding of the ConA to the mannose present on the surface of the prepared liposomes. Thus, it has been shown that the adamantyl moiety from mannosylated 1-aminoadamantane and mannosylated adamantyltripeptides can be used as an anchor in the lipid bilayer for carbohydrate moiety exposed on the liposome surface.	Stimac et al. 2012

Effectiveness and safety Amantadine Rimantadine Children/elderly	Background The effectiveness and safety of amantadine (AMT) and rimantadine (RMT) for preventing and treating influenza A in adults has been systematically reviewed. However, little is known about these treatments in children and the elderly. Objectives To systematically review the effectiveness and safety of AMT and RMT in preventing and treating influenza A in children and the elderly. Search methods We searched the Cochrane Central Register of Controlled Trials (CENTRAL) (The Cochrane Library 2011, Issue 2) which contains the Cochrane Acute Respiratory Infections (ARI) Group's Specialised Register, MEDLINE (1966 to June week 3, 2011) and EMBASE (1980 to June 2011). Selection criteria Randomised controlled trials (RCTs) or quasi- RCTs comparing AMT and/ or RMT with placebo, control, other antivirals or different doses or schedules of AMT or RMT, or both, or no intervention, in children and the elderly. Data collection and analysis Two review authors independently selected trials for inclusion and assessed methodological quality. We resolved disagreements by consensus. In all comparisons except for one, we separately analysed the trials in children and the elderly using ReviewManager software. Main results A total of 12 studies involving 2494 participants (1586 children and adolescents and 908 elderly) compared AMT and RMT with placebo, paracetamol (one trial; 69 children) or zanamivir (two trials; 545 seniors). All studies were RCTs but most were still susceptible to bias. Two trials in the elderly had a high risk of bias because of incomplete outcome data. In one of those trials there was also a lack of outcome assessment blinding. Risk of bias was unclear in 10 studies due to unclear random sequence generation and allocation concealment. Only two trials in children were considered to have a low risk of bias. AMT was effective in preventing influenza A in children. A total of 773 participants were included in this outcome (risk ratio (RR) 0.11; 95% confidence interval (CI) 0.04 to 0.30). The assumed risk of influenza in the control group was 10 per 100 and the corresponding risk in the RMT group was one per 100 (95% CI 0 to 3). The quality of the evidence was considered low. For treatment purposes, RMT was beneficial for abating fever on day three of treatment. For this purpose one study was selected with low risk of bias and included 69 children (RR 0.36; 95% CI 0.14 to 0.91). The assumed risk was 38 per 100 and the corresponding risk in the RMT group was 14 per 100, 95% CI 5 to 34. The quality of the evidence was moderate. RMT did not show a prophylactic effect against influenza in the elderly, but the quality of evidence was considered very low. There were 103 participants (RR 0.45; 95% CI 0.14 to 1.41, for an assumed risk of 17 per 100 and a corresponding risk in the RMT group of 7 per 100, 95% CI 2 to 23). We did not identify any AMT trials in the elderly that met our inclusion criteria. There was no evidence of adverse effects of AMT and RMT in children or an adverse effect of RMT in the elderly. We did not identify any AMT trials in the elderly that met our inclusion criteria. Authors' conclusions AMT is effective in preventing influenza A in children but the NNTB is high (NNTB: 12 (95% CI 9 to 17). RMT probably helps the abatement of fever on day three of treatment, but the quality of the evidence is poor. Due to the small number of available studies, we could not reach a definitive conclusion on the safety of AMT or the effectiveness of RMT in preventing influenza in children and the elderly.	Galvao et al. 2012

Resistance to adamantanes 281 influenza A isolates Australia Europe Asia	The adamantanes (amantadine and rimantadine) were the initial antivirals licensed for use against influenza A viruses and have been used in some countries to control seasonal influenza and have also been stockpiled for potential pandemic use. While high rates of resistance have been observed in recent years with A(H3) viruses, the rates of resistance with A(H1) viruses has varied widely. In this study we analysed 281 human influenza A viruses isolated in 2007 that were referred to the WHO Collaborating Centre for Reference and Research in Melbourne, mainly from Australia and the surrounding regions, for evidence of resistance to adamantanes and a subset of these was examined for resistance to the neuraminidase inhibitors (NIs). We found that the rates of acdamantane resistance in A(H3) viruses continued to increase in most countries in 2007 but a distinct variation was seen with A(H1) resistance levels. A(H1) viruses from Australia, New Zealand and Europe had low rates of resistance (2–9%) whereas viruses from a number of South East (SE) Asian countries had high rates of resistance (33–100%). This difference can be attributed to the spread of A/Brisbane/59/2007-like viruses to many parts of the world with the exception of SE Asia where A/Hong Kong/2652/2006-like viruses continue to predominate. When these two A(H1) subgroups were compared for their in vitro sensitivity to the other class of influenza antiviral drugs, the neuraminidase inhibitors, no difference was seen between the groups with both showing normal levels of sensitivity to these drugs, The finding of reducing A(H1) resistance rates in Australia and rising levels in SE Asia in 2007, reverses the trend seen in 2006 when A(H1) resistance levels were rising in Australia and elsewhere but remained low in most of SE Asia.	Barr et al. 2008
Resistance to adamantanes Pyrosequencing Confirmatory sequence analysis Phenotypic testing	Background adamantanes have been used to treat influenza A virus infections for many years. Studies have shown a low incidence of resistance to these drugs among circulating influenza viruses; however, their use is rising worldwide and drug resistance has been reported among influenza A (H5N1) viruses isolated from poultry and human beings in Asia. We sought to assess adamantane resistance among influenza A viruses isolated during the past decade from countries participating in WHO's global influenza surveillance network. Methods we analysed data for influenza field isolates that were obtained worldwide and submitted to the WHO Collaborating Center for Influenza at the US Centers for Disease Control and Prevention between Oct 1, 1994, and Mar 31, 2005. We used pyrosequencing, confirmatory sequence analysis, and phenotypic testing to detect drug resistance among circulating influenza A H3N2 (n = 6524), H1N1 (n = 589), and H1N2 (n = 83) viruses. Findings More than 7000 influenza A field isolates were screened for specific aminoacid substitutions in the M2 gene known to confer drug resistance. During the decade of surveillance a significant increase in drug resistance was noted, from 0.4% in 1994-1995 to 12.3% in 2003–2004. This increase in the proportion of resistant viruses was weighted heavily by those obtained from Asia with 61% of resistant viruses isolated since 2003 being from people in Asia. Interpretation our data raise concerns about the appropriate use of adamantanes and draw attention to the importance of tracking the emergence and spread of drug-resistant influenza A viruses.	Bright et al. 2005

Resistance to adamantanes USA S31N substitution Influenza A and B	This report describes new findings regarding the resistance to adamantanes of influenza A viruses currently circulating in the United States and provides interim recommendations that these drugs not be used during the remainder of the 2005–2006 influenza season. Amantadine also is used to treat symptoms of Parkinson disease and may continue to be used for this indication. Resistance of influenza A viruses to adamantanes can occur spontaneously or emerge rapidly during treatment. A single point mutation in the codons for amino acids at positions 26, 27, 30, 31, or 34 of the M2 protein can confer cross-resistance to both amantadine and rimantadine. Neither replication, transmission, nor virulence of adamantane-resistant influenza A viruses are impaired by the point mutations conferring resistance. A recent report on the global prevalence of adamantane-resistant influenza A viruses indicated a significant increase of drug resistance, from 1.8% during the 2001–2002 influenza season to 12.3% during the 2003–2004 season. In the United States, the frequency of adamantane resistance increased from 1.9% during the 2003–2004 influenza season to 11% during the 2004-05 season (CDC, unpublished data, 2005). In contrast to adamantane resistance, neuraminidase inhibitor resistance remains rare worldwide. Since the beginning of the 2005–2006 influenza surveillance season, WHO and NREVSS laboratories have tested a total of 38,932 specimens for influenza viruses; 1557 (4.0%) tested positive. Among the 1557 influenza viruses, 1499 (96.3%) were influenza A viruses, and 58 (3.7%) were influenza B viruses. A total of 765 (51.0%) of the 1499 influenza A viruses have been subtyped; 760 (99.3%) were influenza A (H3N2) viruses, and five (0.7%) were influenza A (H1N1) viruses. During October 1, 2005-January 14, 2006, a total of 123 influenza A viruses collected from 23 states were tested at CDC for adamantane resistance. Among the 120 influenza A (H3N2) viruses tested, 109 (91%) demonstrated the S31N substitution in the M2 protein that confers resistance to amantadine and rimantadine. Conventional sequencing on a subset of 20 viruses confirmed this substitution. Among the three influenza A (H1N1) viruses tested, none contained any mutations associated with resistance. As of January 14, all U.S. influenza viruses screened for antiviral resistance at CDC had demonstrated susceptibility to neuraminidase inhibitors. Procedures for virus propagation, RNA extraction, and pyrosequencing for adamantane resistance have been described previously.	Bright et al. 2006

Table 4.3. Inhibitors of viral RNA polymerase

Polymerase basic protein 2 Cap-binding domain Cap analogs	Influenza viruses cause a significant level of morbidity and mortality in the population every year. Their resistance to current anti-influenza drugs increases the difficulty of flu treatment. Thus, development of new anti-influenza drugs is necessary in regards of prevent the tragedy of influenza pandemic. The Polymerase basic protein 2 (PB2) subunit of influenza virus RNA polymerase is one of potential targets for new drugs because the binding of PB2 with the 5' cap of the host pre-mRNAs is the initial step of the virus' protein synthesis. In this study, we compared the binding potency of PB2 cap binding domain with two small molecules, i.e., RO and PPT28, with that of PB2 with cap analog m7GTP. The calculated binding energies showed that RO and PPT28 had higher binding affinity with PB2. Further interaction analysis showed that the important parts for binding were the five-and six-member heterocyclic rings (the 6/5-member rings) of small molecules, as well as the hydrophobic parts of RO and PPT28 which had good interactions with the hydrophobic residues in the binding cavity. Thus, RO and PPT28 are two potential anti-influenza drugs targeted PB2, which may inhibit the growth of influenza virus by competitively binding with the cap structure binding domain of PB2.	Lv et al. 2011

Polymerase/ endonuclease Cap-binding Host adaptation mechanisms	The heterotrimeric RNA-dependent RNA polymerase of influenza viruses catalyzes RNA replication and transcription activities in infected cell nuclei. The nucleotide polymerization activity is common to both replication and transcription processes, with an additional cap-snatching function being employed during transcription to steal short 5'-capped RNA primers from host mRNAs. Cap-binding, endonuclease, and polymerase activities have long been studied biochemically, but structural studies on the polymerase and its subunits have been hindered by difficulties in producing sufficient quantities of material. Recently, because of heightened effort and advances in expression and crystallization technologies, a series of high resolution structures of individual domains have been determined. These shed light on intrinsic activities of the polymerase, including cap snatching, subunit association, and nucleocytoplasmic transport, and open up the possibility of structure-guided development of new polymerase inhibitors. Furthermore, the activity of influenza polymerase is highly host- and cell type-specific, being dependent on the identity of a few key amino acid positions in the different subunits, especially in the C-terminal region of PB2. New structures demonstrate the surface exposure of these residues, consistent with ideas that they might modulate interactions with host- specific factors that enhance or restrict activity. Recent proteomic and genome-wide interactome and RNA interference screens have suggested the identities of some of these potential regulators of polymerase function.	Boivin et al. 2010
Adaptive evolution Host adaptation mechanisms	Adaptive evolution is characterized by positive and parallel, or repeated selection of mutations. Mouse adaptation of influenza A virus (IAV) produces virulent mutants that demonstrate positive and parallel evolution of mutations in the hemagglutinin (HA) receptor and non-structural protein 1 (NS1) interferon antagonist genes. We now present a genomic analysis of all 11 genes of 39 mouse adapted IAV variants from 10 replicate adaptation experiments. Mutations were mapped on the primary and structural maps of each protein and specific mutations were validated with respect to virulence, replication, and RNA polymerase activity. Mouse adapted (MA) variants obtained after 12 or 20–21 serial infections acquired on average 5.8 and 7.9 nonsynonymous mutations per genome of 11 genes, respectively. Among a total of 115 nonsynonymous mutations, 51 demonstrated properties of natural selection including 27 parallel mutations. The greatest degree of parallel evolution occurred in the HA receptor and ribonucleocapsid components, polymerase subunits (PB1, PB2, PA) and NP. Mutations occurred in host nuclear trafficking factor binding sites as well as sites of virus-virus protein subunit interaction for NP, NS1, HA and NA proteins. Adaptive regions included cap binding and endonuclease domains in the PB2 and PA polymerase subunits. Four mutations in NS1 resulted in loss of binding to the host cleavage and polyadenylation specificity factor (CPSF30) suggesting that a reduction in inhibition of host gene expression was being selected. The most prevalent mutations in PB2 and NP were shown to increase virulence but differed in their ability to enhance replication and demonstrated epistatic effects. Several positively selected RNA polymerase mutations demonstrated increased virulence associated with >300% enhanced polymerase activity. Adaptive mutations that control host range and virulence were identified by their repeated selection to comprise a defined model for studying IAV evolution to increased virulence in the mouse.	Ping et al. 2011

Polymerase PB2 subunit Temperature-dependent polymerase activity	Most avian influenza A viruses, which preferentially replicate at the high temperatures found in the digestive tract of birds, have a glutamic acid at residue 627 of the viral RNA polymerase PB2 subunit (Glu-627), whereas the human viruses, which optimally replicate at the low temperatures observed in the human respiratory tract, have a lysine (Lys-627). The mechanism of action for this mutation is still not understood, although interaction with host factors has been proposed to play a major role. In this study, we explored an alternative, yet related, hypothesis that this PB2 mutation may alter the temperature-dependent enzymatic polymerase activity of the viral polymerase. First, the avian polymerase protein, which was purified from baculovirus expression system, indeed remained significantly active at higher temperatures (i.e. 37 and 42 °C), whereas the human E627K mutant drastically lost activity at these high temperatures. Second, our steady-state kinetics data revealed that the human E627K mutant polymerase is catalytically more active than the avian Glu-627 polymerase at 34 °C. Importantly, the E627K mutation elevates apparent K(cat) at low temperatures with little effect on K_m, suggesting that the E627K mutation alters the biochemical steps involved in enzyme catalysis rather than the interaction with the incoming NTP. Third, this temperature-dependent kinetic impact of the human E627K mutation was also observed with different RNA templates, with different primers and also in the presence of nucleoprotein. In conclusion, our study suggests that the amino acid sequence variations at residue 627 of PB2 subunit can directly alter the enzyme kinetics of influenza polymerase.	Aggarwal et al. 2011
Polymerase PB2 subunit Human-to-human transmission	Background: The identification of mutations that confer unique properties to a pathogen, such as host range, is of fundamental importance in the fight against disease. This paper describes a novel method for identifying amino acid sites that distinguish specific sets of protein sequences, by comparative analysis of matched alignments. The use of mutual information to identify distinctive residues responsible for functional variants makes this approach highly suitable for analyzing large sets of sequences. To support mutual information analysis, we developed the AVANA software, which utilizes sequence annotations to select sets for comparison, according to user-specified criteria. The method presented was applied to an analysis of influenza A PB2 protein sequences, with the objective of identifying the components of adaptation to human-to-human transmission, and reconstructing the mutation history of these components. Results: We compared over 3,000 PB2 protein sequences of human-transmissible and avian isolates, to produce a catalogue of sites involved in adaptation to human-to-human transmission. This analysis identified 17 characteristic sites, five of which have been present in human-transmissible strains since the 1918 Spanish flu pandemic. Sixteen of these sites are located in functional domains, suggesting they may play functional roles in host-range specificity. The catalogue of characteristic sites was used to derive sequence signatures from historical isolates. These signatures, arranged in chronological order, reveal an evolutionary timeline for the adaptation of the PB2 protein to human hosts. Conclusion: By providing the most complete elucidation to date of the functional components participating in PB2 protein adaptation to humans, this study demonstrates that mutual information is a powerful tool for comparative characterization of sequence sets. In addition to confirming previously reported findings, several novel characteristic sites within PB2 are reported. Sequence signatures generated using the characteristic sites catalogue characterize concisely the adaptation characteristics of individual isolates. Evolutionary timelines derived from signatures of early human influenza isolates suggest that characteristic variants emerged rapidly, and remained remarkably stable through subsequent pandemics. In addition, the signatures of human-infecting H5N1 isolates suggest that this avian subtype has low pandemic potential at present, although it presents more human adaptation components than most avian subtypes	Miotto et al. 2008

Inhibitor of polymerase PA(C) subunit Chlorogenic acid	The avian influenza (H5N1) viral RNA polymerase protein PA(C) was used as a target to screen nine chlorogenic acid derivatives for their polymerase inhibitor activity. Among them, seven compounds were PAC ligands, and four inhibited influenza RNA polymerase activity. These results aid in the design of anti-influenza agents based on caffeoylquinic acid.	Li et al. 2012b
Inhibitor of polymerase PA(C) subunit Licorice-derived compounds	PA(C) subunit from avian influenza (H5N1) viral RNA polymerase was used in this work as a target in the screening for anti-influenza agents from licorice-derived compounds. As a result, 18 beta-glycyrrhetinic acid was suggested to be PA(C) ligand by flexible docking, and was then confirmed by relaxation-edited NMR. The result of ApG primer extension assay indicated that this PA(C) ligand can inhibit the polymerase activity, and thus may potentially be valuable as anti-influenza lead compound. This work validated the possibility of screening polymerase inhibitors by using PAc as a target, and provided a starting point for the further discovery of new anti-influenza drugs.	Li et al. 2012a
Nucleoside analogue T-705 Antiviral activity evaluation	T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) has been found to have potent and selective inhibitory activity against influenza virus. In an in vitro plaque reduction assay, T-705 showed potent inhibitory activity against influenza A, B, and C viruses, with 50% inhibitory concentrations (IC(50)s) of 0.013 to 0.48 μg/ml, while it showed no cytotoxicity at concentrations up to 1000 μg/ml in Madin-Darby canine kidney cells. The selectivity index for influenza virus was more than 2000. It was also active against a neuraminidase inhibitor-resistant virus and some amantadine-resistant viruses. T-705 showed weak activity against non-influenza virus RNA viruses, with the IC(50)s being higher for non-influenza virus RNA viruses than for influenza virus, and it had no activity against DNA viruses. Orally administered T-705 at 100 mg/kg of body weight/day (four times a day) for 5 days significantly reduced the mean pulmonary virus yields and the rate of mortality in mice infected with influenza virus A/PR/8/34 (3×10^2 PFU). These results suggest that T-705 may be a compound that is useful and highly selective against influenza virus infections and that has a mode of action different from those of commercially available drugs, such as amantadine, rimantadine, and neuraminidase inhibitors.	Furuta et al. 2002
Nucleoside analogue T-705 Dose-dependent effect GTP-competition	T-705, a substituted pyrazine compound, has been found to exhibit potent anti-influenza virus activity in vitro and in vivo. In a time-of-addition study, it was indicated that T-705 targeted an early to middle stage of the viral replication cycle but had no effect on the adsorption or release stage. The anti-influenza virus activity of T-705 was attenuated by addition of purines and purine nucleosides, including adenosine, guanosine, inosine, and hypoxanthine, whereas pyrimidines did not affect its activity. T-705-4-ribofuranosyl-5'-triphosphate (T-705RTP) and T-705-4-ribofuranosyl-5'-monophosphate (T-705RMP) were detected in MDCK cells treated with T-705. T-705RTP inhibited influenza virus RNA polymerase activity in a dose-dependent and a GTP-competitive manner. Unlike ribavirin, T-705 did not have an influence on cellular DNA or RNA synthesis. Inhibition of cellular IMP dehydrogenase by T-705RMP was about 150-fold weaker than that by ribavirin monophosphate, indicating the specificity of the anti-influenza virus activity and lower level of cytotoxicity of T-705. These results suggest that T-705RTP, which is generated in infected cells, may function as a specific inhibitor of influenza virus RNA polymerase and contributes to the selective anti-influenza virus activity of T-705.	Furuta et al. 2005

Nucleoside analogues T-705 Peramivir Antiviral activity evaluation Mouse model	Favipiravir (T-705), an influenza virus RNA polymerase inhibitor, and peramivir, an influenza virus neuraminidase inhibitor, were evaluated alone and in combination against pandemic influenza A/California/04/2009 (H1N1) virus infections in mice. Infected mice were treated twice daily for 5 days starting 4 h after virus challenge. Favipiravir was 40%, 70%, and 100% protective at 20, 40, and 100 mg/kg/day. Peramivir was 30% protective at 0.5 mg/kg/day, but ineffective at lower doses when used as monotherapy. Combinations of favipiravir and peramivir increased the numbers of survivors by 10–50% when the 0.025, 0.05, and 0.1 mg/kg/day doses of peramivir were combined with 20 mg/kg/day favipiravir and when all doses of peramivir were combined with 40 mg/kg/day favipiravir. Three-dimensional analysis of drug interactions using the MacSynergy method indicates strong synergy for these drug combinations. In addition, an increase in lifespan for groups of mice treated with drug combinations, compared to the most effective monotherapy group, was observed for the 0.025, 0.05, and 0.1 mg/kg/day doses of peramivir combined with favipiravir at the 20 mg dose level. Therefore, the 20 mg/kg/d dose of favipiravir was selected for further combination studies. Increased survival was exhibited when this dose was combined with peramivir doses of 0.1, 0.25 and 0.5 mg/kg/day (1 mg/kg/day of peramivir alone was 100% protective in this experiment). Improved body weight relative to either compound alone was evident using 0.25, 0.5, and 1 mg/kg/day of peramivir. Significant reductions in lung hemorrhage score and lung weight were evident on day 6 post-infection. In addition, virus titers were reduced significantly on day 4 post-infection by combination therapy containing favipiravir combined with peramivir at 0.25 and 0.5 mg/kg/day. These data demonstrate that combinations of favipiravir and peramivir perform better than suboptimal doses of each compound alone for the treatment of influenza virus infections in mice.	Tarbet et al. 2012
Nucleoside analogues Peramivir Phase I of clinical research	Peramivir is a novel influenza neuraminidase inhibitor. In this article, hydrophilic interaction chromatography coupled with tandem mass spectrometry was developed to determine peramivir in human plasma. The positive ion MRM mode was performed and the precursor to the product ion transitions of m/z 329 ≥ 100 and 285 ≥ 138 were used to measure peramivir and Ro 64-0802 (I.S.). Chromatographic separation was performed on an Amide-80 column with acetonitrile-water-formic acid (70:30:0.1, v/v/v, 0.5 ml/min). The method was linear over a concentration range of 10–10 000 ng/ml. The average inter-day/intra-day precision values were 3.7 ± 1.8% and 4.3 ± 1.8%, respectively, while the accuracy values were 97.0 ± 4.8%. This method has been Successfully applied to Phase I of clinical research of peramivir.	Li et al. 2009
Nucleoside analogues Viramidine Ribavirin Antiviral activity evaluation Mouse model	Viramidine, the 3-carboxamidine derivative of ribavirin, was effective against a spectrum of influenza A (H1N1, H3N2 and H5N1) and B viruses in vitro, with the 50% effective concentration (EC50) ranging from 2 to 32 μg/ml. The mean 50% cytotoxic concentration (CC50) in the MDCK cells used in these experiments was 760 μg/ml. Ribavirin, run in parallel, had a similar antiviral spectrum, with EC50 values ranging from 0.6 to 5.5 μg/ml; the mean CC50 for ribavirin was 560 μg/ml. Oral gavage administrations of viramidine or ribavirin to mice infected with influenza A/NWS/33 (H1N1), A/Victoria/3/75 (H3N2), B[Hong Kong/5/72 or B/Sichuan/379/99 viruses were highly effective in preventing death, lessening decline in arterial oxygen saturation, inhibition Of lung consolidation and reducing lung virus titers. The minimum effective dose of viramidine in these studies ranged from 15 to 31 mg/kg/day, depending upon the virus infection, when administered twice daily for 5 days beginning 4 h pre-virus exposure. The LD50 of the compound was 610 mg/kg/day. Ribavirin's minimum effective dose varied between 18 and 37.5 mg/kg/day with the LD50 determined to be 220 mg/kg/day. Viramidine's efficacy was also seen against an influenza A/NWS/33 (H IN I) virus infection in mice, when the compound was administered in the drinking water, the minimum effective dose being 50 dose of each, was protective 100 mg/kg/day. Delay of the initiation of either viramidine or ribavirin therapy, using the approximate 1/3 LD50 dose of each, was protective as late as 48 h after exposure to the A/NWS/33 virus. While both compounds appear to have similar efficacy against influenza virus infections, when one considers the lesser toxicity, viramidine may wan-ant further evaluation as a possible therapy for influenza.	Sidwell et al. 2005

Nucleoside analogue 2'-deoxy-2'-fluoroguanosine Antiviral activity evaluation Chicken embryo model	The nucleoside analog 2'-deoxy-2'-fluoroguanosine (2'-fluorodGuo) is phosphorylated by cellular enzymes and reversibly inhibits influenza virus replication in chick embryo cells within the first 4 h of infection, RNA hybridization studies revealed that primary and secondary transcription of influenza virus RNA were blocked at a compound concentration of 10 μM, but no inhibition of cell protein synthesis was seen even at high compound concentrations (200 μM). In vitro, the triphosphate of 2'-fluorodGuo is a competitive inhibitor of influenza virus transcriptase activity from disrupted virus, with a K-i of 1.0 μM. The cellular polymerases DNA polymerase alpha and RNA polymerase II were only weakly inhibited or were insusceptible to 2'-fluorodGTP. In kinetic studies with the influenza virus transcriptase, 2'-fluorodGTP, in the absence of GTP, blocked elongation of the virus RNA chain. Similarly, by using purified ribonucleoprotein complexes it was found that the addition of a single nucleotide of 2'-fluorodGTP to the virus RNA caused chain termination, which resulted in the blockage of further virus transcription, Furthermore, the specificity for influenza virus transcriptase was confirmed when the transcriptase from partially resistant virus was found to be 10-fold less susceptible to 2'-fluorodGTP (K-i = 13.1 μM).	Tisdale et al. 1995
Endonuclease inhibitors 33 different types of phytochemicals Marchantins Plagiochin A Perrottetin F Docking simulation	Influenza A possesses an endonuclease within its RNA polymerase which comprises PA, PB1 and PB2 subunits. To identify potential new anti-influenza compounds in our current study, we screened 33 different types of phytochemicals using a PA endonuclease inhibition assay in vitro and an anti-influenza A virus assay. The marchantins are macrocyclic bisbibenzyls found in liverworts, and plagiochin A and perrottetin F are marchantin-related phytochemicals. We found from our screen that marchantin A, B, E, plagiochin A and perrottetin F inhibit influenza PA endonuclease activity in vitro. These compounds have a 3,4-dihydroxyphenethyl group in common, indicating the importance of this moiety for the inhibition of PA endonuclease. Docking simulations of marchantin E with PA endonuclease suggest a putative "fitting and chelating model" as the mechanism underlying PA endonuclease inhibition. The docking amino acids are well conserved between influenza A and B. In a cultured cell system, marchantin E was further found to inhibit the growth of both H3N2 and H1N1 influenza A viruses, and marchantin A, E and perrotein F showed inhibitory properties towards the growth of influenza B. These marchantins also decreased the viral infectivity titer, with marchantin E showing the strongest activity in this assay. We additionally identified a chemical group that is conserved among different anti-influenza chemicals including marchantins, green tea catechins and dihydroxy phenethylphenylphthalimides. Our present results indicate that marchantins are candidate anti-influenza drugs and demonstrate the utility of the PA endonuclease assay in the screening of phytochemicals for anti-influenza characteristics.	Iwai et al. 2011
Endonuclease and cap-binding inhibitors Co-crystal structures Cap-snatching mechanism Diketo compounds Green tea catechin	The viral polymerase, which performs transcription and replication of the RNA genome, is an attractive target for antiviral drugs since potent polymerase inhibitors could directly stop viral replication at an early stage. Recent structural studies on functional domains of the heterotrimeric polymerase, which comprises subunits PA, PB1 and PB2, open the way to a structure based approach to optimise inhibitors of viral replication. In particular, the unique cap-snatching mechanism of viral transcription can be inhibited by targeting either the PB2 cap-binding or PA endonuclease domains. Here we describe high resolution X-ray co-crystal structures of the 2009 pandemic H1N1 (pH1N1) PA endonuclease domain with a series of specific inhibitors, including four diketo compounds and a green tea catechin, all of which chelate the two critical manganese ions in the active site of the enzyme. Comparison of the binding mode of the different compounds and that of a mononucleotide phosphate highlights, firstly, how different substituent groups on the basic metal binding scaffold can be orientated to bind in distinct sub-pockets within the active site cavity, and secondly, the plasticity of certain structural elements of the active site cavity, which result in induced fit binding. These results will be important in optimising the design of more potent inhibitors targeting the cap-snatching endonuclease activity of influenza virus polymerase.	Kowalinski et al. 2012

Transcriptase and endonuclease inhibitors Flutimide 3-substituted 2,4-dioxobutanoic acids	A novel anti-influenza virus compound, flutimide, was identified in extracts of a recently identified fungal species, Delitschia confertaspora. The compound, a substituted 2,6-diketopiperazine, selectively inhibited the cap-dependent transcriptase of influenza A and B viruses and had no effect on the activities of other polymerases. Similar to the 3-substituted 2,4-dioxobutanoic acids, a series of transcriptase inhibitors which we described previously, this inhibitor, which is a natural product, affected neither the initiation nor the elongation of influenza virus mRNA synthesis, but it specifically targeted the cap-dependent endonuclease of the transcriptase. Additionally, the compound was inhibitory to the replication of influenza A and B viruses in cell culture. The selective antiviral properties of this compound further demonstrate the utility of influenza virus endonuclease as a target of antiviral agents.	Tomassini et al. 1996
Phosphorothioate oligonucleotides	In a previous study a 15-mer phosphorothioate oligonucleotide (S-ON) derived from the packaging signal in the 5' end of segment 1 (PB2) of influenza A virus (designated 5-15b) proved markedly inhibitory to virus replication. Here we investigated whether analogous inhibitory S-ONs targeting the 5' end of segments 2 (PB1) and 3 (PA) could be identified and whether viral resistance to S-ONs can be developed. Similar to our earlier result, 20-mer S-ONs reproducing the 5' ends of segments 2 or 3 (complementary to the 3'-coding regions of PB1 and PA, respectively) exerted a powerful antiviral activity against a variety of influenza A virus subtypes in MOCK cells. Serial passage of the A/Taiwan/1/86 H1N1 strain in the presence of S-ON 5-15b or its antisense as5-15b analogue showed that mutant viruses with reduced susceptibility to the S-ON could indeed be generated, although the resistant viruses displayed reduced replicative fitness. Sequencing the resistant viruses identified mutations in the PB1, PB2, PA and M1 genes. Introduction of these changes into the A/PR/8/34 H1N1 strain by reverse genetics, suggested that alterations to RNA function in the packaging regions of segments 2 and 3 were important in developing resistance to S-ON inhibition. However, many of the other sequence changes induced by S-ON treatment were markedly deleterious to virus fitness. We conclude that packaging signals in the influenza A virus polymerase segments provide feasible targets for nucleic acid-based antivirals that may be difficult for the virus to evade through resistance mutations.	Giannecchini et al. 2011
5'-capped short phosphorothioate RNA fragments Cap decoy Liposome encapsulation	We have shown previously that the 5'-capped short phosphodiester RNA fragments, Cap decoy, (Gm 12 nt) are potent inhibitors of influenza virus RNA polymerase gene expression. Here we investigate the modified capped RNA derivative containing phosphorothioate oligonucleotides (Cap decoy) as a potential influenza virus RNA polymerase inhibitor. The modified 5'-capped short phosphorothioate RNA fragments (Gms 12–15 nt) with the 5'-capped structure (m7GpppGm) were synthesized by T7 RNA polymerase. The 5'-capped short RNA fragments (Gms 12–15 nt) were encapsulated in liposome particulates and tested for their inhibitory effects on influenza virus RNA polymerase gene expression in the clone 76 cells. The 12–15 nt long Gms RNA fragments showed highly inhibitory effects. By contrast, the inhibitory effects of the 13 nt long short RNA fragments (Gm 13 nt) were considerably less in comparison with the 5'-capped short phosphorothioate RNA fragments (Gms 12–15 nt). In particular, the various Gms RNA chain lengths showed no significant differences in the inhibition of influenza virus RNA polymerase gene expression. Furthermore, the capped RNA with a phosphorothioate backbone was resistant to nuclease activity. These phosphorothioate RNA fragments exhibited higher inhibitory activity than the 5'-capped short RNA fragments (Gm 12 nt). These decoys may prove to be useful in anti-influenza virus therapeutics.	Tado et al. 2001

Short capped oligonucleotides	The RNA-dependent RNA polymerase of influenza virus transcribes messenger RNA through a unique cap scavenging mechanism. Viral enzyme binds to the cap structure of host mRNA, cleaves the molecule 9-15 bases downstream of the cap, and uses the short capped oligonucleotide as a primer for mRNA synthesis. Previously, we have shown that the viral polymerase can efficiently bind capped RNAs shorter than 9 nucleotides in length, but the viral enzyme cannot utilize these RNAs as primers. For this reason, these short capped oligonucleotides are potent inhibitors of influenza virus transcription. In these studies, it is now shown that short capped oligomers inhibit capped-RNA dependent transcription at the initial step of cap binding. In contrast, low concentrations of these short capped RNAs can actually stimulate viral transcription primed with high concentrations of the dinucleotide ApG. Another capped RNA derivative containing phosphorothioate oligonucleotides was also investigated as a potential polymerase inhibitor. This longer capped RNA was able to bind to the polymerase, but could not be cleaved to primer length by the enzyme associated endonuclease. Thus, the capped phosphorothioate RNA inhibited cap-primed transcription at the step of cap binding. However, in contrast to the short capped oligonucleotide, it also inhibited ApG primed viral transcription.	Cianci et al. 1997
Endonuclease inhibitors 4-substituted 2,4-dioxobutanoic acids	We previously identified a series of compounds which specifically inhibited the transcription of influenza A and B viruses. The compounds, 4-substituted 2,4-dioxobutanoic acids, selectively targeted the cap-dependent endonuclease activity of the transcriptase complex. Additionally, several of these compounds effectively inhibited the replication of influenza virus but not other viruses in cell culture assays. Here, we report on the anti-influenza virus activities of other potent derivatives of the series evaluated in both in vitro and in vivo infectivity assays. These compounds inhibited the replication of influenza virus in yield reduction assays, with 50% inhibitory concentrations ranging from 0.18 to 0.71 μM. These 50% inhibitory concentrations were similar to those observed for inhibition of in vitro transcription (0.32 to 0.54 μM). One selected compound also elicited a dose-dependent inhibition of influenza virus replication in mice following an upper respiratory tract challenge. These studies demonstrate the antiviral efficacy of this inhibitor class and thereby establish the utility of influenza virus endonuclease as a chemotherapeutic target.	Hastings et al. 1996
Replication/ transcription inhibitors Computational and experimental screening Compound library	In this study, computational and experimental screening of an extensive compound library identified THC19, which was able to suppress influenza virus replication. This compound had no cytotoxic effects and did not disrupt cell cycle progression or induce apoptosis in MDCK cells as confirmed by WST-1 assays, flow cytometry analysis, and caspase-3 assays. Time-of-addition experiments showed that THC19 acts at a relatively early stage of the viral lifecycle. Subsequent mini-genome assays revealed that THC19 inhibited viral genome replication and/or transcription, suggesting that it interferes with one or more of the viral components that form the ribonucleoprotein complexes, namely polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), nucleoprotein (NP) and viral RNA. Finally, mini-genome assays where PB2, PB1, PA or NP from A/WSN/33 (H1N1) virus were replaced with those from A/Udorn/307/1972 (H3N2) virus effectively demonstrated that THC19 inhibited viral multiplication in a manner dependent upon the PA subunit. Taken together, these results suggest that influenza virus PA protein is a potential target for, and may aid the development of, novel compounds that inhibit influenza A virus replication.	Yamada et al. 2012

Short interfering RNA	RNA polymerase of influenza virus is a specific enzyme necessary for the viral replication. A siRNA against the RNA polymerase and the RNA polymerase inhibitor L-742,001 reduced accumulation of viral RNAs in the infected cells. L-742,001 strongly inhibited virus re-growth after removal of the agent from the culture, whereas the neuraminidase inhibitor zanamivir did not. L-742,001-resistant mutants showed a Thr-20 to Ala substitution in the PA subunit of RNA polymerase. The drug-resistant virus showed a slight reduction in the susceptibility to L-742,001 in both the plaque assay (threefold reduction) and enzyme assay (two- to three-fold reduction). The resistance levels were lower than those of zanamivir-resistant mutants in the plaque assay. Against zanamivir-resistant mutants, L-742,001 retained the same antiviral activity as against the wild-type strain. These results indicate that L-742,001 is most likely to act at the PA subunit, and possesses a unique profile. It is suggested that PA subunit of RNA polymerase is a promising target for anti-influenza virus agents.	Nakazawa et al. 2008

Table 4.4. Inhibitors of neuraminidase

Neuraminidase Release and spread of progeny virions Virus entry into cell	Influenza virus neuraminidase (NA) plays an essential role in release and spread of progeny virions, following the intracellular viral replication cycle. To test whether NA could also facilitate virus entry into cell, we infected cultures of human airway epithelium with human and avian influenza viruses in the presence of the NA inhibitor oseltamivir carboxylate. Twenty- to 500-fold less cells became infected in drug-treated versus nontreated cultures ($P < 0.0001$) 7 h after virus application, indicating that the drug suppressed the initiation of infection. These data demonstrate that viral NA plays a role early in infection, and they provide further rationale for the prophylactic use of NA inhibitors.	Matrosovich et al. 2004
Neuraminidase Enzymatic activity Endocytic pathways Virus replication	N2 neuraminidase (NA) genes of the 1957 and 1968 pandemic influenza virus strains possessed avian-like low-pH stability of sialidase activity, unlike most epidemic strains. We generated four reverse-genetics viruses from a genetic background of A/WSN/33 (H1N1) that included parental N2 NAs of 1968 pandemic (H3N2) and epidemic (H2N2) strains or their counterpart N2 NAs in which the low-pH stability of the sialidase activity was changed by substitutions of one or two amino acid residues. We found that the transfectant viruses bearing low-pH-stable sialidase (WSN/Stable-NAs) showed 25- to 80-times-greater ability to replicate in Madin-Darby canine kidney (MDCK) cells than did the transfectant viruses bearing low-pH-unstable sialidase (WSN/ Unstable-NAs). Enzymatic activities of WSN/Stable-NAs were detected in endosomes of MDCK cells after 90 min of virus internalization by in situ fluorescent detection with 5-bromo-4-chloro-indole-3-yl-ct-N-acetyineuraminic acid and Fast Red Violet LB. Inhibition of sialidase activity of WSN/Stable-NAs on the endocytic pathway by pretreatment with 4-guanidino-2,4-dideoxy-N-acetylneuraminic acid (zanamivir) resulted in a significant decrease in progeny viruses. In contrast, the enzymatic activities of WSN/Unstable-NAs, the replication of which had no effect on pretreatment with zanamivir, were undetectable in cells under the same conditions. Hemadsorption assays of transfectant-virus-infected cells revealed that the low-pH stability of the sialidase had no effect on the process of removal of sialic acid from hemagglutinin in the Golgi regions. Moreover, high titers of viruses were recovered from the lungs of mice infected with WSN/Stable-NAs on day 3 after intranasal inoculation, but WSN/Unstable-NAs were cleared from the lungs of the mice. These results indicate that sialidase activity in late endosome/lysosome traffic enhances influenza A virus replication.	Suzuki et al. 2005

FANA Antiviral activity evaluation	Susceptibility to the neuraminidase-inhibiting effects of a synthetic analog of neuraminic acid, 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA) was found to vary among different strains of influenza A virus according to the neuraminidase they contain. In particular, NWS (H0N1) virus and recombinant strains which derive their neuraminidase from NWS are especially susceptible to FANA as measured by the concentrations of inhibitor which reduce rates of elution from red cells, in vitro neuraminidase activity using fetuin as a substrate, and virus replication in cell culture under agar. Growth in tissue culture of X-7 (H0N2) virus and all recombinant viruses containing N2 neuraminidase is approximately 50–200 times less susceptible to inhibition by FANA.These results provide further evidence that the inhibitory effects of FANA on the replication of influenza viruses are mediated by its specific neuraminidase-inhibitory activity and confirm that neuraminidase activity is necessary for the replication of influenza viruses.	Schulman and Palese 1975
Zanamivir **DANA** **4-amino-DANA activity** Neuraminidase acitivity Receptor binding Cell fusion	4-GU-DANA (zanamivir) (as well as DANA and 4-AM-DANA) was found to inhibit the neuraminidase activity of human parainfluenza virus type 3 (HPF3). The viral neuraminidase activity is attributable to hemagglutinin-neuraminidase (HN), an envelope protein essential for viral attachment and for fusion mediated by the other envelope protein, F. While there is no evidence that HN's neuraminidase activity is essential for receptor binding and syncytium formation, we found that 4-GU-DANA prevented hemadsorption and fusion of persistently infected cells with uninfected cells. In plaque assays, 4-GU-DANA reduced the number (but not the area) of plaques if present only during the adsorption period and reduced plaque area (but not number) if added only after the 90-min adsorption period. 4-GU-DANA also reduced the area of plaques formed by a neuraminidase-deficient variant, confirming that its interference with cell-cell fusion is unrelated to inhibition of neuraminidase activity. The order-of-magnitude lower 50% inhibitory concentrations of 4-GU-DANA (and also DANA and 4-AM-DANA) for plaque area reduction and for inhibition in the fusion assay than for reducing plaque number or blocking hemadsorption indicate the particular efficacy of these sialic acid analogs in interfering with cell-cell fusion. In cell lines expressing influenza virus hemagglutinin (RA) as the only viral protein, we found that 4-GU-DANA had no effect on hemadsorption but did inhibit HA2b-red blood cell fusion, as judged by both lipid mixing and content mixing. Thus, 4-GU-DANA can interfere with both influenza virus- and HPF3-mediated fusion. The results indicate that (i) in HPF3, 4-GU-DANA and its analogs have an affinity not only for the neuraminidase active site of HN but also for sites important for receptor binding and cell fusion and (ii) sialic acid-based inhibitors of influenza virus neuraminidase can also exert a direct, negative effect on the fusogenic function of the other envelope protein, HA.	Greengard et al. 2000
Zanamivir Multicenter clinical study Antiviral activity Safety Tolerability China 2010–2011 Adolescents/adults	Background It is the first multicenter clinical study in China to investigate zanamivir use among Chinese adolescents and adults with influenza-like illness (ILI) since 2009, when inhaled zanamivir (RELENZA) was marketed in China. Methods An uncontrolled open-label, multicentre study to evaluate the antiviral activity, and safety of inhaled zanamivir (as Rotadisk via Diskhaler device); 10 mg administered twice daily for 5 days in subjects ≥ 12 years old with ILI. Patients were enrolled within 48 hours of onset and followed for eight days. Patients were defined as being influenza-positive if the real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) test had positive results. Results A total of 400 patients ≥ 12 years old were screened from 11 centers in seven provinces from March 2010 to January 2011. Three hundred and ninety-two patients who took at least one dose of zanamivir were entered into the safety analysis. The mean age was 33.8 years and 50% were male. Cardiovascular diseases and diabetes were the most common comorbidities. All the reported adverse events, such as rash, nasal ache, muscle ache, nausea, diarrhea, headache, occurred in less than 1% of subjects. Mild sinus bradycadia or arrhythmia occurred in four subjects (1%). Most of the adverse events were mild and did not require any change of treatment. No severe adverse events (SAE) or fatal cases were reported. Bronchospasm was found in a 38 years old woman whose symptoms disappeared after stopping zanamivir and without additional treatment. All the 61 influenza virus isolates (43 before enrollment, 18 during treatment) proved to be sensitive to zanamivir. Conclusions Zanamivir is well tolerated by Chinese adolescents and adults with ILIs. There is no evidence for the emergence of drug-resistant isolates during treatment with zanamivir.	Cao et al. 2012

Oseltamivir Avian influenza N6 subtype Susceptibility testing	Avian influenza viruses are a source of genetic material that can be transmitted to humans through direct introduction or reassortment. Although there is a wealth of information concerning global monitoring for antiviral resistance among human viruses of the N1 and N2 neuraminidase (NA) subtypes, information concerning avian viruses of these and other NA subtypes is limited. We undertook a surveillance study to investigate the antiviral susceptibility of avian influenza N6 NA viruses, the predominant subtype among wild waterfowl. We evaluated 73 viruses from North American ducks and shorebirds for susceptibility to the NA inhibitor oseltamivir in a fluorescence-based NA enzyme inhibition assay. Most (90%) had mean IC50 values ranging from < 0.01 to 5.0 nM; 10% were from 5.1 to 50.0 nM; and none were > 50.0 nM. Susceptibility to oseltamivir remained stable among all isolates collected over approximately three decades ($P \leq 0.74$). Two isolates with 1222V NA substitution had moderately reduced susceptibility to oseltamivir in vitro (IC50, 30.0 and 40.0 nM). One field sample was a mixed population containing an avian paramyxovirus (APMV) and H4N6 influenza virus, as revealed by electron microscopy and hemagglutination inhibition assays with a panel of anti-APMV antisera. This highlights the importance of awareness and careful examination of non-influenza pathogens in field samples from avian sources. This study showed that oseltamivir-resistant N6 NA avian influenza viruses are rare, and must be tested both phenotypically and genotypically to confirm resistance.	Stoner et al. 2012
Oseltamivir carboxylate **Zanamivir** Production of viral particles Protein synthesis Long tubular virus-like structures	Background: Neuraminidase (NA) inhibitors used for influenza therapy are believed to prevent the release of progeny virus from the surface of an infected cell. In this study, we found that NA inhibitors have a novel antiviral function against an avian influenza virus. Results: Madin-Darby canine kidney cells, commonly used for the isolation and propagation of the influenza virus, were infected with an avian influenza viral strain A/chicken/German/N/49(H10N7) (H10/chicken) or a human influenza viral strain A/Osaka/981/98(H3N2) (H3/Osaka) virus. Cells were incubated in a medium without or with a NA inhibitor, oseltamivir carboxylate (GS4071), from 1 to 13 h post infection (p.i.). Infected cells were washed 12 h p.i. to remove GS4071, incubated for 1 h without GS4071, and assayed for virus production. Incubation with GS4071 decreased the production of infectious viruses. When H10/chicken virus-infected cells were incubated with GS4071 from 12 to 13 h p.i. (i.e., 1 h before the virus production assay), the inhibitory effect was clearly observed, however, the same was not evident for H3/Osaka virus-infected cells. Furthermore, viral protein synthesis in infected cells was not affected by GS4071. Using a scanning electron microscope, many single spherical buds were observed on the surface of H3/Osaka virus-infected cells incubated without GS4071, whereas many aggregated particles were observed on the surface of cells incubated with GS4071. However, many long tubular virus-like structures, with no aggregated particles, were observed on the surface of H10/chicken virus-infected cells incubated with GS4071. The same results were obtained when another NA inhibitor, zanamivir, was used. Conclusions: These results indicate that NA inhibitors interfered with virus particle formation in the H10/chicken virus-infected cells, in which the inhibitor caused the formation of long tubular virus-like structures instead of spherical virus particles.	Ushirogawa and Ohuchi 2011

Peramivir Evaluation of antiviral activity Mouse model	New and emerging influenza virus strains, such as the pandemic influenza A (H1N1) virus require constant vigilance for antiviral drug sensitivity and resistance. Efficacy of intramuscularly (IM) administered neuraminidase (NA) inhibitor, peramivir, was evaluated in mice infected with recently isolated pandemic A/California/04/2009 (H1N1, swine origin, mouse adapted) influenza virus. A single IM injection of peramivir (four dose groups), given 1 h prior to inoculation, significantly reduced weight loss ($P < 0.001$) and mortality ($P < 0.05$) in mice infected with LD(90) dose of pandemic A/California/04/2009 (H1N1) influenza virus compared to vehicle group. There was 20% survival in the vehicle-treated group, whereas in the peramivir-treated groups, survival increased in a dose-dependent manner with 60, 60, 90 and 100% survivors for the 1, 3, 10, and 30 mg/kg doses, respectively. Weight loss on day 4 in the vehicle-treated group was 3.4 gm, and in the peramivir-treated groups was 2.1, 1.5, 1.8 and 1.8 g for the 1,3, 10 and 30 mg/kg dose groups, respectively. In the treatment model, peramivir given 24 h after infection as a single IM injection at 50 mg/kg dose, showed significant protection against lethality and weight loss. There was 13% survival in the vehicle-treated group while in the peramivir-treated group at 24, 48, and 72 h post infection, survival was 100, 40, and 50%, respectively. Survival in the oseltamivir groups (10 mg/kg/day twice a day, orally for 5 days) was 90, 30 and 20% at 24, 48 and 72h, respectively. These data demonstrate efficacy of parenterally administered peramivir against the recently isolated pandemic influenza virus in murine infection models.	Bantia et al. 2010
Peramivir **Oseltamivir** **Zanamivir** Efficacy of a single intramuscular injection Mouse model	In the event of an influenza outbreak, antivirals including the neuraminiclase (NA) inhibitors, peramivir, oseltamivir, and zanamivir may provide valuable benefit when vaccine production is delayed, limited, or cannot be used. Here we demonstrate the efficacy of a single intramuscular injection of peramivir in the mouse influenza model. Peramivir potently inhibits the neuraminidase enzyme N9 from H1N9 virus in vitro with a 50% inhibitory concentration (IC50) of 1.3 ± 0.4 nM. On-site dissociation studies indicate that peramivir remains tightly bound to N9 NA ($t_{1/2} > 24$ h), whereas, zanamivir and oseltamivir carboxylate dissociated rapidly from the enzyme ($t_{1/2} = 1.25$ h). A single intramuscular injection of peramivir (10mg/kg) significantly reduces weight loss and mortality in mice infected with influenza A/H1N1, while oseltamivir demonstrates no efficacy by the same treatment regimen. This may be due to tight binding of peramivir to the NI NA enzymes similar to that observed for N9 enzyme. Additional efficacy studies indicate that a single injection of peramivir (2–20 mg/kg) was comparable to a q.d. × 5 day course of orally administered oseltamivir (2–20mg/kg/day) in preventing lethality in H3N2 and H1N1 influenza models. A single intramuscular injection of peramivir may successfully treat influenza infections and provide an alternate option to oseltamivir during an influenza outbreak.	Bantia et al. 2006
Laninamivir Evaluation of antiviral activity Mouse/ferret model	We have reported on laninamivir (code name, R-125489), a novel neuraminidase inhibitor, and have discovered that the laninamivir prodrug CS-8958 worked as a long-acting neuraminidase inhibitor in a mouse influenza virus infection model when it is intranasally administered. In this study, CS-8958 was administered just once 7 days before infection and showed significant efficacy in vivo. The efficacy of a single administration of CS-8958 after viral infection was then compared with that of repeated administrations of oseltamivir phosphate or zanamivir in mice and ferrets. CS-8958 showed efficacy superior or similar to the efficacies of the two licensed NA inhibitors. CS-8958 also significantly reduced the titers of an oseltamivir-resistant H1N1 virus with a neuraminidase H274Y substitution in a mouse infection model. These results suggest that since CS-8958 is characteristically long lasting in the lungs, it may be ideal for the prophylaxis and treatment of influenza.	Kubo et al. 2010
Zanamivir Multimeric conjugates	A set of trimeric and tetrameric derivatives 6-11 of the influenza virus neuraminidase inhibitor zanamivir 1 have been synthesized by coupling a common monomeric zanamivir derivative 3 onto various multimeric carboxylic acid core groups. These discrete multimeric compounds are all significantly more antiviral than zanamivir and also show outstanding long-lasting protective activity when tested in mouse influenza infectivity experiments. (C) 2004 Elsevier Ltd. All rights reserved.	Watson et al. 2004

Polyvalent zanamivir conjugates Synthesis Antiviral effect	Polyvalent sialidase inhibitors bearing 4-guanidino-Neu5Ac2en derivatives on a poly-L-glutamine backbone are described. Aiming for a longer retention time of 4-guanidino-Neu5Ac2en (zanamivir) in bronchi and lungs, we focused on supermolecules bearing 4-guanidino-Neu5Ac2en derivatives bound at their C-7 position through non-cleavable alkyl ether linkages. We first found that alkylation of the 7-hydroxyl group of sialic acid derivative 8 proceeded smoothly, and produced 7-O-alkyl-4-guanidino-Neu5Ac2en derivatives 13, which exhibited equipotent inhibitory activity against not only influenza A virus sialidase but also influenza A virus in the cell culture. Next, we synthesized poly-L-glutamine bearing 7-O-alkyl-4-guanidino-Neu5Ac2en derivatives linked by amide bonds, 26, which showed enhanced antiviral activity against influenza A virus and more potent efficacy in vivo relative to a monomeric sialidase inhibitor.	Masuda et al. 2003
Dual-targeted bifunctional anti-influenza drugs Caffeic acid-zanamivir conjugate Suppression of pro-inflamatory cytokines	Influenza therapy with a single targeted compound is often limited in efficacy due to the rapidly developed drug resistance. Moreover, the uncontrolled virus-induced cytokines could cause the high mortality of human infected by H5N1 avian influenza virus. In this study, we explored the novel dual-targeted bifunctional anti-influenza drugs formed by conjugation with anti-inflammatory agents. In particular, the caffeic acid (CA)-bearing zanamivir (ZA) conjugates ZA-7-CA (1) and ZA-7-CA-amide (7) showed simultaneous inhibition of influenza virus neuraminidase and suppression of pro-inflammatory cytokines. These ZA conjugates provided remarkable protection of cells and mice against influenza infections. Intranasal administration of low dosage (< 1.2 μmol/kg/day) of ZA conjugates exhibited much greater effect than the combination therapy with ZA and the anti-inflammatory agents in protection of the lethally infected mice by H1N1 or H5N1 influenza viruses.	Liu et al. 2012
Theaflavins Neuraminidase activity assay Haemagglutination inhibition assay Real-time quantitative PCR Anti-inflammatory properties	The theaflavins fraction (TF80%, with a purity of 80%) and three theaflavin (TF) derivatives from black tea have been found to exhibit potent inhibitory effects against influenza virus in vitro. They were evaluated with a neuraminidase (NA) activity assay, a hemagglutination (HA) inhibition assay, a real-time quantitative PCR (qPCR) assay for gene expression of hemagglutinin (HA) and a cytopathic effect (CPE) reduction assay. The experimental results showed that they all exerted significant inhibitory effects on the NA of three different subtypes of influenza virus strains [A/PR/8/34(H1N1), A/Sydney/5/97(H3N2) and 8/Jiangsu/10/2003] with 50% inhibitory concentration (IC50) values ranging from 9.27 to 36.55 μg/ml, and they also displayed an inhibitory effect on HA; these inhibitory effects might constitute two major mechanisms of their antiviral activity. Time-of-addition studies demonstrated that TF derivatives might have a direct effect on viral particle infectivity, which was consistent with the inhibitory effect on HA. Subsequently, the inhibitory effect of TF derivatives on the replication of the viral HA gene as assayed by qPCR and on the nuclear localization of the influenza virus vRNP further demonstrated that they may primarily act during the early stage of infection. Interestingly, besides the activity against functional viral proteins, TF derivatives also decreased the expression level of the inflammatory cytokine IL-6 during viral infection, expression of which may result in serious tissue injury and apoptosis. Our results indicated that TF derivatives are potential compounds with anti-influenza viral replication and anti-inflammatory properties. These findings will provide important information for new drug design and development for the treatment of influenza virus infection.	Zu et al. 2012

Traditional Chinese medicines Water extracts from plants Mouse model	Etnopharmacological relevance: Neuraminidase (NA) inhibitors are currently the most effective drugs to treat influenza A viruses infection. Many traditional Chinese medicines (TCMs) have been used in the clinics to treat influenza. The anti-viral mechanisms of these TCMs and their inhibitory effects towards NA need to be systematically tested. Aim of the study: To evaluate the anti-NA activity of the TCMs and the anti-influenza A virus effects of the NA inhibitory TCMs in vitro and in vivo. Material and methods: We tested the inhibitory activity of water extracts from 439 TCMs towards NA. The in vitro anti-influenza virus activities of the 5 TCMs were evaluated using the strain A/California/7/2009 (H1N1) NYMC X-179A of influenza A virus. A randomly selected TCM with NA inhibitory activity. Melia toosendan extract, was further evaluated using a mouse model infected with influenza A virus. Results: Five TCMs, Duchesnea indica, Focke (Fragaria indica), Liquidambar formosana, Lithospermum erythrorhizon, Melia toosendan, and Prunella vulgaris, exerted potent inhibitory activity towards NA. These TCMs in the range of 25–250 μg/ml had the ability to reduce virus-induced cytopathic effect (CPE) and the virus yield in MDCK cells. Melia toosendan significantly reduced death rate and prolonged mean day to death (MDD) of the viral infected mice. Conclusions: This study describes five TCMs exerted strong inhibitory activities towards NA, and exhibited antiviral effect against influenza A virus by reducing viral reproduction and reduced CPE of the viral infected cells. Melia toosendan, significantly reduced death rate and prolonged survival of the H1N1 viral infected mice.	Tian et al. 2011
Plant flavonols Gossypetin Kaempferol Evaluation of antiviral activity	Five flavonols (3, 5, and 9–11) were isolated from Rhodiola rosea, and compared with commercially available flavonoids (1, 2, 4, 6-8, and 12–14) to facilitate analysis of their structure-activity relationship (SAR). All compounds (1–14) showed neuraminidase inhibitory activities with IC_{50} values ranging from 0.8 to 56.9 μM. The in vitro anti-influenza virus activities of flavonoids 1-6, 8–12, and 14 were evaluated using two influenza viral strains, H1N1 (A/PR/8/34) and H9N2 (A/Chicken/Korea/MS96/96), testing their ability to reduce virus-induced cytopathic effect (CPE) in MDCK cells. We found that the activity of these compounds ranged from 30.2 to 99.1 μM against H1N1- and 18.5 to 133.6 μM against H9N2-induced CPE. Of compounds 1–14, gossypetin (6) exhibited the most potent inhibitory activity, with IC_{50} values of 0.8 and 2.6 μM on neuraminidases from Clostridium perfringens and recombinant influenza virus A (rvH1N1), respectively. In contrast, kaempferol (3) exhibited the highest activity against two influenza viruses, H1N1 and H9N2 with EC_{50} values of 30.2 and 18.5 μM, respectively. Activity depended on the position and number of hydroxy groups on the flavonoids backbone. In kinetic studies, all isolated compounds behaved as noncompetitive inhibitors.	Jeong et al. 2009
1,3-diarylprop-2-en-1-one derivatives Synthesis Evaluation	A series of 1,3-diarylprop-2-en-1-one derivatives 3a-v have been synthesized and evaluated for their ability to inhibit neuraminidase (NA). Among the prepared compounds, the less lipophilic derivative 3k showed the most potent in vitro inhibitory activity against NA with an IC50 value of 1.5 μ M.	Kinger et al. 2012

Cyclopentane derivatives	A novel series of cyclopentane derivatives have been found to exhibit potent and selective inhibitory effects on influenza virus neuraminidase, These compounds, designated RWJ-270201, BCX-1827, BCX-1898, and BCX-1923, were tested in parallel with zanamivir and oseltamivir carboxylate against a spectrum of influenza A (H1N1, H3N2, and H5N1) and influenza B viruses in MDCK cells, inhibition of viral cytopathic effect ascertained visually and by neutral red dye uptake was used, with 50% effective (virus-inhibitory) concentrations (EC50) determined. Against the H1N1 viruses A/Bayern/07/95, A/Beijing/262/95, A/PR/8/34, and A/Texas/36/91, EC(50)s (determined by neutral red assay) of the novel compounds were less than or equal to 1.5 μM. Twelve strains of H3N2 and two strains of avian H5N1 viruses were inhibited at < 0.3 μM Influenza B/Beijing/184/93 and B/Harbin/07/94 viruses were inhibited at < 0.2 μM, with three other B virus strains inhibited at 0.8 to 8 μM, The novel inhibitors were comparable in potency to (or slightly more potent than) zanamivir and oseltamivir carboxylate. No cytotoxicity was seen with the compounds at concentrations of less than or equal to 1 mM in cell proliferation assays. The antiviral activity of RWJ-270201, chosen for clinical development, was studied in greater detail. Its potency and that of oseltamivir carboxylate decreased with increasing multiplicity of virus infection. Time-of-addition studies indicated that treatment with either compound needed to begin 0 to 12 h after virus exposure for optimal activity. Exposure of cells to RWJ-270201 caused most of the virus to remain cell associated, with extracellular virus decreasing in a concentration-dependent manner. This is consistent with its effect as a neuraminidase inhibitor. RWJ-270201 shows promise in the treatment of human influenza virus infections.	Smee et al. 2001
Resistance to neuraminidase inhibitors Conserved residues Viral fitness	Neuraminidase inhibitors (NAIs) are antivirals designed to target conserved residues at the neuraminidase (NA) enzyme active site in influenza A and B viruses. The conserved residues that interact with NAIs are under selective pressure, but only a few have been linked to resistance. In the A/Wuhan/359/95 (H3N2) recombinant virus background, we characterized seven charged, conserved NA residues (11118, R371, E227, R152, R224, E276, and D151) that directly interact with the NAIs but have not been reported to confer resistance to NAIs. These NA residues were replaced with amino acids that possess side chains having similar properties to maintain their original charge. The NA mutations we introduced significantly decreased NA activity compared to that of the A/Wuhan/359/95 recombinant wild-type and R292K (an NA mutation frequently reported to confer resistance) viruses, which were analyzed for comparison. However, the recombinant viruses differed in replication efficiency when we serially passaged them in vitro; the growth of the R118K and E227D viruses was most impaired. The R224K E276D, and R371K mutations conferred resistance to both zanamivir and oseltamivir, while the D151E mutation reduced susceptibility to oseltamivir only (similar to 10-fold) and the R152K mutation did not alter susceptibility to either drug. Because the R224K mutation was genetically unstable and the emergence of the R371K mutation in the N2 subtype is statistically unlikely, our results suggest that only the E276D mutation is likely to emerge under selective pressure. The results of our study may help to optimize the design of NAIs.	Yen et al. 2006
Resistance to adamantanes Central/South America	Background Recent influenza antiviral resistance studies reveal an alarming increase in both adamantanes and neuraminidase inhibitors (NAIs) resistant viral strains worldwide, particularly in Asia, Europe and the United States. Objectives In this study, we have evaluated influenza virus resistance in Central and South America. Methods Influenza viruses, isolated from symptomatic patients throughout Central and South America in 2005-2008 were analyzed for inhibitor resistance. The M2 and NA genes of influenza viruses were sequenced and resistance was inferred by comparison with published sequences and known resistant mutations. Results Our results indicate that: (i) resistance to adamantanes was seen in the majority (95.5%) of the influenza A/H3N2 isolates but only in one isolate of the influenza A/H1N1 viruses; (ii) resistance to NAIs began to be detected in A/H1N1 isolates from Central America in 2008; and (iii) none of the influenza B viruses analyzed were resistant to NAIs. Conclusions These findings suggest a limited effectiveness of influenza inhibitors due to the detection of resistance among A/H1 and A/H3 viruses.	Garcia et al. 2009

Pharmacokinetic studies Oseltamivir Bioavailability Renal clearance Steady-state plasma concentration	Oseltamivir is an ethyl ester prodrug of Po 64-0802, a selective inhibitor of influenza virus neuraminidase. Oral administration of oseltamivir delivers the active antiviral Po 64-0802 to the bloodstream, and thus all sites of influenza infection (lung, nasal mucosa, middle ear) are accessible. The pharmacokinetic profile of oseltamivir is simple and predictable, and twice daily treatment results in effective antiviral plasma concentrations over the entire administration interval. After oral administration, oseltamivir is readily absorbed from the gastrointestinal tract and extensively converted to the active metabolite. The absolute bioavailability of the active metabolite from orally administered oseltamivir is 80%. The active metabolite is detectable in plasma within 30 minutes and reaches maximal concentrations after 3 to 4 hours. After peak plasma concentrations are attained, the concentration of the active metabolite declines with an apparent half-life of 6 to 10 hours. Oseltamivir is eliminated primarily by conversion to and renal excretion of the active metabolite. Renal clearance of both compounds exceeds glomerular filtration rate, indicating that renal tubular secretion contributes to their elimination via the anionic pathway. Neither compound interacts with cytochrome P450 mixed-function oxidases or glucuronosyltransferases. The pharmacokinetic profile of the active metabolite is linear and dose-proportional, with less than 2-fold accumulation over a dosage range of oseltamivir 50 to 500 mg twice daily. Steady-state plasma concentrations are achieved within 3 days of twice daily administration, and at a dosage of 75 mg twice daily the steady-state plasma trough concentrations of active metabolite remain above the minimum inhibitory concentration for all influenza strains tested. Exposure to the active metabolite at steady state is approximately 25% higher in elderly compared with young individuals; however, no dosage adjustment is necessary. In patients with renal impairment, metabolite clearance decreases linearly with creatinine clearance. A dosage reduction to 75 mg once daily is recommended fbr patients with creatinine clearance < 30 ml/min (1.8 l/h). The pharmacokinetics in patients with influenza are qualitatively similar to those in healthy young adults. In vitro and in vivo studies indicate no clinically significant drug interactions. Neither paracetamol (acetaminophen) nor cimetidine altered the pharmacokinetics of Ro 64-0802. Coadministration of probenecid resulted in a 2.5-fold increase in exposure to Ro 64-0802, however, this competition is unlikely to result in clinically relevant effects. These properties make oseltamivir a suitable candidate for use in the prevention and treatment of influenza.	He et al. 1999

Table 4.5. Host cell factor targeting

Signalling cascade blockers C-Jun kinase Blocking of transcription and RNA synthesis	C-Jun N-terminal kinases (JNK) are activated in course of many viral infections. Here we analyzed the activity of JNK inhibitors on influenza A virus (IAV) amplification. Human lung epithelial cells were infected with either the highly pathogenic avian virus strain A/FPV/Bratislava/79 (H7N7) or the pandemic swine-origin influenza virus A/Hamburg/4/09 (H1N1v). The application of the JNK inhibitors SP600125 and AS601245 reduced IAV amplification by suppressing viral protein and RNA synthesis. Although AS601245 appeared to generally block the transcription of newly introduced genes, SP600125 specifically affected viral RNA synthesis. Overexpression of a dominant negative mutant of SEK/MKK4 and siRNA-mediated suppression of JNK2 expression confirmed that specific manipulation of the JNK pathway attenuates virus propagation. An IAV minigenome replication assay revealed that SP600125 did not directly affect the activity of the viral RNA polymerase complex but seems to suppress an anti-influenza nonstructural protein 1-mediated virus supportive function. Finally, when H7N7-or H1N1v-infected mice were treated with SP600125, the viral load is reduced in lungs of treated compared with untreated mice. Our data suggest that this class of ATP competitive inhibitors once optimized for antiviral action potentially represent novel drugs for antiviral intervention.	Nacken et al. 2012

Signalling cascade blockers Raf/MEK/ERK signalling pathway Oseltamivir resistant strains Mouse model	The emergence of the 2009 H1N1 pandemic swine influenza A virus is a good example of how this viral infection can impact health systems around the world in a very short time. The continuous zoonotic circulation and reassortment potential of influenza A viruses (IAV) in nature represents an enormous public health threat to humans. Beside vaccination antivirals are needed to efficiently control spreading of the disease. In the present work we investigated whether the MEK inhibitor U0126, targeting the intracellular Raf/MEK/ERK signaling pathway, is able to suppress propagation of the 2009 pandemic IV H1N1v (v = variant) as well as highly pathogenic avian influenza viruses (HPAIV) in cell culture and also in vivo in the mouse lung. U0126 showed antiviral activity in cell culture against all tested IAV strains including Raf/MEK/ERK signaling pathway. Furthermore, we were able to demonstrate that treatment of mice with U0126 via the aerosol route led to inhibition of MEK activation in the lung reduction of progeny IAV titers compared to untreated controls protection of IAV infected mice against a 100× lethal viral challenge. Moreover, no adverse effects of U0126 were found in cell culture or in the mouse. Thus, we conclude that U0126, by inhibiting the cellular target MEK, has an antiviral potential not only in vitro in cell culture, but also in vivo in the mouse model.	Droebner et al. 2011
Cathelicidin peptides Decrease of pro-inflamatory cytokines Protection against influenza	The extensive world-wide morbidity and mortality caused by influenza A viruses highlights the need for new insights into the host immune response and novel treatment approaches. Cationic Host Defense Peptides (CHDP, also known as antimicrobial peptides), which include cathelicidins and defensins, are key components of the innate immune system that are upregulated during infection and inflammation. Cathelicidins have immunomodulatory and anti-viral effects, but their impact on influenza virus infection has not been previously assessed. We therefore evaluated the effect of cathelicidin peptides on disease caused by influenza A virus in mice. The human cathelicidin, LL-37, and the murine cathelicidin, mCRAMP, demonstrated significant anti-viral activity in vivo, reducing disease severity and viral replication in infected mice to a similar extent as the well-characterized influenza virus-specific antiviral drug zanamivir. In vitro and in vivo experiments suggested that the peptides may act directly on the influenza virion rather than via receptor-based mechanisms. Influenza virus-infected mice treated with LL-37 had lower concentrations of pro-inflammatory cytokines in the lung than did infected animals that had not been treated with cathelicidin peptides. These data suggest that treatment of influenza-infected individuals with cathelicidin-derived therapeutics, or modulation of endogenous cathelicidin production may provide significant protection against disease.	Barlow et al. 2011
Vacuolar proton-ATPase inhibitor Concanamycin A	The selective inhibitor of the vacuolar proton-ATPase, concanamycin A, powerfully blocks influenza virus entry into cells, if present during the initial times of virus infection. Attachment of virus particles to cells is not prevented by concanamycin A, rather the exit of influenza virus from endosomes is the step blocked by this macrolide antibiotic. Inhibition of influenza virus entry into cells by concanamycin A or by nigericin takes place under acidic conditions. Moreover, if the pH gradient is abolished by pre-incubation of cells in acidic pH, influenza virus entry does not occur even in the absence of any inhibitors. These results indicate that acidic conditions per se are not sufficient to promote virus entry into cells; rather this step of virus infection requires a pH gradient.	Guinea and Carrasco 1994

Inhibitor of vacuolar-type proton pump Bafilomycin Al Endosome acidification	We studied the effect of bafilomycin Al (Baf-A1), a novel and highly specific inhibitor for vacuolar-type proton (V-H+) pump, on the growth of influenza A and B viruses in Madin-Darby canine kidney cells. Vital fluorescence microscopic study showed that Baf-A1 induced the complete disappearance of acidified compartments such as endosomes and lysosomes both in infected and uninfected cells by the treatment with 0.1 mu M inhibitor for 1 h at 37 °C. In addition, virus growth was inhibited when Baf-A1 was present from 1 h before infection to the end of incubation, or added within as early as 5–10 min after infection. Conversely, the virus growth was recovered in correlation with the reappearance of acidified compartments after removal of Baf-A1. These data suggest that Baf-A1-sensitive V-H+ pumps are solely responsible for the acidification of endosomes and lysosomes, and thus Baf-A1 inhibits the growth of influenza A and B viruses by affecting the acidified compartments in which low pH is essential for the uncoating process of influenza virus growth at an early stage of infection.	Ochiai et al. 1995
Proteasome inhibitor	Recently it has been shown that the proinflammatory NF-kappa B pathway promotes efficient influenza virus propagation. Based on these findings, it was suggested that NF-kappa B blockade may be a promising approach for antiviral intervention. The classical virus-induced activation of the NF-kappa B pathway requires proteasomal degradation of the inhibitor of NF-kappa B, I kappa B. Therefore, we hypothesized that inhibition of proteasomal I kappa B degradation should impair influenza A virus (IAV) replication. We chose the specific proteasome inhibitor PS-341, which is a clinically approved anticancer drug also known as Bortezomib or Velcade. As expected, PS-341 treatment of infected A549 cells in a concentration range that was not toxic resulted in a significant reduction of progeny virus titers. However, we could not observe the proposed suppression of NF-kappa B-signaling in vitro. Rather, PS-341 treatment resulted in an induction of I kappa B degradation and activation of NF-kappa B as well as the JNK/AP-1 pathway. This coincides with enhanced expression of antiviral genes, such as interleukin-6 and, most importantly, MxA, which is a strong interferon (IFN)-induced suppressor of influenza virus replication. This suggests that PS-341 may act as an antiviral agent via induction of the type I IFN response. Accordingly, PS-341 did not affect virus titers in Vero cells, which lack type I IFN genes, but strongly inhibited replication of vesicular stomatitis virus (VSV), a highly IFN-sensitive pathogen. Thus, we conclude that PS-341 blocks IAV and VSV replication by inducing an antiviral state mediated by the NF-kappa B-dependent expression of antivirus-acting gene products.	Dudek et al. 2010
Proteasome inhibitor Reduction of cytokine release	In the present study we show that the proteasome inhibitor VL-01 leads to reduction of influenza virus replication in human lung adenocarcinoma epithelial cells (A549) as demonstrated with three different influenza virus strains, A/Puerto Rico/8/34 (H1N1) (EC_{50} value of 1.7 μM), A/Regensburg/D6/09 (H1N1v) (EC_{50} value of 2.4 μM) and A/Mallard/Bavaria/1/2006 (H5N1) (EC_{50} value of 0.8 μM). In in vivo experiments we could demonstrate that VL-01-aerosol-treatment of BALB/c mice with 14.1 mg/kg results in no toxic side effects, reduced progeny virus titers in the lung (1.1 ± 0.3 $\log_{10}$ pfu) and enhanced survival of mice after infection with a 5-fold MLD_{50} of the human influenza A virus strain A/Puerto Rico/8/34 (H1N1) up to 50%. Furthermore, treatment of mice with VL-01 reduced the cytokine release of IL-alpha/beta, IL-6, MIP-1 beta, RANTES and TNF-alpha induced by LPS or highly pathogen avian H5N1 influenza A virus. The present data demonstrates an antiviral effect of VL-01 in vitro and in vivo and the ability to reduce influenza virus induced cytokines and chemokines.	Haasbach et al. 2011
NF-kappa B inhibitor Pyronepolyene C-glucoside	A new pyronepolyene C-glucoside, named iso-D8646-2-6 (1) together with the known related compound D8646-2-6 (2), was isolated from the sponge-associated fungus Epicoccum sp. JJY40. They showed NF-kappa B inhibitory and anti-influenza A viral (H1N1) activities.	Peng et al. 2012

Protease inhibitors Aprotinin Leupeptin Camostat	Efforts to develop new antiviral chemotherapeutic approaches are focusing on compounds that target either influenza virus replication itself or host factor(s) that are critical to influenza replication. Host protease mediated influenza hemagglutinin (HA) cleavage is critical for activation of virus infectivity and as such is a chemotherapeutic target. Influenza pathogenesis involves a "vicious cycle" in which host proteases activate progeny virus which in turn amplifies replication and stimulates further protease activities which may be detrimental to the infected host. Aprotinin, a 58 amino acid polypeptide purified from bovine lung that is one of a family of host-targeted antivirals that inhibit serine proteases responsible for influenza virus activation. This drug and similar agents, such as leupeptin and camostat, suppress virus HA cleavage and limit reproduction of human and avian influenza viruses with a single arginine in the HA cleavage site. Site-directed structural modifications of aprotinin are possible to increase its intracellular targeting of cleavage of highly virulent H5 and H7 hemagglutinins possessing multi-arginine/lysine cleavage site. An additional mechanism of action for serine protease inhibitors is to target a number of host mediators of inflammation and down regulate their levels in virus-infected hosts. Aprotinin is a generic drug approved for intravenous use in humans to treat pancreatitis and limit post-operative bleeding. As an antiinfluenzal compound, aprotinin might be delivered by two routes: (i) a small-particle aerosol has been approved in Russia for local respiratory application in mild-to-moderate influenza and (ii) a proposed intravenous administration for severe influenza to provide both an antiviral effect and a decrease in systemic pathology and inflammation.	Zhirnov et al. 2011
Protease inhibitors Camostat mesilate Nafamostat mesilate	Six nucleoside analogues, two sulfated polysaccharides, and four protease inhibitors were evaluated in vitro as inhibitors of influenza virus replication. Four guanosine analogues (mizoribine, ribavirin, pyrazofurin, and 5-ethynyl-1-beta-D-ribofuranosylimidazole-4-carboxamide), the sulfated polysaccharide dextran sulfate (molecular weight 500 000), and two protease inhibitors (camostat mesilate and nafamostat mesilate) were inhibitory to the replication of strains of influenza virus types A and B at concentrations down to 0.3 μg/ml. Of these seven compounds, ribavirin, camostat mesilate, and nafamostat mesilate were efficacious in both reducing the virus titer and increasing the survival rate of influenza virus-infected chick embryos. For camostat mesilate, the ED50 (required to improve the survival rate of influenza virus-infected chick embryos by 50%) was 0.80 μg/g, and its selectivity index, based on the ratio of the 50% toxic dose (required to reduce the viability of chick embryos by 50%) to ED50, was 280. Camostat mesilate deserves further exploration for its potential in the treatment of influenza virus infection.	Hosoya et al. 1993
Protease inhibitors Nafamostat mesilate Camostat mesilate Gabexate mesilate Aprotinin Chicken embryos	We studied the effects of eight protease inhibitors on the multicycle replications of various orthomyxoviruses and paramyxoviruses. Among the compounds, nafamostat mesilate, camostat mesilate, gabexate mesilate, and aprotinin, which are widely used in the treatment of pancreatitis, inhibited influenza virus A and B replication at concentrations that were significantly lower than their cytotoxic thresholds in vitro. None of the protease inhibitors had activity against respiratory syncytial virus, measles virus, or parainfluenza virus type 3 at the highest concentrations tested. Camostat mesilate was found to be the most selective inhibitor. Its 50% effective concentration for influenza virus A replication was 2.2 μg/ml, and the selectivity index, which was based on the ratio of the 50% inhibitory concentration for host cell proliferation to the 50% effective concentration for influenza virus A replication, was 680. When the in ovo antiviral activity of the compounds was tested by using chicken embryos, camostat mesilate at a dose of 10 μg/g markedly reduced the hemagglutinin titers of influenza viruses A and B.	Hosoya et al. 1992

Haemagglutinin folding Glutathione derivative	Aim: The aim of this study was to determine whether GSH-C4, a hydrophobic glutathione derivative, affects in vitro and in vivo influenza virus infection by interfering with redox-sensitive intracellular pathways involved in the maturation of viral hemagglutinin (HA). Results: GSH-C4 strongly inhibited influenza A virus replication in cultured cells and in lethally infected mice, where it also reduced lung damage and mortality. In cell-culture studies, GSH-C4 arrested viral HA folding; the disulfide-rich glycoprotein remained in the endoplasmic reticulum as a reduced monomer instead of undergoing oligomerization and cell plasma-membrane insertion. HA maturation depends on the host-cell oxidoreductase, protein disulfide isomerase (PDI), whose activity in infected cells is probably facilitated by virus-induced glutathione depletion. By correcting this deficit, GSH-C4 increased levels of reduced PDI and inhibited essential disulfide bond formation in HA. Host-cell glycoprotein expression in uninfected cells was unaffected by glutathione, which thus appears to act exclusively on glutathione-depleted cells. Innovation: All currently approved anti-influenza drugs target essential viral structures, and their efficacy is limited by toxicity and by the almost inevitable selection of drug-resistant viral mutants. GSH-C4 inhibits influenza virus replication by modulating redox-sensitive pathways in infected cells, without producing toxicity in uninfected cells or animals. Novel anti-influenza drugs that target intracellular pathways essential for viral replication ("cell-based approach") offer two important potential advantages: they are more difficult for the virus to adapt to and their efficacy should not be dependent on virus type, strain, or antigenic properties. Conclusion: Redox-sensitive host-cell pathways exploited for viral replication are promising targets for effective anti-influenza strategies.	Sgarbanti et al. 2011
Proteasome inhibitors	We have demonstrated that influenza A virus (IAV) RNA synthesis depends on the ubiquitin-proteasome system. IAV replication was reduced both by proteasome inhibitors and in E36ts20 cells, which contain the thermolabile ubiquitin-activating enzyme E1. While virus entry was not affected in E36ts20 cells, the proteasome inhibitor MG132 retained viral particles in the cytoplasm. Addition-removal experiments of MG132 in combination with bafilomycin A1, a well-established inhibitor of IAV entry and fusion, showed that MG132 affected IAV infection at a postfusion step. This was confirmed by the lack of inhibition of IAV entry by proteasome inhibitors in a virus-like particle fusion assay.	Widjaja et al. 2010
Host factor targeting Genome-wide RNA interference screening 295 cellular cofactors	Influenza A virus is an RNA virus that encodes up to 11 proteins and this small coding capacity demands that the virus use the host cellular machinery for many aspects of its life cycle. Knowledge of these host cell requirements not only informs us of the molecular pathways exploited by the virus but also provides further targets that could be pursued for antiviral drug development. Here we use an integrative systems approach, based on genome-wide RNA interference screening, to identify 295 cellular cofactors required for early-stage influenza virus replication. Within this group, those involved in kinase-regulated signalling, ubiquitination and phosphatase activity are the most highly enriched, and 181 factors assemble into a highly significant host-pathogen interaction network. Moreover, 219 of the 295 factors were confirmed to be required for efficient wild-type influenza virus growth, and further analysis of a subset of genes showed 23 factors necessary for viral entry, including members of the vacuolar ATPase (vATPase) and COPI-protein families, fibroblast growth factor receptor (FGFR) proteins, and glycogen synthase kinase 3 (GSK3)-beta. Furthermore, 10 proteins were confirmed to be involved in post-entry steps of influenza virus replication. These include nuclear import components, proteases, and the calcium/calmodulin-dependent protein kinase (CaM kinase) II beta (CAMK2B). Notably, growth of swine-origin H1N1 influenza virus is also dependent on the identified host factors, and we show that small molecule inhibitors of several factors, including vATPase and CAMK2B, antagonize influenza virus replication.	Konig et al. 2010

Table 4.6. Other anti-influenza agents

Nucleoprotein inhibitors Nucleozin Fluorescence-quenching effect	Recent studies have shown that NP (nucleoprotein), which possesses multiple functions in the viral life cycle, is a new potential anti-influenza drug target. NP inhibitors reliably induce conformational changes in NPs, and these changes may confer inhibition of the influenza virus. The six conserved tryptophan residues in NP can be used as an intrinsic probe to monitor the change in fluorescence of the tryptophan residues in the protein upon binding to an NP inhibitor. In the present study, we found that the fluorescence of recombinant NP proteins was quenched following the binding of available NP inhibitors (such as nucleozin) in a concentration- and time-dependent manner, which suggests that the inhibitor induced conformational changes in the NPs. The minimal fluorescence-quenching effect and weak binding constant of nucleozin to the swine-origin influenza virus H1N1pdm09 (SOIV) NP revealed that the SOIV is resistant to nucleozin. We have used the fluorescence-quenching property of tryptophans in NPs that were bound to ligands in a 96-well-plate-based drug screen to assess the ability of promising small molecules to interact with NPs and have identified one new anti-influenza drug, CSV0C001018, with a high SI value. This convenient method for drug screening may facilitate the development of antiviral drugs that target viruses other than the influenza virus, such as HIV and HBV.	Hung et al. 2012
Nucleoprotein inhibitors Nucleozin	The influenza virus nucleoprotein (NP) is an emerging target for anti-influenza drug development. Nucleozin (1) and its closely related derivatives had been identified as NP inhibitors displaying anti-influenza activity. Utilizing 1 as a lead molecule, we successfully designed and synthesized a series of 1H-1,2,3-triazole-4-carboxamide derivatives as new anti-influenza A agents. One of the most potent compounds, 3b, inhibited the replication of various H3N2 and H1N1 influenza A virus strains with IC50 values ranging from 0.5 to 4.6 μM. Compound 3b also strongly inhibited the replication of H5N1 (RG14), amantidine-resistant A/WSN/33 (H1N1), and oseltamivir-resistant A/WSN/1933 (H1N1, 274Y) virus strains with IC50 values in sub-mu M ranges. Further computational studies and mechanism investigation suggested that 3b might directly target influenza virus A nucleoprotein to inhibit its nuclear accumulation.	Cheng et al. 2012
Inhibitors of non-structural protein	The innate immune system guards against virus infection through a variety of mechanisms including mobilization of the host interferon system, which attacks viral products mainly at a posttranscriptional level. The influenza virus NS1 protein is a multifunctional facilitator of virus replication, one of whose actions is to antagonize the interferon response. Since NS1 is required for efficient virus replication, it was reasoned that chemical inhibitors of this protein could be used to further understand virus-host interactions and also serve as potential new antiviral agents. A yeast-based assay was developed to identify compounds that phenotypically suppress NS1 function. Several such compounds exhibited significant activity specifically against influenza A virus in cell culture but had no effect on the replication of another RNA virus, respiratory syncytial virus. Interestingly, cells lacking an interferon response were drug resistant, suggesting that the compounds block interactions between NS1 and the interferon system. Accordingly, the compounds reversed the inhibition of beta interferon mRNA induction during infection, which is known to be caused by NS1. In addition, the compounds blocked the ability of NS1 protein to inhibit double-stranded RNA-dependent activation of a transfected beta interferon promoter construct. The effects of the compounds were specific to NS1, because they had no effect on the ability of the severe acute respiratory syndrome coronavirus papainlike protease protein to block beta interferon promoter activation. These data demonstrate that the function of NS1 can be modulated by chemical inhibitors and that such inhibitors will be useful as probes of biological function and as starting points for clinical drug development.	Basu et al. 2009

Function of NS1 protein Inactivation of NF-kappa B Prevention of interferon induction	The alpha/beta interferon (IFN-alpha/beta) system represents one of the first lines of defense against virus infections. As a result, most viruses encode IFN antagonistic factors which enhance viral replication in their hosts. We have previously shown that a recombinant influenza A virus lacking the NS1 gene (delNS1) only replicates efficiently in IFN-alpha/beta -deficient systems. Consistent with this observation, we found that infection of tissue culture cells with delNS1 virus, but not with wild-type influenza A virus, induced high levels of mRNA synthesis from IFN-alpha/beta genes, including IFN-beta. It is known that transactivation of the IFN-beta promoter depends on NF-kappaB and several other transcription factors. Interestingly, cells infected with delNS1 virus showed high levels of NF-kappaB activation compared with those infected with wild-type virus. Expression of dominant-negative inhibitors of the NF-kappaB pathway during deINS1 virus infection prevented the transactivation of the IFN-beta promoter, demonstrating a functional link between NF-kappaB activation and IFN-alpha/beta synthesis in deINS1 virus-infected cells. Moreover, expression of the NS1 protein prevented virus- and/or double-stranded RNA (dsRNA)-mediated activation of the NF-kappaB pathway and of IFN-beta synthesis. This inhibitory property of the NS1 protein of influenza A virus was dependent on its ability to bind dsRNA, supporting a model in which binding of NS1 to dsRNA generated during influenza virus infection prevents the activation of the IFN system. NS1-mediated inhibition of the NF-kappaB pathway may thus play a key role in the pathogenesis of influenza A virus.	Wang et al. 2000
RNA interference Short interfering RNAs Viral mRNA degradation	Highly pathogenic avian influenza (HPAI) H5N1 virus is endemic in many regions around the world and remains a significant pandemic threat. To date H5N1 has claimed almost 300 human lives worldwide, with a mortality rate of 60% and has caused the death or culling of hundreds of millions of poultry since its initial outbreak in 1997. We have designed multifunctional RNA interference (RNAi)-based therapeutics targeting H5N1 that degrade viral mRNA via the RNAi pathway while at the same time augmenting the host antiviral response by inducing host type I interferon (IFN) production. Moreover, we have identified two factors critical for maximising the immunostimulatory properties of short interfering (si) RNAs in chicken cells (i) mode of synthesis and (ii) nucleoside sequence to augment the response to virus. The 5-bp nucleoside sequence 5'-UGUGU-3' is a key determinant in inducing high levels of expression of IFN-alpha, -beta, -lambda and interleukin 1-beta in chicken cells. Positioning of this 5'-UGUGU-3' motif at the 5'- end of the sense strand of siRNAs, but not the 3'- end, resulted in a rapid and enhanced induction of type I IFN. An anti-H5N1 avian influenza siRNA directed against the PB1 gene (PB1-2257) tagged with 5'-UGUGU-3' induced type I IFN earlier and to a greater extent compared to a non-tagged PB1-2257. Tested against H5N1 in vitro, the tagged PB1-2257 was more effective than non-tagged PB1-2257. These data demonstrate the ability of an immunostimulatory motif to improve the performance of an RNAi-based antiviral, a finding that may influence the design of future RNAi-based anti-influenza therapeutics.	Stewart et al. 2011
RNA interference Short interfering RNAs Silence gene expression Viral mRNA degradation	RNA interference (RNAi) is a powerful tool to silence gene expression. Small interfering RNA (siRNA)-induced RNA degradation has been recently used as an antivirus agent to inhibit specific virus replication. Here, we showed that several siRNAs specific for conserved regions of influenza virus matrix (M2) and nucleocapsid protein (NP) genes could effectively inhibit expression of the corresponding viral protein. We also evaluated the antiviral potential of these siRNAs targeting M2 and NP of H5N1 avian influenza virus (AIV), which are essential to viral replication. We investigated the inhibitory effect of M2-specific siRNAs and NP-specific siRNAs on influenza A virus (H5N1, H1N1 and H9N2) replication in Madin-Darby canine kidney (MDCK) cells and BALB/c mice. The results showed that treatment with these siRNAs could specifically inhibit influenza A virus replication in MDCK cells (0.51–1.63 TCID50 reduction in virus titers), and delivery of pS-M48 and pS-NP1383 significantly reduced lung virus titers in the infected mice (16–50-fold reduction in lung virus titers) and partially protected the mice from lethal influenza virus challenge (a survival rate of 4/8 for H1N1 virus-infected mice and 2/8 for H5N1 virus infected mice). Moreover, the treatment of pS-M48 and pS-NP1383 could suppress replication of different subtypes of influenza A viruses, including a H5N1 highly pathogenic avian isolate strain. The results provided a basis for further development of siRNA for prophylaxis and therapy of influenza virus infection in humans and animals.	Zhou et al. 2007

RNA interference Short interfering RNAs siRNA-resistant viruses H1-short hairpin RNA	RNA interference (RNAi) provides a powerful new means to inhibit viral infection specifically. However, the selection of siRNA-resistant viruses is a major concern in the use of RNAi as antiviral therapeutics. In this study, we conducted a lentiviral vector with a H1-short hairpin RNA (shRNA) expression cassette to deliver small interfering RNAs (siRNAs) into mammalian cells. Using this vector that also expresses enhanced green fluorescence protein (EGFP) as surrogate marker, stable shRNA-expressing cell lines were successfully established and the inhibition efficiencies of rationally designed siRNAs targeting to conserved regions of influenza A virus genome were assessed. The results showed that a siRNA targeting influenza M2 gene (siM2) potently inhibited viral replication. The siM2 was not only effective for H1N1 virus but also for highly pathogenic avian influenza virus H5N1. In addition to its M2 inhibition, the siM2 also inhibited NP mRNA accumulation and protein expression. A long term inhibition effect of the siM2 was demonstrated and the emergence of siRNA-resistant mutants in influenza quasispecies was not observed. Taken together, our study suggested that M2 gene might be an optimal RNAi target for antiviral therapy. These findings provide useful information for the development of RNAi-based prophylaxis and therapy for human influenza virus infection.	Sui et al. 2009
Alkaloids from Amaryllidaceae Lycorine Haemanthamine	Influenza A viruses occasionally cause large epidemics and kill thousands every year. While little is known about the mechanism of cell fusion in diseases, especially influenza virus infected and protection from Amaryllidaceae Alkaloids. The two compounds lycorine (AA1) and haemanthamine (AA3) were obtained from bulbs of L. radiate, exhibited anti-influenza activity after influenza virus entry cells. The two compounds were investigated for in vitro displaying different levels of resistance to pro-apoptotic stimuli. Seven influenza viruses were used, A/CK/GD/178/04 (H5N, 178), A/DK/GD/212/04 (H5N1, 212), A/swine/GD/166/06 (H3N2, 166), A/CK/HN/170/03 (H1N1, 170), A/Puerto Rico/8/34 (H1N1, PR8), A/Ck/GD/400/07 (H9N2, 400), A/Ck/GD/228/04 (H9N2, 228), the two compounds exhibited potency in the single-digit micromolar range. The studies also showed that AA1 and AA3 exerted their in vitro anti-influenza activity through cytostatic rather than cytotoxic effects. Many viruses interact with the host cells to change their own growth which they favor the speed. The cells infected with virus, growth of MDCK cells was slowed down by arresting cell cycle at G1/S phase. With the compound treated, the growth cycle was decreased in S phase. With H5N1 influenza virus treated, the cytoskeleton of cells was changed while with the compound treated the protection of cytoskeleton was obviously protected. The data showed differences between drug treated cells and virus infected cells, provided a basis to further explore cell fusion and anti-influenza mechanism of the two compounds.	He et al. 2012
Polysaccharides from seaweeds Sulfated fucans Heteroglycuronans	To explore the polysaccharides from selected seaweeds of Atlantic Canada and to evaluate their potential anti-influenza virus activities, polysaccharides were isolated from several Atlantic Canadian seaweeds, including three red algae (Polysiphonia lanosa, Furcellaria lumbricalis, and Palmaria palmata), two brown algae (Ascophyllum nodosum and Fucus vesiculosus), and one green alga (Ulva lactuca) by sequential extraction with cold water, hot water, and alkali solutions. These polysaccharides were analyzed for monosaccharide composition and other general chemical properties, and they were evaluated for anti-influenza virus activities. Total sugar contents in these polysaccharides ranged from 15.4% (in U. lactuca) to 91.4% (in F. lumbricalis); sulfation level was as high as 17.6% in a polysaccharide from U. lactuca, whereas it could not be detected in an alikali-extract from P. palmaria. For polysaccharides from red seaweeds, the main sugar units were sulfated galactans (agar or carrageenan) for P. lanosa, F. lumbricalis, and xylans for P. palmata. In brown seaweeds, the polysaccharides largely contained sulfated fucans, whereas the polysaccharides in green seaweed were mainly composed of heteroglycuronans. Screening for antiviral activity against influenza A/PR/8/34 (H1N1) virus revealed that brown algal polysaccharides were particularly effective. Seaweeds from Atlantic Canada are a good source of marine polysaccharides with potential antiviral properties.	Jiao et al. 2012

Carrageenans from red algae Kappa-carrageenan oligosaccharides	Carrageenans, the sulphated galactans derived from red algae, are attracting increasing interest in developing potential anti-viral drugs. In this study, low molecular weight kappa-carrageenan oligosaccharides (KCO) and their sulphated derivatives (KCO-S) were prepared, and their anti-influenza A virus (IAV) properties were investigated. The results indicated that KCO and KCO-S could effectively inhibit IAV multiplication in MDCK cells in a dose-dependent manner. Furthermore, a structure-activity relationship study showed that the degree of sulphation and molecular weight were the main factors that influenced the anti-IAV activity of kappa-carrageenan oligosaccharide. The most active kappa-carrageenan oligosaccharide had a sulphate content of 0.8–1.0 mole/mole of disaccharide and a molecular weight of 1–3 kDa. In addition, KCO and KCO-S could significantly improve survival and decrease pulmonary viral titres in IAV-infected mice. Moreover, the antiviral effect of KCO and KCO-S does not seem to be dependent on the interferon system. In conclusion, carrageenan oligosaccharide and its sulphated derivative have good inhibitory actions on IAV replication in vitro and in vivo.	Wang et al. 2012
Substances from Mosla dianthera Phenolic sesquiterpenes Aromatic compounds	Ethnopharmacological relevance: Mosla dianthera as an aromatic herb is used in folk medicine for the treatment of cough, colds, fever, bronchitis, nasal congestion and headache. Aim of the study: To characterize chemical compositions and to evaluate the anti-influenza effects of essential oils of M. dianthera (MDEO) in influenza virus A (IVA) infected mice. Materials and methods: MDEO was obtained by hydrodistillation and analysed by gas chromatography-mass spectrometry (GC-MS). ICR mice were treated with MDEO for 5 consecutive days at doses of 90–360 mg/kg after post-infected. Levels of Serum IL-4 and IFN-gamma were assayed by ELISA. Levels of MOD, SOD, TAOC and GSH-Px in lung tissue were determined by colorimetric method. Results: GC-MS analysis revealed the presence of 29 components that account for 97.74% of phenolic sesquiterpenes and aromatic compounds. The major compounds were elemicin (16.51%), thymol (14.77%), beta-caryophyllene (14.49%), iso-elemicin (9.22%), asarone (6.09%) and alpha-caryophyllene (5.26%). It had significant effects on decreasing lung viral titers, inhibiting pneumonia, reducing levels of serum IFN-gamma and IL-4, and enhancing antioxidant activity in the lung tissue of IVA infected mice. Conclusions: MPE could exhibit therapeutical effects in IVA infected mice as a suppressor of IVA replication and inflammatory mediators and a promoter of antioxidant potentials. Therefore, MDEO could provide a safe and effective therapeutic candidate for treatment of influenza and its subsequent viral pneumonia.	Wu et al. 2012
Inhibitors from herbal extract Berberine	Objective: To explore the potential effects of berberine on influenza virus infection both in vitro and in vivo. Methods: In vitro anti-influenza virus assays were performed by cytopathogenic effect and neuraminidase assays in Madin Darby canine kidney cells. In vivo anti-influenza virus assays were performed on the viral pneumonia model of mice. The numbers of mice that died within day 2 to day 14 postinfection were recorded to calculate the mortality. On days 2, 4, and 6, the viral titers in the lungs were determined by hemagglutination assay; hematoxylin/eosin staining was used to assess the pathogenic changes of lung tissues; the concentrations of tumor necrosis factor-alpha (TNF-alpha) and monocyte specific chemoattractant molecule (MCP-1) were measured by radio immunoassay or enzyme-linked immunosorbent assay; the concentrations of nitric oxide (NO) and inducible nitric oxide synthetase (iNOS) were detected by colorimetric method; reverse transcription polymerase chain reaction was used to detect the mRNA level of TNF-alpha and MCP-1. Results: Berberine showed inhibitory effects on cytopathogenic effects and neuraminidase activity of virus, with the therapeutic index 9.69. In vivo, berberine decreased mice mortality from 90% to 55%, reduced virus titers in the lungs on day 2 postinfection ($P < 0.05$). The lung histology scores were 1.50 ±0.67, 4.50 ± 1.00, and 5.50 ± 1.00 in the berberine group on days 2, 4, and 6, respectively, which were significantly reduced compared to 2.17 ± 0.22, 6.83 ± 0.44, and 8.50 ± 0.33 in the infected group ($P < 0.05$). The productions of NO and iNOS were repressed by berberine compared with those in the infected group ($P < 0.01$). The transcription and expression of TNF- alpha were inhibited by berberine on day 4 ($P < 0.01$) and day 6 ($P < 0.05$), and those of MCP-1 were inhibited on day 6 ($P < 0.01$) compared with the infected group. Conclusions: Berberine exhibited antiviral effects on the influenza virus both in vitro and in vivo. The possible therapeutic mechanism of berberine on influenza-induced viral pneumonia might be inhibiting the virus infection, as well as improving the pathogenic changes by repressing inflammatory substances release.	Wu et al. 2011

Nanobodies Targeting of H5N1hemagglutinin	We assessed the protective potential of monovalent and bivalent Nanobodies (Ablynx) against challenge with this virus. These Nanobodies were derived from llamas and target H5N1 hemagglutinin. Intranasal administration of Nanobodies effectively controlled homologous influenza A virus replication. Administration of Nanobodies before challenge strongly reduced H5N1 virus replication in the lungs and protected mice from morbidity and mortality after a lethal challenge with H5N1 virus. The bivalent Nanobody was at least 60-fold more effective than the monovalent Nanobody in controlling virus replication. In addition, Nanobody therapy after challenge strongly reduced viral replication and significantly delayed time to death. Epitope mapping revealed that the VHH Nanobody binds to antigenic site B in H5 hemagglutinin. Because Nanobodies are small, stable, and simple to produce, they are a promising, novel therapeutic agent against influenza.	Ibanez et al. 2011
Nanobodies M2 protein targeting	Neutralizing antibodies against the highly conserved M2 ion channel is thought to offer broad protection against influenza A viruses. Here, we screened synthetic Camel single-domain antibody (VHH) libraries against native M2 ion channel protein. One of the isolated VHHs, M2-7A, specifically bound to M2-expressed cell membrane as well as influenza A virion, inhibited replication of both amantadine-sensitive and resistant influenza A viruses in vitro, and protected mice from a lethal influenza virus challenge. Moreover, M2-7A showed blocking activity for proton influx through M2 ion channel. These pieces of evidence collectively demonstrate for the first time that a neutralizing antibody against M2 with broad specificity is achievable, and M2-7A may have potential for cross protection against a number of variants and subtypes of influenza A viruses.	Wei et al. 2011
Humanised monoclonal antibodies Haemagglutinin targeting	Humanized monoclonal antibodies (mAbs) that neutralize H5N1 virus could be used as prophylaxis and treatment to aid in the containment of such a pandemic. Methods: Neutralizing mAbs against H5 hemagglutinin were humanized and introduced into C57BL/6 mice (1, 5, or 10 mg/kg bodyweight) one day prior to-, one day post- and three days post-lethal challenge with H5N1 A/Vietnam/1203/04 virus. Efficacy was determined by observation of weight loss as well as survival. Results: Two mAbs neutralizing for antigenically variant H5N1 viruses, A/Vietnam/1203/04 and A/Hong Kong/213/03 were identified and humanized without loss of specificity. Both antibodies exhibited prophylactic efficacy in mice, however, VN04-2-huG1 performed better requiring only 1 mg/kg bodyweight for complete protection. When used to treat infection VN04-2-huG1 was also completely protective, even when introduced three days post infection, although higher dose of antibody was required. Conclusion: Prophylaxis and treatment using neutralizing humanized mAbs is efficacious against lethal challenge with A/Vietnam/1203/04, providing proof of principle for the use of passive antibody therapy as a containment option in the event of pandemic influenza.	Hanson et al. 2006
Human monoclonal antibodies	Pre-existing neutralizing antibody provides the first line of defence against pathogens in general. For influenza virus, annual vaccinations are given to maintain protective levels of antibody against the currently circulating strains. Here we report that after booster vaccination there was a rapid and robust influenza-specific IgG^+ antibody- secreting plasma cell (ASC) response that peaked at approximately day 7 and accounted for up to 6% of peripheral blood B cells. These ASCs could be distinguished from influenza- specific IgG^+ memory B cells that peaked 14–21 days after vaccination and averaged 1% of all B cells. Importantly, as much as 80% of ASCs purified at the peak of the response were influenza specific. This ASC response was characterized by a highly restricted B-cell receptor (BCR) repertoire that in some donors was dominated by only a few B-cell clones. This pauciclonal response, however, showed extensive intraclonal diversification from accumulated somatic mutations. We used the immunoglobulin variable regions isolated from sorted single ASCs to produce over 50 human monoclonal antibodies (mAbs) that bound to the three influenza vaccine strains with high affinity. This strategy demonstrates that we can generate multiple high-affinity mAbs from humans within a month after vaccination. The panel of influenza-virus- specific human mAbs allowed us to address the issue of original antigenic sin (OAS): the phenomenon where the induced antibody shows higher affinity to a previously encountered influenza virus strain compared with the virus strain present in the vaccine (1). However, we found that most of the influenza- virus- specific mAbs showed the highest affinity for the current vaccine strain. Thus, OAS does not seem to be a common occurrence in normal, healthy adults receiving influenza vaccination.	Wrammert et al. 2008

Table 5. Combination therapy of influenza infections

Oseltamivir with amantadine Mouse model	In the present study, the effect of combining anti-influenza drugs active at different steps of the influenza virus replication cycle, oseltamivir as a neuraminidase (NA) inhibitor and amantadine targeting M2 protein, was investigated in vivo by oral administration in a mouse model of aerosol influenza virus infection and in vitro in MDCK cells. In mice, doses of oseltamivir and amantadine providing 50–60% survival against A/Hongkong/1/68 (H3N2) or A/PR/8/34 (H1N1) were capable of conferring complete protection when used simultaneously, suggesting that increased inhibition of influenza virus replication by combining oseltamivir and amantadine in vitro translates into protection from lethal infection of mice. The combination of amantadine with oseltamivir required 15-fold less oseltamivir than monotherapy to confer complete protection against lethal aerosol influenza virus infection. Remarkably, amantadine-based combination chemoprophylaxis was even effective against amantadine-resistant A/PR/8/34 influenza virus. Thus, combination chemotherapy may be more efficacious than monotherapy against newly emerging Influenza A subtypes.	Masihi et al. 2007
Zanamivir with immuno-modulators Celecoxib Gemfibrozil Mesalazine Mouse model	The mortality of human infection by influenza A/H5N1 virus can exceed 80%. The high mortality and its poor response to the neuraminidase inhibitor oseltamivir have been attributed to uncontrolled virus-induced cytokine storm. We challenged BALB/c mice with 1000 LD_{50} of influenza A/Vietnam/1194/04. Survival, body weight, histopathology, inflammatory markers, viral loads, T lymphocyte counts, and neutralizing antibody response were documented in infected mice treated individually or in combination with zanamvir, celecoxib, gemfibrozil, and mesalazine. To imitate the real-life scenario, treatment was initiated at 48 h after viral challenge. There were significant improvements in survival rate ($P = 0.02$), survival time ($P < 0.02$), and inflammatory markers ($P < 0.01$) in the group treated-with a triple combination of zanamivir, celecoxib, and mesalazine when compared with zanamivir alone. Zanamivir with or without immunomodulators reduced viral load to a similar extent. Insignificant prolongation of survival was observed when individual agents were used alone. Significantly higher levels of $CD4^+$ and $CD8^+$ T lymphocytes and less pulmonary inflammation were also found in the group receiving triple therapy. Zanamivir alone reduced viral load but not inflammation and mortality. The survival benefits of adding celecoxib and mesalazine to zanamivir could be caused by their synergistic effects in reducing cytokine dysfunction and preventing apoptosis. Combinations of a neuraminidase inhibitor with these immunomodulators should be considered in randomized controlled treatment trials of patients suffering from H5N1 infection.	Zheng et al. 2008
Oseltamivir with corticosteroids Methylprednisolone Hydrocortisone Lung injury acute respiratory distress syndrome	PURPOSE: During the 2009 H1N1 influenza A virus pandemic, a minority of patients developed rapidly progressive pneumonia leading to acute lung injury (ALI)-acute respiratory distress syndrome (ARDS). A recent meta-analysis provides support for prolonged corticosteroid treatment in ALI-ARDS. We prospectively evaluated the response to oseltamivir and prolonged corticosteroid treatment in patients with ALI-ARDS and suspected H1N1 influenza. METHODS: From June 24 through 12 July 2009, 13 patients with suspected H1N1 pneumonia and ALI-ARDS were admitted to the intensive care unit (ICU) of a tertiary care hospital. H1N1 influenza was confirmed with real-time reverse transcriptase-polymerase chain reaction assay in eight patients. Oseltamivir and corticosteroid treatment were initiated concomitantly at ICU admission; those with severe ARDS received methylprednisolone (1 mg/kg/day), and others received hydrocortisone (300 mg/day) for a duration of 21 ± 6 days. RESULTS: Patients with and without confirmed H1N1 influenza had similar disease severity at presentation and a comparable response to treatment. By day 7 of treatment, patients experienced a significant improvement in lung injury and multiple organ dysfunction scores ($P < 0.001$). Twelve patients (92%) improved lung function, were extubated, and discharged alive from the ICU. Hospital length of stay and mortality were 18.7 ± 9.6 days and 15%, respectively. Survivors were discharged home without oxygen supplementation. CONCLUSIONS: In ARDS patients, with and without confirmed H1N1 influenza, prolonged low-to-moderate dose corticosteroid treatment was well tolerated and associated with significant improvement in lung injury and multiple organ dysfunction scores and a low hospital mortality. These findings provide the rationale for developing a randomized trial.	Quispe-Laime et al. 2010

Laninamivir octanoate with artificial surfactant Mouse model	Background: Patients with influenza virus infection can develop severe pneumonia and acute respiratory distress syndrome (ARDS) which have a high mortality. Influenza virus infection is treated worldwide mainly by neuraminidase inhibitors (NAIs). However, monotherapy with NAIs is insufficient for severe pneumonia secondary to influenza virus infection. We previously demonstrated that mice infected with a lethal dose of influenza virus develop diffuse alveolar damage (DAD) with alveolar collapse similar to that seen in ARDS in humans. Additionally, pulmonary surfactant proteins were gradually increased in mouse serum, suggesting a decrease in pulmonary surfactant in the lung. Therefore, the present study examined whether combination therapy of NAI with exogenous artificial surfactant affects mortality of influenza virus-infected mice. Methodology/Principal Findings: BALB/c mice were inoculated with several viral doses of influenza A/Puerto Rico/8/34 (PR8) virus (H1N1). The mice were additionally administered exogenous artificial surfactant in the presence or absence of a new up to 20 days after inoculation. Viral titer and cytokine/chemokine levels in the lungs, lung weight, pathological analysis, and blood O_2 and CO_2 pressures were evaluated. Infected mice treated with combination therapy of laninamivir octanoate with artificial surfactant showed a significantly higher survival rate compared with those that received laninamivir octanoate monotherapy (P = 0.003). However, virus titer, lung weight and cytokine/chemokine responses were not different between the groups. Histopathological examination, a hydrostatic lung test and blood gas analysis showed positive results in the combination therapy group. Conclusions/Significance: Combination therapy of laninamivir octanoate with artificial surfactant reduces lethality in mice infected with influenza virus, and eventually suppresses DAD formation and preserves lung function. This combination could be effective for prevention of severe pneumonia secondary to influenza virus infection in humans, which is not improved by NAI monotherapy.	Fukushi et al. 2012
Oseltamivir with ketotifen Mast cell degranulation inhibitor In vitro/in vivo tests	Although an important role for mast cells in several viral infections has been demonstrated, its role in the invasion of highly pathogenic H5N1 influenza virus is unknown. In the present study, we demonstrate that mast cells were activated significantly by H5N1 virus (A/chicken/Henan/1/2004) infection both in vivo and in vitro. Mast cells could possibly intensify the lung injury that results from H5N1 infection by releasing proinflammatory mediators, including histamine, tryptase, and gamma interferon (IFN-gamma). Lung lesions and apoptosis induced by H5N1 infection were reduced dramatically by treatment with ketotifen, which is a mast cell degranulation inhibitor. A combination of ketotifen and the neuraminidase inhibitor oseltamivir protected 100% of the mice from death postinfection. In conclusion, our data suggest that mast cells play a crucial role in the early stages of H5N1 influenza virus infection and provide a new approach to combat highly pathogenic influenza virus infection.	Hu et al. 2012
Dual-targeted bifunctional anti-influenza drugs Anti-inflammatory effect Caffeic acid-zanamivir conjugate In vivo/in vitro tests	Influenza therapy with a single targeted compound is often limited in efficacy due to the rapidly developed drug resistance. Moreover, the uncontrolled virus-induced cytokines could cause the high mortality of human infected by H5N1 avian influenza virus. In this study, we explored the novel dual-targeted bifunctional anti-influenza drugs formed by conjugation with anti-inflammatory agents. In particular, the caffeic acid (CA)-bearing zanamivir (ZA) conjugates ZA-7-CA (1) and ZA-7-CA-amide (7) showed simultaneous inhibition of influenza virus neuraminidase and suppression of pro-inflammatory cytokines. These ZA conjugates provided remarkable protection of cells and mice against influenza infections. Intranasal administration of low dosage (<1.2 mu mol/kg/day) of ZA conjugates exhibited much greater effect than the combination therapy with ZA and the anti-inflammatory agents in protection of the lethally infected mice by H1N1 or H5N1 influenza viruses.	Liu et al. 2012

Neuraminidase inhibitors with rimantadine Additive and synergistic effects In vitro tests	There is insufficient information about combination therapy with approved anti-influenza agents. We tested combinations that paired a neuraminidase (NA) inhibitor (zanamivir, oseltamivir carboxylate, or peramivir) with rimantadine against infection of MDCK cells with H1N1 and H3N2 subtypes of influenza A virus and characterized their mode of interaction. When reduction of extracellular virus was analyzed by individual regression models and three-dimensional representations of the data, all three combinations showed additive and synergistic effects with no cytotoxicity. Maximum synergy against A/New Caledonia/20/99 (H1N1) virus infection was observed with < 2.5 μM rimantadine paired with low concentrations of NA inhibitors. All combinations reduced the extracellular yield of A/Panama/2007/99 (H3N2) influenza virus synergistically. However, our findings were different for the cell-associated virus yield. At some drug concentrations, the yield of cell-associated virus was inhibited antagonistically. Therefore, the method of analysis can be a crucial factor in evaluating the interactions of drugs with different mechanisms. We hypothesize that assays based on cell-associated virus yield may underestimate the efficacies of drug combinations that include an NA inhibitor. Taken together, our results suggest that regimens that combine NA inhibitors and rimantadine exert synergistic anti-influenza effects in vitro. These findings provide baseline information for therapeutic testing of the drug combinations in vivo.	Govorkova et al. 2004
Triple-combination antiviral drug therapy Amantadine Ribavirin Oseltamivir Adult patients	A recent in vitro study showed that the three compounds of antiviral drugs with different mechanisms of action (amantadine, ribavirin, and oseltamivir) could result in synergistic antiviral activity against influenza virus. However, no clinical studies have evaluated the efficacy and safety of combination antiviral therapy in patients with severe influenza illness. A total of 245 adult patients who were critically ill with confirmed pandemic influenza A/H1N1 2009 (pH1N1) virus infection and were admitted to one of the intensive care units of 28 hospitals in Korea were reviewed. Patients who required ventilator support and received either triple-combination antiviral drug (TCAD) therapy or oseltamivir monotherapy were analyzed. A total of 127 patients were included in our analysis. Among them, 24 patients received TCAD therapy, and 103 patients received oseltamivir monotherapy. The 14-day mortality was 17% in the TCAD group and 35% in the oseltamivir group (P = 0.08), and the 90-day mortality was 46% in the TCAD group and 59% in the oseltamivir group (P = 0.23). None of the toxicities attributable to antiviral drugs occurred in either group of our study, including hemolytic anemia and hepatic toxicities related to the use of ribavirin. Logistic regression analysis indicated that the odds ratio for the association of TCAD with 90-day mortality was 0.58 (95% confidence interval, 0.24 to 1.42; P = 0.24). Although this study was retrospective and did not provide virologic outcomes, our results suggest that the treatment outcome of the triple combination of amantadine, ribavirin, and oseltamivir was comparable to that of oseltamivir monotherapy.	Kim et al. 2011
Triple-combination antiviral drug therapy Amantadine Ribavirin Oseltamivir Dose-dependent protection Synergistic effect Broad-spectrum activity Mouse model	The limited efficacy of existing antiviral therapies for influenza – coupled with widespread baseline antiviral resistance – highlights the urgent need for more effective therapy. We describe a triple combination antiviral drug (TCAD) regimen composed of amantadine, oseltamivir, and ribavirin that is highly efficacious at reducing mortality and weight loss in mouse models of influenza infection. TCAD therapy was superior to dual and single drug regimens in mice infected with drug-susceptible, low pathogenic A/H5N1 (A/Duck/MN/1525/81) and amantadine-resistant 2009 A/H1N1 influenza (A/California/04/09). Treatment with TCAD afforded > 90% survival in mice infected with both viruses, whereas treatment with dual and single drug regimens resulted in 0% to 60% survival. Importantly, amantadine had no activity as monotherapy against the amantadine-resistant virus, but demonstrated dose-dependent protection in combination with oseltamivir and ribavirin, indicative that amantadine's activity had been restored in the context of TCAD therapy. Furthermore, TCAD therapy provided survival benefit when treatment was delayed until 72 hours post-infection, whereas oseltamivir monotherapy was not protective after 24 hours post-infection. These findings demonstrate in vivo efficacy of TCAD therapy and confirm previous reports of the synergy and broad spectrum activity of TCAD therapy against susceptible and resistant influenza strains in vitro.	Nguyen et al. 2012

Combination of favipiravir and oseltamivir Mouse model	Favipiravir (T-705 [6-fluoro-3-hydroxy-2-pyrazinecarboxamide]) and oseltamivir were combined to treat influenza virus A/NWS/33 (H1N1), A/Victoria/3/75 (H3N2), and A/Duck/MN/1525/81 (H5N1) infections. T-705 alone inhibited viruses in cell culture at 1.4 to 4.3 μM. Oseltamivir inhibited these three viruses in cells at 3.7, 0.02, and 0.16 μM and in neuraminidase assays at 0.94, 0.46, and 2.31 nM, respectively. Oral treatments were given twice daily to mice for 5 to 7 days starting, generally, 24 h after infection. Survival resulting from 5 days of oseltamivir treatment (0.1 and 0.3 mg/kg/day) was significantly better in combination with 20 mg/kg of body weight/day of T-705 against the H1N1 infection. Treatment of the H3N2 infection required 50 mg/kg/day of oseltamivir for 7 days to achieve 60% protection; 25 mg/kg/day was ineffective. T-705 was ≥ 70% protective at 50 to 100 mg/kg/day but inactive at 25 mg/kg/day. The combination of inhibitors (25 mg/kg/day each) increased survival to 90%. The H5N1 infection was not benefited by treatment with oseltamivir (≤ 100 mg/kg/day for 7 days). T-705 was 30 to 70% protective at 25 to 100 mg/kg/day. Survival improved slightly with combination treatments. Increased activity was seen against H5N1 infection by starting treatments 2 h before infection. Oseltamivir was ineffective at ≤ 40 mg/kg/day. T-705 was 100% protective at 40 and 80 mg/kg/day and inactive at 20 mg/kg/day. Combining ineffective doses (20 mg/kg/day of T-705 and 10 to 40 mg/kg/day of oseltamivir) afforded 60 to 80% protection and improved body weights during infection. Thus, synergistic responses were achieved with low doses of T-705 combined with oseltamivir. These compounds may be viable candidates for combination treatment of human influenza infections.	Smee et al. 2010
Oseltamivir with zanamivir Transmission in households	Background: The effectiveness of neuraminidase inhibitors to reduce transmission when used as treatment in influenza-infected patients remains debated. Methods: In a prespecified analysis of a blinded randomized controlled trial on the efficacy of oseltamivir-zanamivir combination therapy versus oseltamivir and zanamivir monotherapy conducted during the 2008–2009 seasonal influenza epidemic, we compared the rate of secondary illness in household contacts of influenza-positive index patients between arms. Secondary illness was defined as occurrence in contacts of fever plus cough within 7 days from randomization of index patients. Analyses were conducted according to the delay between patients' onset of symptoms and intervention. Results: A total of 543 household contacts of 267 index patients were included, of which 466 had follow-up assessment. A secondary illness was reported in 58 (12.5%) contacts with no significant difference between arms overall (P = 0.07). When the analysis was limited to the 232 contacts of 136 index patients with first treatment intake within 24 h of onset of symptoms, a lower rate of secondary illness was reported in the combination therapy arm (2 of 56 [4%]) than in the oseltamivir arm (14 of 81 [17%]; P = 0.014) and the zanamivir arm (14 of 95 [15%]; P = 0.031). Multivariate analysis accounting for intra-household correlation confirmed these findings. Conclusions: Our analysis suggests a greater effectiveness of the combination therapy to reduce transmissibility when given to the index patient within 24 h of onset of symptoms. As the finding was obtained from a subgroup analysis, it should be interpreted with caution.	Carrat et al. 2012

Table 6. Influenza drug resistance

Mechanism of drug resistance Neuraminidase mutants Tamiflu Relenza	New mutants of human influenza virus (A/H1N1) exhibit resistance to antiviral drugs. The mechanism whereby they develop insensitivity to these medications is, however, not yet completely understood. A crystallographic structure of A/H1N1 neuraminidase has been published recently. Using molecular dynamic simulations, it is now possible to characterize at the atomic level the mechanism that underlies the loss of binding affinity of the drugs. In this study, free-energy perturbation was used to evaluate the relative binding free energies of Tamiflu and Relenza with H274Y, N294S, and Y252H neuraminidase mutants. Our results demonstrate a remarkable correlation between theoretical and experimental data, which quantitatively confirms that the mutants are resistant to Tamiflu but are still strongly inhibited by Relenza. The simulations further reveal the key interactions that govern the affinity of the two drugs for each mutant. This information is envisioned to prove useful for the design of novel neuraminidase inhibitors and for the characterization of new potential mutants.	Vergara-Jaque et al. 2012
Resistance to zanamivir and oseltamivir Molecular markers of resistance Pyrosequencing	We report here the design of a pyrosequencing approach for the detection of molecular markers of resistance to the neuraminidase inhibitors zanamivir and oseltamivir in influenza viruses of type B. Primers were designed to analyze the sequences at eight amino acid positions E119, R152, D198. 1222, S250, H274, R371, and G402 (universal A/N2 numbering) in the neuraminidase (NA) which have been previously found to be associated with resistance or reduced susceptibility to oseltamivir and/or zanamivir in the NA inhibition assay. In addition, the designed primers could be utilized to the distinguish between the NAs of influenza B viruses from the two major lineages (Victoria and Yamagata) that have co-circulated globally in recent years, thus providing a valuable tool for virus strain surveillance.	Sheu et al. 2010
Resistance to oseltamivir Five years of non-prescription oseltamivir New Zealand	In 2007 New Zealand (NZ) became the first country to make oseltamivir (Tamiflu) available off-prescription. This study investigated the extent of pharmacist supply of oseltamivir over 5 years, including during the influenza A(H1N1) pandemic, and the impact of pharmacist supply of oseltamivir on influenza virus oseltamivir susceptibility, personal stockpiling and influenza vaccine uptake. Randomly selected community pharmacies in NZ reported oseltamivir provision by prescription and through pharmacist supply from 1 January 2007 to 15 September 2011. Oseltamivir resistance data on influenza viruses isolated during influenza surveillance from 2008 to 2011 were obtained, along with influenza vaccine uptake data from 2005 to 2011 and influenza detection data. Seventy of 85 eligible pharmacies completed the study (82 response rate). Most supplies of oseltamivir throughout the 5 years were dispensed against a prescription rather than pharmacist supplied, with pharmacist supply responsible for 11 of supplies during the pandemic years (2009–2010) versus 27 and 31 during 2007 and 2008, respectively. Pharmacist-supplied oseltamivir did not appear to be associated with the development of resistance, with identified likely stockpiling or with a decline in influenza immunization. Pharmacist supplies largely matched the timing of influenza in the community and peaked in June 2009, as did prescription supplies. Five years of non-prescription oseltamivir in NZ has resulted in no significant change in the development of resistance or rates of influenza immunization. Supplies remained modest and significant consumer stockpiling through pharmacist supply has not occurred, even during the influenza A(H1N1)pdm09 pandemic in 2009 and 2010. Pharmacists could be better utilized in ensuring fast distribution of antivirals to influenza sufferers during a pandemic	Gauld et al. 2012

Resistance to amantadine Amantadine treated children Sequencing	Clinical samples from 15 amantadine-treated children were collected serially-before, during, and/or after treatment and were studied to determine the actual prevalence, timing, and clinical implications of M2 mutational events. After viral RNA extraction and reverse-transcriptase polymerase chain reaction amplification of the viral RNA encoding the M2 protein, the products were cloned into plasmids, and their sequences were determined. Five mutations known to confer amantadine resistance in clinical samples were identified in 12 (80%) of 15 evaluable patients, and 9 patients had > 1 (2–4) mutant virus. The pattern of emergence of mutant strains was clarified from the study of 6 patients with at least 4 serial samples. Although viruses with M2 mutations tended to become the dominant populations, in 2 cases, wild-type viruses became dominant after decreasing to low levels. These results suggest that resistant viruses emerge in a much higher proportion of amantadine-treated patients than has been suggested by previous studies.	Shiraishi et al. 2003
Resistance to amantadine Amino acid substitutions	In two influenza seasons during which H1N1 and H3N2 cocirculated, resistance was more frequent in H3N2 strains than in H1N1 strains after amantadine treatment. Predominant amino acid substitutions in M2 protein occurred at position 31 (serine to asparagine) in H3N2 strains and at position 27 (valine to alanine) in H1N1 strains.	Saito et al. 2003
Resistance to adamantanes 281 influenza isolates Australia Europe Asia	The adamantanes (amantadine and rimantadine) were the initial antivirals licensed for use against influenza A viruses and have been used in some countries to control seasonal influenza and have also been stockpiled for potential pandemic use. While high rates of resistance have been observed in recent years with A(H3) viruses, the rates of resistance with A(H1) viruses has varied widely. In this study we analysed 281 human influenza A viruses isolated in 2007 that were referred to the WHO Collaborating Centre for Reference and Research in Melbourne, mainly from Australia and the surrounding regions, for evidence of resistance to adamantanes and a subset of these was examined for resistance to the neuraminidase inhibitors (NIs). We found that the rates of acdamantane resistance in A(H3) viruses continued to increase in most countries in 2007 but a distinct variation was seen with A(H1) resistance levels. A(H1) viruses from Australia, New Zealand and Europe had low rates of resistance (2–9%) whereas viruses from a number of South East (SE) Asian countries had high rates of resistance (33–100%). This difference can be attributed to the spread of A/Brisbane/59/2007-like viruses to many parts of the world with the exception of SE Asia where A/Hong Kong/2652/2006-like viruses continue to predominate. When these two A(H1) subgroups were compared for their in vitro sensitivity to the other class of influenza antiviral drugs, the neuraminidase inhibitors, no difference was seen between the groups with both showing normal levels of sensitivity to these drugs, The finding of reducing A(H1) resistance rates in Australia and rising levels in SE Asia in 2007, reverses the trend seen in 2006 when A(H1) resistance levels were rising in Australia and elsewhere but remained low in most of SE Asia.	Barr et al. 2008
Resistance to adamantanes and neuraminidase inhibitors Central/South America	Background Recent influenza antiviral resistance studies reveal an alarming increase in both adamantanes and neuraminidase inhibitors (NAIs) resistant viral strains worldwide, particularly in Asia, Europe and the United States. Objectives In this study, we have evaluated influenza virus resistance in Central and South America. Methods Influenza viruses, isolated from symptomatic patients throughout Central and South America in 2005–2008 were analyzed for inhibitor resistance. The M2 and NA genes of influenza viruses were sequenced and resistance was inferred by comparison with published sequences and known resistant mutations. Results Our results indicate that: (i) resistance to adamantanes was seen in the majority (95.5%) of the influenza A/H3N2 isolates but only in one isolate of the influenza A/H1N1 viruses; (ii) resistance to NAIs began to be detected in A/H1N1 isolates from Central America in 2008; and (iii) none of the influenza B viruses analyzed were resistant to NAIs. Conclusions These findings suggest a limited effectiveness of influenza inhibitors due to the detection of resistance among A/H1 and A/H3 viruses.	Garcia et al. 2009

Resistance to adamantanes Pyrosequencing	Background adamantanes have been used to treat influenza A virus infections for many years. Studies have shown a low incidence of resistance to these drugs among circulating influenza viruses; however, their use is rising worldwide and drug resistance has been reported among influenza A (H5N1) viruses isolated from poultry and human beings in Asia. We sought to assess adamantane resistance among influenza A viruses isolated during the past decade from countries participating in WHO's global influenza surveillance network. Methods We analysed data for influenza field isolates that were obtained worldwide and submitted to the WHO Collaborating Center for Influenza at the US Centers for Disease Control and Prevention between Oct 1, 1994, and Mar 31, 2005. We used pyrosequencing, confirmatory sequence analysis, and phenotypic testing to detect drug resistance among circulating influenza A H3N2 (n = 6524), H1N1 (n = 589), and H1N2 (n = 83) viruses. Findings More than 7000 influenza A field isolates were screened for specific aminoacid substitutions in the M2 gene known to confer drug resistance. During the decade of surveillance a significant increase in drug resistance was noted, from 0.4% in 1994–1995 to 12.3% in 2003–2004. This increase in the proportion of resistant viruses was weighted heavily by those obtained from Asia with 61% of resistant viruses isolated since 2003 being from people in Asia. Interpretation Our data raise concerns about the appropriate use of adamantanes and draw attention to the importance of tracking the emergence and spread of drug-resistant influenza A viruses.	Bright et al. 2005
Viral fitness and transmissibility Neuraminidase mutations Oseltamivir carboxylate	Limited antiviral compounds are available for the control of influenza, and the emergence of resistant variants would further narrow the options for defense. The H275Y neuraminidase (NA) mutation, which confers resistance to oseltamivir carboxylate, has been identified among the seasonal H1N1 and 2009 pandemic influenza viruses; however, those H275Y resistant variants demonstrated distinct epidemiological outcomes in humans. Specifically, dominance of the H275Y variant over the oseltamivir-sensitive viruses was only reported for a seasonal H1N1 variant during 2008–2009. Here, we systematically analyze the effect of the H275Y NA mutation on viral fitness and transmissibility of A(H1N1)pdm09 and seasonal H1N1 influenza viruses. The NA genes from A(H1N1)pdm09 A/California/04/09 (CA04), seasonal H1N1 A/New Caledonia/20/1999 (NewCal), and A/Brisbane/59/2007 (Brisbane) were individually introduced into the genetic background of CA04. The H275Y mutation led to reduced NA enzyme activity, an increased K_m for 3'-sialylactose or 6'-sialylactose, and decreased infectivity in mucin-secreting human airway epithelial cells compared to the oseltamivir-sensitive wild-type counterparts. Attenuated pathogenicity in both RG-CA04(NA-H275Y) and RG-CA04 x Brisbane(NA-H275Y) viruses was observed in ferrets compared to RG-CA04 virus, although the transmissibility was minimally affected. In parallel experiments using recombinant Brisbane viruses differing by hemagglutinin and NA, comparable direct contact and respiratory droplet transmissibilities were observed among RG-NewCal(HA,NA), RG-NewCal(HA,NA-H275Y), RG-Brisbane(HA,NA-H275Y), and RG-NewCal(HA) × Brisbane(NA-H275Y) viruses. Our results demonstrate that, despite the H275Y mutation leading to a minor reduction in viral fitness, the transmission potentials of three different antigenic strains carrying this mutation were comparable in the naive ferret model.	Wong et al. 2012
Rimantadine-resistance mutations Growth characteristics and virulence	The influence of rimantadine-resistance mutations on the virulence of human H3N2 viruses in ferrets was examined. The similarities in virulence of the drug-resistant mutants with single amino acid substitutions at three different locations, 27, 30, and 31, within the M2 sequence and their corresponding sensitive wild-type isolates contrasted with differences in virulence between the three pairs of viruses. These data provide further evidence that rimantadine-resistant viruses that emerge during treatment of patients with the drug are unaltered both in their growth characteristics and virulence.	Sweet et al. 1991

Resistance to neuraminidase inhibitors Conserved residues Viral fitness Neuraminidase activity	Neuraminidase inhibitors (NAIs) are antivirals designed to target conserved residues at the neuraminidase (NA) enzyme active site in influenza A and B viruses. The conserved residues that interact with NAIs are under selective pressure, but only a few have been linked to resistance. In the A/Wuhan/359/95 (H3N2) recombinant virus background, we characterized seven charged, conserved NA residues (11118, R371, E227, R152, R224, E276, and D151) that directly interact with the NAIs but have not been reported to confer resistance to NAIs. These NA residues were replaced with amino acids that possess side chains having similar properties to maintain their original charge. The NA mutations we introduced significantly decreased NA activity compared to that of the A/Wuhan/359/95 recombinant wild-type and R292K (an NA mutation frequently reported to confer resistance) viruses, which were analyzed for comparison. However, the recombinant viruses differed in replication efficiency when we serially passaged them in vitro; the growth of the R118K and E227D viruses was most impaired. The R224K E276D, and R371K mutations conferred resistance to both zanamivir and oseltamivir, while the D151E mutation reduced susceptibility to oseltamivir only (similar to 10-fold) and the R152K mutation did not alter susceptibility to either drug. Because the R224K mutation was genetically unstable and the emergence of the R371K mutation in the N2 subtype is statistically unlikely, our results suggest that only the E276D mutation is likely to emerge under selective pressure. The results of our study may help to optimize the design of NAIs.	Yen et al. 2006
Virus transmission Resistance to neuraminidase inhibitors	Three type A influenza viruses, each of which has a distinct neuraminidase-gene mutation and is resistant to the neuraminidase inhibitor oseltamivir, have been isolated. Previously, in the ferret model, an R292K mutant of a type A (H3N2) virus was not transmitted under conditions in which the wild-type virus was transmitted. This model was used to investigate whether the E119V mutant of a type A (H3N2) virus and the H274Y mutant of a type A (H1N1) virus would be transmitted under similar circumstances. Both mutant viruses were transmitted, although the H274Y mutant required a 100-fold-higher dose for infection of donor ferrets and was transmitted more slowly than was the wild type. Both the mutant and the wild-type viruses retained their genotypic characteristics.	Herlocher et al. 2004
Resistance to neuraminidase inhibitors and adamantanes Computational 3D structures Residue mutations	The neuraminidase (NA) and M2 proton channel of influenza virus are the drug-targeting proteins, based on which several drugs were developed. However these once powerful drugs encountered drug-resistant problem to the H5N1 and H1N1 flu. To address this problem, the computational 3D structures of NA and M2 proteins of 2009-H1N1 influenza virus were built using the molecular modeling technique and computational chemistry method. Based on the models the structure features of NA and M2 proteins were analyzed, the docking structures of drug-protein complexes were computed, and the residue mutations were annotated. The results may help to solve the drug-resistant problem and stimulate designing more effective drugs against 2009-H1N1 influenza pandemic.	Du et al. 2010
Molecular markers of resistance Pyrosequencing M2 blockers Neuraminidase inhibitors	In the present study, we describe how a pyrosequencing method can be used to rapidly detect established molecular markers of resistance to M2 blockers and NA inhibitors in influenza A (H5N1) viruses. The residues L26, V27, A30, S31, and G34 in the M2 protein were targeted for pyrosequencing. The NA residues for pyrosequencing analysis included the established markers of drug resistance (H274 and N294), as well as residues of less certain relevance (V116, I117, Q136, K150, and I222). A single pair of pyro-reverse transcription (RT)-PCR primers was designed to allow amplification of an approximately 600-nucleotide-long amplicon of the NA genes of H5N1 viruses from various clades/subclades associated with infections in humans. The sensitivity of the assay was demonstrated by the successful pyrosequencing of RNA extracted from samples of serially diluted (10^{-5} to 10^{-7} virus stocks with initial concentrations ranging from 10^5 to 10^8 PFU/ml. The markers of resistance were detected in samples with threshold cycle values ranging from 32 to 37, as determined by real-time RT-PCR. The pyrosequencing approach may provide a valuable tool for rapid detection of markers of drug resistance in H5N1 viruses and facilitate the elucidation of the role of such changes in natural and acquired drug resistance.	Deyde et al. 2009

Identification of the potential resistance sites Computer-aided method Oseltamivir Zanamivir Molecular basis of drug resistance	The outbreak and high speed global spread of the new strain of influenza A (H1N1) virus in 2009 poses a serious threat to the general population and governments. At present, the most effective drugs for the treatment of 2009 influenza A (H1N1) virus are neuraminidase inhibitors: mainly oseltamivir and zanamivir. The use of these two inhibitors will undoubtedly increase, and therefore it is more likely that drug-resistant influenza strains will arise. The identification of the potential resistance sites for these drugs in advance and the understanding of corresponding molecular basis to cause drug resistance are no doubt very important to fight against the new resistant influenza strains. In this study, first, the complexes of neuraminidase with the substrate sialic acid and two inhibitors oseltamivir and zanamivir were obtained by fitting them to the 3D structure of 2009 influenza A (H1N1) neuraminidase obtained by homology modeling. By using these complexes as the initial structures, molecular dynamics simulation and molecular mechanics generalized Born surface area (MM-GBSA) calculations were performed to identify the residues with significant contribution to the binding of substrate and inhibitors. By analyzing the difference of interaction profiles of substrate and inhibitors, the potential drug resistance sites for two inhibitors were identified. Parts of the identified sites have been verified to confer resistance to oseltamivir and zanamivir for influenza virus of the past flu epidemic. The identified potential resistance sites in this study will be useful for the development of new effective drugs against the drug resistance and avoid the situation of having no effective drugs to treat new mutant influenza strains.	Liu et al. 2010
Prevalence of amantadine-resistant viruses M2 channel mutations Genome sequencing Japan	Background: The prevalence of amantadine-resistant influenza A/H3N2 viruses (belonging to the N-lineage), possessing an S31N mutation in the M2 protein and S193F and D225N substitutions in their HA1 subunit, has significantly increased worldwide since 2005. The aim of this study was to clarify the genomic events contributing to the evolution and continuity of the N-lineage amantadine-resistant viruses. Methods: The fullgenome sequence of A/H3N2 isolates, including both amantadine-resistant and amantadine-sensitive viruses, collected in Japan between 2006 and 2008, was determined and phylogenetically compared with isolates obtained from the database. Results: On the basis of the full genome sequence analysis, the N-lineage could be further divided into three genetically related clades: Ni (A/Wisconsin/67/2005-like amantadine-resistant viruses from years 2005–2007), N2 (amantadine-sensitive viruses from 2007) and N3 (A/Brisbane/10/2007-like amantadine-resistant viruses from 2007 and 2008). The 2006/2007 season showed cocirculation of antigenic variants of amantadine-resistant viruses of clades Ni and N3 in addition to the N2-sensitive viruses. In the 2007/2008 season, the clade N3 amantadine-resistant lineage dominated and replaced other strains. Phylogenetic analysis of each individual segment suggested that N2 and N3 were generated from two independent reassortment events involving clade Ni viruses and pre-N-lineage strains. Conclusions: Our data show that several reassortment events have contributed to the evolution of amantadine-resistant A/H3N2 strains and, consequently, to the successful spread of this lineage. Although amantadine resistance is caused by single amino acid mutations in the M2 protein, genome-wide adjustment involving multiple genes appears to be necessary to obtain efficient replication and transmission of resistant viruses. Such adjustments are attainable through reassortment of segments among different virus lineages.	Zaraket et al. 2010

Prevalence of antiviral resistance Oseltamivir Zanamivir Amantadine Argentina 2005–2008	Objective. To describe the virological characteristics of the influenza strains circulating in Argentina in 2005–2008 and to assess the prevalence of antiviral resistance. Methods. On the basis of their geographical spread and prevalence, influenza Lambda and B isolates grown in Madin-Darby canine kidney cells were selected after antigenic and genomic characterization to be analyzed for antiviral resistance by enzymatic assay and pyrosequencing. Amantadine susceptibility was evaluated by pyrosequencing for known resistance markers on 45 strains of influenza A. Susceptibility to oseltamivir and zanamivir was evaluated by enzymatic assay of 67 influenza Lambda and 46 influenza B strains, some of which were further analyzed by sequencing the neuraminidase gene. Results. Resistance to amantadine was observed only on A(H3N2) strains (29/33); all of them carried the mutation S31N in their M2 sequence. Oseltamivir resistance was observed in 12 (34.3%) of the 35 A(H1N1) strains from 2008; all of them carried the mutation H275Y in their neuraminidase sequence. All these viruses remained sensitive to zanamivir. Conclusions. This study describes a high incidence of amantadine-resistant influenza A(H3N2) viruses since 2006 and an unprecedented increase in oseltamivir resistance detected only in influenza A(H1N1) viruses isolated in 2008. Influenza A and B viruses were more sensitive to oseltamivir than to zanamivir, and influenza A viruses were more sensitive to both neuraminidase inhibitors than the influenza B viruses. The national data generated and analyzed in this study may help increase knowledge about influenza antiviral drug resistance, which is a problem of global concern.	Pontoriero et al. 2011
Resistance to haemagglutinin inhibitors Stachyflin In silico screening	In this study we performed molecular dynamics simulations on a spike protein on the viral envelop, hemagglutinin for the wild type and three kinds of mutants using a model system consisting of a trimeric hemagglutin complex, viral lipid membrane. solvation waters, and ions . A natural product stachyflin, which shows a high level of antiviral activity specific to some subtypes of influenza viruses, was examined on binding to the wild-type hemagglutinin was clarified. Next, 8 compounds were selected from a chemical database by in silico screening, considering the findings from the simulations. Inhibitory activities to suppress the proliferation of influenza virus were measured by cell-based antiviral assays, and chemical scaffolds were found to be potent for an inhibitor. More than 30 derivatives bearing either of these two chemical scaffolds were synthesized, and cell culture assays were carried out of evaluate the compound potency. Several derivatives displayed a high compound potency, and 50% effective concentrations of two synthesized compounds were below 1 μM.	Yanagita et al. 2012
Resistance to phosphoro-thioate oligonucleotide	In a previous study a 15-mer phosphorothioate oligonucleotide (S-ON) derived from the packaging signal in the 5' end of segment 1 (PB2) of influenza A virus (designated 5-15b) proved markedly inhibitory to virus replication. Here we investigated whether analogous inhibitory S-ONs targeting the 5' end of segments 2 (PB1) and 3 (PA) could be identified and whether viral resistance to S-ONs can be developed. Similar to our earlier result, 20-mer S-ONs reproducing the 5' ends of segments 2 or 3 (complementary to the 3'-coding regions of PB1 and PA, respectively) exerted a powerful antiviral activity against a variety of influenza A virus subtypes in MOCK cells. Serial passage of the A/Taiwan/1/86 H1N1 strain in the presence of S-ON 5-15b or its antisense as 5-15b analogue showed that mutant viruses with reduced susceptibility to the S-ON could indeed be generated, although the resistant viruses displayed reduced replicative fitness. Sequencing the resistant viruses identified mutations in the PB1, PB2, PA and M1 genes. Introduction of these changes into the A/PR/8/34 H1N1 strain by reverse genetics, suggested that alterations to RNA function in the packaging regions of segments 2 and 3 were important in developing resistance to S-ON inhibition. However, many of the other sequence changes induced by S-ON treatment were markedly deleterious to virus fitness. We conclude that packaging signals in the influenza A virus polymerase segments provide feasible targets for nucleic acid-based antivirals that may be difficult for the virus to evade through resistance mutations.	Giannecchini et al. 2011