Taking aim at a moving target – inhibitors of influenza virus Part 1: virus adsorption, entry and uncoating

Nicholas A. Meanwell and Mark Krystal

Annual epidemics of influenza virus infection are responsible for considerable morbidity and mortality, and pandemics are much more devastating. Considerable knowledge of viral infectivity and replication has been acquired, but many details have yet to be elucidated and the virus remains a particularly challenging target for drug design and development. In this two part review, the current status of influenza research is summarized in the context of inhibitor design and discovery, and recent advances in the search for clinically effective drug therapy are detailed. The first part addresses virus adsorption, entry and uncoating; the second part, to be published in the September issue of *Drug Discovery Today*, discusses aspects of virus replication, packaging and release.

Annual epidemic outbreaks of influenza virus infection continue to be an important cause of morbidity and mortality, with the young and elderly populations being particularly vulnerable[1,2]. Because the options for the therapeutic treatment of influenza infections are currently limited, a prophylactic strategy has been adopted that relies on the annual implementation of a vaccination program. However, vaccination has provided only limited control over influenza infectivity due to incomplete immunization coverage of individuals considered to be at risk and the propensity for the virus to escape the immune system. The latter is a property inherent to the structural composition and mode of replication of influenza viruses, which are members of the Orthomyxoviradae family of enveloped viruses.

The viral envelope contains two major surface antigenic proteins, hemagglutinin (HA) and neuraminidase (NA), along with a minor component, the M2 protein, which is a small hydrophobic protein found in type A influenza virus strains and which functions as an ion channel (Figure 1). Type B influenza strains contain an analogous protein, designated NB, which is encoded by a second reading frame on the NA gene. Under the electron microscope[3], the HA and NA proteins appear as spikes on the virion surface, and recurrent influenza epidemics are associated with antigenic changes in these molecules. Layered immediately beneath the lipid envelope of the virus particle is the matrix (M) protein, which surrounds the core of ribonucleoprotein (RNP). The RNP consists of a genome of eight different RNA segments of negative polarity which are coated with nucleoprotein (NP), forming helical structures that contain, as minor components, the three viral polymerase proteins PB2, PB1 and PA (Ref. 4). The virus genome also encodes two proteins, NS1 and NS2, which are not found in mature virions (Figure 1).

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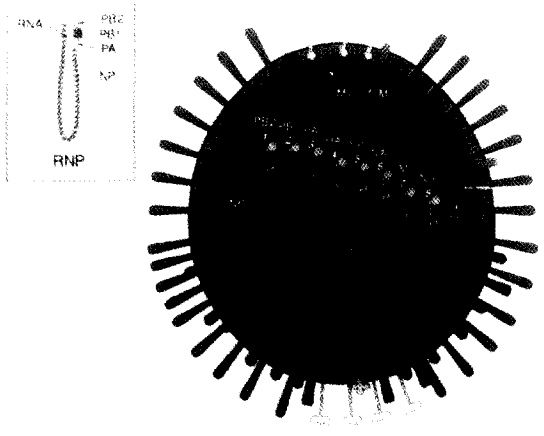


Figure 1. *Influenza virion particle. The eight ribonucleoprotein (RNP) segments are surrounded by a layer of matrix (M) protein and the lipid bilayer. The lipid bilayer contains three viral proteins: hemagglutinin (HA), neuraminidase (NA) and, for type influenza A strains, the M2 protein. Influenza type B viruses contain an analogous protein, designated NB, which is coded by a second reading frame on the NA gene. Although NA is depicted as occurring in patches on the surface of the virus, this distribution pattern is speculative. PB2, PB1 and PA are the three viral polymerase proteins. Inset: Depiction of the helical RNP-containing RNA surrounded by nucleoprotein (NP) and associated with the polymerase complex consisting of PB1, PB2 and PA. (Courtesy of Dr Robert G. Webster, St Judes Children's Hospital, Memphis, TN, USA.)*

The classification of influenza viruses is based on the serologic cross-reactivity of the abundant internal proteins M and NP. Three clinically infectious influenza virus types have been identified, of which A and B are commonly associated with disease, whilst C strain infections are rarely symptomatic[5]. Influenza A viruses have been associated with periodic pandemic episodes in humans, the result of antigenic shift, whereas types A and B both cause annual influenza epidemics as a consequence of antigenic drift.

The structural composition and properties of the influenza viruses have several important implications that relate to this pathogenicity. The influenza virus RNA polymerase is prone to a high error rate which, in conjunction with the single strand genomic structure, manifests as a poor editing function. The result is a high mutation rate[6–8], which promotes the production of mutant viruses that escape antibody recognition, causing antigenic drift and allowing infection in individuals with prior exposure to earlier viral strains.

However, an event that has been associated with more serious clinical consequences is the antigenic shift that results from the introduction into circulating virus of new genes coding for the surface HA and NA proteins. Antigenic shifts have been responsible for the periodic pandemic infections associated with exceptionally high levels of morbidity and mortality[9]. For example, the Spanish influenza pandemic of 1918 resulted in 20 million deaths worldwide, with 2 million in the USA alone[4]. Antigenic shift has been limited to influenza A, as a consequence of a host range that extends beyond man to animals and birds. One explanation that has been proposed to account for the occurrence of antigenic shift involves a susceptible animal, such as swine, being coinfected with a human and an avian influenza strain[10]. Acting as a mixing vessel, the segmented nature of the virus allows reassortants to occur, whereby the surface proteins of the avian strain replace those of the human strain. Gene reassortment of this type is believed to have been responsible for the introduction of H2N2 virus in 1957 and H3N2 virus in 1968. Another example of antigenic shift is when older human strains are reintroduced into a naive population that has not been previously exposed to this strain. This is thought to have been the case in 1977 when H1N1 strains nearly identical to those circulating around 1950 were reintroduced[11,12].

Current vaccination programs use formalin-inactivated virus, although live virus vaccines are currently being tested[13]. This strategy requires that viral strains predicted to be the most likely to circulate in the upcoming season are produced on a large scale several months in advance[13]. The success of a vaccination program is clearly dependent on both an element of speculation and the extent of immunization within the community at risk. However, these factors, coupled with the propensity for the virus to escape immune surveillance due to antigenic drift, mean that influenza infection is a perennial problem requiring the familiar annual vaccination campaigns.

Antigenic drift arises when, by chance, mutations occur in antigenic regions of the viral surface protein HA, and antigenically distinct viruses are selected for because they evade the host immune response. The underlying mechanism of antigenic drift is also a factor that, at least in part, limits the widespread application of the only approved influenza antiviral therapies, amantadine and rimantadine. These agents, discussed in more detail below, are structurally closely related bicyclic amines that interfere with the function of the viral M2 protein ion channel. Treatment of index cases with these drugs

results in the rapid emergence of infectious, drug-resistant virus due to single amino acid changes in the transmembrane domain of the M2 protein[14–16]. A further limitation associated with amantadine and rimantadine therapy is the fact that their spectrum of activity is confined to type A influenza viral strains; type B influenza viruses, which cause significant morbidity during epidemic seasons, are insensitive. The use of amantadine has also been associated with several undesirable side-effects, the most prominent of which is interference of functions within the CNS[17]. In fact, amantadine is an approved therapeutic treatment for Parkinson's disease[18].

The need for better agents for the treatment and prevention of influenza infections sustains the search for more efficacious drugs, and several influenza virus inhibitors with novel modes of action have been developed and advanced into clinical trials in recent years. Several of these have been discovered using empirical screening techniques which, particularly in high-throughput modes, continue to be applied to identify lead structures in the search for potential drug candidates. However, the development of GG 167 (see Part II) as a potent and selective inhibitor of influenza NA provides a compelling and successful example of the utility and application of X-ray crystallographic data of target proteins in the process of contemporary rational drug design and refinement.

Although the influenza virus life-cycle provides a number of opportunities for therapeutic intervention, the complexity of the genetic background places constraints on the identification and development of therapeutic agents, which must demonstrate efficacy against all relevant viral subtypes. Nevertheless, much progress has been made in recent years in understanding the biochemistry of virus infectivity and function, and the subtle interplay between virus and host cell. This has translated into the identification of several new potential targets for drug discovery and development. Whilst viral targets offer the inherent advantage of selectivity, interference with host cell processes provides a method of diminishing the problems associated with the development of resistance.

In the following sections, we summarize the status and progress of many of the current strategies directed towards the identification of potential influenza-inhibiting drug candidates. These will be discussed broadly from a historical perspective and in the context of the order in which they interfere with the virus life-cycle, which is summarized in Figure 2.

Virus adsorption

Influenza virus infects epithelial cells of the upper respiratory tract, gaining entry by binding to cell surface receptors, which are subsequently endocytosed via coated pits into the endosomal pathway. Once incorporated into endosomes, the influenza virion must extricate itself before reaching the lysosome, where it would be destroyed. This is accomplished by fusion of the viral and endosomal membranes, an event that is triggered by the declining pH of the endosome. Fusion releases the RNP core into the cytosol, from which it migrates into the nucleus, the site of viral transcription and replication. While viral entry comprises several distinct steps, it is fundamentally mediated by two influenza cell surface proteins: HA and the M2 protein. Binding of influenza virus to cell surface receptors is mediated by HA, a protein complex comprising three identical subunits which projects in a spike-like fashion from the viral membrane[19] (Figure 1). Each subunit possesses a binding site, located distal to the viral membrane, that recognizes sialic acid (*N*-acetylneuraminic acid), a carbohydrate that is the terminal residue of many cell surface glycoproteins. Prevention of binding of HA to sialic acid residues has been explored as a method of preventing viral entry and infection, a strategy that is thought to mimic that adopted by the immune system since antigenic sites have been mapped to the surface of HA surrounding the sialic acid binding pocket[20]. The recent determination of the solid-state structure of a complex of the antigenic domain of HA with a neutralizing antibody has provided a clearer understanding of the nature and extent of the intermolecular interactions[21]. Viral escape from the immune system is postulated to be a consequence of single amino acid mutations in regions remote from the sialic acid binding site which markedly reduce antibody binding affinity without affecting crucial elements involved in receptor recognition[21].

Inhibitors of influenza virus hemagglutinin binding to sialylated proteins

Attempts to identify small-molecule inhibitors of HA binding to cell membranes have focused on the synthesis and evaluation of sialic acid derivatives; this effort has only recently begun to meet with some success. The crystal structure of influenza H3 HA, cleaved from viral membranes by treatment with bromelain, has been solved at high resolution, and the sialic acid binding site has been mapped in sufficient detail to be of use in the design of ligands[22–24]. However, the identification of effective sialic acid receptor antagonists has been thwarted by the low affinity of the individual binding sites for simple sialic acid derivatives, defined by the prototypical methyl ether derivative, Neu5Ac2Me (**1**; Figure 3), which displays a dissociation constant in the order of only 2–3 mM (Refs 24,25). Attempts to identify more potent antagonists by

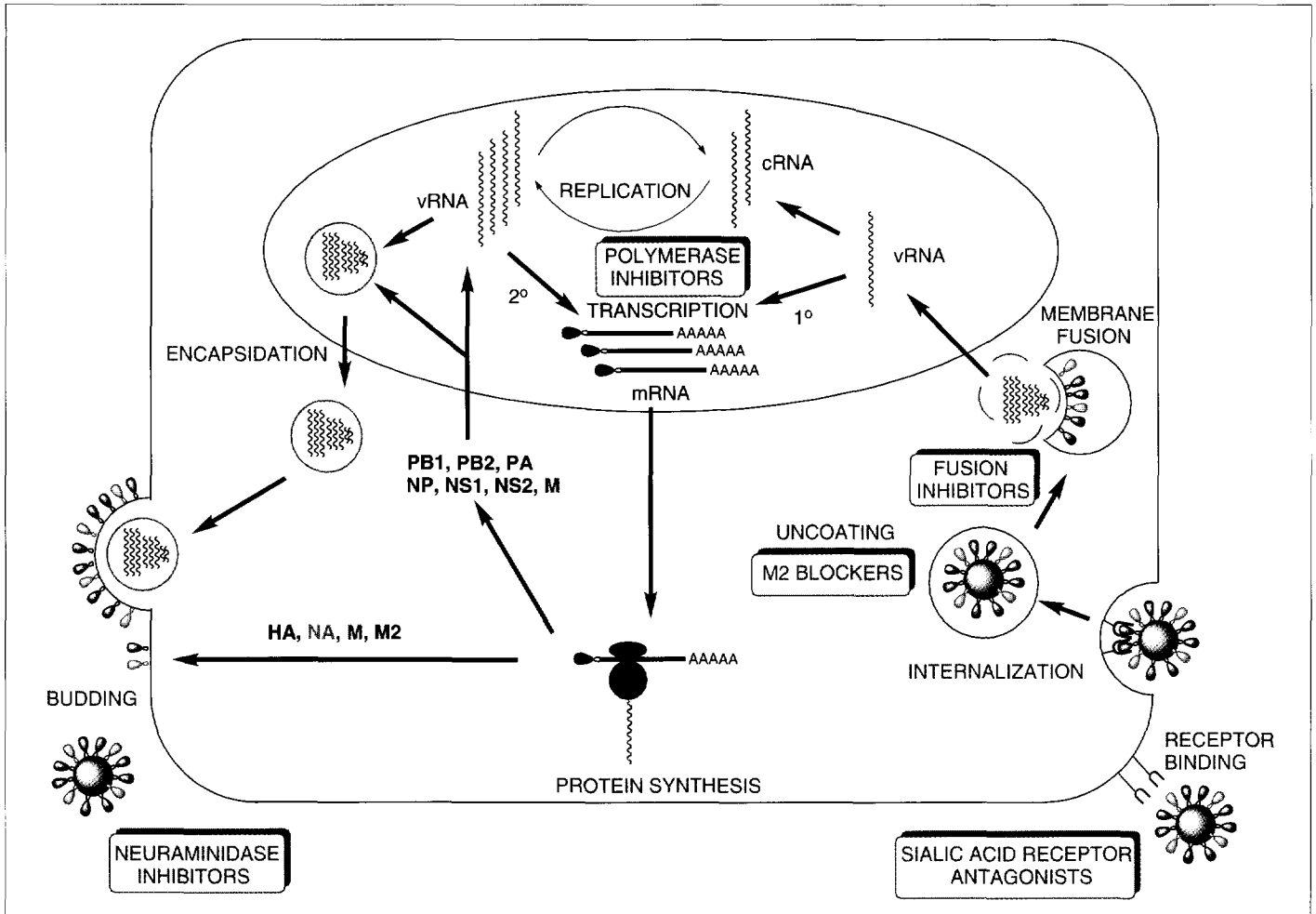


Figure 2. *Influenza life-cycle and defined sites for therapeutic intervention. PB1, PB2, PA, polymerase complex; NP, nucleoprotein; NS1, NS2, proteins not found in mature virions; M, matrix protein; M2, M2 ion channel protein; HA, hemagglutinin; NA, neuraminidase.*

introducing hydrophobic substituents[24,26] have met with only limited success, with **2** (Figure 3; K_i = 40 μM)[26] being the optimum example of this structural class.

A far more effective strategy has been to take advantage of the nature of the interaction of the virus with its receptor, which depends on the thermodynamic advantage offered by engaging in multiple simultaneous binding interactions between HA and sialic acid residues clustered on the surfaces of epithelial cells. Whilst bivalent sialic acid ligands provide some advantage[27], the most potent and effective sialic acid receptor antagonists that have shown activity *in vitro* incorporate sialic acid moieties onto polymeric templates[28–34], a strategy used to advantage by nature[35]. The structure and physical properties of the template employed to project the sialic acid residues are of importance, and hydrophobic components appear to be necessary for high affinity binding. For example, the heptasaccharide derivative **3** (Figure 3), a rather complex bivalent ligand, inhibits influenza virus absorption to erythrocytes (hemagglutination) with an IC_{50} of 0.18 mM (Ref. 28), whereas the structurally simpler dimeric ligand **4** (Figure 3) inhibits hemagglutination with an IC_{50} of 3 μM (Ref. 27). In the latter compound, the sialic acid moieties are separated by approximately 55 Å, a distance determined to be optimal and thought to approximate the distance between the individual receptors on the surface of the HA protein complex. Ligands with markedly increased affinity have been attained through the preparation of higher polymeric species. Branched, dendrite-like amino acid backbones capped with sialic acid moieties afforded molecules that cluster 4, 8 or 16 residues and are effective inhibitors of hemagglutination of erythrocytes[30].

An alternative approach focused on the incorporation of differing concentrations of the sialoside lipid **5** (Figure 3) into

liposomes, followed by irradiation-induced polymerization. This process afforded liposomal preparations that optimally inhibit hemagglutination at submicromolar concentrations of the sialic acid ligand[29]. However, somewhat surprisingly, the activity of these liposomal preparations in a plaque reduction infectivity assay did not correlate closely with inhibition of hemagglutination, a finding that remains unexplained.

The most potent compounds to emerge from this strategy have been polyacrylamide derivatives incorporating sialic acid moieties[32–34]. Derivatization of an acrylic acid polymer containing active ester leaving groups afforded a NA-resistant polymeric species containing 20% of the C-glycoside **6** (Figure 3) and 10% of benzylamine on a polyacrylamide backbone. This polymeric species inhibited influenza-mediated hemagglutination of erythrocytes with an IC_{50} of 0.6 nM at 36°C (Ref. 33) and interacted in a synergistic fashion with a NA inhibitor[34]. However, the potential therapeutic utility of these antagonists remains to be determined, since data accumulated thus far have been restricted to evaluation *in vitro* under highly controlled conditions.

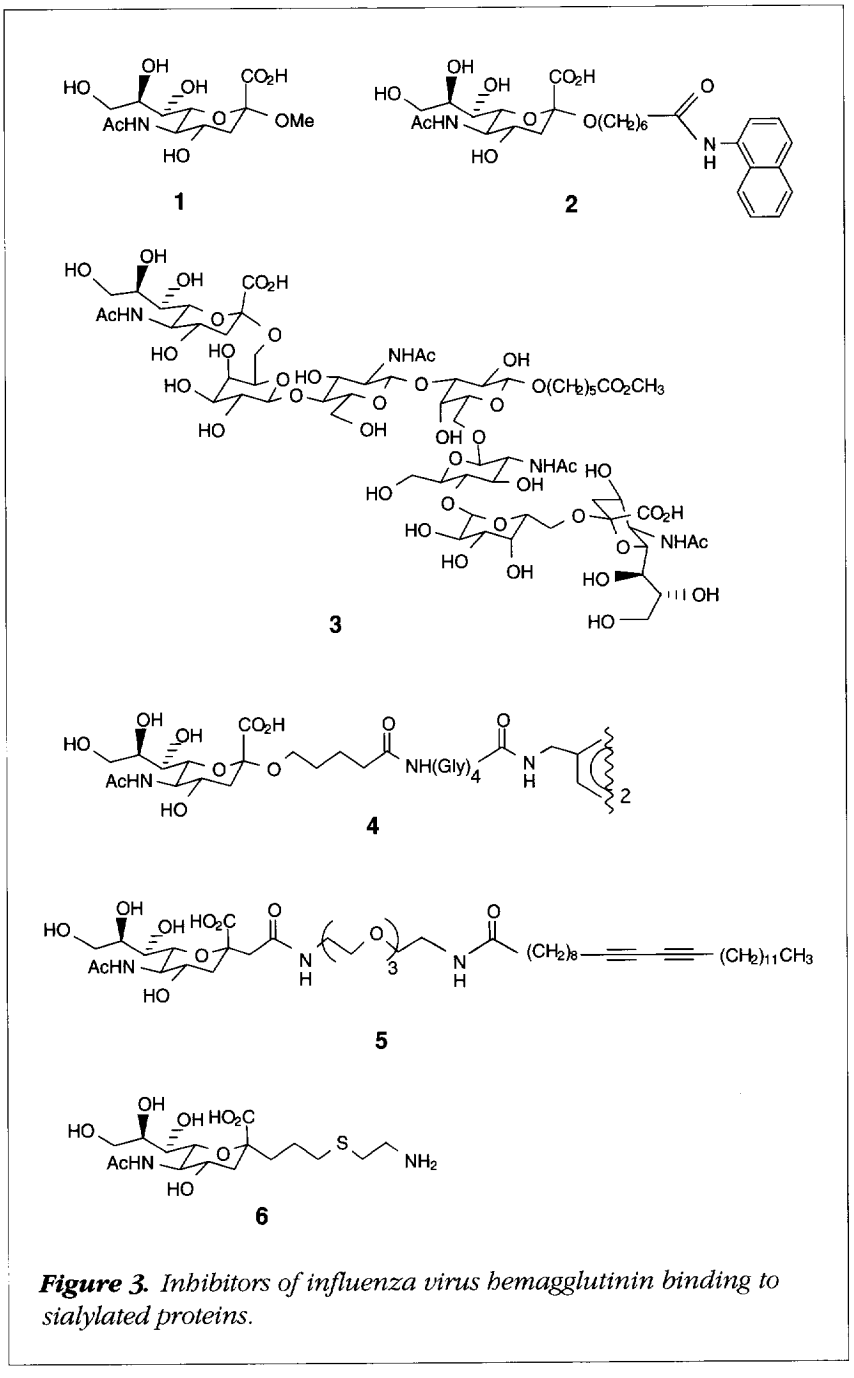


Figure 3. *Inhibitors of influenza virus hemagglutinin binding to sialylated proteins.*

Viral uncoating

Inhibitors of endosomal acidification

After internalization, influenza virus bound to cell surface receptors progresses into the endosomal pathway. The incremental acidification of the endosome is an essential step in the process of viral entry, because it catalyzes dismantling of the RNP core and activates the fusion apparatus. Endosomal acidification can be prevented in a relatively unsophisticated fashion by agents that act as general bases to buffer the gradually declining pH. These are typically basic amine derivatives, which are only effective at concentrations too high to be considered of physiological relevance. For example, millimolar concentrations of the antiparkinsonian agent norakin (**7**; Figure 4)[36] and chloroquine (**8**; Figure 4)[37] are thought to function in this fashion. Alternatively, blocking the proton pump that mediates endosomal acidification can interfere with viral uncoating, because the vacuolar-type proton pump inhibitor bafilomycin A1 has been shown to prevent influenza A and B infectivity in Madin–Darby canine kidney (MDCK) cells *in vitro*[38].

M2 ion channel blockers

After endocytosis of the influenza virus, there is a delay of approximately 25 min before viral and endosomal membrane fusion occurs. During this period, the lowered pH affects two viral proteins essential to the uncoating process, the M2 ion channel protein and HA. The M2 ion channel protein, which is present in relatively low amounts in the viral membrane, is activated by the declining pH. This allows protons to be introduced into the viral particle, which facilitates dissociation of the RNPs from interactions with the viral matrix protein and

the viral envelope[39]. This acid-sensitive activation of the M2 channel explains the selectivity of its function at specific points in the virus life-cycle. The influenza virus M2 ion channel is a 97-residue protein with a single membrane-spanning domain that functions as a homotetramer stabilized by intermolecular disulfide bonds[40]. Blockade of the M2 channel is the primary mode of action of amantadine (**9**; Figure 4) and rimantadine (**10**; Figure 4)[41], which results in the accumulation of M1-associated RNPs in the cytoplasm which are unable to penetrate the nucleus. Details of the precise molecular mechanism of amantadine and rimantadine action remain to be clarified, but open channel blockade, analogous to that of classical ion channel pore blockers, has been postulated[41] and modelled[42]. However, electrophysiological studies have been inconclusive[43], and speculation has recently focused on an alternative mode of action in which these bicyclic amines cause a constriction of the channel[44].

Amantadine and rimantadine are the only drugs currently approved in the USA for the prevention and treatment of influenza infections, and both have demonstrated some value in a clinical setting[17,45–48]. In spite of their close structural similarity, the two compounds show markedly different dispositions *in vivo*, pharmacokinetic properties that have an impact on the efficacy and incidence of side-effects[48]. Both compounds are orally bioavailable, but peak plasma levels of rimantadine are three times lower than those achieved with the same dose of amantadine. However, this is offset by the fact that rimantadine is three times more potent than amantadine and concentrates much more effectively in nasal mucosa[49]. Amantadine is excreted unchanged in the urine, but over 90% of an administered dose of rimantadine is metabolized by hydroxylation at multiple sites of the molecule, with only one metabolite showing significant antiviral activity[49]. Consequently, rimantadine has the more favorable profile with a reduced incidence of the CNS side-effects that are frequently associated with therapeutic doses of amantadine[17].

In addition to unwanted side-effects and a high degree of specificity for influenza A viral strains, more extensive clinical application of these drugs has been restricted by the spectre of resistance[16]. Influenza viruses resistant to amantadine exhibit mutations that map to one of four amino acids in a hydrophobic sequence of the transmembrane domain of the M2 protein, specifically located in the region that resides in the membrane outer leaflet[41,50]. Since single amino acid changes in M2 markedly reduce the efficacy of these bicyclic amines, amantadine-resistant viruses are quickly selected, both *in vitro* and *in vivo*, as determined from clinical studies of infected individuals[15]. Interestingly, though, the development of resistant virus has not proven to be a major problem in the population as a whole, as most clinical isolates described to date remain sensitive to amantadine and rimantadine[51].

Following the original disclosure of the antiviral properties of amantadine[52], extensive SAR studies were described[53–56]. Two compounds, the spiropyrrolidine derivative **11** (Figure 4)[57,58] and ICI 130685 (**12**; Figure 4)[59], were advanced into clinical trials but neither has emerged as an approved drug, while the spiropyrrolidine **13** (Figure 4)[60], an isomer of **11**, and the norbornylamine **14** (Figure 4)[61] have recently been described as influenza virus inhibitors.

However, there remains continued interest in the identification of more effective M2 channel blocking agents[62,63] because drugs that show efficacy against resistant strains may be useful therapeutic agents when used in combination with amantadine and rimantadine. The implementation of high-throughput assays is a very effective method to discover structurally novel drug leads, and two such assays based on M2 channel function have recently been described[62,63]. In several systems, high level expression of the influenza M2 channel is toxic to cells[62–65]. In yeast, expression of the M2 channel protein causes growth impairment that can be restored

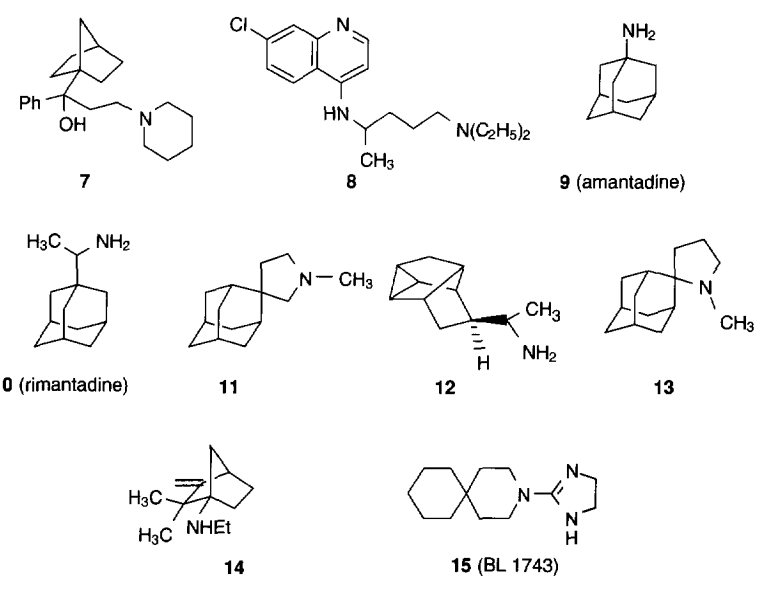


Figure 4. *Inhibitors of influenza uncoating.*

by the addition of amantadine. This formed the basis of a high-throughput assay that resulted in the identification of BL 1743 (**15**; Figure 4) as a structurally novel inhibitor of influenza M2 channel function[62]. A more detailed evaluation of BL 1743 revealed that the compound effectively blocked the activity of the influenza M2 ion channel expressed in *Xenopus laevis* oocytes[66]. However, BL 1743 exhibited an overlapping spectrum of resistance with amantadine, and all four of the single amino acid changes in M2 that result in resistance to amantadine also confer resistance to BL 1743. In addition, a fifth amino acid change was identified within the transmembrane region of M2 that uniquely conferred resistance to BL 1743. Interestingly, BL 1743 exhibited reversible inhibition of M2 function within the time frame of the electrophysiological procedures, which contrasts with amantadine where inhibition was found to be only slowly reversible[66]. These observations suggest that amantadine and BL 1743 interact differently with the transmembrane pore region of the M2 protein. Because BL 1743 shows cross-resistance with amantadine and rimantadine, it is not a viable drug candidate, and a more fruitful endeavour may be broad screening using an amantadine-resistant M2 channel. This tactic has been adopted using an assay in which expression of the M2 channel in *Xenopus* oocytes reduces cell survival[63].

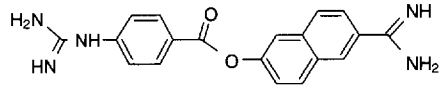
Fusion of influenza virus and host cell membranes

The second effect of lowered endosomal pH on virus uncoating is the triggering of the fusion of viral and endosomal membranes. Once this occurs, the viral RNPs, which have been dissociated from membrane interactions through the action of the M2 protein, are released into the cytosol. Membrane fusion is mediated by the viral HA protein, which undergoes an extensive, pH-dependent conformational rearrangement as part of the fusion process. A broad understanding of this process has emerged from biochemical[67–70] and X-ray crystallographic studies[22,71], although many details still remain to be elucidated. Hemagglutinin is synthesized as a single polypeptide chain, approximately 550 residues long in A strain viruses[72] and slightly longer in influenza B viruses[73]. The protein encodes a cleavable signal sequence, which directs insertion into the endoplasmic reticulum membrane. After removal of the signal peptide, the protein folds as a homotrimer that is anchored to the membrane via the C-terminus transmembrane domain. Fusion-competent HA is produced through a proteolytic cleavage that occurs at Arg328, with the Arg frequently removed by exopeptidases in a subsequent step. This process generates a metastable heterodimeric HA molecule comprising the amino terminal HA1 subunit and the C-terminal HA2 subunit, which are held together by disulfide bonds and constrained in the native state by a network of ionic and hydrogen bonding interactions that are stable when the pH is above 6.0 (Ref. 19). Through a mechanism that remains to be defined, the low pH of the endosome induces dissociation of the membrane-distal portion of HA1 from HA2. Subsequently, HA2 undergoes an irreversible conformational change, a process driven by the strong intrinsic propensity of a 36-residue peptide present as a loop between two α-helical segments in the native HA2 subunit to adopt a more stable coiled-coil arrangement[74]. The end-result is that the hydrophobic amino terminus of HA2, the so-called fusion peptide, which is highly conserved across all viral strains, is projected away from the viral membrane and into the endosomal membrane, thereby drawing viral and host cell membranes into close proximity. Whilst details of the exact nature and sequence of the events that ultimately lead to membrane fusion remain to be clarified at a molecular level, the formation of a pore between the membrane-spanning domains of aggregated HAs appears to be an essential step that facilitates mixing of virion and endosomal constituents[75–77].

Against this background there are several opportunities for therapeutic intervention to disrupt the carefully orchestrated role of HA in influenza infectivity. One strategy that has been explored is inhibition of the cellular enzymes responsible for the essential proteolytic cleavage of HA, an important determinant of pathogenicity and cell tropism[78]. However, progress in this area has been confounded by the multitude of cellular and extracellular trypsin-like enzymes capable of performing this step[79–81], including bacterial proteases, which as coinfectants can potentiate influenza infections[82]. Nevertheless, both the trypsin inhibitor Futhan (**16**; Figure 5) and an anticathepsin B IgG antibody have been shown to possess influenza inhibitory activity in an *in vitro* setting[83,84].

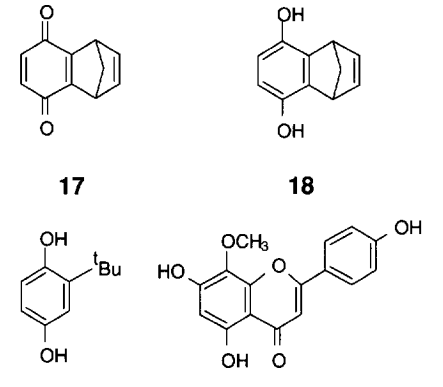
An alternative antiviral strategy would be to destabilize HA, thereby promoting the conformational change prematurely. This approach is based on the fact that HA protein that has undergone the irreversible conformational change in the absence of a target membrane is both fusion and receptor binding incompetent[85]. While this is clearly the case with H1- and H3-containing viral strains, H2 HA appears to maintain fusion competency for a considerable period of time after exposure to low pH conditions and it is not yet clear whether the low pH induced conformational change of the H2 HA is an irreversible one[86]. While compounds (other than protons) which promote the conformational change of the HA have not yet been described, amantadine and rimantadine can indirectly

affect HA stability. During the later stages of viral growth, HA is synthesized and transported through the Golgi network. However, since the Golgi network is mildly acidic, a proteolytically cleaved HA would potentially be unstable and may conformationally rearrange during transport, an irreversible event that would inactivate the protein. This is especially true for HA molecules that are inherently less stable, such as that from the A/fowl plague virus/Rostock viral strain, and which undergo the conformational change at a higher pH than most other HA molecules. It is believed that, in order to ensure that a fully functional HA trimer is delivered to the membrane surface, the influenza M2 channel functions to increase the Golgi pH by pumping protons from inside the Golgi to the cytosol[87–90]. The M2 channel in the Golgi can be blocked by high concentrations of amantadine and rimantadine, resulting in an antiviral effect that is manifested at a late stage in the virus life-cycle[88,91–93]. Viruses resistant to these extremely high concentrations of amantadine that have been isolated can have mutations in the HA protein. These changes have been shown to provide additional stability compared to the native structure of the trimer such that it undergoes the conformational change at a lower pH (Refs 88,94).



16 (Futhan)

Figure 5. *Inhibitor of influenza hemagglutinin processing.*



17 **18** **19** **20**

Figure 6. *Inhibitors of influenza virus fusion.*

A third antiviral strategy directed towards the HA protein is through the use of compounds that would stabilize HA and prevent the acid-induced conformational rearrangement. Evidence for the viability of this as a mechanistic approach is derived from studies in which the introduction of disulfide bonds that link HA monomers act to stabilize the structure and impair fusogenic activity[95]. Small molecules that can reversibly stabilize HA in the native state have also been described recently[96]. These influenza inhibitors were identified using a computer-based program that effectively maps binding sites in proteins and identifies complementary functionality in compounds available in commercial databases[96]. Exploiting the crystal structure of native H3 HA (Refs 19,22), invaginations in the surface of the protein proximate to the fusion peptide were identified and mapped using the DOCK (Ref. 97) molecular modelling paradigm and candidate molecules selected for biological evaluation[96]. From a total library of 55,000 compounds, 48 were chosen for screening in an assay in which the acid-induced conformational rearrangement of purified H3 HA cleaved from viral membranes by bromelain was detected by an antibody recognizing the exposed fusion peptide. The quinone **17** (Figure 6) was identified as a specific inhibitor of the conformational change (IC_{50} = 250 μM), but the hydroquinone form **18** (Figure 6) proved to be considerably more potent, with an IC_{50} of 25 μM. Evaluation of a series of closely related compounds revealed **19** (Figure 6) to be the most potent conformational change inhibitor (IC_{50} = 5 μM) that weakly prevented influenza-induced hemolysis of erythrocytes (IC_{50} = 100 μM). This compound also demonstrated antiviral activity *in vitro* (IC_{50} = 20 μM), while cellular toxicity was manifest only at more than fivefold higher concentrations. Although the chemical properties of **17–19** make them unlikely drug candidates, these compounds represent the first of a new mechanistic class of influenza inhibitors that demonstrate the feasibility of preventing the conformational change of HA with small molecules of limited complexity. More recently, the flavone **20** (Figure 6), which contains some structural elements in common with **17–19**, has also been postulated to interfere with the fusion of influenza viral and endosomal membranes[98].

Summary

There continues to be a high level of contemporary interest in the design and evaluation of inhibitors of influenza virus adsorption, viral uncoating and fusion of the virus and endosomal membranes. As a consequence, considerable progress has been made in understanding the fundamental biochemical mechanisms of these processes and this, in turn, has been applied to the design and identification of agents with potential therapeutic value. Nevertheless, much remains to be achieved if this enhanced understanding is to be translated into the development of clinically effective therapeutic agents that interfere with these events in the influenza virus life-cycle. In the second part of this review, inhibition of later steps in

the virus life-cycle, including replication, viral packaging and viral release, will be discussed.

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