Taking aim at a moving target – inhibitors of influenza virus Part 2: viral replication, packaging and release

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In this two part review, the current status of influenza research is summarized in the context of inhibitor design and discovery. In Part 1, published in the August edition of *Drug Discovery Today*, the authors considered therapeutic approaches to influenza through interference with early phases of the virus life cycle. In Part 2, the authors describe the current level of understanding of the biochemical aspects of viral replication, packaging and release, and recent progress in the design and identification of potent and selective inhibitors of these crucial stages in the life cycle. Recent developments at both the preclinical and the clinical level highlight the considerable potential of this area of influenza inhibitor drug discovery.

After release of the viral ribonucleoprotein (RNP) into the cytosol, the genome migrates to the nucleus, where the process of transcription and translation of the viral genome is initiated. Three polymerase proteins, designated PB1, PB2 and PA, comprise the polymerase complex. Together these coordinate primary transcription, which produces the positive strand RNAs from the viral genome that act as mRNAs, and replication, in which progeny negative-strand RNAs are amplified from complete positive-strand templates (Figure 1). Primary transcription occurs in the nucleus and proceeds in the absence of viral protein synthesis, which makes this a relatively easy process to study *in vitro*. By contrast, viral replication is a more complex and much less well characterized process because it is regulated both by viral proteins[1] and cellular factors[2].

Viral replication

Primary transcription by the influenza polymerase complex, which functions as a trimeric enzyme, proceeds by a unique mechanism that is dependent upon host cell RNA synthesis for priming. The PB2 polymerase subunit specifically binds to the 7-methylated guanosine 5′-triphosphate (m^7GpppNm) that caps the 5′ end of host mRNA. A viral protein, possibly PB2, then cleaves host cell mRNA 9–15 nucleotides downstream from the 5′ end, generating a primer suitable for viral mRNA synthesis. The overall process is commonly referred to as cap snatching[3,4]. It has been established that a minimum of nine nucleotides are required to prime the polymerase for subsequent elongation, which is mediated by the PB1 polymerase subunit[1], but shorter oligonucleotides still bind efficiently to the PB2 polymerase[5]. A pool of oligonucleotides comprising only four nucleotides attached to the

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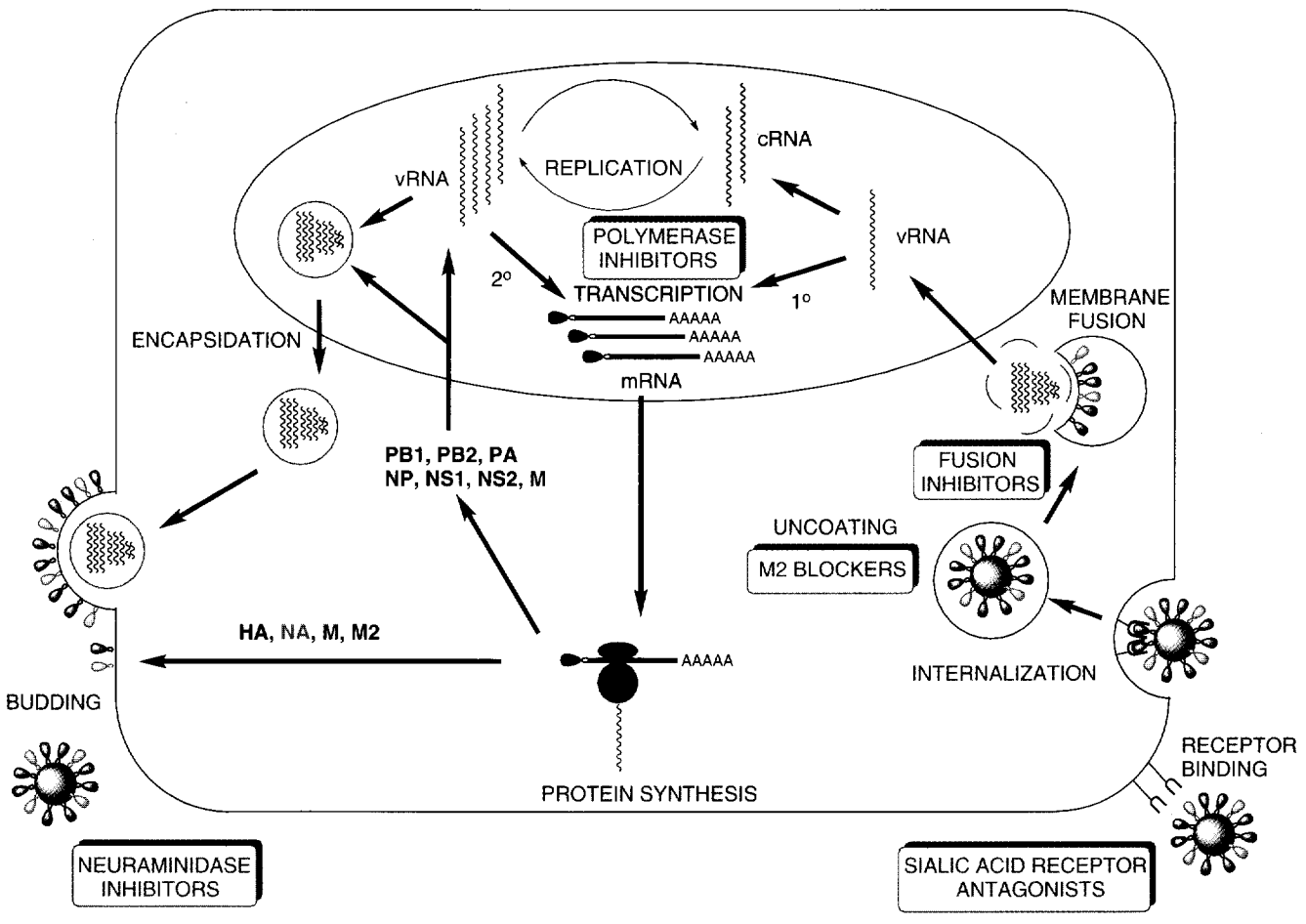

Figure 1. *Influenza life cycle and defined sites for therapeutic intervention. PB1, PB2, PA, polymerase complex; NP, nucleoprotein; NS1, NS2, proteins not found in mature virions; M, matrix protein; M2, M2 ion channel protein; HA, hemagglutinin; NA, neuraminidase.*

m^{7}GpppNm cap can still bind to the polymerase but, because they are too short to function as primers, they behave as potent and competitive inhibitors of the polymerase *in vitro*[5].

Simple phosphate derivatives have been described as nonselective inhibitors of viral DNA and RNA polymerases, including influenza polymerase[6–8]. Phosphonoformic acid (foscarnet, **1**; Figure 2) is prototypical, but the acetic acid homologue **2** (Figure 2), the tetrazole analogue **3** (Figure 2) and the squarate derivative **4** (Figure 2)[9] are all active inhibitors, although less potent. Because the activity of these compounds correlates with binding to zinc ions, they are postulated to coordinate to an essential zinc ion in the active site of the polymerase and sterically interfere with nucleotide triphosphate binding or prevent the release of pyrophosphate after chain extension[7].

Because primary transcription is readily performed *in vitro*, it is amenable to high-throughput screening techniques, which have been employed in the search for effective inhibitors of the viral polymerase. A cap-dependent transcription assay was recently used to identify a dioxobutanoic acid derivative as representative of a new structural class of inhibitors[10]. The initial discovery was refined by SAR studies into L 735882 (**5**; Figure 2), a potent (IC_{50} = 1.1 μM) and selective inhibitor of the influenza endonuclease[10]. L 735882 inhibits the replication of influenza A and B viruses in cell culture with an IC_{50} of 6 μM, while exhibiting little cellular cytotoxicity at significantly higher concentrations[11]. The acid **6** (Figure 2), an isomer of L 735882, is three times more potent as an endonuclease inhibitor (IC_{50} = 0.33 μM), and has also shown good anti-influenza efficacy in a mouse model of infection following intranasal administration. At

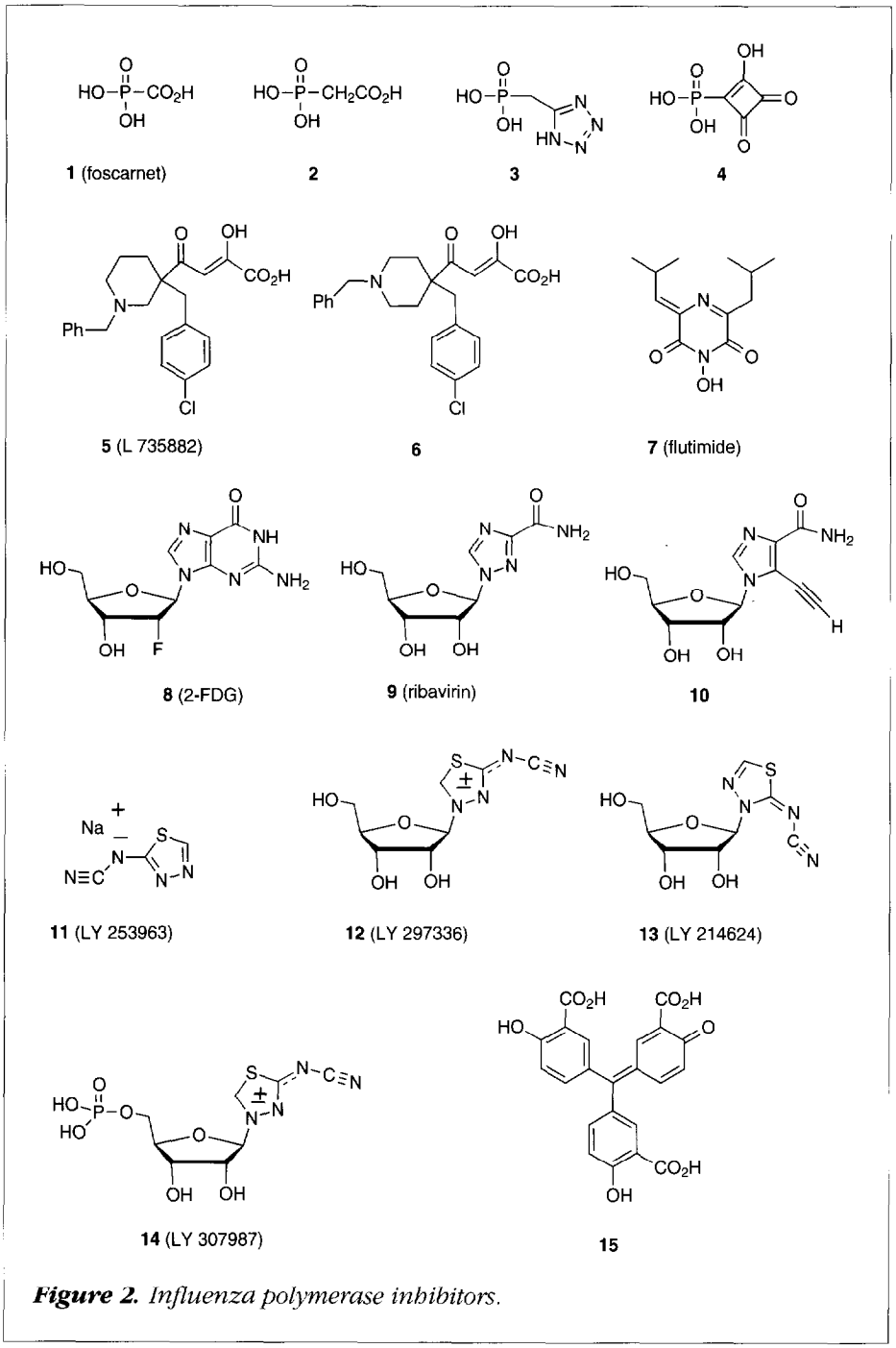

Figure 2. *Influenza polymerase inhibitors.*

doses ranging from 1 to 9 mg/kg, initiated 14 h before inoculation with influenza virus and continued until 21 h after inoculation, the acid **6** was able to significantly reduce viral titres[11].

Another selective influenza endonuclease inhibitor is the structurally unusual *N*-hydroxy imide derivative flutimide (**7**), which was isolated from the fungal species *Delitschia confertaspora*[12]. Flutimide inhibits transcriptase enzymes isolated from several influenza A and B virus strains with IC_{50} values that range from 3.5 to 5.5 μM. In cell culture, flutimide inhibited influenza virus infectivity in Madin–Darby canine kidney (MDCK) cells with an IC_{50} of 5.9 μM, with overt cytotoxicity not observed at drug concentrations as high as 100 μM (Ref. 12). Structure–activity relationships demonstrate the critical importance of the *N*-hydroxy moiety and the level of unsaturation, while increasing the bulk of one or both of the isopropyl moieties to that of a simple phenyl ring does not alter the activity. The introduction of substituted phenyl rings resulted in increased inhibitory potency in the transcriptase assay but these compounds were also significantly more cytotoxic than flutimide, precluding a more detailed evaluation of their antiviral properties.

Nucleoside analogues have long proven to be effective inhibitors of viruses that encode their own polymerases or protein cofactors required for viral replication. A series of purine nucleoside derivatives, prepared by enzyme-catalyzed transfer of the modified ribose from 2′-deoxy-2′-fluoridine to other bases, were evaluated as influenza A inhibitors using a tissue culture assay in MDCK cells[13]. 2′-Deoxy-2′-fluoroguanosine (2-FDG, **8**; Figure 2) emerged as the most potent inhibitor (IC_{50} = 19 μM), and showed a similar level of activity against a panel of influenza A and B viral strains, as well as against parainfluenza virus, in MDCK and chick embryo fibroblast cells[14]. This nucleoside also demonstrated excellent influenza inhibitory activity in human cell lines, including respiratory epithelial cells[15]. Serial passage of virus in the presence of 2-FDG led to the isolation of virus with increased resistance, implicating a viral protein as the likely target[14,15]. Further analysis of these viruses identified mutations in the PB1 protein, the subunit believed to be responsible for elongation[16]. Mechanistic studies confirmed that 2-FDG is phosphorylated by host cell kinases and that the triphosphate is a competitive

and selective inhibitor of influenza transcriptase with a K_i of 1 μM (Refs 14,17). Incorporation of 2-FDG triphosphate (2-FDGTP) by influenza transcriptase resulted in chain termination, and the transcriptase enzyme isolated from partially resistant virus showed reduced susceptibility to inhibition by 2-FDGTP (Ref. 17). Both primary and secondary transcription are inhibited by 2-FDG, and influenza inhibitory activity *in vitro* was found to be additive with amantadine[18]. In a ferret model of influenza, 2-FDG reduced fever and nasal inflammation following intraperitoneal administration as a single dose 1 h after inoculation with virus[19]. However, drug administration at later time points after infection proved to be less efficacious[17]. While single doses of 2-FDG ranging from 5 to 40 mg/kg markedly reduced viral titres in nasal washings after infection with influenza A virus, the compound showed reduced efficacy against an influenza B infection at the highest dose (40 mg/kg) tested[19]. Although 2-FDG would appear to be a viable antiviral drug candidate, as a nucleoside analogue it has the potential liability associated with cellular toxicity, an aspect that needs to be examined closely.

Host cell enzymes that play a crucial role in the virus life cycle represent an alternative target for the development of influenza inhibitors. Although indirect, the approach of targeting a cellular enzyme has the distinct advantage that it is less likely to lead to the formation of resistant virus. However, offsetting this potential advantage is the ever-present question of host cell toxicity. The most familiar representative of this class of compound is probably the broad-spectrum antiviral agent ribavirin (**9**; Figure 2). The triphosphate derivative of ribavirin[20] has been reported to inhibit capping of mRNA by inhibiting guanyltransferase enzymes[21]. In addition, ribavirin triphosphate inhibits influenza polymerase[22], interfering with both primer generation and elongation[23]. However, the dominant effect of ribavirin is indirect inhibition of influenza polymerase by depleting cellular pools of GTP (Refs 24–26). Ribavirin 5′-monophosphate is a potent inhibitor of inosine 5′-monophosphate dehydrogenase (IMPDH), which converts inosine monophosphate (IMP) to xanthosine monophosphate (XMP), the biosynthetic precursor to guanosine monophosphate (GMP). Ribavirin is of clinical value in the treatment of a wide range of viral infections, especially respiratory syncytial virus infection in children[27,28], as well as demonstrating efficacy in the treatment of influenza[29,30]. However, its toxicity and possible immunosuppressive effects suggest that it may be too toxic for routine and widespread use[29,30].

The imidazole nucleoside derivative **10** (Figure 2) is an analogue of ribavirin that shows broad-spectrum antiviral activity, including inhibition of influenza A and B infections *in vitro*[31]. The antiviral effects of **10** are reversed by addition of excess guanosine, which is consistent with IMPDH as the target. This was subsequently confirmed by more detailed biochemical studies[32,33], which revealed that the 5′-monophosphate derivative of **10** is an irreversible inhibitor of both human type II and *Escherichia coli* IMPDH, covalently modifying a cysteine residue of the enzyme thought to be functionally involved in the catalytic cycle[33].

LY 253963 (**11**; Figure 2), the sodium salt of LY 217896, has been found to inhibit representative influenza A and B strain infections in MDCK cells at concentrations ranging from 1 to 4 μg/ml (Refs 34,35). However, LY 253963 also inhibits normal cell growth[34], does not select for resistant virus mutants and shows broad-spectrum antiviral properties[36], all of which suggest a mechanism that involves inhibition of host cell processes. The observation that LY 253963 reduced GTP pools in MDCK cells and that influenza inhibitory activity was reversed by adding excess GTP, a profile analogous to that of ribavirin, implicated IMPDH inhibition as the potential mechanism of action[37,38]. Elevation of IMP levels in cells treated with LY 253963 added further support to this postulation[39]. Studies using ^{14}C-labelled material established that LY 253963 itself was not the active species but acted as a prodrug, proceeding through a series of metabolic transformations before producing the active IMPDH inhibitor[37–39]. Using MS and NMR techniques, the ribosylated derivative **12** (Figure 2), a meso-ionic compound, was identified as a urinary metabolite following administration to mice. Incubation of LY 253963 with ribose-1-phosphate and purine nucleoside phosphorylases from several species produced the meso-ionic ribosylated derivative **12**, a process that was irreversible under the reaction conditions[37]. At high enzyme concentrations, the isomeric ribosylated product **13** (Figure 2) was observed but, because its formation was reversible, the isomer **12** was ultimately produced. The monophosphate derivative **14** (Figure 2) was identified as an additional metabolite, and was shown to be a potent inhibitor of IMPDH and the likely mediator of the biological activity of **11** (Ref. 39). It appears that **14** is formed directly from **11** by phosphoribosylation, rather than sequential metabolism involving the phosphorylation of **12**. LY 297336 (**12**) did not demonstrate significant antiviral activity at high concentrations, suggesting that it was a secondary metabolite formed by dephosphorylation of **14** (Ref. 39).

Although LY 253963 (**11**) showed protective activity in animal models of influenza following oral administration[35,36], a clinical study was disappointing. The drug was administered as daily single doses of 75 mg for seven days, beginning 24 h before inoculation with an influenza A virus. However, viral titres in nasal washings, the rate and duration of viral shedding and the incidence of upper respiratory tract illness were not significantly different between drug- and placebo-treated individuals[40].

Several other agents that interfere with the function of the influenza polymerase have also been described. Aurin tricarboxylic acid (**15**; Figure 2) was identified from a series of triphenylmethane dyes as a nonselective polymerase inhibitor that reversibly inhibited influenza infectivity in Madin–Darby bovine kidney (MDBK) cells[41]. Viral replication is dependent upon the action of various cellular methyltransferases that methylate the guanosine present at the 5′ end of host cell RNA, which is used to prime viral RNA synthesis, and also methylate internal adenosine residues in viral RNA (Ref. 42). 3-Deaza-adenosine and neplanocin A are methyltransferase inhibitors that inhibit influenza virus replication in tissue cell culture, although the two nucleoside analogues appear to target different enzymes[43].

An alternative method of inhibiting influenza virus translation depends upon the design and characterization of antisense oligonucleotides[44]. This is an especially attractive strategic approach towards inhibiting influenza replication because of the single-stranded RNA genomic structure of the virus. In theory, all of the replicative phases of the virus life cycle (transcription, translation and replication) should be susceptible to inhibition by antisense oligonucleotides. Although antisense oligonucleotides containing normal phosphodiester bonds were not effective inhibitors in tissue culture[45], oligonucleotides bound to other agents or phosphorothioate oligonucleotides did exhibit antiviral properties[45–47]. However, while antisense oligonucleotides are being evaluated in a wide variety of disease states, they remain an unproven therapeutic strategy[48]. Consequently, the continued development of antisense oligonucleotides as antiviral agents will probably depend upon the success of the entire antisense field[48].

Virion packaging

After viral replication, progeny virus is packaged and released to repeat the cycle of infection. As part of this process, the viral matrix protein migrates to the nucleus and surrounds the viral RNP, which may be a mechanism for shutting down replication[49–51]. Simultaneously, the viral surface proteins hemagglutinin (HA), neuraminidase (NA) and M2 protein are synthesized and transported to the apical membrane of the cell. Ribonucleoproteins coated with matrix (M) protein are subsequently transported to the apical membrane, where virus formation ensues, presumably through a budding mechanism (Figure 1)[49]. Because the process through which packaging occurs is not well understood, targets for therapeutic intervention have been difficult to identify. However, the capacity of the matrix protein to inhibit transcription by RNPs *in vitro*[52–54] has prompted studies on the mechanism and potential utility of this property. While high concentrations of matrix protein are required for inhibition, monoclonal antibodies that map to amino acids 70–140 of the matrix protein have been found to neutralize this activity[52,53], and studies of mutants implicate an essential function for the region between residues 91 and 111 (Ref. 55). An extract from *Pinus parviflora* Sieb bark that contains acidic polysaccharides and other high-M_r compounds has recently been described as an inhibitor of this stage of virus growth[56]. Although not characterized at the molecular level, these extracts suppress the growth of influenza virus in MDCK cells and interfere with M1–RNA complex formation *in vitro*[56].

Virus release

Recent studies suggest that the release of viral particles may ultimately prove to be the most effective target for antiviral drug discovery and development. The influenza NA is the second major viral surface protein and one of only two enzymes encoded by the virus. It plays a functional role in the release of virus by cleaving sialic acid residues from glycoproteins and contributes to viral infectivity and virulence in several ways[57]. Both influenza HA and NA are glycosylated after synthesis and during transport to the cell membrane. Neuraminidase removes the terminal sialic acid residues from these proteins, eliminating an element of self-recognition and reducing the propensity of the virus particles to aggregate[58,59]. Neuraminidase also cleaves sialic acid moieties from host cell proteins, which facilitates release of the virus from host cells and increases virus mobility in mucus *in vivo*, thereby enhancing accessibility to epithelial cells.

Neuraminidase has long been recognized as a potential target for therapeutic intervention in influenza infections, but it is only recently that inhibitors with sufficient potency and selectivity to make them viable candidates for drug

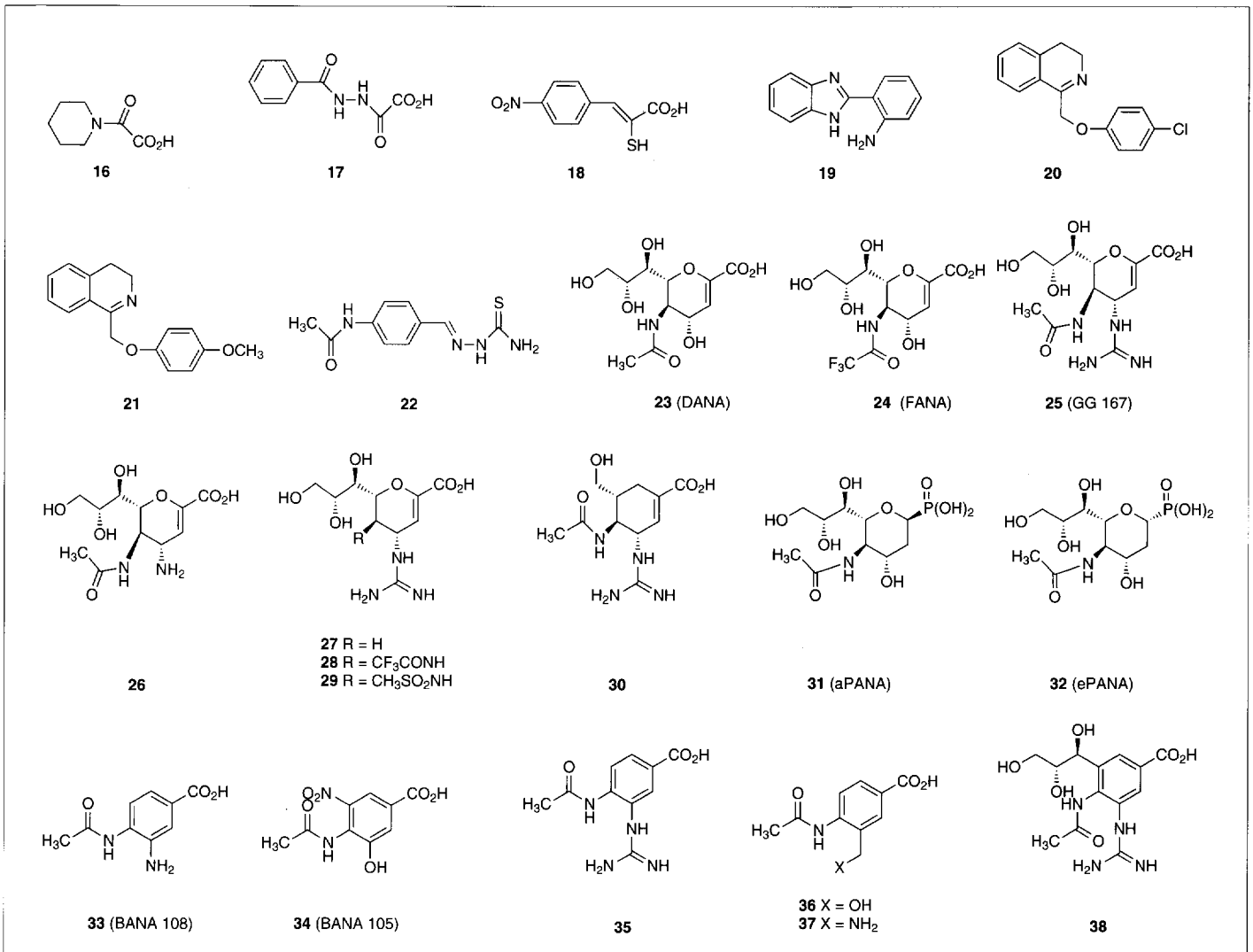

Figure 3. *Influenza neuraminidase inhibitors.*

development have been identified. Oxamic acid derivatives were one of the earliest studied classes of influenza NA inhibitors, the most potent of which were **16** and **17** (Figure 3)[60]. The inhibitory activity of these compounds was rationalized by comparison with sialic acid, using the carboxylate moiety as a common structural element[60]. The thiopyruvic acid **18** (Figure 3) is an inhibitor of bacterial NA (IC_{50} = 0.14 μg/ml), and shares some obvious common structural elements with **16** and **17** (Ref. 61). However, the SARs associated with **18** suggest that the two series are distinct. Although **18** and a close analogue showed activity in a plaque reduction assay using a strain of parainfluenza 3 virus, high doses of **18**, given orally or intraperitoneally, failed to protect mice against the lethal effects of an influenza infection[61].

Three distinct structural classes of influenza NA inhibitors that do not contain a carboxylic acid have been characterized[61–64]. The benzimidazole **19** (Figure 3) is the most potent representative of a series of structurally related heterocycles characterized as competitive NA inhibitors, although it failed to exhibit antiviral activity *in vivo*[61]. Isoquinoline (**20**; Figure 3) is a relatively weak (IC_{50} >500 μg/ml) and nonselective influenza NA inhibitor that inhibits replication of a broad range of viruses in cell culture assays[62]. Nevertheless, the homologous compound **21** (Figure 3) was advanced into clinical studies, where it showed some efficacy against influenza B viral infection[63]. The thiosemicarbazone **22** (Figure 3) was identified as an influenza NA inhibitor by broad screening techniques using an N1-containing viral strain[64]. Although **22** is a reasonably potent inhibitor of

N1 NA (IC_{50} = 8–15 μM), it is of limited potential as a therapeutic agent because its activity is restricted to this enzyme subtype. This appears to be related to the allosteric nature of inhibition that was found to be competitive with Ca^{2+} binding to a regulatory site of the enzyme[64].

Substrate-based neuraminidase inhibitors

A far more successful strategy that has led to the characterization of very powerful inhibitors of influenza NA has been a substrate-based approach that integrates an understanding of enzyme mechanism[65,66] with the type of insight available only from X-ray crystallographic studies of influenza NAs[67–69]. This method has been implemented as an iterative process, consisting of the design, synthesis and evaluation of potential inhibitors, coupled with the determination of the molecular structure of the newly synthesized inhibitors bound to the enzyme. While this process has frequently confirmed the predicted mode of binding, the importance of this step should not be underestimated because recent findings have provided unique insights into unanticipated aspects of molecular recognition.

DANA, FANA and GG 167

DANA (2-deoxy-2,3-dihydro-*N*-acetylneuraminic acid, Neu5Ac2en; **23**; Figure 3) was the first substrate-based NA inhibitor described[70] in which the planar sp^2-hybridized olefin was proposed to mimic the structure of the cationic transition state[65,66]. This compound inhibited NAs from representative influenza A and B viruses with IC_{50} values ranging from 20 to 90 μM (Ref. 70), and inhibited virus replication in tissue cell culture[71]. The *N*-trifluoroacetyl analogue, FANA (**24**; Figure 3), was ten times more potent than **23** (IC_{50} = 2–5 μM), and kinetic studies indicated competitive inhibition with respect to substrate[70]. However, the potential therapeutic value of NA inhibitors was not realized with these compounds because they demonstrated poor selectivity for influenza enzymes and were not protective in animal models of influenza.

The determination of the crystal structure of influenza virus NAs in 1983 (Ref. 72) and subsequent studies[73,74], particularly those with inhibitors bound in the active site, have provided a basis for structural refinement of DANA and FANA into much more potent and selective agents. GG 167 (**25**; Figure 3) has emerged from this work as a rationally designed, potent and effective influenza NA inhibitor that is protective in animal models of influenza infection and has advanced into clinical trials[67]. The successful development of GG 167 has stimulated a high level of interest in the design and synthesis of influenza NA inhibitors, an effort that focuses on improving several deficiencies associated with the prototype. One particular facet that is attracting attention is the less than desirable pharmacokinetic properties of GG 167, which is not orally bioavailable and so must be administered as intranasal drops or an intranasal spray.

Influenza NAs are homotetramers comprising glycosylated 60 kDa polypeptides that can be enzymatically cleaved from the viral membrane surface and crystallized. The surface amino acid residues of NAs differ across viral subtypes and are subject to constant immune response pressure. However, the active site of the enzyme is buried below the surface of the protein and is highly conserved, being lined with amino acids that are invariant across all influenza A and B strains.[67] A careful analysis of the interactions of DANA with active site residues and the application of molecular modelling paradigms identified a region in the vicinity of the 4-hydroxyl substituent that contained several acidic residues, leading to the postulate that a basic amine at the 4-position of DANA should increase binding affinity[67,75]. This was indeed the case: the amino derivative **26** (Figure 3) was found to inhibit an influenza N2 NA with an IC_{50} of 50 nM, a nearly 20-fold improvement over DANA. The guanidino homologue GG 167 was an even more potent inhibitor (IC_{50} = 0.2 nM), which displayed slow-binding inhibition of NAs from influenza A strains, with steady state reached after approximately 20 min (Ref. 67). The latter is thought to be a consequence of the displacement of a molecule of water from the active site by the guanidine moiety and the steady state K_i has been determined to be 0.03 nM after 150 min (Refs 67,76). By contrast, NA from an influenza B virus is inhibited by GG 167 in a simple competitive fashion[76]. GG 167 potently inhibits a wide range of clinically relevant influenza A and B virus NAs[77] and avian influenza NAs[78,79], but it is a considerably weaker inhibitor of bacterial, mammalian and other viral sialidases[80]. In plaque reduction assays in cell culture, GG 167 half-maximally inhibited the replication of a broad range of human[67,77] and avian[78] influenza viruses at concentrations 100-fold lower than amantadine, rimantadine and ribavirin, and amantadine-resistant viral strains remained sensitive to GG 167. The compound effectively inhibits influenza replication in human respiratory epithelial cells[81] and demonstrates an excellent therapeutic index *in vitro*, because cytotoxicity was not observed in several mammalian cell types at concentrations as high as 10 mM (Refs 77,81).

In vivo *studies*

GG 167 demonstrates efficacy in animal models of influenza conducted in mice[67,82] and ferrets[83] following intranasal administration, but fails to provide prophylactic protection of chickens against the lethal effects of a highly pathogenic strain of avian influenza[84].

A consequence of the highly polar, zwitterionic nature of GG 167, a physical property that contributes to its low cytotoxicity, is poor bioavailability following intraperitoneal or oral administration of the drug and an abbreviated 10 min half-life following intravenous administration to mice[82,83]. In man[85], the drug is rapidly eliminated in the urine without metabolic modification following intravenous dosing, and it exhibits a $t_{1/2}$ of 1.6 h. In contrast, plasma levels of GG 167 are sustained for longer periods following intranasal or inhalation administration, with terminal phase half-lives of 2.9 and 3.4 h (Ref. 85). This observation was interpreted to be a consequence of a relatively slow absorption of the drug from bronchial tissue, reflecting an extended residence time in the target tissue. In a preliminary clinical study conducted in 166 volunteers, GG 167 was evaluated for both its prophylactic and therapeutic properties in an experimental influenza infection[86], with the latter study arm designed to evaluate the efficacy of early and delayed drug intervention following challenge with virus. The initial dosing regimen, intranasal administration of 16 mg of GG 167 in drops six times daily, was identical in both arms of the study but was initiated 4 h before inoculation with an A strain of influenza in the prophylactic trial and either 26 h or 50 h post-inoculation in the treatment group. Subsequently, lower doses of drug, 3.2 mg and 7.2 mg, administered twice daily were evaluated using the prophylactic paradigm. The drug was well tolerated and efficacy was demonstrated in both the low- and high-dose groups treated prophylactically, measured as a statistically significant reduction in virus titres and duration of viral shedding, compared to the placebo-treated control group. Drug treatment completely protected against febrile illness, and the twice daily dosing schedule was as effective as the six times a day regimen. In the early treatment group, the duration of viral shedding was significantly lower than in drug-free controls, although viral titres were similar in both groups, and the incidence of influenza-like symptoms was reduced, with the absence of fever a prominent benefit.[86] In the delayed treatment study, the drug was considerably less effective, with clinical symptoms similar in both the drug-treated and control groups.

Recent studies of GG 167 have focused on the question of resistance development[87–90] and aspects of structure–activity and synthetic accessibility[80,91–96]. Interestingly, resistance mutations arising from passage of virus in the presence of GG 167 map to both the NA enzyme and HA (Refs 87–90). A single amino acid change in NA, in which Glu119 is replaced by a Gly or Ala residue, is responsible for the emergence of resistance to GG 167 (Refs 88–90). The substitution of Glu119 by Gly leads to a 250–1,000-fold reduction in viral sensitivity to GG 167 and a loss of the slow-binding inhibitory kinetics[89]. The mutant NA has been characterized by X-ray crystallographic analysis and the observed resistance rationalized based on the fact that Glu119 resides in the active site of NA and is one of the residues that interacts with the guanidine moiety of GG 167. Interestingly, the Gly119 mutant NA and the Glu119 wild-type enzyme are equally susceptible to inhibition by the amino analogue (**26**; Figure 3). Mutations in HA map to regions proximate to the sialic acid receptor binding pocket and presumably alter the binding affinity of the HA for its receptor, thereby favouring viral release from cells in the absence of an effective NA (Ref. 87). Other HA mutations appear to involve potential glycosylation sites[90].

Inhibitors derived from GG 167

Medicinal chemistry studies have defined fundamental structural elements associated with the inhibitory properties of GG 167 and have simultaneously led to the development of improved synthetic approaches to this class of sialic acid derivative[80,91–96]. From the SARs explored to date, the C-4 guanidino moiety of GG 167 appears to be optimal. Substitution of the guanidino nitrogens[91] or the introduction of other substituents[80], both basic and non-basic, at C-4 resulted in considerably weaker NA inhibitors. Abbreviation of the C-6 glycerol side-chain by removing the terminal CH_2OH resulted in a 100-fold reduction in potency, while a simple CH_2OH at C-6 gave a compound 2,000-fold weaker than GG 167 (Ref. 92). The 5-CH_3CONH moiety is crucial, because **27** (Figure 3) shows a 25,000-fold reduced affinity for NA. However, the CF_3CONH group of **28** is an effective substitute (Figure 3), replicating the SAR observed for DANA, and the sulfonamide **29** (Figure 3) also retains potent NA inhibitory activity[95]. The effect of replacing the pyran heterocycle with a carbocyclic template was examined in the context of analogue **30** (Figure 3), which incorporates a synthetically more accessible abbreviated glycerol side-chain and was found to be equipotent with the corresponding

pyran analogue[93]. Phosphonate **31** (aPANA; Figure 3), in which the phosphonic acid moiety is axial, is a generally poor inhibitor of influenza NAs[96]. In contrast, the equatorial isomer **32** (ePANA; Figure 3) is 100-fold more potent than sialic acid as an inhibitor of influenza A N2 NA although it is weaker against type A N9 NA (Ref. 96). The increased potency of **32** compared with sialic acid was ascribed to a combination of a stronger electrostatic interaction between the phosphonate and the complementary Arg in the active site, and the reduced distortion of the active site of NA by **32**, as judged from X-ray crystallographic structures of **32** bound to NA (Ref. 96).

Attempts to simplify the structure of these sialic acid based inhibitors have met with limited success so far but, nevertheless, provide several new avenues for drug design that will almost certainly yield more potent compounds after further structural refinement[97–100]. The strategy of replacing the pyran ring of sialic acid derivatives with a planar benzene ring as the nuclear template – a design based on an analysis of the conformation of DANA bound to NA – results in considerable structural simplification and, correspondingly, increases synthetic accessibility[97,99,100]. BANA 108 (**33**; Figure 3) is a structural analogue of **26** that is a poor inhibitor of influenza NA (Ref. 97), presumably because it lacks the glycerol side-chain, which in the sialic acid series is known to engage in important interactions with the enzyme. BANA 105 (**34**; Figure 3) is more potent, with IC_{50}s of 0.75 mM against type A and B NAs, but this is still considerably weaker than DANA and GG 167. These compounds bind to the active site of influenza NAs in a predictable fashion, with the carboxylate and acetamido moieties mimicking the same functionality in DANA. In the case of BANA 105, the hydroxyl fills the pocket occupied by the C-4 hydroxyl of DANA (Refs 97,98). Further structural modification, in which functionality designed to increase the similarity with GG 167 was introduced, led to increased potency, and **35** (Figure 3) inhibits influenza N2 and B NAs with IC_{50}s of 10 μM. This compound, which is the most potent NA inhibitor based on this chemotype to be reported to date, is an effective inhibitor of virus replication in MDCK cells in culture at concentrations ranging from 1 to 10 μM (Refs 99,100). Determination of the crystallographic structure of **35** bound to an influenza NA enzyme provided a surprise: the guanidino moiety was found to occupy the site normally occupied by the glycerol side-chain of sialic acid based inhibitors, rather than binding in the pocket occupied by the same element of GG 167 (Ref. 99). The analogues **36** and **37** (Figure 3) were found to project their hydroxymethyl and aminomethyl groups into the same binding pocket. Consistent with this as the preferred binding mode, introduction of the glycerol side-chain to **35** afforded an inactive compound (**38**; Figure 3)[100].

Summary

As the search for clinically effective and selective inhibitors of influenza virus infection enters its fourth decade, the pace of discovery has noticeably quickened. Progress is being driven as much by advances in the understanding of viral biochemistry and its integration with host cell processes, leading to the development of several new strategic approaches to control viral infectivity, as it is by viewing previously identified targets from a different perspective. While modern influenza inhibitor research began 30 years ago with the serendipitous discovery of amantadine, the discovery of the NA inhibitor GG 167 represents an elegant application of contemporary methods of structure-based drug design. The preclinical profile of this compound and the results of preliminary clinical studies are extremely encouraging and suggest that influenza inhibitor drug discovery may be on the threshold of a new era. A broader and more rigorous clinical evaluation of GG 167 under nonexperimental conditions is ongoing and the results are anxiously anticipated. In the meantime, the apparent success of GG 167 is stimulating a great deal of interest in the design and development of NA inhibitors, and compounds with improved pharmacokinetic properties will almost certainly emerge in the near future.

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In short ...

JoAnne Stubbe and **David E. Housman**, two renowned scientists, have been appointed to Ciba-Geigy chairs at the Massachusetts Institute of Technology (MIT). JoAnne Stubbe, who is noted for her contributions to the understanding of the mechanism of enzymes and natural products that bind to and cleave DNA molecules, has been named as the Ciba-Geigy Professor of Chemistry. David Housman, a molecular biologist and a member of the Department of Biology and the Center of Cancer Research at MIT, known for his significant contributions toward understanding human genetic diseases, has been named as the Ciba-Geigy Professor of Biology. MIT Dean of Science, Robert J. Birgeneau, announced: 'They will undoubtedly continue to carry out innovative, frontier research and will at the same time help our ties with one of the world's great pharmaceutical companies.'