

Review

Pandemic influenza: its origin and control

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Abstract

A “new” influenza virus will appear at some time in the future. This virus will arise by natural processes, which we do not fully understand, or it might be created by some bioterrorist. The world’s population will have no immunity to the new virus, which will spread like wild-fire, causing much misery, economic disruption and many deaths. Vaccines will take time to develop and the only means of control, at least in the early stages of the epidemic, are anti-viral drugs, of which the neuraminidase inhibitors currently seem the most effective.

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1. Introduction

Early accounts of influenza epidemics are few and far between. Although the birthplace of modern influenza pandemics seems to be China, no records of ancient influenza epidemics in China, Central Asia or Russia have been unearthed. In Europe, the first epidemic with a reasonably clear description was in Italy and France in 1173 (F.B. Smith, unpublished). Further epidemics were said to have occurred in 1414, 1557 and 1675–1676. Flu epidemics occurred in France in 1788–1790, in 1789–1790 and in 1830–1832 [1].

The first truly global epidemic (pandemic) of influenza seems to have occurred in 1847. Deaths of the unfortunate flu victims in this pandemic would have been increased by the therapy of severe repeated venesection so beloved of the surgeons of the time. The first great flu pandemic which was widely recorded was the Russian flu of 1889–1893, which returned annually until 1901. In Great Britain, the winters of 1891 and 1892 were the worst. Symptoms varied, but victims commonly experienced sudden fever which lasted 3–5 d, sometimes for a fortnight, chills, especially in the back, thumping muscular pains, runny nose and eyes, sneezing or dry coughing, prostration for up to a fortnight, loss of appe-

tite and photophobia [1]. “Headache” and “melancholia” were also reported.

The epidemic was characterised by huge morbidity. London, one of the worst affected cities had, at one stage, one-third of its population incapacitated by the flu. Economic disruption was immense. There were also deaths. In 1891, 125,000 died from influenza, and in 1892, there were 250,000 flu deaths in Great Britain.

Then, in 1918–1919, there occurred the greatest recorded influenza pandemic. “Spanish flu” killed more than 20,000,000 people world-wide, though some historians believe that this number may have died in India alone, where no one kept reliable records. Why was this virus so virulent? So far, there is no answer to this question. Lung samples from Eskimos who died from flu at Brevig Mission in Alaska in 1918 have been preserved, buried in the permafrost ever since, and lung samples from Spanish flu victims preserved in pathology museums have yielded enough viral RNA fragments for Jeffrey Taubenberger and his colleagues to sequence the haemagglutinin, neuraminidase and non-structural protein genes of the 1918 virus [2]. There was nothing obvious in these sequences to suggest what genetic changes caused the virus to be highly pathogenic, and the answer may lie in some of the other genes which are still being sequenced at this time.

However, a novel phylogenetic analysis [3] has suggested that the 1918 haemagglutinin gene was a recombinant and that the recombination event occurred at about the same time as the virus became so virulent. There is no reason to sup-

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pose, however, that it was this genetic change which caused the highly pathogenic properties of the virus. The conclusion, that true recombination had occurred, has also been questioned [4]. Recent sequencing of flu RNA recovered from birds preserved in alcohol since 1917 suggests that the 1918 Spanish flu virus was not directly derived from an avian influenza virus [41].

Type A influenza viruses can be subdivided into subtypes with serologically distinct haemagglutinin and neuraminidase surface antigens. So far, 15 haemagglutinin (H) subtypes and nine neuraminidase (N) subtypes have been discovered in nature. The 1918 Spanish flu virus was influenza type A, subtype H1N1 and, as far as we know, this subtype persisted until 1933, when the first human influenza virus was isolated.

Then, in 1957, a new influenza virus suddenly appeared in Southeast China. This type A influenza virus, subtype H2N2, caused the "Asian flu" pandemic during which H1N1 strains disappeared completely from the human population. Asian (H2N2) influenza persisted until 1968, when another new virus suddenly appeared in China. This virus, subtype H3N2 had a new haemagglutinin and caused the pandemic of "Hong Kong" influenza during which H2N2 viruses disappeared completely from people. In 1977 the H1N1 virus re-appeared, apparently in Anshan in northern China, on February the 4th of that year. Today, variants of H3N2 and H1N1 viruses are co-circulating, together with influenza type B strains. Recently, it was reported that reassortant H1N2 strains were also infecting people [5].

The Asian (H2N2) and the Hong Kong (H3N2) viruses were both re-assortants. In 1957, the H2 haemagglutinin and the N2 neuraminidase, as well as one of the polymerase proteins (PB1) apparently came from an avian influenza virus, while the other proteins were from the "old" H1N1 human virus [6]. The "new" H3 haemagglutinin [7] of the 1968 Hong Kong virus was found to have most likely come from an avian influenza virus [8], as did the PB1 polymerase protein [6], while the other proteins were from the "old" Asian H2 N2 human flu virus.

Pigs can be infected by both avian and human strains of type A influenza virus, and it has been proposed that pigs are the "mixing vessels" where the re-assortant events occur [9]. Although this is an attractive idea, no evidence exists which shows that the Asian and Hong Kong viruses (or any other "new" human influenza virus) were formed in pigs in this way.

Why H1N1 (Russian flu) re-emerged in 1977 is a mystery. All the genes of the 1977 virus were virtually identical to those of H1N1 viruses circulating in 1950 [10]. Where had this virus been hiding, unnoticed and unchanging for 27 years? The answer is not known.

In 1997, an H5N1 avian influenza virus killed chickens in the live bird markets in Hong Kong. During a short period at the end of 1997, this virus began infecting people, with disastrous results. Of 18 people with confirmed H5N1 infections, six died, a huge mortality rate. No evidence of human-

to-human transmission was found. Following the slaughter of all of the chickens in Hong Kong, no more human cases were identified. All the genes of this H5N1 virus were of avian virus origin and the genetic change which enabled the virus to infect humans has not been identified.

The H5N1 viruses could, however, be divided into two groups. One virus (HK483) was highly pathogenic for mice, while another (HK486), had low pathogenicity. Re-assortant experiments showed that it was the PB2 gene (coding one of the internal polymerase proteins) in association with a readily cleavable haemagglutinin which seemed to be responsible for the difference in virulence (for mice) of these two avian influenza viruses [11]. There are eight amino acid differences between PB2 of HK483 and HK486 viruses, and the residue responsible for the difference in virulence seems to be that at position 627, which is lysine in HK483 and glutamic acid in HK486 [12].

2. The origin and spread of pandemic influenza

The last three influenza pandemics originated in China, and although historical records of earlier pandemics have not been discovered, it is likely that these may have also originated in this part of the world.

All the known subtypes of type A influenza exist in water birds in China and the following is a possible future scenario. One day, somewhere in Southeast China, one of these avian influenza viruses suddenly acquires, by mutation or by genetic re-assortment, the ability to infect and spread in people. Although the virus is highly infectious and readily transmissible, the disease it causes is fairly mild and, probably, because of this, the fact that a "new" influenza virus has appeared in the human population escapes notice until hundreds of people are infected.

The infection is at first confined to the small village where the new virus arose, but after a few days, it begins to spread along the railway lines by infected travellers. In this way, the virus reaches Beijing and Hong Kong, where an explosive epidemic occurs. The virus by now has been recognised as a new virus by the local surveillance labs and samples have been sent to WHO Reference Centres at Mill Hill, London, and CDC in Atlanta for identification. The answer comes back. It is type A influenza of subtype H9N6 (for example). This means that no one in the world has any immunity to the virus and it is inevitable that a huge pandemic will occur.

But then, something more sinister takes place. The H9N6 virus, which so far has caused fairly mild influenza illness, suddenly mutates to a form which is highly pathogenic. Patients start being admitted to hospital with severe pneumonia. There is an increasing number of deaths. Such an event happened in 1983 when a benign H5N2 avian influenza virus, which was present in commercial poultry sheds in Pennsylvania, suddenly acquired pathogenicity and killed chickens and turkeys with 100% mortality. In the end, 17

million chickens died as a result of a single base change in the haemagglutinin gene.

The mutation which caused the avian H5N2 virus to change in this way was identified as threonine to lysine at position 13 in the haemagglutinin. This change abolished a glycosylation site at position 11, where the carbohydrate was protecting a pathogenic HA cleavage sequence. This produced a readily cleavable haemagglutinin, thus enabling the virus to infect every organ in the chicken's body [13]. So with the spectre of unimaginable world-wide disaster imminent, what could be done to contain a new, lethal H9N6 virus if one arose in this way?

Vaccines against the new virus could never be made in time and, in any case, a mass vaccination campaign against the new virus would be fraught with difficulties. Such a mass influenza vaccination was, in fact, attempted in 1976, when a swine flu outbreak occurred among army recruits at Fort Dix, New Jersey [14]. As it was thought that the Spanish flu virus had returned, President Ford authorised the expenditure of \$350,000,000 to "...vaccinate every man, woman and child in the USA".

This mass vaccination campaign experienced a number of problems – low antibody titres, vaccine side-effects and litigation tangles. The expected epidemic never materialized but the problems could be repeated if such an exercise were ever attempted again. So, at the moment, it seems that the first line of defence against a new virus will be anti-virals, and at the moment the neuraminidase inhibitors, although costly, seem to be the drugs of choice.

3. Controlling influenza by inhibiting the virus' neuraminidase

The neuraminidase story started in the 1940s, when George Hirst, working at the Rockefeller Institute in New York, found that if red blood cells were mixed in the cold with allantoic fluid from chick embryos which had been infected with influenza virus, the cells were very heavily agglutinated. When the agglutinated cells were warmed to 37°, they dispersed and could not be re-agglutinated in the cold with fresh virus [15]. Hirst interpreted these results to mean that the virus possessed an enzyme which was destroying receptors for the virus on the red cells. Then, MacFarlane Burnet, working in the Walter and Eliza Hall Institute in Melbourne, Australia, showed that *Vibrio cholerae* secreted an enzyme which did the same thing, and this activity was called receptor-destroying enzyme, or RDE.

Alfred Gottschalk, also working in the Hall Institute, reasoned that if RDE was destroying receptors on cells, there was probably a "split product" from the action of the enzyme. He therefore digested purified ovomucin with cholera RDE, dialysed the reaction mixture and isolated and eventually characterised the "split product" as sialic acid, or *N*-acetyl neuraminic acid [16]. Thus the viral enzyme became known as "sialidase" or "neuraminidase".

These experiments led Burnet to suggest that an effective "competitive poison" of the virus' enzyme might be useful in controlling infection by the virus [17], but how this could be done was not realised for another 50 or so years. In 1948, it was thought that haemagglutination by influenza virus was due to the enzyme, neuraminidase, on the surface of the virus, binding to its substrate, sialic acid, on the surface of the red cell. This idea gained some credence when it was found that the sialidase secreted by *V. cholerae* could adsorb to red cells in the cold, a finding which was used in purification of this enzyme.

Then, in 1961, soluble sialidase was isolated from particles of PR8 influenza virus, and this did not adsorb to red cells [18]. In other experiments, treatment of influenza B virus with trypsin liberated almost all of the virus' neuraminidase (NA) as a soluble product and left the haemagglutinin (HA) activity associated with the virus particle. The liberated neuraminidase had a molecular weight of about 200,000 [19]. This was the best evidence so far that the haemagglutinin activity of flu virus was not due to enzyme-substrate binding.

Trypsin, of course, cleaves covalent bonds and the Noll experiment [19] did not prove that the HA and NA activities resided in separate molecules. When purified influenza virus particles were disrupted by detergent, however, and electrophoresed on cellulose acetate strips, a clear separation of HA and NA activities was obtained [20].

Further experiments with detergent-disrupted re-assortant influenza virus particles [21] resulted in pure preparations of intact haemagglutinin and neuraminidase molecules. Electron micrographs showed that these two surface "spikes" on the influenza virus particle had different shapes; the HA was a triangular rod-shaped object, while the NA had a box-like head atop a long thin stalk [22]. Later, when pure NA "heads" were obtained, some elegant electron micrographs by Nick Wrigley showed very clearly the square box-like nature of the NA and that the molecule was probably a tetramer [23].

4. Function of influenza virus neuraminidase

The first clue as to the function of influenza virus neuraminidase came from experiments by Seto and Rott which showed that sialidase activity was associated with the release of virus from infected host cells [24]. It was then shown that antibody directed specifically against influenza virus neuraminidase did not prevent infection of susceptible cells, but prevented the release of detectable levels of haemagglutinin [25].

But perhaps the most elegant explanation of the function of flu virus' neuraminidase came from experiments with temperature-sensitive influenza virus mutants defective in neuraminidase. Electron micrographs of cells infected with wild-type virus showed newly formed virus particles which had budded off the cells, roaming freely away to infect other cells. Similar images of cells infected with a mutant lacking

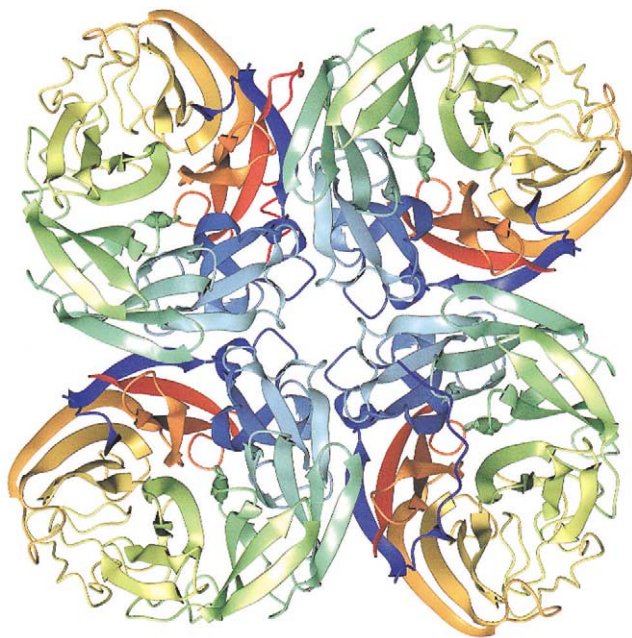


Fig. 1. Ribbon diagram of an N2 neuraminidase tetramer [PDB entry 1NN2]. The colours change from blue (N terminus) to red (C terminus) through green along the amino acid sequence. Figure drawn with AESOP.

NA activity showed huge aggregates of virus particles which had budded from the cells and then remained attached to or near the cell surface. These virus particles were obviously not going anywhere, and the infection was effectively terminated [26].

5. Structure of influenza virus neuraminidase

It has already been described how influenza B virus neuraminidase “heads” can be released from the virus by trypsin digestion [19]. Neuraminidase of type A influenza viruses was not readily released from virus particles by trypsin, but another protease, pronase, did this very effectively [27].

Pronase-released neuraminidase “heads” from a number of type A influenza viruses were crystallised [28]. This was achieved more or less by accident during experiments which showed that the NA of the H2N2 Asian influenza viruses was similar in sequence as well as in antigenic properties to the NA of the H3N2 Hong Kong viruses. The crystals obtained in this way diffracted X-rays very well [29] and the 3-D structure of influenza virus N2 neuraminidase was solved by Peter Colman and his colleagues in Melbourne, Australia [30]. The structure (Fig. 1) shows a tetrameric molecule, which when “...viewed from above the head, each monomer consists of six of the four-stranded sheets arrayed like the petals of a flower but twisted like the blades of a pinwheel” [31]. In each of the monomers was a deep cleft, or canyon.

Although the amino acid sequences of a number of influenza A and B neuraminidases differed greatly, some residues at various widely separated positions along the linear

neuraminidase polypeptide chain were totally conserved among all sequences of flu A and flu B neuraminidase which had been deposited in the data bank. Remarkably, when the neuraminidase polypeptide folded into its final 3-D structure, these conserved residues which were so widely separated in the primary sequence, clustered together, lining the walls of the deep cleft or canyon present in each of the four monomers in the tetrameric neuraminidase “head”.

This indicated that the cleft was the catalytic site of flu neuraminidase and that this site was conserved among all flu strains. So it was clear that if an inhibitor, a “plug-drug”, could be created which was effective against one influenza virus, it should be effective against all flu strains, even those which have not yet appeared in man. Confirmation that the conserved site was the catalytic site of the flu enzyme came when sialic acid was soaked into N2 neuraminidase crystals and its location in the site determined by X-ray crystallography.

Could this conserved site form the basis for a universal flu vaccine? A vaccine which could induce antibodies against all flu strains? The answer is: “Probably not”. Neuraminidase heads were complexed with monoclonal antibody Fab fragments, the complexes were crystallised and the 3-D structures were determined by X-ray crystallography [32]. The results showed that the epitope on the neuraminidase comprised five separate peptide segments which contained about 17 amino acids that were in contact with a similar number of amino acids in the binding site of the antibody. Single amino acid sequence changes in the epitope totally abolished antibody binding [33]. Since the “footprint” of the antibody was considerably larger than the conserved catalytic site, any antibody raised against this site would inevitably contact variable amino acids around the rim of the site, making a universal vaccine based on the conserved site difficult, if not impossible, to develop.

Research then concentrated on the rational design of potent and specific inhibitors of influenza virus neuraminidase, in the hope that these would be useful agents for controlling influenza. Two inhibitors, amantadine and rimantadine, drugs which blocked the ion channel activity of the M2 protein of type A influenza viruses, already existed. These compounds, however, suffered from several disadvantages. They were not effective against influenza B, they allowed resistant viruses to form easily and they caused unpleasant side effects when administered to people.

Attempts to identify other inhibitors by random screening of many thousands of compounds failed miserably. However, in the 1970s, Peter Palese and his colleagues found that dehydrated sialic acid, DANA, Neu5 Ac2en, and its fluorinated derivative (FANA), were good inhibitors of flu neuraminidase, as well as of neuraminidases from other sources. DANA and FANA inhibited influenza virus replication in tissue culture but failed to prevent disease in flu-infected animals [34].

X-ray crystallography of flu N2 neuraminidase crystals soaked in sialic acid showed that adjacent to the 4-hydroxyl

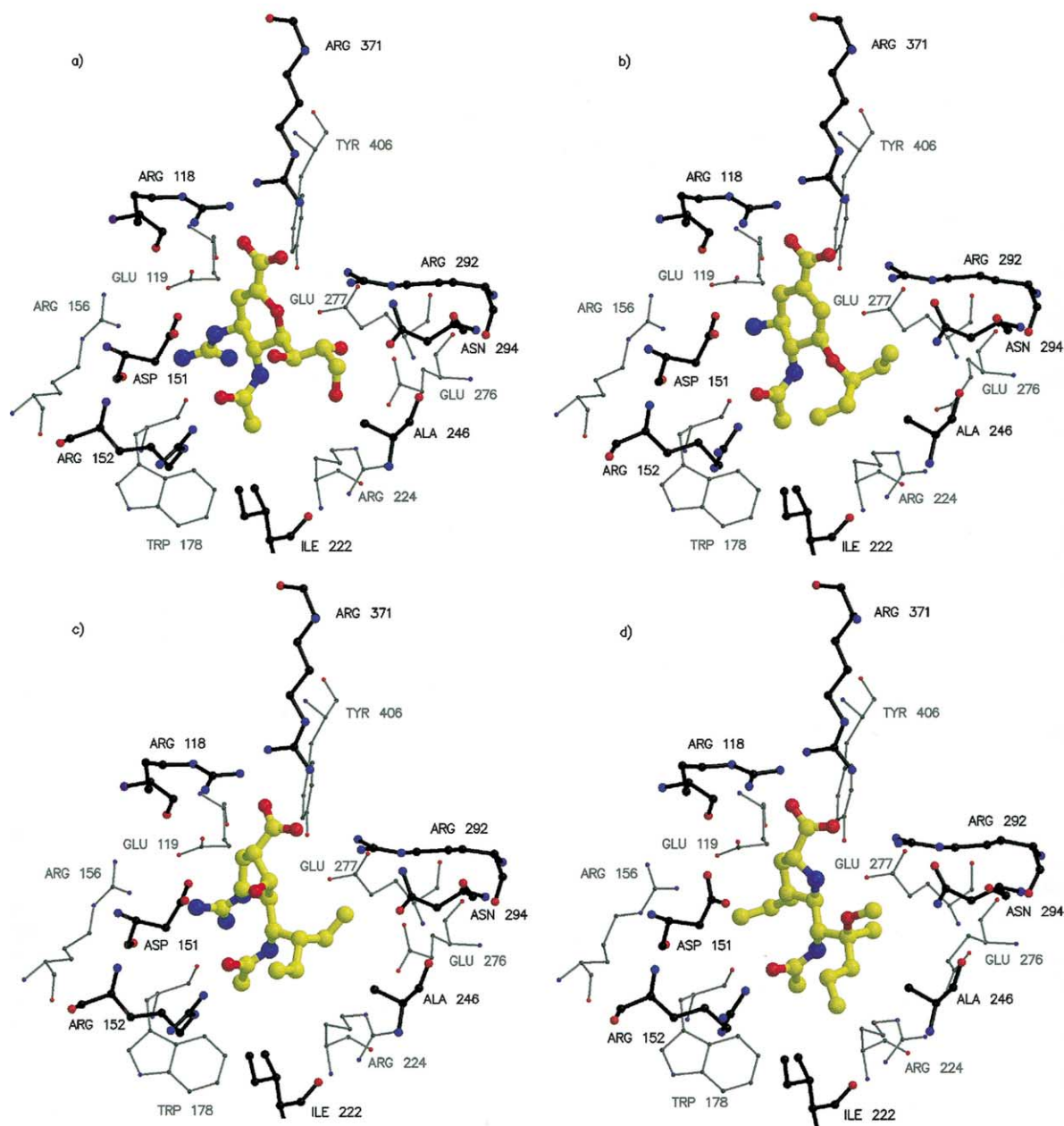


Fig. 2. Crystallographic structures of influenza virus neuraminidase (N9 subtype) with four different rationally designed inhibitors bound in the active site of the enzyme. The inhibitors are shown as atom-coloured ball and stick models (yellow, carbon; blue, nitrogen; red, oxygen). The catalytic site of the enzyme is shown with the closer carbon atoms in black and those further away in grey. This catalytic site is conserved across all flu neuraminidases. (a) and (b) Anti-flu drugs approved for use. (a) Relenza (4-guanidino-Neu5 Ac2en). [Reprinted, with permission, from G. Laver and E. Garman, *Science*, 293, 7 September 2001, pp. 1776, Copyright 2001 American Association for the Advancement of Science]. (b) De-esterified Tamiflu (4-acetamido-5-amino-3(1-ethylpropoxy)-1-cyclohexane-1-carboxylic acid). (c) and (d) Two further drugs which are being developed. (c) BCX-1812 [Biocryst] (1 S, 2S, 3R, -4R, 1'S)-3-(1'-acetyl-amino-2'-ethyl)butyl-4-[(aminoimino)-methyl]amino-2-hydroxycyclopentane-1-carboxylic acid). (d) A315675 [Abbott] (-)-(2R, 4S, 5R, -1'R, 2'S)-5-(1-acetyl-amino-2-methoxy-2-methyl-pentyl)-4-propenyl-pyrrolidine-2-carboxylic acid. The figures were drawn with Molscrip and rendered with Raster3d.

on the sialic acid there was a pocket in the neuraminidase at the bottom of which were two conserved glutamic acid residues. Replacing the hydroxyl at position 4 in DANA by an amino group produced a better inhibitor, but when 4-guanidino DANA was prepared, this was found to be an extremely potent and specific inhibitor of influenza neuraminidase, but not for neuraminidases from other

sources. [35]. Binding of 4-guanidino DANA in the neuraminidase active site is shown in Fig. 2a .

4-Guanidino DANA, marketed under the name Relenza, was shown to be an effective inhibitor of influenza virus replication in tissue culture, in ferrets and finally in human clinical trials. It has been approved for human use in a number of countries including Australia, USA, Canada,

Great Britain and Japan. Relenza is administered as a powder which is puffed into the lungs. The very chemical group which makes Relenza such a good inhibitor of flu neuraminidase (the guanidino group) also makes Relenza unable to cross membranes. It is not orally bioavailable.

In a further development currently under way, it was found that by linking two Relenza molecules through the 7-hydroxyl on the glycerol side chain, a compound was created which was 100-fold better than Relenza in its ability to inhibit the neuraminidase on the influenza virus particle. Furthermore, if the linker contained a hydrophobic benzene group, this, in conjunction with the highly charged guanidino group, enabled the Relenza dimer to be retained in the respiratory tract much longer than the Relenza monomer. The reason for this is not at all clear, but it should allow a once-a-week dosage, which is more acceptable than the present daily regime [36].

6. Avian influenza viruses from pelagic birds

Relenza was developed from knowledge of the crystal structure of influenza virus neuraminidase of the N2 subtype. At about the same time as this work was being done, other projects were also under way. One of these involved expeditions to some of the coral islands on Australia's Great Barrier Reef. These uninhabited islands, remote from human habitation, were the home of thousands of pelagic birds which included vast numbers of short-tailed shearwaters, booby gannets and white-capped noddies.

From one of these white-capped noddies, caught by Dr. Adrian Gibbs, an influenza virus was isolated which had a novel neuraminidase. This NA was subtype N9, and it gave the best crystals obtained so far of any influenza virus neuraminidase (Fig. 3). N9 neuraminidase crystals have now been used by three pharmaceutical companies in the rational design of neuraminidase inhibitors which are orally bioavailable and can be administered as a pill, which is swallowed.

Gilead Sciences have produced a carbocyclic compound (GS4071). This lacked a guanidino group and had a lipophilic side chain in the same position as the glycerol side chain of sialic acid [37]. Binding of GS4071 in the neuraminidase active site is shown in Fig. 2b. It was found, however, that GS4071 was not orally bioavailable, but when the carboxyl group was esterified, this problem disappeared and the ester could be administered as a pill. After absorption from the gut, the ester is hydrolysed and the active compound is delivered to the respiratory secretions. The pro-drug is now marketed under the name Tamiflu.

Relenza and Tamiflu are both effective at stopping virus replication. They cannot, however, repair the damage the virus has already done to the body. In order to be effective, therefore, these drugs need to be administered as soon as possible after the initial infection. This requirement, and the obvious fact that the drugs are only effective against flu A and B, and not against any of the other viruses or bacteria which

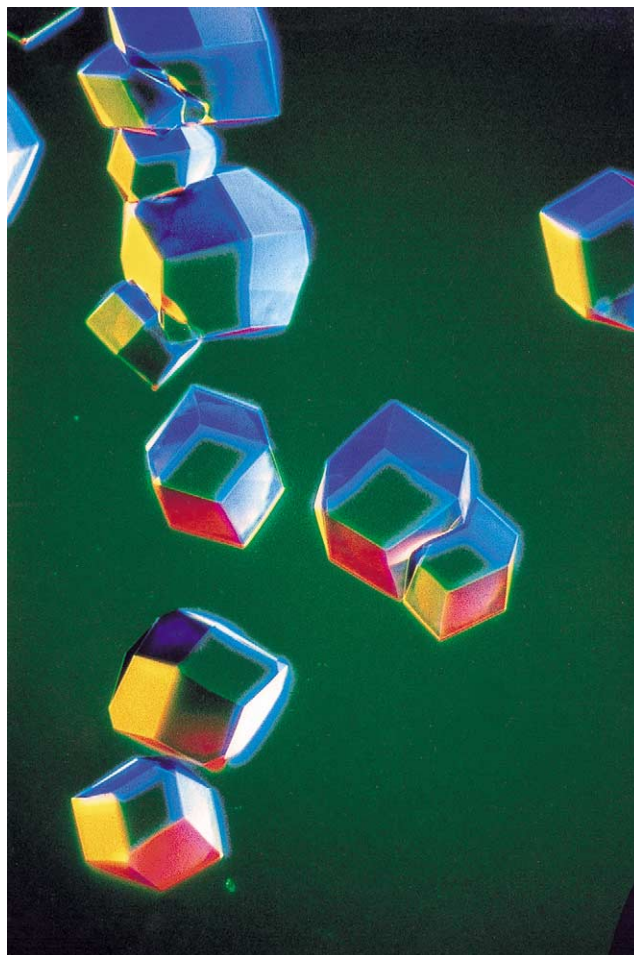


Fig. 3. N9 neuraminidase crystals of approximate edge size 0.6 mm. The crystals are colourless: the colours are due to internal reflections of the coloured lights shone onto the crystals to illuminate them.

have their own neuraminidases, make correct use of the drugs in the community difficult to achieve.

One solution to this problem would be the easy availability of rapid, cheap, sensitive diagnostic tests for influenza. These tests should preferably be able to be performed in the local pharmacy, or even in the home. Several flu diagnostic tests are currently being used, and doubtless others will be developed in the future.

Two other inhibitors of influenza virus neuraminidase are also in development. One of these, BCX-1812, was discovered by BioCryst pharmaceuticals [38], and is orally active against both A and B influenza viruses. It has a cyclopentane ring as a central scaffold, with a carboxylic acid and a guanidino group attached, which both occupy the same pockets as the same groups on Relenza, although the guanidino group takes up a different orientation within the pocket. In place of the glycerol and *N*-acetyl side chains of Relenza, BCX-1812 has an acetylamino-ethylbutyl group, which binds in the neuraminidase active site as shown in Fig. 2c. The other inhibitor, A315675, is being researched by Abbott Laboratories, following the screening of α - and β -amino acid inhibitors designed to mimic the transition state of the enzy-

matic reaction [39]. The lead compound is a pyrrolidine carboxylic acid, illustrated in Fig. 2d.

7. Drug resistance

When influenza viruses have been passaged in the presence of the neuraminidase inhibitors, two distinct classes of mutant viruses have been selected. In one, changes are seen in the haemagglutinin gene with no change in the neuraminidase and in the other, changes have occurred in the neuraminidase with no changes in the HA.

HA mutants can be selected fairly easily *in vitro*, but selection of drug-resistant mutants with changes in the neuraminidase often requires many passages in the presence of the drug. The reason for this is not at all clear. The mutants with changes in the haemagglutinin which are resistant to the drugs *in vitro* (and in some cases have even been found to be drug-dependent) seem not to be resistant when tested in animals. The mutants with changes in the neuraminidase which have so far been isolated have been found to be compromised in some way and do not affect patient recovery [40].

8. Conclusions

Influenza pandemics have occurred at irregular intervals in the past, and there is no reason to suppose another pandemic will not occur at some future date. That this will happen, seems inevitable. The next pandemic will be caused by a virus with a novel haemagglutinin and, possibly, a novel neuraminidase. Although the new virus may be readily transmitted, it may not be highly pathogenic. Nevertheless, many deaths will occur worldwide. Millions of people will fall victim to the virus, and consequently much human misery, social disruption and economic loss will occur. Attempts to prevent spread of the virus from the location where it arose are not likely to succeed, and a vaccine will take some time to develop, manufacture and distribute.

The most promising first defence against the new virus will be anti-viral drugs, and of these, the neuraminidase inhibitors seem at the moment to be the most effective. These are, however, likely to be in woefully short supply. The question will then arise: "Who should get these drugs?" The elderly, who might be most at risk from death? Healthcare workers? Police and others in essential services? And what about the politicians? Who will decide priorities and who will enforce them? If the new virus is particularly virulent, there may also be a thriving black market for the drugs. Then, there are the developing countries which will be just as susceptible to the new virus but without the resources to pay for anti-viral drugs. We believe there are no immediate answers to these questions and the new virus, when it comes, might well take the world by surprise.

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