THE ORIGIN AND CONTROL OF PANDEMIC INFLUENZA

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In 1918 an epidemic of influenza killed 20 million people worldwide. Spanish flu, as it was called, was a horrific disease. The flu would start with headaches, muscular pain, and fever. These would be rapidly followed by vomiting, dizziness, labored breathing, and profuse sweating. Sometimes purple blisters would appear on the skin, and often blood would spurt out of the nose from hemorrhages in the lungs. Some of the victims of this dreadful, sudden, and unexpected illness went into violent fits of coughing. Death often followed, sometimes only hours after the first symptoms appeared.

Influenza viruses infect a number of different animals, and some of these viruses can cause very serious disease indeed, particularly in domesticated chickens and turkeys. Avian influenza viruses sometimes rapidly kill these birds, with 100 percent mortality, and the symptoms resemble, at least to some extent, those of the Spanish flu in 1918. You can imagine, therefore, the concern felt when, in late 1997, a virulent bird flu virus, which had never before been seen in man, started infecting and killing people in Hong Kong [1]. This virus, designated H5N1, killed six of the 18 people it infected. The virus seems to have been transmitted to people from infected chickens in the live bird markets, but so far there has been no evidence that the virus had learned how to spread from person to person. But there is also no reason to suppose that this might not happen, some time in the future. Killing off all the chickens in Hong Kong seems to have stopped the epidemic, at least for the time being. What could be done to control such a virulent influenza virus, which, if it took off, would spread through

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today's crowded communities with explosive violence? Vaccines probably could not be prepared in time, and the two anti-flu drugs available at the moment, amantadine and rimantadine, not only have undesirable side effects, but mutant viruses, resistant to these drugs, develop very rapidly.

Recently, a new approach to preventing and treating influenza in humans has been tested in clinical trials. These trials involved compounds that inhibit an enzyme on the virus (called neuraminidase) which the virus needs in order to complete its replication cycle in the body. These compounds are not vaccines, which prime the body's immune system; instead, they act directly on the virus itself to stop it replicating, in much the same way antibiotics act to prevent the replication of susceptible bacteria. This article tells the story behind the development of these new flu drugs.

The Virus

Viruses, unlike bacteria, can only grow inside living cells, using much of the cellular machinery to make thousands of new virus particles. This intimate relationship between virus and cell has made the development of anti-viral drugs very difficult, since many of the substances which "kill" the virus will also kill the host cell.

Unlike most viruses, which are regular in shape, flu virus particles may exist as long, spaghetti-like filaments, round balls, or any shape in between. They consist of a lipid membrane, inside which is the genome of the virus, associated with five different viral proteins. Flu therefore belongs to the "enveloped" group of viruses [2]. The influenza virus genome consists of eight separate pieces of ribonucleic acid (RNA) of negative sense each of which specifies the amino acid sequence of one (and sometimes two) of the virus's proteins. This segmented nature of the RNA allows different flu viruses to easily "mate" with each other, forming hybrid progeny viruses with bits of RNA from each parent virus.

Stuck onto the lipid envelope of the virus, like pins in a pincushion, are two glycoprotein molecules which play a vital role in the life-cycle of the virus. One of these surface "spikes" is a triangular, rod-shaped molecule called *hemagglutinin*, and one of its functions is to attach the virus to cells by way of specific receptors on the cell. The name *hemagglutinin* was originally used because the virus was found to agglutinate red blood cells, and this formed the basis of an assay for flu viruses. If the cell is a host cell which the virus is about to infect, instead of a red blood cell (which the virus cannot infect), the hemagglutinin spike will attach the virus to the cell through receptors on the cell, containing sialic acid, and then fuse the cell membrane with the membrane of the virus, allowing the RNA of the virus to get inside the cell and instruct the cell to make thousands of new virus particles.

The other "spike" on the surface of the virus is a mushroom-shaped enzyme, called *neuraminidase*. This enzyme removes the sialic acid recep-

tors from the host cell (and from other newly made virus particles), and allows the virus to escape from the cell in which it grew and spread in the body to infect other cells. If this neuraminidase is blocked in some way, then the virus particles pile up in great clumps on the cell surface, unable to go anywhere, and the infection is effectively terminated [3].

The Influenza Virus Keeps Changing All the Time

There are two distinct kinds of influenza, types A and B. These two groups of flu viruses have similar structures, but all of the A virus proteins are completely different from those of the B viruses as far as recognition by the immune system goes. It is flu A which infects pigs, horses, seals, whales, and many kinds of birds, as well as humans, while flu B has been found only in people.

Type A flu can be subdivided further into subtypes, according to how their hemagglutinin (H) and neuraminidase (N) antigens are recognized by antibodies. So far, 15 different H and nine different N antigens have been discovered, and Type A influenza viruses are made up of various combinations of these, such as H1N1, H3N2, H5N1, and so on. Of these, H1N1, H2N2, H3N2, and, recently, H5N1 have been found to infect people. Many other combinations of H and N have been found in animal flu viruses.

If you catch the flu, or receive flu vaccine, antibodies are formed in your body to the virus's proteins. Some of these antibodies recognize small areas (epitopes) on the hemagglutinin and neuraminidase of the virus. If the same virus then attempts to reinfect you, those antibodies will bind tightly to its hemagglutinin and prevent the virus from infecting cells in the respiratory tract. You have developed immunity to that flu virus. With most viruses, that is the end of the story. In the case of measles, mumps, or smallpox, for example, infection or vaccination with these viruses results in immunity which may last all your life. These are stable viruses and change very little, if at all, over the years.

The influenza virus, on the other hand, is changing all the time. Major antigenic "shifts" occur in influenza Type A from time to time. In these, "new" flu viruses suddenly appear in the human population. The first human influenza virus, isolated in 1933, was designated H1N1. This remained until 1957, when a new virus, H2N2, suddenly appeared in China. No one had any immunity to this new virus and it caused a pandemic of Asian flu. Other pandemics known to have occurred in this century were Spanish flu in 1918, Hong Kong flu in 1968, and Russian flu in 1977.

Between these shifts, the virus undergoes a series of small changes in its two surface proteins, H and N, called "drift." What is the molecular basis of "shift" and "drift"? In the early 1960s it was thought that each virus contained a number of hemagglutinin antigens common to strains of the type, and that strain variation represented rearrangement of these anti-

gens either quantitatively or spatially. Our colleague, Stephen Fazekas de St. Groth, pointed out that such an array of antigens on the surface of the virus implied the existence in the virus of an equivalent number of different protein molecules, all of which would need to be coded for by the viral genome.

This was too small to code for more than a very few proteins, and so antigenic variation had to occur by some other mechanism. We therefore selected antigenic mutants of a couple of influenza viruses in the laboratory by passaging the viruses several times in the presence of sublimiting concentrations of neutralizing antibodies. We had already developed techniques for isolating hemagglutinin molecules from the wild-type and mutant viruses and also by this time had perfected a method for comparing the amino acid sequences of flu virus proteins. Nowadays methods are available for rapidly and accurately determining the sequences of very small amounts of proteins, but in 1970 such techniques had not been fully developed. We used a method called "peptide mapping," in which the proteins were digested with trypsin to give a mixture of small peptides. These peptides were then separated by two-dimensional electrophoresis and chromatography, and stained to give a two-dimensional pattern or "map." Identical proteins gave identical peptide maps. Proteins with one or two differences in their sequence gave slightly different maps, while proteins which differed greatly gave vastly different peptide maps.

When we looked at peptide maps of hemagglutinin molecules from the "escape mutants" (antigenic variants which escaped neutralization by antibody), we saw that while most of the spots were in the same location, one or two had shifted dramatically [4]. This was the first indication that antigenic drift occurred by small changes in the amino acid sequences of the surface antigens of the virus. Later, more sophisticated experiments using monoclonal antibodies confirmed this early work, and when the crystal structure of the hamagglutinin, the neuraminidase, and a complex of the neuraminidase with antibody Fab became available [5–7], it was clear that single sequence changes in epitopes recognized by individual antibodies were able to make those epitopes quite unable to bind the antibodies which previously bound very well. These experiments proved that drift results from mutations in the pieces of RNA coding the hemagglutinin and neuraminidase. These in turn cause small changes in the regions (epitopes) on the H and N molecules which bind antibodies, so that these antibodies can no longer bind and the virus is able to "escape" and cause an infection in a previously immune person.

The Major Shifts: Where Do the New Viruses Come From?

But what about the major shifts? What is the origin of the "new" viruses which cause the major influenza pandemics? How would one go about try-

ing to find out where these viruses come from? Why do they always seem to come from China?

In February 1957, a virus suddenly appeared somewhere along the road between Kutsing and Kweiyang in southern China which was quite unlike the H1N1 viruses previously causing flu infections in that region. The virus was designated H2N2 and caused a major pandemic of Asian flu. Where had the virus come from? At the time, Sir Christopher Andrews suggested it may have come from some reservoir of influenza viruses existing in animals. Then, in 1965, Helio Pereira and Bela Tumova, working in the National Institute for Medical Research at Mill Hill in London, showed that genetic interaction could occur between influenza viruses of human and animal origin [8]. This was followed by their discovery that a turkey flu virus contained the same neuraminidase as the human Asian H2N2 virus.

In July 1968, again in China, another "new" virus made its appearance. This virus had the same neuraminidase (N2) as the old Asian flu viruses, but the hemagglutinin was so different from that of the Asian flu virus that antibodies to the Asian (H2) hemagglutinin did not bind at all to the new hemagglutinin. The virus was therefore called H3N2, and it caused the Hong Kong flu pandemic of 1968. Again we used peptide mapping to examine the sequences of the Asian (H2) and Hong Kong (H3) hemagglutinins. It was immediately clear that the two hemagglutinins differed greatly in their amino acid sequence and that there was no way one could have arisen from the other by mutation in such a short period of time. The Hong Kong H3 hemagglutinin had to have come from somewhere else. But where? And how?

At an international virology conference in Budapest, we heard that the hemagglutinin of flu viruses from ducks and horses cross-reacted antigenically with the hemagglutinin of the Hong Kong virus. We then showed, by peptide mapping, that H molecules from Hong Kong flu and from a duck influenza virus were very similar in amino acid sequence. It was now clear how the Hong Kong virus had originated: the new virus was a reassortant (hybrid) virus with the N2 spike from the "old" Asian flu virus and the "new" H3 spike from an animal or bird flu virus [9].

Later, more sophisticated sequence analyses confirmed these findings and showed that the Asian H2N2 virus was also a hybrid virus.

Why do these hybrid viruses always seem to arise in China? One possibility is the close contact between people, poultry, and pigs in this country, increasing the chances for reassortant events between viruses to occur. However, conditions like this do exist elsewhere in the world, and it remains a mystery why China should be the birthplace of flu. It is clear that the recent H5N1 bird flu virus, which infected 18 people and caused six deaths in Hong Kong at the end of 1997, was not a hybrid virus (all its RNA segments were of avian virus origin), and it may be that other factors will allow certain animal influenza viruses to cross the species barrier and infect people.

A Single Mutation Can Turn a Harmless Flu Virus into a Killer

There is no doubt that mutations in a fairly harmless flu virus can turn it into a killer. In Pennsylvania in 1983, a benign avian influenza virus was infecting chickens in the large commercial chicken sheds, giving them a mild dose of the flu. Then, suddenly, this benign virus mutated and turned into a killer, devastating the poultry industry in that area. Seventeen million birds died, and the outbreak was contained only by slaughter and quarantine. No humans were infected by the virus.

The flu team at St. Jude Children's Research Hospital in Memphis examined the virus before and after it became virulent. They found that a single base change in the RNA segment coding the H spike, which in turn caused a single amino acid sequence change in the hemagglutinin, caused the virus to become a killer [10]. That such a tiny change in the virus could enable it to wreak so much havoc was an awesome discovery.

Although drift occurs in both flu A and B, the major shifts have only been seen in flu A. Possibly this is because the influenza Type A virus is found in a variety of hosts, while flu B has only ever been found in human infections.

The Natural Host of Influenza Type A Virus May Be Wild Water Birds

The widespread distribution in nature of influenza Type A viruses was discovered more or less by accident. It has been known for a long time that chickens and turkeys suffer from flu; in fact, fowl plague was the first influenza virus to be isolated. Terns also had been found dying from flu in South Africa in 1961. Helio Pereira in London and Martin Kaplan in Geneva had long been stressing the importance of trying to find out if animal influenza viruses were involved in any way in human flu epidemics, and many light-hearted suggestions to look for flu viruses in some of the more exotic regions of the world were made from time to time.

However, until the early 1970s, no one had ever looked for influenza viruses in wild birds in their natural state, remote from human habitation. With Martin's help, we organized an expedition to the remote and beautiful coral islands of Australia's Great Barrier Reef to see if we could isolate influenza viruses from any of the thousands of wild sea birds which nest on these islands.

In the first instance, rather than trying to isolate the virus, we took antibody samples from the birds to see if there was evidence of past flu infections. We tested the bird serum samples for their ability to inhibit the activity of human influenza virus N2 neuraminidase. While most of the samples gave full enzyme activity, in one sample the enzyme activity had been totally inhibited by the bird serum. It did not take us very long to show that this was due to specific antibody in the bird, which meant that at some time the bird had been infected with a virus that carried this enzyme. In this case the enzyme was the same as that of the human Asian H2N2 flu virus.

This, of course, was very exciting. It was clear that flu viruses were infecting these birds, so we had to return to the Great Barrier Reef to try to isolate the virus.

Now, if you want to isolate influenza virus from people, the normal process is to take a throat swab. This involves swabbing a person's throat and then placing the swab into tissue culture to see if any virus grows. Naturally we tried to do the same thing with the birds. We tested the throat swabs in tissue culture and eggs without much luck. It was then discovered that flu in birds is usually not a respiratory infection. Rather, the virus replicates in the lower intestine. Hence we needed to concentrate on the other end of the bird and take a cloacal swab. We took a number of cloacal swabs and, to our great delight, we finally isolated a number of different influenza viruses [11].

This discovery led to a proliferation of expeditions to many parts of the world. One of these was to the lakes in northern Canada, where populations of ducks were found which carried most kinds of influenza type A virus. The flu viruses do the ducks no harm at all—the ducks are infected with the viruses, they excrete them into the lake, other ducks gobble up the lake water, get infected, and so the infection goes on. Despite this, the ducks remain completely healthy. In fact, if you go up there you can not only isolate all these viruses from the ducks, you can even isolate them from the lake water. All you have to do is take a spoonful of lake water, put it into tissue culture and you will grow flu viruses. Thus, the ducks in northern Canada and other water birds around the world seem to be the main host of influenza virus, at least of Type A flu, and they may have been for many millions of years. The virus may have only crossed into humans recently when the population became large enough to sustain epidemics.

The Reef trips also paid off in another way. One of the viruses we isolated had neuraminidase of a new subtype: N9. We later found that N9 neuraminidase produced the best crystals of any flu virus neuraminidase (see cover illustration), and it has proved to be valuable in the rational design of anti-flu drugs [12]. (A photo of N9 neuraminidase crystals adorned the cover of *Perspectives* in Winter 1989.)

Discovery of Neuraminidase Inhibitors

It seems unlikely that influenza vaccines will ever cope adequately with antigenic drift. In fact, they may not be effective at all in the case of a major antigenic shift, because of the time needed to develop, manufacture, test, safety-test, and distribute the new vaccine. Anti-viral drugs—similar to antibiotics for bacterial infections—might be a much better way to control flu epidemics. The remainder of this article will describe the development of a novel class of anti-flu drugs, effective against all flu viruses, which are showing promise of fulfilling this objective.

The influenza virus has a number of activities which could be used as targets for anti-viral drugs. The hemagglutinin has receptor-binding and membrane-fusing activities. Flu A has an ion channel (the M2 protein) which is the target for amantadine and rimantadine. The replication complex in the virus is another target, but we chose the virus's receptor-destroying enzyme, neuraminidase.

The story starts in the 1940s, when George Hirst, working in the Rockefeller Institute in New York, noticed that when fluids from chicken embryos infected with flu virus were mixed with red blood cells in the cold, the cells were very heavily agglutinated by the virus. If the agglutinated red cells were warmed up, they dispersed and could not be re-agglutinated in the cold by fresh virus. Hirst's interpretation of this finding was that the flu virus possessed an enzyme which destroyed receptors for the virus on the red cell. Cells lacking receptors for the virus could, of course, no longer be agglutinated by the virus.

Alfred Gottschalk, working in the Walter and Eliza Hall Institute in Melbourne, Australia, reasoned that if the virus's enzyme was destroying receptors on the red cells, there had to be a "split product," and he finally discovered what this split product was. It was a kind of sugar called sialic or neuraminic acid (Fig. 1), and the enzyme on the virus was therefore called sialidase (or neuraminidase) [13]. At the time it was thought that it was the neuraminidase itself which was responsible for the virus's ability to stick to and agglutinate red cells, but in collaboration with Robin Valentine at the National Institute for Medical Research at Mill Hill in London, we showed that the hemagglutinin (receptor-binding) and neuraminidase (receptor-destroying) activities of the virus resided in two quite different spikes on the surface of the virus. The hemagglutinin was a long, triangular rod-shaped molecule, while the neuraminidase consisted of a square box-shaped head atop a long thin stalk which attached the neuraminidase to the lipid envelope of the virus [14].

Much of the work on reassortant flu viruses which has been described resulted from a key finding we made in Ed Kilbourne's laboratory at Cornell University in New York. Here we discovered that if cells were infected at the same time with two different Type A flu viruses—say H1N1 and H2N2—the RNA pieces coding the various virus proteins reassorted to give hybrid viruses, some of which contained the hemagglutinin from one parent and the neuraminidase from the other [15]. Thus H1N2 and H2N1 viruses could be obtained from this "mating" of the two parents. This discovery not only provided an explanation of how new pandemic strains of flu A could form, it also led to a very neat way to produce influenza viruses "to order," having any desired combination of hemagglutinin and neuraminidase spikes [16]. Such viruses were essential to the production of pure neuraminidase for the crystal growth and drug design experiments described later.

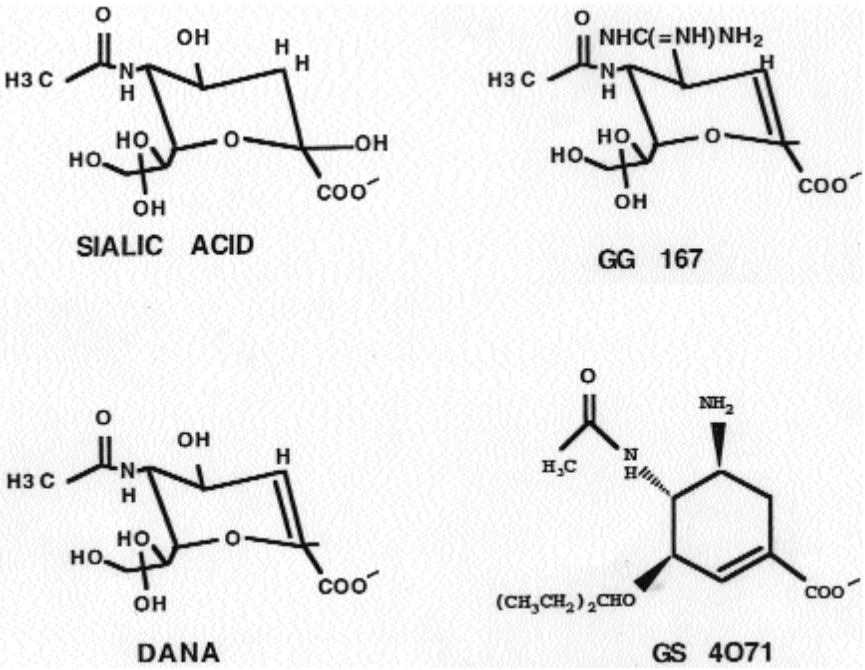

FIG. 1.—The lefthand side and top right show stages in the development of the antiviral flu drug GG167 (Zanamivir, Relenza). Sialic acid, the natural substrate of flu neuraminidase, is also a weak inhibitor of this enzyme. The dehydrated compound, DANA (which has been known for a long time), is a very much better inhibitor but has no antiviral activity in vivo. 4-guanidino DANA (GG167) inhibits flu neuraminidase about a thousand times better than DANA, and it is this compound which has been tested in clinical trials by GlaxoWellcome.

On the bottom right is shown the structure of the carbocyclic sialic acid analogue with potent anti-influenza activity developed by Gilead Sciences and tested in clinical trials by Hoffmann La Roche. The drug is given as the neutral ester GS4104.

NOTE.—The figures were drawn by Dr. Robert Esnouf using BobScript and rendered with Raster3D.

Crystallization of Flu Virus Neuraminidase

The discovery that flu virus neuraminidase could be crystallized was made quite by accident. We have already described how work done in the 1970s in Canberra and at St. Jude Children's Research Hospital provided the first biochemical evidence that the H3N2 virus, which caused the 1968 Hong Kong flu pandemic, was a hybrid reassortant virus, having hemagglutinin spikes from a duck flu virus and neuraminidase which was similar to that of the old Asian H2N2 flu virus [9]. We wanted to confirm, by peptide mapping, that the N2 of Hong Kong flu did indeed have the same sequence as that of the old Asian flu strain.

First, we had to isolate pure preparations of neuraminidase from each of these viruses. The procedure was to inject a small amount of seed virus into

a thousand or more 11-day-old living embryonated chicken eggs, incubate these embryos for two days to allow the virus to grow, and then suck off the allantoic fluid surrounding the embryo, which was full of newly formed virus particles. These were then concentrated and purified, the end-product from a couple of thousand eggs consisting of 2 to 3 milliliters of a creamy white fluid containing perhaps 100 billion new virus particles.

This purified virus was then used to make pure neuraminidase. The purified virus particles were incubated with an enzyme, such as trypsin, capable of digesting proteins. The virus used was chosen so that digestion with protease would split the "heads" of the neuraminidase off the virus particle without destroying them, while at the same time either leaving the hemagglutinin spike intact and still stuck to the virus, or else totally destroyed. In either case, the only material released by the protease would be the heads of the neuraminidase spikes. These were then concentrated by high-speed centrifugation, in which very small particles in solution are spun down in the high gravity created in the centrifuge.

The first time this was done with N2 neuraminidase, and the tiny pellet of neuraminidase heads was examined. Lo and behold, it had a crystalline appearance. Soon afterwards, a suspension of N2 neuraminidase crystals in water was obtained. When stirred, these showed a typical crystalline sheen, and under the microscope hundreds of tiny plate-like crystals were visible [17]. This was the accidental discovery which eventually led to the rational design of neuraminidase inhibitors. Initially, of course, some people scoffed at the notion that flu neuraminidase had been crystallized ("All you've got are salt crystals—don't be such an idiot"), but very soon X-ray diffraction analysis of larger crystals showed they were indeed made of protein, and some time later the three-dimensional structure of N2 neuraminidase was solved by Peter Colman and his colleagues in Melbourne [6].

The Three-Dimensional Structure of Influenza Virus Neuraminidase

Flu neuraminidase is a tetrameric molecule, made up of four identical protein subunits. Each of these consists of a single long polypeptide chain, containing 467 amino acids, folded to give an almost spherical object with a deep cleft or cavity that is clearly visible and a long tail which forms the stalk of the enzyme. Four of these objects come together to form the square, box-like neuraminidase.

Many variants of flu neuraminidase exist: N1 to N9, and also flu Type B neuraminidase. Each of these contains about the same number of amino acids, but the sequence differs considerably from one neuraminidase to the next. However, scattered along the sequence are a number of amino acids which are conserved in all flu viruses.

When the neuraminidase polypeptide folded up to form the active neuraminidse enzyme, it could be seen that many of those conserved amino

acids came together, lining the walls of the central cavity. This indicated that the cleft or cavity was probably the active catalytic site of the neuraminidase, and that the conserved residues were conserved because they were essential for the enzyme to carry out the activity it was designed to do. Also, because the active site was so well conserved, it was clear that if a compound could be made which fitted precisely into the active site of the neuraminidase from one strain of flu virus (a plug-drug), it would bind tightly and inhibit strongly the neuraminidase activity from all other influenza viruses, even those which have not yet appeared in man. Since influenza viruses in which the activity of the neuraminidase is suppressed cannot easily spread from cell to cell in the body, could it be that a universal cure for the flu might be possible, after all?

Nearly 30 years ago, it was demonstrated that a synthetic analog of sialic or neuraminic acid, referred to as Neu5Ac2en (DANA) (Fig. 1) could inhibit bacterial and mammalian neuraminidases, as well as influenza virus neuraminidase with Ki values of approximately 100mM [18]. Subsequently, numerous derivatives of DANA have been made, but none have shown sufficient activity in animal models of flu infections to indicate they might be useful anti-viral agents in humans [19].

Design and Synthesis of Small Molecules to Act as Potent Inhibitors of Flu Virus Neuraminidase

Mark von Itzstein and his colleagues at the Victorian College of Pharmacy synthesized a sialic acid–like compound, in which the OH at the 4 position was replaced by a basic, positively charged amino group. This was a much better inhibitor than sialic acid, or its dehydro-deoxy transition state analogue, DANA. But when the amino group at the 4 position was replaced by a guanidino group, the resulting compound was an extraordinarily potent inhibitor of flu neuraminidase, but with little activity for bacterial or mammalian neuraminidases (Fig. 1). It was given the name GG167 or Zanamivir. When the substrate for the neuraminidase (sialic acid) was soaked into neuraminidase crystals by Peter Colman and the three-dimensional structure of the soaked crystals was determined by X-ray crystallography, the sialic acid could be seen anchored in the central cavity by some of the conserved amino acids lining its walls [20] (Fig. 2). At the bottom of the cavity, another small pocket could be seen, at the base of which were two glutamic acids (E119 and E227). These glutamics also were totally conserved among all flu viruses, even though they were too far away from the sialic acid to be involved in substrate binding.

When GG1C7 was soaked into flu neuraminidase crystals, the guanidino group could be seen close to E119 and E227. There is no doubt that the strong binding of GG167 by flu neuraminidase is due to the anchoring of the positively charged guanidino group on GG167 by these negatively

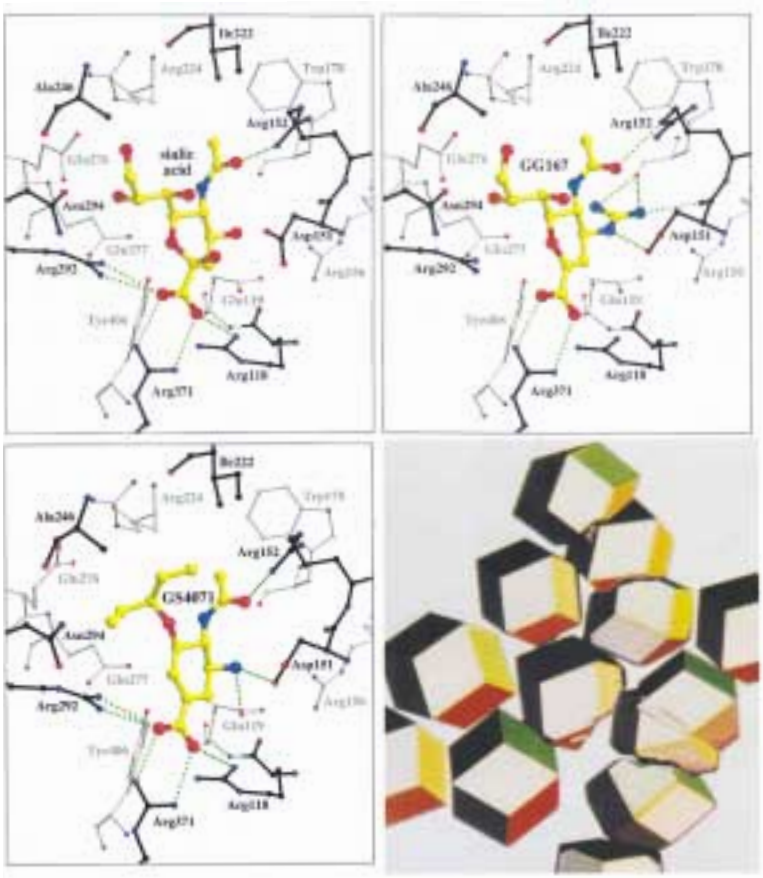

FIG. 2.—The catalytic site of wild-type tern N9 neuraminidase showing how sialic acid and the inhibitors GG167 and GS4071 bind in it. In each figure, the substrate/inhibitor is shown as an atom-colored ball-and-stick model (with carbon atoms in yellow), the protein surrounding the binding site is shown by thin atom-colored ball-and-stick models (with black carbon atoms for residues towards the front of the view, and grey carbon atoms for those residues towards the rear). Hydrogen-bonding interactions involving substrate or inhibitor are shown by dashed green bonds. Residue Glu227 forms the back face of the pocket in each case but is omitted for clarity. With sialic acid and the inhibitor GS4071, residue Glu 227 forms hydrogen bonds to two water molecules nearest the center of each figure (not shown). With the inhibitor GG167, Glu227 forms similar hydrogen bonds to one water molecule and to one of the nitrogen atoms of the guanidinium group of GG167.

Bottom right shows a photograph of crystals of N9 neuraminidase which were obtained from the influenza virus found to infect wild birds on the Great Barrier Reef of Australia. Neuraminidase crystals enabled the three-dimensional structure of the enzyme to be determined, and this led to the synthesis of drugs to plug its active site. The hues are reflections of colored lights.

charged glutamic acids in the pocket of flu neuraminidase which is not present in other neuraminidases [21] (Fig. 2).

GG167 was an effective inhibitor of influenza virus replication in tissue cultures at very low concentrations. In ferrets, which are a good model for flu infections, GG167 was effective in reducing fever and virus shedding without showing any toxic symptoms. In phase 2 human trials, in which volunteers were given the drug and deliberately infected with flu virus, GG167 was effective in preventing any symptoms of the flu if given early enough, and in reducing their severity if given later after infection [22].

GG167, however, does not work if taken by mouth (it has poor bioavailability), and it is best administered as a nasal spray, or as a powder which must be sniffed into the respiratory tract. This is because of its chemical nature. By a twist of fate, the very guanidino group which is responsible for the tight binding of GG167 to the neuraminidase also prevents the drug from readily crossing cell membranes.

In 1995, scientists at Gilead Sciences in California initiated work on inhibitors of neuraminidase, with the goal of identifying a compound which was not only potent, but which also was orally bioavailable and could be swallowed as a pill. Such a task, while conceptually straightforward and simple, in practice presents a formidable challenge. As was evident from the crystal structure of GG167 bound to the active site of neuraminidase, the potency exhibited by GG167 derives mainly from strong ionic interactions of charged residues (i.e., the negatively charged carboxylates and the positively charged guanidine) and from strong hydrogen bonding of polar functionalities (i.e., the three hydroxyl groups of the glycerol side chain) with amino acids of the neuraminidase enzyme. However, these ionic and hydrogen bond interactions are also the reason why GG167 is not orally bioavailable.

So this was seemingly a paradox: how could one preserve the tight-binding interaction of the inhibitor with the neuraminidase, while at the same time getting rid of the ionic functionalities and hydrogen bonds? A large number of compounds were synthesized and evaluated, and this paradox eventually was solved with the realization that a new analog, GS4071 (Fig. 1), exhibited potency comparable to GG167. Interestingly, GS4071 lacked the ionic guanidine function and the hydrogen-bonding glycerol side chain. The explanation for why GS4071 binds so strongly to the neuraminidase became apparent when the crystal structure of GS 4071 bound to the neuraminidase enzyme was later solved: a change had occurred in one of the amino acids (glutamate 276) of the neuraminidase. It had flipped 90°, and as a consequence a new hydrophobic pocket had been created which allowed the lipophilic side chain of GS4071 to bind strongly (Fig. 2). Even though some binding energy had been lost by replacing the guanidine function, this was compensated for by a gain in energy by the lipophilic side chain. Evaluation of GS4071 in various in vitro assays was encouraging. The

compound was a potent inhibitor of all neuraminidase types tested (N1 through N9). It inhibited the replication of a variety of influenza strains in vitro, and furthermore, it exhibited good selectivity. No inhibition of related mammalian neuraminidases was observed, even at concentrations a million-fold higher than what was necessary to inhibit influenza neuraminidases.

Unfortunately, when subsequently evaluated in preclinical models, GS4071 was not orally absorbed. That is it could not be given as a pill. This dilemma was solved by converting GS4071 into a prodrug, i.e., masking the negatively charged carboxylate by converting it into a neutral ester. This neutral ester, GS4104, is labile in biological fluids and tissues, and after absorption from the gut it is converted back to the parent drug GS4071. This was confirmed in animals. When administered orally, the prodrug GS4104 was readily orally absorbed and efficiently converted in the body to the active neuraminidase inhibitor GS4071 [23]. Figure 1 shows the structures of some of these inhibitors, and Figure 2 shows how they bind in the catalytic site.

When further evaluated in preclinical models, GS4104 showed encouraging properties. It had a relatively long half life in the body, suggesting the possibility of once or twice daily dosing. It proved to be very safe, with very few side effects observed when the compound was administered to animals at high doses. Furthermore, it was also efficacious when administered orally to influenza virus–infected ferrets, preventing fever and symptoms of influenza infection [24].

With such promising preclinical data in hand, a human clinical study was initiated in March 1997. Healthy volunteers received oral GS4104 in order to assess oral bioavailability, pharmacokinetics, and safety. The results of these studies were again encouraging: 60 percent of the orally administered GS4104 was delivered to the blood and converted efficiently to the parent GS4071. GS4071 had a prolonged half life, suggesting once or twice daily oral dosing. And finally, GS4104 was safe and well tolerated.

The efficancy of oral GS4104 was assessed in subsequent studies where human volunteers were infected experimentally with a strain of influenza virus which is known to cause mild symptoms. One study assessed the utility of GS4104 for prevention, with the drug being administered prior to infection, and a second study assessed the potential of GS4104 for treatment, where infected individuals were given the drug. Again, GS4104 was found to be effective when dosed once or twice a day, both in preventing symptoms of influenza when administered prophylactically and in reducing the severity and duration of symptoms when administered as a treatment.

Drug-Resistant Mutant Viruses

It has been stated that "plug-drugs," inhibitors of flu neuraminidase, which bind tightly in the active site cavity of this enzyme, will be effective against all influenza viruses, even those which have not yet appeared in

man. This is because the three-dimensional structure of the neuraminidase active site is the same for all flu viruses so far examined, and the amino acids making up the site are totally conserved. Furthermore, it was believed that these amino acids were conserved because they were required by the neuraminidase to carry out its enzymic function, and that any change in these conserved amino acids would result in a "dead" enzyme.

This concept was, perhaps, a trifle naïve. For many thousands—maybe millions—of years, the influenza virus has learned very well how to escape from antibodies by changing its structure. In all this time, because the active site cavity is not accessible to antibodies, this has remained unchanged. It has now been found, however, that it is not unchangeable. When influenza viruses are grown in cell cultures in the presence of GG167 or GS4071, drug-resistant viruses can be isolated.

This resistance seems to be of two kinds. In the first kind, the resistant viruses, which grow very well in cell culture in the presence of high concentrations of GG167, have no changes in the neuraminidase, but have a changed hemagglutinin spike. The sequence changes in the hemagglutinin of the resistant mutants clustered around the receptor-binding site, suggesting that the ability of the mutant virus to grow in the presence of GG167 was due to a decrease in the affinity of the hemagglutinin for its cellular receptor, so that the virus could escape from infected cells even though the neuraminidase activity was almost totally eliminated. These hemagglutinin mutants, however, seemed to be resistant to GG167 only in cultured cells; they were still susceptible to the drug in animal infections [25].

The second kind of mutant virus which grew in the presence of GG167 had changes in the neuraminidase. Viruses with amino acid sequence changes at position 119 or 292 in the neuraminidase have been selected after growing viruses in cultured cells in the presence of GG167 or GS4071 [26–28]. When the glutamic acid at position 119 changed to glycine, alanine, or aspartic acid, the neuraminidase was fully active in the presence of concentrations of GG167 which totally inhibited the wild-type enzyme. This made a lot of sense, since it was the glutamic acid at position 119 which was partly responsible for anchoring GG167 in the active site of the neuraminidase. Remove the glutamic acid, and this anchoring effect was reduced.

But this mutant neuraminidase had some strange properties. It was much less stable than the wild-type neuraminidase, and the mutant neuraminidase heads, in which glutamic acid 119 had changed to glycine, rapidly lost enzyme activity. It could be seen in the electron microscope that the tetrameric neuraminidase heads had fallen apart into monomers. Just how this change—glutamic acid to glycine—caused this to happen is not known, but what is clear is that the conserved glutamic acid at position 119 is not there simply in order to provide an anchoring point for GG167, but that it plays a vital role in the stability of the neuraminidase molecule [29].

The other mutant neuraminidase, in which an arginine at position 292 had changed to lysine, was resistant to both GG167 and GS4071, although resistance to the latter inhibitor was considerably greater. Although the arginine to lysine mutant was just as stable as the wild-type neuraminidase, the mutant enzyme was crippled in some way and was less active than the wild type. This change in the activity of the neuraminidase, as well as its resistance to the inhibitors, was due to a change in the shape of the active site which occurred when the conserved arginine at position 292 changed to lysine [30].

These findings show that the virus is having some trouble in escaping from the inhibitors, and it may be that viruses resistant to the new flu drugs may not be too much of a problem after all.

Crystal Growing in Space: An Adventure

In an attempt to grow better neuraminidase crystals, and so to have a better chance of designing better inhibitors, we were at one stage part of a NASA project to grow protein crystals in conditions of weightlessness on the orbiting space shuttle.

What are the advantages of growing crystals under conditions of weightlessness? When a crystal is growing, the solution close to the growing crystal face will be depleted of protein, and this region will have a lower density than the rest of the solution. On Earth, this region will be lighter and will rise, and as it rises it may interfere with the growing crystal lattice. However, this distortion should not occur under microgravity conditions, and therefore a much more perfect lattice should develop in space, leading to a more perfect crystal. Our attempts to grow crystals on the shuttle produced some neuraminidase crystals that were absolutely beautiful. They looked as though they had been machined, although they were too small to do anything with. But then the Challenger shuttle was destroyed, and our program had to cease.

However, the Russians had a permanently orbiting space station called *Mir*. So I popped over to Moscow and asked: "Can I grow some neuraminidase crystals on your space station?" They were a bit astounded at this, because they were used to dealing with government delegations, not some guy who comes in off the street and wants to use the space station. Nevertheless, they said: "Okay! It's a great idea. Let's do it." So we signed agreements: they would provide the rocket, the space station, and all the equipment, while I would provide the neuraminidase protein.

The Russians had never done this before, and had no suitable equipment. Yet in three months they invented, designed, and built some apparatus for growing crystals on *Mir*. The apparatus was made of titanium, which is the heaviest metal known, but the Russians had very powerful rockets and weight was not a problem, although space on board was. The

apparatus for growing the neuraminidase crystals was produced just before the rocket was due to blast off, so it was never tested, but nevertheless we sent it up to *Mir* in June 1988 with a couple of Soviet cosmonauts and a Bulgarian.

Everything was going fine until the American Pentagon heard about the experiment. They said that the experiment had to be stopped because the Soviet Union would gain vital knowledge that would assist them in germ warfare. One individual, an expert on the transfer of technology to the Soviet Union, went so far as to say that the ramifications of the Australian experiment were "absolutely awesome" and threatened the security of the United States. Luckily, we were able to straighten things out with the Pentagon, convincing them that growing neuraminidase crystals had no sinister ramifications, and the experiment went ahead.

The crystals were left to grow in space for three months. When the mission was completed I went to Moscow to collect the crystals. My excitement at being taken to mission control turned to dismay when I discovered that the Russians were having major problems. First the reentry vehicle, with two Afghans in it, went into the wrong orbit and they nearly lost it. The two poor cosmonauts were whizzing around without any water, and getting short of air, with the neuraminidase crystals sitting on the seat next to them. The cosmonauts almost perished.

At the last minute the Russian team brought the reentry vehicle down with a big bang in the Gobi Desert and we got some crystals back. They were battered and covered with hairs, but even so, they were better than anything we had grown on Earth. The crystals were used by BioCryst Pharmaceuticals in Birmingham Alabama, to redetermine the neuraminidase structure as a first step in the rational design of their own neuraminidase inhibitors [31].

While the *Mir* crystals were used to get a good data set, they weren't so much better than the crystals we grew on Earth as to make the whole thing worthwhile in terms of expense and trouble. Even so, these experiments are still being done by NASA. The general experience is that most proteins do not produce better crystals in space, but some do, so NASA is going ahead with these experiments and will probably continue to grow crystals on the space station when it is constructed.

This article has related some of the story behind the discovery and development of neuraminidase inhibitors as anti-viral drugs for the control of influenza. In clinical trials so far, the two leading compounds, BiotaGlaxoWellcome's GG167 (Zanamivir or Relenza) and Gilead-Roche's GS 4104 (Tamiflu) have behaved well. How they behave when prescribed by physicians and used by the general public remains to be seen.

There seem to be two main problems to be overcome, apart from the possible emergence of drug-resistance. First, the drugs seem to be most

effective when given before infection, or shortly after the first symptoms appear. Second, the drugs are only effective against the influenza virus and are totally useless against other virus infections (the common cold, adenoviruses, etc.) or against bacterial infections, all of which might be mistaken for the flu. However, these drawbacks to the use of neuraminidase inhibitors might be overcome if very rapid, simple, and inexpensive diagnostic kits for influenza were on the market. Such kits are already in development.

Conclusions

Influenza is the most widespread of the many serious infectious diseases suffered by mankind, and up to the present no effective treatment has ever existed. However, basic research carried out over the years on the biology of influenza viruses has led to the identification and characterization of the essential enzyme neuraminidase on the virus particle. Based on insights gained from this work, a large effort by academic and industrial organizations has resulted in the identification of potent and selective inhibitors of this enzyme. Two of these inhibitors have shown promising results in human clinical studies by alleviating the symptoms and shortening the duration of influenza virus infections.

These drugs will be of greatest usefulness if a new pandemic virus suddenly appears. It is unlikely that vaccines could be developed in time to deal with a new virus, and if the virus were also virulent, the neuraminidase inhibitors might be the only way to avert a worldwide calamity. Even if the new virus were relatively benign, many people would suffer much misery, and the community would experience great economic losses. These new treatments, available for the first time, might go a long way in preventing such things from happening.

Note added in proof: Zanamivir (Relenza®) has been approved in Europe, Australia, the United States, and Japan, and GS 4104 (Tamiflu®) has been approved in Switzerland, the United States, and Canada. Hundreds of thousands of prescriptions for these drugs were written during the 1999–2000 winter influenza epidemic. To date, however, no follow-up studies of the acceptability or effectiveness of these drugs, or of the emergence of drug-resistant strains, have been reported.

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