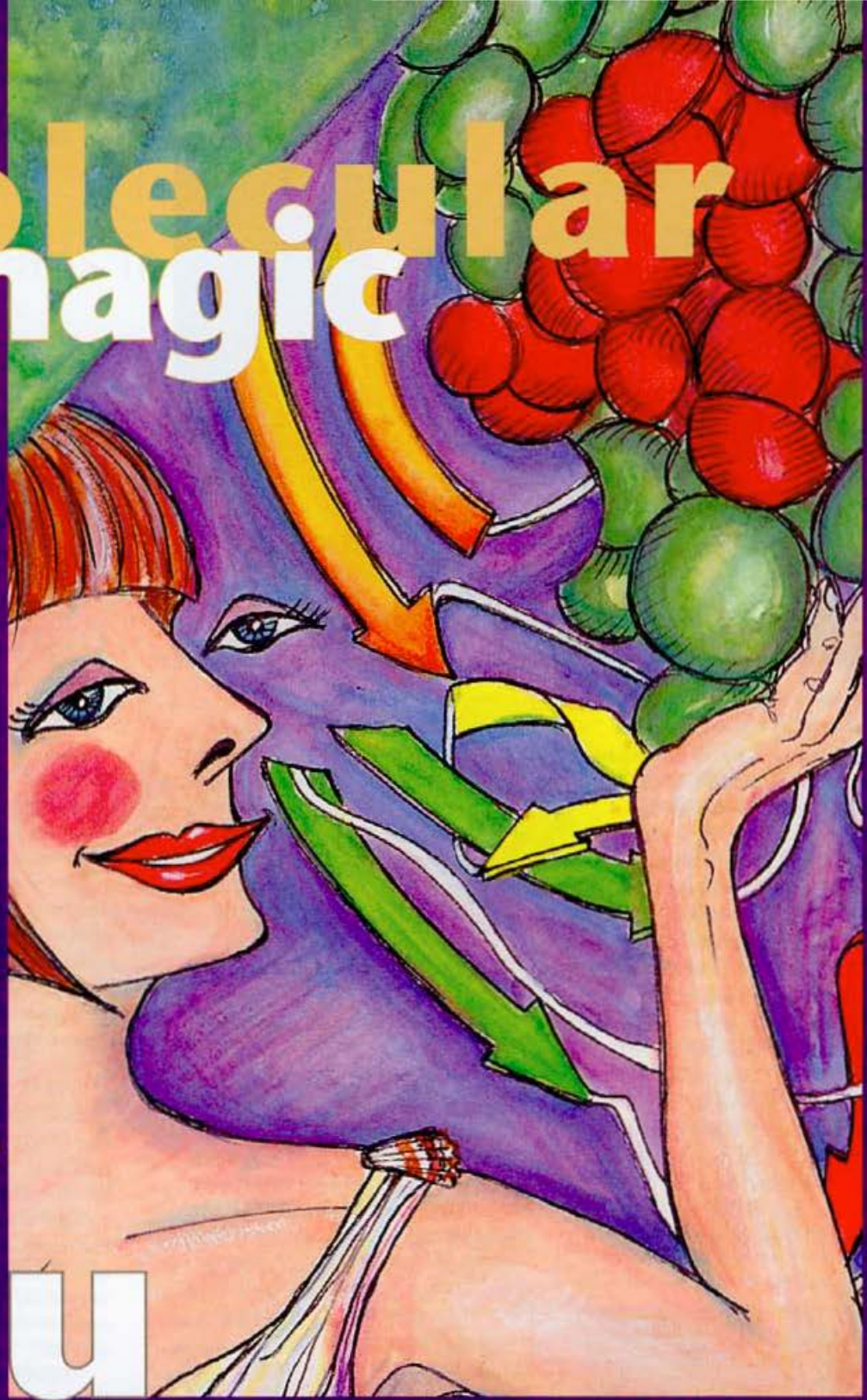
Molecular magic sets a trap for the 'flu



A VIRUS is one of nature's simplest but most efficient inventions: a sub-microscopic package of genes in a protein capsule, programmed to invade its host's cells and convert them into factories for new virus particles. While vaccines can prevent certain virus diseases, a century of medical research has not yielded a genuine cure. But three Australian scientists have now shown that one of the world's most dangerous viruses can be stopped in its tracks by jamming a carefully designed molecular spanner in its works.

For their pioneering efforts in developing a potential cure and a therapy for influenza, Dr Peter Colman, chief of CSIRO's Division of Biomolecular Engineering, Professor Mark von Itzstein of Monash University's Victorian College of Pharmacy, and Dr Graeme Laver of the Australian National University's John Curtin School of Medical Research, were awarded the 1996 Australia Prize for scientific research benefiting humanity.

Now in its final testing phase, the anti-influenza drug developed by Australian scientists appears set for release by the turn of the century. CSIRO's Dr Peter Colman attributes the breakthrough to a mixture of curiosity and luck, but as **Graeme O'Neill** reports, the research was world-class.

The pharmaceutical company Glaxo Australia, which is developing the anti-influenza drug under an agreement with the Melbourne-based biotechnology company Biota Holdings, expects to begin marketing the anti-influenza drug by decade's end. Phase 2 trials in human volunteers in the United States suggest that the compound, code-named GG167, confers a high level of protection against influenza and reduces the severity and duration of an established infection.

In January this year the deputy chairman of Glaxo Wellcome, Sir Richard Sykes, nominated the Australian anti-influenza compound as potentially the most valuable of a dozen candidate drugs his company now has in clinical trials. Each could achieve sales of at least $1 billion annually within five years of launch.

Colman says the designer anti-influenza compound was conceived in simple scientific curiosity, and chance played a prominent role in its early development. But in honouring him and his colleagues with the Australia Prize, his peers have paid homage not to chance, but to keen scientific intuition and persistence.

Knowing the enemy

The influenza virus survives by presenting a shifting target to the immune system, preventing its human and animal victims from developing durable immunity (see boxed story on page 11). In 1983, however, Colman and Laver announced in the international research journal *Nature* that they had identified an unchanging molecular 'bull's eye', common to all strains of the virus during the past half century.

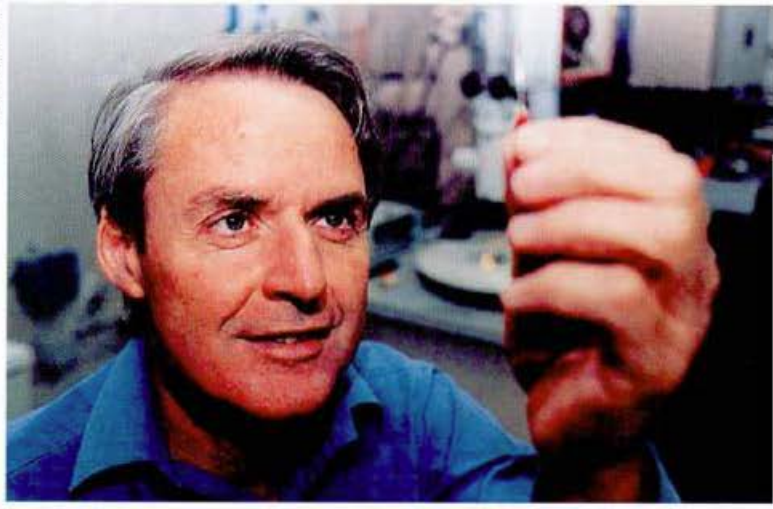
The groundwork for the discovery had been laid six years earlier, when Laver succeeded in crystallising the influenza virus's neuraminidase protein. He offered crystals to Colman to determine their structure by X-ray crystallography. The crystals were not of good quality, so the first X-ray images of neuraminidase's structure came from crystals that Colman grew in his own laboratory.

Working with his CSIRO colleague Dr Jose Varghese, Colman found that the structure of neuraminidase, which is an enzyme, varied from strain to strain. But all strains had a cavity, deeply recessed in the surface of the virus, that was rigorously conserved, hinting that it played some crucial role in the virus's replication cycle.

Neuraminidase is a tetramer: a molecule consisting of four identical sub-units, radially arranged around a common centre so each neuraminidase molecule has four active sites. The amino acid chains of each sub-unit fold into a shape not unlike a six-bladed version of the simple plastic propellers sold at fairgrounds. The active pocket is recessed at the point where the 'blades' converge, where its location and depth render it inaccessible to antibodies that might otherwise zero in on the pocket and provide cross-protection against different strains of the influenza virus.

As early as 1974, American virologist and influenza expert Professor Peter Palese conducted experiments which indicated that neuraminidase, one of a class of enzymes called sialidases, allows newly synthesised virus particles or virions to exit the cells in which they have just been synthesised. The new Australian influenza drug effectively confirmed this theory.

By this time, however, Palese and other influenza researchers had given up on the idea of using neuraminidase as a target for drugs. Haemagglutinin, the virus's passkey into the cell, seemed to offer a better target; it seemed more logical to lock the virus out of the cell, rather than to lock it in.



Virginia Valko-Roub

Dr Peter Colman: the designer anti-influenza compound was conceived in simple scientific curiosity.

A spanner in the works

The virus begins its replication cycle by attaching itself to the surface of cells lining the host's respiratory tract. Haemagglutinin molecules in the virus's coat react with strands of sialic acid on the host cell's outer membrane, holding the virus fast. It then releases its genetic blueprint, which reprograms the cell's own genetic machinery to make new virions.

As newly formed virions begin to bud from the cell membrane, their own haemagglutinin molecules snag on the sialic acid molecules that earlier facilitated the parent virus's attachment. Neuraminidase now comes into play, cleaving the sialic acid bonds to free the virions.

Colman and his colleagues suspected that the invariant pocket was the enzyme's active site: the region that envelops and cleaves the cell's sialic acid molecules. If a mutation in the neuraminidase gene changed the shape or chemistry of the pocket, the virion would be left stuck to the host cell's membrane, unable to transmit the mutation to its progeny.

Although it was hidden from the immune system's antibodies, the pocket offered a promising target for an anti-influenza drug. It is now known that sialic acid, attached to a very large molecule, goes into the pocket. What comes out of the pocket is free sialic acid. During the band-breaking process, sialic acid is distorted to look like Neu5Ac2en. Since nature designed the pocket to complement the specific shape and chemistry of the Neu5Ac2en residue, it should be an ideal template for a molecule that would jam itself tightly into the pocket, inhibiting the enzyme's activity. Scientists had actually tried this approach in 1966, but Neu5Ac2en showed no useful activity as a neuraminidase inhibitor.

CSIRO sold the intellectual rights for its discovery to a small, Melbourne-based biotechnology company called Biota Holdings in 1986. Biota decided to sponsor further research that might lead to a neuraminidase inhibitor. Colman's CSIRO group would continue its X-ray crystallography studies to refine its understanding of the shape and chemistry of the neuraminidase molecule and its invariant pocket, while von Itzstein's team at the Victorian College of Pharmacy would design and synthesise a 'plug': a molecule with the right shape and chemistry to inhibit the enzyme's activity.

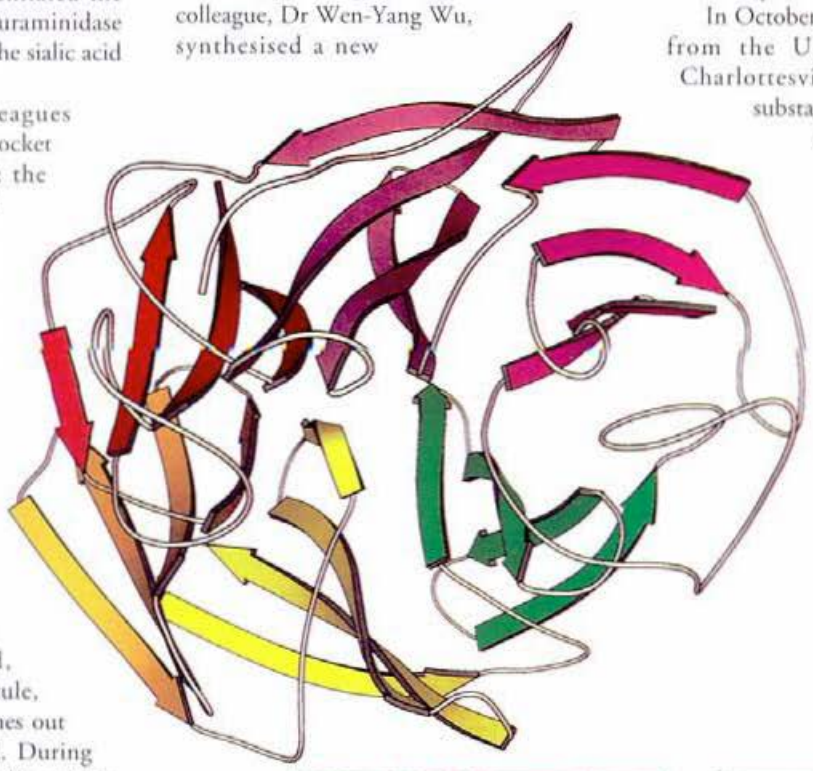
'GG167 is in the vanguard of a new generation of anti-viral agents that will make it possible to treat and even cure formerly intractable virus diseases.'

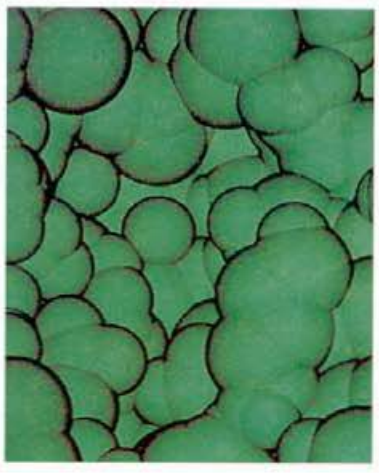
Armed with the CSIRO group's increasingly precise data on the shape and chemistry of the pocket, von Itztein and a colleague, Dr Wen-Yang Wu, synthesised a new variant of the Neu5Ac2en molecule in 1988. When Biota sent samples to the British pharmaceutical company Glaxo for testing in its United Kingdom laboratories in 1989, the samples strongly suppressed infection in ferrets, a species highly susceptible to influenza.

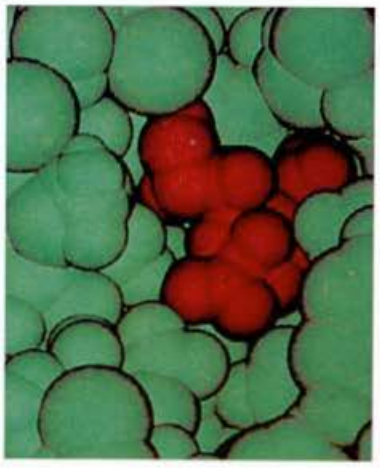
By late 1989, von Itzstein and Wu had produced another compound, the so-called 4-amino variant, which protected ferrets against influenza at a concentration five orders of magnitude lower. A few months later they developed a third molecule, the 4-guanadino variant, which was 100 times more potent again.

Biota signed an agreement with Glaxo Australia in 1990 to test both compounds. Glaxo subsequently selected the more active guanadino variant, now code-named GG167, for continuing development. It began trialling the drug in human volunteers in the United States, Australia and Europe in 1994.

In October 1994 Dr Frederick Hayden, from the University of Virginia in Charlottesville, reported that GG167 substantially ameliorated influenza infection in healthy male volunteers. Some took the drug in a nasal spray before being challenged with the virus. Others







Left: An illustration of the protein polymer neuraminidase. The starting point is to the rear and the ending point is the front of this view.

Below: The active site of neuraminidase without (left) and with a molecular plug inserted.

inhaled GG167 24 hours after being challenged. It proved to be highly effective in preventing infection in the first group, and in the second group, virus-shedding was reduced 100-fold.

Rational design or serendipity?

GG167 is in the vanguard of a new generation of anti-viral agents that will make it possible to treat and even cure formerly intractable virus diseases. It confirms the promise of rational drug design: developing synthetic molecules that interact with specific, well-defined molecular targets.

But according to Colman, rational drug design is unlikely to prove a panacea for virus diseases. He says in some respects the choice of neuraminidase as a molecular target for an anti-influenza drug was a lucky one. 'One of the things we had going for us was that we were confronted with a protein structure that was quite rigid in certain regions,' he says. 'And our drug doesn't have to get inside the cell to do what it does.'

Colman's initial interest in neuraminidase was purely academic. He wanted to see how human antibodies locked onto the influenza virus's antigens. Laver's successful crystallisation of neuraminidase in 1977 determined which influenza virus protein he would study. But even in the early 1980s, after haemagglutinin had been crystallised and medical researchers began to seriously consider the possibility of designing an anti-influenza drug around the virus's entry key, Colman stuck with neuraminidase on a scientific hunch.

Colman says he persisted with neuraminidase because, unlike haemagglutinin, it is an enzyme, and enzymes mediate high-energy biochemical reactions. If that energy could be exploited to lock a synthetic molecule tightly into the enzyme's pocket, it might inhibit neuramindase and prevent the virus from completing its replication cycle.

The project's success also hinged on the decision to revisit an unpromising, 20-year old compound. It didn't take von Itzstein's team long to show that relatively minor changes to its shape and chemistry vastly enhanced the biological activity of Neu5Ac2en. In consequence, the modified compound works at very low concentrations, at which it exhibits very low toxicity. The compound's toxicity is inherently low in any case, and is virtually free of side effects in mammals because it is directed at a specific target on the virus' surface.

Of the team's Australia Prize win, Colman says: 'Everyone knows all prizes are lotteries. We're delighted to have shared it, but I could point to other examples of similar work that would be very strong competitors. In science, there may be only small differences between things that get up and things that don't.'

More about the 'flu virus

Colman PM (1994) Influenza virus neuraminidase: Structure, antibodies, and inhibitors. *Protein Science* 3: pp1687-96. Cambridge University Press.

A global killer in many a guise

IF THE United States Food and Drug Administration approves GG167 for human use after Phase Three trials, Biota and Glaxo could launch their new anti-influenza drug sometime after 1997. It would provide long-needed insurance against the virus disease that many virologists consider more dangerous than much-publicised African haemorrhagic diseases such as Ebola, Marburg and Lassa fever.

In a New York Times article last January, eminent biologist and Nobel laureate Professor Joshua Lederberg drew attention to the fact that, in 1995, a 'garden variety' of influenza had killed 70 000 people in the US. Influenza is responsible for between 5 and 10% of all mortality in the US in an average year.

These figures pale against the toll taken by influenza pandemics during the past 2000 years. In the late eighth century, a burning fever decimated Charlemagne's armies, delaying the Frankish king's conquest of Europe; its symptoms were those of influenza. In 1580 another influenza pandemic followed new global trading routes to take a heavy toll of human life in Africa, Europe and the Americas. The Spanish 'flu pandemic of 1918-19 killed an estimated 25 million people in six continents.

For most common virus diseases, a single episode of infection – or vaccination with a weakened or 'killed' virus strain – normally induces lifetime immunity. Not so with influenza. This is because the virus mutates rapidly, changing the shape of the two protein molecules, haemagglutinin and neuraminidase, that form its coat.

The immune system rapidly recognises and subdues any virus to which it has previously been exposed, but it cannot anticipate the influenza virus's antigenic shifts: it cannot recognise what it has not previously seen. A person may thus suffer multiple bouts of influenza during his or her lifetime.

A successful immune response relies on the immune system's antibodies to make antibodies that precisely complement the shape of alien antigens. Minor mutations that subtly change the genes encoding haemagglutinin and neuraminidase can alter the shape of the proteins sufficiently to render existing antibodies useless. While the immune system is designing new antibodies, the patient suffers another bout of influenza.

Several times a century, the influenza virus undergoes major antigenic shifts. The amino acid sequence of its coat proteins may differ by as much as 50 per cent from preceding strains. Such strains are believed to involve genetic recombinations between different influenza virus strains adapted to humans, ducks and pigs, which live in close proximity on farms in Asia. Such strains have caused three pandemics this century: the Spanish 'flu of 1918-19, the Hong Kong 'flu of 1957, and the Asian 'flu of 1968.

Influenza also takes a heavy economic toll. At the height of the 1918-19 pandemic, up to 20% of workers in the US were immobilised. Absenteeism during the 1967-68 Hong Kong 'flu pandemic is estimated to have cost the US economy nearly $3.9 billion. The world's population has quadrupled since the Spanish 'flu epidemic nearly 80 years ago. Modern jetliners could now transport a lethal new mutant influenza virus around the globe and into densely crowded cities within 48 hours.

A US Federal working group which recently reviewed strategies for countering the next influenza pandemic concluded it would take at least six months after detecting a virulent new strain to produce enough conventional vaccine doses to immunise 250 million Americans. Professor Joshua Lederberg warns that secondary bacterial infections would still take a heavy