Antiviral adhesion molecular mechanisms for influenza: W. G. Laver’s lifetime obsession

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Infection by the influenza virus depends firstly on cell adhesion via the sialic-acid-binding viral surface protein, haemagglutinin, and secondly on the successful escape of progeny viruses from the host cell to enable the virus to spread to other cells. To achieve the latter, influenza uses another glycoprotein, the enzyme neuraminidase (NA), to cleave the sialic acid receptors from the surface of the original host cell. This paper traces the development of anti-influenza drugs, from the initial suggestion by MacFarlane Burnet in 1948 that an effective ‘competitive poison’ of the virus’ NA might be useful in controlling infection by the virus, through to the determination of the structure of NA by X-ray crystallography and the realization of Burnet’s idea with the design of NA inhibitors. A focus is the contribution of the late William Graeme Laver, FRS, to this research.

1. Introduction

Influenza remains a serious threat to global health. The first influenza pandemic to be reliably recorded occurred in 1580. It originated in Asia, spread to Europe via Africa and the whole of Europe was infected during a six-month period. It eventually reached America [1]. Huge numbers of people were ill and 8000 deaths were reported from Rome alone with some Spanish cities being decimated [2]. In the twentieth century, epidemics have occurred regularly with more serious pandemics striking in 1918, 1957, 1968, 1977 and 2009. Epidemics kill thousands of people annually worldwide, whereas during pandemics the number of deaths can reach millions. As well as infecting humans, the influenza virus has an economic impact on the domestic poultry industry, where it can annihilate an entire turkey or chicken battery in a matter of days.

Influenza A and B viruses are 80–120 nm diameter pleomorphic, irregularly shaped members of the Orthomyxoviridae family and contain eight segments of negative sense RNA. They are composed of a lipid membrane into which are inserted two glycoprotein molecules, haemagglutinin (HA) and neuraminidase (NA). The triangular trimers of rod-shaped polypeptide molecules of HA form spikes protruding from the virus surface. HA cleavage into HA1 and HA2 is necessary for virus infectivity and occurs during virus maturation. It is normally expedited by enzymes in the respiratory tract, where the virus replicates. In contrasting morphology, four NA molecules form a tetramer making a square, box-like head sitting at the end of a long thin stalk. Its end is embedded in the lipid bilayer of the viral membrane.

Three distinct types of influenza virus have been classified as influenza A, B and C. Humans are commonly infected by types A and B, but C occurs much more rarely in humans and pigs, has only 7 RNA segments and no NA. Humans can suffer multiple bouts of influenza A in a lifetime, since the virus is liable to both ‘drift’ and ‘shift’ so that the immune system of the victim is required to mount a renewed antibody response for most infections by the virus. In drift, point mutations arise in the amino acid chains constituting the two glycoproteins, HA and NA. Owing to the lack of error checking mechanisms, RNA viruses are much more likely than DNA viruses to drift in this way and to produce progeny...
viruses which are unfaithful to the parent virus. This results in minor changes in the HA and NA surface antigenic footprint and perhaps a new infection for a patient who has already had influenza. Thus, vaccination against influenza is of limited effectiveness, since the vaccine strains have to be chosen around nine months before the ‘flu season’ and the virus can drift meanwhile.

In shift, a major change in the HA and/or NA surface antigens responsible for virus fusion and spreading occurs. For HA, 16 antigenically distinct subtypes (H1–H16) have thus far been identified in influenza isolated from wild waterfowl, as have nine for NA (N1–N9). Additionally, two more subtypes each of HA and NA have been recently found in bats (H17N10 and H18N11). Reassortment resulting in new H or N subtypes in viruses infecting humans has historically sometimes resulted in global human influenza A pandemics (1918: Spanish flu, H1N1 more than 20 million deaths; [3]; 1957: Asian flu, H2N2 more than 1 million; 1968: Hong Kong flu, H3N2 more than 0.8 million; 1977: Russian flu, H1N1; 2009: H1N1pdm more than 60.8 million cases, more than 0.25 million hospitalizations, more than 18 000 confirmed deaths but the Centre for Disease Control in Atlanta, USA, estimated it at 284 000). The last had a low death rate of around 0.7% but is on the increase again and is now circulating as one of the seasonal flu viruses. However, the 1918 pandemic was the most widespread of these, with an estimated 33% of the world population infected and a mortality rate of 3–5% resulting in the death of more than 1.3% of the total global inhabitants. A feared H5N1 ‘bird flu’ pandemic was avoided in 1997 by the slaughter of all the birds in the Hong Kong live bird markets following the deaths of six of the 18 cases. This subtype combination persists, and by the end of January 2014 had claimed the lives of 386 of the 650 people infected, a 59.4% mortality rate. Current concern centres round a new shifted virus which has jumped from birds to humans: H7N9. In 2013 and 2014 (to May), it has caused 146 deaths from 402 infections in mainland China. This year an H10N8 (with six genes derived from H9N2) virus has also been reported in mainland China and an H6N1 in Taiwan. Currently, both H7N9 and H9N2 coexist in chickens in Chinese live bird markets, and these could re-assort into a new influenza virus at any time [4].

This paper presents a summary of the research behind the development of the anti-influenza NA inhibitors now commercially available, zanamivir (‘Relenza’) and oseltamivir (‘Tamiflu’), particularly highlighting the contribution of the late William Graeme Laver, FRS (3/6/23–26/8/08).

2. Brief history

The infection causing ‘fowl plague’ in chicken houses was first isolated in 1900, but not recognized as being related to influenza until 1955 [5]. In 1931, swine influenza was isolated from pigs [6], and then human H1N1 from ferrets in 1933 [7]. Another human influenza virus was isolated in 1940 and found to be completely unrelated, serologically, to previous isolates [8]. Thus, the already discovered viruses were designated influenza type A and the new strain, type B. Type B viruses only infect humans (apart from one report of the infection in a fur seal [9]) and as yet has only exhibited ‘drift’, not ‘shift’.

In 1941, George Hirst, working in New York, performed a pivotal experiment on influenza A virus in which he found that red blood cells were agglutinated in the cold by allantoic fluid of chick embryos in eggs infected with virus. He then heated the cells causing them to disperse, and added new virus, only to find that the cells could no longer be agglutinated in the cold. He reasoned that there must thus be a receptor destroying enzyme (RDE) on the surface of the virus which had disabled the ability of the red blood cell to agglutinate [10]. Alfred Gottschalk and his technician at the Walter and Eliza Hall Institute of Medical Research (WEHI) in Melbourne then identified the substrate of RDE as sialic acid (or N-acetyl neuraminic acid: Neu5Ac2en (NANA); figure 1). The RDE was thus a sialidase or NA. Gottschalk’s technician at the time was called William G. Laver (Graeme) who as a 17-year-old school leaver, was paid 1 shilling/hour. In 1948, while Director of WEHI, McFarlane Burnet, realized that NA inhibitors might be useful anti-viral agents even before anything was known about the molecular biology of the influenza virus:

An effective competitive poison for the virus enzyme might be administered which, when deposited on the mucous film lining the respiratory tract, would render this an effective barrier against infection from without and the spreading surface infection of the mucosa which follows the initiation of infection. (Burnet quoted in [11, pp. 410–411]).

This idea finally bore fruit more than 50 years later, as now, at least four ‘competitive poisons’ of flu NA have been developed, two of which have so far been approved for clinical use (see §5).

Hirst initially assumed that the agglutination of red cells by influenza virus particles was mediated by the NA on the surface of the virus, binding to its substrate, sialic acid, on the surface of the cells and thus linking them together in large clumps [10]. The first indication that the NA was not responsible for agglutinating red cells came from the discovery that when some strains of influenza virus were heated to 55°C, the NA was inactivated but the HA was still fully active [12,13].

However, in 1961, Mayron and his colleagues found that a soluble sialidase could be separated from particles of influenza virus and that the soluble enzyme had no HA activity and did not adsorb to red cells [14]. Around the same time, Hans Noll found that if influenza type B virus was treated with trypsin, almost 100% of the NA was liberated as a soluble enzyme with a sedimentation coefficient of 9S (MW about 200 kDa) leaving all of the HA activity associated with the virus particles [15]. It was then clear that it was the HA, not the NA that was causing the agglutination.

Inspired by his work as a laboratory technician, Laver went to night school and qualified to undertake a part time biochemistry degree at Melbourne University which he completed in 1954. Following a 2 year full time MSc with Victor Trikojas in....
the Melbourne University Biochemistry Department, he went to St Mary’s Hospital, London, and gained a PhD in 2 years working on porphyrin biosynthesis. Returning to Australia, he took up a position at the John Curtin School of Medical Research (JCSMR) at the A.N.U. in Canberra, and returning to his original interest in influenza viruses, spent the next 50 years there engaged in various aspects of research on them.

Laver’s first notable contribution in 1964 was to describe efficient methods for the disruption of particles of influenza B virus (Lee strain) at room temperature with the detergent sodium dodecyl sulfate (SDS), without cleavage of covalent bonds, into subunits very much smaller than the original virus. Furthermore, some strains disrupted in this way retained HA activity, others retained NA activity and with others, both HA and NA were still active after SDS treatment [16]. Using these techniques, he then carried out structural studies on the protein subunits from three strains of influenza virus.

His discovery that the virus particles could be disrupted by detergents led to the invention, with Rob Webster, of the non-toxic, split-virus or subunit influenza vaccine used throughout the world today [17]. From detergent-disrupted virus, Laver also isolated pure, intact HA and NA molecules. These were the ‘spikes’ on the surface of the virus, and electron micrographs obtained by Robin Valentine showed that they had different morphology. The HA was a triangular rod-shaped object while the NA consisted of a square box-like head on top of a long thin stalk [18]. When NA ‘heads’ liberated from virus particles by protease digestion then became available, some beautiful electron micrographs by Nick Wrigley showed that the square box-shaped heads were, in all probability, tetramers [19].

In 1966 using techniques developed in Canberra, Laver discovered that antigenic hybrid viruses, isolated after the infection of cells with two different influenza viruses by Ed Kilbourne in New York, had the HA from one parent and the NA from the other [20]. This same genetic reassortment was then shown to be possible in animals by Webster et al. [21]. The segregation of the HA and NA antigens led to the production of monospecific antisera, now used widely in the identification of flu virus isolates.

Laver and Webster then showed, for the first time, that antigenic drift in influenza resulted from small changes in the amino acid sequence of the HA and not from a shuffling of antigens on the virus as previously thought [22]. Laver later (1982) also demonstrated, with Rob Webster and Gillian Air, that flu virus variants which were selected with monoclonal antibodies (‘escape mutants’) had single amino acid sequence changes, and that these were located in discrete sites at the tip of the HA spike [23].

Experimental work by Laver & Webster [24] proved the avian origin of human influenza. Laver’s initial isolation of a type A influenza virus from an Australian pelagic bird [25] followed as a result of several three week field trips by Laver and collaborators (including his teenage children who had to catch the ‘mutton birds’ (shearwaters and terns)) to Tryon Atoll (figure 2a), an uninhabited outcrop of the Great Barrier Reef (GBR). The first expedition in 1969 was funded by an AU$8500 research grant from WHO, after his grant application elsewhere was met with ‘Laver must be hallucinating’ (if he thinks there is flu in GBR birds). Antibodies to H2N2 were detected in the sera of the aquatic birds in which the disease seemed to be asymptomatic and virus was isolated from cloacal swabs. This work led to the discovery by Webster that it is the aquatic birds of the world that are the source of all type A flu viruses [26], the virus being shed in faecal droppings.

3. Neuraminidase and cell adhesion

So what is the function of the NA in the influenza virus life cycle if the HA was responsible for the agglutination by binding to sialic receptors on the cell surface and then being involved in the fusion of the virus with the cell?

In 1966, Sato et al. [27] described the isolation of a low molecular weight sialidase (NA) by pronase treatment of the H2N2 virus, its partial purification and determination of its sedimentation coefficient. In the same year, Sato & Rott [28] also demonstrated that the function of the NA was probably to release virus particles from infected cells. Antibody directed specifically against the NA did not prevent virus from infecting cells but did stop the release of newly formed virus particles [29].

This inhibition of progeny virus release by NA was demonstrated most convincingly by Peter Palese, Dick Compan and their colleagues who obtained electron micrographs of sections of cells infected with a wild-type influenza virus and also of cells infected with a mutant which lacked NA activity [30]. For the mutant, the newly formed virus particles which had budded off from the surface of the infected cell formed large aggregates still attached to the cell surface. Without NA to release them, these virus particles were not mobile and could not migrate to infect new cells, effectively terminating the infection. Palese et al. [30] postulated that NA is essential for the replication of influenza virus and is required to remove neuraminic (sialic) acid from the viral envelope to avoid aggregation of the progeny virus. Thus, if the enzyme activity of NA could be inhibited, the infection would be prevented from spreading.

4. Influenza neuraminidase crystals and structures

Laver had become fascinated with crystals during his time with Alfred Gottschalk and Gordon Ada at the WEHI; following his purification of the ‘RDE’ from the medium in which Vibrio cholerae had grown, he observed long thin crystals in the solution of enzyme [31]. Forty years later, he again crystallized this protein, resulting in the determination of the structure of the Vibrio cholerae sialidase [32].

In 1978, Laver released NA ‘heads’ by pronase treatment of reassortant influenza viruses containing the N2 subtype. The heads were purified by sucrose density gradient centrifugation and fractions from the gradient containing the NA were dialysed against water to remove the sucrose. The NA crystallized as thousands of small square plates [33], reportedly during a journey down the corridor at the JCSMR while carrying a tube of the purified enzyme. Larger crystals were then grown in an idiosyncratic ‘large-scale’ crystallization system that Laver used for the next 30 years, consisting of a 7 cm diameter round glass dish with a ground glass top rim in which the reservoir solution was placed, topped with an upturned Perspex plate. This plate had been preloaded with a mixture of several millilitres of equal volumes of protein and reservoir solution and was sealed on with vacuum grease (figure 2b). By comparison, current crystallization
robots dispense between 50 and 200 nl per drop in 96 well arrays (figure 2b). Laver successfully grew subtypes of N2 (human), N6 (English duck), N8 (Ukrainian duck) and N9 (noddy tern) NA crystals in this way (figure 2c), as well as other NAs including those from *Vibrio cholerae* mentioned above and from *Salmonella typhimurium* sialidase. He transported or mailed them round the globe to collaborators in thick glass walled tubes which were flame sealed at both ends (figure 2b(ii)), and extracting the crystals from these was an art in itself. However, crystals have been successfully stored in these tubes for at least 10 years and still diffracted. Laver also negotiated as an individual with the Soviets (causing consternation in the Pentagon) to send NA up to the MIR space station to try to grow higher quality crystals in zero gravity, but the results were only very marginally and rather unconvincingly better.

The larger N2 crystals were of a quality good enough for X-ray crystallographic three-dimensional structure determination by Jose Varghese and Peter Colman in 1983 at CSIRO in Melbourne: a true tour de force in its time [34]. Each monomer in the NA tetramer was composed of six topologically identical beta sheets, arranged as in the blades of a propeller (figure 3a). The tetrameric enzyme has circular fourfold symmetry stabilized in part by calcium ions bound on the symmetry axis. The catalytic sites were identified by soaking sialic acid substrate (figure 1) into N2 NA crystals...
and solving the structure of the complex [35] (figure 3b). The catalytic site was located in deep pockets that occur on the upper corners of the box-shaped tetramer.

Diffraction quality crystals of N2, N6, N8 and N9 [36] are shown in the top row of figure 2c. Subtypes N1, N3 and N5 (figure 2c, bottom row) were also crystallized by Laver but were unsuitable for structure solution. His attempts to crystallize subtypes N4 and N7 were never successful. In 1987, Laver also crystallized antibody Fab fragments bound to flu NA (figure 2c, bottom right). The structure of the complex [35] showed how antibodies recognized an epitope on a viral protein and how this epitope changed its structure during antigenic drift [37].

Additionally, the structures of N9 [38], N1, N4 and N8 [39], N6 [40], and influenza type B [41] NA have all now been determined. They all have a similar overall topology to that of N2 despite having differences in amino acid sequence of the NA polypeptide chain, which in the case of flu B NA is as high as 75%. The 18 active site residues (figure 4) are conserved in N1–N9 and this suggested that the catalytic site of influenza NA was totally conserved among all flu strains, and that a drug which could effectively block one NA would also be effective at blocking all other flu NAs, even those on viruses which have not yet appeared in people.

However, further more recent phylogenetic analysis of the amino acid sequences [42] has revealed that the NAs fall into two distinct groups, as do the three-dimensional shapes of their active sites. Group 1 consists of N1, N4, N5, N8 and group 2 of N2, N3, N6, N7, N9, with the differences being around the ‘150s loop’. This variability has opened up new opportunities for anti-influenza drug discovery [39].

5. ‘Competitive poisons’ of neuraminidase

As early as 1966, NA inhibitors were being investigated, and it was found by Palese and colleagues from random screening that DANA (dehydroxy-NANA) (figure 5) and FANA (a fluorinated DANA derivative) [43] were mildly effective with inhibition constants of around $K_i = 10^{-4}$ M. More mechanistic drug discovery was pursued between 1969 and 1974, and the drugs amantadine and rimantidine were approved for use. These drugs blocked the ion channel protein, M2, that spans the lipid bilayer of influenza A viruses with inhibition constants about 100 times lower than FANA. However, resistant virus strains arose quickly and the drugs also had unwanted side effects, although amantadine is now used in the long-term treatment of neurological disorders.

After the three-dimensional structure of NA was elucidated in 1983, it was possible to take a more considered approach to identifying NA inhibitors, resulting in the design of McFarlane Burnet’s ‘competitive poison’ in the form of a small molecule ‘plug drug’ for the NA active site. This was achieved by Mark von Itzstein and colleagues at the Victorian School of Pharmacy in Melbourne in 1993, when the first NA inhibitor wasrationally designed using computer modelling. They then synthesized and tested it in both cell culture and animal models. The molecule, which had a $K_i$ of around $10^{-11}$ M, was 4-guanidino-DANA (figures 5 and 6a) or Zanamivir and was effective against all flu viruses. It is now marketed under the name Relenza [44]. It has only two differences from sialic acid, the natural substrate of NA: the OH group on carbon 4 on the ring is replaced by a basic guanidino group, in order to bind in an empty pocket in the active site near the negatively charged Glu119 (N9 numbering), and there is a double bond between carbons 2 and 3 rather than the single bond in sialic acid. This double bond mimics the transition state analogue. Figure 6a shows Relenza bound in the N9 NA active site. Relenza is not orally bioavailable; it is not absorbed from the gut and is administered as a powder which is puffed into the lungs. Interestingly, however, if Relenza is administered intravenously, it seems to be able to cross from the blood into the respiratory secretions.

In order to ensure that alternative inhibitors of flu NA were developed, Laver supplied N9 NA crystals to a number of pharmaceutical companies worldwide. This N9 subtype had been isolated from a GBR noddy tern cloacal swab in 1973. As a result of the availability of these N9 crystals, three new such ‘plug drugs’ were developed [45–47] and one, synthesized by Choung Kim and his associates at Gilead Sciences in California is called Oseltamivir (Tamiflu) [45]. For its design, the presence of a large hydrophobic pocket in the active site region of NA that accommodates the glycerol side chain of the substrate, sialic acid, was exploited in the synthesis of carbocyclic analogues with hydrophobic alky1 side chains. These carbocyclic compounds are not sugars and have no oxygen in the ring. The final compound chosen, GS 4071, with a 3-pentyl side chain (figure 5) was a potent and specific inhibitor of influenza type A and type B NAs. The X-ray crystallographic structure of GS 4071 bound in the NA active site is shown in figure 6b.
However, GS4071 was not orally bioavailable. This problem was overcome by converting the carboxylate to the ethyl ester. The resulting compound, GS4104 (figure 5), now marketed as ‘Tamiflu’ could be administered as a pill. Following absorption of this prodrug from the gut, the ester was hydrolysed in the liver and the resulting NA inhibitor found its way into the respiratory secretions. It is not clear why GS4071 is able to cross the membranes in the respiratory tract when it could not cross the membrane in the gut.

Following Phase 3 clinical trials, both Tamiflu and Relenza were approved for clinical use, but due to issues of the emergence of drug resistance (see §6), further work to identify other NA inhibitors and alternative drugs which target other components of the flu virus is currently an area of ongoing research.

6. Use of anti-neuraminidase inhibitors in public health

Although clinical trials showed the efficacy of Relenza and Tamiflu in infected patients so that the drugs were approved for widespread use, the validity of these trials have recently been questioned [48]. Many governments have acquired stockpiles in the event that H5N1 acquired human-to-human transmissibility and led to a global pandemic. This strategy was, in fact, suggested by Laver and Garman in 2001 [49].

However, various issues have arisen with the use of NA inhibitors in patients. Among these is the identification of resistant strains to Relenza identified in tissue culture (although not in animals or clinical trials). These strains are somewhat compromised: for instance a Glu119 to Gly119 (N9 numbering) mutation decreases the electrostatic interaction between Glu119 and the guanidino group of Relenza, but makes for weaker intermolecular monomer contacts, decreasing the number of tetramers and causing destabilization of the virus [50]. More resistant mutants have been reported for Tamiflu with changes in HA but none in NA, and vice versa. For instance, in NA, an Arg292Lys mutant causes Glu276 to be anchored to Lys 229 by an ionic link so that it can no longer rotate to accommodate Tamiflu (see figure 6b), and a His274Tyr mutation destabilizes the H-bond network [51]. Some of these mutants have been isolated from infected patients [52]. In the case of HA mutations, the HA seemed to have reduced sialic acid binding affinity, so reducing the need for ‘escape’ of the viruses from sialic receptors on the infected cell. This has raised the possibility that NA has some additional function(s) in the life cycle of the virus.

More mutations to Tamiflu have arisen because it is a molecule further from the shape of the natural ligand, sialic acid, than is Relenza (figure 5) and so the virus can more easily mutate in response to Tamiflu but still remain functional. This is unfortunate, since patients generally prefer taking pills (Tamiflu) rather than inhaling (Relenza). In Japan, there were reports of teen suicides and mental instability associated with taking Tamiflu, and other side effects were also attributed to this drug [53].
However, the main challenge with administering the NA inhibitors, is that because of their mode of action (inhibiting virus budding from the surface of a host cell by causing clumping of the viruses to each other), to be effective they must be given within the first 24 h after influenza symptoms start. This presents public health difficulties relating to
diagnosis happening in time for the drugs to be effective. Laver strongly favoured over-the-counter availability for the drugs and the development of rapid detection methods (e.g. lab-on-a-chip diagnostics) so that people who suspected they were getting flu could seek immediate medication. The NA inhibitors are, of course, ineffective against respiratory tract or bacterial infections, or against the common cold, since they are exquisitely designed to bind to the NA active site.

It should be noted that the seasonal H1N1 viruses in 2007–2008 (which disappeared after the emergence of the pandemic 2009 H1N1) became completely resistant to all of the NA inhibitors [54,55]. Since the majority of influenza viruses are already resistant to amantadine we are dependent on a single class of anti-influenza drugs, the NA inhibitors, and are thus in a monotherapy situation. It is inevitable that influenza will eventually develop escape mutants and become resistant to the NA inhibitors. This highlights the pressing need for the development of additional anti-influenza drugs.

7. Summary
Graeme Laver, working both alone and with various collaborators worldwide, made a number of significant contributions to influenza research over 50 years. These included: his use of mild detergent to remove influenza external subunits which led to the development of subunit vaccines still used today, the successfully isolation of pure intact HA and NA and the identification of their morphology, the identification of an antigenic hybrid virus in cells, and the production of monospecific antisera. Additionally, Laver was the first to isolate influenza strains from pelagic wild waterfowl. He established that antigenic drift could be due to single amino acid changes in HA. He crystallized the second antibody/enzyme complex (the first was chicken egg white lysozyme) allowing the three-dimensional structure to be solved and thus showing how antibodies recognized an epitope on a viral protein. He is pictured in figure 7.

He was a highly original thinker as well as a brilliant bench scientist, but he scorned all bureaucracy and any procedural impediments that in any way obstructed what he wanted to do. He did not suffer people he regarded as fools at all. He was famously described as someone who ‘Poured oil on troubled waters and then set fire to it.’ He knew it and was proud of it.

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