

## Study of Specific Oligosaccharide Structures Related with Swine Flu (H1N1) and Avian Flu, and Tamiflu as Their Remedy

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The infection of pandemic influenza viruses such as swine flu (H1N1) and avian flu viruses to the host cells is related to the following two factors: First, the surface protein such as HA (hemagglutinin) and NA (neuraminidase) of the influenza virus. Second, the specific structure of the oligosaccharide [sialic acid( $\alpha$ 2-6) galactose( $\beta$ 1-4)glucose or sialic acid( $\alpha$ 2-3)galactose( $\beta$ 1-4)glucose] on the host cell. After recognizing the specific structure of the oligosaccharide on the surface of host cells by the surface protein of the influenza virus, the influenza virus can secrete sialidase and cleave the sialic acid attached on the final position of the specific structure of the oligosaccharide on the surface of host cells. Tamiflu (oseltamivir), known as a remedy of swine flu, has a saccharide analog structure, especially the sialic acid analog. Tamiflu can inhibit the invasion of influenza viruses (swine flu and avian flu viruses) into the host cells by competition with sialic acid on the terminal position of the specific oligosaccharide on the surface of the host cell. Because of the emergence of Tamiflu resistance, the development of new potent anti-influenza inhibitors is needed. The inhibitors with positive-charge groups have potential as antiviral therapeutics, and the strain specificity must also be resolved.

**Keywords:** Swine flu (H1N1), Tamiflu, structure, sialidase, sialic acid, ( $\alpha$ 2-3) linkage

In March and April 2009, H1N1 (swine-origin influenza A) virus appeared in Mexico and the United States. Within one month, the virus had spread worldwide to over 30 countries including Korea by human-to-human transmission. The worldwide spread of H1N1 with terrible mortality has raised concerns that the H1N1 influenza virus had mutated and caused a pandemic of influenza in humans [13, 22, 30, 33, 36, 38].

The current swine influenza virus belongs to the influenza A virus H1N1 subtype (A/H1N1). The three historic pandemics in humans were caused by A/H1N1 (in 1918), A/H2N2 (in 1957), and A/H3N2 (in 1968), [17, 37, 40, 48]. Moreover, there are significant changes in both surface proteins such as HA (hemagglutinin) and NA (neuraminidase) of H1N1 (2009), 27.2% and 18.2% of the amino acid sequence, from prior H1N1 isolated in 2008 and current vaccine. Such a degree of change qualifies as an “antigenic shift.” It may give influenza H1N1 (2009) significant pandemic potential [13]. The purpose of this paper is to provide information to understand the events surrounding the emergence and spread of the new influenza H1N1 strain (2009), and Tamiflu as the remedy.

### How are Humans Infected by Swine Flu Virus? What is Swine Flu (H1N1)?

The influenza virion, in other words, infectious particle, has a spherical shape, as shown in Fig. 1 [14]. It is an enveloped virus with a single-stranded RNA gene [14, 32].

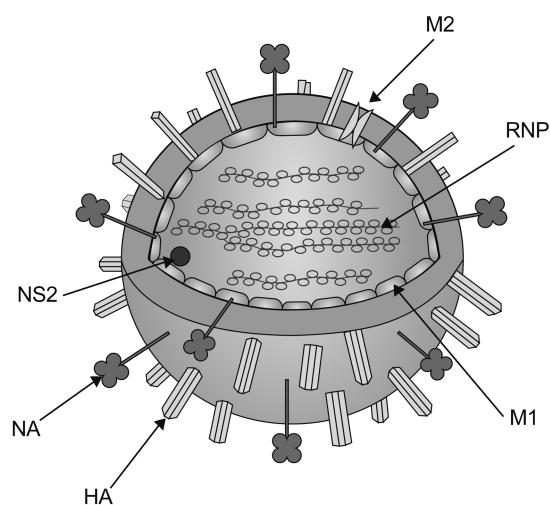


Fig. 1. Schematic representation of influenza A virion [14].

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The outer layer of the influenza virion is a lipid membrane, which is taken from the host cell in which the virus multiplies.

Three proteins, HA (hemagglutinin) trimers, NA (neuraminidase) tetramers, and M2 protein, form “spikes” on the surface of the virion. These are glycoproteins that determine the type of influenza virus (A, B, or C) and the subtype (*e.g.*, A/H1N1, A/H2N2, or A/H3N2). The NA protein is the target of antiviral drugs such as Tamiflu (oseltamivir) and Relenza (zanamivir).

The M2 protein (Fig. 1) is also embedded in the lipid membrane. It is the target of the antiviral adamantanes (amantadine and rimantadine). Beneath the lipid membrane is a viral protein called M1 (matrix protein). The M1 protein forms a shell, which gives strength and rigidity to the lipid membrane. Within the interior of the virion, there are the viral RNAs; they code for one or two proteins (RNP such as B1, PB2, PA, and NP). Both influenza A and B viruses have eight RNA segments, whereas the influenza C viruses have seven RNA segments [6, 14, 24, 31, 41]. The interior of the virion also contains the NS2 (NEP) protein, which is associated with M1 [14, 33].

### Structures of the A, B, and C of Influenza Virus

Although it would be difficult to distinguish influenza A and B viruses by electron microscopy, there are differences in the compositions of membrane proteins. The influenza A virions have four kinds of membrane proteins: three membrane proteins including HA, NA, and M2; a matrix protein (M1) just below the lipid bilayer; a ribonucleoprotein core (consisting of eight viral RNA segments and three proteins: PA, PB1, PB2); and the NEP (NS2) protein.

The influenza B virions have four membrane proteins (HA, NA, NB, and BM2). Like the M2 protein of influenza A virus, the BM2 protein is a proton channel. The NB protein is an ion channel. Influenza B viruses cause the same aspect of disease as influenza A. However, influenza B viruses do not cause pandemics because of the limited hosts such as humans and seals [1, 14].

Influenza C viruses are very different from influenza A or B viruses [33, 34]. The influenza C virions have hexagonal structures on the surface and form the cord-like structure with a long tail (500 microns). Like the influenza A and B viruses, the core of influenza C virus consists of a ribonucleoprotein made up of viral RNA and four proteins. The M1 protein in the influenza C virus also lies below the membrane, like in the influenza A and B viruses. The CM2 protein functions as an ion channel. The major difference between influenza viruses A/B and C is the surface glycoprotein. Influenza virus C has HEF (hemagglutinin-esterase-fusion). Because HEF has the functions of both the HA and the NA, the influenza C virus contains seven RNA segments, not eight RNAs like in influenza A and B viruses [29, 33, 34].

### Kinds of Subtypes

The influenza A virus membranes contain two glycoproteins; HA and NA (which is also called sialidase) [10, 13, 18]. HA attaches to cellular receptors of host cells, penetrates, and then promotes fusion of viral and cellular membranes. After virus replication, NA removes sialic acid from cellular glycoproteins to facilitate the virus release and the spread of infection to new cells.

The distinct antigenic properties of different HA and NA molecules are used to classify influenza A viruses into subtypes; sixteen for hemagglutinin (H1–H16) and nine for neuraminidase (N1–N9). Numerous combinations of HA and NA subtypes are found in avian species [such as H5N1 (2006)] and in humans [such as H1N1 (1918), H2N2 (1957), and H3N2 (1968)]. The H1N1 (2009) pandemic strain is a reassortment of avian, human, and swine influenza viruses. NAs of viruses currently circulating in humans belong to two phylogenetically distinct groups: group 1 (composed of the N1, N4, N5, and N8 subtypes) and group 2 (composed of N2, N3, N6, N7, and N9) [5]. The percentage sequence identities of group 1, group 2 of influenza A and B are shown in Table 1 [15, 19, 21, 28, 31, 35].

There is a strong correlation between the extent of sequence identity and the similarity of the crystal structures.

### Infection Mechanism

Viruses cannot reproduce outside of a cell. The production of new infectious particles must take place within a cell. Upon entering cells, viruses parasitize the host cell to produce new viral progeny. The infection mechanism of a virus includes five steps (Table 2).

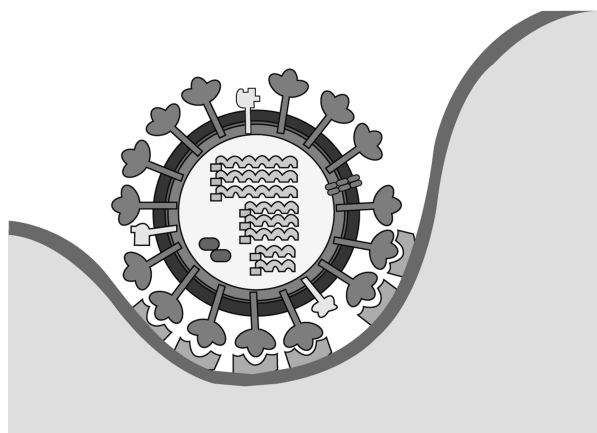
**Table 1.** Statistics for the similarities of sequences and structures between two members of group 1 (N1 and N8) and two forms of group 2 (N2 and N9) of influenza A with the NA from influenza B [35].

Type of NA	N1	N8	N2	N9	B
N1	-	0.54	0.90	0.84	1.40
N8	56%	-	0.85	0.92	1.48
N2	43%	43%	-	0.71	1.64
N9	48%	43%	46%	-	1.75
B	31%	33%	32%	27%	-

The percentage sequence identities are shown on the left-hand side and root mean square deviations of  $c_{\alpha}$  positions between monomers is given on the right-hand side.

**Table 2.** The five steps of virus infection mechanism [1, 5, 6, 7, 14, 16, 24, 31, 33, 41].

Step 1	Attachment and entry of the virus
Step 2	Translation of mRNA into protein
Step 3	Genome replication producing more RNA or DNA
Step 4	Formation of assembly of new particles
Step 5	Release of particles from the cell



**Fig. 2.** Illustration of an influenza virion binding to its cell receptor [33].

The virions must attach to a receptor on the plasma membrane of the host cell in order to enter the cell. Every virus has a specific receptor and there is a particular viral protein that binds this receptor (Fig. 2) [29, 33].

The influenza viral spike (HA protein) attaches to the cell receptor. The cell receptor is sialic acid (neuraminic acid), in other words, a small carbohydrate that is attached to many proteins or lipids on the cell surface.

### HA, NA, and SA (Sialic Acid)

The two surface glycoproteins of influenza virus are HA (called haemagglutinin, because of its ability to agglutinate erythrocytes) and NA (Fig. 1) [which interact with receptors that contain terminal sialic acid (neuraminic acid) residues]. HA consists of two structural domains, with the peripheral, globular domain having a binding cleft for attachment to cells. HA attaches to cellular receptors to initiate virus penetration and promotes fusion of viral and cellular membranes. NA is a tetramer enzyme composed of a cytoplasmic tail, a transmembrane domain, a stalk region, and a globular head. NA destroys receptors recognized by HA

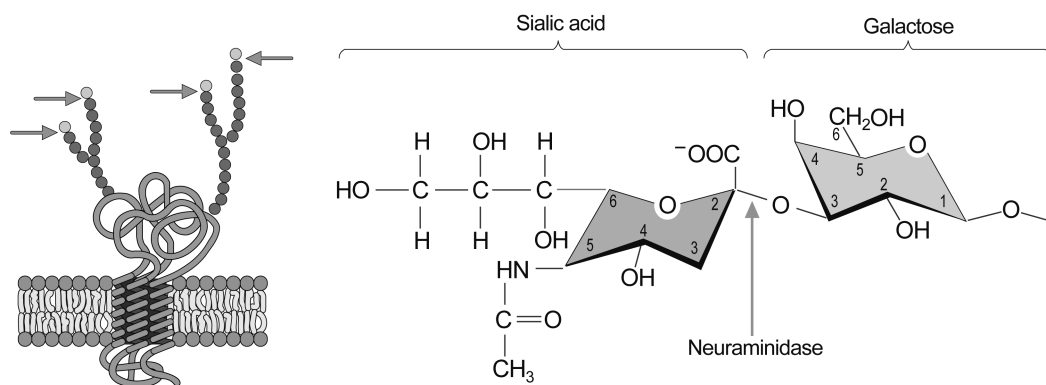
by cleaving the  $\alpha$ -ketosidic bond linking a terminal sialic acid (Neu5Ac, neuraminic acid) residue to the adjacent oligosaccharide moiety [sialic acid( $\alpha$ 2-6)galactose( $\beta$ 1-4)glucose or sialic acid( $\alpha$ 2-3) galactose( $\beta$ 1-4)glucose] (Fig. 3). This cleavage facilitates movement of the virus to, and from, the site of infection in the respiratory tract. Respiratory mucins contain sialic acid residues, so the receptor destruction is important for virus penetration through secretions. Because replication of influenza virus in the respiratory tract imposes higher requirements on HA and NA functions, changes in these surface glycoproteins (HA and NA) are likely to decrease influenza virus toxicity and transmissibility *in vivo*.

Sialic acid is always the terminal sugar in a chain that is attached to a protein embedded in the plasma membrane of a host cell (Fig. 3) [3, 20, 25, 33, 42]. Sialic acid is an acidic saccharide containing a carboxyl group ( $-\text{COO}^-$ ). Therefore, it interacts with positive charges of amino acids in the active site of NA.

### $\alpha$ -Ketosidic Bond [( $\alpha$ 2-3) Linkage vs. (A2-6) Linkage]

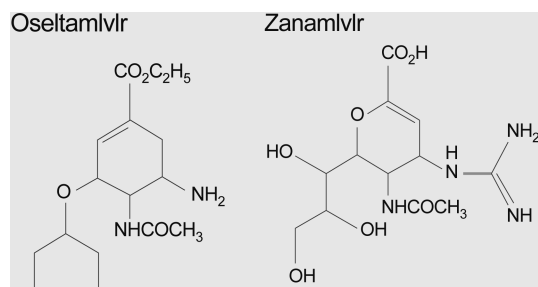
Sialic acids are required to attach all influenza A virus strains to cells. However, there are a number of chemically different linked sialic acids, such as sialic acid ( $\alpha$ 2-3) or sialic acid ( $\alpha$ 2-6) linked forms. The influenza virus strains vary in their affinity for the type of linkages. These differences may determine which animal species can be infected. Fig. 4 shows that sialic acid is linked to the galactose by ( $\alpha$ 2-3) linkage. The ( $\alpha$ 2-3) linkage is a kind of glycosidic bond between the carbon atom at position 2 of the sialic acid and the carbon atom at position 3 of the galactose.

Avian influenza virus strains preferentially bind to sialic acids attached to galactose *via* an ( $\alpha$ 2-3) linkage. This ( $\alpha$ 2-3) linkage is the major sialic acid on epithelial cells of the duct gut. In contrast, human influenza virus strains preferentially attach to sialic acids attached to galactose by an ( $\alpha$ 2-6) linkage. This is the major type of sialic acid



**Fig. 3.** Schematic diagram of the plasma membrane (left).

The spheres are saccharides attached to proteins (glycoprotein). The arrows indicate the sialic acids attached to terminal positions of glycoproteins. The structure of sialic acid ( $\alpha$ 2-3) galactose is presented on the right [33].



**Fig. 4.** Structures of Tamiflu (oseltamivir) and Relenza (zanamivir) [14].

present on human respiratory epithelial cells. The ( $\alpha$ 2-3) linked sialic acids are found on ciliated epithelial cells, which are a minor population within the human respiratory tract, and also on some epithelial cells in the lower tract [3, 25, 28, 32, 34, 42].

Epithelial cells of the pig trachea produce both ( $\alpha$ 2-3) and ( $\alpha$ 2-6) linked sialic acids. This is believed to be the reason why pigs can be infected with both avian and human influenza virus strains. The swine serves as a “mixing vessel” for the emergence of new viruses. The receptor specificities related to the types of linkages have implications for human infection with swine and/or avian influenza virus strains. The highly pathogenic avian H5N1 influenza viruses undergo limited replication in the human respiratory tract because of the presence of some cells with ( $\alpha$ 2-3) linked sialic acids [2, 9, 26]

Efficient human-to-human transmission requires that the swine and avian viruses recognize sialic acids with ( $\alpha$ 2-6) linkages. The results of studies of early influenza virus isolates from the 1918, 1957, and 1968 pandemics suggested that these viruses preferentially recognized ( $\alpha$ 2-6) linked sialic acids [9].

### How Can Tamiflu Act to Protect from the Invasion of Swine Flu Viruses?

As new virions are produced by budding, they would immediately bind to sialic acid receptors on the cell surface. The neuraminidase cleaves sialic acids from the surface of the cell, so that newly formed virions can be released. This requirement explains how the neuraminidase inhibitors such as Tamiflu and Relenza function; they prevent cleavage of sialic acid from the cell surface. In the presence of neuraminidase inhibitors, virions bud from the cell surface, but they remain firmly attached, not released. Therefore, Tamiflu and Relenza block infection by preventing the spread of newly synthesized virus particles to other cells. The sialic acid monomer as antiviral drug becomes a rapid enzymatic breakdown *in vivo*. Thus, a high concentration of monomeric sialic acid must be delivered to inhibit vital infection, and these concentrations are toxic [11]. Free sialic acid monomers cannot be used as an antiviral drug

*in vivo*. The enzyme, neuraminidase has also been targeted in structure-based enzyme inhibitor designs. The antiviral drugs Relenza and Tamiflu are mimics of the transition state of the sialic acid cleavage by neuraminidase.

### Tamiflu vs. M2 Inhibitor

Most viruses use a receptor that binds to a cellular surface component such as M2, HA, and NA as a targeting mechanism. Therefore, blocking the initial interaction of a virus with these host cell receptors can prevent viral infection. The binding between virus and receptor of a host cell may be inhibited by an extracellular therapeutic agent that resembles the surface-binding component of the host.

There are two classes of anti-influenza virus drugs targeting either the M2 or NA proteins for influenza treatment. The two M2 blockers, amantadine and its analog rimantadine, showed a lack of inhibitory effects against the influenza B viruses in which M2 protein does not exist. They also have many side effects and resistance problems [9, 12, 14, 22, 46].

### Tamiflu vs. Relenza

Unlike amantadine and rimantadine that target the M2 protein of influenza A viruses, Tamiflu and Relenza inhibit replication of both influenza A and B viruses. Tamiflu and Relenza are sugar analogs (Fig. 4). They inhibit the viral sialidase (neuraminidase) by competing with the host cell’s sialic acid in the structure of sialic acid( $\alpha$ 2-6)galactose( $\beta$ 1-4)glucose for binding. This prevents the release of viruses from the infected cell and causes viral particles to aggregate, both of which block another cycle of infection.

Relenza is delivered by inhalation because of its low oral bioavailability, whereas Tamiflu is administered by mouth. The highly polar, zwitterionic nature of Relenza results in low oral bioavailability [38]. Tamiflu was synthesized using a cyclohexene ring and replacement of a polar glycerol with lipophilic side chains (Fig. 4) [11, 12, 14, 22, 23, 39, 44–47].

### Future Works

Tamiflu and Relenza are currently the only two anti-influenza drugs targeting the NA of human influenza virus. Because of several reports of the emergence of drug resistance, the development of new potent anti-influenza inhibitors is urgently needed. The inhibitors with positively charged groups have potential as antiviral therapeutics and the strain specificity of these inhibitors must be resolved.

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