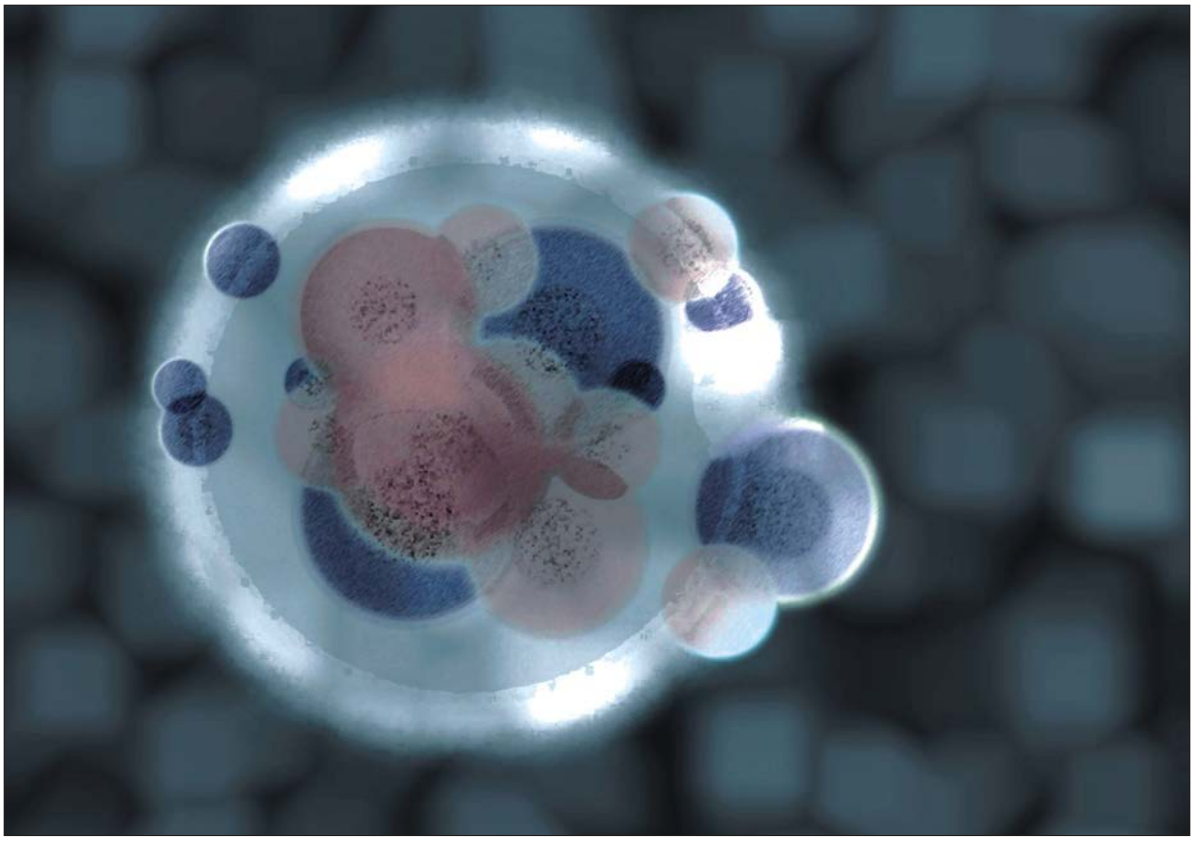

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Structure of the Sialic Acid Binding Site in Influenza A Virus: Hemagglutinin

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Abstract: Influenza A viruses have several key features accounted for public health concerns and for a number of historic pandemics. The genome of the virus is segmented RNA with negative polarity which attributed significantly to the stability of the virus during replication. Furthermore, the virus has two major variable surface glycoproteins Hemagglutinin (HA) and Neuraminidase (NA), which affected by antigenic changes (drift and shift) and recombination leading to recurrent outbreaks of influenza viruses. HA cleavage, intracellularly by host proteases into HA_1 and HA_2 subunits is one of the determinants of tropism and pathogenicity, is characteristics for binding of the virus to cellular sialic acid-containing receptors. Because of the importance of HA in viral infection and as inducer of neutralizing antibodies, this review will focus on the molecular basis of HA-binding site. Molecular and biologically approaches elucidating HA-binding site will advance methods ranging from vaccine development to the designing effective inhibitors.

Key words: Influenza virus, hemagglutinin, sialic acid

INTRODUCTION

Influenz A viruses are an RNA viruses belonging to the family of *Orthomyxovirus* family (Palese and Young, 1982). The Influenza virus particles are spherical with about 1000 A in diameter and consist of eight single-stranded RNA segments of negative polarity (Kash *et al*., 2006; Skehel and Wiley, 1995). Based on the antigenic differences of the nucleoprotein (NP) and matrix (M1) proteins, influenza viruses are classified into three different types A, B and C (Palese and Young, 1982; Tobita, 1997). Furthermore, they are also classified into subtypes based on differences of envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). There are fifteen HA and nine NA (HA-15 and NA-9) subtype have been identified up-to-date (Reid and Taubenberger, 2003; Tobita, 1997; Webster *et al*., 1992). Complete role of the HA in viral infection remains unclear. Infection by the virus begins when viral HA binds to the target cell surface receptors containing sialic acid through receptor mediated-endocytosis. In the other hand, NA is responsible for viral release from infected cells (Air and Laver, 1989; Air *et al*., 1989; Kelm *et al*., 1992; Rogers *et al*., 1983).

Influenza virus (H1, H2 and H3 subtype) belongs to influenza A viruses and have been circulating primarily in human. In addition to human, H3 subtype is also present in other mammalian species such as pigs, horse, etc. (Steinhauer, 1999). Influenza viruses are not considered highly pathogenic. However, rapid evolution due to antigenic changes of surface glycoproteins causes the recurrence of virus and accounted for major public health concerns particularly Influenza A viruses (Kash *et al*., 2006; Zambon, 1999; Zambon, 2001). Pandemic diseases of influenza A viruses have occurred several time killing millions people world wide; for example: Spanish flu in 1918 (H1N1 subtype), the Asian flu in 1957 (H2N2 subtype) and the Hong Kong flu in 1968 (H3N2 subtype) (Hsieh *et al*., 2006; Kollerova and Betakova, 2006; Reid and Taubenberger, 2003; Turkulov and Madle-Samardzija, 2000). Recently, a new life threat of avian influenza A virus (H5N1 subtype), has been transmitted from bird to human causing fatal disease (Hayden, 2006; Kash *et al*., 2006; Olsen *et al*., 2006; Webster *et al*., 2006). More attention, effective vaccine and antiviral treatment are needed to prevent transmission of the virus from human to human which, if occurred, may become source of global pandemics.

Crystal structure and atomic model determination of the HA (H3 serotype) of influenza A virus ascribe location and molecular rearrangement of the receptor binding site (Wiley and Skehel, 1987). In addition, proposed location of HA-antibody binding site and specificity of the binding is influenced by single-amino acid substitution (Rogers *et al*., 1983; Wiley and Skehel, 1987). Binding site of influenza A virus HA (H3 subtype) will be reviewed in details in this study.

HA is a homotrimer glycoprotein, which occurs as mushroom-like shapes projecting from the surface of the viral particles and extends 135 Å from the membrane (Weis *et al*., 1988; Wilson *et al*., 1981) (Fig. 1). The molecular size of the HA protein is approximately 75 kDa, which consists of HA_1 and HA_2 subunits linked by a disulphide bond. The top of the HA molecule contains the HA_1 part as the globular domain, which consists of antiparallel β-sheet. This domain is an important region

in this protein, which has a receptor-binding site. HA is the major component of the virus that is recognized by the human immune system (Marshall-Clarke *et al*., 2006). The stem region consists of HA_1 and HA_2 subunits forming a triple stranded coiled-coil of α-helices that projects 76Å from the membrane. In the course of translation, HA is modified by addition of several oligosacchrides. HA precursor (HA0) is first synthesized and assembled at the surface of the viral particles as an inactive form. HA0 precursor is cleaved intracellularly by host proteases at conserved arginine 329 to form HA_1 and HA_2 subunits (Chen *et al*., 1998). Efficient infection requires cleavage of the HA, however some influenza viruses, such as 1918 (H1N1) virus, can replicate in the cell culture without the need for trypsin in the cell media, as required for replication of modern viruses. It is not yet known whether this means the virus can replicate without cleavage or if the virus has the ability to cleave itself. Therefore, HA cleavage is an essential step for HA function and activated HA binds to the cell receptors, facilitating viral infection.

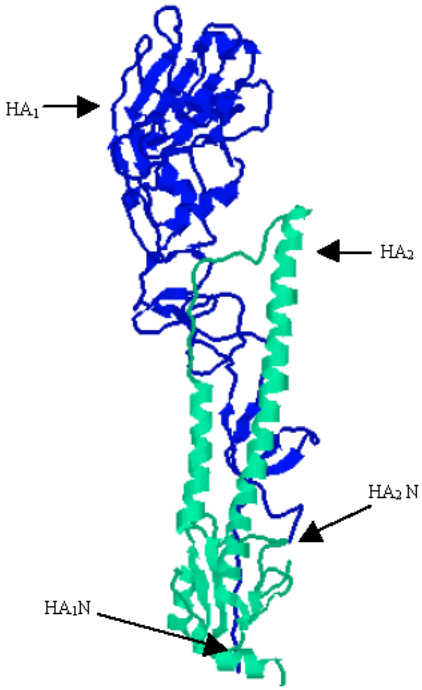

Fig. 1: Ribbon diagram of the influenza virus hemagglutinin monomer. The top consists of the HA_1 globular domain (represented in blue color) and the stem, which contains both HA_1 and HA_2 domain (HA_2represented in green color). It shows also the amino terminal residues for both HA_1 and HA_2 domains

Sialic acids, derivatives of neuraminic acid, are components of oligosaccharide chains of glycoproteins and glycolipids. The HA_1 globular domain has a sialic acid binding site located in the distal top of the molecule and it is defined as a pocket of amino acids that are highly conserved among influenza virus strains (Rohm *et al*., 1996) (Fig. 2). Moreover, the three dimensional structure of the sialic acid binding site was crystallographically determined (Skehel and Wiley, 2000; Weis *et al*., 1988; Wiley and Skehel, 1987). In this review the structure of the sialic acid binding site of influenza HA will be evaluated at the molecular level. Furthermore, amino acids that are conserved in almost all influenza virus strains that are involved in the binding activity and effects of mutations and inhibitors on the binding affinity will be examined.

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H1N1   SENGTCYPGE FIDYEELREQ LSSISSFEKF EIFPKASSWP NHETTKGVTA
H6N5   AQNGICYPGV LNEVEELKAL IGSGERVERF EMFPK.STWT GVDTSSGVTR
H12N6  EMEGVCYPGS IENQEELRSL FSSIKKYERV KMFDF.TKWN ..VTYTGTSK
H2N2   .AFSNCYPYD VPDYASLRSL VASSGTLEFI TEG...FTWT G.VIQNGGSN
H3N8   .AFSNCYPYD IPDYASLRSL VASSGTLEFI TEG...FTWT G.VTQNGGSS
H7N7   .GNDVCYPGK FVNEEALRQI LRESGGINKE TTG...FTYS G.IRTNGVTS

H1N1   ACSYSGAS.S FYRNLLWITK ..KGTSYPKL SKSYTNNKGK EVLVLWGVHH
H6N5   ACPYNSGS.S FYRNLLWIIK .TKSAAYSVI KGAYNNTGNQ PILYFWGVHH
H12N6  ACNNTSNQGS FYRSMRWLTL ..KSGQFPVQ TDEYKNTRDS DIVFTWAIHH
H2N2   ACKRGPGS.G FFSRLNWLTK S..GSTYPVL NVTMPNNDNF DKLYIWGIHH
H3N8   ACKRGPAN.G FFSRLNWLTK S..ESAYPVL NVTMPNNDNF DKLYIWGVHH
H7N7   ACRR.LGS.S FYAEMKWLLS NTDNAAFPQM TKSYKNTRNE PALIVWGIHH

H1N1   PPSVSEQQSL YQNADAYVSV GSSKYNRRFA PEIAARPEVR GQAGRMNYYW
H6N5   PPDTNEQNTL YGSGDRYVRM GTESMNFAKS PEIAARPAVN GQRGRIDYYW
H12N6  PPTSDEQVKL YKNPDTLSSV TTVEINRSFK PNIGPRPLVR GQQGRMDYYW
H2N2   PSTNQEQTSL YVQASGRVTV STRRSQQTII PNIGSRPWVR GLSSRISIYW
H3N8   PSTNQEQTNL YVQASGRVTV STRRSQQTII PNIGSRPWVR GQPGRISIYW
H7N7   SGSATEQTKL YGSGNKLITV GSSNYQQSFV PSPGARPQVN GQSGRIDFHW
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Fig. 2: Amino acid sequence alignment of HA_1 of several influenza virus subtypes. Conserved amino acid residues, which form the binding site, are represented in red color

Three-dimensional structure of the receptor-binding site

Structure of unbound receptor-binding site: The structure of the receptor-binding site of influenza virus HA without silaic acid binding was studied crystallographically revealing that the site is defined as a pocket localized in the top subunit of the trimeric HA glycoprotein (Weis *et al*., 1988). This pocket contains several conserved amino acids that are present in most influenza virus strains (Fig. 2). As shown in Fig. 3, this pocket is a depression surrounded primarily by a α-helix strand at the back of this site that is short and has different amino acid residues contributing to the binding site (Fig. 3A and B). These amino acid residues project toward the site and interact through hydrogen bonds with the bottom amino acid residues of the site. The left side of the binding site is composed of amino acid side chains forming β-strands. The right side also consists of amino acid residues forming the right edge of this site that interact primarily with the receptors (Fig. 3B). All of these amino acid residues, as well as Tyr-98 and His-183, project from the back of the site forming the basic structure of this site. Crystallographic study of unbound sialic acid receptors suggested that Tyr-98, His-183 and Trp-153 interact by hydrogen bond interaction forming the base surface of the receptor-binding site (Fig. 3C).

Structure of the receptor-binding site and amino acid residues involved in the binding: The structure of the binding site with its receptor sialic acid was determined using X-ray crystallography (Weis *et al*., 1988). Influenza virus strain X31 was treated with bromelain to produce a soluble HA trimer (BHA), which contains all amino acid residues of HA_1 and the first 175 amino acid resides of HA_2. BHA-NeuAc complex was crystallized and the three dimensional structure was determined. All of the amino acid residues, which are involved in binding with sialic acid receptor (NeuAc), are from the HA_1 chain of the HA molecule. Examination of the BHA-NeuAc complex structure confirmed that the sialic acid binding site is a part of the large pocket located at the top of the HA molecule. The back of the pocket as shown in Fig. 4 is formed by Ser-228 from the left and a short α-helix strand containing Glu-190, Tyr-195 and Leu-194 projecting into the site. The right side of the pocket is composed of Gly-135, Ser-136 and Asn-137. The surface of the site is formed by Tyr-98, Trp-153, Thr-155 and His-183. All these amino acid residues are in direct contact either with receptors or with each other by hydrogen bond and/or by van der Waals interactions. His-183, Tyr-98 and Glu-190 are polar side chains, which are in position in the binding site to form hydrogen bonds with the receptor. Trp-153, Leu-194 and Leu-226 are non-polar side chains that interact with hydrophobic regions of the sialic acid receptor by van der Waals interaction. Also important in this pocket is the orientation of the pyranose ring of the sialic acid, which is in position to form hydrogen bonds with conserved amino acid residues, playing a role in the three-dimensional fit and binding in this site. C8-OH and C9-OH of the glycerol side chain, oxygen carboxylate and acetamido nitrogen of the sialic acid form hydrogen bonds with Tyr-98, Glu-190, Ser-136, Asn-137 and Gly-135, respectively (Fig. 4).

Evaluation of the active atoms of the sialic acid involved in the binding: Synthetic sialic acid polymers were studied to demonstrate the receptor atoms that play an important role in the binding (Kelm *et al*., 1992). This was achieved by evaluating the binding affinity of multiple hemagglutinin-sialic acid interactions. Virus adsorption inhibition assay was used to evaluate several sialic acid polymers as inhibitors of influenza virus mediated hemagglutination. Carbon atoms of the pyranose ring were subjected to modifications. The changes were either in the side chain of the glycerol, the C5 of the *N-acetyl* group, or the C1 carboxy group of the sialic acid (Table 1). Removing C8 and C9 of the glycerol side chain dramatically decreased their activity as inhibitors (Table 1A1 and Fig. 5B). Substituting the hydroxyl of C7, C8, or C9 with a deoxy group caused significant change in

Table 1: Effect of functional modification on the binding of sialic acid analogue to HA

Sialic acid analogues	50% inhibition mM	Relative inhibition potency
A) Potential inhibition effect of sialic acid polymers with modification at glycerol side chain		
1) Benzyl-α-Neu5Ac	8.0	1.0
Benzyl-α-Neu5Ac-C7-aldehyde	100	<0.08
2) 2-d-2Heq-Neu5Ac	4.8	1.0
2,7-d_2-$2H_{eq}$-Neu5Ac	25	<0.14
2,8-d_2-$2H_{eq}$-Neu5Ac	14	0.27
3) Methyl- -Neu5Ac	3.5	1.0
Methyl-α-9-d-Neu5Ac	3.5	1.0
Methyl-α-9-cyano-9-d-Neu5Ac	20	<0.17
B) Potential inhibition effect of sialic acid polymers with modification at the N- acetyl group		
Methyl-α-Neu5Ac	3.5	1.0
Methyl-α-KDN	50	<0.07
Methyl-α-5-azido-5-d-KDN	50	<0.07
Methyl-α-5-amino-5d-KDN	22	<0.36
C) Potential inhibition effect of sialic acid polymers with modification at C2		
Methyl-α-Neu5Ac	3.5	1.0
(4-Isothiocyano) benzyl-α-Neu5Ac	1.7	2.0
2-Naphthyl-α-Neu5Ac	5.5	1.2

Binding of each ligand analogues were examined using virus adsorption assay. Inhibition of the reference was used to express the relative inhibitory potencies. (a) Potential inhibition effect of sialic acid polymers with modification at glycerol side chain. (b) Potential inhibition effect of sialic acid polymers with modification at the N- acetyl group. (c) Potential inhibition effect of sialic acid polymers with modification at C2. Modified table was taken from Kelm *et al*. (1992)

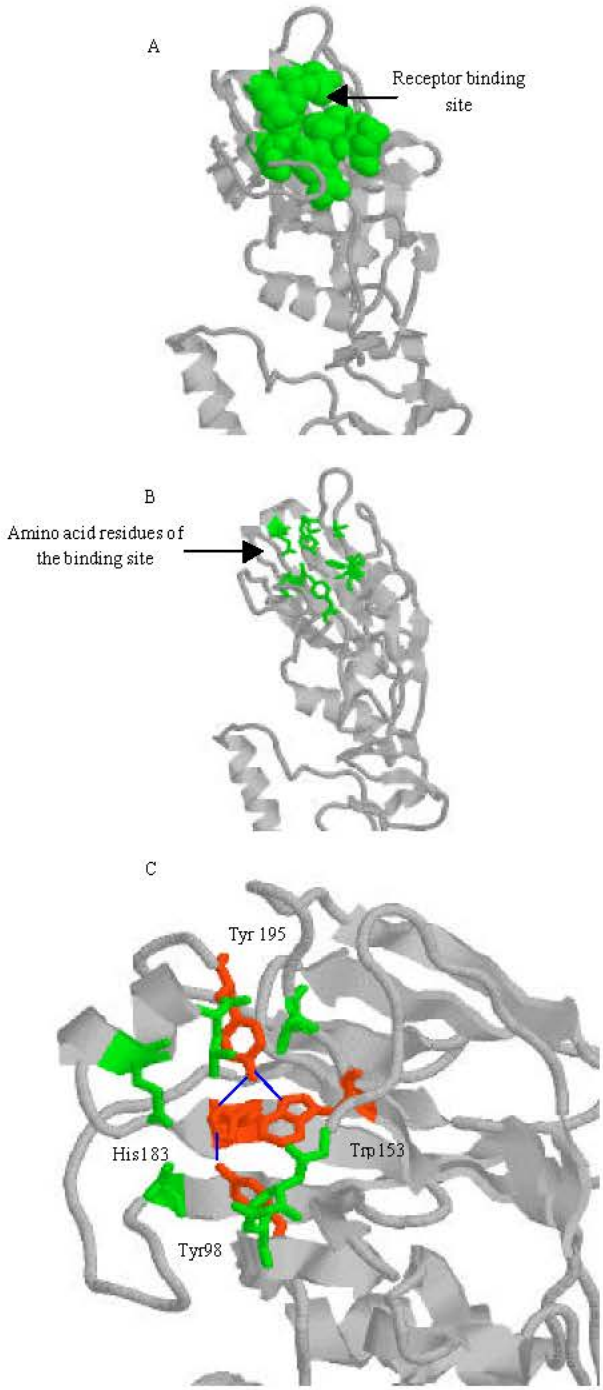

Fig. 3: Location and Schematic diagram of the unbound receptor-binding site. (A). Space-fill model of unbound receptor-binding site. It shows conserved amino acid residues represented in green forming the binding site at the top of HA_1 subunit. (B). Schematic diagram of unbound receptor-binding site. The pocket as shown is a depression surrounded by α-helix and β-strands of conserved amino acid residues represented in green forming the base of the binding site. (C). It shows conserved amino acids residues, Tyr-98, His-183, Trp-153 and Tyr-195 represented in red color, interacting with each other through hydrogen bond interaction forming the base of the pocket

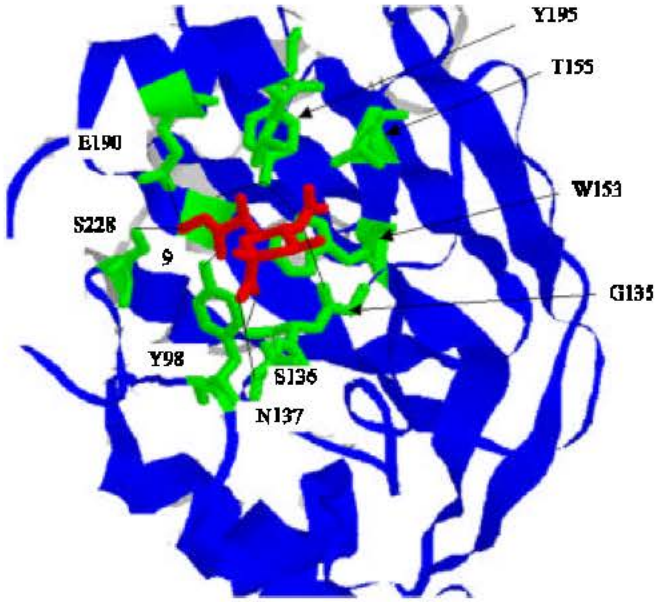

Fig. 4: Diagram of the receptor-binding site. It shows the possible hydrogen bond interaction represented in black dots lines between amino acid residues of HA and sialic acid. Sialic acid represented in red, HA_1 represented in blue and amino acids residues involved in the binding shown in green

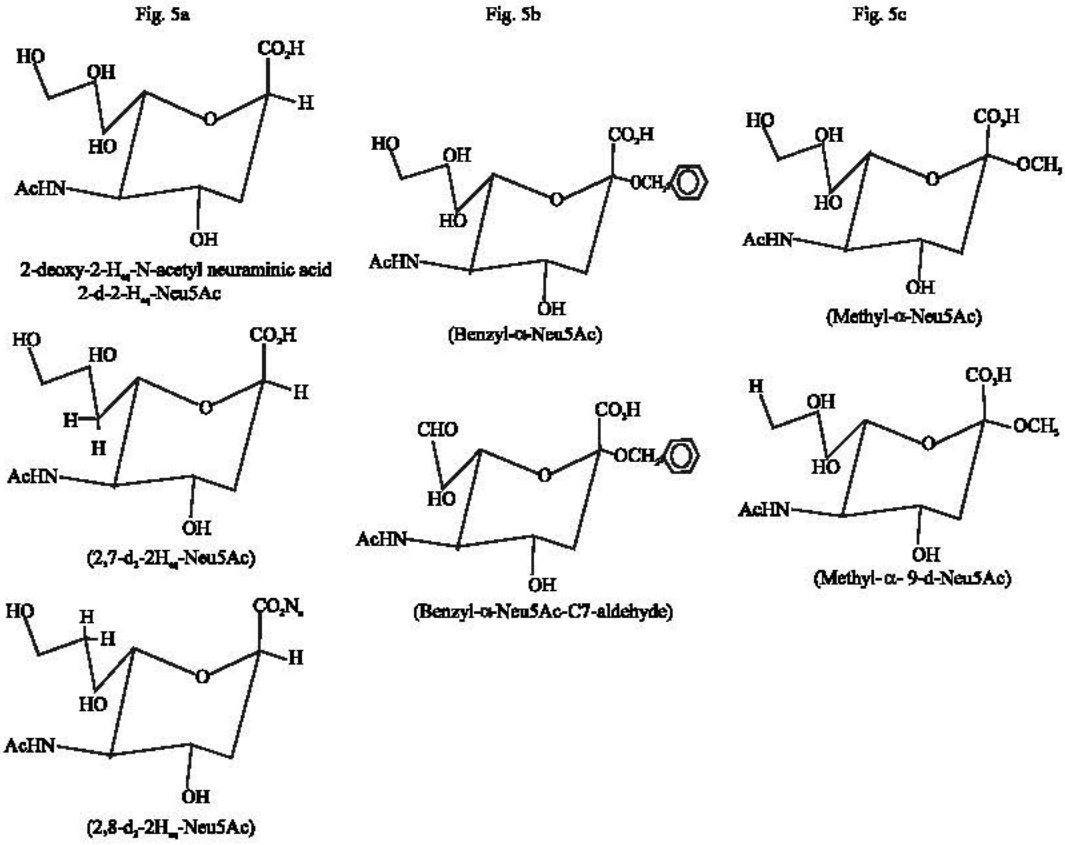

Fig. 5: Structural formulas for the Neu5Ac analogues studied. Structure of the sialic acid polymers with modification at glycerol side chain, which used to study potential inhibition effect, is shown in A; analogue with modification at C8 and C9, B; polymers with shortened glycerol side chain and C; polymer with modification at C9. The symbol AcHN represents the acetamido side chain group of the sialic acid

their hemagglutination inhibition activities (Table 1A2 and Fig. 5A). Substitution of the-OH of C7 resulted in a higher affinity for binding HA than C8, which indicated its importance for interaction with conserved amino acid in the receptor-binding site. Replacing the C9-OH with C9-H had no effect in the hydrogen binding interaction in the binding site (Table 1A3 and Fig. 5C). It was demonstrated also that modification at C9 using bulky molecule residues decreased sialic acid analogue inhibition affinity. These residues are expected to have an effect due to their large size in the vicinity of the binding site. Inhibitory effects of the *N-acetyl* group at the C5 carbon were also demonstrated by substituting with OH, azido, or amino groups (Table 1B). All of these modifications at the *N-acetyl* group had inhibitory effects illustrating their role in binding affinity. Potential inhibition on the C2 constituent indicated two compounds that can be used as candidates for inhibiting the binding: 1) (4-Isothiocyano) benzyl-α-Neu5Ac and 2) 2-Naphthyl-α-Neu5Ac (Table 1C). This study showed the importance of the glycerol side chain, particularly C7 and C8, in the binding of the sialic acid to the HA. C9 binding affinity contrasts with crystallographic study in which it describes the H-bond interaction of this atom with Glu-190 or Ser-228. The acetamido side chain and carboxyl group are also critical for binding with HA.

Mutations of the receptor-binding site: The first mutation of the receptor-binding site of influenza virus showed that single amino acid substitution of hemagglutinin gene alters the binding specificity (Rogers *et al*., 1983). Influenza virus strains could be distinguished based on their ability to agglutinate erythrocyte derivatives. Influenza virus strain X-31/HS that has Gln-226 changed for Leu-226, agglutinates erythrocytes containing a NeuAcα (2→3) Gal linkage of the sialic acid receptor. In contrast, the X-31 strain has the normal Leu-226 of the HA_1 gene and it preferentially agglutinates erythrocyte containing a NeuAcα (2→6) Gal linkage. It was not demonstrated whether the NeuAcα (2→3) or NeuAcα (2→ 6) linkage orientations and their atoms play a role in the binding. This finding was further investigated by crystallographic studies, which confirmed that the sialic acid binding-site is a pocket located in the top of the HA molecule (Weis *et al*., 1988). In this study, the HA of wild type virus, which has Leu-226 and the HA of mutant virus, which has Gln-226, were complexed with receptor sialyllactose and crystallized. Comparison of the electron density maps at 3 Å resolution demonstrated that wild type and mutant viruses bound similarly to the receptor-binding site. In both cases, the orientation of the pyranose ring of the silaic acid was similar. The functional atoms of the sialic acid interacted with conserved amino acid in the binding site through hydrogen bonding of both wild type and mutant type viruses. The position of the amino nitrogen and carbonyl oxygen of the Gln-226 mutant is out of plane with Leu-226 methyl groups when superimposed at different electron density. It was also described that the binding affinity of the mutant was decreased compared to the wild type suggesting that structural changes occurred because of the substitutions and not because of sialic acid linkage. This observation describes the specificity of the binding at the amino acid level.

Subsequent study has also shown that other mutations in the sialic acid binding site can alter the affinity of influenza virus binding (Martin *et al*., 1998). Mutant hemagglutinins were generated to probe the role of designed mutations in the receptor-binding site (Table 2). This technique required the generation of mutant viruses that are expressed in vaccinia virus using reverse genetics systems. The ability of constructed mutant viruses to agglutinate human erythrocytes, which have sialic acid, was examined. Expression of HA of Trp-153 mutant failed, an amino acid residue located in the surface of the binding site (Fig. 3C). This observation suggests that mutation at this position may affect the folding and overall structure of the binding site. Hemagglutination activities of mutants Tyr-98, His-183 and Leu-194, other important residues in the binding site, were significantly decreased (Table 2A). These residues are essential for binding and they form the network contact in the binding site. Tyr-98 forms a hydrogen contact with His-183 and C8-OH of the sialic acid receptor. His-183 and Leu-194 form hydrophobic interactions with C7 and C9 of the glycerol side chain of the sialic acid receptor. Mutations at Ser-136, Tyr-195 and Gly-225, right side, α-helix and left side of the binding site, respectively, had very little impact on the binding activity as demonstrated by their average hemagglutination activities compared with wild type used in this study.

Table 2: Hemagglutination and hemagglutination inhibition assays of the wild type and mutant vimses

Mutation	A) Human RBC binding	B) Inhibition of HRBC binding
WT	100	99
Y98F	5	(-)
S136	30	74
H183	12	(-)
L194	3	(-)
Y195	36	94
G225D	53	96
G225R	136	57
L226P	42	30

A) Average percentage of human erythrocyte binding (% of wild type) was reported. B) Percentage of hemagglutination inhibition test of human RBC binding by 10% horse semm was read. (-); Specificity of the binding mutants were not determined due to their decreased agglutination activity. Modified table was taken from Martin *et al*. (1998)

Also in this study, inhibition assay was used to evaluate binding specificity of the mutants to erythrocytes in the presence of horse serum, which is rich in sialic acids in α-2→6 linkage (Table 2). Binding specificity of mutants Tyr-98, His-183 and Leu-194 were not determined due to their decreased agglutination activity (Table 2B). Mutants Tyr-195 and Gly-225D were completely inhibited. Mutants Gly-225R and Leu-226P were resistant for the inhibition, demonstrating the role of these residues in the binding, particularly the Leu-226P, which was described before, to agglutinate erythrocytes containing sialic acids in (α-2→3) linkage.

Inhibition of receptor binding of the influenza virus HA by neutralizing antibody: Blocking binding of complex carbohydrates with terminal sialic acids and their bindingsite on HA is an important step for neutralizing virus infectivity. Antibody directed against the receptor-binding site is essential for neutralization. Several studies have shown that some influenza virus strains were able to escape from neutralization, suggesting that mutations occur on the antibody-binding site. In a crystallographic study by Bizebard *et al.* (1995), a complex of HC19 Fab antibody fragments with HA of the influenza virus X31 strain was determined. In this study, the receptor-binding site was covered by HC19 antibody and all conserved residues of the receptor-binding site were blocked. Mutants that allow the virus to escape from neutralization by HC19 antibody were mapped to be on the rim of the receptor-binding site. Some structural changes were detected upon HC19 Fab-HA complex formation allowing new hydrogen bonds to form.

In another study, the importance of inhibition of the receptor-binding site for virus neutralization was confirmed (Fleury *et al.*, 1999). Here, the X-ray structure of the HC45-HA complex was evaluated and compared with the previously determined HC19-HA complex. The HC45 Fab antibody fragment bound outside of the HA receptor-binding site and the HC45-HA complex structure was not affected by this binding (Fig. 6). It was indicated that neutralization by these antibodies caused minor structural changes to HA receptor-binding site. Also, the HC45-IgG affinity, in comparison with HC19, was higher for HA but its avidity to the virus was weaker. Although the avidities of both Fab fragments were similar, the neutralization efficiencies were different, indicating the

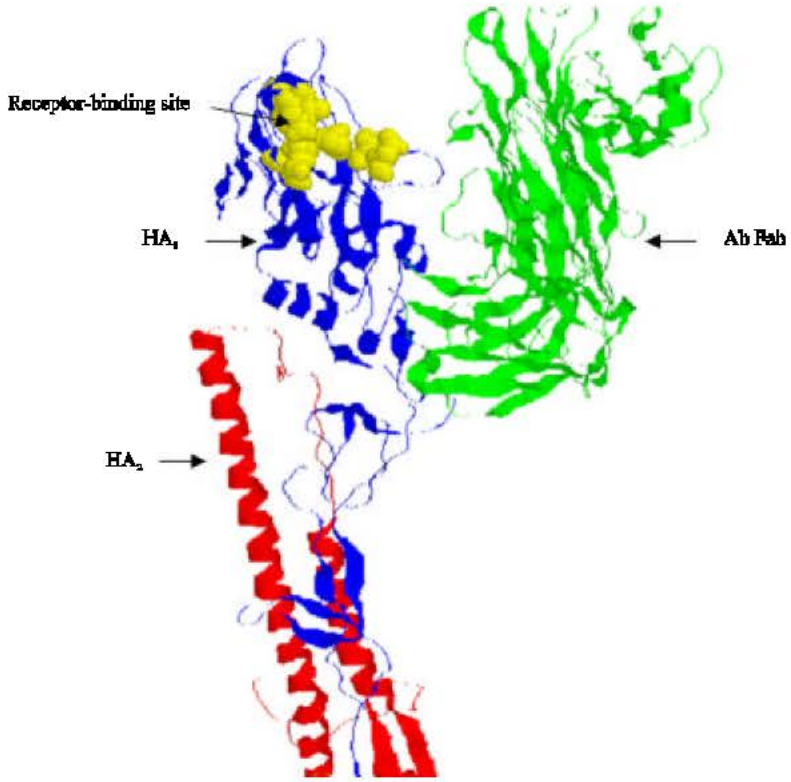

Fig. 6: Antibody-binding site of the influenza virus X31 strain HA. The antibody Fab fragment of HA-HC45 Fab complex is shown in green, receptor binding site represented in yellow, HA_1 is shown in blue and HA_2 is shown in red

importance of inhibiting the receptor-binding site for neutralization. It was concluded that HC19 neutralizes the virus infectivity more efficiently than HC45.

CONCLUSION

Since the RNA genome of influenza viruses is segmented, the virus undergoes antigenic drift and antigenic shift (reassortment) which can create new pandemic strains produced by new viral progeny. This is mainly demonstrated why recent outbreaks of influenza A virus subtype H5N1 occurred! Reassorted virus was probably generated by co-infection of pigs by human and avian influenza viruses (Reid and Taubenberger, 2003; Scholtissek, 1995). To prevent global outbreaks and human fatalities caused by influenza virus infection, it is indispensable to prepare universal safe vaccines.

There are growing concerns regarding influenza virus infection in particular when avian influenza (H5N1) has spread world wide causing fatal disease. Therefore, a accurate view at the molecular level of how the virus initiates infection should not only enhance our understanding of virus entry but also it may encourage and shed alight for design inhibitors design to avert virus infection.

The first study of the structure of influenza virus hemagglutinin revealed that conserved amino acids, Tyr-98, His-183, Glu-190, Trp-153 and Leu-194 located at the top of the HA_1 domain have a potential for being the active site for binding with cell-receptors (Wilson *et al*., 1981). This important finding was followed by several studies to identify the properties of the active site and possible ligands that interact in this site. The identification of the binding site for sialic acid as a pocket at the distal end of the HA molecule was confirmed by crystallographic and point mutation studies (Martin *et al*., 1998; Rogers *et al*., 1983; Weis *et al*., 1988).

The receptor-binding site was determined to be a shallow surface pocket containing conserved amino acid residues. These amino acid residues are located at the top of the HA molecule, in the HA_1 globular domain. The structure and folding pattern of the HA_1 domain, which consists of α-helix and α-strands and its location at the distal end of the HA molecule influences the binding with sialic acid receptors. The binding affinity depends on the influenza virus strains and the nature of the linkage of the sialic acid containing oligosaccharides. Ser-228, Glu-190, Tyr-195, Leu-194, Gly-135, Ser-136, Asn-137, Tyr-98, Trp-153, Thr-155 and His-183 are the most important amino acids forming the structure of the binding site. It was demonstrated that mutation of Trp-153 may cause conformational change in the structure of the HA.

The results obtained from these studies enhance our knowledge of how the sialic acids interact in the binding site and atoms that are involved in the interaction. The base of the binding site was mainly supported by the interaction of Tyr-98, His-183, Trp-153 and Tyr-195 in hydrogen bond interaction (Fig. 3C). However, there are other amino acid residues, which are involved in supporting and stabilizing the binding site, are not in direct contact with binding site. Evidence for this observation derived from crystallographic study where the sialic acid bind to the pocket and make hydrogen bond interaction with several amino acid residues (Fig. 4). His-183, Trp-153 and Tyr-195 are still in hydrogen bond interaction with each other stabilizing the structure of binding site but when sialic acid binds to Tyr-98, Glu-190, Ser-228, Asn-137 and Ser136 the overall structure was not affected suggesting a possible of other amino acid residues play role in structure and stability of binding site.

In the present study, interesting results were observed confirming the importance of sialic acid side chains and their influence in the binding. One observation that is not convincing is the substitution of C9-OH with C9-H where it was demonstrated that replacing the C9-OH with C9-H had no effect in the hydrogen binding interaction in the binding site (Table 1A3 and Fig. 5C). Crystallographic structure study of the binding site with this analogue may reveal important property of this pocket, which could be utilized for designing inhibitors (which will be discussed latter in this review).

It was demonstrated that mutation at position 226 of HA might alter the binding specificity with its receptors. Even though this observation is very interesting and described the specificity of the binding at the amino acids level! Therefore, the binding affinity between wild type and mutant viruses was different because of the substitution and not because of the sialic acids linkage. First, it was not demonstrated whether the linkage NeuAc(2-3) or NeuAc(2-6) orientation and their atoms plays a role in the binding. Second, several studies suggested that the amino acid residue at position 226 does not play a role in binding with sialic acid (Fig. 4).

Also, it was demonstrated that mutation of Trp-153 may cause conformational changes in the structure of the HA. This was not the only plausible explanation for this finding. Therefore, failure of expression of Trp-153 virus mutant may have been due to the reverse-genetic system used to generate this virus mutant. Another approach could be utilized to overcome this problem, which is not attempted in this study.

In spite of the development of new vaccines and antiviral drugs, influenza remains a serious life threat. Extensive studies in previous years of the biochemical

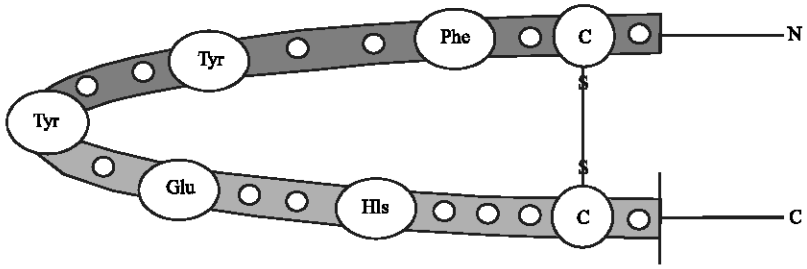

Fig. 7: Schematic drawing of the single side chain of the peptide inhibitor. Single peptides linked by disulphide bonds and candidate amino acid residues that should interact in the binding site are represented along the peptide side chain

structure of influenza virus advances our knowledge, particularly by the use of high-resolution X-ray crystallographic techniques. These studies shed some insight into the life cycle of the influenza virus and possible control of the virus infection at any stage.

Synthesis of smaller, nonimunogenic peptides that mimic the receptor-binding site and inhibit or at least reduce the spread of the viral infection in the body could be utilized for future research. One approach to block the binding function of HA is to design effective inhibitors of the HA binding site. As described above, the sialic acid binding site (the biological receptors for influenza viruses) was identified on HA, containing amino acid residues conserved among all influenza viruses. Recombinant single-chain peptides containing approximately 20 amino acid residues representing the functional atoms of sialic acid that play a role in binding to HA will be constructed (Fig. 7). The properties of the engineered circular peptide that is linked at C-terminal and N-terminal by disulphide bonding should have amino acids residues actively similar to the functional group of sialic acid which forms a direct H-bond with conserved amino acid residues in the binding site. The proposed carton diagram (Fig. 7) is only to illustrate the position of functional group of the amino acid residues. As shown in Fig. 7, the phenylalanine amino acid residue should be in positioned to make hydrophobic interaction with Trp-153 in the binding site. Two tyrosine amino acid residues, which have a hydroxyl group at the side chain, will be synthesized in position to make H-contact with the Glu-190 and Ser-228 amino acid residues of HA. Glutamic acid residue will be constructed in position to interact with Tyr-98 through H-bond interaction. Histidine residue of the synthetic peptide will be in position to donate a proton to the Asn-137 and Ser137 amino acid residues of the binding site. This peptide will undergo several modifications particularly in the order of the amino acid residues in the chain that should have direct contact with conserved amino acid residues in the binding site. Recombinant peptides will be evaluated *in vitro* to test their binding inhibition activity. Hemagglutination inhibition testing should be used to determine the inhibition affinity of these peptides based on the inhibition of influenza virus to agglutinate erythrocytes. Promising approaches such as this may be used to overcome this disease. For blockade therapy to be successful, intensive studies and careful evaluation of new pharmacological agents using the advanced techniques described herein will be required

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