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New insights into influenza A specificity: an evolution of paradigms

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Abstract

Understanding the molecular origin of influenza receptor specificity is complicated by the paucity of quantitative affinity measurements, and the qualitative and variable nature of glycan array data. Further obstacles arise from the varied impact of viral glycosylation and the relatively narrow spectrum of biologically relevant receptors present on glycan arrays. A survey of receptor conformational properties is presented, leading to the conclusion that conformational entropy plays a key role in defining specificity, as does the newly reported ability of biantennary receptors that terminate in Siaα2-6Gal sequences to form bidentate interactions to two binding sites in a hemagglutinin trimer. Bidentate binding provides a functional explanation for the observation that Siaα2-6 receptors adopt an open-umbrella topology when bound to hemagglutinins from human-infective viruses, and calls for a reassessment of virus avidity and tissue tropism.

Introduction

Wild birds are the primary natural reservoir for influenza A viruses [1], and the 1918 Spanish Flu pandemic that killed an estimated 50 million people [2] is believed to have originated from spontaneous mutations in an avian influenza virus that conferred human-to-human transmissibility [3,4]. While zoonotic influenza can infect humans [5], close contact with infected animals is required [6]. Subsequent human-to-human transmission, leading to pandemics, requires that the virus undergo additional genetic alterations [5,6]. As noted by Reper-ant *et al.* [5], in order for a zoonotic virus to become human-infective, it must overcome three sets of barriers: animal-to-human transmission, virus-cell interaction, and human-to-human transmission. Seasonal influenza epidemics arise from human-to-human transmission of circulating strains that have undergone sufficient mutation (antigenic drift) to circumvent established immunity within the population [7].

In contrast to the Spanish Flu, the Swine Flu pandemic of 2009 was relatively mild [8]. Nevertheless it raised concerns within the World Health Organization because of the rapidity with which it spread [9]; within 6 weeks of the first case, Swine Flu had spread to over 70

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countries [10] and required the development of a new vaccine. Human adaptation is of particular concern in the case of highly pathogenic avian influenza (HPAI) subtypes, such as H5N1. Although infrequent, human infection by avian H5N1 has been reported in 16 countries, resulting in approximately 60% mortality [11]. Preparedness for pandemics therefore necessitates anticipation of the virulence of emerging strains, providing motivation for developing a deeper understanding of the basis for influenza specificity. Here, we reassess the relationship between host glycan structure and influenza specificity in light of recent data that indicates critical roles for glycan substructure and dynamics.

Influenza A classification is based on the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) envelope proteins. Influenza HA is a homotrimeric glycoprotein whose protomers each comprise a globular head domain (HA1) and stalk region (HA2) [12]. Each HA1 domain contains a receptor binding site (RBS), through which the virus adheres to the host cell via binding to host glycans that contain sialic acid (Sia, neuraminic acid, Neu5Ac). There are currently 18 hemagglutinin subtypes, which are classified into two groups based on their antigenic properties: group 1 consists of H1-2, H5-6, H8-H9 H11-13, and H16; group 2 contains H3-4, H7, H10, H14, and H15. The most extensively studied HAs include H1, H3 and H5 [13•,14]. The NA protein mediates the cleaving of Sia from the host receptor glycan post cellular infection, enabling progeny virus to escape from the host cell surface [15]. Cryoelectron tomography indicates that there are approximately 300 HA proteins in the viral envelope [16], with the ratio of HA to NA varying between different strains from 4 to 6:1 [16,17]. Compound factors affect the ability of a particular strain of influenza to infect humans, including the level of exposure, the replication rate in newly infected individuals, the glycan binding preferences of the viral surface HA, and the activity of the viral surface NA [15,18–23]. Further, the enzyme activity of the NA must balance with the affinity of the HA [15,22]. If the NA is too active, relative to the affinity of the HA, it will attenuate the ability of the virus to infect the host cell. Conversely, a relatively weak NA will impair shedding of the progeny virus.

In addition to receptor specificity, zoonotic infection is also sensitive to differences in the susceptibility of the HA to pH-mediated endosomal fusion [24], and differences in the efficiency of translocation of the viral ribonucleoprotein complex to the host nucleus [25] (host adaptation). Moreover, ease of transmission and replication appears to be dependent on the distribution and composition of the receptors on host tissue. Viral attachment studies have shown that human influenza viruses adhere more strongly to human trachea and bronchi than avian viruses, and attach to different cell types [26]. Thus, the lack of a suitable receptor has been invoked as being responsible for the inefficient transmission [27] and replication of avian viruses in humans [28,29]. Much work has been done to elucidate the molecular basis for the observed tissue tropism [28,30,31,32••].

Defining receptor specificity

The canonical view of the relationship between HA receptor specificity and species infectivity is that the HA in human-infective viruses prefers to bind to glycans present on the cell surface that terminate with the Siaα2-6Gal (α2-6) sequence; in avian-infective viruses, the HA prefers to bind to glycans that terminate in Siaα2-3Gal (α2-3). Some species, such

as swine, can be co-infected by viruses that prefer either α2-3 or α2-6 structures, leading to the potential for genetic reassortment (antigenic shift) in swine that results in the introduction of α2-6 binding preference (enhanced human infectivity) into a zoonotic framework [5].

The discovery of the α2-6/α2-3 infectivity relationship originated not from quantitative biophysical studies, but from more qualitative, yet robust, hemagglutination assays [33]. Rogers and Paulson's [34••] seminal work on enzymatically modified red blood cells (RBCs) established that influenza receptor specificity depends, to an extent, on the species from which the virus was isolated. They reported that isolates of human subtype H3N2 agglutinated RBCs whose modified surface glycans terminated in the α2-6 sequence, but that these isolates did not agglutinate RBCs with α2-3 glycans. Conversely, avian isolates preferentially agglutinated RBCs containing the α2-3 linkage. While hemagglutination by influenza is a general phenomenon not limited to chicken RBCs [35], not all virus strains agglutinate all types of RBCs equally well [35,36]. Unmodified chicken RBCs contain a diversity of multiantennary glycans, roughly in an equal ratio of α2-3:α2-6 [36], but these represent only a limited subset of the glycans found on human epithelial tissue, which also include multiple lactosamine repeats in the antennae. The observation that the necessary human-type receptors are not present provides an explanation of the inability of certain human-adapted influenza strains to agglutinate chicken RBCs [36]. As noted by Ovsyannikova *et al.* [35], species selection of red blood cells (RBCs) is critical to determine antibody titers to influenza viruses reliably, however, further glycomics analyses are required to elucidate the origin of the differences in RBC agglutination behavior.

Affinity versus avidity

Monomeric binding affinities for HA-glycan interactions confirm the canonical view of HA specificity, but show remarkably modest differences between α2-3 and α2-6 receptors (Table 1). Avidity arising from interactions between multiple host glycans and multiple trimeric HAs on the viral surface has been invoked to explain the difference between the weak (mM) monomeric affinities for HA-glycan interactions and the sub-μM binding for whole virus [37,38•,39,40••]. Indeed, models of binding kinetics [38•,39] have shown that avidity can exponentially amplify the subtle differences in monomeric affinities, resulting in agreement with experimental virus binding kinetics.

In 2012, Lin *et al.* [44••] reported that the avidity of H3N2 viruses for an α2-6 trisaccharide receptor decreased approximately fourfold between 1968 and 2001, then progressively decreased a further 200-fold from 2001 to 2010, to such an extent that higher virus concentrations were required to observe any binding for the 2010 strains. This decrease in binding avidity was shown to be the result of mutations (antigenic drift) that weakened specific interactions between the RBS and the glycan receptors [44••]. Recently, Peng *et al.* [45••] screened the HAs from a number of H3N2 viruses against a custom glycan array that included multiantennary glycans of the type found in the human respiratory tract [46••], and confirmed that binding to short, or linear, glycans had steadily decreased, consistent with the observations of Lin *et al.* [44••]. However, strong binding to long biantennary sialoglycans was observed that was relatively insensitive to the effects of antigenic drift.

Recently, Peng *et al.* [45••], and de Vries *et al.* [47•] have raised the intriguing possibility that both branches in a biantennary glycan could bind simultaneously to two RBSs in an HA trimer, provided the branches were sufficiently long to reach two RBSs (Figure 1). Such bidentate binding would amplify the affinity of the glycan, potentially resulting in an apparent affinity of as much as the square of the monovalent K_D ($K_{D,mono}^2$) [40••], although this would likely be reduced by entropic penalties. This binding enhancement would enable the HA to continue to retain affinity for certain biantennary glycans despite the overall negative impact of antigenic drift on receptor binding at a monovalent level. This hypothesis provides a basis for explaining the observation that, despite the general decrease in avidity displayed by H3N2 viruses [44••,48], they retain the ability to bind to biantennary glycans [45••] and, thus, to infect and transmit in the human population [48].

Impact of HA glycosylation on specificity

Glycosylation of HA proteins varies both in location and composition depending on the strain of the virus [45••,50], as well as on the cell-type in which the virus was produced [51]. Over time, the number of glycosylation sites in circulating influenza strains has increased [50,52], presumably shielding the protein surface from antibody recognition and assisting the virus in evading host immune surveillance [50,53–56]. However, the more heavily glycosylated an HA1 domain, the more likely that its receptor binding ability will be impaired, either because the glycosylation directly blocks access to the RBS [57,58], or because it forms a shield through which short receptor glycans may not be able to penetrate. Increased glycosylation, thus, potentially decreases affinity and virulence [59•]. Three decades ago, it was observed that passaging of an avian infective H1N1 strain (A/WSN/1933) in mammalian (MDBK) cells led to the loss of glycosylation at N129 in the HA1 domain, leading to an increased affinity for host receptors, whereas passaging in chicken cells had no effect on glycosylation [60]. More recently, based on an analysis of 3D structures of HAs, Jayaraman *et al.* [57] predicted that, because of its proximity to the RBS, the loss of glycosylation at N91 in the HA from an H1N1 (A/South Carolina/1/18 and two variants, D225G and D190E/D225G) should affect receptor-binding properties. While loss of glycosylation at N91 was found to have no affect on the binding of the D190E/D225G (avian-like) variant to immobilized α2-3 oligosaccharides, it completely abrogated binding of the D225G variant to α2-3 and α2-6 oligosaccharides, and attenuated binding of wild-type HA to α2-6 oligosaccharides. The mechanism underlying the negative impact of loss of glycosylation on α2-6 binding was not identified.

In H5N1 strains, the N158 glycosylation site occupies a similar spatial position to that of N129 in H1N1 strains, and appears to produce similar effects when glycosylated (attenuation of antigenicity, reduction of affinity for α2-6 receptors [61]). H5N1 viruses lacking glycosylation at N158 transmit efficiently by direct contact among guinea-pigs [62]. In 2015, Zhang *et al.* [63] examined the impact of glycosylation at three sites in the HA1 of an H5N1 virus (A/Mallard/Huadong/S/2005) and reached the conclusions that loss of glycosylation at N158 was a prerequisite for binding to α2,6-modified RBCs, and viruses with a loss of glycosylation at N158 or N169 had higher lethality in mice. In 2010, Liao *et al.* [64••] showed that deletion of glycosylation sites in an H5 derived from a consensus-

based sequence [65] led to no major change in the glycan binding profiles for α2-3 oligosaccharides.

Yang *et al.* [54] noted in a study of H3N2 strains that the viruses had evolved to prefer longer linear glycans, and hypothesized that this preference was related to an increase in the number of glycosylation sites in the HA1. Alymova *et al.* [66] also recently examined H3N2 with varying glycosylation levels, and concluded that glycosylation of the HA1 could decrease binding affinity, without reducing virulence. They further introduced the hypothesis, based on the consistent binding of the HAs to linear α2-6 sialylated polylactosamine glycans, that physiologically relevant receptor binding had not changed over the past 40 years. However, their array did not include the large biantennary glycans used by Peng *et al.* [45••], who concluded that H3N2 had evolved specificity for extended, branched α2-6 glycans.

While the current data regarding the impact of HA1 glycosylation show strain dependence, binding to α2-6 receptors generally appears to be markedly sensitive to variations in HA1 glycosylation. Further studies will be required to develop a clear understanding of the conditions under which HA1 glycosylation alters receptor binding and or virulence.

Relating HA structure to receptor specificity

Examination of pandemic HA sequences permits the identification of mutations in the RBS that appear to play a role in switching the virus specificity. A pair of mutations identified as E190D and G225D in H1N1 viruses has been shown to be critical for switching the binding preference from α2-3 to α2-6 glycans [3,4,56], and appears to have been responsible for the Spanish Flu pandemic [67]. Mutation at only one of these sites within an H1 typically leads to dual α2-3 and α2-6 receptor binding [3,56,68]. A different pair of mutations (Q226L and G228S) enabled the H2N2 and H3N2 pandemic viruses to gain specificity for α2-6 glycans [69]. However, these observations should not be considered to be specificity ‘rules’ — as part of a study to engineer α2-6 specificity into an H5N1 (A/Vietnam/1203/04), introduction of the E190D and G225D double mutations remarkably eliminated binding to all α2-3 and α2-6 glycans examined [70]. Additional host-adaptation is required in order to achieve this specificity switch in H5N1 viruses [71–74]. Very recently de Vries *et al.* [47•] have shown that three mutations (V186K/G, K193T, and G228S) switch H7N9 influenza to human-type receptor specificity, with a binding profile practically identical to pandemic H1N1 A/California/04/2009.

The 3D structures of HA-oligosaccharide complexes are essential for understanding, and potentially predicting, the effect of mutations in HA on receptor specificity, and the structural features of influenza HA-glycan co-complexes have been well described [13•, 15,75,76•]. However, despite the large and growing number of co-crystal structures of HA-oligosaccharide complexes, rationalization of the observed specificity preferences in terms of 3D structural properties is far from straightforward [77•]. As a case in point, consider the complexes of HAs from avian-infective (A/Wild Duck/JX/12416/2005) and human-infective (A/California/04/2009) H1N1 viruses (Figure 2). These HAs have been co-crystallized with both α2-3 and α2-6 glycans, and therefore provide an opportunity to illustrate the

differences in hydrogen-bonding patterns in human-adapted or avian-adapted HAs. It is clear from an examination of the hydrogen bond patterns between α2-3 and α2-6 oligosaccharides with the human-adapted HA (Figure 2, lower panels) that the α2-6 receptor makes several additional interactions (involving D190, D225 and K222) relative to the α2-3. These interactions are consistent with the observed α2-6 preference for human-adapted HAs. Why avian-adapted HAs generally bind more weakly, if at all, to α2-6 oligosaccharides is far less clear from these structures (Figure 2 upper panels). Indeed, as noted by Lin *et al.* [78••], the mode of binding observed for the avian-adapted HA is not consistent with the view that the avian HA favors α2-3 receptors over α2-6.

The answer to these structural riddles must lie in properties that are not as readily apparent as hydrogen bond networks. These include van der Waals contacts, as well as contributions from conformational entropy, which may be significantly different for the two types of ligand.

Conformational entropy — the missing link

Given the relatively plastic nature of glycans, binding to a protein incurs an entropic penalty proportional to the degree of conformational constriction, and this has been proposed as an unfavorable contribution in HA-glycan binding [79]. Notably, computational analyses, based on molecular dynamics simulations of crystallographic HA co-complexes, fail to reproduce the observed binding specificities unless entropic contributions are explicitly included [80,81••]. The magnitude of the entropy penalty S for each rotatable bond W that becomes constrained upon binding can be estimated from Boltzmann's expression ($S = R \ln W$) [82], or calculated from observed conformational populations [80,81••,83]. In α2-3 linkages the φ-angle (C1′–C2′–O3–C3) typically populates two rotamers in solution (anti and –gauche with respect to C1′) [84], but only one when bound to an HA, resulting in an estimated entropic penalty of approximately 0.4 kcal/mol (at 25°C). The α2-6 linkage has an additional rotatable bond that leads to multiple conformations, giving rise to an estimated entropic penalty of at least 1.5 kcal/mol [80,81••,83,85]. Furthermore, in the case of α2-6 glycans, a curled or open-umbrella topology places more of the glycan substructure in contact with the HA surface than in the case of α2-3 glycans that adopt linear or cone-like topologies. These additional glycan-HA interactions can result in entropic penalties for α2-6 glycans that are larger than those for α2-3 glycans by as much as 5 kcal/mol [81••].

Bidentate binding would also be expected to lead to a heightened entropic penalty, due to the overall restriction of motion for such large, flexible glycans, and in particular for the 1–6 linkage in the glycan core. Additionally, interactions between the amino acid side chains and the receptor in the RBS [82,86] may be entropically disfavored. For example, for K222 to form its hydrogen bond with the receptor, the long flexible side chain pays an entropic penalty of up to 2 kcal/mol (using $S = R \ln W$) [82]. The more constrained a flexible ligand is by enthalpically favorable interactions, such as hydrogen bonds and van der Waals contacts, the higher the entropic penalty paid by the system [87], leading to the key concept of enthalpy–entropy compensation [88].

In order to prefer binding to α2-6 glycans over α2-3, the HA must evolve to form proportionally more or stronger interactions with the α2-6 receptor. Thus, although crystallography demonstrates that an avian-adapted HA can form as many (or more) interactions with an α2-6 glycan [78••], the resultant entropically disfavored stiffening of the α2-6 receptor results in a net free energy preference for the α2-3 glycan. For this reason, the number of receptor-HA interactions (Figure 2) is a poor metric for assessing subtle differences in affinity/specificity.

Relating glycan structure to specificity

Glycan array screening has been extensively applied to help define the specificity of influenza hemagglutinins. Overall, the data support the view that HAs from avian-adapted strains prefer α2-3 glycans, while human-infective strains generally prefer α2-6 [44••,45••, 46••,48,70,89–97]. Nevertheless, glycan array screening has also brought to light many exceptions to the accepted view of specificity, and raised new and unanswered questions, particularly related to variations in response as a function of monosaccharide modifications (sulfation, acetylation, etc.) and glycan substructure [48,97]. Common modifications to the Sia residue include acetylation of the glyceryl side chain (typically at the 9-position), or 5-N-glycolylation (Neu5Gc), which generally attenuate binding to HA from human-infective virus [98•,99]. Remarkably, in contrast to the effect of acetylation, a 9-*O*-lactoyl group appears to restore affinity (H1N1 and H3N2) to levels comparable to the non-derivatized sialoside [98•]. Neu5Gc is not produced in humans [100], but can be abundant in non-human species; for example, Neu5Gc-containing glycans are the dominant moieties on epithelial cells from equine trachea [101]. Not surprisingly therefore, HAs from some (but not all) equine-infective influenza strains bind preferentially to glycans containing this modification [102], whereas HA from human-infective strains generally do not [98•], explaining the equine/ human zoonotic transmission barrier [101].

The sensitivity of binding to glycan substructure is an essential component when defining influenza specificity, but its assessment is complicated by the diversity of possible glycan structures, the influence of glycan substructure on the 3D structure of the sialylated terminus (Figure 3), and the differential impact of mutations in the RBS on interactions with glycan modifications [103]. It is impossible to separate the impact of modifications in the glycan from the overall context of the glycan 3D shape, just as it is impossible to discuss the significance of mutations in the HA independently from the context of the particular subtype. For example, the Gal-2 residue may be linked to GlcNAc-3 at either the 3-position or 4-position (Galβ1-3GlcNAc or Galβ1-4GlcNAc). This chemically subtle difference is often left undefined in glycomic analyses [36], and yet has a dramatic impact on the orientation of the GlcNAc-3 residue relative to Gal-2, flipping the positions of the NAc and O6 moieties in the GlcNAc by approximately 180° degrees in the RBS (Figure 3). This difference in glycan substructure would be expected to have a noticeable influence on binding when the HA has evolved to prefer a receptor in which the GlcNAc is modified by sulfation at O6. For example, the HA from an equine H3N8 binds preferentially to 6-sulfated sialosides, but only when the Gal-2-GlcNAc-3 linkage is present in the β1-4 form [102]. For H5 subtypes, 6-O-sulfation of the receptor enhances binding [104] and was predicted to lead to the formation of a salt bridge between the sulfate moiety and K193 [105], which was

recently confirmed by crystallography [106]. Similar favorable electrostatic interactions were observed between the same sulfated receptor and K158A in an avian H10 [43].

Another common modification of α2-3 sialosides is α-fucosylation at the 3-position or 4-position of GlcNAc-3. The site of fucosylation depends on the nature of the Gal-2-GlcNAc-3 linkage (β1-4 or β1-3), generating the well-known sialyl Le^X (SLe^x) and SLe^a motifs, respectively. Whether or not fucosylation attenuates affinity has been suggested to depend on the presence or absence of steric collisions with bulky side chains at positions 222 and/or 227 [102,107–109]. Given the prevalence of SLe^x in mucins, they have been proposed as providing a barrier to infection [110].

In contrast to the 3D properties of the RBS, less attention has been given to a systematic analysis of the conformations of the receptors in the complexes, although it has frequently been observed that the α2-3 linkage adopts a '*trans*' orientation, resulting in a cone-like topology of the glycan relative to the HA surface [111•]. The '*cis*' orientation of the α2-6 linkage [112,113] has been further noted to lead such ligands to form a compact, curled, or folded conformation [114] that results in the receptor spanning a larger region of the HA surface, referred to as an open-umbrella topology [111•]. The use of the '*cis*-' descriptor for the Siaα2-6Gal φ-angle has become widespread, however it is not useful when comparing the conformation of such linkages in HA complexes, as to date all such linkages adopt this conformation when co-complexed with HAs (Table 2). The conformation of the ψ angle in Siaα2-6Gal linkages does however vary, populating only two states, herein denoted 'anti-ψ' or 'eclipsed-ψ'. Moreover, the terms '*cis*' and '*trans*' imply that the orientation of the bond is fixed, as in a double bond. As this is not the case for α2-6 or α2-3 linkages, we will refer to the so-called '*cis*' orientation as 'gauche', and the '*trans*' as 'anti'. The receptor conformational properties extracted from well-resolved HA-oligosaccharide co-complexes are presented in Table 2.

An examination of the data in Table 2 indicates that α2-6 linkages adopt two conformations when bound to HAs, which can be defined by the value of the ψ (C2′–O6–C6–C5) angle. Two shapes are also adopted by bound α2-3 linkages, which vary in the φ (C1′C2′O3-C3) angle. The significance of these shapes, with respect to the presentation of the receptor in the RBS is illustrated in Figure 4. Figure 4 illustrates that the open-umbrella topology is associated with the 'curled' anti-ψ conformation of an α2-6 linkage (panel A), while the cone-like topology results from the 'extended' anti-φ conformation of the α2-3 linkage (panel C). Presented in panels B and D are the alternative conformations of α2-6 (eclipsed-ψ) and α2-3 (–gauche-φ) linkages. The trisaccharides in the crystal structures presented in Figure 4 have each been extended to contain three lactosamine repeats to clearly illustrate the impact of the Sia-Gal linkage conformation on the orientation of the glycans. This analysis is consistent with the observations by Xu *et al.* [81••] that the division of the glycan topologies into only cone-like or open-umbrella is insufficient to capture the diversity of glycan conformations in HA complexes.

The recent proposal by Peng *et al.* [45••] that multi-antennary α2-6 glycans can form bidentate interactions with trimeric HAs casts new light on the origin of glycan substructure differences. There are several constraints on the ability of a glycan to exhibit bidentate

binding. One such constraint is the ability of the antennae to span the distance between two RBSs without steric blocking by HA surface residues, or by HA glycosylation. Another constraint arises from the topologies of the termini of individual glycan branches, which must facilitate orientations conducive to bidentate binding. As shown in Figure 4, *only α2-6 receptors in a curled anti-ψ conformation satisfy this latter requirement; no known conformations of the α2-3 receptors promote bidentate binding.* Although the α2-3 oligosaccharides in the –gauche φ-conformation (panel D) reach upward from the RBS rather than away (as in panels B and C), their spatial divergence from each other precludes their origination as branches of a single bian-tennary glycan. Biantennary binding requires that the bound oligosaccharides converge toward a common point in the glycan core (as in panel A). Ultimately, the inability of biantennary α2-3 receptors to form bidentate complexes arises from the linear shape of the α2-3 linkage, which controls the relative orientation of the Siaα2-3Gal disaccharide.

The observation that the α2-3 linkage precludes bidentate binding provides further insight into the functional significance of the cone like (α2-3) versus open-umbrella (α2-6) topologies [111•]. The curled anti-ψ conformation of the α2-6 glycans promotes the formation of a bidentate complex, which may also be stabilized by glycan-protein interactions associated with the larger contact area of the open-umbrella topology. Thus, while both α2-3 and α2-6 glycans may in principle form multimeric interactions with an HA, only the α2-6 receptors appear to be able to form bidentate interactions. When glycan density is sufficiently high that two or more glycans can bind simultaneously to the same HA, bidentate binding may offer little enhancement to affinity [95]. However, the ability to form bidentate interactions provides a unique opportunity for the virus to achieve avidity-enhanced binding to α2-6 receptors on a single glycan. This unique capability explains why, despite the overall loss of avidity [44••], human-adapted H3N2 viruses retain affinity for a subset of long biantennary α2-6 glycans [45••]. Tissue tropism therefore needs to be interpreted not only in terms of composition and spatial distribution of the glycans, but also in the relative density of α2-3 and α2-6 glycans.

Conclusions

Although glycan array screening is a convenient method for examining specificity, developing structure–activity relationships solely on the basis of such data is perilous. Glycan array data should generally be treated qualitatively given that the data are sensitive to numerous factors, including glycan density, glycan linker chemistry, analyte concentration, and detection method [115]. While it is possible to determine surface binding constants ($K_{D,surf}$) using glycan arrays [64••,116], offering an important advantage by quantifying the binding properties of each of the glycans in an array, these protocols are not yet in widespread use. A further factor that significantly complicates the interpretation of array data is the extremely limited diversity of even the largest arrays [46••]. This limitation has obvious consequences for ligand discovery [45••], and for the elucidation of structure-specificity relationships. Although at present, data from glycan array screening need generally to be treated qualitatively, community-wide standards are being developed [117], which together with more quantitative approaches to data processing [64••] and computational analysis [118•,119•], will enhance the interpretability of such data. A

powerful example of the generation and use of quantitative surface K_D values from glycan array screening was reported by Wong *et al.* [120••]. They were able to dissect the energetic contributions made by each monosaccharide, including the sulfate moieties, in an array of sialosides binding to HAs, showing that the sulfate could enhance binding by nearly 100-fold. Further, by comparing the relative binding energies for each receptor, they were able to conclude that there is likely a competition between favorable binding interactions in the RBS, which the sulfate group maximizes and the fucose sterically blocks.

Crystallographic studies provide unique and crucial atomic-level insight into HA-receptor interactions, but in the absence of entropic considerations, do not necessarily enable a clear rationalization of specificity. Such interpretations would greatly benefit from the generation of additional quantitative monomeric affinity measurements, as well as from modeling, which may guide the choice of targets for crystallography and array screening. Lastly, variations in HA glycosylation [45••,50,51] can impact affinity and virulence [59•], and should be considered in any analysis of specificity.

On the basis of agglutination data, glycan array screening, and (albeit limited) biophysical affinity measurements, avian-infective HAs have a clear preference for α2-3 glycans, consistent with the inability of these HAs to compensate for the entropic penalty associated with binding α2-6 glycans. The specificity of human-adapted HAs for α2-6 glycans is more complex, in part because the virus may retain residual affinity for α2-3-receptors, while evolving the ability to bind to α2-6-receptors. For preferential binding of α2-6 linked glycans, mutations must occur in the RBS that overcome the entropic penalty associated with binding to the more flexible α2-6 receptor, and/or which favor the formation of bidentate interactions with multiantennary glycans. The preference for bound α2-6 glycans to adopt an anti-ψ angle (required for bidentate binding) is seen in all well-resolved crystal structures of HAs from human transmissible viruses. This suggests that bidentate binding may be a general mechanism adopted by influenza A to boost affinity for α2-6 receptors, enabling human-to-human transmission.

This review has hopefully illustrated that, despite the challenges in reconciling all of the data relating to influenza A specificity, a molecular interpretation is emerging. The implications of glycan linkage α2-3 or α2-6 on specificity extend beyond the direct interactions between the terminal Sia-Gal sequence and the HA to more macroscopic features, such as the ability to form multi-dentate complexes and the need to overcome the inherent entropic penalty associated with binding to α2-6 glycans. A complete understanding of specificity requires a continuous reevaluation of the paradigms with a view to integrating all available data into a holistic analysis.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

• of special interest

•• of outstanding interest

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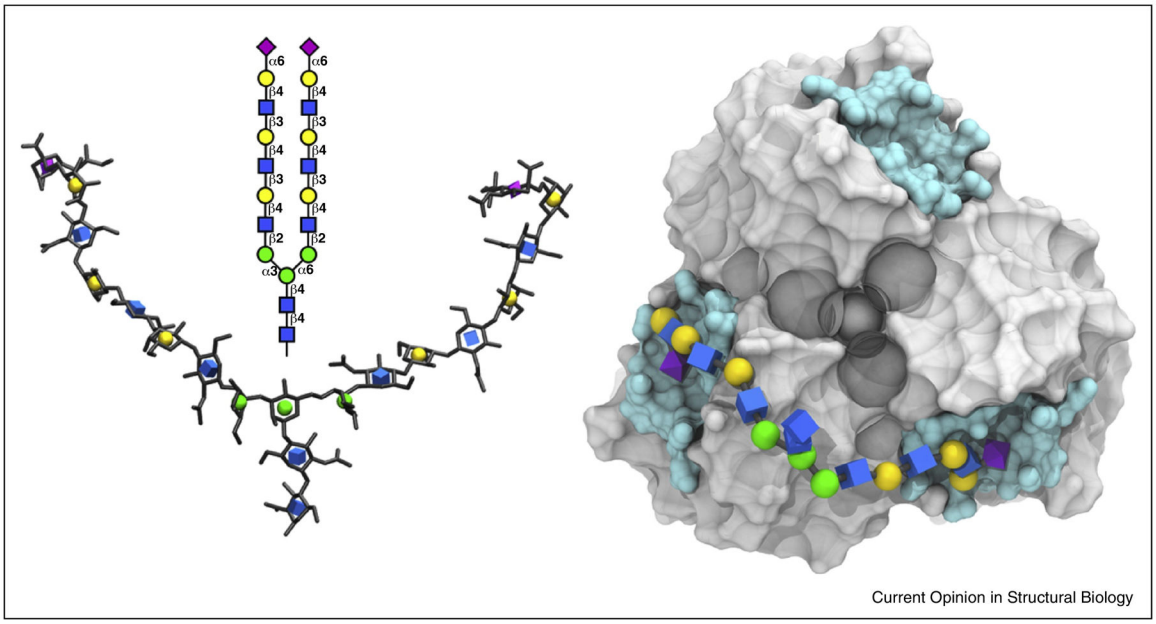

Figure 1.

Right: proposed [45••] bidentate binding of a biantennary α2-6 glycan (**left**, 3D-SNFG representation [49]) to the HA (grey surface) from a pandemic H1N1 (A/California/04/2009), residues lining the RBS are shown in cyan. The glycan is shown in the conformation required for bidentate binding.

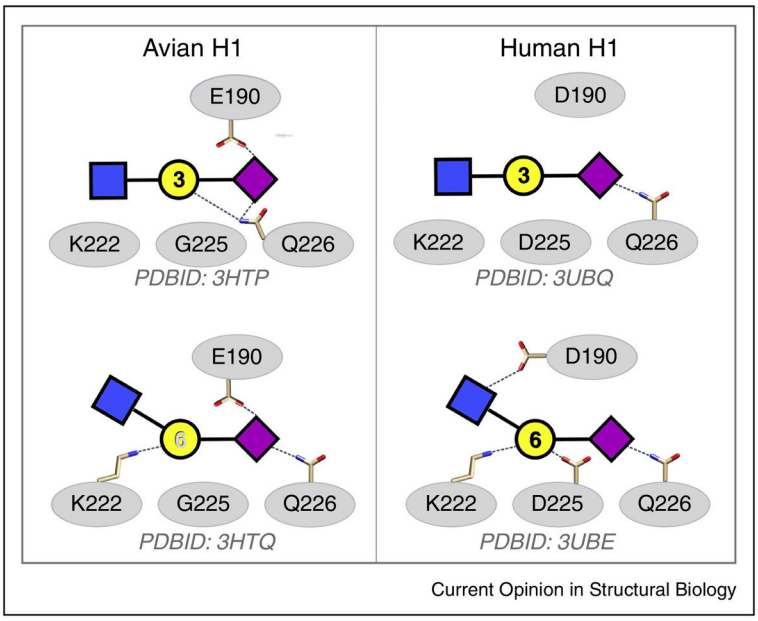

Figure 2.

Schematic representations of the binding modes for representative avian-adapted **(left)** and human-adapted **(right)** HAs from H1N1 viruses binding to α2-3 (upper) or α2-6 (lower) receptor analogs.

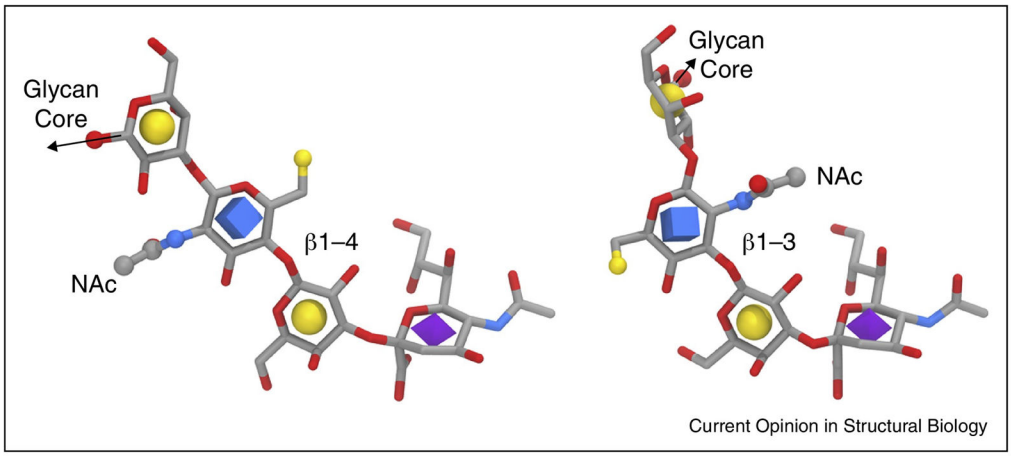

Figure 3.

HA receptor structures indicating the influence of the Gal-2 — GlcNAc3 linkage type (**left**: β1-4, **right**: β1-3) on conformation and presentation. The structures were retrieved from PDB IDs 4YYA and 4NRL, respectively, and aligned relative to the Sia residues. Note the reversal of the *N*-acetyl moieties relative to the Sia residues. The GlcNAc 6-position, which may be sulfated, is shown as a small yellow sphere.

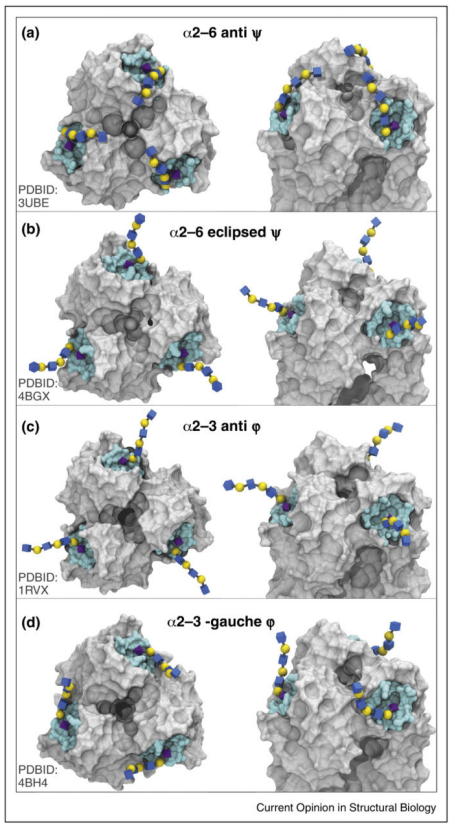

Figure 4.

Top **(left)** and side **(right)** views of the HA1 domains of four HA co-complexes that illustrate the four common ligand conformations seen in HA-oligosaccharide co-complexes.

Table 1

Monomeric oligosaccharide — HA binding affinities

HA viral strain	Canonical specificity	Ligand	K_D (mM)	ΔG (kcal/mol)[a]
H3N2				
A/Hong Kong/1/1968 [38•,41•] (X-31)	α2-6	SiaαOMe	2.8 ± 0.3	−3.5
		3′SLN	3.1 ± 0.4	−3.4
		3′SL	3.6 ± 0.7, 3.2 ± 0.6	−3.3, −3.4
		LSTa	3.8 ± 0.8	−3.3
		6′SLN	2.0 ± 0.2	−3.7
		6′SL	1.7 ± 0.5, 2.1 ± 0.3	−3.8, −3.6
A/Memphis/102/72 [42•]	α2-6	SiaαOMe	2.0 ± 1.1	−3.7
		LSTa	8.0	−2.9
		LSTc	1.2	−4.0
H3N2				
A/Hong Kong/1/1968 (X-31) L226Q [41•]	α2-3	SiaαOMe	4.7 ± 0.5	−3.2
		3′SL	2.9 ± 0.3	−3.4
		6′SL	5.9 ± 0.7	−3.0
H5N1				
A/Vietnam/1194/04[38•]	α2-3	3′SLN	1.1 ± 0.2	−4.0
		3′SL	0.7 ± 0.4	−4.3
		6′SLN	17 ± 3	−2.4
		6′SL	21 ± 6	−2.3
A/Vietnam/1194/04 (ferret transmissible) N158D/N224K/Q226L/T318I [38•]	α2-6	3′SLN	32 ± 8	−2.0
		3′SL	43 ± 12	−1.9
		6′SLN	12 ± 2.5	−2.6
		6′SL	17 ± 5	−2.4
H10N8				
A/Jiangxi-Donghu/346/2013[43]	α2-6	3′SLN	1.8 ± 0.39	−3.7
		6′SLN	1.4 ± 0.32	−3.9

[a] At 25°C.

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Table 2

Ligand conformation and HAs co-complexed to α2-6 and α2-3 oligosaccharides (resolution ≤ 2.5 Å)

PDB ID, ligand	α2-6 ψ^a	PDB ID, ligand	α2-3 ϕ^b	HA virus strain	Amino acids at 190,193,222, 225, 226, 227, 228
H1N1					
1RVT, LSTc	anti	1RV0, LSTa	_[c]	A/Swine/Iowa/15/1930	D,S,K,G,Q,A,G
1RVZ, LSTc	anti	1RVX, LSTa	anti	A/Puerto Rico/8/1934	E,D,K,D,Q,A,G
3UBE, LSTc	anti	3UBJ, LSTa	anti	A/California/04/2009	D,S,K,D,Q,E,G
3UBN, 6′SLN	anti	3UBQ, 3′SLN	anti	A/California/04/2009	D,S,K,D,Q,E,G
3HTQ[d], LSTc	anti	3HTP[d], LSTa	anti	A/Wild Duck/JX/12416/2005	E,T,K,G,Q,A,G
H2N2					
2WR1, LSTc	anti	2WR2, LSTa	anti	A/Chicken/New York/29878/91	E,T,K,G,Q,G,G
2WR4, LSTc	anti	2WR3, LSTa	anti	A/Duck/Ontario/1977	E,T,K,G,Q,G,G
2WR7, LSTc	anti	–		A/Singapore/1/57	E,T,K,G,L,G,S
H3N2					
2YP3, 6′SLN	anti	2YP5, 3′SLN	_[c]	A/Finland/486/2004	D,S,R,D,I,P,S
2YP4, LSTc	anti			A/Finland/486/2004	D,S,R,D,I,P,S
2YP8, 6′SLN	_[c]	2YP9, 3′SLN	_[c]	A/Hong Kong/4443/2005	D,F,R,N,I,P,S
2YPG[d], LSTc	anti			A/Aichi/2/1968-X31	E,S,W,G,L,S,S
H3N8					
		4WA2, 3′SLN	anti	A/harbor seal/Massachusetts/1/2011	E,N,L,G,Q,S,G
H5N1					
1JSO, LSTc	_[c]	1JSN, LSTa	anti	A/Duck/Singapore/3/1997	E,K,K,G,Q,S,G
4BGX, 6′SLN	eclipsed[b]	4BGY[d], 3′SLN	anti	A/Vietnam/1194/2004	E,K,K,G,Q,S,G
4BH0, 6′SLN	eclipsed	4BH1, 3′SLN	anti	A/Turkey/Turkey/1/2005	E,R,K,G,Q,S,G
4BH3, 6′SLN	anti	4BH4, 3′SLN	–gauche[b]	A/Vietnam/1203/2004 (N158D,N224Q,Q226L,T318I)	E,K,K,G,L,S,G
4KDO, LSTc	anti	4KDN, LSTa	_[c]	A/Vietnam/1203/2004 (N158D/N224K/Q226L/T318I)	E,K,K,G,L,S,G
4CQR, 6′SLN	eclipsed	4CQQ[d], 3′SLN	−gauche	A/Vietnam/1194/2004 (S227N,Q196R)	E,K,K,G,Q,R,G
4CQU, 6′SLN	eclipsed	5AJM, 3′SLN	–gauche	A/Vietnam/1194/2004 (N186K)	E,K,K,G,Q,S,G
		4CQY, LSTa	anti	A/turkey/Turkey/1/2005 (Δ133/I155T)	E,R,K,G,Q,S,G

PDB ID, ligand	α2-6 ψ^a	PDB ID, ligand	α2-3 ϕ^b	HA virus strain	Amino acids at 190,193,222, 225, 226, 227, 228
4CQX, 6′SLN	eclipsed	4CQW, 3′SLN	anti	A/turkey/Turkey/1/2005 (Δ133/I155T)	E,R,K,G,Q,S,G
H6N1					
5BR6[a], LSTc	anti	5BR3[d], LSTa	−gauche	A/Taiwan/2/2013	V,N,A,G,Q,R,S
		4XKF, LSTa	−gauche	A/Taiwan/2/2013	V,N,A,G,Q,R,S
4XKG, 6′SLN	eclipsed	4XKE, 3′SLN	−gauche	A/Taiwan/2/2013	V,N,A,G,Q,R,S
H7N3					
4BSH, 6′SLN	eclipsed	4BSI, 3′SLN[d]	anti	A/Turkey/Italy/214845/2002	E,K,Q,G,Q,S,G
H7N9					
4BSB, LSTc	anti			A/Anhui/1/2013 (L20,T135)	E,K,Q,G,L,S,G
4BSC[d], 6′SLN	anti	4BSD, 3′SLN	−gauche	A/Anhui/1/2013 (L20,T135)	E,K,Q,G,L,S,G
4BSE[d], LSTc	anti			A/Anhui/1/2013 (V20,A135)	E,K,Q,G,L,S,G
4LKK[a], 6′SLNLN	anti	4LKJ[d], 3′SLNLN	anti	A/Anhui/1/2013 (L226Q)	E,K,Q,G,Q,S,G
		4N62, 3′SL(6S)N	+gauche[e]	A/Shanghai/2/2013 (L226)	E,K,Q,G,L,S,G
H9N2					
1JSI, LSTc	anti	1JSH, LSTa	−gauche	A/Swine/Hong Kong/9/1998	V,N,L,G,L,H,G
H10N2					
		4CYZ, LSTa	anti	A/mallard/Sweden/51/2002	E,D,Q,G,Q,S,G
H10N8					
4D00, 6′SLN	anti and eclipsed[f]			A/Jiangxi-Donghu/346/2013	E,D,Q,G,Q,S,G

[a]For α2-6 linkages, ψ (C2′–O6–C6–C5) adopts either an anti (188° ± 23) or eclipsed (113° ± 7) conformation. The remaining glycosidic angles adopt a single conformation characterized by average φ (C1′–C2′–O6–C6) = −55° ± 11 (–gauche, a.k.a. '*cis*') and average ω (O6–C6–C5–O5) = 63° ± 17, with the exceptions of 5BR6, where φ = 201°, and ω = −41°, 4LKK, where ω = 157°.

[b]For α2-3 linkages, φ (C1′–C2′–O3–C3) adopts either an anti (185° ± 10, a.k.a. '*trans*') or –gauche (−57° ± 8, a.k.a. '*cis*') conformation. The ψ (C2–O3–C3–C4) glycosidic angle adopt a single conformation average ψ = 100° ± 11.

[c]Only the Sia residue is resolved.

[d]Resolution > 2.5 Å.

[e]Distorted Sia-1 and GlcNAc-3 rings, high B-factors.

[f]Eclipsed in chain E.