NMR interaction studies of Neu5Ac-α-(2,6)-Gal-β-(1-4)-GlcNAc with influenza-virus Hemagglutinin expressed in transfected human cells.

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Abstract

The emergence of escape-mutants of Influenza Hemagglutinin following vaccination compels the yearly re-formulation of flu vaccines. Since binding the sialic acid receptor remains in all cases essential for infection, small-molecule inhibitors of hemagglutinin binding to sialic acid could be interesting therapeutic complements or alternatives to immuno-prophylaxis in the control of flu epidemics. In this work, we made use of NMR spectroscopy to study the interaction between a derivative of sialic acid (the Neu5Ac-α-(2,6)-Gal-β-(1-4)-GlcNAc trisaccharide) and hemagglutinins (H1 and H5) from human and avian strains of influenza virus, directly expressed on the surface of stable transfected 293T human cells. The hemagglutinins were shown to retain their native trimeric conformation and binding properties. Exploiting the magnetization transfer between the proteins and the ligand, we obtained evidence of the binding event and mapped the (non-identical) sugar epitopes recognized by the two hemagglutinin species. The rapid and reliable method for screening sialic acid-related hemagglutinin ligands we have developed could yield useful information for an efficient drug design.

Introduction

Influenza viruses are important respiratory pathogens causing significant morbidity, mortality and considerable economic losses in the recurrent yearly epidemics and much more devastatingly in the sporadic pandemic spreads. Influenza seasonal epidemics result in the death of 250,000 - 500,000 people every year (https://www.cdc.gov/flu), up to millions in specific pandemic years. Influenza viruses belong to the *Ortomyxoviridae* family and are formed by several genera: the most common being A, B and C. Most influenza serotypes are of avian origin. Although several of them have sporadically infected humans, only a few serotypes of influenza A virus (IAV) (bearing hemagglutinin variants H1, H2 and H3) have caused influenza pandemics in the last 100 years (Stevens *et al*., 2006a). Three viral surface proteins are embedded in the cell-derived virion membrane: hemagglutinin (HA), neuraminidase (NA) and the M2 proton channel protein. Based on the antigenicity of HA and NA, IAV can be further classified into different subtypes including 16 HA (H1–H16) and 9 NA (N1–N9) subtypes.

M2 proton channel maintains pH across the viral envelope during cell entry and viral maturation. The first anti-influenza virus drug, amantadine, is a specific blocker of the M2 H+ channel. Amantadin and its derivative have been widely abandoned due to virus resistance.

HA binds to sialic-acid containing glycans on the surface of target cells. The binding triggers internalization of the virion into the host cell; NA releases the newly formed virus particles from an infected cell by nicking sialic acid residues to which the budding virion is bound (Skehel and Wiley, 2000; Xu *et al*, 2012).

Based on structural studies of its highly conserved catalytic site (Colman *et al*, 1983; Varghese *et al*,1983), several competitive inhibitors of NA were developed. Two of them, oseltamivir (Tamiflu) and zanamivir (Relenza), have been approved worldwide as drugs. However, drug resistance due to the emergence of NA escape mutants is becoming widespread and the development of alternative anti-influenza drugs targeted to other components of the virus (including HA) are urgently needed (Jiang *et al*, 2010).

To date, sialic acid-containing lipids and polymers have been studied as entry blockers (Matsubara *et al*, 2010). In particular, many types of sialic acid-containing polymers have been developed on the basis of a cristallographyc HA structures, including sialic acid-conjugated dendritic polymers, sialyloligosaccharides containing poly L-glutamic

acid backbones, and sialyllactose-carrying polystyrene (Gambaryan *et al*., 2005; Tanaka *et al*., 2014). Unfortunately they suffer from subtype-dependent antiviral activity and a low barrier for resistance selection (Tang *et al*., 2016). Taking into account these attempts, it is important the development of universal antagonists, which are able to bind into the sialic binding site with high affinity so that single protein mutations should not be able to escape their inhibition. In this context, the detailed analysis of the binding features of syalic derivatives is very important and in this paper a new procedure to perform the interaction studies will be proposed.

The HA receptor glycans display a different glycosidic linkage between sialic acid -(Neu5Ac: N-acetyl neuraminic acid) and the penultimate sugar of the chain. Among these, influenza viruses recognize Neu5Ac(α2-3) or Neu5Ac (α2-6) linked to galactose (Gal) or N-acetyl galactosamine (GalNAc), (α2-6) linked to N-acetyl glucosamine (GlcNAc) containing oligosaccharides (Layne *et al*, 2009, Paulson *et al*., 2006). Until now, the general paradigm for the species selectivity of avian-and human-virus infection is that human-flu viruses preferentially bind to sialyl(α2-6)-linked disaccharides that is predominant in the upper human respiratory tracts, whereas avian flu viruses bind to sialyl(α2-3)-linked disaccharide moieties of the host receptor binding sites, which predominates in the avian enteric tract (Paulson *et al*., 2006; Viswanathan *et al*., 2010) (Figure 1). The paradigm of classifying viruses as only sialyl(α2-3) or (α2-6) linkage types is not sufficient to explain the infection and transmission of various strains of avian and mammalian influenza viruses. In fact, in the case of H5N1, H7N9, and other mutated flu viruses, classification based on linkage specificity does not correlate with the tendencies of human infection and intra- and inter-species transmissions of flu viruses. For example, highly pathogenic H5N1 viruses show strong sialyl(α2-6)-binding properties and weak sialyl(α2-3)-binding properties, but they have shown inefficient human infection and human-to-human transmission (Haselhorst *et al*, 2008; Chandrasekaran *et al*, 2008).

To date, most efforts to study viral membrane proteins (HA and NA) have utilized solubilized versions of recombinant proteins (Elli *et al*., 2014), and there are concerns that the recombinant protein structure may not accurately reflect the native structure due to the absence of transmembrane domains or membrane components (Stevens *et al*, 2006b). Virus Like Particles (VLP) expressing HA have been used to overcome this problem (Haselhorst *et al*, 2008). VLP present the possibility to study the biochemical and biophysical properties of viral membrane proteins in their native environment, but they can contain significant amounts of serum proteins, which

exhibit high potential for non-specific interactions, thereby complicating NMR studies of ligand-target interactions (Antanasijevic *et al*, 2016).

In this paper we describe a new model system where HAs is expressed on the surface of stably transfected human HEK-293T cells. The expression of HAs at high density on the cell surface was confirmed by Fluorescence-activated cell sorting (FACS) with anti-HA antibodies. The proteins were shown to be recognized by monoclonal antibodies specific for the native trimeric conformation and to maintain their binding properties. The interaction of HA expressing HEK-293T cells with the Neu5Ac-α-(2,6)-Gal-β-(1-4)-GlcNAc HA receptor is demonstrated here for the first time, using STD-NMR experiments.

We chose Neu5Ac-α-(2,6)-Gal-β-(1-4)-GlcNAc trisaccharide (**1**) (Figure 1A) as target molecule to investigate the binding. The complex was analyzed by Saturation Transfer Difference (STD) NMR (Meyer and Peters, 2003), which is an ideal tool to map the epitope and to describe the target-ligand interactions (Vasile *et al*, 2014; Heggelund *et al*, 2012; Haselhorst *et al*, 2008). We have already shown that the interactions between small ligands and membrane-bound proteins can be observed by STD NMR and trNOESY techniques directly in suspensions of living cells (Potenza *et al*, 2011; Guzzetti *et al*, 2013 and 2017) or platelets (Potenza *et al*, 2008) without the need of isolating the protein receptor. These experimental conditions are particularly valuable in the case of membrane proteins, because the structural and thermodynamic features of purified proteins can differ significantly from those attained *in vivo*.

Figure 1.

Results

Characterization of H1- and H5-293T cell lines.

Stable H1- and H5-transfectants of HEK-293T cells were constructed as detailed in the Methods. After transfection, HA-positive cells were sorted by FACS (Figure 2A.) The presence of HA DNA in the transfectants was monitored by PCR (Figure 2B). The proteins are exported to the cell surface as demonstrated by flow cytometry, which shows that more than 85% of the cells are HA-positive (Figure 2C). Western blot analysis of whole cell lysates in reducing and non-reducing conditions shows that the proteins are synthetized as monomers and then aggregated into dimers

and trimers (Figure 2D). Immuno-precipitation with CR6261 of membrane proteins solubilized by Triton X-100 extraction, followed by western blot analysis in non-reducing conditions, demonstrates that the surface proteins are correctly conformed as trimers and then dissociated into dimers and monomers during the electrophoresis (Figure 2E). Furthermore, the HA molecules are shown to be able to bind sialic acid, because they agglutinate chicken red cells forming rosettes (Figure 2F). We repeated hemagglutination test on cells in buffer and we confirmed that HAs maintain their conformation after 15h (which are the NMR condition and experiments time),

Figure 2.

Conformational analysis of the ligand

The glycan conformation and topology is important in governing the receptor specificity of the human and avian hemagglutinin subtypes H1 and H5. The NMR spectra of **1** were measured in deuterated buffer solution and the assignment of compound is reported in Table I.

The analysis of the conformational features of compound **1** at 298K has been made difficult by the absence of inter-residual NOEs, due to high mobility of this small saccharide. At 283K, the NOESY spectrum, recorded with 700 ms mixing time, shows one weak inter-residual NOE between H5 Neu5Ac and CH3CO GlcNAc corresponding to an estimated distance of 4-5 Å (Figure 1B). So we performed a molecular dynamic simulation and energy minimization with Gromacs. Analyzing the resulting structures we observed that the molecule assume different conformations (Figure 1C): about 15% among these are satisfying the NMR constraint, indicating a bent conformation (Figure 1B).

This kind of conformation (Figure 1B) is termed as “umbrella” in the literature and was reported for several derivatives with α(2-6) linkage at the nonreducing end, with different lengths and different second glicosidic linkages (Sassaki *et al*, 2013). This conformation is also reported (Chandrasekaran *et al*, 2008) for a tetrasaccharide cocrystallized with a human HA subtype, in which the mobility of the backbone of the ligand allows it to explore conformations similar to an umbrella progressing from a fully open form to a fully closed form.

The tr-NOESY experiment (data not shown) in presence of transfected cells has few and weak NOE cross peaks similar to that observed in the NOESY experiment of the free ligand. The absence of inter-residual NOEs does not

allow us to identify a preferred bound conformation and not even a significant conformational change upon binding to the protein.

NMR Interaction studies

Making use of STD-NMR technique, we analyzed the interaction of 6'-α-sialyl-N-acetyllactosamine (**1**) with three different cell lines: H1-293T and H5-293T, stably transfected with human (H1) and aviary (H5) hemagglutinin genes, respectively, and untransfected 293T cells as a negative control (Figure 3). The use of STD technique permits to obtain evidence of binding and information about the protons involved in the epitope.

No binding evidence was obtained in the experiment with control cell line (untransfected 293T), while,on the contrary, clear STD signals are shown for the trisaccharide in the presence of both HA-expressing cell lines (Figure 3). The absence of signal in control experiments suggests that we are observing a specific binding of the ligand to HAs.The spectra also show that the binding epitopes of the saccharide differ in presence of the two HAs.

Figure 3.

For a better comparison of the epitope of the molecule **1** with different HA subtypes, atoms interacting with H1-293T cells are labelled with red dots in Figure 4A, while those involved in the interaction with H5-293T cells are marked with blue dots in Figure 4B. Absolute STD % (Meyer and Peters, 2003) were quantified and reported in Figure 4C-D.

Figure 4.

The analysis of the ligand in presence of cells expressing H1 suggests that the epitope (Figure 4A) comprises the acetyl group and H7 of the Neu5Ac mojety, the anomeric and H2 protons of Gal and only the acetyl group of GlcNAc. All the saccharides have protons interacting with the protein, but it is a quite shallow interaction with low values of absolute STD percentage (ranging from 0.36% for H2 Gal and H7 NeuAc, to 0.68% for Acetyl groups, Figure 4C). This epitope is compatible with the interactions suggested in the literature for similar compounds (i.e. PDB structure 2WRG, Liu *et al*, 2009) and H1 hemagglutinin.

The same ligand in presence of cells expressing H5 exhibits a different epitope. Several peaks of Neu5Ac mojety can be detected in STD experiment (Figure 3 and 4B), such as the acetyl group, and the H3, H5, H6, H7 and H8

protons, suggesting that it is more strongly involved in the interaction with the target HA-H5. Also the GlcNAc interaction is more effective involving the acetyl group, the anomeric and H2 protons. Also in this case, H1 and H2 protons of Gal residue contribute to epitope. In this case the number of protons involved in the epitope and the absolute STD percentage (Figure 4D) of signals (ranging from 1.6% for H2 Gal and H7 NeuAc, to 2.8% for Acetyl groups) suggest a stronger interaction of the ligand with a particular involvement of the sialyl group.

A strong interaction of Acetyl groups and a weak involvment of the Galactose residue are common point with both cell lines. These results recapitulate the known features of the sialyl(α2-6) derivatives. In particular, the binding mode observed by X-ray in presence of H1 (Liu *et al*, 2009) and the strongest affinity of short α2-6 saccharides for H5 subtype (Chandrasekaran *et al*., 2008). Our data also confirm that: i) hemagglutinin is expressed in its native/active conformation on the HEK cell membrane and ii) that these cells are good tools for rapid screening and structural optimization of HA antagonists.

Discussion

The rapid genomic variability of the influenza virus requires a continuous effort in the development of anti-viral molecules, to circumvent the emergence of resistant strains. A key tool for the rational design of such anti-viral leads is STD-NMR, which can map the interactions of the epitope as a function of variation of the target molecule at atomic scale. In this paper, we developed a rapid and reliable new method for screening sialic acid-related hemagglutinin ligands in an environment which is much more similar to that *in vivo* than using the purified protein or the popular VLP.

We tested the method to analyze the interaction of sialyl trisaccharide **1** with 293T cells transfected with H1 and H5 subtypes of hemagglutinin, responsible for human and bird flu respectively. The key feature provided by this method is that HA molecules transfected on HEK-293T cells membrane retain their native conformations (as evidenced by the reactivity with monoclonal antibodies specific for conformational stem epitopes) and their binding properties (evidenced by the rosettes formed with chicken erythrocytes). With this approach, we are able to detect the binding event and the epitope, both valuable informations for drug design and we suggest that this could be a suitable effective method to screen other ligands which might act as antagonists of possible pharmacological relevance. Three different cell lines (untransfected 293T, H1-293T and H5-293T), suspended in phosphate buffer,

were tested to produce control STD-NMR spectra, suggesting that no subtraction is necessary. This is a remarkable improvement over the use of VLPs, which produced noise signals from nonbinding ligands (i.e. unavoidable medium components) (Haselhorst *et al*, 2008). The absence of cells residual effect makes the STD signals clearer and easily detectable.

Making use of STD-NMR spectra of Neu5Ac-α-(2,6)-Gal-β-(1-4)-GlcNAc in presence of human H1 and avian H5 we could rationalize at atomic level the different binding properties to the two strains. Currently, avian H5N1 viruses have high α2-3 glycan specificity but some strains also show mixed α2-3 /α2-6 binding (Yamada *et* al., 2006), but have not yet transmitted between humans at epidemic levels. Recent studies based on analysis of HA–glycan cocrystal structures (Bewley, 2008; Chandrasekaran *et al*, 2008) have highlighted the importance of glycan conformation and topology in governing the receptor specificity of avian- and human-adapted HAs. Our results suggested that α2-6 human receptor is able to bind avian H5 with a wider epitope relative to human H1. In fact, in presence of H5, the Neu5Ac residue at the nonreducing end is strongly involved in the interaction. These data are in agreement with the observation, reported by Chandrasekaran *et al*., that also avian viruses are able to bind α2-6 saccharides, since they conclude that human adapted viruses (H1) may be able to bind long (α2-6) derivatives preferentially while avian viruses (H5) bind (α2-3) and short (α2-6) saccharides.

We think that further applications of this studies can bring to a better understanding of HAs – glycans binding features and can be useful for the design of new anti-influenza drugs.

Materials and Methods

Compound Neu5Ac-α-(2,6)-Gal-β-(1-4)-GlcNAc was purchased by Carbosynth Ltd, Berkshire (UK).

Construction of stable hemagglutinin-expressing transfectant cell lines

Constructs pcDNA3.1-H1 and pcDNA3.1-H5 plasmids, carrying the hemagglutinin genes from influenza viruses A/California/7/2009_H1N1 and A/*Cygnus olor*/Italy/724/2005_H5N1, were obtained by subcloning the genes (Di Lullo et al., 2009) into the MCS of the commercial plasmid pcDNA3.1(+) (Invitrogen), in which gene expression is dierected by the CMV promoter. The human cell line HEK-293T (ATCC CRL-3216) was transfected with plasmid

DNA (10 μg/ml. Transfectants were selected in G418-containing medium and passaged 3 times in the same medium. HA-transfectants show unaffected growth rate and mortality as compared to untransfected HEK-293T. Cells expressing HA on the cell surface were sorted (by FACS) with anti-HA stem monoclonal antibody CR6261 (Friesen et al., 2010) (Acrobiosystems, USA), specific for the native conformation, and expanded as the stable transfectant cell lines H1-293T and H5-293T. PCR (with serotype-specific primers), Western blot (in reducing conditions, with chicken anti-HA antisera), flow cytometry (with CR6261), immuno-precipitation with CR6261 (followed by western blot analysis in non-reducing conditions) were carried out by standard methods. For the hemagglutination rosette assay with chicken erythrocytes (ChRBC), H1-293T, H5-293T, or untransfected 293T cells were mixed with ChRBC, spun into a pellet, resuspended and spun through a Histopaque cushion (293T cells float on Histopaque-1.077 (Sigma-Aldrich), while ChRBC form a pellet). HA-dependent 293T rosettes were recovered from the red pellet.

NMR experiments

All NMR spectra were registered on Bruker Avance 600 MHz using 7–9 mM solutions of the ligand in deuterated phosphate buffer (pH 7.4). The assignment was performed through one- and two-dimensional ^{1}H and ^{13}C NMR spectra by standard manual method (Wuthrich, 1986; Vasile *et al*, 2008). The complete assignment of the molecule is reported in Table I. The proton resonances did not show significant shifts when the compound was analyzed in the presence of the cells suspension. For the conformational analysis, we recorded NOESY with 700 ms and tr-NOESY with 200 ms mixing time. ^{1}H and NOESY experiments were performed using an excitation sculpting sequence for solvent suppression. The STD–NMR spectra were acquired in the presence of about $10x10^{6}$ T293 untransfected and transfected cells in a total volume of 200 mL, using the Watergate sequence for water suppression and varying the saturation times from 0.98 to 2.94 s. The on-resonance irradiation of the protein was performed at a chemical shift of -0.05 ppm. Off-resonance irradiation was applied at 200 ppm, where no protein signals were visible. Negative controls were performed to avoid the presence of signals in the blank or artifacts. STD spectra of the free ligands and of the cells in absence of ligand did not show any signals. Velocity of sedimentation of the cells in the NMR tube was not studied but the cells are maintained in suspension thanks to the rotation into the NMR probe(the spin rate is 20 revolutions/second = 20 Hz).

NMR experiments were acquired at 298 K and repeated at 283K on free ligand in order to reduce the mobility and to observe NOEs useful for conformational analysis. At 298 K, due to the high mobility of this small saccharide, we detected only an intra-residual NOE (NeuAc H3eq/H3ax), at 283K instead we detected several intra-residual NOEs (NeuAc H3eq/H3ax, NeuAc H3eq/H4, NeuAc H5/H3ax, Gal H1/H3) and one inter-residual NOE (NeuAc H5/ GlcNAc $COCH_3$) and we used it for the conformational analysis.

STD experiments were performed at 298K and also in this case they were repeated at 283K as attempt to slow down the magnetization exchange and to obtain an enhancement of the signals. The STD experiments discussed herein were conducted at 298 K with a total irradiation time of 2.94 s.

Conformational analysis

The molecular dynamic simulations are performed with Gromacs 4.6.5 (Hess *et al*, 2008). The interactions of the molecule are modelled with the GAFF force field (Wang *et al*, 2004, Vasile *et al,* 2017) in explicit water. After energy minimization and water thermalisation, we performed 50ns of simulation at 300K, recording 1000 conformations to be analyzed.

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Figure 1. Structure of Neu5Ac-α-(2,6)-Gal-β-(1-4)-GlcNAc **1** (**A**)**.** Representative structure of the bent conformation in which the distance between NeuAc H5/ GlcNAc $COCH_3$ is lower than 0.5 nm, compatible with the presence of the NOE (the population of this family of structure is estimated around 15%) (**B**). Representative structure of a more extended conformation (**C**).

Figure 2. (A) Fluorescence-Activated Cell Sorting with Mab CR6261 of H1- and H5-transfectants of HEre collected from "R4" sorting windows. (B) PCR analyis of HA DNA from H1-293T and H5-293T cell lines; subtype-specific primers reveal a short internal fragment of the H1 gene and a full-length fragment of the H5 gene. Positive and negative controls (C^+ an C^-) are the donor plasmids and untransfected 293T cells, respectively. (C) Flow cytometry analysis, with Mab CR6261, shows that more than 85% of the cells are HA-positive in both cell lines. (D) Western blot analysis of whole cell lysates, in reducing (+DTT) and non-reducing (-DTT) conditions, shows that the proteins are synthetised as monomers and aggregated into dimers and trimers. (E) Immuno-precipitation with CR6261 of membrane proteins solubilized by Triton X-100 extraction, followed by western blot analysis in non-reducing conditions. (F) H5-293T cells (and H1-293T cells, not shown) agglutinate chicken red cells forming rosettes.

Figure 3. **A)** ^{1}H-NMR of 6'-α-sialyl-N-acetyllactosamine in deuterated phosphate buffer pH=7.4 (T=298K). **B and C)** STD-NMR experiments of 6'-α-sialyl-N-acetyllactosamine in presence of H5-293T and H1-293T cells respectively. **D)** STD-NMR experiments of 6'-α-sialyl-N-acetyllactosamine in presence of untransfected cells. NMR experiments were performed using a Bruker 600 MHz spectrometer, STD spectra were obtained with - 0.05 ppm as irradiation frequency and 2.94 s of saturation time.

Figure 4. The protons belonging to the epitope of compound **1** in the presence of H1-293T cells are marked with dots (**A**) and the absolute STD percentage for each protons is reported (**C**). The same compound analyzed in presence of H5-293T shows a stronger affinity: the epitope comprises several protons (**B**) with a higher intensity of absolute STD % (**D**).

Table I. NMR assignment of Neu5Ac-α-(2,6)-Gal-β-(1-4)-GlcNAc in deuterated phosphate buffer (pH=7.4).

		^{1}H (ppm)*	^{13}C (ppm)
NeuAc	3	2.58 (eq), 1.63 (ax)	39.4
	4	3.56	67.5
	5	3.74	54.9
	6	3.62	71.6
	7	3.48	67.6
	8	3.89	69.0
	9	3.79, 3.56	61.9
	CH_3	1.94	21.8
Gal	1	4.36	102.6
	2	3.46	70.0
	3	3.6	71.8
	4	3.84	67.9
	5	3.74	72.9
	6	3.91, 3.46	62.8
GlcNAc (α)	1	5.12	89.7
	2	3.85	52.6
	3	3.81	70.9
	4	3.58	80.2
	5	3.54	73.9
	6	3.79	59.3
	CH_3	1.98	22.0
GlcNAc (β)	1	4.66	94.0
	2	3.64	55.3
	3	3.81	70.9
	4	3.58	80.2
	5	3.54	73.9
	6	3.83, 3.73	59.6
	CH_3	1.98	22.0

* The spectrum is referenced through the solvent lock (2H) signal according to IUPAC recommended secondary referencing method.