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REVIEW

Carbohydrate–protein interactions and multivalency: implications for the inhibition of influenza A virus infections

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ABSTRACT

Introduction: Protein–carbohydrate interactions play a very important role in many biological processes. A single interaction between a protein and a carbohydrate is usually weak, but multivalent ligands can compensate for this deficiency by binding multiple binding sites to one biological entity simultaneously. Over the past few years, numerous efforts have been made for the design and synthesis of carbohydrate-based multivalent ligands thereby serving as potent inhibitors for pathogens such as the influenza A virus.

Areas covered: In this review, the authors cover a variety of multivalent systems from small to large molecules which showed a potent inhibitory effect against several pathogens.

Expert opinion: Scaffold structure, linker type, and ligand density are important parameters that need to be optimized for potent multivalent inhibitors. The challenges of multivalent glycodrugs include issues such as bioavailability, pharmacokinetics, and immunogenicity which greatly depend on where the compounds are used in the body. Anti-flu (influenza) applications in the lungs using multivalent carbohydrates particularly has potential because of the high binding affinities. With much more research focusing on Influenza A virus inhibition, therapeutic applications may be achieved in the near future.

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Carbohydrate-protein interactions; influenza A virus; hemagglutinin; multivalent inhibitors

1. Introduction

Carbohydrate–protein interactions are at the heart of many important biological processes [1–3]. These interactions play a key role in the binding of bacteria, viruses, and toxins to cell surfaces. The numerous glycan-binding proteins on the surface of these microbes enable the binding or adherence to glycosylated cell surfaces as the first step of the infection process, and constitute a major threat to human health. One of the infectious diseases that are transmitted using carbohydrate–protein interactions is the influenza infection (known as the flu), where an initial sialic acid-dependent binding to the host cell surface is a necessary requirement to trigger the infection [4].

A single interaction between a protein and a carbohydrate is usually weak, but multivalent ligands can compensate for this deficiency by binding two or more binding sites simultaneously. Over the past few years, advanced by numerous biochemical studies, we obtained a better understanding of multivalent interactions which are of importance to the modulation of many critical biological systems [5]. In this review, we will discuss the importance of the multivalency effect in carbohydrate–protein interactions and how this could help to discover new drugs to treat or prevent the influenza infection.

2. Multivalency effects in carbohydrate–protein interactions

2.1. Mechanisms of multivalent binding

There are several possible mechanisms that can contribute to the potency of multivalent interactions: the chelate effect, subsite binding, statistical rebinding and also aggregation (Figure 1) [6,7].

In a multivalent system, lowered entropic barriers may favor binding between the ligand and the protein and thus lead to higher affinity or inhibitory potency [8]. In the binding of a multivalent ligand, the translational entropic cost is paid by the first binding event and all the subsequent binding interactions can proceed with smaller entropic penalties. As a result, due to the lower entropic cost in total, stronger binding may occur. This mechanism involving simultaneous binding of two or more (sub)ligands is commonly named as the chelate effect [9]. Subsite binding is another type of chelation that involves secondary binding interactions with a receptor other than the primary binding site. With the additional binding event, the multivalent ligands also become more effective [10]. When only one binding site is involved in an interaction, multivalent ligands can still show increased inhibitory potencies due to the high local concentration of subligands around that binding site, which is referred to as the

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Article highlights

- Carbohydrate-protein interactions are involved in many processes including those that cause disease.
- Multivalency in the design of inhibitors can dramatically increase inhibitory potencies.
- Multivalent carbohydrates haven been demonstrated effective in selected animal models and have progressed in one case to clinical trials.
- The hemagglutinin (HA) protein of Influenza virus A is an important and challenging target protein.
- Multivalency approaches are starting to become successful in addressing HA.

This box summarizes key points contained in the article.

mechanism of statistical rebinding. This effect is caused by the slower off-rate of the multivalent ligand, due to the non-bound subligands rapidly replacing the bound subligand after it releases. This may result in a higher binding affinity. Instead of intramolecular association, large aggregation (cross-linking) of intermolecular binding is often observed. This effect is dependent on factors like concentrations, valencies, binding affinities, different kinds of interactions, etc. Together, these different modes of binding give us unique information of multivalent carbohydrate–protein interactions.

2.2. Carbohydrate-based multivalent inhibitors

Carbohydrates play a central role in living organisms as recognition markers to enable biological processes like cell adhesion, fertilization, differentiation, and tumor-cell metastasis through carbohydrate–protein interactions [11–13]. Pathogens take advantage of the carbohydrates for cell adhesion and the subsequent infection or invasion of the host. Furthermore, certain microbes mimic the host cell glycans which facilitates their evasion from the immune response.

The discovery of the diverse biological roles of carbohydrate–protein interactions is increasingly of interest in the search for applications in drug discovery. By using ligands to mimic the carbohydrate binding sites and block the recognition or adhesion process, many infectious diseases might be prevented. However, the binding affinity between an individual carbohydrate and a target protein is relatively weak; therefore, inhibitor design is directed towards using the multivalency effect. As there are multiple copies of the glycoligands presented on the surface, an optimized multivalent ligands could bind to the target protein much stronger than the monovalent ligand. By taking advantage of this effect, recent progress has been made in the design and synthesis of carbohydrate-based multivalent ligands thereby serving as powerful inhibitors.

This can be exemplified with the lectin LecA, one of the virulence factors of the problematic pathogen *Pseudomonas aeruginosa*. LecA is a tetrameric lectin specific for binding with oligo- and polysaccharides containing a terminal α-D-galactose and an interesting target for the design of multivalent carbohydrate-based inhibitors. The shortest distance between two neighboring binding sites of LecA is *ca*. 26 Å [14]. Guided with the structural information, several multivalent inhibitors like glycopeptide dendrimers [15], calixarenes [16], resorcinarenes [17], fullerenes [18] and cyclooligosaccharides [19] were designed and tested which resulted in potent inhibitors. A series of divalent ligands was reported by our group, presenting galactose residues with variations in the spacer length between them to fit the divalent binding mode (Figure 2) [20–22]. Linear and relatively rigid spacers based on glucose-1,2,3-triazole alternating units linked via 'click' chemistry were developed. Divalent galactoside **1** exhibited an IC_{50} value of 0.12 μM for LecA, which was 484-fold stronger in relative potency per sugar compared to a monovalent reference compound. A slightly shorter and more rigid version (compound **2**) exhibited a much more potent inhibition, which was over 7500-fold (IC_{50} = 2.7 nM) stronger than that of the monovalent galactose derivative (IC_{50} 22 μM). This finding could be explained by the design of compound **2** which contains the perfect spacing between the two galactosides to match to the dimeric geometry of LecA.

Cholera Toxin, an AB_5-subunit toxin, consists of a single disease-initiating A-subunit and five lectin-like B-subunits [23]. The ring-shaped pentameric B-subunits play an important role to trigger the attachment of the toxin to the cell surfaces of the intestines by recognizing the exposed part of the GM1-ganglioside, i.e. the GM1os (GM1 oligosaccharide) moieties and then facilitate the endocytosis process [24]. Multivalent glycoconjugates based on different carrier platforms like pentacyclen [25], calixarenes [26], corannulenes [27], even CTB (cholera toxin B-subunit) itself [28] have successfully shown effective inhibition of the CTB binding.

It was reported by our group that highly effective dendrimers targeting CTB were prepared, which combined three

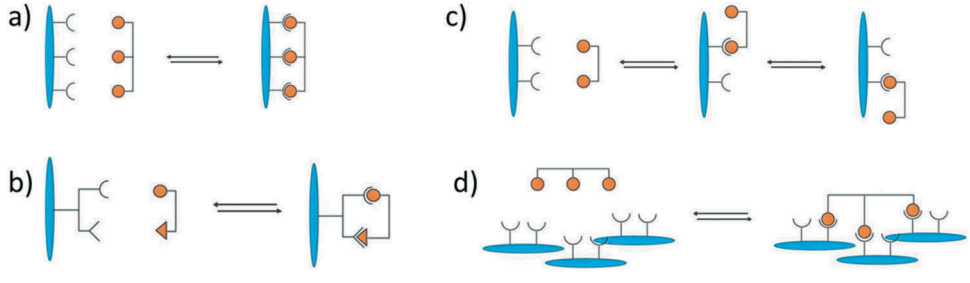

Figure 1. Mechanisms of multivalent binding. (a) chelate effect; (b) subsite binding; (c) statistical rebinding; (d) aggregation (cross-linking).

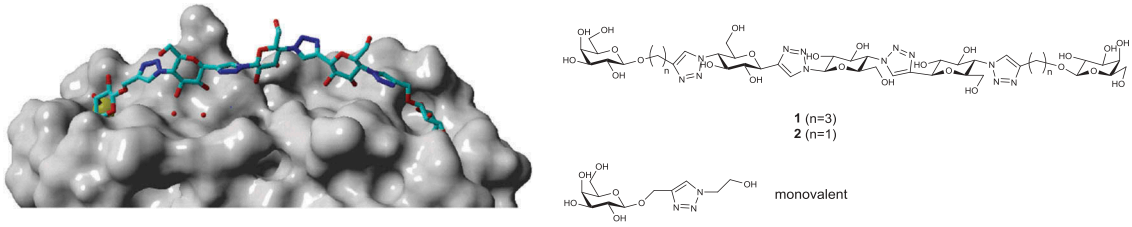

Figure 2. Divalent inhibitors based on the structure of lectin LecA (PDB ID: 4YWA).

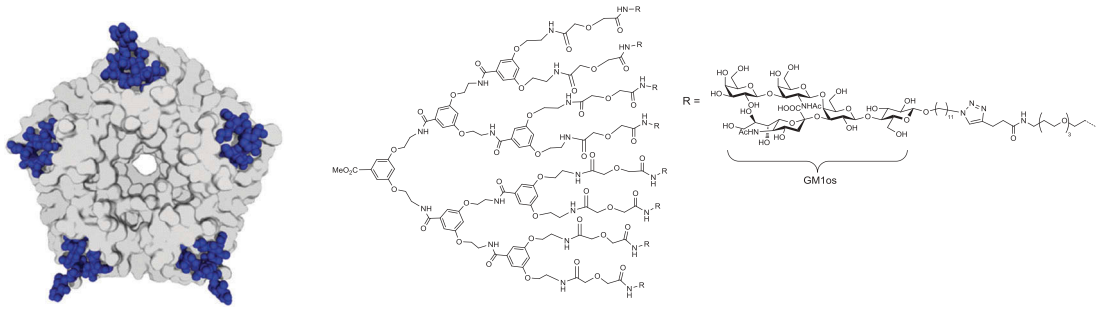

Figure 3. Glycodendrimer targeting the cholera toxin B-subunit (PDB ID: 3CHB, CTB with 5 bound GM1os molecules).

important parts in the design for improving binding affinity: multivalent dendritic scaffolds, the GM1os ligand, and elongated flexible linkers with optimal length (Figure 3). The building blocks were efficiently coupled via 'click' chemistry to give a variety of dendrimers which showed different effects. The best dendrimer which contained eight GM1os ligands exhibited a 47,500-fold enhancement per GM1 derivative compared to monovalent GM1os [29–31].

These results of multivalent inhibitor design strategy is strongly to encourage the use of multivalent glycoconjugates in other carbohydrate-related therapies such as viral and fungal infections. A water-soluble globular fullerene [32] was employed as a biocompatible scaffold and decorated with 120 peripheral carbohydrate subunits using 'click' chemistry to build a multivalent inhibitor for the Ebola virus. This giant molecule was produced in a minimum of synthetic steps, and infection assays showed that the fullerene inhibited the cell infection in the subnanomolar range (IC_{50} = 0.667 nM). Multivalent carbohydrates have also been moved forward towards medical applications. The glycopeptide MK-2640 contains four fucoside moieties on an insulin molecule that modulate its availability by binding to the mannose receptor C-type 1 depending on blood glucose levels [33,34]. Furthermore, two studies involving multivalent versions of the Gb_3 carbohydrate were shown to be effective in mouse studies [35,36], while related compounds showed promising effects against *Streptococcus suis* in mice [37].

Meanwhile, as one of the most common diseases all over the world, flu is caused by an infection of the influenza virus, which draws more and more attention to researchers to investigate how to prevent the viral infection by using multivalent carbohydrate-based inhibitors. We are going to discuss these studies in detail.

3. Multivalent strategies for the inhibition of the influenza A virus

3.1. Influenza A viruses

Influenza A viruses (IAVs), which infect the epithelial cells of the respiratory tract, cause the highly contagious flu to humans. The regularly recurring seasonal influenza results in substantial morbidity and the deaths of 250,000–500,000 people each year, with children and the elderly representing the majority of the victims. Worldwide pandemics can cause even more severe mortality, such as in 1918 when approximately 50 million deaths were attributed to the Spanish flu [38].

Different subtypes of IAVs are labeled according to the combinations of different virus surface proteins: 18 hemagglutinin (HA) subtypes and 11 neuraminidase (NA) subtypes. For example avian influenza 'bird flu' virus H5N1 and H7N9 or swine influenza 'swine flu' virus H1N1 and H3N2. Occasionally, viruses are transmitted from wild aquatic birds to domestic poultry, and this may also cause an outbreak or give rise to a human influenza pandemic.

3.2. Infection process of IAVs

Typically, to initiate an infection, the virus adheres to the target host cell by using its surface glycoprotein HA to recognize glycoconjugates that contain terminal α-(2,3)- or α-(2,6)-linked sialic acid residues. Following receptor binding, the viruses are endocytosed, leading to their residency in intracellular vesicles. Then, the low pH-induced fusion of viruses occurs from the endosomal compartment, releasing the uncoated viral genome into the cytoplasm, followed by replication in the nucleus [39]. Budding of new virion takes place after the replication, where the membrane is generated from

the host plasma membrane and contains the viral transmembrane proteins. Finally, The glycosidase enzyme (NA) removes the sialic acid group from the glycan which allows the newly produced viruses to release from the cell surface and help the viruses to spread [40,41].

3.3. Current treatment for IAV infection

Interference with the effects of IAV is of importance for both humans and animals. The current options to combat IAV infection include vaccination and two classes of antiviral compounds, the M2 ion channel blockers and the NA inhibitors. Vaccination is a valuable clinical approach for the seasonal IAV variants which are usually life-threatening for children and the elderly. The more dangerous IAV variants are commonly seen in epidemics when we need other kinds of anti-viral agents to tackle these more severe cases. In those cases, oseltamivir (Tamiflu®) and zanamivir (Relenza®) are successful carbohydrate-based drugs that block the function of NA and significantly inhibit the release of IAV [42]. The M2 ion channel has also been targeted by a class of drugs referred to as the adamantanes, which include amantadine (Symmetrel®) and rimantadine (Flumadine®) (Figure 4).

Unfortunately, a major problem that has been observed with both classes of antiviral drugs is the rapid emergence of drug-resistant strains of IAVs. And the vaccines that were developed also can't prevent the flu epidemics effectively. Thus, novel IAV inhibitors with new mechanisms of action to combat the virus would be of great value [43,44].

3.4. Multivalent interactions between HA and the host cell

As we have already gained the knowledge of the life cycle of IAV, several therapeutic targets should be paid more attention to. Among them, HA is one of the most appealing targets present with ca. 300–400 copies on the viral surface. This adhesion protein contains three symmetrical binding pockets (Figure 5). The spacing between HA trimers is ca. 100 Å from center to center, while the distance between binding pockets within a trimer is ca. 42 Å [45]. HA binds to sialylated glycans: α2,6 linked sialyl N-Acetyllactosamine (α2,6-SiaLacNAc) binds specifically to human-type-specific HA while α2,3-SiaLacNAc binds to avian-type-specific HA [40,45,46], both with weak affinities in the millimolar range. In order to increase the overall strength of the interaction between the virus and the host cell surface, multiple HA molecules on the virion surface bind to various glycoproteins based on the multivalency effect [5]. In that sense, it is a logical and attractive strategy to block the viral infection with a multivalent sialic-acid-based inhibitor to mimic the binding between the HA and the cell surface glycans, to prevent the virus infection.

3.5. Multivalent inhibitors targeting HA

3.5.1. Polymeric inhibitors

To increase the potency of synthetic HA inhibitors, several approaches for polyvalent sialoside systems have been tested. Large molecular entities, e.g. functionalized polymers [47–53], chitosan [54], liposomes [55,56], nanostructures [52], and dendrimers [57], showed their inhibitory advantages by interacting with multiple HA trimers simultaneously and/or binding to more than one binding site within an HA trimer.

Matrosovich and coworkers [47] synthesized a polyvalent inhibitor composed of anomeric aminobenzylglycosides of N-Acetylneuraminic acid (Neu5Ac) linked to a polyacrylate carrier, which is the first example of a totally synthetic and comparatively potent inhibitor of HA. The macromolecular carrier containing 10 mol% of Neu5Ac showed a 2040-fold potency increase per sugar compared to the corresponding monovalent benzylsialoside.

The group of Whitesides designed an α-sialoside linked to acrylamide [48,50], which formed high molecular weight copolymers. These polymeric inhibitors showed 10^4–10^6 times enhanced potencies when compared to the reference α-methyl sialoside. They concluded that for polymers containing a high density of sialic acid, the inhibition happened both by binding the HA binding pockets and the NA active sites.

The group of Wong reported a lysoganglioside GM3 (lyso-GM3) conjugated to the polymer poly-L-glutamic acid [58]. This polyvalent inhibitor contained a hydrophobic part, which could interfere with the fusion process and gave a picomolar inhibition (IC_{50} = 1.9 pM). They also designed a series of *p*-nitrophenyl α-*O*-glycosides of *C*-3 modified sialic-acid liposomes which acted as inhibitors for both of HA and NA [56]. A 1000-fold enhancement was observed in the binding affinity assay against A/Puerto Rico/8/1934 (H1N1) and A/Aichi/2/1968 (H3N2), indicating that liposomes are effective platforms for the development of multivalent IAV inhibitors.

Yeh and coworkers [55] also developed an efficient multivalent system based on the conjugation of phospholipids to the thio(*S*)-linked glycoligand (*S*-α2,6-SiaLacNAc). The self-assembled liposomes were found to have potent inhibitory activity. Effects were shown in both the virus neutralization assay (EC_{50} = 71 ± 5.5 μM) and the hemagglutination inhibition assay (K_i = 125 μM) where sialic acid did not inhibit, and they could also effectively interfere with the entry of the H1N1 into MDCK cells.

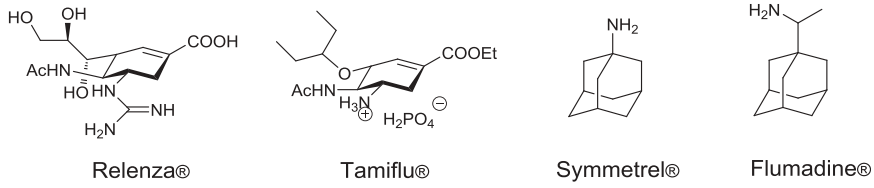

Figure 4. Current anti-viral drugs approved for clinical use.

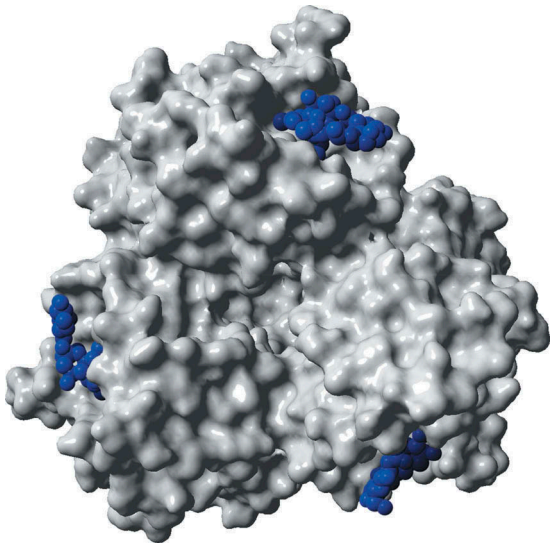

Figure 5. Top view of the binding between the trimeric HA and 3 bound α2,6-SiaLacNAc molecules (PDB ID: 3UBN).

The group of Rainer Haag used biocompatible nanoparticles as scaffolds for multivalent HA inhibitors. They first reported sialic-acid-coated glycerol dendrons immobilized on 2 nm and 14 nm gold nanoparticles [59]. The 14 nm particles exhibited inhibition in the nanomolar range in a hemagglutination assay while the 2 nm particles had no inhibitory effect. Later, they also described a series of sialic acid-conjugated, hyper-branched polyglycerol-based nanoparticles [52] with diameters in the 1–100 nm range. Their 50 nm particle were 7000-fold more effective than the 3 nm one which had similar sugar concentrations. The further increase of particle size did not lead to the corresponding increase of inhibition effect but to a reduced activity. Their work emphasized the importance of matching particle sizes and ligand densities to mimic the virus surfaces and achieve maximum efficiency.

Kwon and coworkers [60] designed multivalent 6′-sialyllactose-(6′SL) moieties by linking them to polyamidoamine (PAMAM) dendrimers with a 1.2–3.1 nm distance between multiple sialic acid ligands. The conjugates with spacings of around 3 nm showed the strongest inhibition effect of HA trimer binding and H1N1 infection both *in vitro* (IC_{50} = 1.7 μM) and *in vivo*. As the distance between 6′SL binding sites in the trimeric HA is around 4 nm, 3.1 nm distance within a flexible framework, seemed fit to the binding sites of HA the best within this series and enhanced the binding affinity the most. Therefore, modulating the interligand spacing was shown to be an important aspect, more so than the valency of the HA inhibitors.

Li and coworkers [54] presented two novel 3′-sialyllactose (3′SL) modified chitosan-based materials: a water-soluble polymer and a functionalized fiber, targeting viral adhesion inhibition and decontamination. A surface plasmon resonance study revealed that the monovalent 3′SL had no visible binding with the HA at 1 mg/mL (1.57 mM) or less, while the 3′SL-chitosan conjugates showed higher avidity (K_d = 0.59–1.93 μM). The result demonstrated that multivalent presentation of 3′SL ligands on a chitosan skeleton is effective in enhancing the binding of HA.

Dendrimers are alternative scaffolds of potential synthetic inhibitors to prevent IAV infection. Reuter and coworkers [61] evaluated a series of dendrimers such as linear, linear-dendron copolymers, comb-branched, and dendrigraft polymers, for their ability to inhibit HA and to block virus infection *in vitro*. The dendrigraft inhibitors showed up to 50,000-fold increased activity against the IAVs (IC_{50} = 0.1 μM) and do not exhibit cytotoxicity to MDCK cells at therapeutic concentrations. Roy and coworkers [57] synthesized water-soluble dendritic α-thiosialosides using hyperbranched L-lysine scaffolds which achieved inhibition of HA at a concentration of 19 μM. These dendritic macromolecules could mimic multi-antennary glycoproteins and showed inhibition properties of HA and NA. Bhatia and coworkers [53] compared the linear and dendritic polyglycerol sialosides (LPGSA and dPGSA) *in vitro* and *in vivo*, which differed with respect to molecular weight and number of sialic acid units. The linear LPGSA inhibited IAV infection with an IC_{50} in the lower nanomolar range, which was better than the dendritic dPGSA. LPGSA also exhibited potent activity against two avian influenza strains and showed low toxicity. This study revealed that high ligand densities are not necessary for designing effective IAV inhibitors. Similarly, a carbosilane dendrimers with valencies of four was shown to be 60-fold more potent than a monovalent reference compound [3].

Xiao and coworkers [62] prepared several pentacyclic triterpene – cyclodextrin conjugates using the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction. The heptavalent cyclodextrin conjugate bound tightly to HA with a K_d of 2.08 μM, which also showed a 125-fold potency enhancement of its IC_{50} value (1.60 μM) over the corresponding monomer. These multivalent inhibitors exhibited potent antiviral activity against H1N1 virus (A/WSN/33), even equivalent or superior to oseltamivir, with a lower cytotoxicity, and also exhibited broad-spectrum inhibitory activity against the two other human influenza viruses A/JX/312 (H3N2) and A/HN/1222 (H3N2) with IC_{50} values of 2.47–14.90 μM.

In many of the mentioned cases, the sialic acid moieties have the natural O-linkages. These can in principle be cleaved by the neuraminidase also present on the virus. This is not reported in all studies, as e.g. only HA may have been used in the assay. There are however several studies in which S-linked sialic acids are used, and these are the most likely ones to become part of therapeutics, as they are stable to the neuraminidases [52,53,55,57,63–66], however, C-glycosides have also been used [49].

3.5.2 'Small' molecule inhibitors

In the macromolecular approaches, structural information on the spatial arrangement of the trimeric binding sites of the HA protein is not usually taken into account for the design of the multivalent inhibitors. It was found that smaller molecules containing proper carbohydrate units can in some cases be effective IAV inhibitors depending on whether their topological design allows them to bind simultaneously to several binding sites of a single HA trimer [6,67], or even to binding sites on nearby trimers.

Bridging binding sites within an HA trimer is a challenging task when using relatively small molecules, while their activity may also come from bridging HA trimers on the densely packed viral surface. The latter phenomenon was supposed to be the cause of enhanced inhibition in early experiments with divalent sialosides linked by spacers with varying lengths and flexibilities, since the enhancements (100-fold) would only be observed in the assay with whole viruses and not with just the HA trimer [68]. Similarly, a 10-fold enhancement was exhibited with a system containing two Neu5Acα2-6Galβ1-4GlcNAc (i.e. α2,6-SiaLacNAc) units linked to a single galactoside moiety [69], the bivalent trisaccharide ligands would give the structures more rigidity when anchored to the sugar hydroxyls of a galactose residue. Marra and coworkers [64] synthesized tetra- and octavalent sialoside clusters via multiple 'click' reactions of a propargyl thiosialoside with calix [4]arene polyazides. The calix-sugars were shown to inhibit the hemagglutination and the viral infection at submillimolar concentrations.

Ohta and coworkers [70] reported a chemoenzymatic synthesis of mono-, bi-, and tri-substituted (schematically shown as **6**, Figure 6) involving cyclic peptides presenting three α2,3-SiaLac units. Only the bi-, and tri-substituted derivatives showed significant affinity to HA with K_d's of 1.6 mM and 0.63 mM, respectively.

Feng and coworkers [71] designed trivalent sialyllactoside inhibitors against IAV strain A/Puerto Rico/8/1934 (H1N1) based on trisphenol and trisaniline skeletons. The flexible hydrophilic spacer between 3'SL and the scaffold was made up of polyethylene-glycol units. Trisphenol-sialyllactose showed a significant inhibitory effect at 400 μM in a plaque reduction assay with MDCK cells while unexpectedly, both of the trisphenol and trisaniline sialyllactosides exhibited no hemagglutination inhibition activity.

Waldmann and coworkers [72] described the computer design, chemical synthesis and binding analysis of a trivalent glycopeptide inhibitor **4**, which has a scaffold of trisubstituted benzene ring linked to sialic acid residues via peptide-based spacers. This compound bound much stronger to avian influenza H5N1 (A/Vietnam/1203/2004) (K_d = 446 nM) comparing to 2-O-methyl-α-Neu5Ac, but only 4.9-fold better than the monovalent glycopeptide. Most of the effect was not due to multivalency but to a combination of sialic acid and peptide binding, the latter of which was shown to bind HA even stronger that the sialic acid derivative.

Yang and coworkers [65] prepared a series of multivalent conjugates based on thiosialoside ligands and human serum albumin or bovine serum albumin to enhance the binding affinity to HA and NA. The synthetic glycoproteins with a higher density of the S-sialoside on the protein surface had a higher affinity for HA (K_i = 3.13–50 μM) than the di-, tri-, tetravalent clusters they synthesized, likely due to the higher valency and larger size of the protein scaffold vs the small molecules. The low valency sialoside design with short spacer arms was not able to reach three binding sites of HA indicating that proper spacing between the trivalent inhibitor arms is an important design aspect.

The three-armed system **5** was based on three-way junction DNA as a well-defined rigid scaffold for the display of α2,3-SiaLac units [73], This DNA molecule had a similar topology to the sialic acid binding sites on HA. Evaluation in whole virus hemagglutination inhibition experiments indicated strong enhancement, i.e. 8.0×10^4-fold higher potency for IAV A/Puerto Rico/8/1934 (H1N1) over free 3'SL, but only a factor of 8 (i.e. less than 2 per sugar) over a reference compound that only has a glycoligand in one of its three arms. This indicates that multivalency effects were minor but that HA binding to the DNA component was an important factor contributing to the observed enhancements.

A system based on rigid self-assembled peptide nucleic acid (PNA)-DNA complexes displaying two α2,6-SiaLacNAc units at various distances was also reported [45]. An optimal distance between the glycoligands of approximately 50 Å was observed with a 30-fold enhancement (15-fold per sugar) over a DNA-PNA reference architecture containing only a single glycoligand. The data were also in agreement with experiments using whole virus and hemagglutination inhibition assays. Notably, more flexible

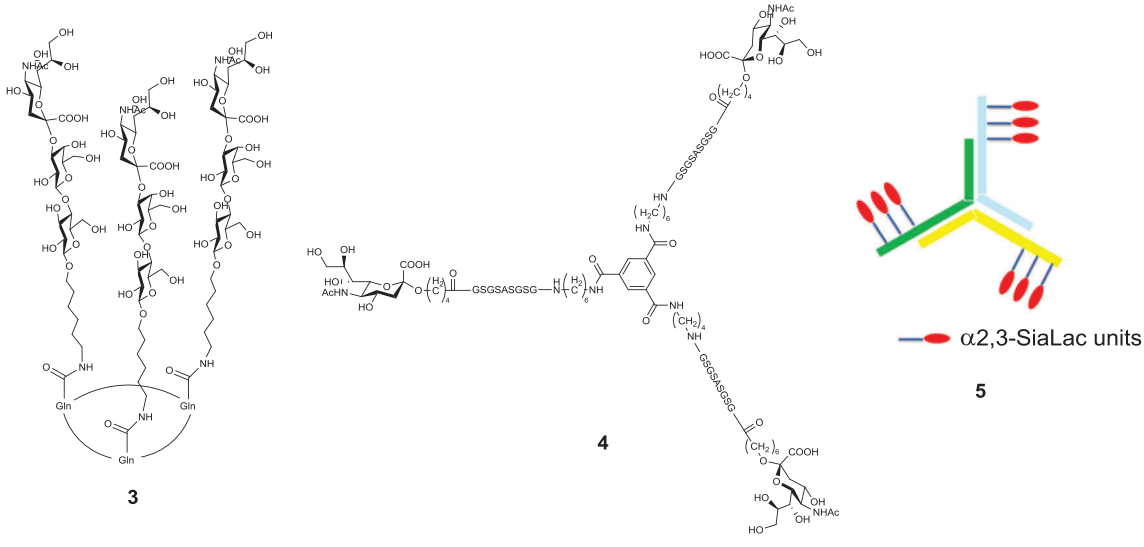

Figure 6. Trivalent inhibitors of HA protein.

polyethylene glycol linked molecules showed no enhancement which was supported by a theoretical model indicating that the distance-affinity relationships for interactions of the HA with bivalent SiaLacNAc ligands.

4. Conclusions

Multivalent protein-carbohydrate interactions play a very important role in the pathogen adhesion to cells. Understanding the unique interactions on the molecular level is of prime importance for developing optimized multivalent ligands to achieve strong inhibitory effects. Suitable protein targets with relatively close binding sites such as the influenza A virus HA have been investigated.

Although there are already two families of antiviral drugs being used to treat IAV infections, the emergence of drug-resistant viral strains makes it more urgent to find other ways to block the infection. Despite the research focusing on vaccines [74–76] and NA [77–81], HA is an important target for investigating new IAV inhibitor strategies. Larger molecular entities such as glycopolymers, nanoparicles, dendrimers, DNA moieties, taking advantage of their size to bridge multiple HA trimers, have exhibited high inhibitory potencies. On the other hand, relatively small trivalent molecules also showed potential based on proper structural design, which may have advantages for development into clinical drugs. All these examples provide inspiration for investigating multivalent ligands as new antiviral drugs and also as drugs for other diseases.

5. Expert opinion

Proteins that bind to carbohydrate structures based on a multivalency effect represent a vast potential for the development of clinical therapeutics. In order to design a successful multivalent carbohydrate-based inhibitor, we need to have a detailed understanding of the biological process of pathogen infection. As for the blocking the influenza A virus infection, we first need to gain knowledge of the IAV virus itself and also the infection process of IAV [82]. There are 300–-400 HA proteins on the surface of the virus which determine the host recognition and are controlled by the multivalency effect. The HA protein has a trimeric symmetrical structure, so making use of multivalent carbohydrate-based agents to block the recognition process is a promising strategy to inhibit the entry of virus into cells and could lead to the discovery of novel antiviral agents.

It is difficult to make compounds with an exact size and multivalency to match natural systems such as viruses, which is one of the challenges. Scaffold structure, molecule size, linker between scaffold and ligand, ligand density, and also mechanical properties are important parameters that need to be optimized for the design of multivalent inhibitors. Besides high binding affinities, other mechanisms such as steric shielding and clustering effects are also important for successful pathogen inhibition.

The barriers to design large sized multivalent inhibitors may be an incomplete understanding of the delicate balance between entropy and enthalpy, and also the efficient tools to investigate multivalent interactions. The scaffold size, the flexibility degree of the arms and the whole molecule, the hydration extent, are important for designing successful inhibitors. Glycopolymers, nanoparticles, dendrimers, liposomal systems already provided highly potent inhibitors. These larger polyvalent based systems may engage more sialic acid binding proteins, while smaller trivalent systems may improve the specificity for binding strongly to a single HA-trimer.

Small multivalent inhibitors also show great potential for the treatment of viral diseases. The reasonable scaffold size, spacer length and shape should be considered into the design to match the geometry of trimeric HA and try to connect multiple binding sites simultaneously, which should also give strong inhibition even though they may not yet yield the same inhibitory potencies when compared to large molecule entities, capable of bridging HA binding sites within trimers as well as from different trimers. Even after successful inhibition of HA *in vitro*, there are still several challenges that remain [83]. However, the urgency of preparation for large IAV pandemics make this the right time to keep the investigation of multivalent inhibitor design going towards the clinics, either as a stand-alone antiflu drug but more likely in combination with other options such as vaccines and NA inhibitors [84,85].

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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