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Siglec-7 in complex with sialosides

The structure of Siglec-7 in complex with sialosides: leads for rational structure-based inhibitor design

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Abstract

Siglecs are transmembrane receptors for sialylated glycoconjugates and modulate cellular interactions and signaling events in the hematopoietic, immune and nervous systems. Siglec-7 is a structural prototype for the recently described family of immune inhibitory CD33-related siglecs and is predominantly expressed on natural killer cells and monocytes as well as subsets of CD8 T cells. Siglec-specific inhibitors are desirable for detection of masked and unmasked forms of siglecs, to aid in dissection of signaling pathways and as tools to investigate siglecs as potential therapeutic targets. As a first step towards this, we present a crystal structure of Siglec-7 in complex with a sialylated ligand, the ganglioside analogue $\alpha(2,3)/\alpha(2,6)$ disialyl lactotetraosyl 2-(trimethylsilyl)ethyl (DSLc4), allowing the detailed description of the binding site required for structure-guided inhibitor design. Mutagenesis and binding assays were used to demonstrate a key structural role for Lys131, a residue that changes conformation upon sialic acid binding. Differences between the binding sites of siglec family members were then exploited using α -methyl Neu5Ac as a basic scaffold. A co-crystal of Siglec-7 in complex with the sialoside inhibitor methyl α -9-(aminooxalyl-amino)-9-deoxyNeu5Ac and inhibition data of sialosides gives clear leads for future inhibitor design.

Abbreviations: Neu5Ac, *N*-Acetylneuraminic acid; Siglec, Sialic acid binding Ig-like lectin; Me, Methyl; NK, Natural killer cell; DSLc4, disialyl lactotetraosyl; ITIM, immune tyrosine-based inhibitory motif; Gal, galactose; GalNAc, *N*-Acetyl galactosamine; Glc, glucose; oxamido-Neu5Ac, methyl α -9-(aminooxalyl-amino)-9-deoxyNeu5Ac; OPD, *O*-phenylenediamine; BIP, biphenyl.

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Introduction

The sialic acid binding immunoglobulin-like lectin (siglec) family is a group of transmembrane receptors with the ability to recognize sialic acids, a family of nine-carbon sugars which often cap the non-reducing ends of glycoconjugates in higher animals. Eleven siglecs have been described in humans, predominantly expressed on cells of the immune and hematopoietic systems [1, 2]. Each siglec has a characteristic cell type-dependent expression pattern and a distinct binding specificity for sialylated glycoconjugates [3]. Siglecs are often expressed in an overlapping manner on cells of the immune system and this may be important in allowing a given cell type to respond to a spectrum of sialylated ligands.

With the exception of MAG (Siglec-4) and sialoadhesin (Siglec-1), siglecs are thought to play a role in the suppression of immune cell activation [4], possessing consensus immunoreceptor tyrosine based inhibitory motifs (ITIMs) and ITIM-like motifs in their cytoplasmic tails. ITIMs have been described in a growing number of inhibitory receptors of the immune system (Staub E, Rosenthal A, Hinzmann B (2004). Their phosphorylation creates a high affinity binding site for Src-homology 2 (SH2) domain containing phosphatases (SHP-1, SHP-2 and SHIP-1) and subsequent antagonism of activatory signals [5, 6].

The human CD33-related siglecs (CD33, Siglec-5 to 11) and CD22 can interact with sialylated ligands presented on the same cell (*cis* interaction) [7], a phenomenon referred to as 'masking'. This prevents the binding of exogenously-added ligands. For CD22 and some CD33-related siglecs expressed on blood leukocytes, B cell activation results in partial unmasking [7] (Razi N, Varki A (1999). Cryptic sialic acid binding lectins on human blood leukocytes can be unmasked by sialidase treatment or cellular activation. However, for other siglecs such as Siglec-7 expressed on natural killer (NK) cells, a variety of activation signals tested did not lead to unmasking [8]. The development of siglec-specific inhibitors based on a sialic acid template

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would provide useful tools for studying these receptors in their natural context. This has been achieved recently for CD22 by the modification of Neu5Ac acid at the C9 position with a biphenyl (BIP) moiety, leading to a >200-fold improvement in relative inhibitory potency compared with the unmodified sugar [9]. The utility of this inhibitor was clearly demonstrated when it was used to reveal that *cis* interactions are important in the inhibitory function of CD22 during B cell activation [10].

Siglec-7 represents a good candidate for structure-based inhibitor design. It is the only CD33-related siglec for which the structure has been determined so far and therefore provides a template on which other members of this family may be modeled [11]. It displays a unique ligand binding preference, binding internally branched $\alpha(2,6)$ -linked sialic acid and $\alpha(2,8)$ -linked disialic acids [12]. Siglec-7 is expressed predominantly on NK cells and it has been shown to inhibit NK cell cytotoxicity towards target cells over-expressing the $\alpha(2,8)$ -disialic acid-bearing ganglioside, GD3 [8]. The elevation of GD3 levels described for certain tumours (such as malignant melanoma and neuroblastoma) may therefore serve as an evasion strategy from NK killing [13]. A Siglec-7 blocking compound could therefore have potential therapeutic value.

In this paper we present the first structure of Siglec-7 in complex with sialylated ligands: a ganglioside analogue, $\alpha(2,3)/\alpha(2,6)$ disialyl lactotetraosyl 2-(trimethylsilyl)ethyl (DSLc4) [14], and a derivative of sialic acid, methyl α -9-(aminooxalyl-amino)-9-deoxyNeu5Ac (oxamido-Neu5Ac). In combination with binding assays, these structures give us an insight into what governs ligand binding and possible routes for rational inhibitor design.

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Results and Discussion

The DSLc4 complex Sparse matrix sampling screening for Siglec-7 co-crystals with the DSLc4 ganglioside analogue (Fig. 1A) yielded an unusually high number of hits, over 20 out of 144 conditions within a period of 2 days. All crystals were of a bi-pyramidal morphology, appearing very similar to that of an uncomplexed crystal form of Siglec-7. Synchrotron diffraction data were collected to a resolution of 1.90 Å (Table I). The unit cell dimensions were also similar to apo-Siglec-7 and indexed in the same space group, P4₁2₁2. Interpretation of the ligand density revealed an ordered tetrasaccharide unit Neu5Ac α (2,3)Gal β (1,3)GlcNAc[α (2,6)Neu5Ac] bridged the binding sites of Siglec-7 in adjacent asymmetric units across a crystallographic two-fold axis of rotation, with two-fold disorder (Fig. 1C & D). The net result is the stabilization of the same crystal packing observed for native Siglec-7, perhaps explaining the unusual promotion of crystal formation. Comparison of the native and complexed form (RMSD = 0.73 Å on all C α atoms) reveals that there is little deformation in crystal packing with only a 0.9 Å decrease in the c axis of the unit cell.

Within the ligand, intramolecular hydrogen bonding between the Gal C4 hydroxyl and the Neu5Ac (α (2,3) linkage) C7 hydroxyl stabilize the tetrasaccharide (Table II), tethering it between the two symmetry related Siglec-7 molecules (Fig. 1C & D). Although no direct contacts are made to the protein molecules beyond the terminal sugars, water-mediated hydrogen bonds are made between Asn133 and Lys135 and the C3 nitrogen of the GlcNAc and the oxygen of the glycosidic bond between Gal and GlcNAc (in the α (2,3) linkage orientation) (Fig. 1D and Table II).

It is not clear if the contacts observed outside the primary sialic acid binding site in this crystal structure represent 'natural' or artificial contacts which arise from crystal packing. Binding studies have shown that both the sialic acid linkage and the adjacent sub-terminal sugar are key

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contributors to siglec ligand specificity. Siglec-7 displays only a weak interaction with ligands bearing $\alpha(2,3)$ -linked sialic acids [12]. Comparison of the binding of Siglec-7 to branched $\alpha(2,3)$ / $\alpha(2,6)$ disialyl ligands, such as disialylgalactosyl-globoside and disialyl Lewis^a motifs and their monosialylated, $\alpha(2,3)$ sialic acid bearing equivalents, show that it is the $\alpha(2,6)$ branch that is required for higher affinity binding [15, 16]. We would therefore expect to observe a selective binding of the $\alpha(2,6)$ branch, rather than equal binding, as in this crystal structure. Furthermore, the C-C' loop, which has been implicated in directing ligand binding specificity, is disordered in the Siglec-7-DSLc4 complex. There are two possible explanations for the absence of direct contacts between side chain and sub-terminal sugars in this crystal structure. Firstly, regions of Siglec-7, such as the C-C' loop, may adopt different conformations to interact with the different linkages and two-fold disorder in the ligand may make such flexible protein interactions difficult to see in the electron density maps. Secondly, the interaction of the capping sialic acids drives cross-linking and stabilization of crystal packing, and the ligand is held in an artificial manner. Nevertheless, the water-mediated interaction between the ligand and residues Asn133 and Lys135, suggests that they may play a genuine role in ligand binding.

The ligand bridges two binding sites which are relatively close together, possibly resulting in conformational strain in the ligand. This can be examined by comparing the dihedral bond angles of the DSLc4 ligand and those of the same linkages in other previously determined glycan structures. The ψ and ϕ angles of Neu5Ac $\alpha(2,3)$ Gal and Neu5Ac $\alpha(2,6)$ GlcNAc linkages lie within regions seen in other glycans deposited in the PDB (Table II). The distortion lies in the Gal $\beta(1,3)$ GlcNAc linkage. In the PDB all ψ angles fall within the synclinal conformation, +28 to +68. In DSLc4 this lies in the opposite orientation at -41°. The ϕ angle of this bond is again different, -59° (synclinal), compared with a synperiplanar conformation (-27 to +25°) adopted by other structures. The net result is that the ring faces of Gal and GlcNAc in the DSLc4 glycan

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bound to Siglec-7 lie at a 90° angle relative to each other, whilst in the other structures the rings are co-planar. The straining of the ligand is also reflected in the contacts made by each Neu5Ac residue and, overall, the $\alpha(2,6)$ -linked Neu5Ac is more intimately associated with the binding site (Table II)

Sialic acid binding by Siglec-7. Siglec-7 makes several contacts with the DSLc4 terminal sialic acid that are similar to those observed in the crystal structure of Sialoadhesin complexed with 3' sialyllactose (Fig. 2). Arg124 forms a salt bridge with the sialic acid carboxylate, an interaction that is essential for sialic acid binding in all siglecs [17, 18] (Fig. 2A). Hydrogen bonds between the glycerol hydroxyls at the C8 and C9 position and the nitrogen of the C5 *N*-acetyl and the protein backbone are conserved [19]. In both structures a tryptophan makes hydrophobic contacts with the glycerol moiety of the sialic acid. Sialoadhesin makes additional contacts with the primary sialic acid via a hydrogen bond between of the C4 hydroxyl and the backbone carbonyl of Ser103 and hydrophobic interactions between the *N*-acetyl methyl and Trp2 (Fig. 2B). In the Siglec-7/DSLc4 structure these contacts differ. An amino acid insertion in the F-G loop of Sialoadhesin projects it toward the binding site, bringing the carbonyl oxygen of Ser103 to within hydrogen bonding distance of the C4 hydroxyl. In Siglec-7, the equivalent residue (Asn129), is 4.7 Å from the C4 hydroxyl. Instead, this interaction is replaced by a water-mediated hydrogen bond. The tryptophan which interacts with the *N*-acetyl methyl in Sialoadhesin is replaced by a tyrosine (Tyr26) in Siglec-7. In the Siglec-7/DSLc4 complex the orientation of the acetyl group is rotated 180°, allowing the oxygen to form a weak hydrogen bond with the tyrosine hydroxyl.

Flexibility within the binding site Comparison of the apo structures (PDB codes 1o7S and 1NKO; 1o7V is excluded from this analysis as a loop from a symmetry related molecule occupies the binding site) of Siglec-7 with that of the complexed form reveal that there is little overall

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positional change in the residues that form the binding site (RMSD = 1.2 Å on all atoms, Fig. 3A). One notable difference is that in the apo structures the side chain of Lys131 obscures access to the guanidinium group of Arg124. In the complexed form this residue moves away to reveal the primary arginine allowing it to interact with the carboxyl of sialic acid (Fig. 3A). A similar phenomenon is observed when comparing the apo (PDB code 1QFP) and liganded (PDB code 1QFO) form of Sialoadhesin, in which the “essential” arginine, Arg97, is masked by the side chain of Arg105 (Fig. 3B). Upon ligand binding the Arg105 moves away (by 3.8 Å), and Arg97, no longer blocked by Arg105, moves 1.8 Å, into a position where it can engage the carboxylate of sialic acid. With the exception of siglec-4 (myelin-associated glycoprotein, MAG), in all the human siglecs, the residue equivalent to Lys131 in Siglec-7 is either an arginine or a lysine residue (in MAG it is a Gln). When Lys131 was substituted with Ala by site-directed mutagenesis, this unexpectedly abolished binding of Siglec-7-Fc to red blood cells (Fig. 3C), an effect comparable to that seen when the essential Arg124 was substituted with Ala (Fig. 3C). Both mutant proteins appeared to be correctly folded as judged by their normal reactivity with anti-Siglec-7 monoclonal antibodies (data not shown). These results suggest that rather than masking Arg124 from ligands, Lys131 is essential for ligand engagement. What biological significance this has is unclear; for example this residue may protect the primary arginine from non-productive or low affinity interactions with organic or non-organic anions, which may disrupt binding site integrity.

Sialic acid based inhibitor design Siglec-7 only weakly interacts with a Neu5Ac moiety. This has also been observed in hapten inhibition assays (Table II) where 3 mM Me- α -Neu5Ac leads to only 35% inhibition. However, hydroxylation of the *N*-acetyl group to *N*-glycolyl enhances binding, since Me- α -Neu5Gc inhibits at least 2-fold better. In contrast, this sialic acid is not

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bound by Sialoadhesin or Siglec-4 (myelin-associated glycoprotein) (Kelm *et al.*, 1994). Molecular modeling (data not shown) suggests that this additional hydroxyl group could lie within hydrogen bonding distance (between 2.7 – 2.6 Å) with the side chain of Tyr26. In Sialoadhesin or Siglec-4, where this position is occupied by a Trp, such a modification may be sterically unfavorable.

In hapten inhibition assays, Neu5Ac derivatized with an oxamido group at C9 of the glycerol side chain (Fig. 4B), was found to be at least a 3-fold better inhibitor than Neu5Ac (Table II). In contrast, this sialic acid derivative was an approximately 5-fold weaker inhibitor for Sialoadhesin compared with Neu5Ac (data not shown). To examine this differential binding, Siglec-7 was co-crystallized with 20-fold molar excess of oxamido-Neu5Ac, synchrotron diffraction data were collected to 1.6 Å, the structure solved by molecular replacement and refined (RMSD = 0.83 Å on all C α atoms compared to the apo structure, Table I). The structure of the oxamido-Neu5Ac complex reveals that many of the important contacts are maintained: a salt bridge formed between the carboxylate and Arg124, hydrogen bonding between Asn133 carbonyl and the C5 *N*-acetyl group, hydrophobic interactions between the derivatized glycerol chain and Trp132, and hydrogen bonding between the C8 hydroxyl and the backbone nitrogen of Asn133 (Fig. 4A). The hydrogen bond formed between the C9 hydroxyl of sialic acid and Asn133 carbonyl is directly replaced by the nitrogen of oxamido group. An additional hydrogen bond is formed between C11 oxygen and the backbone nitrogen of Lys131. Superposition of oxamido-Neu5Ac with Sialoadhesin structures suggests that the weaker binding of this compound could be due to a steric clash of the side chain of Val109 with terminal amide of the oxamido group (Fig. 4D).

Derivatization of Neu5Ac at the C9 position by hydrophobic moieties has yielded increased binding to other siglecs, namely human and murine CD22 and CD33, and Sialoadhesin

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[9, 10]. Crystal structures of Sialoadhesin in complex with sialosides demonstrated that this was predominantly due to the presence of a “hydrophobic gate” formed by Val109 and Leu107 [9], which sandwich the hydrophobic substituents. By sequence alignment and modeling of members of the siglec family, Zaccari *et al.* (2003) proposed that variations in the residues which create this cleft (which are not well conserved) might contribute to the differences in binding affinity. In Siglec-7 the walls of this hydrophobic groove are formed by Lys135 and Tyr136 (Fig 4C), and differ significantly from those of Sialoadhesin (Fig. 4D). The hydrophobic gate of Siglec-7 is deeper, with much steeper sides, and the axis of the cleft is skewed by approximately 45° compared to that of Sialoadhesin.

Such differences provide promising leads for the design of siglec-specific sialosides: of the human siglecs, Siglec-7 is unique in having Tyr and Lys forming the hydrophobic gate. In the Siglec-7-oxamido-Neu5Ac structure, the oxamido group is anchored by a hydrogen bond with its terminal amide, aligning it with the axis of the channel. Therefore, the addition of aromatic moieties to the C11 nitrogen of oxamido-Neu5Ac may enhance binding and specificity. This could be complemented by the use of Neu5Gc as the template for derivatization.

Conclusions We have described the structure of Siglec-7 in complex with a ganglioside analogue of DSLc4. This structure has allowed detailed analysis of the primary sialic acid binding site of Siglec-7. Further, a sialic acid analog carrying an oxamido group at C9 was studied in complex with Siglec-7. On the basis of this structure, the 3-4 fold increase in relative inhibitory potency when compared to the Me- α -Neu5Ac has been explained in molecular detail. This compound will serve as a lead for further substitutions, with the aim of creating a ligand of high affinity and high selectivity toward Siglec-7.

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Materials and Methods

Expression and Purification of Siglec-7 The Siglec-7 V-set domain was expressed and purified as described by Alpey *et al.* (2003). Briefly, the region encoding the Siglec-7 V-set domain was cloned into the pDEF expression vector and transfected into CHO Lec1 cells [20]. A stable cell line was established using selection with hygromycin B. Cells were cultured in α -MEM containing 5 % FCS and 1 % penicillin/streptomycin mix (Life Technologies). Siglec-7 was purified from the medium using an anti-Siglec-7 polyclonal antibody affinity column followed by size exclusion chromatography. The protein was concentrated to 6 mg/ml in 25 mM Tris pH 8.0 and 75 mM NaCl.

Synthesis of Methyl α -9-(aminooxalyl-amino)-9-deoxy-Neu5Ac (oxamido-Neu5Ac) This compound was prepared from the methyl α -glycoside of 9-amino-9-deoxy-Neu5Ac by reaction with activated oxamic acid. Experimental details will be reported elsewhere. The analogue was characterized by high-resolution nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry.

Crystallization and data collection For co-crystallization experiments, Siglec-7 was incubated on ice for 10 min with 20-fold molar excess of ligand (either DSLc4 or oxamido-Neu5Ac). Crystallization was performed using sitting drop vapour diffusion, using 0.5 μ l reservoir solution added to a 0.5 μ l drop of protein-ligand complex, equilibrated with 100 μ l of reservoir solution at 20 °C. The DSLc4-Siglec-7 co-crystals used for data collection grew in crystal screen CS CRYO condition forty-one (8.5 % iso-propanol, 0.095 M HEPES, pH 7.5, 17 % PEG 4000, 15 % glycerol). A data set was collected to 1.90 Å at the ESRF, beamline ID14 EH4 at 100 K using a ADSC Q4 CCD detector.

Co-crystals for Siglec-7 and oxamido-Neu5Ac grew in a screen containing 30 % PEG 4000, 0.1 M sodium acetate, pH 4.6, 0.2 M ammonium acetate. The crystal was cryogenically

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protected by soaking in the mother liquor containing 10 % glycerol before freezing in a stream of nitrogen gas. A dataset was collected on ESRF beamline ID14 EH4 using a ADSC Q4 CCD detector at 100 K. Data were processed and scaled using the HKL suite of programs [21].

Structure determination and refinement The structures for Siglec-7/DSLc4 and Siglec-7/oxamido-Neu5Ac were solved by molecular replacement in AMoRe [22] using the Siglec-7 V-set domain structure (PDB code 1o7S [11]) as a search model. For both, a single solution was found with R-factors of 0.33 and 0.32 and correlation coefficients of 0.70 and 0.72, respectively. The model phases were used in WarpNTrace which built 102 and 95 out of 127 residues, respectively [23]. The models were refined using CNS [24] interspersed with manual model building in O [25]. Final refinement was performed using Refmac 5 [26].

After model building and the inclusion of water molecules in refinement, the terminal sialic acid was placed into the unbiased $|F_o|-|F_c|, \phi_{calc}$ electron density and further refinement was carried out. Strong connective $|F_o|-|F_c|, \phi_{calc}$ electron density was observed across the two-fold symmetry axis between the sialic acids of adjacent asymmetric units (Fig. 1). The mode of binding which could best account for this density is the ligand bridging two siglec molecules. Although the DSLc4 ligand is not symmetric, it does possess a degree of pseudosymmetry in the tetrasaccharide unit NeuAc α (2,3)Gal β (1,3)GlcNAc β [NeuAc α (2,6)], the plane of pseudosymmetry being about the Gal β (1,3)GlcNAc β bond. The density was interpreted to consist of the DSLc4 tetrasaccharide, as described above, spanning two Siglec-7 monomers in no preferential orientation. The density between the sialic acid residues is therefore made up of both Gal and GlcNAc occupying the same space at 50% occupancy each. The tetrasaccharide was accommodated in refinement by modeling it across the asymmetric unit at 50% occupancy, allowing the symmetry operation to generate the ligand in the opposite orientation. There is thus two-fold disorder combined with a two-fold crystallographic axis. As these two orientations are

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mutually exclusive, interactions were turned off between these molecules. This satisfied the electron density between the binding sites. No assignable density for the other sugar residues was observed.

For the oxamido-Neu5Ac/Siglec-7 complex, after the inclusion of water molecules and model building, the oxamido-Neu5Ac was modeled into the unbiased $|F_o|-|F_c|, \phi_{calc}$ density and further refined.

Site directed mutagenesis and expression of chimeras Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) on a siglec-7-Fc chimeric construct in a modified pEE14 plasmid. Successful mutagenesis was confirmed by sequencing. The plasmids were transiently transfected into COS-1 cells using FuGene 6 transfection reagent (Roche). The cells were cultured for a further five days in X-VIVO 10 serum-free medium (BioWhitaker) and the media harvested. Siglec-7-Fc concentration was assayed by ELISA and correct folding was confirmed by ELISA using anti-siglec-7 mAbs (7.7a and 7.5a[27]).

Red Blood Cell binding assay Siglec-7-Fc chimeras were immobilized on microtitre plates to create a surface for human red blood cell (RBC) capture [28]. For this goat anti-human Fc antibody (Sigma) was coated on immulon 4 HBX microtitre plates. The plates were washed with PBA (PBS, 0.1% BSA, 10 mM sodium azide) and blocked with 5 % milk powder. 50 μ l of Siglec-7-Fc chimeras serial dilutions per well were allowed to bind for 2 h at room temperature, followed by three washes with PBA. 100 μ l of a RBC suspension were added to each well and allowed to bind for 30 min at room temperature. Unbound cells were removed by gentle washing and the cell layer was allowed to air-dry, followed by methanol fixation and permeabilization. The extent of RBC binding was assayed using *O*-phenylenediamine peroxidase (OPD) substrate [28], measuring OD₄₅₀ with a Cytofluor (PerSeptive Biosystems) plate reader.

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Hapten inhibition assays with sialic acid derivatives For hapten inhibition assays, Fc-chimeras containing the N-terminal three domains of Siglec-7 were purified in protein A agarose from tissue culture supernatants of stably transfected Chinese Hamster Ovary cells as described by Yamaji *et al.* (2002). As a binding target, microtitre plates with covalently linked Neu5Ac (GlycoWells from Lundonia, Lund Sweden) were used. Prior to the binding assay, the Fc-chimeras (final concentration 200 ng/ml) was complexed with anti-Fc antibody coupled to alkaline phosphatase (1 in 1000 dilution, supplied by Dianova, Hamburg, Germany) in HBS-T (10 mM HEPES, 150 mM NaCl, pH 7.4, 0.05% Tween20) for 15 to 30 minutes at room temperature. 30 µl of this pre-complex were added per well and incubated for 4 h at 4 °C in the presence or absence of sialic acid derivatives. After five washes with HBS-T, the amount of bound alkaline phosphatase was quantified by determination of the initial reaction velocity using fluorescein disphosphate (15 µM) as substrate. To determine the concentration required for 50% inhibition (IC₅₀), seven concentrations of inhibitor spanning two orders of magnitude were used. Each concentration was done in triplicates and assays were repeated at least three times.

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Tables

Table I. Data collection and refinement statistics. The coordinates and structure factors have been deposited with the PDB (entries 2DF3 and 2G5R).

	Sig7-DSLc4	Sig7-oxamido-Neu5Ac
Data Collection		
Wavelength (Å)	0.933	0.933
Resolution (Å)	25 –1.90 (1.97-1.90)	25 –1.60 (1.66-1.60)
Space group	P4 ₁ 2 ₁ 2	P4 ₁ 2 ₁ 2
Unit cell (Å)	a=b=53.07, c=92.88	a=b=52.93, c=93.24
Reflections: Observed	46855	74536
Unique	11014	17748
Redundancy	4.3 (3.8)	4.2 (2.2)
R_{merge}	0.078 (0.445)	0.043 (0.338)
$I/\sigma I$	15.5 (3.1)	21.5 (2.0)
Completeness (%)	99.3 (94.0)	97.5 (78.9)
R_{free} reflections	523	893
R_{cryst} (%)	19.7	21.4
R_{free} (%)	23.7	22.8
Total number of atoms	1119	1053
Protein	944	904
Water	95	102
Glycan	14	14
Ligand	66	27
 Protein (Å ²)	20	18
 Water (Å ²)	27	27
 Glycan (Å ²)	33	27
 Ligand (Å ²)	24	44
RMSD from ideal geometry		
Bond lengths (Å)	0.014	0.015
Bond angles (°)	1.65	1.35
Main chain B (Å ²)	1.55	1.77

statistics for the highest resolution shell are shown in brackets

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Table II. Hydrogen bond network.

Potential hydrogen bonds are shown and the distances between atoms.

Ligand – protein hydrogen bonds

<i>DSLc4 atom</i>	<i>Protein atom</i>	<i>Distance (Å)</i>
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Neu5Ac (α2,6)

O1A	Arg 124 NH2	3.0
O1B	Arg 124 NH1	2.8
N5	Lys 131 CO	2.7
O5	Tyr 26 OH	3.4
O8	Asn 133 N	2.9
O9	Lys 135 NZ	2.8
O9	Asn 133 CO	2.8

Neu5Ac (α2,3)

O1A	Arg 124 NH2	2.7
O1B	Arg 124 NH1	2.9
N5	Lys 131 CO	3.3
O5	Tyr 26 OH	4.3
O8	Asn 133 N	3.0
O9	Lys 135 NZ	3.1
O9	Asn 133 CO	3.1

DSLc4-protein hydrogen bonds mediated by water

<i>Atom</i>	<i>Water molecule</i>	<i>Distance (Å)</i>
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Gal (α2,3) O3	105	3.4
GlcNAc (α2,3) N2	105	3.4
Asn 133 ND2	105	2.6
Lys 135 NZ	105	2.7
O4 Neu5Ac (α2,6)	110	3.1
O4 Neu5Ac (α 2,3)	110	3.8
Asn 129 CO	110	2.6

Intramolecular hydrogen bonds

<i>DSLc4 atom</i>	<i>DSLc4 atom</i>	<i>Distance (Å)</i>
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Neu5Ac (α2,3) O7	Gal O4	2.7
Neu5Ac O8 (α2,6)	Neu5Ac O9	2.8
Neu5Ac O8 (α 2,3)	Neu5Ac O9	3.4

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Table III. Glycosidic bond angles.

Dihedral angles for the glycosidic bonds of DSLc4 and equivalent linkages in the PDB were analysed using the CARbohydrate Ramachandran Plot (CARP) tool of the Carbohydrate Structure Suite (CSS) [29] (<http://www.dkfz.de/spec/css/index.php>). The dihedral angles for the Gal β (1,3)GlcNAc-linkage are defined as: ϕ = O5-C1-O3'-C3' and ψ = C1-O3'-C3'-C4'. For the Neu5Ac α (2,3)Gal linkage: ϕ = C1-C2-O3'-C3' and ψ = C2-O2-C3'-C4'. For the Neu5Ac α (2,6)GlcNAc linkage: ϕ = C1-C2-O6'-C6', ψ = C2-O2-C6'-C5'. The stereochemical conformation is given after the torsion angle, and are defined as: 0 to 30 synperiplanar (*sp*); 30° to 90° and -30° to -90° synclinal (*sc*); 90° to 150° and -90° to -150° anticlinal (*ac*); and -/+ 150° to 180° antiperiplanar (*ap*). The No. column refers to the number of ligand examples in the PDB.

Linkage	DSLc4		Survey of PDB		
	ϕ	Ψ	ϕ	ψ	No.
Neu5Ac α (2,6)GlcNAc	-84 (<i>ac</i>)	-169 (<i>ap</i>)	-85 to -82 (<i>sc</i>), +22 to +27 (<i>sp</i>)	+190 to +144 (<i>ap</i>)	5
Gal β (1,3)GlcNAc	-41 (<i>sc</i>)	-59 (<i>sc</i>)	+28 to +63 (<i>sc</i>)	-27 to +25 (<i>sp</i>)	12
Neu5Ac α (2,3)Gal	-133 (<i>ac</i>)	-42 (<i>sc</i>)	-35 to -75 (<i>sc</i>), +175 to +190 (<i>ap</i>)	+26 to -44 (<i>sp</i> , <i>sc</i>)	62

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Table IV: Hapten Inhibition Assay Data

Sialic acid derivative	IC ₅₀ (μM)
Me-α-Neu5Ac	>3000 (35%)
Me-α-oxamido-Neu5Ac	1600
Me-α-5-N-glycolyl-Neu	2000

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Figure legends

Figure 1: The Siglec-7-DSLc4 interaction.

(A) A schematic representation of the DSLc4 analogue is shown. (B) The structure of the N-terminal, ligand-binding domain of Siglec-7 is shown. β -strands are shown as blue arrows. The intrasheet disulphide bond is shown in green and the primary sialic acid binding Arg124 with carbons in mauve. Panels C and D show the DSLc4 ligand bridging two Siglec-7 molecules related by a 2-fold crystallographic symmetry axis. Both orientations of the DSLc4 tetrasaccharide (green sticks) are shown modeled into electron density that spans symmetry-related molecules. Panel C shows the ligand in the orientation: Neu5Ac α (2,6)GlcNAc β (3,1)Gal[Neu5Ac α (2,3)], while D in the Neu5Ac α (2,3)Gal β (1,3)GlcNAc[α (2,6)Neu5Ac] orientation. Unbiased (i.e. before inclusion of any ligand) $[F_o]-[F_c], \phi_{calc}$ density contoured at 2.25σ is shown as a yellow meshwork. The side chains of residues that interact with the ligand are labeled, and waters bridging the protein and the ligand are depicted as magenta spheres. The potential hydrogen bonding network is shown as black dashed lines.

Figure 2: Comparison of sialic acid binding by Siglec-7 (A) and Sialoadhesin (B).

An intra-sheet disulfide bond (green sticks) results in the widening of the β sandwich, exposing hydrophobic residues that contribute to the primary sialic acid binding site. In both structures the sialic acid carboxylate is anchored by an interaction with a conserved arginine. In siglec-7 the replacement of a tryptophan (Trp2) with tyrosine (Tyr26), results in the rotation of the C5 *N*-acetyl group to allow hydrogen bonding with the side chain hydroxyl.

Figure 3: The role of Lys131.

The binding of sialic acid is accompanied by small, conserved conformational changes. Superposition of the apo and liganded crystal structures of Siglec-7 (A) and Sialoadhesin (B),

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show that there is a conserved movement upon ligand binding. Side chains which remain static are shown in cream (apo) and grey (liganded), whilst those that move are shown with orange (apo) and pink (liganded) carbons. Arrows indicate the conformational change that accompanies ligand binding. A water molecule (green sphere) which hydrogen bonds with the backbone nitrogen of Asn 133 of siglec-7 is displaced by the sialic acid C8 hydroxyl. C. A RBC binding assay was used as a measure of sialic acid-dependent binding. Serial dilutions of Siglec-7-Fc chimeras, wild type (■), and mutants, R124A (◇), and K131A (●), were immobilized on microtitre plates and the extent of RBC binding was measured by an OPD assay.

Figure 4: Binding of oxamido-Neu5Ac by Siglec-7.

The methyl- α -glycoside of 9-amino-9-deoxy-Neu5Ac was derivatized with an oxamido group at the C9 position (B). Siglec-7 is shown in complex with oxamido-Neu5Ac (A) with unbiased $[F_o] - [F_c]$, ϕ_{calc} density contoured at 2.25σ . A surface representation of Siglec-7 (C) and Sialoadhesin (D) shows that both possess a hydrophobic gate, formed by Lys135 and Tyr136, and Leu107 and Val109, respectively. However, these channels lie at divergent angles. The Sialoadhesin9-BIP-Neu5Ac (D, BIP is shown with cream carbon atoms) is shown to highlight the orientation of the hydrophobic gate. Additionally, residues which participate in BIP binding are shown. Superimposition of oxamido-Neu5Ac (cyan carbons) onto Sialoadhesin (D), show that Val109 could sterically hinder binding.







