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Screening of Neu5Acα(2–6)gal isomer preferences of siglecs with a sialic acid microarray†

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Sialic acids (Sias) are important terminal sugars on cell surfaces involved in a wide range of protein–carbohydrate interactions. Hence, agents modulating sias-mediated protein interactions are promising inhibitors or vaccine candidates. Here, we report the synthesis of Neu5Acα(2–6)Gal structural analogs and their binding to a series of siglecs. The results showed distinct binding patterns with conserved siglecs (hCD22 and mCD22) compared to rapid evolving siglecs (Siglecs -3 & -10).

Sialic acids are the terminal sugars of glycoproteins and glycolipids on cell surfaces that are characterized by a 9-carbon backbone.[1] To date, nearly fifty different forms of Sias have been identified. Among them, *N*-acetylneuraminic acid and its *N*-glycolyl derivatives are the most prominent.[2] Given their structural diversity, Sias act in several biological processes. Sias on the cell surfaces are involved in regulation of the cell cycle and autoimmune disorders, and are also involved in pathogen recognition.[3] Sialic acid binding lectins (Siglecs) are the major homologous subfamily of I-type lectins that mediate Sia recognition *via* immunoglobulin (Ig)-like domains.[4] Expression of specific siglecs modulate immune responses, inflammation, tumorigenesis and apopotosis.[5]

Siglecs are classified into two subgroups, CD33-related subgroups, (Siglec-3, -5, -6, -7 & -10), which appear to be rapidly evolving with complex expansion in different mammalian lineages and the second subgroup, which includes the evolutionarily conserved ones (Siglec-1, -2 and -4).[6] In general, siglecs bind to common epitopes containing Neu5Acα(2–6)gal and/or Neu5Acα(2–3)gal linkages. Various siglecs exhibit overlapping actions as a result of similar glycan binding preferences that complicate the assessment of distinct siglec functions. Hence, identifying the distinct Sias glycans' recognition patterns is essential for revealing the siglec functions. To this end, the C-9 and C-4 positions of sialic acids were modified with aromatic and sulphate derivatives to increase the Siglec-2 binding affinity.[7] The synthetic glycan microarray analysis of these glycans revealed that biphenyl substitution at the C-9 position and sulphate at the C-4 position revealed 750 000-fold higher affinity to hCD22 compared to the native sialic acid ligands.[8] Similarly, potential modification on sialic acid has been studied to elucidate the role of siglecs in a biological system. However, the basic differences between the conservative and evolutionary siglec selectivity remains largely unclear and it is unknown what factors are responsible for the specificity. To understand how siglecs generate specificity for sias glycans, it is essential to construct structural analogs of Neu5Acα(2–6)Gal to modulate the distinct binding patterns.

Here, we have systematically investigated the effect of sialic acid as terminal sugar and galactose/glucose/mannose as penultimate sugar to study the major human siglecs' binding patterns. Based on the binding affinities, it could be possible to deduce how nature differs with the evolutionary and conserved siglec-ligand selectivity.

The sialic acid disaccharides (**1**, **3** and **5**) were obtained from fully protected disaccharides **8**, **9** and **10** respectively. The esters, ether and azide functionalities on **8–10** were successively removed by the use of a global deprotection protocol (Scheme 1). The sialic acid donor (**11**) and galactose/mannose/glucose acceptors (**12–14**) were synthesized according to a modified literature procedure.[9] The glycosylation of a donor and acceptor were carried out by NIS/TfOH at −40 °C which gave a protected form of disaccharides in moderate to good yield. The 4,6-benzylidine protection of galactose and glucose thiotoluene pentahydroxy derivatives and their successive C-2, C-3 benzoylation afforded a protected form of galactose and glucose thiotoluene. The stereodirecting benzoyl group at the C-2 position was chosen to take advantage of the beta configuration which is usually found on the natural linkage of glycans. The glycosylation of galactose and glucose derivatives with a linker in the presence of NIS/TfOH at −40 °C and successive regioselective ring opening by $PhBCl_2/Et_3SiH$ afforded acceptors **12** and **13** with good yield. Simultaneously the regio-

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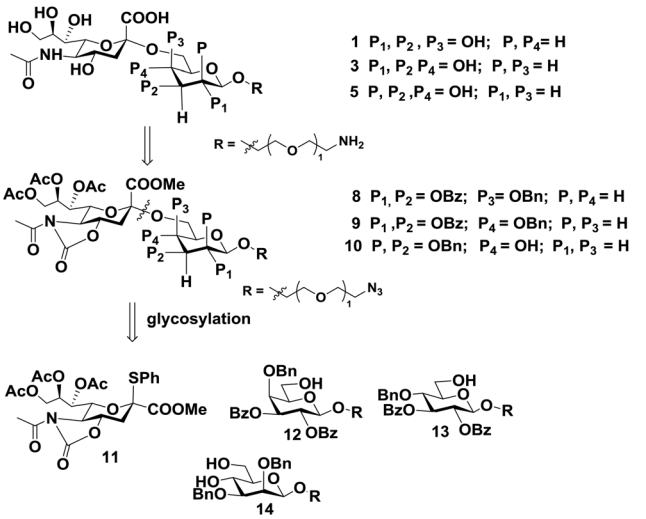
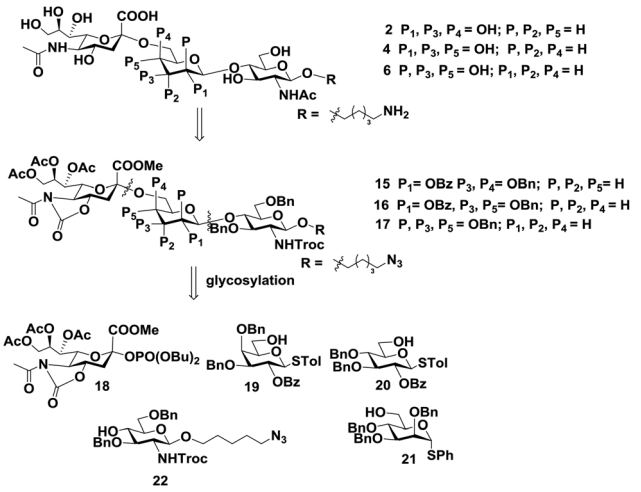


Scheme 1 Retrosynthesis of sialic acid disaccharides.



Scheme 2 Retrosynthesis of sialic acid trisaccharides.

selective 4,6 benzylidine protection and 2,3 benzylation gave a thiophenol mannose derivative which was then converted into its sulfoxide in the presence of *m*-CPBA at lower temperature to afford the highly active mannose-sulfoxide thiophenol donor. The mannose donor was glycosylated with a linker in the presence of Tf_2O/TTBP, at −78 °C and the benzylidine ring cleavage by an acid catalyst afforded acceptor **14** in moderate yield.

The trisaccharide analogs of Neu5Acα(2–6)Galβ(1–4)GlcNAc were obtained by global deprotection of fully protected trisaccharides **15–17** in moderate yield. A synthetic convergent approach involves a sialic acid dibutylphosphate donor (**18**) and C6-OH thiotoluene, thiophenol acceptors of galactose (**19**) glucose (**20**), mannose (**21**) and glucosamine (**22**) at the non-reducing end (Scheme 1). The donor **18** was synthesized from *N*-acetylneuraminic acid in 7 steps according to the literature protocol.[10] Synthesis of acceptors **19** and **20** was achieved in four steps involving 4,6-benzylidine protection and then selective C-3 benzylation with nBu$_2$SnO/BnBr followed by C-2 benzoylation and finally, regioselective selective benzylidine ring opening with $PhBCl_2$/Et_3SiH afforded the C6-OH acceptor in good yield. Similarly mannose C6-OH acceptor (**21**) was synthesized from its pentahydroxy thiophenol derivative by following temporary C6-OH TBDMS protection, then benzylation of the remaining hydroxyl-groups, and finally the removal of temporary silyl protection by p-TSA gave the desired compound in good yield. The synthesis of building block **22** was challenging and successfully achieved in 11 steps by following the published protocol.[11] Sialic acid donor **18** was glycosylated with acceptors **19–21** in the presence of TMSOTf at −78 °C and gave the expected disaccharides, which subsequently glycosylated with acceptor **22** in the presence of NIS/TfOH at −40 to −20 °C yielding fully protected trisaccharide analogs (**15–17**). Finally ester, Troc, and oxazolidinone protecting groups were removed by the global deprotection protocol and later the removal of benzyl ether and simultaneous azide to amine reduction was achieved by hydrogenolysis to afford fully deprotected trisaccharides (**2**, **4** and **6**) in moderate yield (Scheme 2).

Next, the synthetic di- and tri-saccharides were printed onto epoxide-functionalized microarray slides at 100 μM in replication of **4** as described in the Experimental section. Human (H) and mouse (m) Siglec-Fc chimeras (H-Siglec-2, m-Siglec-2, H-Siglec-3 and -10) were incubated on the slide at three concentrations (5 ng μl^{-1}, 10 ng μl^{-1} and 20 ng μl^{-1},) in PBS with 1% ovalbumin, followed by the secondary antibody (Cy3-anti-human IgG). Slides were scanned and the binding was determined by the fluorescence intensity as described in the Experimental section. Initially, the sialic acid microarray was analyzed by incubating with control lectins of a known binding specificity. As expected, sambucus nigra lectin (SNA), which is specific of Neu5Acα(2–6)gal bound to **1** and **2** and displayed no binding with **3–6** (Fig. 1A). On confirming the binding affinity with control lectin, H-Siglec-2 and m-Siglec-2 binding in the microarray was established. H-Siglec-2 displayed weak binding with **1** and **2** compared to m-Siglec-2 (Fig. 1B and C). Interestingly, isomers of compounds **1** and **2** did not show any binding while H-siglec-3 and H-siglec-10 bound to **1** and **2**. Remarkably, the isomers of **1** and **2** displayed better binding to Siglec-3 and -10 than the native form (Fig. 1D and E). According to the microarray studies, compounds **1–6** sialic acid glycans possess two distinct binding patterns. The evolutionarily conserved H-Siglec-2 or m-Siglec-2 displayed selective binding towards **1** and **2**, indicating that the interaction between the penultimate sugar (galactose) and the specific amino acid sequence in Siglec-2 is conserved, thus generating the specific binding motif. On the other hand, the rapidly evolving siglec-3 and 10 displayed spatial flexibility, allowing them to bind galactose isomers (mannose and glucose). Overall, the identification of these detailed binding preferences provides the basis for understanding and targeting siglec selectively. The mannose isomers of sialic acid glycans are the common side products of β-mannosidosis[12] disease and the binding between **5** and **6** with siglecs-3 and 10 reveals the possible mechanism of immune responses in the human system.

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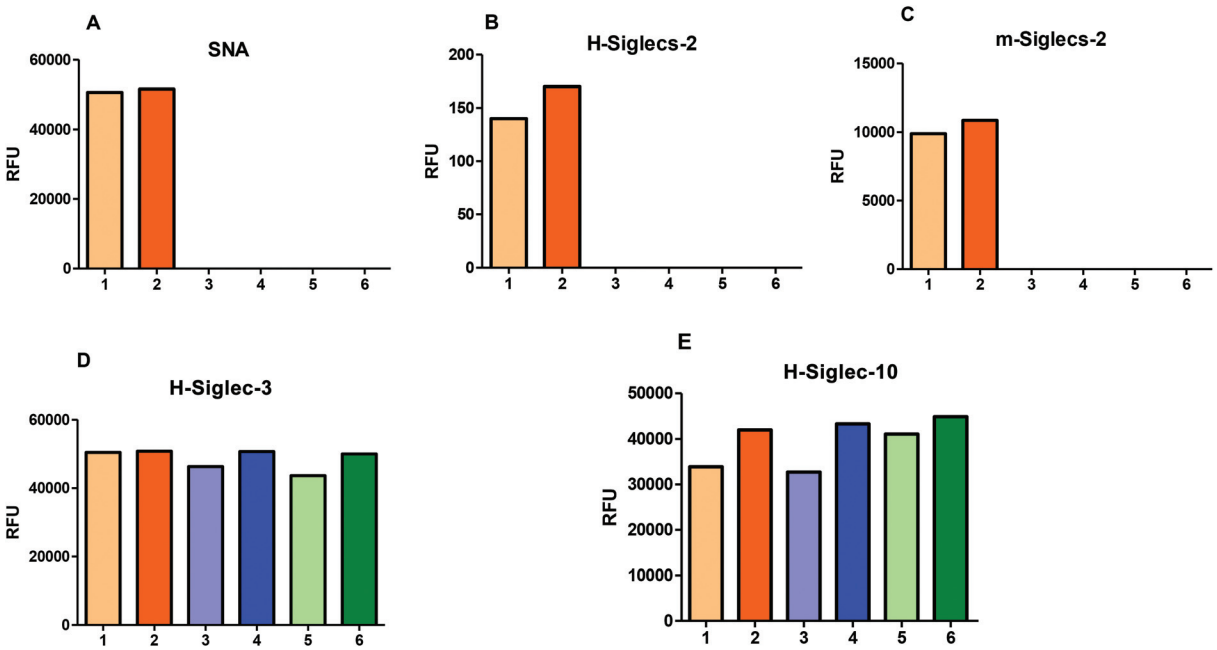

Fig. 1 Microarray analysis of sialic acid-binding lectin derivatives (**1–6**) (at 20 ng μl^{-1}).

Conclusion

We have synthesized a library of sialic acid glycans by modifying the penultimate sugar and immobilized them on the glycan microarray to determine siglec binding preferences. Comparison of the siglec binding patterns allows further understanding of the basic differences between the conserved and evolving siglecs, and constitutes a valuable tool for designing siglec specific molecules for therapeutic applications.

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