Cell Targeting

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Click and Pick: Identification of Sialoside Analogues for Siglec-Based Cell Targeting **

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The siglec family of sialic-acid binding proteins is comprised of 15 human and 9 murine members that are primarily expressed by white blood cells, which mediate innate and adaptive immune functions.[1] Their restricted expression pattern and activity as endocytic receptors has made these proteins attractive molecular targets for directed therapy for immune-cell-mediated diseases.[2] Although anti-siglec antibodies are already in clinical use, nanoparticles having sialoside ligands show promise for targeting siglecs in vivo, thus providing alternatives for delivery of therapeutic cargo.[3]

The difficulty in identifying siglec ligands of suitable affinity and selectivity has limited the potential of ligand-bearing nanoparticles.[1a,2,4] Previous reports have demonstrated that modification of sialic acid (NeuAc) at the C9-position can produce both increased affinity and selectivity for sialoadhesin (siglec-1), CD22 (siglec-2), and myelin-associated glycoprotein (siglec-4).[5] It has also been suggested that modifications at the C5-position can modulate affinity and selectivity for several siglecs, however, sialoside analogues modified at this position have not been fully explored for these properties.[6] Thus, although modifications to sialic acid at C5 and C9 have potential for yielding promising sialoside ligands, and these positions are relatively straightforward to modify using an enzymatic synthetic approach, the lack of methods to robustly generate sialoside analogue libraries and systematically screen them against a library of siglecs has hampered progress.

To address this issue, we devised a facile "click and pick" strategy involving high-throughput synthesis of a sialoside analogue library using click chemistry, coupled with microarray technology to pick high-affinity "hits" for human and murine siglecs (Figure 1). To generate the library, eight sialoside parent compounds with ethyl amine linkers were synthesized by a convergent chemoenzymatic approach (Scheme 1, and in the Supporting Information, Scheme S1);

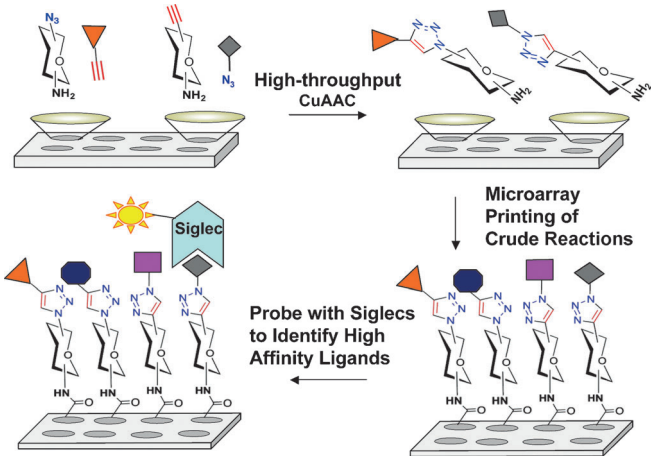

Figure 1. A schematic representation of the click and pick strategy for identification of high-avidity siglec ligands. This strategy involves the synthesis of the analogue library by parallel Cu^{I}-catalyzed azide–alkyne cycloaddition (CuAAC), glycan microarray printing, and screening with fluorescently labeled siglec-Fc chimeras to identify high-affinity ligands.

these compounds have azide or alkyne substituents at the 5-position (**A0–D0**) or the 9-position (**E0–H0**) of the sialic acid moiety, and are attached through an α2-3 or α2-6 linkage to the penultimate galactose, the two most common linkages in mammalian glycans. These parent scaffolds were then subjected to high-throughput Cu^{I}-catalyzed azide–alkyne cycloaddition[7] (CuAAC, click chemistry) with 24–30 coupling partners (Supporting Information, Figures S1 and S2) to generate a library of 224 sialoside analogues (Supporting Information, Tables S1–S8), with quantitative conversion for nearly all couplings. The sialoside products could then be printed directly onto *N*-hydroxysuccinimide (NHS) activated microarray slides without prior purification owing to the orthogonality of the click reaction with amine acylation chemistry, thus allowing for far higher screening throughput and library diversity than in previous efforts.[6b,8]

To identify high-affinity ligand analogues of individual siglecs, fluorescently labeled siglec-Fc chimeras were overlayed on the microarrays (Figure 1). At optimal concentrations of the Fc chimeras there was no binding to native sialoside controls or the parent scaffolds **A0–H0**, thus ensuring that any hits correspond to higher-affinity ligands.[8–9] Representative microarray data obtained using this approach is shown for a panel of human and murine siglecs in Figure 2, Figure 3a, and in the Supporting Information, Figure S3.

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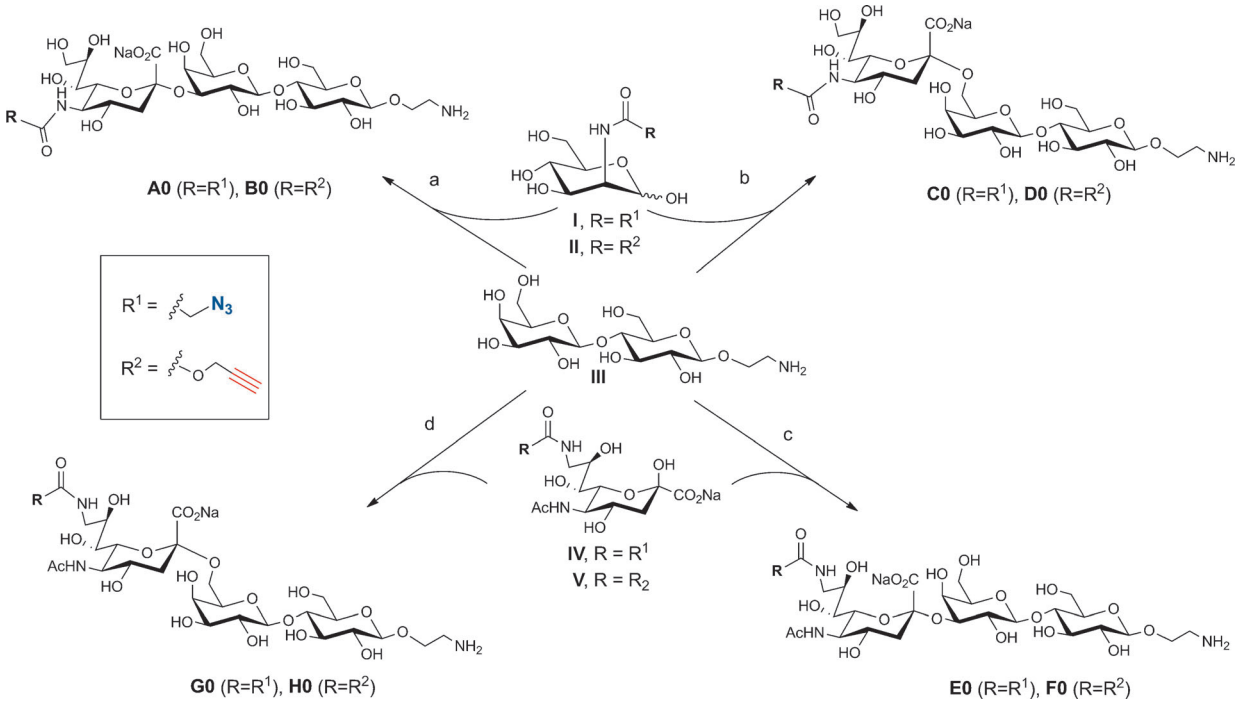

Scheme 1. Synthesis of parent azide and alkyne sialosides **A0-H0**. Reagents and conditions: a) **I** or **II**, pyruvate, *C. Perfrigens* NeuAc aldolase, CTP, *N. Meningitidis* CMP-NeuAc synthetase, *P. Multocida* 2,3-sialyltransferase, 75–90%; b) **I** or **II**, pyruvate, *C. Perfrigens* NeuAc aldolase, CTP, *N. Meningitidis* CMP-NeuAc synthetase, *P. Damsella* 2,6-sialyltransferase, 85–95%; c) **IV** or **V**, CTP, *N. Meningitidis* CMP-NeuAc synthetase, *P. Multocida* 2,3 Sialyltransferase, 70–85%; d) **IV** or **V**, CTP, *N. Meningitidis* CMP-NeuAc synthetase, *P. Damsella* 2,6-sialyltransferase, 85–95%. CMP = cytidine monophosphate, CTP = cytidine triphosphate.

Remarkably, each siglec exhibits a distinct binding pattern towards the analogue library, and analogues based on seven of the eight parent structures **A0–H0** yielded high-avidity ligands for one or more siglec.

In general we found, with few exceptions, that individual siglecs bound preferably to analogues with substituents at either C9 or C5, but not both, and the most promising were those having relatively bulky and hydrophobic substituents. Furthermore, there appeared to be little preference for the sialoside linkage (e.g. α2-3 and α2-6) suggesting that the modified sialic acid, and not the underlying lactose core, provides most of the binding affinity. We should note that the sialic acid scaffold, and not the substituent alone, is a key determinant for binding. Evidence for this is the fact that analogues with the same substituent linked at either C5 or C9 give drastically different results (for example, siglec-E with an adamantyl azide at C9, **F9** and **H9**, is a hit but at C5, **B9** and **D9**, shows no binding).

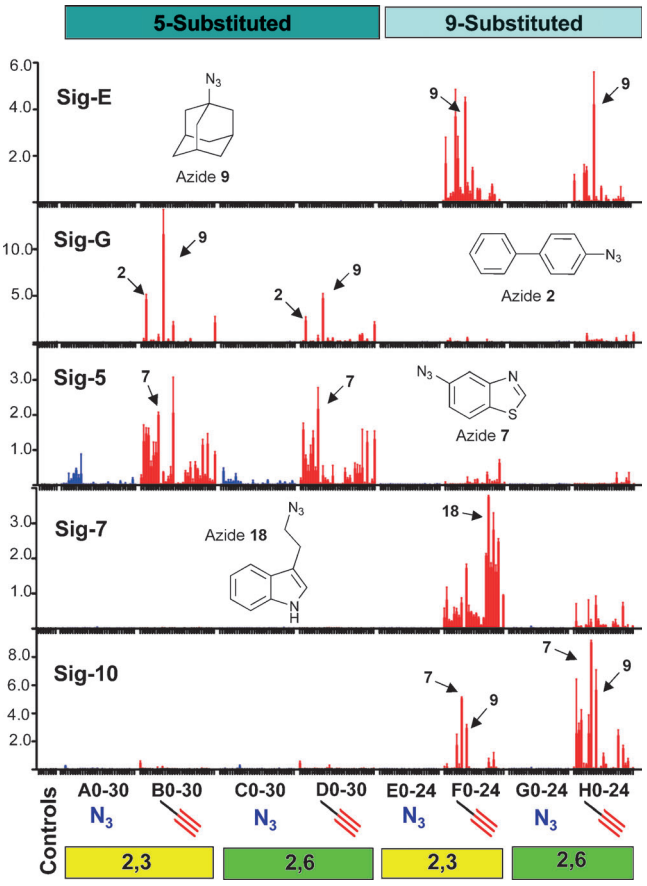

Figure 2. Siglec screening reveals unique specificity profiles for sialoside analogues. Fluorescently labeled murine (siglec-E, siglec-G) and human (siglec-5, siglec-7, siglec-10) siglecs were applied to the sialoside analogue glycan microarray to identify high-affinity analogues. Exemplary analogue hits are highlighted and denoted with the library number of the corresponding azide or alkyne substitutent (Tables S1–S8). The compound nomenclature combines the letter corresponding to the parent sialoside (Scheme 1, **A0-H0**) with the number of the azide or alkyne that it was reacted with (Figure S1 and S2). Controls include native sialosides and previously identified high-affinity analogues for hCD22, mSn, and rMAG (Figure S7).

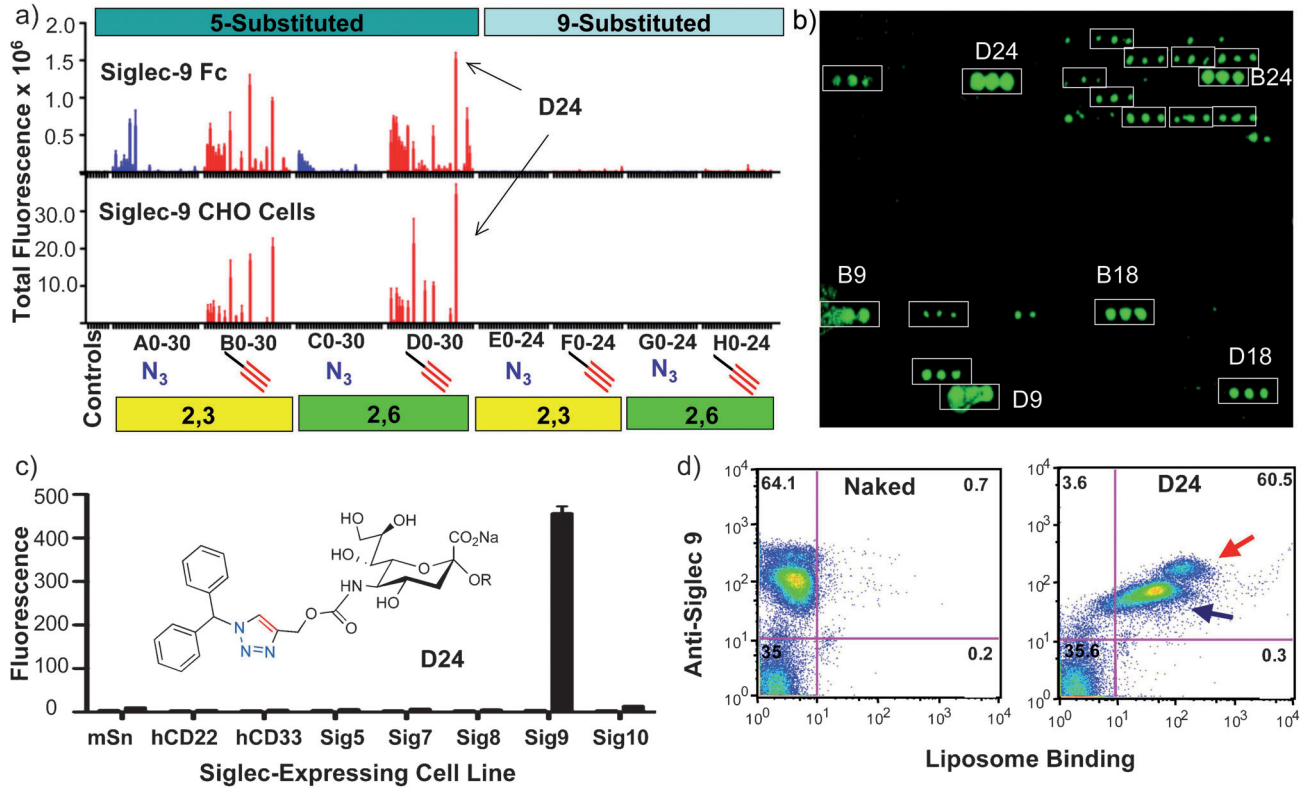

Figure 3. A siglec-9 specific analogue for cell targeting applications was identified through high-throughput screening. a) Comparison of the binding specificity of fluorescently labeled siglec-9-Fc chimera (top) and siglec-9-expressing CHO cells (bottom). b) An image of fluorescent siglec-9 CHO cells bound to the analogue microarray. c) The specificity of liposomal nanoparticles incorporating **D24**-PEG-lipid (siglec-9 targeting; filled blocks), or no ligand (naked; empty blocks), was assessed, by flow cytometry, for binding to a panel of siglec-expressing cell lines in triplicate. Inset shows the structure of **D24**. d) The siglec-9-targeting and naked liposomes were tested for binding to white blood cells isolated from peripheral human blood and co-stained with an anti-siglec-9 antibody. The two siglec-9-expressing subsets are granulocytes (blue arrow) and monocytes (red arrow) as shown by forward and side scatter properties.

To explore the potential to use the glycan array to screen the specificity of siglecs expressed on the surface of intact cells, we examined the binding of several fluorescently labeled siglec-expressing cell lines. As shown in Figure 3a, siglec-9-expressing Chinese hamster ovary (CHO) cells bound with nearly the same specificity as the siglec-9-Fc chimera, that is, binding primarily to C5-substituted compounds, and with highest apparent avidity to **D24** (see Figure 3c, inset). Similar results were obtained with human-CD22-expressing CHO cells and even a human B-cell line that expresses CD22 (Figure S4). Consistent with the binding of the hCD22-Fc chimera (Supporting Information, Figure S3), hCD22-expressing cells bound with highest avidity to **G1**, **G6**, and 9-*N*-biphenylcarboxamido-NeuAcα2-6Galβ1-4Glc (^{BPC}NeuAc), which is a known high-affinity ligand of CD22.[3e,5] Thus, it is clear that arrays can be probed with whole cells to identify high-avidity siglec ligands.

As the ultimate goal is to identify ligands that are suitable for targeting a single human or murine siglec when incorporated into synthetic multivalent sialoside probes, we tested exemplary leads for their ability to facilitate selective binding to their respective siglecs. We selected **D24** as a candidate ligand for siglec-9 owing to the fact that it facilitated binding to siglec-9 on CHO cells, even in the presence of competing *cis* ligands (Figure 3a,b),[3b–e] and it was not recognized by any other siglec tested in the screen. As a multivalent platform we chose ligand-bearing liposomal nanoparticles because of their demonstrated utility for targeting siglecs in vivo.[3b] Accordingly, **D24** was covalently attached to a PEGylated lipid (Supporting Information, Scheme S2), incorporated into liposomal nanoparticles (siglec-9-targeting liposomes), and these liposomes were assessed for binding to a panel of siglec-expressing cell lines (Figure 3c and the Supporting Information, Figure S5). The siglec-9-targeting liposomes avidly bound to and were rapidly endocytosed by siglec-9-expressing CHO cells, whereas the control (naked) liposomes exhibited no detectable binding. In contrast, no binding of the siglec-9-targeting liposomes was observed to any of the other seven siglec-expressing cell lines, thus demonstrating their high selectivity for siglec-9 (Figure 3c).

To assess the ability of the siglec-9-targeting liposomes to bind to native human leukocytes, additional experiments were carried out with white blood cells isolated from peripheral human blood (Figure 3d). While no binding of the control (naked) liposomes was observed to any cell population, the siglec-9-targeting liposomes bound avidly to two siglec-9 positive cell populations in proportion to the amount of siglec-9-expressed, and exhibited no detectable binding to cells that were siglec-9 negative. Forward- and side-scatter analysis showed that the two cell populations were

granulocytes (blue arrow) and monocytes (red arrow), consistent with previous reports documenting the expression of siglec-9 on these cell populations.[10]

To further address the general utility of this approach for identifying high-avidity siglec ligands for cell targeting, we selected **F9**, a ligand that bound to human siglec-10, and prepared the corresponding pegylated lipid (Supporting Information, Scheme S3) for incorporation into liposomal nanoparticles. When tested with a panel of human siglec-expressing cell lines, it was found that these liposomes were entirely specific for siglec-10 over any other human siglec (Figure 4a). When these liposomes were incubated with

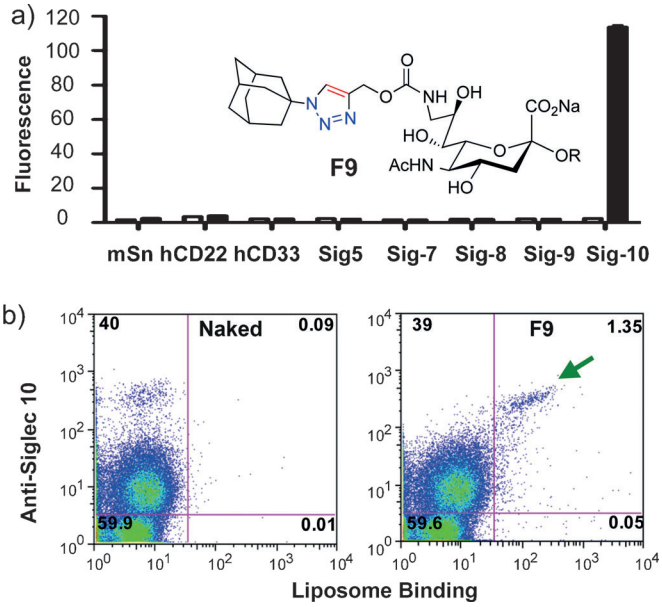

Figure 4. Identification of **F9** as a siglec-10-specific analogue for cell-targeting applications. a) The specificity of **F9**-bearing liposomal nanoparticles was assessed, by flow cytometry, for binding to a panel of siglec-expressing cell lines in triplicate. Naked: empty blocks, **F9**: filled blocks. b) These liposomes were further tested for binding to white blood cells obtained from peripheral human blood and co-stained with an anti-siglec-10 antibody. The high expressing siglec-10 cells that bind the **F9**-bearing liposomes selectively are indicated with a green arrow and are a subpopulation of monocytes as shown by forward- and side-scatter properties.

peripheral human blood cells, it was found that they bound only to a unique monocyte subpopulation that expresses particularly high levels of siglec-10 (Figure 4b).[11]

We next expanded the scope of these specificity studies to the mouse system and found, as expected from the microarray data (Figure 2), that **F9**-bearing liposomes, also bind avidly to recombinant siglec-E-expressing cells, but not other murine siglec-expressing cell lines (Supporting Information, Figure S6). In primary bone marrow isolates, it was found that **F9**-bearing liposomes bind to mouse neutrophils (Supporting Information, Figure S6); a result which is consistent with the documented expression of this siglec.[12] The lack of siglec-E-deficient mice or a suitable siglec-E antibody for flow cytometry applications have precluded further specificity studies, which will be necessary before in vivo studies can be carried out. Nonetheless, **F9** appears to be a promising new siglec ligand for both mouse and human siglec studies.

As illustrated from these examples, the click and pick strategy has provided numerous leads for the development of multivalent ligand-based probes for human and murine siglecs even in the absence of structural information for the majority of the siglec family members. Such agents could be used to explore the functions of siglecs,[3a,13] and for applications involving targeting of leukocytes in vivo.[3b] Moreover, the method may be applicable for identifying high-affinity ligands for other families of glycan-binding proteins of biological and therapeutic interest such as the C-type lectins,[14] which are broadly expressed on antigen-presenting cells (APCs) involved in innate and adaptive immunity. Recently, chemoenzymatic approaches have been used to generate azide and alkyne bearing ligands for these receptors,[15] which could serve as starting points for analogue library generation and subsequent screening efforts to identify new chemical probes for this protein family and as vaccine delivery agents to APC subsets.[16]

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