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'Click and Pick' Identification of High Affinity Sialoside Ligands for Siglec-based Targeting of Leukocytes**

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

Limiting the potential of ligand targeted nanoparticles has been the difficulty in identifying siglec ligands of suitable affinity and selectivity.^[1a, 2, 4] Previous reports have demonstrated that modification of sialic acid (NeuAc) with substituents at C9 can produce both increased affinity and selectivity for sialoadhesin (Siglec-1), CD22 (Siglec-2) and myelin associated glycoprotein (Siglec-4).^[5] Modifications at the C5-position have also been suggested to modulate affinity and selectivity of several siglecs, however, sialoside analogs modified at this position have not been fully explored for these properties.^[6] Thus, while modifications 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

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methods to robustly generate sialoside analog libraries and systematically screen them against a library of siglecs has hampered progress.

To address this, we devised a facile ‘click and pick’ strategy involving high-throughput synthesis of a sialoside analog 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 via a convergent chemoenzymatic approach (Scheme 1 and Scheme S1) with azide or alkyne substituents at the 5-position (**A0-D0**) or the 9-position (**E0-H0**) of the sialic acid moiety, each attached in α 2–3 or α 2–6 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 (Figure S1–S2) to generate a library of 224 sialoside analogs (Tables S1–S8) with quantitative conversion of nearly all couplings. The sialoside products could then be printed directly onto NHS-activated microarray slides without prior purification due to the orthogonality of the click reaction with amine acylation chemistry, allowing for far higher screening throughput and library diversity than previous efforts towards this end.^[6b, 8]

To identify high-affinity ligand analogs of individual siglecs, fluorescently labeled siglec-Fc chimeras were overlaid 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**, 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 Figure S3. Remarkably, each siglec exhibits a distinct binding pattern towards the analog library, and analogs based on seven of the eight parent structures **A0-H0** yielded high-avidity ligands for one or more siglecs.

In general we found, with few exceptions, that individual siglecs preferred substituents at either C9 or C5, but not both, and the most promising were those that are relatively bulky and hydrophobic. Furthermore, there was greater preference for the substituent on the sialic acid than for the sialoside linkage (e.g. 2–3 and 2–6). While there is relaxed linkage specificity, the sialic acid scaffold, and not the substituent itself, is a key determinant for binding. Evidence for this is the fact that the same substituent linked at C5 or C9 gives drastically different results (for example, Siglec-E with an adamantyl azide at C9 is a hit, **F9** and **H9**, but at C5 shows no binding, **B9** and **D9**).

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, binding primarily to C-5 substituted compounds, and with highest apparent avidity to **D24** (see inset). Similar results were obtained with human CD22 expressing CHO cells and even a human B-cell line which expresses CD22 (Figure S4). Consistent with the binding of the hCD22 Fc-chimera (Figure S3), hCD22 expressing cells bound with highest avidity to **G1**, **G6**, and 9-*N*-biphenylcarboxamido-NeuAc α 2–6Gal β 1–4Glc (^{BPC}NeuAc), printed as 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.

Since 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 support selective binding to their respective siglecs. We selected **D24** as a candidate ligand for Siglec-9 due to the fact that it supported binding of

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 decorated liposomal nanoparticles based on the demonstrated utility of this platform for targeting siglecs *in vivo*.^[3b] Accordingly, **D24** was covalently attached to a PEGylated lipid (Scheme S2), incorporated into liposomal nanoparticles ('Siglec-9 targeted liposomes'), and these liposomes were assessed for binding to a panel of siglec expressing cell lines (Figure 3c, Figure S5). The Siglec-9 targeted liposomes avidly bound to and were rapidly endocytosed by Siglec-9 expressing CHO cells, while the control ('Naked') liposomes exhibited no detectable binding. In contrast, no binding of the Siglec-9 targeted liposomes was observed to any of the other seven siglec-expressing cell lines, demonstrating high selectivity for Siglec-9 (Figure 3c).

To assess the ability of the Siglec-9 targeted 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 targeted 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 a ligand detected by human Siglec-10, **F9**, and prepared the corresponding pegylated lipid (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 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]

Expanding the scope of these specificity studies to the mouse system it was found, as expected from the microarray data (Figure 2), that **F9**-displaying liposomes, also bind avidly to recombinant Siglec-E expressing cells, but not other murine siglec-expressing cell lines (Figure S6). In primary bone marrow isolates, it was found that **F9**-liposomes bind to mouse neutrophils (Figure S6), 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 to move this candidate ligand forward into *in vivo* studies. 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 developing multivalent ligand-based probes for human and murine siglecs even in the absence of structural information for the majority of siglec family members. Such agents will have value as tools for exploring 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 analog library generation and subsequent screening efforts to identify new chemical probes for this protein family and as vaccine delivery agents to APC subsets.^[16]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Click and pick strategy for identification of high avidity siglec ligands

Work flow involving parallel Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) synthesis of the analog library, glycan microarray printing, and screening with fluorescently labeled siglec-Fc chimeras to identify high affinity ligands.

Figure 2. Siglec screening reveals unique specificity profiles for sialoside analogs
Fluorescently labeled murine (Siglec-E, Siglec-G) and human (Siglec-5, Siglec-7, Siglec-10) siglecs were applied to the sialoside analog glycan microarray to identify high affinity analogs. Exemplary analog hits are highlighted and denoted with the library number of the corresponding azide or alkyne substituent (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 (Figures S1 and S2). Controls include native sialosides and previously identified high affinity analogs for hCD22, mSn, and rMAG (Figure S7).

Figure 3. High-throughput screening identifies a Siglec-9 specific analog for cell targeting applications

a) Comparison of the binding specificity of fluorescently labeled Siglec-9 Fc-chimera (*top*) and Siglec-9 expressing CHO cells (*bottom*). The structure of the strongest hit, **D24**, is shown in the inset. b) An image of fluorescent Siglec-9 CHO cells bound to the analog microarray. c) The specificity of liposomal nanoparticles incorporating **D24**-PEG-lipid (Siglec-9 targeted), or no ligand (Naked), was assessed by flow-cytometry for binding to a panel of Siglec-expressing cell lines in triplicate. d) The Siglec-9 targeted and naked liposomes were tested for binding to white blood cells isolated from peripheral human blood and costained with an anti-Siglec 9 antibody. The two Siglec-9 expressing subsets are granulocytes (red arrow) and monocytes (blue arrow) as shown by forward and side scatter properties.

Figure 4. Identification of F9 as a Siglec-10 specific analog for cell targeting applications
 a) The specificity of **F9**-displaying liposomal nanoparticles was assessed by flow-cytometry for binding to a panel of Siglec-expressing cell lines in triplicate. 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 which bind the **F9**-liposomes selectively are noted with a green arrow and are a subpopulation of monocytes as shown by forward and side-scatter properties.

Scheme 1.

Synthesis of Parent Azide and Alkyne Sialosides **A0-H0**. Reagents and conditions: a) **1** or **2**, Pyruvate, *C. Perfringens* NeuAc Aldolase, CTP, *N. Meningitidis* CMP-NeuAc Synthetase, *P. Multocida* 2,3 Sialyltransferase, 75–90%; b) **1** or **2**, Pyruvate, *C. Perfringens* NeuAc Aldolase, CTP, *N. Meningitidis* CMP-NeuAc Synthetase, *P. Damsella* 2,6 Sialyltransferase, 85–95%; c) **4** or **5**, CTP, *N. Meningitidis* CMP-NeuAc Synthetase, *P. Multocida* 2,3 Sialyltransferase, 70–85%; d) **4** or **5**, CTP, *N. Meningitidis* CMP-NeuAc Synthetase, *P. Damsella* 2,6 Sialyltransferase, 85–95%.