Azido Sialic Acids Can Modulate Cell-Surface Interactions

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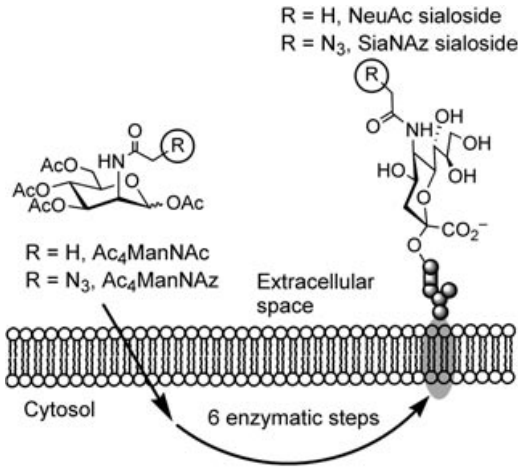
Carbohydrate–receptor interactions govern cell–cell recognition events that are crucial in development, the immune response, and microbial infection.[1–3] An understanding of the structural basis of these interactions is paramount for the development of chemical probes and therapeutic approaches. Conventional structure–activity relationship (SAR) studies have involved chemical synthesis of modified carbohydrates and in vitro binding assays to identify those functional groups most critical for molecular recognition. However, the specificities of carbohydrate-binding proteins for their ligands can be affected by the context in which those ligands are presented. When displayed on cell surfaces, analogues of L-selectin ligands exhibit a different rank order of receptor-binding affinities than observed in simple solution binding assays.[4,5] Similarly, the relative lectin-binding affinities of carbohydrate analogues in solution can change when the compounds are immobilized on solid phase.[6] These examples highlight the importance of studying carbohydrate–receptor interactions in the more native environment of the cell surface. To perform SAR studies in this context requires a means to alter cell-surface carbohydrate structures.

We and others have previously shown that cell-surface carbohydrate structures can be modulated by the biosynthetic introduction of unnatural monosaccharides, a method termed "metabolic oligosaccharide engineering".[7,8] Structural modifications to sialic acid,[9–11] *N*-acetylgalactosamine,[12,13] and *N*-acetylglucosamine[14,15] can be achieved in this fashion, thus providing a means to probe their contribution to specific receptor–ligand interactions. Indeed, Reutter, Pawlita and co-workers have applied unnatural sialic acid biosynthesis to modulate viral receptor and lectin binding at the cell surface.[16–18]

Here we probe the tolerance of numerous sialic acid-binding proteins for unnatural modification to their cell-surface ligands. We replaced natural cell-surface sialic acids with an *N*-azidoacetyl analogue (SiaNAz) by feeding to the cells the precursor peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz), an analogue of *N*-acetylmannosamine (ManNAc; Scheme 1).[11] The effects of this modification on recognition by sialic acid-binding lectins, the sialic acid-dependent antibody HECA-452, and the receptors E-selectin and Siglec-1 were determined to provide insight into their ligand promiscuity. These results have implications regarding the physiological effects of unnatural sialic acids presented on cells in vivo.



Scheme 1. *Metabolic oligosaccharide engineering permits structural modulation of cell-surface sialic acids. Peracetylated* N*-azidoacetylmannosamine (Ac_4-ManNAz) is taken up by cells, converted into the unnatural sialoside,* N*-azidoacetyl sialic acid (SiaNAz), and delivered to glycoconjugate chains destined for the cell surface. The peracetylated derivatives are typically used because they cross the cell membrane more readily[42,43] than free sugars and are deacetylated in situ by cytosolic esterases.*

For these studies, we required a cell line with low basal levels of natural sialic acid and the ability to transform the unnatural precursor Ac_4ManNAz to cell-surface SiaNAz at relatively high levels. The mutant cell lines K20 (human B-cell lymphoma varient),[19] HL60-I (human myeloid leukemia variant) and Lec3 (Chinese hamster ovary (CHO) variant)[20] are ideal for this purpose. These cells are deficient in UDP-GlcNAc 2-epimerase activity, the enzyme that produces ManNAc, and have low cell-surface sialic acid levels, thereby minimizing background activity in receptor binding studies. K20 and HL60-I cells have previously been shown to deliver unnatural sialic acids to cell-surface glycoconjugates, a modification that disrupted sialic acid–receptor interactions.[18] We sought to determine whether Lec3 cells are also suitable hosts for unnatural cell-surface sialosides.

To determine if Lec3 cell-surface glycans could be modified with SiaNAz, we treated the cells with Ac_4ManNAz and analyzed their surfaces for azides by Staudinger ligation with a phosphine-FLAG probe, as previously described.[15,21] The cells were labeled with a fluorescein-conjugated anti-FLAG antibody and analyzed by flow cytometry. The Lec3 cells showed robust metabolic incorporation of azides into their cellular glycoconjugates at levels comparable to previously studied cell lines (see Supporting Information).[15]

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Although Lec3 cells are deficient in endogenous sialic acid production, they are able to scavenge sialic acid from glycoproteins in their serum-containing media and are thus not totally devoid of the natural sugar, even while in media lacking serum for the duration of the experiments presented here.[20] We therefore sought to quantify the representation of SiaNAz among the total sialic acids resident within Lec3 glycans. Direct analysis of sialic acids was achieved by mild acid hydrolysis of cell lysates followed by HPLC analysis compared to authentic sialic acid standards.[22,23] As shown in Table 1, untreated Lec3 cells possess the natural sialic acids *N*-acetyl sialic acid (NeuAc, Scheme 1) and *N*-glycolyl sialic acid (NeuGc). Supplementation of the media with peracetylated ManNAc (Ac_4ManNAc) increased the level of NeuAc, and the overall sialic acid level, as expected. When Lec3 cells were treated with Ac_4ManNAz, SiaNAz was produced at a level corresponding to ~30% of the total sialic acid pool. The overall sialic acid level was elevated 1.5-fold under these conditions. Both the representation of SiaNAz and the absolute levels of total sialic acids produced were deemed appropriate for receptor binding studies.

Table 1. *Degree of natural and unnatural sialylation of Lec3 cells.*

Media supplement		Sialic acid analysis			
NAc[a]	NAz[b]	NeuGc[c]	NeuAc[c]	SiaNAz[c]	total sia[c]
–	–	123	754	0	877
+	–	74	2011	0	2085
–	+	183	865	530	1578

[a] Cells were supplemented with Ac_4ManNAc (35 μM) where indicated (+). [b] Cells were supplemented with Ac_4ManNAz (35 μM) where indicated (+). [c] pmol sialic acid per mg protein.

With an experimental system in hand, we next probed the effect of the *N*-acyl-group modification on binding to well-characterized sialic acid-specific lectins. These included *Maackia amurensis* (MAA), *Limax flavus* agglutinin (LFA) and *Sambucus nigra* agglutinin (SNA). Lec3 cells were untreated, or exposed to either Ac_4ManNAc or Ac_4ManNAz for three days. The cells were washed and labeled with fluorescein-conjugated LFA (LFA–FITC, linkage-independent) or MAA (MAA–FITC, specific for Sia-α(2,3)-Gal). Untreated Lec3 cells bound LFA–FITC at low levels (Figure 1 A). Treatment with Ac_4ManNAc caused a dramatic increase in LFA–FITC binding. By contrast, treatment with Ac_4ManNAz had no measurable effect on lectin binding. Independent detection of azides by Staudinger ligation with phosphine-FLAG indicated that SiaNAz was indeed present on the cell surface (not shown). Since overall sialylation of Lec3 cells increased in the presence of SiaNAz by ~50% (Table 1), we conclude that LFA–FITC does not recognize sialic acid with the unnatural *N*-acyl group. This result is consistent with the previous observation that LFA recognizes NeuAc but not NeuGc, a natural *N*-acyl modified sialic acid.[24] The inability of LFA to bind either SiaNAz or NeuGc suggests that LFA has a stringent requirement for the *N*-acetyl group of NeuAc.

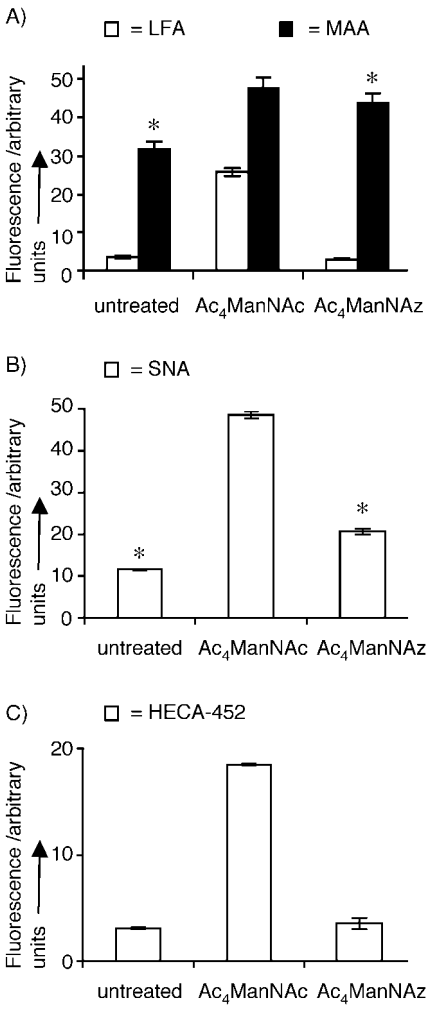

Figure 1. *Sialic acid-binding proteins exhibit different specificities for SiaNAz-modified glycoconjugates. A) Lec3 cells were treated with Ac_4ManNAc (35 μM), Ac_4ManNAz (35 μM), or untreated, for 3 d in serum-free media. Cells were washed, labeled with LFA–FITC or MAA–FITC, and analyzed by flow cytometry. B) Lec3 cells were transfected with ST6Gal I and treated with the indicated monosaccharides as in A. The cells were labeled with SNA–FITC and analyzed by flow cytometry. C) Lec3 cells were transfected with FucT7 and treated with the indicated monosaccharides as in A. The cells were labeled with HECA-452 and analyzed by flow cytometry. In both B and C, mock-transfected cells showed background levels of fluorescence equivalent to untreated cells (not shown). In A, B and C, error bars represent the standard deviation of three replicate experiments. The asterisks indicate that lectin binding to untreated and Ac_4ManNAz-treated cells is significantly different ($P < 0.005$, two-tailed t test).*

We performed a similar study with MAA–FITC. Despite their low levels of sialic acid, untreated Lec3 cells bound MAA–FITC at significant levels that were unaffected by treatment with sialidase (see Supporting Information). This sialic acid-independent binding was presumably due to the cross-reactivity of MAA with Gal-β(1,4)-GlcNAc/Glc.[25] However, MAA–FITC binding increased by 1.5- and 1.4-fold when Lec3 cells were treated with Ac_4ManNAc or Ac_4ManNAz, respectively (Figure 1 A), and this response was abrogated by sialidase treatment (see Supporting Information). Thus, MAA–FITC appears to recognize SiaNAz, consistent with its known binding activity with NeuGc.[25,26]

Like MAA, SNA is also promiscuous, binding NeuGc as well as NeuAc.[26] To determine whether SNA recognizes SiaNAz, we introduced the gene encoding the sialyltransferase ST6Gal I[27] into Lec3 cells to produce α-2,6-linked sialylated epitopes. Following transient transfection, cells were treated with Ac_4ManNAc or Ac_4ManNAz, labeled with fluorescein-conjugated SNA (SNA–FITC), and analyzed by flow cytometry. Like MAA–FITC, SNA–FITC stained untreated cells at significant background levels that were essentially unaffected by sialidase treatment (Figure 1B and Supporting Information). However, Ac_4ManNAc-treated cells showed a fourfold increase in SNA–FITC binding over untreated cells. The fluorescence of Ac_4ManNAz-treated cells increased 1.8-fold over untreated cells; this suggests that SNA binds SiaNAz in addition to NeuAc. Furthermore, these data imply that SiaNAz can be introduced into Sia-α(2,6)-Gal linkages by ST6Gal I.

Carbohydrate-specific antibodies are important tools in glycobiology, and the structural basis of antigen binding is a subject of considerable attention.[28,29] We sought to test the effects of cell-surface sialic acid modifications on binding to an antibody known to require this residue. HECA-452 is a commercially available IgM that recognizes sialyl Lewis x (Sia-α(2,3)-Gal-β(1,4)[Fuc(α-1,3)]-GlcNAc, sLe^x), a motif found in selectin ligands. To determine whether HECA-452 can bind sLe^x possessing SiaNAz, we introduced the gene encoding fucosyltransferase VII (FucT7)[30,31] into Lec3 cells by transient transfection to generate the sLe^x epitope. The transfected cells were treated with Ac_4ManNAc, Ac_4ManNAz, or left untreated, then labeled with biotinylated HECA-452 followed by fluorescein-conjugated avidin (FITC-avidin) and analyzed by flow cytometry. Untreated Lec3 cells did not bind HECA-452; this is consistent with the antibody's requirement for the sialylated tetrasaccharide (Figure 1C). Treatment with Ac_4ManNAc significantly increased HECA-452 binding whereas treatment with Ac_4ManNAz had no effect. These data indicate that HECA-452 does not bind sLe^x bearing the unnatural sialic acid, SiaNAz, and therefore must have a strict requirement for the *N*-acetyl group of NeuAc.

The dramatic effect of the *N*-azidoacetyl modification on HECA-452 binding prompted us to explore its impact on selectin recognition. Synthetic sLe^x analogues of the tetrasaccharide have been probed with respect to selectin binding,[32] and these studies suggest considerable tolerance of modifications to the *N*-acyl group of sialic acid. To confirm this with respect to SiaNAz, we transfected cells with genes encoding FucT7 and a second glycosyltransferase, core 2 *N*-acetylglucosaminyltransferase (C2GnT),[33] in order to elaborate both *N*- and *O*-linked glycans with sLe^x. The cells were incubated with Ac_4ManNAc, Ac_4ManNAz, or left untreated, and probed with a soluble form of E-selectin conjugated to the constant region of a human IgM (E-selectin-IgM[34,35]). The IgM portion of the molecule enabled flow cytometry analysis with a fluorescein-labeled anti-human IgM antibody (anti-IgM–FITC). E-selectin-IgM bound to untreated Lec3 cells to a minimal extent (Figure 2A). E-selectin binding was dramatically increased when Lec3 cells were exposed to either Ac_4ManNAc or Ac_4ManNAz. Control cells treated with a class-matched antibody (human IgM) in place of E-selectin-IgM showed only background levels of fluorescence. To confirm that the observed cellular fluorescence was due to specific E-selectin-ligand binding, we treated cells with EDTA. Chelation of the calcium ions required for E-selectin binding caused a total loss of cell-surface fluorescence (Figure 2A). These data indicate that E-selectin can bind cell-surface sLe^x bearing SiaNAz in place of natural sialic acid.

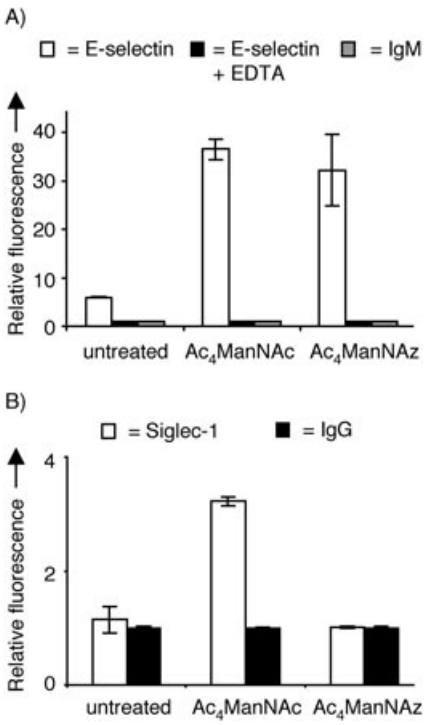

Figure 2. *E-selectin recognizes SiaNAz-terminated glycans whereas Siglec-1 does not. A) Lec3 cells were transfected with FucT7 and C2GnT and then incubated with Ac_4ManNAc (35 μM) or Ac_4ManNAz (35 μM) for 3 d in serum-free media. Cells were then labeled with human IgM (isotype control) or E-selectin-IgM that had been preincubated with anti-human IgM–FITC in the presence or absence of EDTA. The cells were analyzed by flow cytometry. B) Lec3 cells were incubated with the compounds indicated for 3 d in serum-free media. The cells were subsequently labeled with Siglec-1-Ig or human IgG (isotype control) that had been preincubated with biotinylated anti-human IgG. Labeled cells were further stained with FITC-avidin and analyzed by flow cytometry. In both A and B, the data are reported as fluorescence intensity relative to the isotype control. Error bars represent the standard deviation of three replicate experiments.*

Siglec-1 (sialoadhesin) is a sialic acid-specific lectin found primarily on macrophages.[36] Its function remains unknown, but its restricted expression pattern suggests a role in the immune system. The ability to perturb Siglec-1 binding would provide a means to study its biological roles. Murine Siglec-1 discriminates between NeuAc and NeuGc, with a preference for the smaller *N*-acyl group.[37] To determine if Siglec-1 binds to SiaNAz, we incubated Lec3 cells with Ac_4ManNAc or Ac_4ManNAz. The cells were then exposed to Siglec-1-Ig, a soluble version of murine Siglec-1 fused to the Fc domain of a human IgG.[38,39] The cells were then treated with a biotinylated anti-human IgG antibody followed by FITC-avidin and analyzed by flow cytometry. Cells treated with Ac_4ManNAc showed an increase in cellular fluorescence over untreated cells (2.8-fold, Figure 2B), whereas cells treated with Ac_4ManNAz showed only

background levels of binding. Control cells treated with human IgG in place of Siglec-1-Ig also showed background levels of binding. These data indicate that SiaNAz is not recognized by murine Siglec-1-Ig, and suggest an avenue for disrupting Siglec-1 binding to cells, that is, metabolic replacement of NeuAc with an unnatural *N*-acyl variant.

In summary, we have applied metabolic oligosaccharide engineering to probe the effects of structural modifications to sialic acid on the binding of carbohydrate receptors. The approach was validated with well-characterized lectins, and then used to define the unnatural sialic acid tolerance of the widely used antibody HECA-452, a reporter of sLe^x expression, and the macrophage-specific adhesion molecule Siglec-1. We also probed the unnatural sialic acid tolerance of E-selectin in the context of the cell surface. These results provide a means to probe Siglec-1 function in cell-based systems. In addition, they enable predictions regarding the physiological effects of unnatural sialic acid expression in living organisms, a major frontier of metabolic oligosaccharide engineering.[40,41]

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