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Modified GM3 gangliosides produced by metabolic oligosaccharide engineering

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ABSTRACT

Metabolic oligosaccharide engineering is powerful approach to altering the structure of cellular sialosides. This method relies on culturing cells with *N*-acetylmannosamine (ManNAc) analogs that are metabolized to their sialic acid counterparts and added to glycoproteins and glycolipids. Here we employed two cell lines that are deficient in ManNAc biosynthesis and examined their relative abilities to metabolize a panel of ManNAc analogs to sialosides. In addition to measuring global sialoside production, we also examined biosynthesis of the sialic acid-containing glycolipid, GM3. We discovered that the two cell lines differ in their ability to discriminate among the variant forms of ManNAc. Further, our data suggest that modified forms of sialic acid may be preferentially incorporated into certain sialosides and excluded from others. Taken together, our results demonstrate that global analysis of sialoside production can obscure sialoside-specific differences. These findings have implications for downstream applications of metabolic oligosaccharide engineering, including imaging and proteomics.

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Glycoconjugates are displayed on the surface of mammalian cells where they play critical roles in both normal and pathological recognition events. Efforts to better delineate glycoconjugates' functions are hindered by the fact that the glycan components are not primary gene products, limiting the utility of site-directed mutagenesis to alter glycan structures in predictable ways. Metabolic oligosaccharide engineering^{1,2} has provided an important alternative approach to studying glycoconjugate function. By introducing small structural modifications to monosaccharides, and then adding these analogs to cultured cells, it is possible to alter the structure of cellular glycoconjugates in a predictable and controlled way. Metabolic oligosaccharide engineering relies on the abilities of mammalian cells to internalize analogs, metabolize them, and incorporate the unnatural metabolites into glycan structures in place of natural sugars. Early work demonstrated that sialic acid analogs in which the *N*-acyl chain was lengthened with additional methylene units could be readily tolerated and incorporated into cell surface glycan structures in cells and living animals.^{3,4} Since then, metabolic oligosaccharide engineering has expanded in scope and now enables the introduction of a variety of functional groups with specialized reactivity (e.g., ketone,⁵ azide,^{6,7} alkyne,⁸ diazirine⁹) into various glycoconjugates, but most successfully into sialic acid-containing glycoconjugates, also known as sialosides.¹ Metabolic oligosaccharide engineering has found widespread utility, including the exploration of host–virus

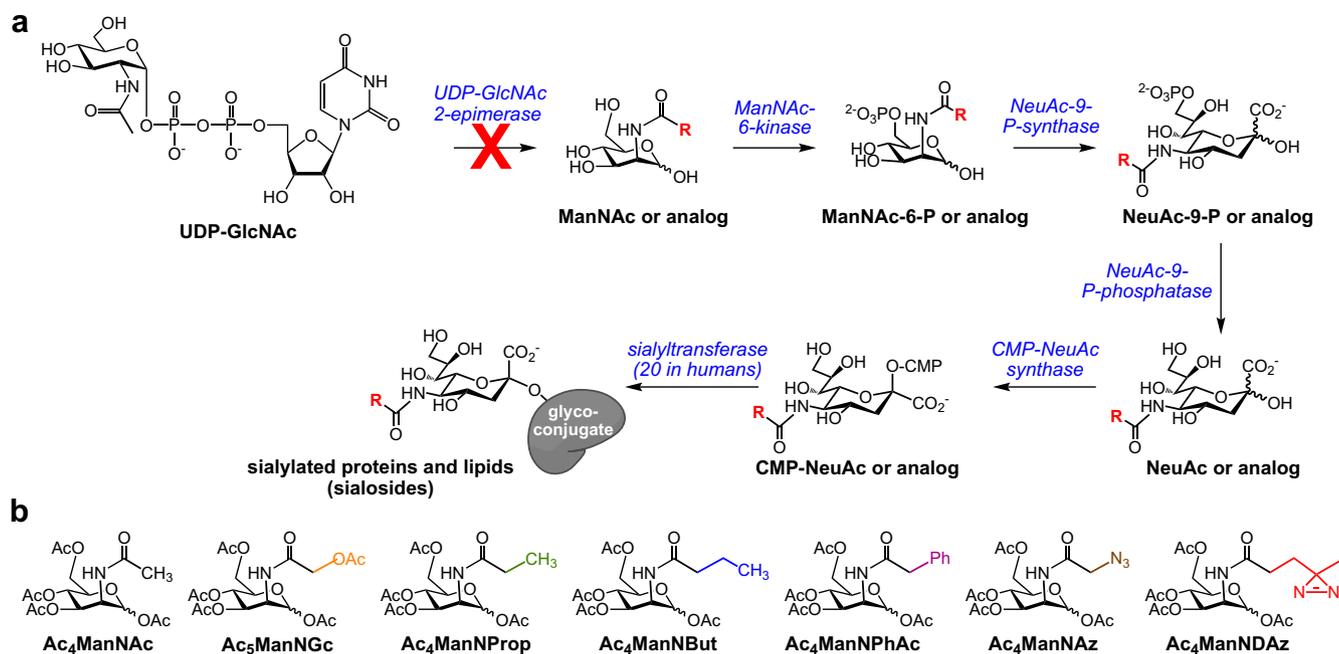
interactions,¹⁰ the regulation of neuronal cell differentiation,¹¹ and the visualization of developmental changes in glycosylation.¹²

While metabolic oligosaccharide engineering has proved to be a highly effective method to alter sialoside structure, the majority of studies measure the success of the engineering at a global level and do not distinguish among the types of sialosides that are modified by this technique. In humans, production of sialosides is controlled by twenty different sialyltransferases.¹³ Sialyltransferases transfer sialic acid from CMP-sialic acid, the nucleotide sugar donor, to acceptor glycoconjugates. Sialyltransferases are classified into families that catalyze the formation of the α 2–3-, α 2–6-, or α 2–8-linked sialic acid and each sialyltransferase has unique acceptor specificity. Because of this diversity at the sialyltransfer step, it is possible that metabolically incorporated sialic acid variants are not equally well incorporated into all types of sialosides. Therefore, we decided investigate the linkage-specific and acceptor-specific incorporation of sialic acid analogs.

For these experiments, we employed analogs of *N*-acetylmannosamine (ManNAc), the biosynthetic precursor of sialic acid. In mammalian cells, ManNAc is metabolized to *N*-acetylneuraminic acid (NeuAc), a common form of sialic acid, in three enzymatic steps (Scheme 1a). Similarly, *N*-acyl modified ManNAc analogs can be metabolized to their sialic acid counterparts.¹ Sialic acids and sialic acid analogs are activated to CMP-sialic acids, then transported into the Golgi, where the sialic acids are transferred to glycoconjugates destined for secretion or cell surface display. Based on previous reports of successful metabolic oligosaccharide engineering, we pre-

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Scheme 1. Metabolism of protected ManNAc analogs to sialosides. (a) BJAB K20 and CHO *Lec3* cells lack UDP-GlcNAc 2-epimerase activity, rendering them unable to biosynthesize ManNAc. When these cells are cultured in serum free conditions, they fail to produce sialosides. The UDP-GlcNAc 2-epimerase-deficient cells maintain the ability to transform ManNAc to sialosides and can also metabolize exogenously supplied ManNAc analogs to their sialoside counterparts. (b) *N*-acyl-modified ManNAc analogs were prepared and added to the media of cultured cells. All analogs were peracetylated to facilitate their entry into cells. All acetyl groups, including the one on the *N*-glycolyl side chain of Ac_5ManNGc , are believed to be removed by intracellular esterases.

pared a small panel of ManNAc analogs (Scheme 1b). ManNAz, a widely-used azide-containing analog, is known to be metabolized into SiaNAz, which has been incorporated into many cell surface glycoconjugates,⁶ including gangliosides of Jurkat cells.¹⁴ ManNProp and ManNPhAc have also been successfully metabolized to SiaNProp and SiaNPhAc, respectively, and incorporated into cell surface gangliosides in several cancer lines. These modified gangliosides have been utilized as immunogenic targets for antibodies.^{15–18} ManNBut is incorporated into glycoconjugates as SiaNBut, but acts as a chain terminator in polysialylation.¹⁹ ManNGc, which can be metabolized into the naturally occurring sialic acid NeuGc, has been used to metabolically produce hydroxylated cell surface gangliosides in neuronal cells to investigate myelin–axon interactions.²⁰ Finally, ManNDAz is metabolized to SiaDAz, which contains a photoactivable crosslinking group that enables covalent crosslinking of sialosides to their binding partners.^{9,21} In the experiments described here, all ManNAc analogs were peracetylated to enable their facile transport into the cell.²² Protecting groups are assumed to be removed by intracellular nonspecific esterases.

To specifically track the metabolism of exogenously introduced ManNAc analogs, we employed two cell lines, human BJAB K20^{23,24} and hamster CHO *Lec3*,²⁵ that are impaired in sialic acid biosynthesis due to lack of UDP-GlcNAc 2-epimerase activity (Scheme 1a). When these epimerase-deficient cell lines are cultured in serum-free conditions, where they cannot scavenge sialic acid, they are unable to synthesize sialosides. When the media is supplemented with Ac_4ManNAc , the cells regain the ability to generate sialylated glycoconjugates. Similarly, when the cells are cultured with unnatural ManNAc analogs, any sialoside production is interpreted to reflect successful metabolism of these unnatural analogs to sialosides. As a control, we also examined sialoside production in similar cell lines, BJAB K88 and wt CHO, that maintain UDP-GlcNAc 2-epimerase activity.

BJAB K20 and CHO *Lec3* cells were cultured in serum-free conditions with each of the protected ManNAc analogs. First, we exam-

ined total cell surface sialoside levels using the recently reported periodate oxidation and aniline-catalyzed oxime ligation (PAL).²⁶ Consistent with previous reports, we found that Ac_4ManNAc , $\text{Ac}_4\text{ManNProp}$, and $\text{Ac}_4\text{ManNBut}$ were robustly metabolized to sialosides, both in human BJAB K20 cells and hamster CHO *Lec3* cells (Fig. 1a and b). Ac_4ManNAz and $\text{Ac}_4\text{ManNDAz}$ were also metabolized to sialosides in both cell lines, albeit at lower efficiency. Our data do not clearly demonstrate that $\text{Ac}_4\text{ManNPhAc}$ is metabolized to sialosides, but this may reflect the sensitivity limits of the PAL assay since SiaNPhAc-containing glycoconjugates have been detected using antibodies by others.^{16–18} We noted one significant difference between human BJAB K20 cells and hamster CHO *Lec3* cells cultured with Ac_5ManNGc : *Lec3* cells produce high levels of NeuGc-containing glycoconjugates, while these levels are lower in K20 cells.

Next, we used lectin binding to examine whether the incorporation of variant sialic acids was linkage-dependent (Fig. 1c–e). α 2–3-Linked sialosides were detected with the *Maackia amurensis* lectin (MAA) and α 2–6-linked sialosides were detected with the *Sambucus nigra* lectin (SNA). In human BJAB K20 cells, we detected both α 2–3- and α 2–6-linked sialosides. Generally, the linkage-specific incorporation trends were similar to the global incorporation trend observed by PAL. However, while a relatively low level of MAA binding was observed in BJAB K20 cells cultured with Ac_5ManNGc , the level of SNA binding was high and exceeded the level of SNA binding observed for BJAB K20 cells cultured with Ac_4ManNAc . In CHO *Lec3* cells, we examined only α 2–3-linked sialoside production, since these cells do not produce detectable levels of α 2–6-linked sialosides.²⁷ In CHO *Lec3* cells, the trends we observed for MAA binding for different ManNAc analogs were similar to the trends for global sialoside production detected by PAL. However, we once again noticed a difference between BJAB K20 and CHO *Lec3* cells: our MAA binding data suggested that CHO *Lec3* cells produce high levels of NeuGc-containing α 2–3-linked sialosides, while these levels are lower in BJAB K20 cells. We considered the possibility that the observed differences SNA and MAA binding that we observed for BJAB K20 cells cultured with

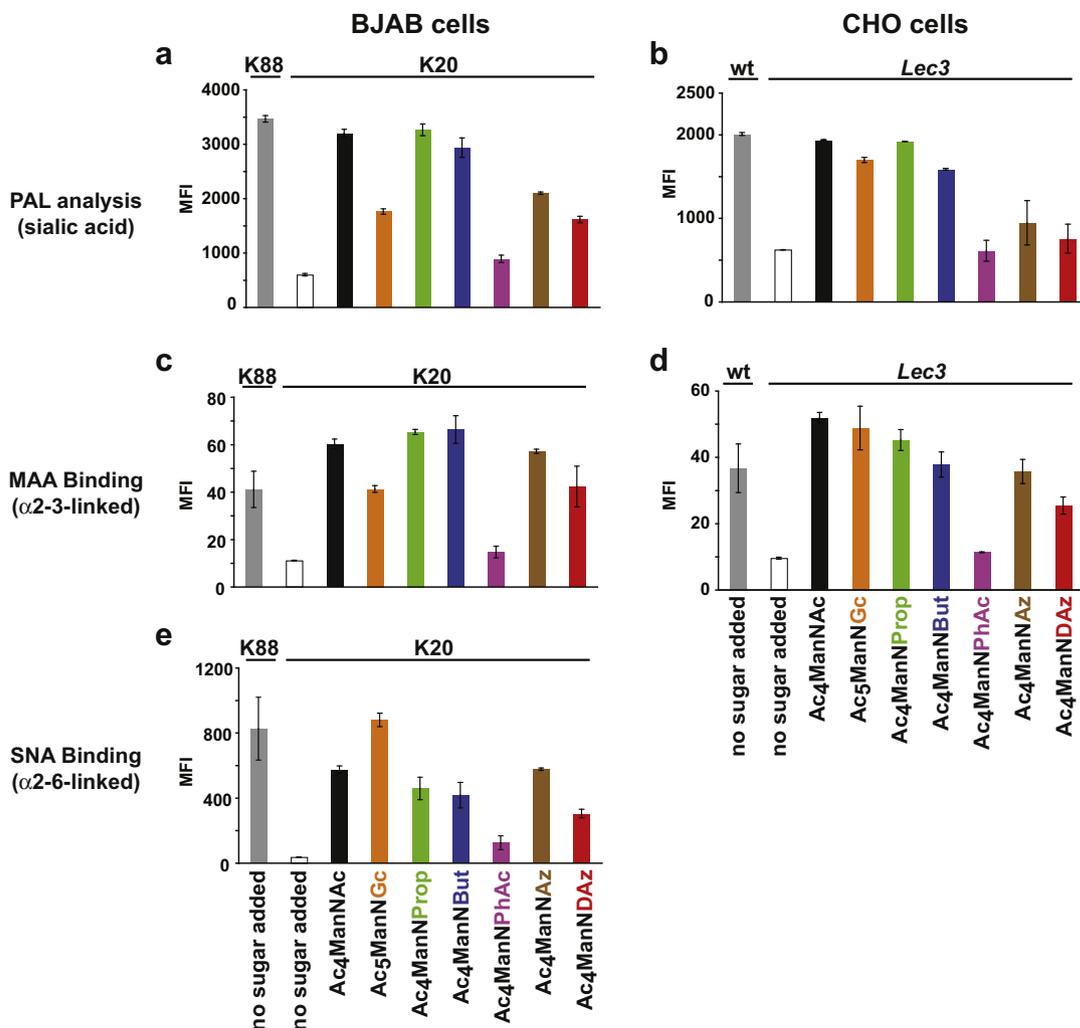


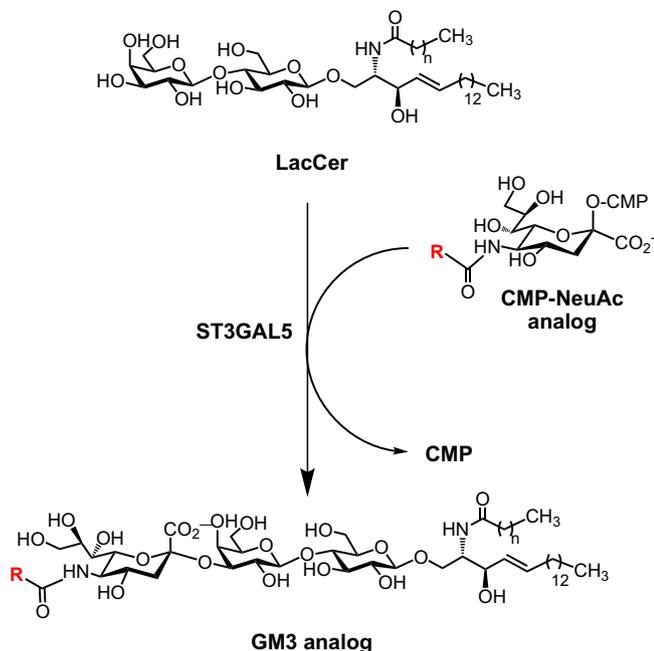
Figure 1. Cell surface display of modified sialic acids. Cell surface sialylation of engineered BJAB K20 and CHO *Lec3* cells was analyzed by periodate oxidation and aniline-catalyzed oxime ligation (PAL; measures total cell surface sialylation) and by binding of fluorescently-labeled lectins (MAA recognizes α 2–3-linked sialic acid; SNA recognizes α 2–6-linked sialic acid). Fluorescence of individual cells, reporting on cell surface sialylation, was measured by flow cytometry. The mean fluorescence intensity (MFI) of at least 10,000 live cells is shown. BJAB K88 and wt CHO cell lines possessing UDP-GlcNAc 2-epimerase activity were used as positive controls. Experiments were performed in biological triplicate; duplication of the entire experiment yielded similar results. (a) PAL analysis of BJAB cells. (b) PAL analysis of CHO cells. (c) MAA lectin analysis of BJAB cells. (d) MAA lectin analysis of CHO cells. (e) SNA lectin analysis of BJAB cells. SNA binding was not assayed in CHO cells because they lack production of α 2–6-linked sialosides.

Ac₅ManNGc reflect an altered lectin affinity for NeuGc-containing sialosides, but feel that this is unlikely. First, previous reports demonstrated that SNA and MAA lectins do not discriminate between NeuAc and NeuGc.²⁸ Second, CHO *Lec3* cells cultured with Ac₅ManNGc produce high levels of MAA binding, suggesting that MAA is capable of binding NeuGc-containing glycoconjugates with high affinity. Therefore, we favor the interpretation that BJAB K20 cells produce NeuGc-containing α 2–3-linked sialosides less efficiently than CHO *Lec3* cells do.

To further investigate the differences in α 2–3-linked sialoside production in these two cell lines, we chose to examine production of a single α 2–3-linked sialoside produced by a single sialyltransferase. GM3 is a sialylated glycolipid, also known as a ganglioside, produced when the sialyltransferase ST3GAL5 adds sialic acid in an α 2–3-linkage to lactose ceramide (LacCer) (Scheme 2).²⁹ Both BJAB K20 and CHO *Lec3* cells express ST3GAL5 and, when provided with sialic acid, are capable of producing GM3. Moreover, GM3 is not elaborated to more complex gangliosides at significant levels in either of these cell lines, simplifying the analysis of unnatural sialic acid incorporation levels. Again, we cultured BJAB K20 and CHO

Lec3 cells in serum-free conditions with each of the protected ManNAc analogs. Metabolically-labeled cells were harvested and the gangliosides were extracted using established methods.^{30–32} Gangliosides were resolved by high performance thin layer chromatography (HPTLC) and visualized by resorcinol staining, which specifically detects sialic acid-containing molecules.

BJAB K20 cells readily produced GM3 analogs containing a variety of modifications to the *N*-acyl side chain of sialic acid (Fig. 2a). Ac₄ManNAc, Ac₄ManNProp, Ac₄ManNBut, and Ac₄ManNAz were efficiently metabolized to the corresponding GM3 analogs. Ac₄ManNDaz was also metabolized successfully, albeit less efficiently. However, when BJAB K20 cells were cultured with Ac₅ManNGc, production of NeuGc-containing GM3 was significantly lower than production of NeuAc-containing GM3. This result is consistent with the MAA binding experiments that suggest that NeuGc is not efficiently incorporated into the α 2–3-linkage in this cell line. Only one analog (Ac₄ManNPhAc) was not metabolized to a GM3 analog at a level detectable by resorcinol staining. MALDI-TOF-MS analysis (Supplementary data) of extracted gangliosides from BJAB K20 cells cultured with Ac₄ManNAc, Ac₅ManNGc, Ac₄ManNAz, and Ac₄ManN



Scheme 2. Biosynthesis of GM3 gangliosides. GM3 is produced by the action of a single sialyltransferase, ST3GAL5, that transfers sialic acid from a CMP-sialic acid to LacCer.

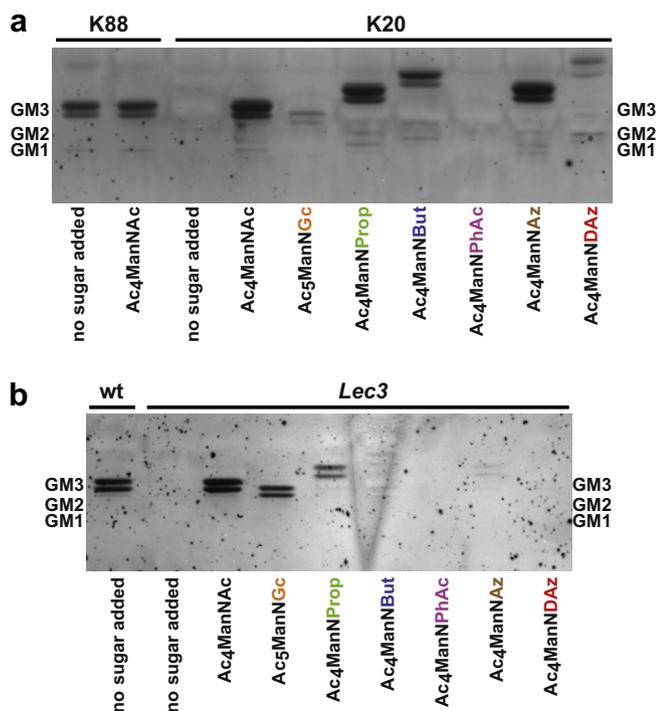


Figure 2. HPTLC analysis of gangliosides produced by BJAB and CHO cells cultured with protected ManNAc analogs. BJAB K88 and wt CHO cell lines containing UDP-GlcNAc 2-epimerase activity were used as positive controls. Gangliosides were extracted, resolved by HPTLC, and visualized by resorcinol staining. Mobilities of standard gangliosides are indicated on either side of the HPTLC images. Gangliosides appear as doublets, reflecting two different fatty acid lengths that are incorporated into LacCer in these cell lines. (a) BJAB cells generate GM3 and small quantities of GM1a gangliosides. (b) CHO cells produce only GM3 gangliosides. Gangliosides incorporating modified sialic acids have altered mobilities. Incorporation of the modified sialic acids was confirmed by mass spectrometry (Supplementary data).

Daz confirmed the identity of metabolically engineered GM3 molecules.

We observed a different pattern of GM3 analog production in CHO *Lec3* cells (Fig. 2b). CHO *Lec3* cells readily metabolized Ac₄ManNAC, Ac₅ManNGc, and Ac₄ManNProp to the corresponding GM3 analogs. Increasing the side chain length (Ac₄ManNB_{ut} and Ac₄ManNAz) led to diminished levels of incorporation and larger *N*-acyl side chains, found on Ac₄ManNPh_{Ac} and Ac₄ManND_{Az}, were not tolerated. MALDI-TOF-MS analysis of extracted gangliosides from CHO *Lec3* cells cultured with Ac₄ManNAC, Ac₅ManNGc, and Ac₄ManNAz confirmed the identity of metabolically engineered GM3 molecules (Supplementary data). In comparison to BJAB K20 cells, CHO *Lec3* cells appear to be more efficient at incorporating NeuGc into GM3 and less efficient at incorporating SiaNB_{ut} and SiaNAz.

The experiments presented here allowed us to compare the global versus specific sialoside production in two mammalian cell lines. By using cell lines deficient in sialic acid biosynthesis, we specifically tracked production of modified sialosides. Our data reveal sialoside-specific incorporation patterns that depend on the form of sialic acid. For example, BJAB K20 cells seem to discriminate against incorporating NeuGc into α 2–3-linked sialosides, including GM3. The low levels of NeuGc incorporation into α 2–3-linked sialosides are unlikely to be explained solely by lower levels of CMP-NeuGc production, since the BJAB K20 cells cultured with Ac₅ManNGc still show robust levels of SNA binding. Similarly, CHO *Lec3* cells produce relatively high levels of cell surface SiaNB_{ut}, but are ineffective at producing SiaNB_{ut}-containing GM3. These differences between global and specific sialoside production may reflect differences in the activity or expression level of one or more of the enzymes involved in sialoside metabolism. Perhaps certain sialyltransferases discriminate among different CMP-sialic acid analogs, resulting in variant sialic acids being more readily incorporated into some sialosides, but excluded from others. Alternatively, discrimination may occur at the level of sialoside catabolism, with sialidases exhibiting substrate specificity for different forms of sialic acid. In addition, differing rates at which different sialosides are synthesized and degraded may affect our observations.

Our findings also highlight key differences in the utility of different cell lines for performing metabolic oligosaccharide engineering experiments, particularly those aimed at producing modified gangliosides. The BJAB K20 cell line demonstrates the substrate flexibility to introduce larger modifications (such as the *N*-butanoyl substituent and the diazirine) into gangliosides, but shows attenuated ability to introduce a naturally occurring sialic acid variant (NeuGc). Conversely, the CHO *Lec3* cell line exhibits restrictions in substrate scope but, unlike the BJAB cell line, is able to utilize Ac₅ManNGc as a precursor for NeuGc-GM3 production. We do not know the molecular basis of the specificity differences between the BJAB K20 and CHO *Lec3*; these cell lines differ in both their tissue and species of origin. However, we are intrigued by these findings in light of the fact that BJAB K20 cells are derived from humans, an organism that produces NeuAc but not NeuGc, while CHO *Lec3* cells are derived from hamsters, an organism that produces both NeuAc and NeuGc.³³

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.128.

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