Glycan engineering using synthetic ManNAc analogues enhances sialyl-Lewis-X expression and adhesion of leukocytes

Anam Tasneem[1], Shubham Parashar[1, 2], Tanya Jain[1], Simran Aittan[1], Jyoti Rautela[1], Kaisar Raza[2], and Srinivasa-Gopalan Sampathkumar[1*]

[1]Laboratory of Chemical Glycobiology, National Institute of Immunology (NII), New Delhi 110067 India

[2]Department of Pharmacy, School of Chemical Sciences and Pharmacy, Central University of Rajasthan (CURAJ), Bandar Sindri, Ajmer, Rajasthan 305817 India

*Corresponding author

E-mail: gopalan@nii.ac.in

Abstract

Cell surface glycans, depending on their structures and dynamic modifications, act as the first point of contact and regulate cell-cell, cell-matrix, and cell-pathogen interactions. Particularly, the sialyl-Lewis-X (sLeX, CD15s) tetrasaccharide epitope, expressed on both gangliosides and glycoproteins, participates in leukocyte extravasation *via* interactions with selectins expressed on endothelial cells, lymphocytes, and platelets (CD62-E/L/P). Neutrophils carrying sLeX epitopes are thought to be responsible for chronic inflammatory diseases resulting in plaque formation and atherosclerosis. Intense efforts have been devoted to the development of sLeX mimetics for inhibition of cell adhesion. On the other hand, dysregulated expression of sLeX and poor extravasation are the major underlying causes of leukocyte adhesion deficiency-II (LAD-II) disorders that result in frequent infections and poor immune response. We hypothesized that metabolic processing of peracetyl *N*-(cycloalkyl)acyl-D-mannosamine derivatives, through the sialic acid pathway, might result in the expression of sialoglycans with altered hydrophobicity which in-turn could modulate their binding to endogenous lectins, including selectins. Herein, we show that treatment of HL-60 (human acute myeloid leukemia) cells with peracetyl *N*-cyclobutanoyl-D-mannosamine (Ac_4ManNCb), at 50 microM for 48 h, resulted in a robust three to four fold increase in the binding of anti-sLeX (CSLEX1) antibody and enhanced cell adhesion to E-selectin coated surfaces; while the corresponding straight-chain analogue, peracetyl *N*-pentanoyl-D-mannosamine (Ac_4ManNPent), and peracetyl *N*-cyclopropanoyl-D-mannosamine (Ac_4ManNCp) both resulted in ~2.5-fold increase compared to controls. The ability to enhance sLeX expression using small molecules has the potential to provide novel opportunities to address challenges in the treatment of immune deficiency disorders.

Cell surface glycans, presented by both proteins and lipids, regulate and participate in biological processes both directly and indirectly. Examples include ABO blood group antigens for direct effects and conformational dynamics in viral glycoproteins for indirect effects[1-2]. Chronic inflammatory diseases such as plaque formation, atherosclerosis, thrombosis, and aneurysm, are critically initiated by glycan-endothelial cell interactions. There has been considerable research effort, by the groups of Ernst, Paulson, Magnani, and others, for the development of sialyl-Lewis-X (sLeX; CD15s) mimetics for intervention in diseases caused by dysregulated cell adhesion[3-6]. Also, there is growing interest on the sialoglycans-selectins and sialoglycans-siglec axis in the context of immune suppression as checkpoints and cancer immunotherapy[3, 7-8]. On one hand the enhanced intravascular adhesion of leukocytes and platelets, driven by glycan-protein interactions, causes inflammatory diseases. On the other hand, dysregulated glycan-protein interactions are responsible for leukocyte adhesion deficiency (LAD) disorders, which results in recurrent infections and poor immune response against pathogens. In LAD-II it is established that low levels of sialyl-Lewis-X, which is attributed to a dysfunctional GDP-Fuc transporter, results in poor extravasation of neutrophils to the site of injury and infection. Under such pathological conditions, enhancing the expression of sLeX through genetic, enzymatic, and pharmacological means might provide physiological insights and novel therapeutic avenues. In this context, we wanted to explore the potential of metabolic glycan engineering approaches for modulating glycan-protein interactions using small molecules.

During the past three decades, the ability to modulate glycan structures by exploiting endogenous metabolic pathways has proven to be a powerful tool for study of structure and functions of glycans on glycoproteins and glycolipids[9]. While template driven biosynthesis of nucleic acids and proteins could be readily addressed using genetic methodologies, their application to the study of glycans is complicated by the non-template driven and metabolism dependent biosynthesis. In this context metabolic glycan engineering (MGE), wherein synthetic monosaccharide analogues are processed by living systems and expressed on the glycoconjugates, provides the much-needed complementary approach to gain insight into glycan-dependent cellular processes in development and diseases[8].

The metabolic processing of *N*-propanoyl-D-mannosamine (ManNProp) to *N*-propanoyl-D-neuraminic acid (NeuProp) in rats by Reutter and coworkers in 1992 set the stage for direct modulation of glycans in living systems[10]. ManNProp, a synthetic analogue of the natural substrate, *N*-acetyl-D-mannosamine (ManNAc), was processed through multiple biosynthetic steps to yield the nine-carbon sialic acids which were then utilized readily by the sialyl transferases in the Golgi apparatus. Initially, free ManNAc analogues were employed in millimolar concentrations but the efficiency of cellular utilization was improved to micromolar concentrations using the peracetyl derivatives[11]. Earlier studies reported simple hydrocarbon extension at the *N*-acyl chain of ManNAc analogues, such as *N*-propanoyl, *N*-butanoyl, *N*-pentanoyl, *N*-hexanoyl, *N*-heptanoyl, and *N*-octanoyl, and their ability to engineer, intercept, and inhibit glycoconjugate biosynthesis and alter cell-cell, cell-pathogen, and cell-matrix interactions[12-13]. Subsequently, several research groups have reported various chemical modifications on the *N*-acetyl-D-hexosamine (HexNAc) scaffold and their applications for tagging, isolation, imaging, glycoproteomic profiling, and characterization of glycans attached to proteins and lipids[9]. The scope of MGE was

expanded with the introduction of chemical reporter groups, by Bertozzi, Wong, and others, which readily undergo bioorthogonal ligations with reagents carrying biotin, FLAG-tags, or fluorophores[14]. The chemical functionalities presented on ManNAc analogues include ketone, azido, alkynyl, sulfhydryl, diaziridinyl, and cyclopropenyl which undergo orthogonal ligations, respectively, with hydrazide, phosphine / alkyne derivatives, azides, maleimide, light, and tetrazine[9]. ManNAc analogues carrying *N*-glycolyl, *N*-phenylacetyl, and halogen substituents at various positions have been reported for their glycan modulating properties[15]. Recently, Wittmann and coworkers reported the *N*-cyclopropanoyl-D-mannosamine analogue as a stable variation to the *N*-cyclopropenyl derivative[16].

While various chemical functional groups have been studied for MGE, the potential chemical space of the HexNAc scaffold, particularly ManNAc for sialic acid engineering, is relatively large. The restriction on chemical structure of ManNAc analogues would be due to steric hindrance for processing by the intracellular enzymes involved in the sialic acid biosynthetic pathway. In this context, we wanted to explore the *N*-(cycloalkyl)acyl derivatives of ManNAc and their effect on glycan modulation in mammalian cells. We hypothesized that, unlike their straight chain counterparts, the processing of ManNAc analogues carrying *N*-(cycloalkyl)acyl-moieties would result in the expression of corresponding sialic acids with tunable hydrophobicity which might exhibit interesting properties and have the potential to alter interactions with sialic acid binding proteins such as selectins, siglecs, and viral hemagglutinins. The presentation of cycloalkyl moieties at the *N*-acyl side chain of sialic acids on the cell surface glycans would modulate the interaction of sialic acid binding proteins – either *via* direct binding or by altering the preferred conformational presentation of sialoglycans – and hence have the potential to modify cell adhesion and cellular response. Herein we describe our results on the synthesis of a panel of derivatives on the HexNAc scaffold and their biological evaluation in mammalian cells. Our results show that the ManNAc analogues were efficiently metabolized by mammalian cells and resulted in the enhancement of expression of sialyl-Lewis-X epitopes and cell adhesion in a ManNAc analogue structure dependent manner.

Results and Discussion

In order to evaluate the effect of modulating the hydrophobicity of the *N*-acyl group, through cycloalkyl moieties on sialic acids on the cell surface, we designed and synthesized a panel of peracetylated ManNAc analogues containing *N*-cyclopropanoyl (**1a**, Ac_4ManNCp)[16], *N*-cyclobutanoyl (**1b**, Ac_4ManNCb), *N*-azidoacetyl (**1c**, Ac_4ManNAz)[17], *N*-acetyl (**1d**, Ac_4ManNAc)[18], *N*-propanoyl (**1e**, Ac_4ManNProp)[18], *N*-butanoyl (**1f**, Ac_4ManNBut)[19], and *N*-pentanoyl (**1g**, Ac_4ManNPent)[20] derivatives (**Fig. 1A**). Corresponding GlcNAc analogues, *viz*., *N*-cyclopropanoyl (**2a**, Ac_4GlcNCp)[16] and *N*-cyclobutanoyl (**2b**, Ac_4GlcNCb) were employed as controls for sialic acid pathway. The GalNAc analogues, *N*-azidoacetyl (**3**, Ac_4GalNAz)[21] was employed as a control for differentiation of carbohydrate metabolic pathways.

Endogenous sialic acid biosynthesis begins from the conversion of UDP-GlcNAc to ManNAc by the enzyme bifunctional UDP-N-acetylglucosamine 2-epimerase / *N*-acetylmannosamine kinase (GNE/MNK) followed by aldol reaction with phosphoenol pyruvate to yield *N*-acetyl-D-neuraminic acid (NeuAc). NeuAc is further converted to

the activated sugar nucleotide donor in the form of CMP-NeuAc, which is utilized by sialyl transferases stationed in the Golgi apparatus (**Fig. 1B**). Intracellular concentrations of sialic acids are maintained at homeostatic levels by feedback inhibition of GNE/MNK by CMP-NeuAc. However, exogenous supply of ManNAc or its analogues could bypass this feedback inhibition and increase the flux of biosynthesis of NeuAc or the corresponding analogues. Depending on the chemical structure and their steric hindrance, ManNAc analogues are processed to varying degrees of incorporation by the enzymes of the sialic acid pathway.

ManNAc analogues carrying *N*-(cycloalkyl)acyl moieties, **1a** and **1b**, if converted to sialic acids, are not amenable to direct estimation in contrast to those carrying bio-orthogonally reactive functional groups. Hence, we resorted to an indirect approach wherein cells were treated with Ac_4ManNAz (**1c**) in the presence or absence of **1a** or **1b**. The control (untreated and vehicle treated) cells would express the wild-type NeuAc and the cells treated with **1c** would express both NeuAc and *N*-azidoacetyl-D-neuraminic acid (NeuAz); whereas the cells treated with either **1a** or **1b**, in the presence of **1c**, if amenable to metabolism, would result in reduced expression of NeuAz along with *N*-cyclopropanoyl-D-neuraminic acid (NeuCp) or *N*-cyclobutanoyl-D-neuraminic acid (NeuCb), respectively, in a competitive manner (**Fig. 2A**).

In order to evaluate the metabolism, we chose Jurkat (human T-lymphoma) cells and incubated with ManNAc analogues either alone (50 μM) or in combination with **1c** (50 μM) for 48 h. Expression of NeuAz on cell surface was estimated by flow cytometry through the copper-free bioorthogonal strain-promoted azide-alkyne cycloaddition (SPAAC) reaction using dibenzocyclooctyne-Cy5 (DBCO-Cy5)[22-23]. The conditions for click reaction were optimized for various concentrations of DBCO-Cy5 and time course of SPAAC reaction in Jurkat cells (**Supporting Figure 1**). Based on the optimization results, we chose incubation time of 30 min at 37 °C for 5.0×10^5 cells (pre-incubated with **1c** for 48 h) with 20 μM of DBCO-Cy5 in 100 μL total volume for experiments with ManNAc analogues. As expected, cells treated with **1c** alone showed robust expression of NeuAz (taken as 100 %), but the NeuAz expression was reduced to 30 %, 60% and 65 % upon co-incubation with **1d**, **1a**, and **1b** respectively. Control conditions of untreated cells and cells treated with vehicle (DMSO, D), **1a**, **1b**, or **1d** alone showed negligible signals upon reaction with DBCO-Cy5 (**Fig. 2B** and **Supporting Figure 2**). These results indicated that the **1d** was able to compete and interrupt metabolism of **1c** to a maximum extent with a 70 % reduction while **1a** and **1b** effected ~ 35-40 % reduction. In order to test the dose-dependency of this inhibitory effect on NeuAz expression and to establish competitive metabolic processing, Jurkat cells were first treated with either **1a** or **1b** (0 – 50 μM) followed by addition of **1c** (50 μM) at 12 h and estimation of NeuAz at 48 h. As expected, the suppression of NeuAz expression was substantially higher when added with a 12 h delay compared to simultaneous addition, in a dose-dependent manner. At 10 μM, delayed addition resulted in 30% and 50% suppression upon treatment with **1a** and **1b**, respectively. By contrast simultaneous addition resulted only in a 10% reduction of NeuAz for both **1a** and **1b** (**Fig. 2C** and **Supporting Figure 2**). These results suggested that both **1a** and **1b** are amenable to sialic acid biosynthesis and that **1b** showed better competitive ability compared to **1a**.

Next, we studied whether the reduction of NeuAz expression is specific to ManNAc analogues. Jurkat cells were incubated with **1a**, **1b**, **2a**, or **2b** (0 – 50 μM) in

combination with **1c** (50 μM; simultaneous addition) for 24 h. At 50 μM, NeuAz expression was suppressed, in a dose-dependent manner, to 60% and 50% of controls upon treatment, respectively, with **1a** and **1b**. In contrast, only negligible reduction in NeuAz expression was observed upon co-incubation with the C-2 GlcNAc epimers **2a** or **2b** (**Fig. 2D**). These results confirmed the specificity of ManNAc analogues for processing through the sialic acid pathway and the inability of GlcNAc analogues to interfere in the biosynthesis of NeuAz from **1c**.

The high degree of selectivity of ManNAc analogues for processing by the sialic acid biosynthetic pathway was further confirmed by competitive studies using Ac_4GalNAz (**3**) which is known to be processed through both the mucin-type *O*-glycosylation (MTOG) and β-*O*-GlcNAc-ylation pathways[21, 24-25]. The expression of NeuAz by **1c** was suppressed by **1a** or **1b** (50 μM) to an extent of 50% upon co-incubation. By contrast, no reduction in the expression of *N*-azidoacetyl-D-galactosamine (GalNAz) on the cell surface was noticed upon co-incubation of **3** (50 μM) with **1a** or **1b** (50 μM) (**Fig. 2E** and **Supporting Figure 2**). These results confirmed that the ManNAc analogues did not interfere or suppress GalNAz processing through MTOG and β-*O*-GlcNAc.

Having confirmed metabolic acceptability in Jurkat cells, we tested the analogues on HL-60 (human acute myeloid leukemia) cells which are a reliable model for neutrophil adhesion[26]. The effect on competitive suppression of NeuAz expression was studied using **1c** as a reporter. HL-60 cells were treated with **1c** either alone (50 μM) or in combination with ManNAc analogues (50 μM), including the straight chain derivatives, for 24 h followed by DBCO-Cy5 ligation. Flow cytometry analysis showed that **1a**, **1b**, **1d**, **1e**, **1f**, and **1g** reduced the NeuAz expression, respectively, to 60 %, 55 %, 40 %, 65 %, 85 %, and 90 % with respect to **1c** alone (taken as 100 % for control) (**Fig. 3A**). Maximum suppression was observed for the *N*-acetyl (wild-type) analogue **1d**, followed by the cycloalkyl derivatives **1a** and **1b**; the straight chain analogues **1e** showed significant suppression, **1f** showed moderate suppression, and **1g** showed mild effect on NeuAz expression. The decreasing ability of linear alkyl moieties (**1d** – **1g**), as a function of chain length, for suppression of NeuAz expression is in contrast to the cycloalkyl moieties. While **1b** induced a reduction of 45 %, the corresponding straight chain analogue with five carbons **1g** showed only a mild reduction of 10 % with respect to controls. Similarly, **1a** induced a reduction of 40% compared to only a 15 % reduction by **1f**. These results demonstrated that the cycloalkyl groups at the *N*-acyl chain of ManNAc are significantly more acceptable for metabolic processing compared to their straight chain analogues.

Next, in order to verify metabolic processing to sialic acids we measured both total and glycoside bound sialic acid levels using the periodate-resorcinol assay in HL-60 cells (**Supporting Figure 3**)[11, 27]. Total sialic acid levels were comparable to untreated cells for treatment with vehicle (D), the GlcNAc analogues **2a**, or **2b** (100 μM, 48 h); an increase of 1.14 to 1.3-fold was observed for treatment with **1a**, **1b**, **1c**, and **1f**. An increase of 2.0-fold and 2.3-fold was observed for **1d** and **1e**, while **1g** showed a decrease by 0.7-fold. The glycoside bound sialic acid levels were comparable to untreated cells for D, **1c**, **1f**, **2a**, and **2b**. About ~10% increase was observed for both **1d** and **1e** while both **1a** and **1b** showed a decrease of ~10%. The ratios of total sialic acids to glycoside bound sialic acids were similar to untreated cells for D, **1c**, and **1f**; higher ratios of free sialic acid were observed for **1a**, **1b**, **1d**, and **1e**

while lower ratios were observed for **1g**, **2a**, and **2b**. Consistent with literature, treatment with **1d** and **1e** resulted in large increase in the metabolic flux of free sialic acid with modest gains in glycoside-bound sialic acids[11]; whereas treatment with **1a** and **1b** resulted in a gain in free sialic acids with a moderate decrease in glycoside-bound sialic acids. Our results are in agreement with the recent reports by Wittmann and coworkers wherein **1a** increased the total sialic acid levels by two folds in HEK293 cells with more than 50% being the modified *N*-cyclopropanoyl-D-neuraminic acid[28]. In the case of treatment with **1g**, **2a**, and **2b** almost all the sialic acids were glycoside bound.

Comparative analysis revealed that the *N*-cyclobutanoyl analogue **1b** resulted in increased flux of sialic acid biosynthesis whereas the corresponding five carbon straight chain *N*-pentanoyl analogue **1g** did not show any effect, suggesting that steric effects played a prominent role; whereas both the *N*-cyclopropanoyl analogue **1a** and the corresponding straight chain *N*-butanoyl analogue **1f** showed similar total sialic acid levels. Cell density measured at 24, 48, and 72 h, as a measure of proliferation, upon incubation with **1a** – **1g**, **2a**, and **2b** (100 μM, 48 h) exhibited negligible toxicity in all cases, with respect to controls, with the exception of **1c**, **1g**, **2a**, and **2b** which exhibited a mild decrease of ~10 % in cell density (**Supporting Figure 3**).

With the ability to modulate the structure of sialic acids on the cell surface using *N*-(cycloalkyl)acyl analogues we ventured to investigate changes to established sialic-acid carrying epitopes of immunological importance, *viz*., sialyl-Lewis-X (sLeX, CD15s) using anti-CD15s (CSLEX1) antibody. Optimal conditions for flow cytometry staining for cells, including isotype and secondary antibody only controls, were obtained by titration at various concentrations of anti-CD15s for saturating concentrations. Consistent with the literature, HL-60 cells were positive and Jurkat cells were negative for CSLEX1 epitopes (**Supporting Figure 4**). Interestingly, HL-60 cells upon incubation with **1b** (50 μM, 24 h) showed nearly a four-fold increase in the binding of CSLEX1 antibody, compared to controls (untreated, vehicle-treated, and **1d**) (**Fig 3B**). Treatment with **1f** resulted in a three-fold increase; the three analogues, *viz*., **1a**, **1e**, and **1g** resulted in an increase of ~2.5-fold while **1c** showed a moderate increase of 1.4-fold. Notably, *N*-butanoyl analogue **1f** showed higher CSLEX1 binding compared to the four-carbon *N*-cyclopropanoyl analogue **1a**; whereas the *N*-pentanoyl analogue **1g** showed lower CSLEX1 binding compared to the five-carbon *N*-cyclobutanoyl analogue **1b**. These results suggest unique structure-based differential effects of the *N*-acyl moiety for metabolic processing and sLeX display.

We tested the likelihood that the increase in sLeX / CD15s levels is due to the utilization of its precursor Lewis-X (LeX / CD15). Measurement of cell surface CD15 levels using anti-CD15 (HI98) in HL-60 cells treated with ManNAc analogues (**1a** – **1g**, 50 μM for 24 h) by flow cytometry revealed no major changes, compared to controls, under all conditions (**Fig. 3B**). This result suggested that there is no trade-off or a reduction in the LeX levels and rather the increase in sLeX levels could be attributed to enhanced biosynthesis.

We next investigated the reversibility of the effect of **1b** on increased sLeX levels. Time course studies revealed that incubation of HL-60 cells with a single dosage of **1b** or **1g** (50 μM) resulted in peak sLeX expression on day 2 and returned to normal levels by day 6. There was a maximum of 3.0-fold and 2.2-fold increase in sLeX levels

upon treatment, respectively, with **1b** and **1g** on day 2 while no change was observed in cells treated with controls either **1d** or vehicle (D). By day 6 it was noticed that cells treated with **1g** showed a slightly faster recovery to native levels while **1b** treatment still maintained approximately 2.3-fold higher levels of sLeX (**Fig. 3C**). These results confirmed the advantages of pharmacological approaches, with dosage control and reversibility, over genetic methods for modulation of sLeX.

In addition to investigating the sLeX levels on surface of intact cells by flow cytometry, we studied the sLeX epitopes by western blotting. HL-60 cells were incubated with **1a**, **1b**, **1d**, **1e**, **1f**, or **1g** (50 μM) along with untreated (U) and vehicle-treated (D) controls, for 72 h and lyzed. Total lysates were resolved by SDS-PAGE, blotted on to nitrocellulose membranes, probed using anti-sLeX (CSLEX1) antibody followed by AF488-conjugated secondary antibody, and imaged using a fluorescence scanner. Robust increase in sLeX bands were observed between 150-100 kDa ranges in cells treated with **1b**, with moderate increase with **1f** and **1g**, faint increase with **1a** and **1e**. Cells treated with controls (U, D, or **1d**) were negative for sLeX epitopes (**Fig. 3D**). Consistent with flow cytometry results, the *N*-cyclobutanoyl analogue **1b** showed maximum gain in sLeX levels compared to the corresponding straight chain *N*-pentanoyl analogue **1g**. In contrast, the *N*-cyclopropanoyl analogue **1a** showed lower levels of sLeX compared to its straight chain *N*-butanoyl analogue **1f**. The western blotting data showed the interesting differential property of the *N*-cyclobutanoyl derivative **1b**, which was not captured by the *N*-cyclopropanoyl derivative **1a**, thus highlighting the significance of ManNAc analogue-structure dependent biological outcomes.

Low levels of sLeX epitopes in HL-60 cells (both untreated and vehicle treated) upon western blotting with CSLEX1 antibody is consistent with the fact that HL-60 tend to spontaneously differentiate to hyposialylated phenotype during culture[29]. Also, sLeX epitopes were not detectable upon treatment with either **1d** or **1e** (50 μM, 72 h), notwithstanding the two-fold increase in total sialic acid levels. Earlier work has shown that HL-60 cells showed enhanced expression of sLeX epitopes (probed with KM93 antibody), particularly on P-selectin glycoprotein ligand-1 (PSGL-1/CD162) upon treatment with the free monosaccharide ManNProp at 10 mM[30]. No such effect was observed with its peracetylated counterpart **1e** at 50 μM. The sLeX epitopes were observed to be increased moderately for both **1f** and **1g**, slightly for **1a**, and maximally for **1b**. The *N*-butanoyl analogue **1f** has been shown to be an efficient inhibitor of polysialic acid attachment to neural cell adhesion molecules (NCAM)[19, 31]. However, **1f** showed an increase in sLeX levels in HL-60 cells while the corresponding *N*-cyclopropanoyl analogue **1a** showed only a faint increase. The *N*-pentanoyl analogue **1g** has been shown to alter contact dependent inhibition of cell growth and modulate viral binding to cell surface[20]. We found that treatment with **1g** enhanced expression of sLeX. However, the corresponding five-carbon *N*-cyclobutanoyl analogue **1b** showed the maximum increase in CSLEX1 epitopes (**Fig. 3D**). The observed increase in sLeX levels upon treatment with **1b** could arise due to two plausible reasons, *viz*., (i) enhanced biosynthesis of both wild-type and modified sLeX carrying *N*-cyclobutanoyl-D-neuraminic acid (NeuCb) and (ii) enhanced binding affinity of CSLEX1 antibody to modified sLeX. Many glycoproteins, including CD162/PSGL-1, CD43, CD44, and ESL-1, have been reported to carry sLeX[32]. The paratopes of CSLEX1 have not been well defined and it is possible that the *N*-cyclobutanoyl moiety

of NeuCb makes direct contacts with hydrophobic residues on the antibody or alters the preferred conformations of sLeX for CSLEX1 binding.

Research over decades has established the essentiality of sLeX epitopes on leukocytes and their interaction with selectins (endothelial (E), leukocyte (L), and platelet (P); CD62E/L/P) for cell-cell communication, cellular migration, and extravasation through the high endothelial venules[33-36]. Since treatment with **1b** resulted in a robust enhancement of sLeX, as revealed by CSLEX1 binding, we tested the effects on the binding to the endogenous receptor for sLeX, *viz*., E-selectin (CD62E). Binding of both mouse and human E-selectin-Fc chimera protein to HL-60 cells were studied using flow cytometry and conditions for saturation binding were optimized (**Supporting Figure 5**). Consistent with earlier reports, mouse E-selectin-Fc (mCD62E) was found to show higher binding to HL-60 cells compared to human E-selectin-Fc (hCD62E)[37]. Flow cytometry results revealed a robust and significance increase of 2.3-fold in cells treated with **1b** (50 μM, 72 h) with respect to untreated cells, similar to the results obtained for CSLEX1 binding (**Fig. 3E**). The controls treated with vehicle (D), **1d**, or **1a** showed no significant change with respect to untreated cells.

Thorough structural and kinetic binding studies have established that E-selectin binding to sLeX is determined by the carboxylate moiety of sialic acid and 3-OH/4-OH of the fucosyl moiety with the *N*-acetyl moiety projecting out to the solvent front and not having any direct contact. These aspects were exploited for the development of the minimal pharmacophore and potential inhibitors of sLeX interactions with E-selectin. In our studies we found that the ManNAc analogue **1b**, which is processed through the sialic acid pathway, resulted in enhanced binding of both mCD62E as well as CSLEX1 antibody. Two possible reasons could explain the observed phenomenon with **1b**, *viz*., (i) the *N*-cyclobutanoyl moiety does not make direct contacts with ligand biding pocket of E-selectin but might strongly influence and enhance the preferred conformations and (ii) the *N*-cyclobutanoyl moiety changes the binding drastically through direct contacts in E-selectin with hydrophobic pockets in the vicinity. Notably, the mCD62E showed higher binding to HL-60 cells compared to hCD62E which could be attributed to the expression of *N*-glycolyl-D-neuraminic acid (NeuGc) carrying sLeX in cells cultured in fetal bovine serum. This indicated that modifications to the *N*-acyl side chain, from *N*-acetyl to *N*-glycolyl could have profound effects on shifting conformation population dynamics to favorable binding. Similar effects might be operating for NeuCb carrying sLeX upon metabolic processing of **1b**.

Cutaneous lymphocyte antigens (CLA) carry sLeX glycans and interact with E-selectin and enable T-cell migration. Anti-CLA antibody (HECA452) is a non-function blocking antibody which binds to sLeX epitopes[38]. We tested the effect of ManNAc analogues treatment on HL-60 cells for HECA452 binding through flow cytometry. Surprisingly, a prominent 40% decrease in HECA-452 binding was observed upon treatment with **1a** or **1b** (50 μM, 24 h) compared to untreated cells; while cells treated with vehicle (D) or **1d** showed only a moderate increase (**Fig. 3F**). While a robust increase was found for binding of mCD62E and CSLEX1, a decrease in HECA452 was observed. It is known that HECA452 is specific for human sLeX carrying NeuAc and does not bind to mouse sLeX carrying NeuGc, suggesting the direct relevance of the *N*-acyl moiety for binding[39]. In the case of treatment with **1a** or **1b**, it is possible that the HECA452 binding is abrogated, respectively, due to presence of NeuCp or

NeuCb on sLeX. Additionally, increased sLeX biosynthesis, as a consequence of treatment with **1a** or **1b,** could in turn affect both glycan site occupancy and glycan densities on the glycoconjugates presenting paratopes for the HECA-452 antibody. Since HECA-452 is a non-function blocking antibody, it might depend on additional peptide backbone residues for contact which could be in principle masked by enhanced glycan site occupancy and the presence of engineered sLeX.

Apart from sLeX, the metabolic processing of ManNAc analogues could in principle affect various other types of sialoglycans on *N*- and *O*-linked glycoproteins as well as gangliosides. In order to address this, we probed global sialylation levels using sialic acid binding lectins, *viz*., *Maackia amurensis* lectin (MAL-II) and *Sambucus nigra* agglutinin (SNA) which bind, respectively, to NeuAcα2→3Gal and NeuAcα2→6Gal/GalNAc epitopes[40]. HL-60 cells incubated with vehicle (D), **1a**, **1b**, or **1d** (50 μM, 72 h) were lysed, resolved on SDS-PAGE, transferred to nitrocellulose membranes and probed with biotinylated MAL-II or biotinylated SNA followed by horse radish peroxidase conjugated avidin (HRP-avidin). MAL-II epitopes in control cells (U, D and **1d**) were found to be below detection limit with a slight increase noted upon treatment with **1a** or **1b** (**Fig. 4A**). MAL-II blot results were consistent with earlier observations of spontaneously induced hypo-sialylated phenotype of HL-60 cells in culture[29, 30]. By contrast, treatment of **1a** or **1b** resulted in significant reduction of SNA epitopes compared to controls (U, D, or **1d**) (**Fig. 4B**). A comparison of lectin blots of cells (U, D, and **1d**) reveal striking differential of abundant SNA epitopes compared to low levels of MAL-II epitopes, suggesting differential endogenous activities of α2→3 *vs*. α2→6 sialyl transferases. Further evaluation of lectin binding on intact cells by flow cytometry confirmed significant gain in MAL-II binding upon treatment with **1a** or **1b**, but not in controls, compared to untreated cells (**Fig. 4C**); no significant differences were observed for SNA binding upon treatment with D, **1d**, **1a**, or **1b** compared to untreated cells (**Fig. 4D**). The increase in MAL-II with a concomitant decrease in SNA epitopes upon treatment with **1b** is in agreement with the enhanced binding observed for CSLEX1 and E-selectin-Fc since the sLeX contains the NeuAcα2→3Gal moiety.

Having established the unique properties of **1b** in enhancing the expression of sLeX, we turned our attention to functional studies on cell adhesion. Cell adhesion studies were performed on plastic plates coated with protein-G followed by either mouse E-selectin-Fc (mCD62E) chimera or mouse L-selectin-Fc (mCD62L) chimera, followed by blocking with BSA. Plates coated with only protein-G and BSA were used as controls. L-selectin was selected in order to study the effect of ManNAc analogues on sulfo-sialyl-Lewis-X (sulfo-sLeX) expression[41]. HL-60 cells were incubated with the ManNAc analogues **1a** – **1g** (50 μM, 48 h), along with untreated and vehicle controls, harvested, and allowed to adhere. After 60 min, the cells in suspension were removed and the surface-bound cells were fixed, stained with DAPI, imaged by microscopy, and the numbers of cells were enumerated (**Supporting Figure 6** and **Fig. 4E**). HL-60 cells pre-incubated with **1b** exhibited about 40 % increase in the numbers of adhered cells on mCD62E coated surface compared to all other conditions. Reduced numbers of cells were found to adhere to mCD62L coated surfaces under all the conditions compared to untreated controls; however, no differential effects were observed with respect to Protein-G and BSA only coated surfaces. These results established that treatment with **1b**, but not controls, resulted in increased binding to CSLEX1, mCD62E, and MAL-II, and in turn exhibit improved adherent properties to E-selectin coated surfaces in a static adhesion model.

Conclusions

Sialoglycans are critically involved in the regulation of cellular immune homeostasis through the sLeX-selectin (E/L/P) axis[3, 7] for cell-cell interactions, cell adhesion, and cell migration[6]. Intense research efforts have been devoted to the development of pharmacological agents that could interfere with sLeX-selectin interactions under inflammatory conditions[35]; On the other hand, disease conditions such as LAD-II are highlighted by the low level of expression of sLeX on leukocytes and consequential poor adhesion to E-selectin expressed on endothelial cells. Current approaches for LAD-II are focused on extracellular fucosylation of neutrophils to restore their ability bind E-selectin[42]. In this context our investigations with a panel of HexNAc analogues revealed that the *N*-cyclobutanoyl ManNAc analogue, **1b**, efficiently enhanced sLeX levels in an analogue structure-dependent manner and lead to enhanced adhesion to E-selectin. Using a panel of ManNAc analogues and multiple complementary approaches we have shown the analogue-structure dependent effects on sLeX expression and consequential modulation of their binding to anti-glycan antibodies, glycan-binding proteins, and lectins. The ability to either enhance or suppress sLeX-selectin, sialoglycans-siglec, and sialoglycans-viral hemagglutinin interactions using pharmacological agents has the potential to open up new avenues for future therapeutics for immune deficiency disorders as well as shed light on the intricate mechanisms of functional roles of glycans in immune regulation.

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Author contributions

Synthesis, purification, and characterization of HexNAc analogues were performed by SP and JR. *In vitro* cell culture studies, flow cytometry, western blotting, and microscopy were performed by AT, TJ, and SA. Research was supervised by KR and SGS. SGS conceptualized and directed the research, performed data analysis, and wrote the manuscript with inputs from all the authors.

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Figures and legends

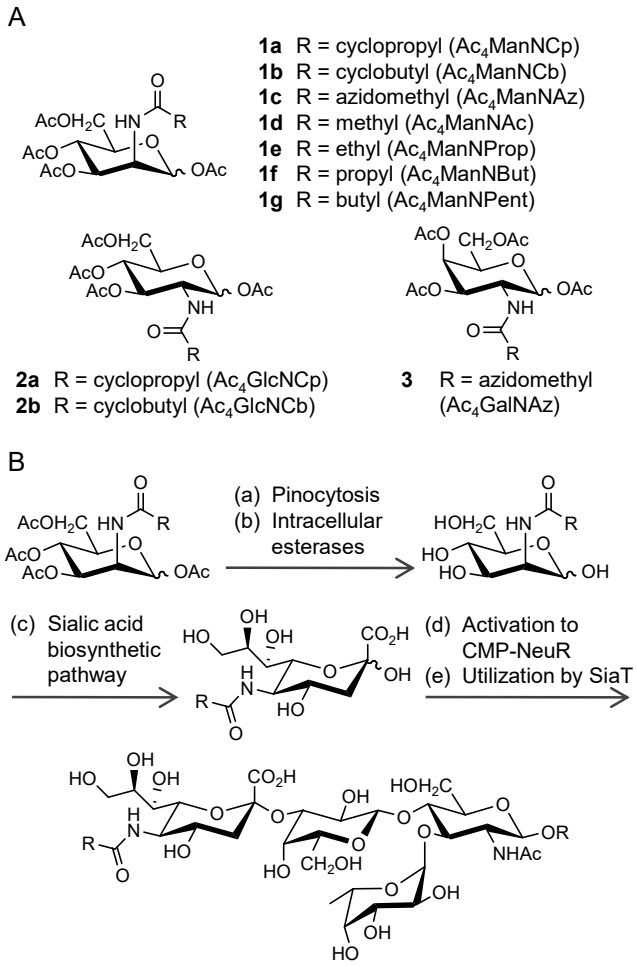

Figure 1. **Modulation of sialoglycoconjugates by synthetic *N*-acetyl-D-hexosamine (HexNAc) analogues**. (A) Chemical structures of the panel of ManNAc analogues (**1a** – **1g**), GlcNAc analogues (**2a** and **2b**), and GalNAc analogues (**3**) employed in this study. (B) Schematic illustration of the metabolic processing of ManNAc analogues through the sialic acid biosynthetic pathway resulting in the expression of modified sialic acids in *N*-linked and *O*-linked glycoproteins and in gangliosides. Expression of modified sialic acids on sialyl-Lewis-X (CD15s), which is critical for cell rolling, adhesion, and migration, is shown as an example. R, glycans attached to proteins or lipids; ManNAc, *N*-acetyl-D-mannosamine; GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; SiaT, sialyl transferases present in the Golgi; CMP-NeuR, cytidine monophosphate derivative of modified *N*-acetyl-D-neuraminic acid (activated sugar nucleotide donors).

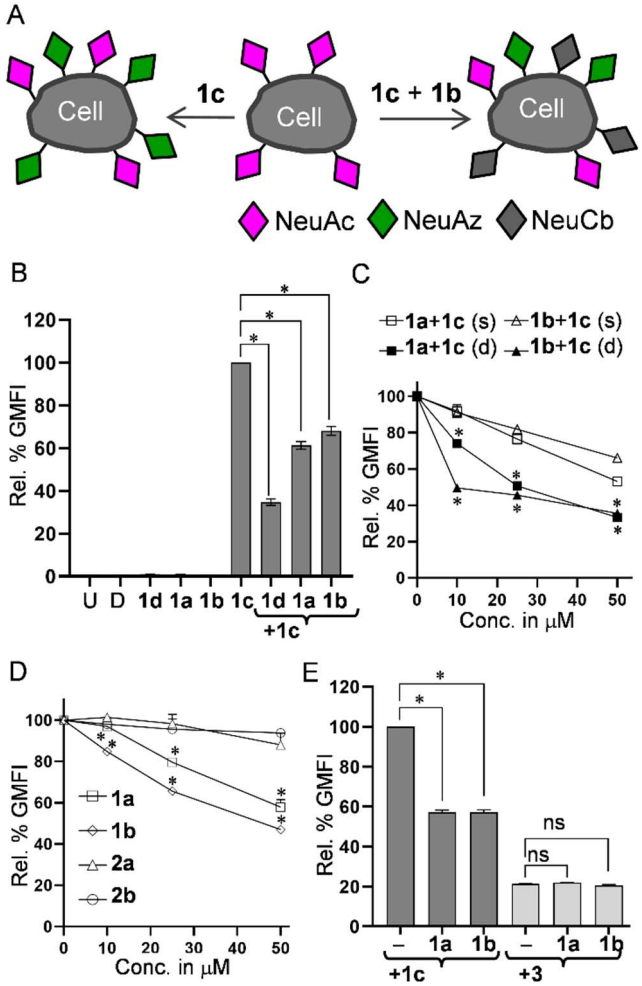

Figure 2. Estimation of metabolic incorporation through competitive processing of HexNAc analogues in Jurkat (human T-lymphoma) cells. (A) Schematic illustration of sialic acids on the cell surface showing expression of wild-type NeuAc (pink diamonds), NeuAz (green diamonds) upon treatment with Ac_4ManNAz (**1c**), and NeuCb (grey diamonds) upon treatment with Ac_4ManNCb (**1b**). Treatment with a mixture of **1b** and **1c** results in competitive metabolic incorporation leading to reduction in NeuAz expression (right) compared to treatment with **1c** alone (left). (B) Estimation of NeuAz expression via SPAAC using DBCO-Cy5 in Jurkat cells upon incubation with U, D, **1a**, **1b**, **1c**, or **1d** separately (50 μM, 24 h) and in combination **1d** + **1c**, **1a** + **1c**, or **1b** + **1c** (50 μM each, 24 h). Relative % GMFI is shown taking NeuAz levels upon treatment with **1c** alone as 100 %. (C) Comparison of NeuAz levels upon either simultaneous addition of **1a** + **1c** or **1b** + **1c** (50 μM each) (s) or addition of **1a** or **1b** (50 μM) first, followed by the addition of **1c** (50 μM) at 12 h (d) and estimation at 48 h. (D) Effect on NeuAz expression by the ManNAc analogues **1a** and **1b** and GlcNAc analogues **2a** and **2b** upon co-incubation with **1c** (50 μM each, 24 h). (E) Effect on NeuAz and GalNAz expression on the cell surface upon incubation, respectively, with **1c** alone, **1a** + **1c** or **1b** + **1c**, and incubation with **3** alone, **1a** + **3**, or **1b** + **3** (50 μM each, 24 h). Bar graphs shown are representative of at least two independent experiments. Error bars shown are standard deviation of two replicates. NeuAc, *N*-acetyl-D-neuraminic acid; NeuAz, *N*-azidoacetyl-D-neuraminic acid; NeuCb, *N*-cyclobutanoyl-D-neuraminic acid; U, untreated; D, dimethyl sulfoxide (DMSO, vehicle); Rel. % GMFI, relative percentage geometric mean fluorescence intensity; Conc., concentration, SPAAC, strain promoted azide alkyne cycloaddition; DBCO-Cy5, dibenzocyclooctyne-Cy5. Statistical analysis was performed using one-way ANOVA for B and E and two-way ANOVA for C and D, along with Bonferroni's multiple comparisons test. ∗ indicates a *p*-value < 0.05; ns, non-significant.

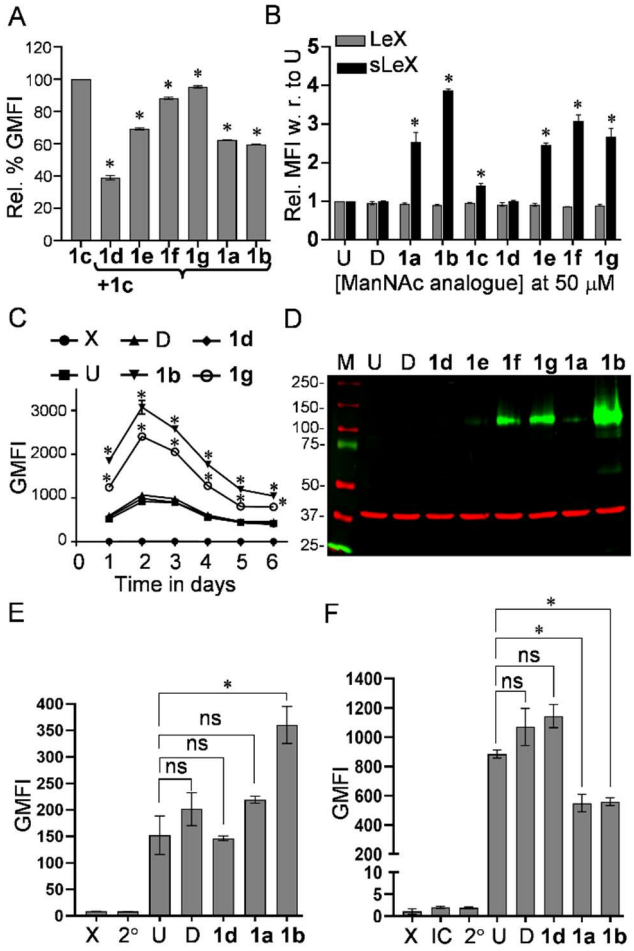

Figure 3. ManNAc derivatives enhance the expression of sialoglycan epitopes in HL-60 cells in an analogue specific manner. (A) Flow cytometry estimation of NeuAz expression via SPAAC using Cy5-DBCO in HL-60 (human acute myeloid leukemia) cells upon incubation with either **1c** (50 μM, 24 h) alone or **1c** in combination with **1a**, **1b**, **1d**, **1e**, **1f**, or **1g** (50 μM each, 24 h). (B) Flow cytometry estimation of expression of LeX and sLeX epitopes on the surface, respectively, using anti-CD15 (HI98) and anti-CD15s (CSLEX1), in HL-60 cells incubated with D, **1a**, **1b**, **1c**, **1d**, **1e**, **1f**, or **1g** (50 μM, 24 h). (C) Time course of surface expression of CD15s (CSLEX1) epitopes in HL-60 cells incubated with D, **1b**, **1d**, or **1g** (50 μM) by flow cytometry. (D) Dual western blots probed using anti-CD15s (CSLEX1) (upper green bands) and anti-β-actin (lower red bands; loading control) of total lysates of HL-60 cells incubated with D, **1d**, **1e**, **1f**, **1g**, **1a**, or **1b** (50 μM, 72 h); M, molecular weight markers. Flow cytometry estimation of (E) binding of E-selectin (CD62E-Fc) chimera protein and (F) anti-cutaneous lymphocyte antigen (CLA) (HECA452) antibody to HL-60 cells incubated with D, **1a**, **1b**, or **1d** (50 μM, 24 h) (along with controls, *viz*., X, unstained; 2°, secondary only; IC, isotype control; U, untreated). Bar graphs and blots shown are representative of at least two independent experiments and error bars shown are standard deviation of at least two replicates. Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple comparisons test for A, E, and F and using paired 't' test for B and C. ∗ indicates a *p*-value < 0.001 for A and < 0.05 for B, C, E, and F; ns, non-significant.

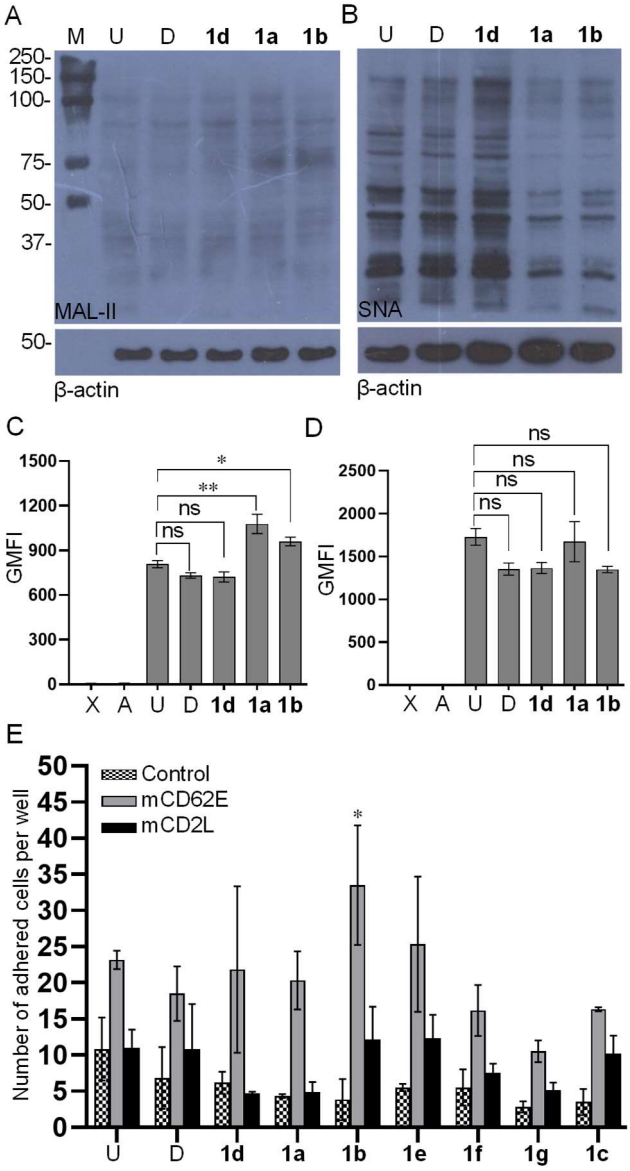

Figure 4. **Modulation of sialoglycans by ManNAc derivatives enhances HL-60 cell adhesion in an analogue-specific manner**. Far western blots of total lysates of HL-60 cells incubated with **1a**, **1b**, or **1d** (50 μM, 72 h) (along with controls – untreated (U) and DMSO (D)) probed using biotinylated (A) *Maackia amurensis* lectin – II (MAL-II) and (B) *Sambucus nigra* agglutinin (SNA) followed by horse radish peroxidase conjugated avidin (HRP-avidin); β-actin blots are shown as loading controls. Flow cytometry estimation of surface epitopes of (C) MAL-II and (D) SNA epitopes in HL-60 cells treated with **1a**, **1b**, or **1d** (50 μM, 24 h), along with controls (X, unstained; A, HRP-avidin alone; U, untreated) probed using biotinylated lectins and FITC-conjugated avidin. (E) Enumeration of number of HL-60 cells adhered to surfaces, coated with (i) protein-G/BSA alone, (ii) protein-G/BSA and E-selectin-Fc chimera, or (iii) protein-G/BSA and L-selectin-Fc-chimera, after incubation with **1a** – **1g**, along with untreated and vehicle treated controls. Each experiment contained three replicates for each condition in 96-wells; two field views were obtained for each well. Bar graphs and blots shown are representative of at least two independent experiments and error bars shown are standard deviation of at least two replicates. Corresponding DAPI stained microscopy images of adhered HL-60 cells are given in Supporting Figure 6. BSA, bovine serum albumin. Statistical analysis was performed using one-way ANOVA for C and D and two-way ANOVA for E with Bonferroni's multiple comparisons test. ∗ indicates a *p*-value < 0.05; ns, non-significant.