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Modulation of Cellular Adhesion by Glycoengineering

Laila Dafik[§], **Marc d'Alarcao**[£,*], and **Krishna Kumar**[§,†,*]

[§]Department of Chemistry, Tufts University, Medford, Massachusetts 02155

[£]Department of Chemistry, San José State University, San José, CA 95192

[†]Cancer Center, Tufts Medical Center, Boston, Massachusetts 02110

Abstract

Aberrant glycosylation of lipid and protein molecules on cellular surfaces is responsible for many of the pathophysiological events in tumor progression and metastasis. Sialic acids in particular, are overexpressed on the glycocalyx of malignant tumor cells and sialic acid-mediated cell adhesion is required for metastasis. We report here that replacement of sialic acids on cell surfaces with fluorinated congeners dramatically decreases cell adhesion to E– and P–selectin-coated surfaces. Comparison of adhesion of fluorinated cells with those modified with non-fluorinated analogues suggests that both reduce binding of the modified sialosides to their cognate lectins to a similar extent on a per molecule basis. The overall reduction in cell adhesion results from greater cell surface presentation of the fluorinated congeners. This work suggests an avenue for inhibition of metastasis by administration of small molecules, and concomitant non-invasive imaging of tumor cells by ^{19}F MRI before they are visible by other means.

Keywords

Glycoengineering; Cell Adhesion; Sialic Acid; Selectins; Fluorination

Introduction

The carbohydrates present on the surface of virtually every eukaryotic cell are responsible for mediating a wide range of interactions between the cell and its environment, ranging from recognition and signaling, to motility and adhesion. The growing appreciation of this central role for the cellular glycocalyx has led to major efforts in mapping differences in cell surface glycans among tissues, and between diseased and normal cells. These efforts have in turn led to carbohydrate-based or carbohydrate-targeted strategies for the detection, treatment, or prevention of a wide range of diseases, ranging from viral infection[1] and malaria[2] to cancer.[3–5]

Cancer progression is accompanied by changes in the glycans expressed on cell surfaces. Tumor cells display more sialic acids as part of the surface carbohydrates as their metastatic potential increases[6, 7] and the overall level of tumor cell polysialylation is correlated with decreased survival time in cancer patients.[8] The sialylated glycans on tumor cells resemble leukocyte glycans necessary for the process of extravasation during inflammation. It is

[*]To whom correspondence should be addressed. Department of Chemistry, San José State University, San José, CA 95192 Phone: 408 924 4962. Fax: 408 924 4945. mdalarcao@science.sjsu.edu (M.D.) and Department of Chemistry, Tufts University, Medford, MA 02155. Phone: 617 627 3441. Fax: 617 627 3443. krishna.kumar@tufts.edu (K.K.).

Supporting Information Available: Typical data set for adhesion, standard curve for DMB labeling and Western blot to quantify SLe^X. This material is available via the Internet at http://pubs.acs.org/.

thought that by expressing leukocyte-like sialylated glycans, a cancerous cell that has broken away from the primary tumor is able to escape the blood stream and form a metastatic tumor at a different site (Figure 1A). Members of two key protein families, the selectins and the integrins, mediate leukocyte rolling and firm adhesion – essential steps in extravasation.[9–12] Sialyl Lewis X (SLe^X) and A (SLe^A), tetrasaccharide epitopes that bind to selectins (Figure 1B), are overexpressed on tumor cells and correlate with poor prognosis.13 For instance, a recent surgical note demonstrated that for cultured cells derived from primary (CHMp) and metastatic (CHMm) lesions of a canine mammary gland tumor from the same animal, adhesion to human umbilical vein endothelial cells that express activated E-selectin was dramatically enhanced in the case of CHMm cells.14 Similarly, expression levels of α2,3-linked sialic acid residues correlated with the metastatic potential of human gastric cancers. 15 The α2–6 sialyltransferase enzyme (ST6Gal-I) is upregulated by oncogenes such as *ras* leading to dramatically increased α2–6 sialylation of the β_1 subunit of integrins, and β_1 from adenocarcinomas exhibits increased α2–6 sialylation relative to normal epithelial cells. ST6Gal-I expression in colon epithelial cells lacking endogenous ST6Gal-I manifests itself in enhanced adhesion to ECM proteins.[16] Consequently, altering selectin- and integrin-mediated adhesion is an emerging strategy for diminishing the metastatic potential of tumor cells. We envisioned that modification of sialic acids on the glycocalyx with fluorinated groups (Figure 1C) would result in reduced cell adhesion, based on prior demonstrations of the bioorthogonal noncovalent behavior of highly fluorinated surfaces.[17–20] Neelamegham and co-workers have recently shown that fluorination of GalNAc alters cell adhesion to selectins[21] and we have reported that cell-surface fluorination leads to modest decrease in adhesion to fibronectin.[22]

Mammalian cells can be induced to display sialic acids possessing unnatural chemical moieties via a glycoengineering strategy. Pioneering work by Reutter and colleagues established that mannosamine derivatives bearing unnatural *N*-acyl groups (e.g. propanoyl) are processed by cells through the sialic acid biosynthetic pathway, leading to cell surface presentation of the foreign group on sialylated glycans.[23, 24] Indeed, cells bearing propanoylated sialic acids exhibit increased adhesion to P– and E–selectin-coated surfaces due to an increase in the amount of SLe^X presented on the cell surface protein PSGL-1,[25] as well as increased adhesion to fibronectin-coated surfaces,[26] establishing sialic acid glycoengineering as a fruitful strategy for altering cell adhesion. The field has been broadened by the discovery that sialic acid derivatives bearing extra-biological chemical functionalities are also taken up by cells and processed through this pathway[27], and that the range of groups tolerated is larger when introduced on sialic acid than on the precursor mannosamine as it is downstream of the most stringent step – phosphorylation of mannosamine catalyzed by Man-6-kinase.[27, 28] Sialic acid glycoengineering has been exploited to attach bioorthogonally reactive functional groups such as azide,[28] ketone,[28] and thiol[29] to cell surfaces. This in turn has permitted highly selective decoration of cell-surface polysialic acid,[30] and attachment of tailored immunogenic sialic acids to tumor-specific antigens.[31] Cell surface modification has been demonstrated in living animals, including rat[32] and mouse[32–34] without apparent toxicity, raising the possibility that glycoengineering precursors may be useful for therapeutically altering cell adhesion.

Experimental Procedures

Cell Culture Conditions

HL60 cells were grown in RPMI-1640 medium supplemented with 10% FBS and penicillin–streptomycin (100 units/mL) in a 5% CO_2, water saturated atmosphere at 37 ℃. Cell densities were maintained between 2.0×10^5 and 2.0×10^6 cells/mL.

Detection of Sialic Acid Analogues on Cell Membranes by HPLC

HL60 cells were grown to cell densities of approximately 2×10^5 cells/mL in 12 mL of media. The cultures were supplemented with the appropriate volume of ethanolic solutions of the peracetylated methyl esters of compounds **2–5** (**2a–5a**) to give a final concentration of 200 μM, and the cells were incubated for 72 h. The cells were harvested, washed three times with PBS, and lysed by three freeze/thaw cycles. The membrane fraction was pelleted by centrifugation at 10,000 μ g for 15 min, and the pellet was washed twice with distilled water. After centrifugation at 10,000 μ g (15 min), the pellet was hydrolyzed for 3 h with 200 μL of 2 M AcOH at 80 °C. The supernatant was passed through 3,000 MW cut-off filters and the filtrate was concentrated. Sialic acid derivatization was performed according to the method of Hara *et al.*[35] and samples were analyzed by reversed-phase HPLC on a C18 column [Tosoh G0005-02E C18 Reverse phase, 4.6 mm × 250 mm, 5μ] using a linear gradient of acetonitrile and water [Solvent A: 98:2 H_2O:CH_3CN; Solvent B: 99:1 CH_3CN:H_2O] at a flow rate 1.0 mL/min. The relative content of sialic acid analogue and NeuAc was quantified by integration of peak areas (Figures 3B and 3C).

Cell Lysate Preparation and Analysis using Antibody-conjugated Beads

Collected cells were suspended in lysis buffer at 4 °C for 30 min (CelLytic Protein Extraction Kit, Sigma). The cell lysate was cleared by microcentrifugation 30 min at 16,000 × g (maximum speed) at 4 °C. Tumble incubation of 30 μL of 50% protein A-Sepharose bead slurry with 5 μL of CD162 IgG (Beckman Coulter M2090) in PBS was carried out for 24 h at 4 °C and the beads were washed twice with lysis buffer. The cleared lysate was added to the antibody-coated beads and further incubated for 12 h at 4 °C while mixing end over end. Microcentrifugation and removal of the unbound proteins was performed for 10 s at 16,000 × g (maximum speed) at 4 °C. The beads were washed three times with ice-cold wash buffer and once with cold PBS. Protein concentrations were determined in 96 well ELISA plates using 100 μL bicinchonic acid protein reagent and 5 μL sample. The absorbance was measured using an InfiniteTM 200 series microplate reader (Tecan Group Ltd, Switzerland) at 562 nm.

Immunoblotting

Samples (20 μL/lane, 400 μg/lane) were separated on SDS polyacrylamide gels (4–20% Pierce) and transferred to nitrocellulose filters. Equal loading was confirmed by actin staining. The blots were blocked with 5% fat-free dry milk powder in Tween PBS, followed by incubation with the primary antibody anti-sialyl-Lewis^x antibody KM93 (Millipore MAB2096) and then the appropriate secondary antibody conjugated with peroxidase (goat anti-mouse IgM polyclonal, Millipore AP128P). Chemiluminescence was measured on Kodak Biomax MS film for 30–45 min. The molecular weights were calculated based on MW marker standards.

Cell Adhesion Assays

Prior to the adhesion, 96-well plates were coated with P– or E–selectin (10 μg/mL) for 16 h at 4 °C. Unspecific binding was blocked with 1% BSA for 4 h at 4 °C. Cells were incubated for 72 h with **2a**–**5a** at 37 °C. Collected cells were washed and incubated under serum-free medium for 2 h before the experiment. Cells were labeled with Calcein-AM at a final concentration of 12.5 μM for 30 min at 37 °C. Cells were washed three times with PBS, resuspended and then added to each well (1.5×10^5 cells in 100 μL) in quadruplicate. The plate was shaken at 611 rpm for 10 s and spun at 411 × g for 2 mins in order to move the cells to the plate surface, then incubated for 2 h at 37 °C. The "before-wash" fluorescence of the samples was measured using a fluorescence plate reader at excitation wavelength 485 nm and emission wavelength 520 nm. The non-adherent cells were gently aspirated and the

wells were washed twice with 100 μL PBS. Finally, 100 μL PBS was added to each well and the "after-wash" fluorescence was measured. The percent adhesion was calculated using the formula: [(RFUafter-wash)/ [(RFUbefore-wash)] × 100. Individual experiments were performed in four to six replicates for each compound and a statistical Student's *t*-test was applied. A typical experimental dataset is shown in the Supplemental Data (Table S1). A set of 3–4 such experiments was then normalized for percentage adhesion for each compound and the averages reported (Figure 4).

Flow Cytometry

Cell were seeded at 2.0×10^5 cells/mL and incubated for three days with compounds **2a**–**5a**. Cells were collected and washed three times with PBS. Immunostaining was carried out by incubation with anti-sialyl-Lewis^x antibody KM93 (Millipore MAB2096) at the optimal concentration (5 μg/1mL) in PBS supplemented with 1% BSA for 1 h at 4 °C. Cell surface binding was detected using the F(ab')2 fragment of FITC-conjugated goat anti-mouse immunoglobulin (affinity-isolated antibody; Dako Cytomation F0479). Flow cytometry was performed on a MoFlo™instrument from Dako Cytomation. Cells were gated by forward and side scatter signals. A total of ~20,000 cells were analyzed in each experiment.

Chemical Synthesis of Sialic Acid Analogues

Compounds **1a**–**7a** were synthesized using modified versions of literature procedures.[27, 36] Purities of the compounds were judged to be ≥95% by HPLC on a reverse phase analytical column (J. T. Baker C_{18}, 5 μ, 4 mm × 250 mm) using linear gradients composed of H_2O and CH_3CN. The anomeric ratios were calculated from relative NMR peak areas.

2,4,7,8,9-Penta-*O*-acetyl-5-*N*-trifluoropropanoyl-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulosonic-1-methyl ester (β–3a)

^{1}H NMR (300 MHz, $CDCl_3$) δ 6.03 (1H, d, *J*=9.01 Hz), 5.37 (1H dd, *J*=1.92 Hz, *J*=5.06 Hz), 5.33-5.27 (1H, m), 5.03–5.27 (1H, m), 4.51 (1H, dd, *J*=2.45 Hz, *J*=12.49 Hz), 4.12–4.21 (3H, m), 3.79 (3H, s), 3.03-2.93 (2H, m), 2.56 (1H, dd, *J*=4.97 Hz, *J*=13.48 Hz), 2.14-2.09 (7H, m), 2.06 (3H, s), 2.03 (3H, s), and 2.02 (3H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 170.91, 170.74, 170.63, 170.41, 168.37, 166.39, 163.10, 124.31 (CF_3, J_{C-f}=276.98 Hz), 97.46, 72.49, 71.54, 67.81, 67.82, 62.09, 53.38, 49.621, 41.81 (CH_2CF_3, J_{C-F}=29.62 Hz), 36.19, 21.02, 20.91, 20.87, 20.83, and 20.75; ESI-MS calcd for $C_{23}H_{30}F_3NNaO_{14}$ (M +Na^+) 624.15, found: 624.18. ESI-HRMS calcd for $C_{23}H_{30}F_3NNaO_{14}$ (M+Na^+) 624.1516, found: 624.1500. Ratio of anomers (%):α: β = 16:84

2,4,7,8,9-Penta-*O*-acetyl-5-*N*-trifluorobutanoyl-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulosonic-1-methyl ester (β–5a)

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.79-5.76 (1H, d, *J*=8.6 Hz), 5.34 (1H, dd, *J*=1.82 Hz, *J*=5.00 Hz), 5.28-5.12 (1H, m), 5.05-5.01 (1H, m), 4.47 (1H, dd, *J*=2.51 Hz, *J*=12.4 Hz), 4.14-4.10 (3H, m), 3.76 (3H, s), 2.57-2.48 (3H, m), 2.30 (2H, m), 2.12 (3H, s), 2.10-2.06 (4H, m), 2.03 (3H, s), 2.01 (3H, s), and 1.97 (3H, s); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 171.05, 170.71, 170.44, 170.35, 170.22, 168.35, 166.40, 126.80 (CF_3, J_{C-F}=276.98 Hz), 97.50, 72.72, 71.41, 68.24, 67.79, 62,11, 53.31, 49.42, 36.07, 28.92, 20.97, 20.93, 20.88, 20.85, 20.82 and 17.66; ESI-MS calcd for $C_{24}H_{32}F_3NNaO_{14}$ (M+Na_+) 638.17, found: 638.17. ESI-HRMS calcd for $C_{24}H_{32}F_3NNaO_{14}$ (M+Na^+) 638.1673, found: 638.1653. Ratio of anomers:α:β (%) = 32:68

2,4,7,8,9-Penta-*O*-acetyl-5-*N*-3-methylbutanoyl-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulosonic-1-methyl ester (β–6a)

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.37-5.33 (4H, m), 5.12-5.08 (1H, m), 4.51 (1H, d, *J*=10.76 Hz), 4.21-4.08 (4H, m), 3.82 (3H, s), 2.59 (1H, dd, *J*=5.34 Hz, *J*=13.20 Hz), 2.19 (3H, s), 2.13 (3H, s), 2.09 (3H, s), 2.06 (3H, s), 2.05 (3H, s), 1.95-1.89 (2H, m), and 0.96 (6H, m); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 173.04, 171.26, 171.06, 170.76, 170.67, 168.73, 166.84, 97.88, 73.29, 71.82, 68.52, 68.31, 62.80, 53.65, 49.59, 46.45, 36.39, 30.15, 26.20, 22.78, 22.72, 21.41, 211.34, 21.29, and 21.23; ESI-MS calcd for $C_{25}H_{37}NNaO_{14}$ ($M+Na^+$) 598.56, found: 598.27. ESI-HRMS calcd for $C_{25}H_{37}NNaO_{14}$ $(M+Na)^+$ 598.2112, found: 598.2103. Ratio of anomers (%):α:β = 46:54

2,4,7,8,9-Penta-*O*-acetyl-5-*N*-4-methylpentanoyl-3,5-dideoxy-D-glycero-β-D-galacto-non-2-ulosonic-1-methyl ester (β–7a)

^{1}H NMR (300 MHz, $CDCl_3$) δ 5.45 (1H, d, *J*=8.08 Hz), 5.34 (2H, d, *J*=5.08), 5.19-5.15 (1H, m), 5.07-5.05 (1H, m), 4.48 (1H, dd, *J*=1.41 Hz, *J*=13.2 Hz), 4.17-4.08 (3H, m), 3.78 (3H, s), 2.54 (1H, dd, *J*=4.80 Hz, *J*=13.17 Hz), 2.17 (3H, s), 2.14 (3H, s), 2.05 (3H, s), 2.03 (3H, s), 2.01 (3H, s), 1.56-1.41 (4H, m), and 0.871 (6H, d, *J*=6.29 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 171.80, 171.25, 171.10, 170.06, 170.67, 168.53, 166.80, 97.58, 73.72, 71.94, 68.80, 67.71, 62.79, 54.05, 49.59, 35.79, 34.71, 30.15, 27.10, 22.80, 22.67, 20.91, 21.30, 21.19, 21.01, and 20.88; ESI-MS calcd for $C_{26}H_{39}NNaO_{14}$ ($M+Na^+$) 612.24, found: 612.36. ESI-HRMS calcd for $C_{26}H_{40}NNaO_{14}$ ($M+H^+$) 590.2449, found: 590.2452. Ratio of anomers (%):α:β = 37:63

Results and Discussion

Incorporation of Unnatural Sialic Acids

In the current study, our goal was to use sialic acid to fluorinate the glycocalyx and thereby reduce selectin-mediated cell adhesion. Accordingly, to modify their surfaces, HL60 cells (human promyelocytic leukemia) were incubated with unnatural sialic acids **2a**–**5a** (Figure 2) that bear pendant groups on the 5-position. After three days of culture, the membrane fractions of the cells were collected. The extent of incorporation of the unnatural sialic acid was determined by acid hydrolysis and labeling with the α-keto acid-specific fluorogenic reagent 1,2-diamino-4,5-methylene-dioxybenzene (DMB)[35] (Figure 3A). All derivatives were incorporated with reasonable efficiencies, with modified sialic acids **2**–**5** comprising 57%, 68%, 24%, and 71% of the total sialic acid on the cell surface. Based on the estimation of the amount of all sialic acids present, in the case of **3** and **5**, there were ~10_8 CF_3 groups on the surface of each cell (Table 1). This is consistent with prior studies where under saturating conditions of mannosamine-derived precursors, ~$2\cdot10^6$ unnatural N-acyl sialic acids were incorporated on Jurkat cells and >10^7 on HL60 cells.[37] Approximating the cross-sectional surface area of a CF_3 group to be 25 Å^2, an estimate of the fractional fluorinated cellular surface area that an apposing adhesion partner would encounter can be made. Dustin and co-workers have determined the total surface area of a Jurkat cell to be ~800 μm^2.[38] If 3 × 10^7 trifluoromethyl groups are displayed per cell, the fractional surface area of the cell occupied by CF_3 groups is 0.9%

Adhesion to ECM Proteins

The ability of the fluorinated sialic acids to alter cellular adhesion to ECM proteins and selectins was assessed by incubating cells with peracetylated methyl esters **2a**–**5a** at 200 μM concentrations for 3 d. The cells were pelleted, washed with buffer, counted, and a known number of cells resuspended in buffer. They were further labeled with the live cell-specific fluorescent dye, Calcein-AM. The cells were then transferred to wells in microtiter plates

coated with E– or P–selectin (Figure 4A) and allowed to adhere for 2 h at 37 °C. The fluorescence emanating from the wells was measured at this stage. The wells were then washed with buffer and the fluorescence was measured once again. The fluorescence before and after washing revealed the number of cells adhered to proteins immobilized on plates. The most dramatic difference in adhesion was observed for P–selectin where cells treated with **3a** and **5a** were 62% and 77% less adherent than untreated cells. Remarkably, cells treated with **2a** showed the same extent of adhesion as those treated with **1a**.[25] A single CH_3 to CF_3 modification on the sialic acid thus had a dramatic effect on the adhesive properties of the cell. The fluorinated derivatives resulted in decreased adhesion to all three of the protein ligands.

The size of a trifluoromethyl group has often been compared to that of an isopropyl moiety. The most compelling evidence for this estimation comes from measurements of rotational barriers in 1,1'-disubstituted biphenyls along the biphenyl axis and the results are suggestive of a $-CF_3$ group imposing steric restrictions equivalent to those of a $-CH(CH_3)_2$ group.[39–41] In order to assess whether the size of the pendant group at the 5-position of neuraminic acid is the key determinant of decreased adhesion, compounds **6a** and **7a** were used as controls for **3a** and **5**. Cells treated with **6a** and **7a** exhibited adhesion to both P- and E-selectin-coated plates to an extent intermediate between untreated cells and those treated with **3a** or **5a**, but **6a** and **7a** were also incorporated less well (10% and 44%, respectively, see Figure 3C, and Table I).

The amount of SLe^X presented specifically on P-selectin glycoprotein ligand-1 (PSGL-1, CD162) and generally on the cell surface was measured by Western blotting and flow cytometry analysis (Figures 4B,C). Assuming that the primary antibody specific for SLe^X binds with the same affinity to the unnatural epitopes, no significant difference was evident in the amount of SLeX on cells treated with **2a**, **3a**, and **5a**. Furthermore, CD162-SLeX levels on cells treated with **2a**–**5a** were either the same, or significantly elevated.

Mechanism of Reduced Cell Adhesion

We have demonstrated that treatment of HL60 cells with fluorinated sialic acid analogues **3a** and **5a** leads to a significant reduction in the ability of these cells to adhere to E- and P-selectin compared with both untreated cells and with cells treated with unfluorinated congeners **2a** and **4a** (Figure 4A). There are at least three possible explanations for the larger reduction in adhesion by the fluorinated analogues: 1) the fluorinated sialosides, including sialyl Lewis X, exhibit specifically reduced binding on the molecular level with their cognate selectins; 2) the amount of the SLe^X-presenting selectin binding partners (e.g. CD162 (PSGL-1) in the case of P-selectin) on the cell surface is reduced compared with untreated cells or cells treated with non-fluorinated sialic acids; and 3) the incorporation of fluorinated analogues on the cell-surface sialosides is enhanced compared to unfluorinated congeners.

To distinguish these possibilities, we measured the ratio of sialic acid analogues to natural N-Ac neuraminic acid present on the cell surface siaolsides using HPLC analysis of DMB derivatives[35] (Figure 3B) as well as the total sialic acid content on the cell surfaces by comparison of fluorescence of the DMB-modified cell hydrolysates with a standard curve (See Supplemental Data, Figure S1). Modified sialic acids **2**–**5** were incorporated to the extent that they comprised 57%, 68%, 24%, and 71% of the total sialic acid on the cell surface, and total sialic acid content on cells treated with **2a**-**5a** varied from 91%–124% of that on untreated cells (Table 1).

Previous work has shown incorporation of **2** on cell surface sialosides was accompanied by an increase in cell adhesion to P-selectin for cells treated with N-propanoyl mannosamine.[25]

In that study, the increased adhesion was found to accompany enhanced expression of CD162-SLe^X (PSGL-1), the principal SLe^X–bearing glycoprotein partner for P-selectin on the cell surface. To determine if the diminished adhesion we observed in cells treated with modified sialic acids **2a**-**5a** was due to a decrease in CD162-SLe^X expression, we measured the amount of CD162-SLe^X on cell surfaces by both Western blot analysis (Figure 4C) and flow cytometry using antibodies specific for CD162 and for SLe^X (Figure 4B). The data show that CD162-SLe^X expression was the same (**4a**) or higher (**2a**, **3a**, and **5a**) than in untreated cells. Therefore, the reduction in cell adhesion that we observe is not due to reduced CD162-SLe^X expression (see also Figure S2).

To distinguish the effect of the increased steric demand of fluorinated analogues **3a** and **5a** from the electronic effects of fluorination, we prepared N-isopropanoyl (**6a**) and N-isobutanoyl (**7a**) analogues of sialic acid and evaluated their effect on HL60 cell adhesion to P- and E- selectin. The data (Table I and Figure 3C) show that incorporation of these analogues is lower (10% for **6a** and 44% for **7a**) than for the fluorinated analogues and that cells treated with these analogues showed less diminution of adhesion to P- and E-selectin than cells treated with the fluorinated analogues.

Since the diminution in P-selectin adhesion is not due to a decrease in the cell-surface presentation of its cognate ligand CD162-SLe^X, the simplest explanation of the observed reduction in cell adhesion upon surface modification (including fluorination) is that glycans bearing unnatural sialic acids are weaker binders of the selectins and other ECM receptor molecules.[42] The extent to which *N*-Ac neuraminic acid is replaced by the unnatural sialic acids correlates reasonably with diminished adhesion, irrespective of the size (Figure 5). The larger the percentage of the unnatural sialic acid on the surface, the lower the adhesion. Fluorinated sialic acids, especially **5a**, are incorporated more efficiently compared to the control molecules, and result in dramatically diminished adhesion.

The molecular basis for the observation that changes in the *N*-acyl group of the sialic acid residue in cell surface sialosides result in reduced binding to P-selectin is difficult to ascertain. A published crystallographic analysis[11] of the CD162-SLe^X:P-selectin complex reveals a distance of 6.87 Å between the acyl carbonyl and the nearest protein loop (Figure 6) and does not indicate an obvious steric conflict between the selectin and the N-acyl group. However, published structural data of the bound and free forms of SLe_x suggest that the Neu5Acα2–3Gal linkage resembles the free-form conformation "A" ($\phi,\psi = -43°,-12°$) when complexed with E-selectin, but is not the global energy minimum.[11, 43] It follows that subtle conformational changes in modified cell-surface CD162-SLe^X structures with larger sialic acid *N*-acyl groups are likely to alter the energy of the optimal binding conformation thereby diminishing the binding affinity.

Conclusion and Outlook

The results described here proffer a new method for a major alteration of the cell glycocalyx with no observed toxicity under cell culture conditions. The introduction of fluorinated groups on cell surfaces resulted in a significant decrease in cell adhesion to ECM receptor molecules and selectins, a property that is thought to be essential for tumor metastasis. This diminished adhesion is in general due to increased efficiency of incorporation, especially that of **5a**, and depletion of the level of *N*-acetyl neuranamic acid on cell surfaces. Furthermore, since fluorine is scarce in soft tissue and cancerous cells are often hypersialylated, cell-surface fluorination may prove valuable for background-free ^{19}F magnetic resonance imaging (MRI) before invasive tumors are visible by other means. The detailed mechanism by which altered adhesion is achieved is the subject of current investigation in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

BSA	bovine serum albumin
DMB	1,2-diamino-4,5-methylenedioxybenzene
ECM	extra cellular matrix
ELISA	enzyme-linked immunosorbent assay
PBS	phosphate buffered saline
PSGL-1	P-selectin glycoprotein ligand-1
SDS	sodium dodecyl sulfate
SLe^A	sialyl Lewis A
SLe^X	sialyl Lewis X
ST6Gal-I	α2-6 sialyltransferase enzyme.

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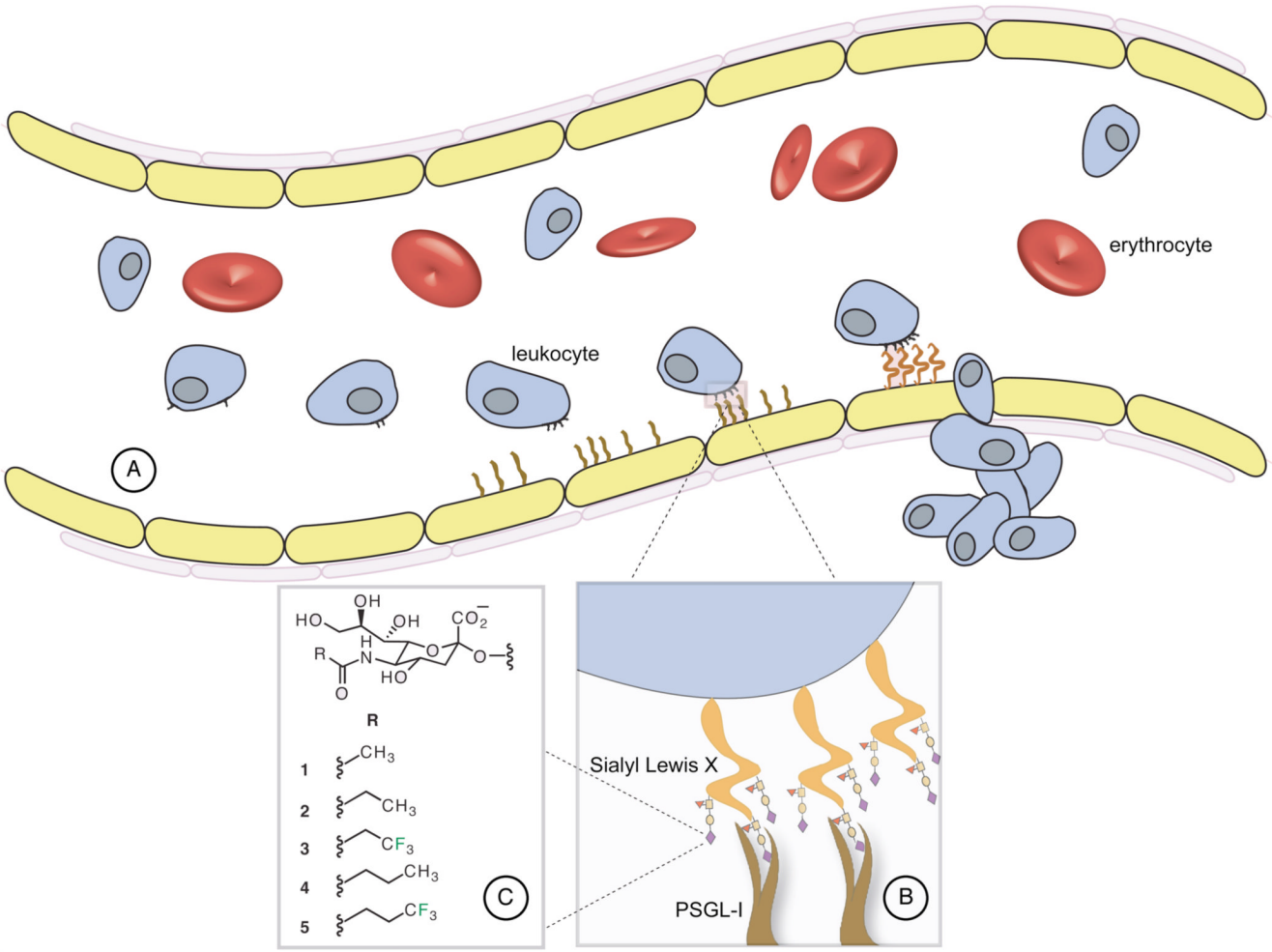


Figure 1.

Hematogenous tumor metastasis mimics leukocyte extravasation. (A) Cell rolling and firm adhesion leading to extravasation are mediated by carbohydrate-protein interactions. (B) Rolling requires interaction of CD162-bound SLe^X on the blood-borne cell with P-selectin on the vascular endothelium. (C) Inset showing the glycoengineered terminal carbohydrate on SLe^X

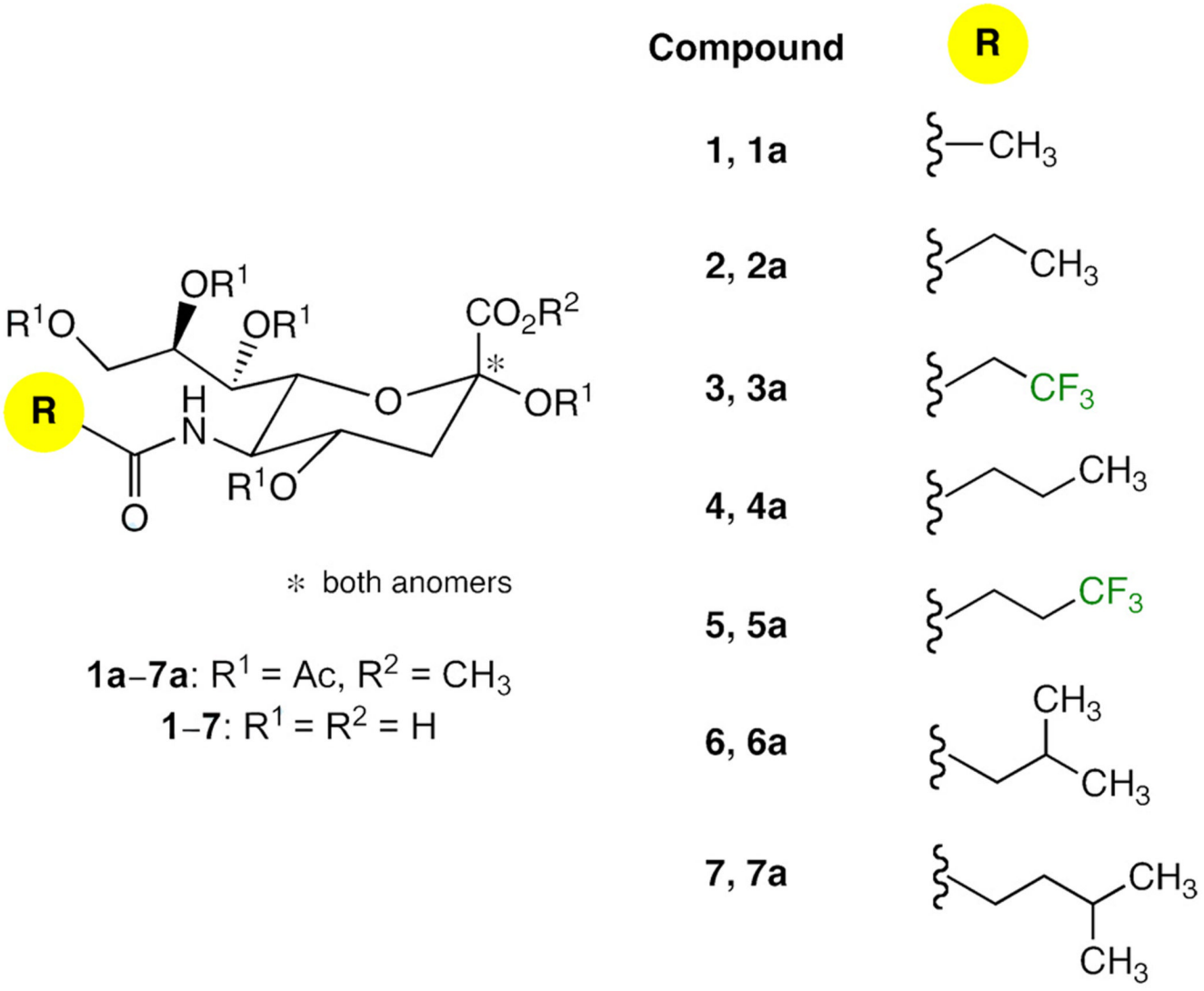
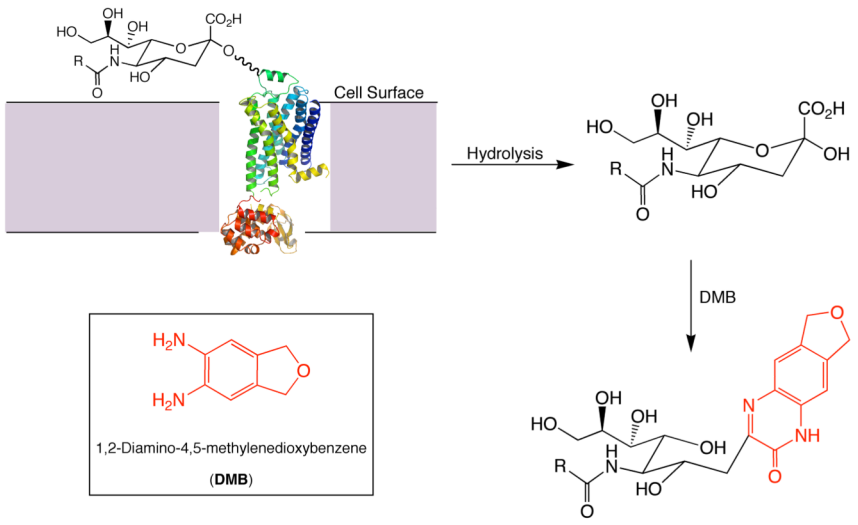


Figure 2.
Structures of sialic acid derivatives used for incubation with mammalian cells in this study. Compounds were fed to the cells in the pentaacetyl methyl ester form (**1a**–**7a**). Presumably these are converted by intracellular lipases to the free hydroxyl (and acid) forms (**1**–**7**) before they enter the biosynthetic pathway. Material isolated from cell surfaces was in the "free" form.

(A)



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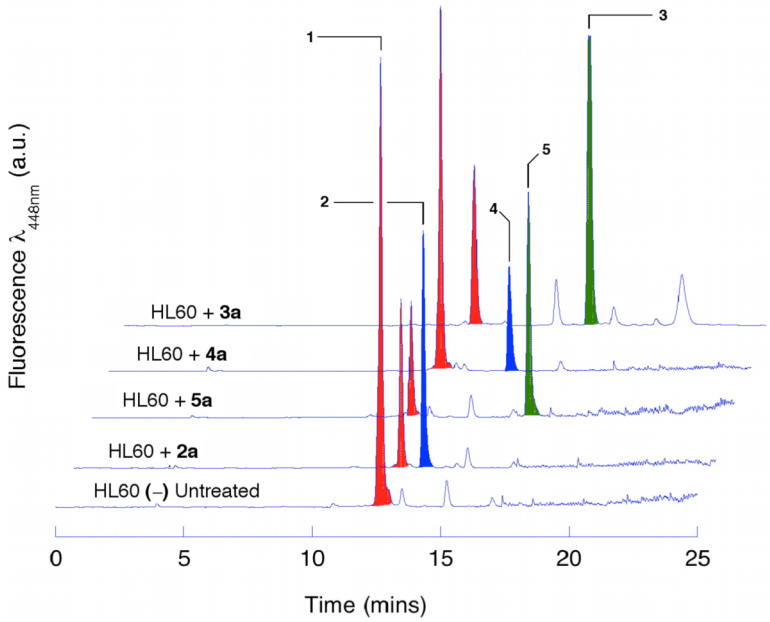

(C)

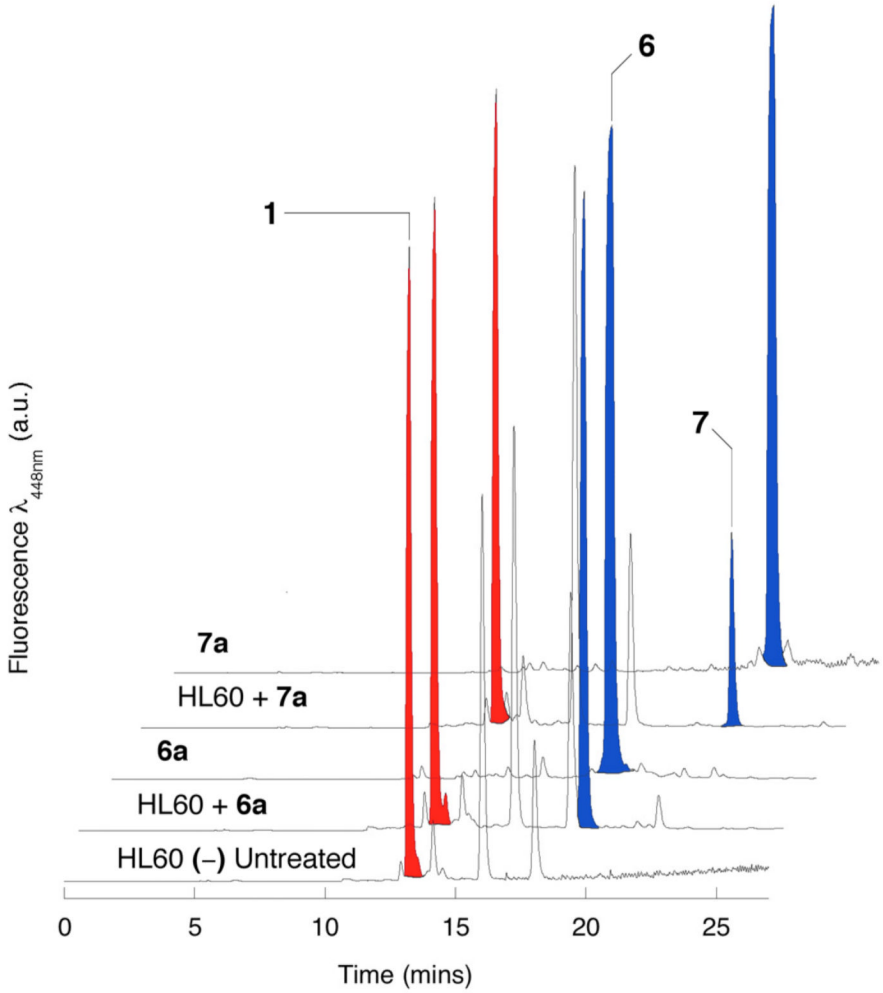

Figure 3.

Analysis of fluorinated sialic acids on cell surfaces. (A) Schematic of the hydrolysis and labeling procedure. Modified cells were lysed (three freeze-thaw cycles), the membranes pelleted (10,000 × g), and subjected to acid hydrolysis (2M HOAc, 80 °C). The hydrolysate was passed through a 3000 Da cutoff molecular weight filter, and released sialic acids were derivatized by treatment with DMB according to literature procedures.[35] (B) HPLC traces of the DMB-labeled cell membrane hydrolysates from cells used for E- and P-selectin cell adhesion assays. (C) HPLC traces of the DMB labeled cell membrane hydrolysates from cells treated with **6a** and **7a** used for E- and P-selectin cell adhesion assays. The numbered peaks correspond to the DMB derivatives of the compounds as confirmed by co-injection with standards.

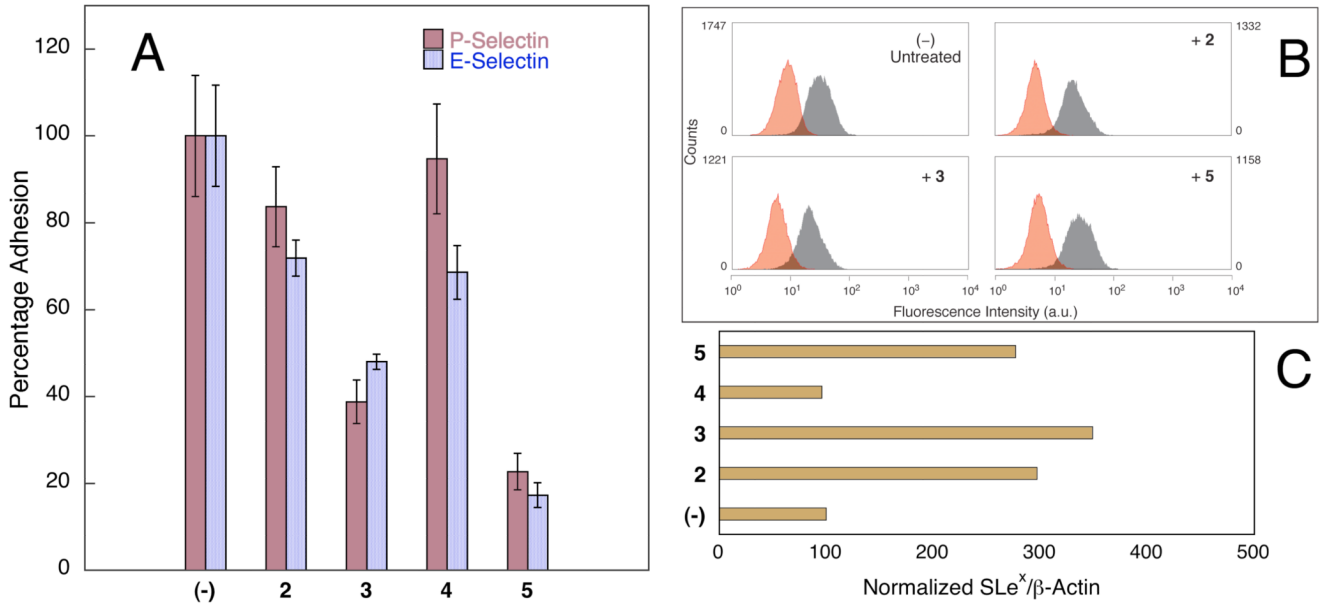

Figure 4.

Effect of cell surface fluorination on adhesion to P– and E–selectin. (A) Introduction on the cellular glycocalyx of fluorinated sialic acids **3** or **5**, but not alkyl congeners **2** or **4**, dramatically reduces the adhesion of cells to selectins. The compounds were fed to cells in the peracetylated, methyl ester forms (**2a**–**5a**, Figure 2) to facilitate passage across the plasma membrane. Error bars represent one S.D. (B) Flow cytometry analysis of modified and native cells with anti-SLex primary and FITC-labeled anti-IgG secondary antibody demonstrates no significant difference in cell surface SLe^X amounts. (C) Western blot analysis of CD162-bound SLe^X demonstrates that decreased adhesion to P–selectin is not due to reduced CD162; treated cells have the same or higher levels. See also Table S1 and Figure S2.

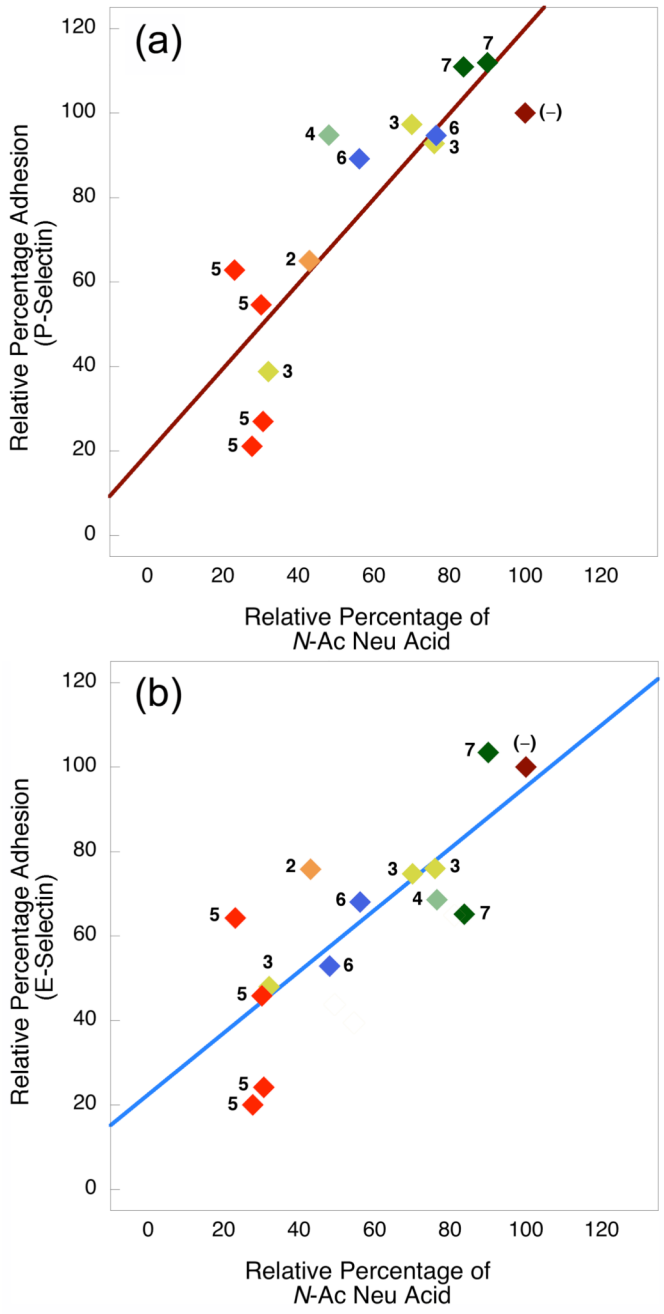

Figure 5.

Plots showing relative percentage of N-Ac neuraminic acid (NANA) versus relative percentage adhesion (A) to P– and (B) E–Selectins (R values of 0.86 and 0.79 respectively) from individual experiments conducted in replicates of 5. The relative percentage of NANA ((NANA)/(NANA+unnatural) × 100) on the surface cell correlates with adhesion. The range of total sialic acid (NANA + unnatural derivative) in all experiments spanned 3.48×10^7 molecules/cell (low) to 1.29×10^8 molecules/cell (high).

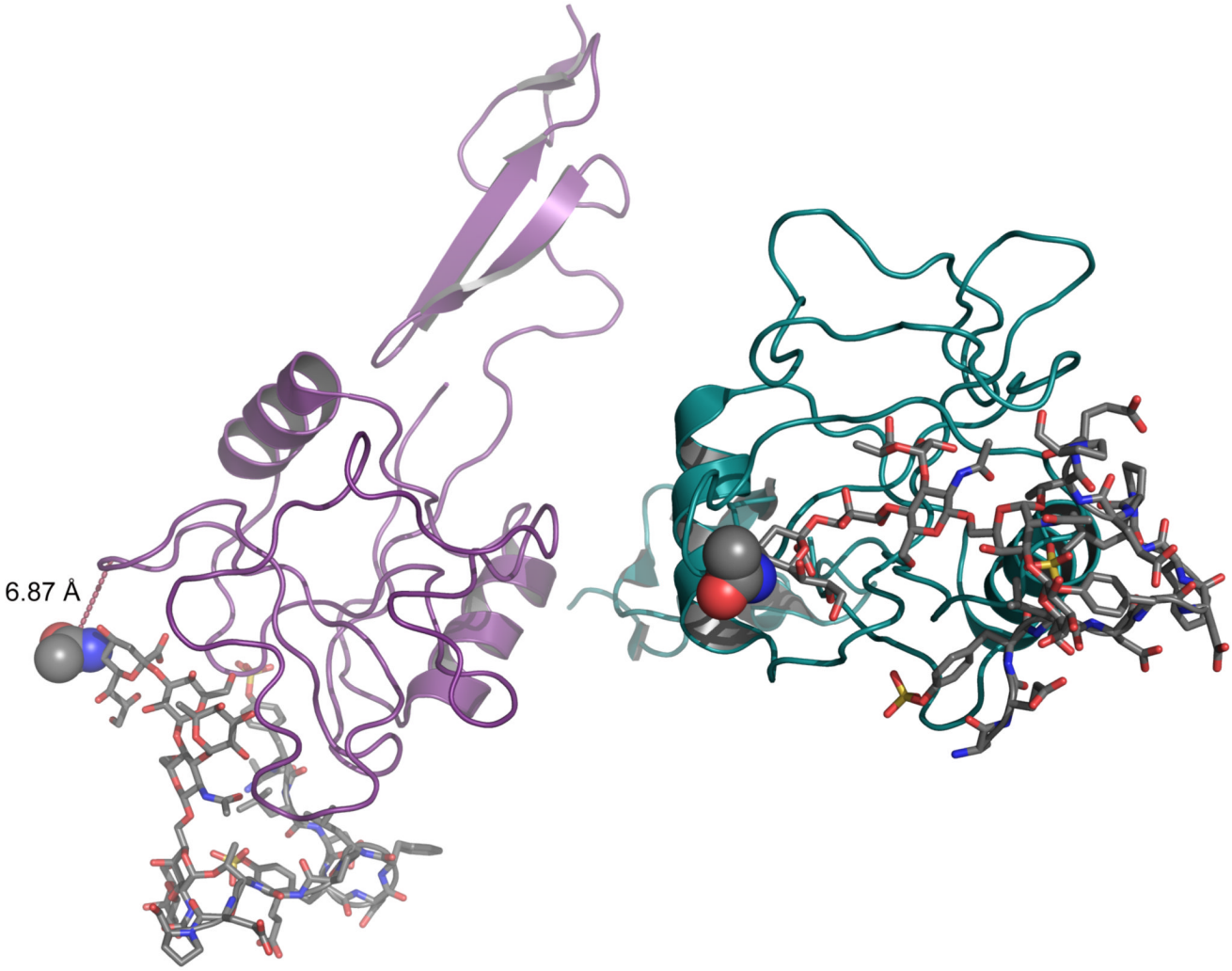

Figure 6.

Cartoon depiction of P-selectin EGF (LE) domain complexed with the N-terminal domain of CD162 modified by tyrosine sulfation and SLex. The acetyl chain at N5 of sialic acid is shown in space filling representation; PDB ID: 1G1S. [11] The nearest neighbor distance from the carbonyl carbon of the acyl (acetyl in this instance) group to any atom in the protein is 6.87 Å (MacPyMOL, Version 0.99, DeLano Scientific, San Carlos, CA). Although the acyl chain in the crystal structure does not seem to play a role in the interaction of P-selectin and SLex, conformational changes in SLe^X are possible upon acylation with unnatural acids, leading to diminished binding.[43, 45]

Table 1

Incorporation of modified sialic acids on HL60 cells treated with compounds **2a–7a**.

Treatment compound	N-Ac-Neu acid	Modified sialic acid	Total sialic acid (% modified)	No. of CF_3 groups/cell
none	1.00	–	1.00 (−)	–
2a	0.52	0.68	1.20 (57)	–
3a	0.29	0.62	0.91 (68)	4.5×10^7
4a	0.84	0.26	1.09 (24)	–
5a	0.35	0.89	1.24 (72)	6.8×10^7
6a	1.04	0.12	1.15 (10)	–
7a	0.78	0.61	1.40 (44)	–

[a]Values are normalized to *N*-acetylneuraminic acid in untreated cells.

[b]Calculated number of CF_3 groups based on total sialic acid as determined by the DMB labeling standard curve (Supplementary Figure S1).

[a,b]The relative amounts were determined by DMB-labeling, integrated areas in HPLC, and the cell count.