A Rapid and Sensitive MicroPlate Assay (MPSA) Using an Alkyne-Modified CMP-Sialic Acid Donor to Evaluate Human Sialyltransferase Specificity

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A Rapid and Sensitive MicroPlate Assay (MPSA) Using an Alkyne-Modified CMP-Sialic Acid Donor to Evaluate Human Sialyltransferase Specificity

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Human sialyltransferases primarily utilize CMP-Sias, especially transferring Neu5Ac from CMP-Neu5Ac to various acceptors. Advances in chemical biology have led to the synthesis of novel CMP-Sia donors suitable for bioorthogonal reactions in cell-based assays. However, the compatibility of these donors with all human enzymes remains uncertain. We synthesized a non-natural CMP-Sia donor with an alkyne modification on the *N*-acyl group of Neu5Ac, which was effectively used by human ST6Gal I and ST3Gal I. A sensitive MicroPlate Sialyltransferase Assay (MPSA) was developed and expanded to a panel of 13 human STs acting on glycoproteins. All assayed enzymes tolerated CMP-SiaNAl, allowing for the determination of kinetic parameters and turnover numbers. This study enhances the biochemical characterization of human sialyltransferases and opens new avenues for developing sialyltransferase inhibitors.

Introduction

Sialic acids (Sias) represent a large family of 9-carbon monosaccharides, which have in common a carboxyl group at the anomeric C-2 position, a glycerol side chain at C-6, an aminoacyl group or hydroxyl group attached to C-5, and a deoxygenated C-3 position.[1] Due to their charged nature and terminal position on glycoconjugates, Sias influence molecular and cellular interactions and are implicated in a number of biological processes. Sialylated molecules found in intercellular spaces[2] and decorating the cell surface[3] are involved in nearly all pathophysiological processes, including neurological disorders and cancers.[4] Numerous studies reported the upregulation of specific sialylated glycans in cancers, including the Sialyl Lewis X (sLe^x) and sialyl Lewis a (sLe^a), sialyl T (sT) and sialyl-Tn (sTn) illustrated in Supplemental Table S1 which are proven signatures of cancer cells with fundamental implication in tumor growth, metastasis, immune evasion and drug resistance.[4a,5] Excess of negatively charged molecules on the cell surface contribute to immune evasion, reduced efficacy of chemo- & radiotherapy and promote tumor metastasis stimulating cell migration, invasion and angiogenesis.[6]

Biosynthesis of these sialylated molecules represents one of the last steps of glycosylation taking place in the *trans*-Golgi network and is achieved by a subset of glycosyltransferases known as sialyltransferases (STs). These evolutionary conserved Leloir enzymes catalyze the transfer of Sia residues from an activated Sia donor (CMP-Sia) to galactose (Gal), *N*-acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc) or Sia residues at the reducing end of glycans on glycoproteins and glycolipids. Twenty STs have been identified in the mammalian tissues[7] and have been classified into four families ST6GAL, ST3GAL, ST8SIA, and ST6GALNAC based on the monosaccharide acceptor and the glycosidic linkage formed.[7a,8] Molecular cloning and biochemical characterization studies are available for a limited number of mammalian STs for which determination of kinetic parameters could be achieved.[7b,9] These studies have underscored their acceptor-substrate specificity,[10] likely relying on evolutionary conserved sequence motifs known as sialylmotifs and family motifs.[7b,11,12,7a,b,13]

STs are generally dysregulated in cancer cells[14] and their increased expression leads to more sialylated Tumor Associated Carbohydrate Antigens (TACAs) and hypersialylation at the cell surface. Therefore, investigating STs represents an avenue to observe and understand the events occurring at the cellular surface in the context of these pathologies and they appear as interesting targets in the search for cancer treatments.[15] However, despite their vital functions, our understanding of their structure-activity relationship remains extremely limited and progress is hampered by a lack of appropriate biochemical tools. Assaying STs activity is still challenging and existing assays are often limited by sensitivity and throughput, hindering comprehensive enzyme characterization and advances in glycobiology and drug discovery. To address this challenge and augment our understanding of the unique characteristic of these enzymes, development of rapid, sensitive and robust high

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throughput screening method for the assessment of STs activities is needed. The human enzymes have long been known to catalyze the addition of sialic acid residues from CMP-Neu5Ac to a variety of glycan acceptors and to show overlapping substrate specificities. Therefore, *in vivo*, the relative contribution of these STs to sialylated molecules biosynthesis depends on their expression level and on their kinetic parameters *i.e.* K_M values. However, comprehensive kinetic data for the twenty human recombinant STs towards CMP-Sia donors are scarce and scattered across multiples studies using different methodologies, making direct comparison of their enzymatic activity challenging.[9a,c,16] In the past, various detection methods were developed to screen substrate specificities of a few STs (reviewed in[17]). Up to now, ST3Gal I and ST6Gal I have been the most extensively studied STs mostly due to their high enzymatic specific activity (ESA) and limitations of methodological approaches to characterize STs. For that purpose, radiolabeled Sias (CMP-[^{14}C]Neu5Ac),[10] or fluorinated labeled Sias (CMP-7FNeu5Ac)[18] or chemoenzymatically synthesized C5- or C9-Sia derivatives modified with a wide range of functionalities like azido (Az), alkyne (Al), biotin or fluorophores were developed to assess STs activities. However, despite their well-studied promiscuity,[19] many of the known human STs utilize a limited repertoire of natural CMP-Sias as sugar donor[20] and it remains to be known whether all human enzymes tolerate the presence of chemical reporter groups on the Sia moiety. Taking advantage of the recent development in chemical biology strategies, a novel synthetic CMP-Sia donor (CMP-SiaNAl) shown in Figure 1A was synthetized by creating an alkyne modification on the *N*-acyl group of Neu5Ac.[21] This small alkyl group is further amenable for bioorthogonal reactions with azido-Biotin in both cell-based or cell-free assays. We demonstrated that the human ST6Gal I and ST3Gal I tolerate CMP-SiaNAl as a donor substrate. Notably, we showed that both enzymes exhibited similar behavior towards the natural radiolabeled donor CMP-[^{14}C]Neu5Ac and the unnatural donor CMP-SiaNAl.[21b] Subsequently, a sensitive and rapid MicroPlate Sialyltransferase Assay (MPSA) was set up (Figure 1B–D) that could be used for the enzymatic characterization of human ST6Gal I and ST3Gal I, which act on *N*- and *O*-glycoproteins respectively.[22] Since it requires small amounts of materials, the MPSA was further used for the characterization of novel fish STs activities.[20a,23] However, up to now, no systematic study with the various human STs has been undertaken.

Here, we expand the capability of MPSA to characterize the enzymatic activities of various STs. We focused on a panel of recombinant human STs acting on glycoproteins, namely ST6Gal I, five ST3Gal (ST3Gal I, ST3Gal II, ST3Gal III, ST3Gal IV and ST3Gal VI), four ST6GalNAc (ST6GalNAc I, ST6GalNAc II, ST6GalNAc IV and ST6GalNAc V) and three ST8Sia (the polysialyltransferases ST8Sia II and ST8Sia IV, and the mono-alpha2,8-sialyltransferase ST8Sia VI). Each ST activity was assessed in various incubation conditions (w.r.t. time, enzyme quantities) using the alkynyl analog of CMP-Neu5Ac (CMP-SiaNAl) and kinetic parameters of each enzyme towards this sugar nucleotide have been established.

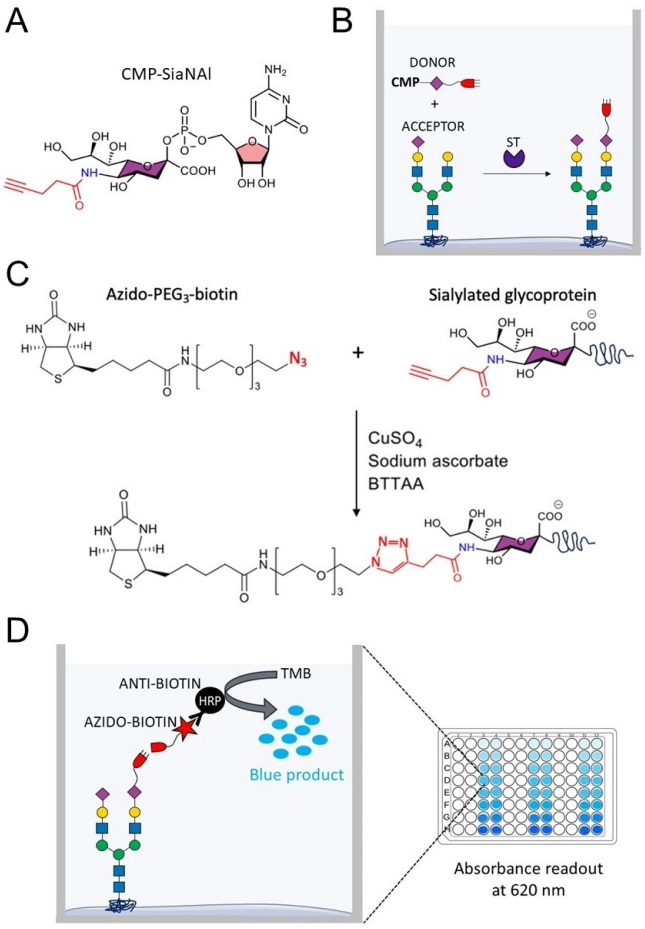

Figure 1. A) Structure of the functionalized CMP-Sia donor (CMP-SiaNAl). B) Schematic illustrating sialylation reaction set up on microplate with CMP-SiaNAl, glycoprotein acceptor (fetuin, asialofetuin or ALCAM) coated in the wells and sialyltransferase. The glycan moiety is represented with the SNFG nomenclature[27] C) Bioorthogonal reaction is conducted with azido-PEG3-biotin, BTTAA and CuAAC in conditions described in Experimental section to detect SiaNAl transfer and (D) schematic representation of biotin detection with the anti-biotin antibody followed by HRP activity detected with TMB colouring the well in blue. Absorbance is red at 620 nm.

Results and Discussion

Utilization of CMP-SiaNAl by Human Sialyltransferases

We firstly investigated whether the 13 commercially available human STs acting on glycoproteins (Supplemental Table S1) could tolerate the presence of a chemical reporter group on the *N*-acyl group of Sia donor substrate and could transfer the non-natural Sia as demonstrated previously for ST3Gal I and ST6Gal I.[21b,22] Since *N*-terminal truncation of STs impacts enzyme production and activity,[16c,24] the commercial ST3Gal I (aa 57–340) and ST6Gal I (aa 44–406) differing in their *N*-terminus from the enzymes produced previously, were included in the study. We then, chemo-enzymatically synthesized CMP-SiaNAl, the alkyne-containing analogue of the sialic acid donor substrate CMP-Neu5Ac (Figure 1A) using CTP, SiaNAl and *N. meningitidis* CMP-Neu5Ac synthase (Supplemental data) as previously described.[25] Fetuin was chosen as the acceptor

substrate as it bears well characterized three mucin-type *O*-glycans and three bi- and triantennary *N*-glycans[26] recognized as a substrate acceptor for the human recombinant STs selected in this study. Fetuin (for ST6GalNAc I, ST6GalNAc IV, ST6GalNAc V and ST8Sia VI) or desialylated fetuin (for ST6Gal I, ST3Gal I, ST3Gal II, ST3Gal III, ST3Gal IV and ST3Gal VI and ST6GalNAc II) was coated on microplates to assay the enzymatic activity of the selected STs. ALCAM, a homologue of NCAM with *N*-glycans was used as a better glycoprotein acceptor for the human polysialyltransferases ST8Sia II and ST8Sia IV, as described previously.[20a] Sialylation reactions were set up on microplates using CMP-SiaNAl as the donor substrate for each sialyltransferase (Figure 1B). Following sialylation, a bioorthogonal reaction was performed using azido-biotin, and a copper-catalyzed azide alkyne cycloaddition (CuACC) was done (Figure C).

We found that all of these enzymes readily used CMP-SiaNAl indicating that it is a universal donor substrate for all the human STs acting on glycoproteins (Supplemental Figures S1 and S2). As illustrated for ST6Gal I in Figure 2, transfer of SiaNAl resulted in a dose-dependent increase in absorbance, which could be inhibited by the pan-sialyltransferase inhibitor CMP. These first data also suggested that in a physiological context, CMP-SiaNAl would similarly be used by these enzymes.

Optimization of Sialylation Conditions

We next sought to characterize systematically the enzymatic activity of each enzyme towards the unnatural donor. For that purpose, the essential conditions of an enzymatic assay as simple and as fast and with the lowest amount of enzyme as possible were set up for each individual enzyme. We set up the optimum conditions of sialylation for each sialyltransferase w.r.t. enzyme concentration and reaction time for which each enzyme exhibits the highest enzyme-specific activity (ESA). To determine the minimum amount of enzyme to use in each case, an assay was performed in duplicate using varying amounts of recombinant enzyme ranging from 0–1000 ng/well with a fixed concentration of substrate. In the initial portion of the resulting curve, where the enzyme concentration is significantly lower than the substrate concentration, the reaction rate is directly proportional to the enzyme concentration and exhibits a linear response (Supplemental Figure S1). Similarly, to determine the minimum sialylation time for each enzyme, a time course of the sialylation was performed in duplicate with incubation time ranging from 15 min to 8 hours. A dependence of the sialylation rate for each enzyme upon the sialylation time could be observed here as well, absorbance values reaching a plateau after 1, 2 or 4 h according to the enzyme (Supplemental Figure S2). Figure 3 illustrates data obtained for fast and slow acting enzymes ST3Gal II (aa 52–350) and ST6GalNAc V (aa 30–336), requiring 1 or 4 h incubation and 8 ng or 100 ng of enzyme to reach a maximal enzyme reaction rate, respectively. Optimum sialylation conditions could be determined for each sialyltransferase and are reported in Table 1 allowing determination for the first time of their ESA. These data gathered in Table 1 provide a useful guideline as to the sialylation time and amount of enzyme required for optimal sialylation reactions.

Enzyme Kinetic Parameters

Since the relative contribution of each sialyltransferase to sialylated glycoconjugates biosynthesis *in vivo* rely on their kinetic parameters, we next sought to compare enzyme activity

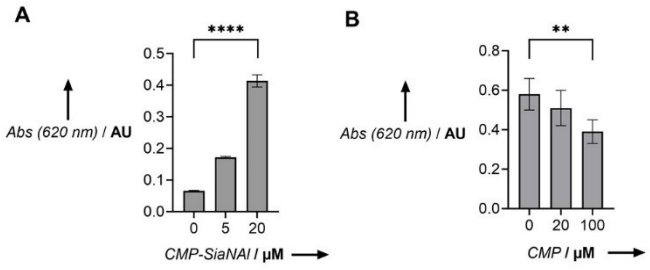

Figure 2. Detection of SiaNAl transfer using ST6Gal I (aa 44–406). A) Sialylation reaction was performed with 400 ng asialofetuin at 37 °C during 1 h, with 4 ng ST6Gal I and increasing concentration of CMP-SiaNAl ranging from 0 to 20 µM. B) Competition of SiaNAl transfer using CMP, a pan-sialyltransferases inhibitor. Sialylation reaction was performed with 400 ng asialofetuin at 37 °C during 1 h, with 4 ng ST6Gal I and 20 µM CMP-SiaNAl with increasing concentration of CMP ranging from 0 to 100 µM. Error bars represent SEM, (n = 2). **** $p \leq 0.0001$ and ** $p \leq 0.01$.

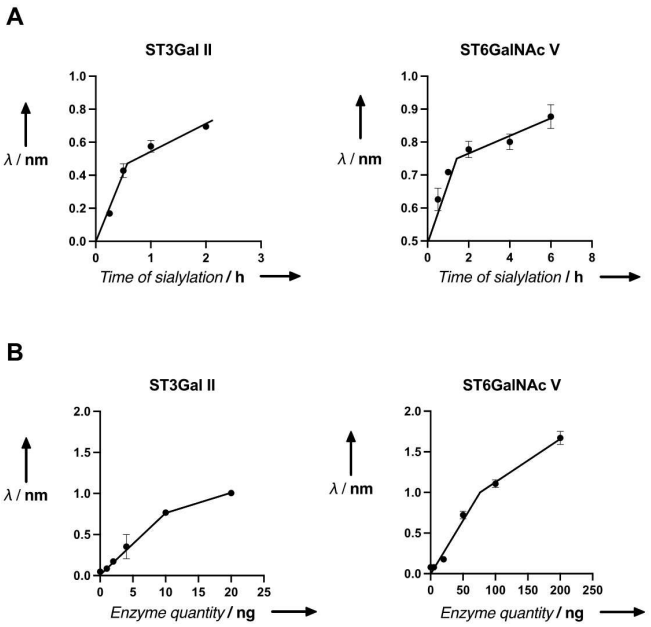

Figure 3. Determination of the optimum conditions of sialylation for ST3Gal II and ST6GalNAc V. A) Time course experiment. For ST3Gal II, the sialylation reaction was performed at 37 °C for incubation times ranging from 15 min to 2 h, with 8 ng of ST3Gal II, 100 µM of CMP-SiaNAl and 400 ng of asialofetuin (left side). For ST6GalNAc V, the sialylation reaction was performed at 37 °C for incubation times ranging from 30 min to 6 h, with 100 ng of ST6GalNAc V, 100 µM of CMP-SiaNAl and 400 ng of fetuin. B) Dependence of absorbance signal and enzyme quantity. For ST3Gal II, the sialylation reaction was performed at 37 °C for 1 h with enzyme amounts ranging from 0 to 20 ng, 20 µM CMP-SiaNAl and 400 ng of asialofetuin. For ST6GalNAc V, the sialylation reaction was performed at 37 °C for 1 h with enzyme amounts ranging from 0 to 200 ng, 100 µM CMP-SiaNAl and 400 ng of fetuin. Error bars represent SEM, (n = 2).

Table 1. Summary of the optimum sialylation conditions for each recombinant sialyltransferase. The catalytic domain (deletion) of each recombinant enzyme used is indicated. The optimum conditions of sialylation for each sialyltransferase refer to best quantity of enzyme, time of sialylation and donor substrate (CMP-SiaNAl) concentration to achieve the highest ESA.

FAMILY	ENZYME	DELETION (aa position)	QUANTITY (ng)	TIME (h)	CMP-SiaNAl CONCENTRATION (μM)	ESA[a] (U[b]/mg)
ST3GAL	ST3Gal I	57–340	4	1	20	54,590
	ST3Gal II	52–350	8	1	20	16,471
	ST3Gal III	29–375	40	1	10	3,717
	ST3Gal IV	41–333	100	4	100	350
	ST3Gal VI	40–331	100	1	100	1,234
ST6GAL	ST6Gal I	44–406	4	1	40	91,699
ST6GALNAC	ST6GalNAc I	36–600	200	2	50	600
	ST6GalNAc II	28–373	50	1	40	4,263
	ST6GalNAc IV	36–302	20	1	50	8,516
	ST6GalNAc V	30–336	100	4	100	637
ST8SIA	ST8Sia II	24–375	100	2	40	1,146
	ST8Sia IV	40–359	100	2	20	909
	ST8Sia VI	28–398	500	4	20	41

[a] ESA: Enzyme Specific Activity. [b] Velocity : U = μM min^{-1} mL.

and determine K_M (apparent) values of each enzyme towards CMP-SiaNAl. To that purpose, we set up titration experiments with increasing concentrations of the CMP-Sia ranging from 0 to 200 μM. The sialylation assays were performed in duplicate on microplate for each sialyltransferase as per the procedure and incubation conditions in terms of enzyme quantities and sialylation time optimized above and reported in Table 1. TMB concentration measurement was achieved to assess the rate of sialic acid transfer on the acceptor substrate and used as initial rate for the Michaelis-Menten plot and apparent kinetic parameters calculation, as described in the Experimental section. The apparent affinity (K_M) and maximal velocity (Vmax) were calculated for each sialyltransferase using non-linear regression analysis with GraphPad Prism (Supplemental Figure S3). Our data, point to the diverse affinity of each enzyme towards CMP-SiaNAl since the steady-state kinetic analyses of these enzymes yielded apparent K_M values ranging from 0.5 ± 0.2 to 117 ± 71.6 μM for ST8Sia IV (aa 40–359) and ST3Gal IV (aa 41–333) respectively and maximal velocity (Vmax) ranging from 50 ± 2 to 60,450 ± 6,774 U/mg for ST8Sia VI and ST6Gal I, respectively. A few ng of enzyme and short sialylation time were needed for ST3Gal I, ST3Gal II and ST6Gal I which also showed low apparent K_M towards CMP-SiaNAl (K_M = 1.2 ± 0.3, 4.7 ± 0.1 and 6.3 ± 0.3 μM, respectively), which reflects high affinity towards this donor substrate. Conversely, larger amounts of enzymes up to 500 ng and longer sialylation times were required for ST8Sia VI and ST3Gal IV activities, which also showed higher apparent K_M values. Figure 4 illustrates data obtained for a slow acting enzyme ST3Gal IV, which shows low affinity for CMP-SiaNAl (K_M = 117 ± 71.6 μM) and also low enzyme specific activity (Vmax = 778 ± 219 U/mg), and a fast-acting enzyme ST6GalNAc IV (aa 36–302) shows greater affinity for CMP-SiaNAl (K_M = 20.2 ± 3.3 μM) and also higher enzyme specific activity (11,546 ± 1,909 U/mg).

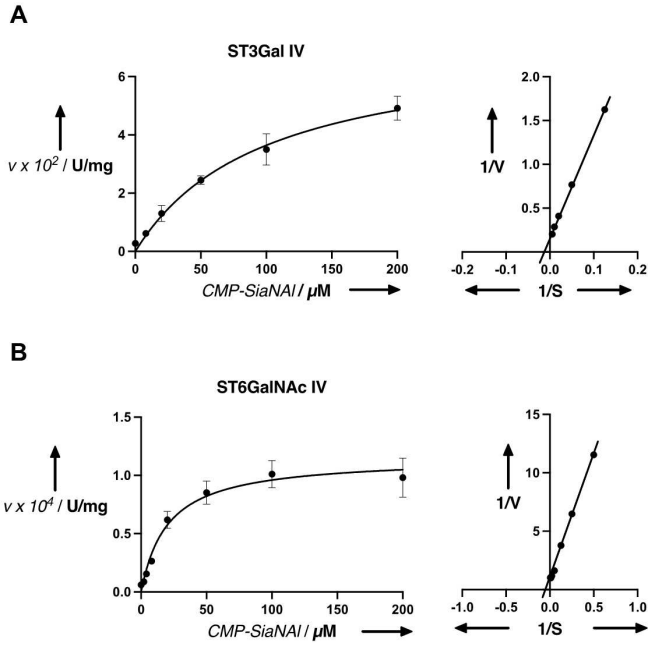

Figure 4. Determination of kinetic properties of recombinant sialyltransferase ST3Gal IV and ST6GalNAc IV towards CMP-SiaNAl according to the Michaelis Menten equation. A) For human ST3Gal IV, the sialylation reaction was performed at 37 °C for 4 h with 100 ng of enzyme at CMP-SiaNAl concentrations ranging from 0 to 200 μM (0, 8, 20, 50, 100 and 200 μM) and 400 ng of asialofetuin. The apparent K_M and Vmax values were determined using non-linear regression analysis (insert) with GraphPad Prism 9. B) For human ST6GalNAc IV, the sialylation reaction was performed at 37 °C for 1 h, with 20 ng of enzyme, CMP-SiaNAl concentrations varying from 0 to 200 μM (0, 4, 8, 20, 50, 100 and 200 μM) and with 400 ng of fetuin. Apparent K_M and Vmax values were determined using non-linear regression analysis (insert) with GraphPad Prism 9. Error bars represent SEM, (n = 2).

The turnover number (Kcat) describing the number of SiaNAl molecules transferred per unit time (min^{-1}) was calcu-

Table 2. Determination of kinetic properties of each enzyme towards CMP-SiaNAl. Apparent K_M (K_M(app)) and Vmax (Vmax(app)) were determined.

Enzyme	K_M (app) (μM)	Vmax (app) (U[a]/mg)	Kcat (min^{-1})	Kcat/K_M ($\mu M^{-1} min^{-1}$)
ST3Gal I	1.2 ± 0.3	60,450 ± 6,774	131,803	109,835
ST3Gal II	4.7 ± 0.1	21,135 ± 3,210	20,519	4,366
ST3Gal III	9.3 ± 1.6	6,454 ± 3,224	9,340	1,038
ST3Gal IV	117 ± 71.6	778 ± 219	1,933	20
ST3Gal VI	3.4 ± 0.4	1,266 ± 44	1,439	479
ST6Gal I	6.3 ± 0.3	94,518 ± 778	443,746	73,958
ST6GalNAc I	1.7 ± 0.5	624 ± 19	1,189	699
ST6GalNAc II	8.7 ± 2.5	5,340 ± 448	12,624	1,403
ST6GalNAc IV	20.2 ± 3.3	11,546 ± 1,909	57,443	2,872
ST6GalNAc V	5.9 ± 0.3	693 ± 59	1,520	253
ST8Sia II	5	1,315 ± 303	1,644	329
ST8Sia IV	0.5 ± 0.2	466 ± 28	994	2,485
ST8Sia VI	5.9 ± 0.6	50 ± 2	110	18

[a] Velocity: U in $\mu M\,min^{-1}\,mL$.

lated for each enzyme (Table 2); ST6Gal I was found to be the most rapid and ST8Sia VI the slowest to transfer SiaNAl. To measure the catalytic efficiency of each enzyme, the Kcat/K_M ratio was determined (Table 2). The most efficient enzyme to catalyze the transfer of SiaNAl was found to be ST3Gal I (109,835 $\mu M^{-1} min^{-1}$) and the less efficient enzymes were ST3Gal IV (20 $\mu M^{-1} min^{-1}$) and ST8Sia VI (18 $\mu M^{-1} min^{-1}$). Altogether, these data are in line with previous reports in the literature on the activity and kinetic parameters of vertebrate STs with various CMP-Sia donors.[9,16b,c]

Interestingly, the human ST8Sia IV, despite requiring a high amount of enzyme and long incubation time, showed high affinity for CMP-SiaNAl (K_M of 0.5 μM) and appeared to accommodate the non-naturally modified Sia donor even better than CMP-Neu5Ac. In a recently published study, molecular dynamics simulation was carried out with the human ST8Sia IV and various CMP-Sia donor substrates.[20b] In particular, the Root Mean Square Deviation (RMSD) of the enzyme and the two donors CMP-Neu5Ac and CMP-SiaNAl were calculated to assess the substrate stability within the ST8Sia IV substrate binding site.[20] These results shown in Figure 5 indicate that CMP-SiaNAl exhibits greater stability compared to CMP-NeuAc, with mean RMSD values of 0.22 and 0.29, respectively. The comparison between the RMSD of the protein C-alpha atoms and the ligands is critical. The close alignment of the RMSD curves for the protein C-alpha atoms and CMP-SiaNAl indicates that the ligand moves in concert with the protein, suggesting a stable binding interaction (Figure 5). Conversely, the divergence between the RMSD curves of the protein C-alpha atoms and CMP-Neu5Ac suggests that this ligand moves away from the protein, indicating a less stable interaction. Altogether, these results shows that ST8Sia IV accommodates CMP-SiaNAl more effectively than CMP-Neu5Ac, which may be attributed to specific interactions between CMP-SiaNAl and the enzyme's active site residues, resulting in a more favorable binding conformation.

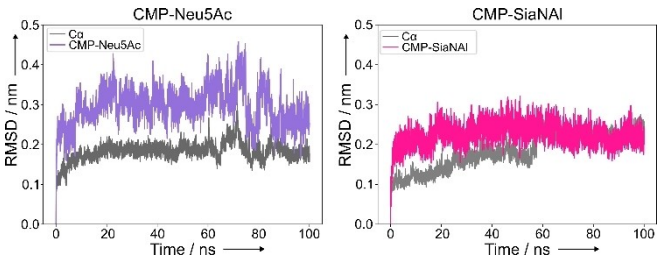

Figure 5. RMSD of the human ST8Sia IV in complex with CMP-NeuAc and CMP-SiaNAl. Protein C-alpa RMSD is show in gray and donor substrate RMSD in color.

Conclusions

This study presents a robust microplate assay for characterizing the enzymatic activities of human sialyltransferases using a novel CMP-Sia donor. The universal compatibility of CMP-SiaNAl with various STs and comparison of their kinetic properties offer significant insights into the biochemical properties of these enzymes. This assay provides a valuable tool for further studies on sialyltransferase activities and the development of targeted inhibitors.

Experimental Section

Material

Fetuin, TMB (3,3′,5,5′-tetramethylbenzidin) and azido-PEG3-biotin were purchased from Sigma. Horseradish peroxidase (HRP) conjugated anti-biotin was purchased from Jackson Immunoresearch. CTP (cytidine 5′-triphosphate disodium salt) was from TCI chemicals.

BTTAA (acid2-(4-((bis 1-(ter-butyl)-1H-1,2,3-triazol-4-yl) methyl)amino)methyl) −1H-1,2,3-triazol-1-yl) acetic) was from Jena Bioscience. Recombinant human sialyltransferases (Supplemental Table S1) ST3Gal I (6905-GT-020), ST3Gal II (7275-GT-020), ST3Gal III (10554-GT-020), ST3Gal IV (10496-GT-020), ST3Gal VI (10591-GT-020), ST6Gal I (7620-GT-010), ST6GalNAc I (9154-GT-020), ST6GalNAc II (6468-GT-020), ST6GalNAc IV (6876-GT-010), ST6GalNAc V (6715-GT-020), ST8Sia II (6590-GT-020), ST8Sia IV (7027-GT-020), ST8Sia VI (9687-GT-020) were from Bio-Techne (R&D Systems, Europe). Sodium cacodylate was from Prolabo.

Synthesis of Donor and Acceptors Substrates

Synthesis of Donor substrate CMP-SiaNAl. SiaNAl (N-4-pentynoyl-neuraminic acid) and CMP-SiaNAl synthesis is detailed in Supplemental data 1. In brief, *N*-mannosamine was converted to *N*-4-pentynoylmannosamine by coupling with succinimidyl 4-pentynoate, which was then reacted with sodium pyruvate in the presence of sialic acid aldolase from *Escherichia coli* K12 in pH 7.5 phosphate buffer and purified by anion exchange chromatography (Dowex 1×8) and gel filtration chromatography (P2 resin) to yield pure SiaNAl (86% overall yield over 2 steps). For the CMP-SiaNAl synthesis, SiaNAl (1 equiv) and CTP (10,6 mg, 0,02 mmol, 1 equiv) were dissolved into Tris-HCl buffer (100 mM, PH 8.5) containing 20 mM $MgCl_2$. Then, 0,3 U CSS (CMP-Sialic acid synthetase) from *Neisseria meningitidis* group B (Sigma Aldrich, EC 2.7.7.43) and 0.5 U PPase (inorganic pyrophosphatase) from baker's yeast (*Saccharomyces cerevisiae*), (Sigma Aldrich, EC 3.6.1.1) were added to the mixture at 37 °C. The reactions were carried out in a 5 mm NMR tube and monitored by ^{31}P NMR spectroscopy in a Brüker Avance II 400 MHz spectrometer. Upon completion, pure CMP-SiaNAl was immediately stored at −80 °C until use.[20a,21a,22]

ALCAM production in HEK293 cells. As described previously,[20a] the p3×FLAG-ALCAM plasmid was transiently transfected in CHO-K1 cells and the recombinant protein was secreted in the UltraMEM medium, which was collected 48 h after transfection and centrifuged 30 min at 10,000 rpm and at 4 °C to remove cell debris. The amount of protein was assessed with the micro-BCA protein assay kit (23,235, Thermo Scientific).

Desialylation of glycoproteins. Bovine fetuin was desialylated with 0.1 M trifluoroacetic acid for 2 h at 80 °C. Released sialic acid residues were removed using MWCO 3500 Spectra/Por3 dialysis membrane (18 mm wide, Spectrum Laboratories) for 24 h, respectively.[21b] The contents were then transferred to a glass tube and lyophilized.

Optimization of Sialylation Assays

Enzymatic assays were performed using the MicroPlate Sialyltransferase Assay as described previously.[22] In brief, 400 ng of glycoprotein acceptors (Bovine fetuin, asialofetuin or ALCAM) in 100 μL of sodium bicarbonate buffer (20 mM, pH 9.6) were adsorbed on the bottom of 96-well plates (F8 MaxiSorp Loose Nunc-Immuno Module ThermoScientific) overnight, at room temperature (RT). After two washes with 125 μL of PBS-T 0.05% (Phosphate buffered saline containing 0.05% Tween 20) and a final rinse with 125 μL of PBS, saturation was carried out for 1 h at RT with 100 μL of 0.05% oxidized BSA in bicarbonate buffer. To set up optimal conditions of sialylation for each sialyltransferase, the sialylation reaction was carried out for 15 min to 8 hours at 37 °C with the chosen recombinant human enzyme (from 4 to 2000 ng/reaction) and various concentrations of CMP-SiaNAl (from 10–100 μM) in 100 mM cacodylate buffer (Sodium cacodylate 250 mM, $MnCl_2$ 25 mM, Triton CF-54 0.5%, pH 6.4) in a final volume (Vf) of 100 μL.

Bioorthogonal Reaction

After sialylation, the wells were washed three times with PBS-T 0.05% and once with PBS 1X. Then click chemistry was carried out using 100 μL of a solution containing 300 μM $CuSO_4$, 600 μM BTTAA, 2.5 mM sodium ascorbate and 250 μM azido-PEG3-biotin in PBS for 1 h at 37 °C.[21b] The reaction was then stopped by washing twice with 150 μl PBS-T 0.05% and once with 150 μl PBS.

Biotin-HRP Detection

Incubation with 100 μl of anti-biotin antibody HRP-conjugated diluted to 1/25000 in PBST-0.05% was carried out for 1 h at 37 °C. After the three washes, 100 μL of TMB was added and incubated for 20 min at RT in the dark. The absorbance was measured at 620 nm with a spectrophotometer (SpectroStar Nano, BMG Labtech). Calibration plots showing that concentration of oxidized TMB is linearly related to quantity of sialylated glycans formed were established (Supplemental Figure S4).

Determination of Enzyme Specific Activity and Kinetic Parameters Towards CMP-SiaNAl

The sialyltransferase activities were analyzed by Michaelis-Menten kinetics with GraphPad Prism 9 and their kinetic properties *i.e.* apparent K_M and Vmax were calculated using a nonlinear regression analysis. The concentration of the product TMB formed was determined using Beer-Lambert's law ($A = \varepsilon \times l \times C$), which gives the relationship between absorbance (A) and concentration of SiaNAl transferred to the acceptor substrate in 1 min (C), l is the optical path or length of the liquid crossed, and ε is the molar extinction coefficient of TMB ($3.2 \times 104\ M^{-1}\ cm^{-1}$ at 620 nm).[28] This concentration measurement is used to assess the transfer rate (vi) of sialic acids on the acceptor substrate and this value used as the initial rate for the Michaelis-Menten plot. vi is determined as follows: $vi = C \times Vf$, where Vf is the final volume of sialylation reaction. The enzyme specific activity (ESA) corresponds to the transfer rate per enzyme quantity (m in mg): $ESA = vi/m$ and the catalytic constant kcat is determined using the following formula $kcat = Vmax/[E]$, [E] being the enzyme concentration used.

Computational Methods: Structural Model and Molecular Dynamic Simulations

In this study, we utilized a structural model of the human enzyme ST8Sia IV (UniProt ID: Q92187) in complex with the donor substrate CMP-Neu5Ac, as well as molecular dynamics (MD) simulations of the complex from a previously published paper.[20b] To conduct a comparative study, we modeled the complex of the same enzyme with the donor substrate CMP-SiaNAl, following the same methodology as in the previous paper regarding the positioning of the donor substrate and the MD simulation. Energy minimizations and MD simulations were executed using the GROMACS-2020.5 program[28] with the CHARMM36 force field.[29] The donor substrates parametrization was accomplished using SwissParam server from the Swiss Institute of Bioinformatics.[30] Briefly, the starting structure was placed in a cubic box with boundaries extending at least 1.2 nm, solvated with TIP4P water molecules, and neutralized with counterions. The system was minimized using the steepest descent algorithm, followed by equilibration under NVT and NPT conditions. Production MD simulations were run for 100 ns under NPT conditions at 300 K. MD analyses were performed using tools from the GROMACS package[28] and the Python library MD Analysis.[31]

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Bioorthognal chemistry · Enzyme assay · Glycobiology · Sialyltransferase · Substrate specificity

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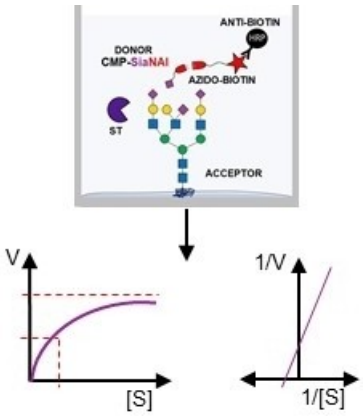
RESEARCH ARTICLE

We developed a sensitive microplate assay to explore the enzymatic activities of human sialyltransferases using an alkyne-modified CMP-sialic acid donor. This method allows for comprehensive kinetic analysis, revealing the substrate tolerance and enzyme specific activities of 13 human sialyltransferases.



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