A Small-Molecule Modulator of Poly-α2,8-Sialic Acid Expression on Cultured Neurons and Tumor Cells

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Poly-α2,8-sialic acid (PSA) has been implicated in numerous normal and pathological processes, including development, neuronal plasticity, and tumor metastasis. We report that cell surface PSA expression can be reversibly inhibited by a small molecule, *N*-butanoylmannosamine (ManBut). Inhibition occurs through a metabolic mechanism in which ManBut is converted to unnatural sialic acid derivatives that effectively act as chain terminators during cellular PSA biosynthesis. *N*-Propanoylmannosamine (ManProp), which differs from ManBut by a single methylene group, did not inhibit PSA biosynthesis. Modulation of PSA expression by chemical means has a role complementary to genetic and biochemical approaches in the study of complex PSA-mediated events.

The biological functions of cell surface oligosaccharides have been difficult to elucidate owing to the complexity of achieving genetic control over a molecule that is the product of multiple enzymes and thus of multiple genes. In a few well-studied cases, the function of a specific oligosaccharide epitope has been determined, enhancing our understanding of cell-cell recognition (*1*). Still, few such structures have been assigned a specific purpose. Small molecules that disrupt or activate a target process in a cellular context have provided insights in systems that are difficult to manipulate with traditional genetic methods (*2*). The ability to block the expression of a specific oligosaccharide epitope by use of a small molecule would facilitate the study of oligosaccharide function.

PSA (Fig. 1A), a linear homopolymer of α2,8-linked sialic acid residues, is found mainly on the neural cell adhesion molecule (NCAM) (*3*, *4*). Its biosynthesis is mediated by polysialyltransferases, the best-characterized human homologs of which are ST8SiaII (STX) and ST8SiaIV (PST) (*5*–*7*). Both enzymes catalyze the iterative formation of α2,8-sialic acid linkages using cytidine 5′-monophosphate (CMP)–sialic acid as a substrate. PSA is abundant in the central nervous system during fetal development but is restricted to those regions of the adult brain associated with synaptic plasticity (*8*–*10*). In addition, PSA is a marker of several tumors including neuroblastomas, small cell lung carcinomas, and Wilms tumor (*11*, *12*). It has been implicated in tumor metastasis and the complex neural processes involved in learning and memory (*3*, *13*). We report here a small-molecule modulator of PSA expression.

The cellular machinery for conversion of *N*-acetylmannosamine (ManNAc, Fig. 1B) to CMP–sialic acid tolerates conservatively altered *N*-acyl substituents (*14*). Thus, administration of *N*-propanoylmannosamine (ManProp, Fig. 1B) or *N*-butanoylmannosamine (ManBut) to cultured cells and laboratory animals results in biosynthesis of the corresponding CMP–sialic acid analogs and the appearance of unnatural sialic acid residues on cell surface glycoproteins. In most sialoglycoconjugates, sialic acid residues occupy terminal α2,3- or α2,6-linkages to galactose; replacement of some fraction of these residues with an unnatural variant has no discernible effect on their abundance (*15*–*17*). By contrast, in PSA sialic acid occupies both terminal and internal positions, prompting us to consider the effects of incorporation of unnatural sialic acids on its biosynthesis.

We treated NT2 neurons (*18*) with ManProp or ManBut and visualized PSA expression by immunofluorescence microscopy with the monoclonal antibody (mAb) to PSA 12F8 (*19*, *20*). At concentrations up to 10 mM in the culture medium, ManProp had no effect on PSA expression (Fig. 1C). However, ManBut abrogated 12F8 staining in a dose-dependent manner, indicating that it functions as a metabolic inhibitor of PSA expression (*21*).

We also analyzed the effects of ManProp and ManBut on the cellular biosynthesis of PSA associated with NCAM. After incubation with various concentrations of ManProp or ManBut, NT2 cells were lysed and subjected to protein immunoblot analysis with the mAb 735 to PSA and the mAb OB11 to NCAM (*19*). Polysialy-

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100 μM Na_3VO_4, 1 μM microcystin LR, 1 mM phenylmethylsulfonylfluoride (PMSF), and 1 mM benzamidine) by trituration through a 25-gauge needle 25 times. The nuclei were removed by centrifugation, and the P100 and S100 fractions were obtained by centrifugation at 100 000*g* for 30 min at 4°C. P100 fractions were resuspended in HES buffer containing 0.1% NP-40, incubated at 4°C for 60 min, and centrifuged at 10000*g* for 5 min to remove insoluble material. Immunoprecipitations were carried out at 4°C for 2 hours using 5 μg of antibody to Flag (Sigma) for transfected COS-1 cells, or overnight using 5 μg of antibody against PKB, or preimmune or antibodies against CTMP for endogenous complexes in untransfected cells. Immune complexes were precipitated using a 1:1 mixture of protein A–protein G–Sepharose (Amersham Pharmacia). The beads were washed, resuspended in 2× Laemmli buffer, and analyzed by SDS-PAGE and immunoblotting.

32. The PKBα mAb A4D6 was generated by M. Thelen, P. Cron, A. Wetterwald, and B. A. Hemmings (unpublished results).
33. HA-PKBα and HA–m/p-PKBα expression vectors were described previously (*17*). pGST-PKB-RD was generated by cloning an Nde I–Eco RI PCR fragment of PKBα into the corresponding sites of the pBC vector (*26*), referred to as pGST in Fig. 2.
34. Cells were starved for 15 hours in DMEM without serum or phosphate, and then incubated for 4 hours in this medium containing 1 mCi of [^{32}P]orthophosphate. Cells were then lysed, and Flag-CTMP immunoprecipitated as described (*14*).
35. Cells were maintained in DMEM supplemented with 10 % fetal calf serum (FBS, Life Technologies) and 50 U/ml Pen/Strep (Gibco) for COS-1, HeLa, HEK293, CCL64, AKT8 cells, or 10 % calf serum for NIH 3T3 cells. Transfections were performed by using the calcium phosphate technique (*27*). Transfected COS-1 cells or HEK293 cells were scraped in NP-40 lysis buffer (*28*), and lysates were cleared by centrifugation at 10 000*g* for 10 min. HA-PKBα was precipitated with the HA mAb 12CA5 absorbed to protein A–Sepharose (Amersham Pharmacia). Immune complexes were washed once with lysis buffer containing 500 mM NaCl, once with lysis buffer, and once with 50 mM Tris-HCl (pH 7.8), 1 mM PMSF, 1 mM benzamidine. In vitro kinase assays were as described (*28*). When required, cells were stimulated with 0.1 mM pervanadate prepared with 0.2 mM H_2O_2 (*17*).
36. RNA was prepared using the Trizol protocol (Gibco) and reverse transcription reactions were performed using the GeneAmp RNA PCR kit (PE Biosystems). The oligonucleotides T7′ and 5′-CTCATCAACACTCTGAACATT-3′ were used in RT-PCR reactions to distinguish the antisense construct from the endogenous CTMP cDNA.
37. Four 10-cm dishes of *AKT8*-transformed CCL64 cells were transfected with 15 μg pSG5 (negative control), or pSG5-FlagNt-puro (empty vector), pF-CTMP or pF-CTMP$_L$ constructs. After transfection, cells were trypsinized and reseeded in three 10-cm dishes in medium containing 2 μg/ml puromycin. Colonies were picked after 7 days and grown to confluency in puromycin-containing medium.
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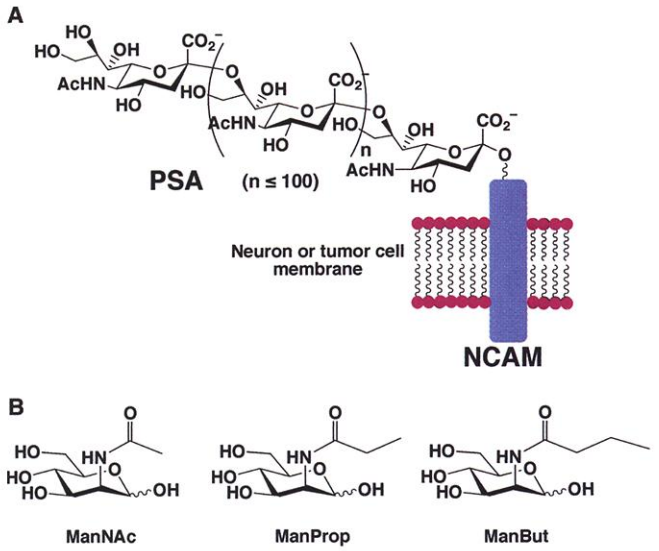

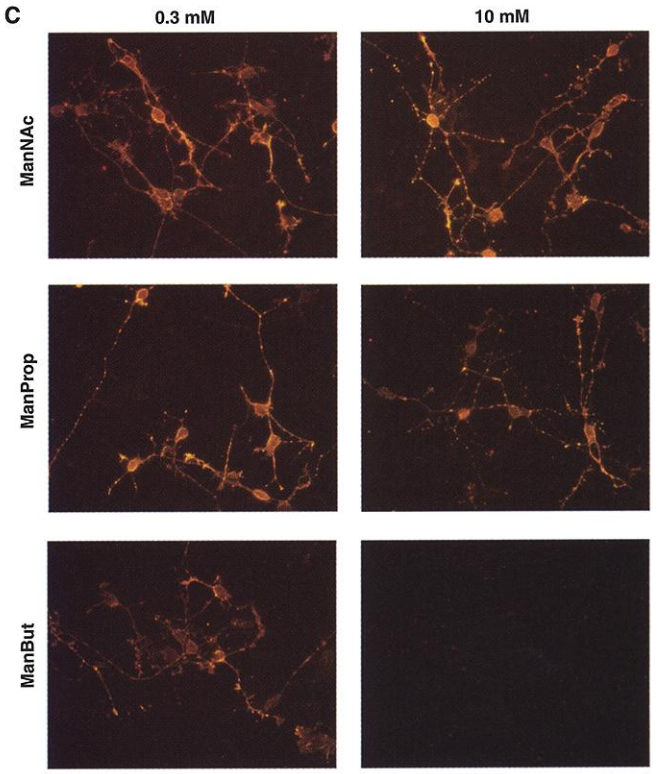

Fig. 1. Effect of *N*-acylmannosamine derivatives on PSA. (**A**) Polysialic acid (PSA) is a posttranslational modification of the neural cell adhesion molecule (NCAM) on neurons and certain tumors. (**B**) Structures of the natural derivative *N*-acetylmannosamine (ManNAc) and unnatural derivatives *N*-propanoyl- and *N*-butanoylmannosamine (ManProp and ManBut, respectively). (**C**) Differentiated NT2 neurons were seeded onto mouse astrocytes on chamber slides and treated for 5 days with either 0.3 or 10 mM ManNAc, ManProp, or ManBut. The cultures were fixed, permeabilized with methanol, and stained with the mAb to PSA 12F8.

lated NCAM normally migrates on SDS–polyacrylamide gel electrophoresis as a diffuse band with an apparent molecular size greater than 200 kD; without PSA chains, NCAM appears as up to three isoforms with apparent molecular sizes of 120, 140, and 180 kD (*22*). ManProp had no effect on the apparent molecular size of polysialylated NCAM (PSA-NCAM) (Fig. 2A), suggesting that the polymer's length, and therefore biosynthesis, is unaffected. NT2 neurons cultured with 10 mM ManProp lost PSA immunoreactivity, but OB11 staining confirmed the presence of the polymer at its normal size. The loss of PSA immunoreactivity is likely due to incorporation of *N*-propanoyl sialic acid into the polymer and concomitant disruption of antibody recognition (*23*).

Unlike ManProp, ManBut did inhibit PSA biosynthesis on NCAM in NT2 neurons. PSA mAb 735 staining was reduced in the presence of 1 mM ManBut (Fig. 2B), and NCAM mAb OB11 staining confirmed a corresponding reduction in the molecular size of the glycoprotein. Inhibition was essentially complete in the presence of 3 mM ManBut, as demonstrated by the appearance of the 140- and 180-kD isoforms of unmodified NCAM.

To determine the effects of ManBut treatment on other cell types, we incubated SH-SY5Y cells (human neuroblastoma) (*12*), H345 cells (human small cell lung carcinoma) (*24*), and HeLa cells (human cervical carcinoma) stably transfected with the 140-kD isoform of NCAM and the human polysialyltransferase STX (HeLa-NCAM-STX) (*25*) with ManProp or ManBut. In all cases, ManProp had no effect on PSA biosynthesis, whereas ManBut inhibited the process (*19*). We confirmed that ManBut is not a general inhibitor of sialylation by analyzing its effects on total cellular sialosides using the periodate-resorcinol assay (*19*, *26*).

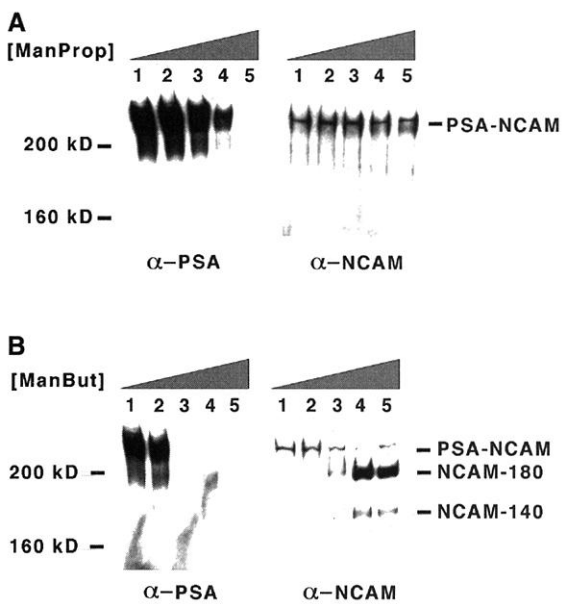

Fig. 2. Western analysis of NCAM from NT2 neurons treated with various concentrations of ManProp or ManBut. NT2 neurons were seeded onto Matrigel and treated with ManProp (**A**) or ManBut (**B**) for 5 days. Western blots of whole-cell lysates were stained with mAb OB11 (α-NCAM) or mAb 735 (α-PSA) followed by the appropriate horseradish peroxidase (HRP)–conjugated secondary antibody, and visualized by chemiluminescence. The positions of the 140- and 180-kD isoforms of NCAM are indicated. (A) ManProp: Lane 1, 0.1 mM; lane 2, 0.3 mM; lane 3, 1 mM; lane 4, 3 mM; lane 5, 10 mM. (B) ManBut: Lane 1, 0.1 mM; lane 2, 0.3 mM; lane 3, 1 mM; lane 4, 3 mM; lane 5, 10 mM. Note that polysialylated NCAM stains less intensely with OB11 than NCAM glycoforms lacking PSA.

Inhibition of PSA biosynthesis by ManBut was reversible. PSA expression was completely inhibited on HeLa-NCAM-STX cells treated with 5 mM ManBut for 24 hours, but returned 24 hours after ManBut was removed from the medium (Fig. 3, A and B). Thus, ManBut disrupts polysialylation in a time-dependent and reversible manner.

Given that ManBut is readily converted to the corresponding unnatural sialoside in other linkage forms (i.e., α2,3 and α2,6), we reasoned that inhibition of PSA biosynthesis is exerted at the level of polysialyltransferase activity. The polysialyltransferases may use unnatural variants of their donor (CMP–sialic acid) and acceptor (NCAM-bound sialic acid) substrates with reduced efficiency according to the size of the *N*-acyl groups. To examine this possibility,

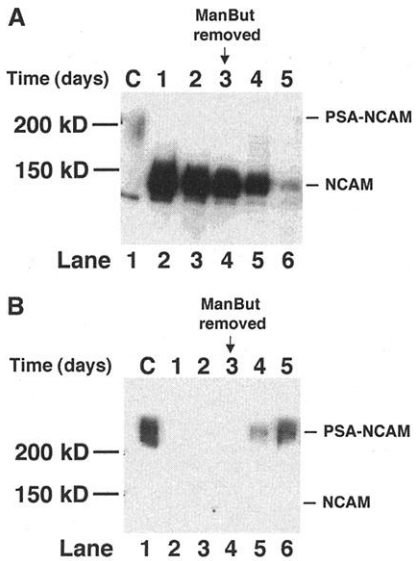

Fig. 3. Inhibition of PSA biosynthesis by ManBut is reversible. HeLa-NCAM-STX cells were incubated with 5 mM ManBut for 2 days, after which ManBut was removed from the medium. Cells were lysed at the indicated times, and NCAM was immunoprecipitated with the mAb ERIC-1. Western blots of immmunoprecipitated samples were stained with either mAb OB11 (**A**) (α-NCAM) or mAb 12F8 (**B**) (α-PSA) followed by the appropriate HRP-conjugated secondary antibody, and visualized by chemiluminescence. Lane C, PSA-NCAM immunoprecipitated from untreated control cells.

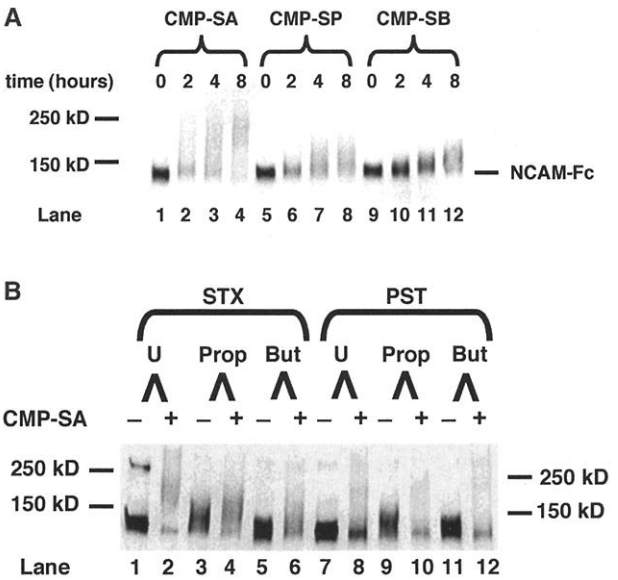

Fig. 4. In vitro PSA biosynthesis with unnatural acceptor and donor substrates. (**A**) NCAM-Fc was treated with STX and 10 nmol CMP-Sia (CMP-SA, lanes 1 to 4), CMP-SiaProp (CMP-SP, lanes 5 to 8), or CMP-SiaBut (CMP-SB, lanes 9 to 12) at 37°C for the indicated times. Samples were analyzed by Western blot, stained with antibody to human immunoglobulin G (Fc-specific)–HRP conjugate, and visualized by chemiluminescence. (**B**) NCAM-Fc was primed with unnatural sialic acids by treatment with STX and either CMP-SiaProp (lanes 3 and 4 and lanes 9 and 10, Prop) or CMP-SiaBut (lanes 5 and 6 and lanes 11 and 12, But). The primed NCAM-Fc was then isolated and reacted with either STX or PST and 10 nmol CMP-Sia (+) or buffer (–) for 4 hours at 37°C, and compared with unprimed NCAM-Fc controls (lanes 1 and 2 and lanes 7 and 8, U). Samples were analyzed by Western blot as before.

we investigated unnatural PSA biosynthesis in vitro using CMP–*N*-propanoyl sialic acid (CMP-SiaProp) or CMP–*N*-butanoyl sialic acid (CMP-SiaBut) (*27*), recombinant protein A fusions of the STX and PST catalytic domains (*28*), and the extracellular domain of NCAM expressed as a Fc fusion (NCAM-Fc) (*29*). The apparent molecular size of NCAM-Fc after enzymatic modification provided a measure of PSA biosynthesis.

CMP-SiaBut was used by the polysialyltransferase STX less efficiently than either natural CMP–sialic acid or CMP-SiaProp (Fig. 4A). Similar results were obtained with PST (*19*). We next tested whether the enzymes could biosynthesize natural PSA starting from an unnatural sialic acid primer on NCAM. To make the primer, we incubated NCAM-Fc with either CMP-SiaProp or CMP-SiaBut in the presence of STX. This primed material was isolated and incubated with native CMP–sialic acid and either STX or PST. NCAM-Fc primed with SiaBut was polysialylated less efficiently by STX than NCAM-Fc primed with SiaProp, and both primed substrates were inferior to unprimed NCAM-Fc (Fig. 4B). By contrast, PSA extension catalyzed by PST appeared to be independent of the primer structure on NCAM-Fc (*19*).

We conclude that STX and PST use CMP-SiaBut less efficiently than CMP-SiaProp, and both are polymerized more slowly than CMP–sialic acid. Furthermore, the activity of STX is diminished by unnatural *N*-acyl groups within the priming sialic acid residues. During cellular PSA biosynthesis, the transit time of NCAM through the Golgi network imposes a limit on the duration of PSA chain extension. In this context, the reduced kinetic efficiencies of CMP-SiaBut and the *N*-butanoyl sialic acid primer may effectively terminate extension of the PSA chain. By contrast, CMP-SiaProp and the corresponding primer may be used by the enzymes at a rate sufficient to polysialylate NCAM in the Golgi compartment.

Until now, PSA could be modulated only by genetic manipulations or by enzymatic digestion. A small-molecule inhibitor such as ManBut has the advantages of temporal control and reversibility. Transient disruption of PSA expression by ManBut might be used to study the roles of PSA in modulating cell adhesion, tumor metastasis, synapse formation, and organ development. The correlation of polysialylation with metastatic potential suggests potential applications of ManBut as an antimetastatic agent (*11*). Given that unnatural sialosides can be generated from the corresponding mannosamine analogs in animals (*14*), the effects of ManBut on PSA biosynthesis in vivo is an important next step.

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