Myelin-associated glycoprotein (MAG, Siglec-4) is one of several endogenous axon regeneration inhibitors that limit recovery from central nervous system injury and disease. Molecules that block such inhibitors may enhance axon regeneration and functional recovery. MAG, a member of the Siglec family of sialic acid-binding lectins, binds to sialoglycoconjugates on axons and particularly to gangliosides GD1a and GT1b, which may mediate some of the inhibitory effects of MAG. In a prior study (Blixt, O., Collins, B. E., van den Nieuwenhof, I. M., Crocker, P. R., and Paulson, J. C. (2003) J. Biol. Chem. 278, 31007–31019), we identified potent monovalent sialoside inhibitors of MAG using a novel screening platform. In the current study, the most potent of these were tested for their ability to reverse MAG-mediated inhibition of axon outgrowth from rat cerebellar granule neurons in vitro. Monovalent sialylglycans enhanced axon regeneration in proportion to their MAG binding affinities. The most potent glycoside was disialyl T antigen (NeuAc2-3Galβ1-3[NeuAc2-6]GalNAc-R), followed by 3-sialyl T antigen (NeuAc2-3Galβ1-3GalNAc-R), structures expressed on O-linked glycosylated proteins as well as on gangliosides. Prior studies indicated that blocking gangliosides reversed MAG inhibition (Vyas, A. A., Patel, H. V., Fromholt, S. E., Heffer-Lauc, M., Vyas, K. A., Dang, J., Schachner, M., and Schnaar, R. L. (2002) Proc. Natl. Acad. Sci. USA 99, 8412–8417). In the current study, blocking O-linked glycoprotein sialylation with benzyl-O-GalNAc had no effect. The ability to reverse MAG inhibition with monovalent sialosides encourages further exploration of glycans and glycan mimetics as blockers of MAG-mediated axon outgrowth inhibition.

The injured adult mammalian central nervous system (CNS) is a highly inhibitory environment for axon regeneration, in large part due to specific axon regeneration inhibitors expressed on residual myelin that persists at sites of CNS injury and on astrocytes recruited to injury sites (1–3). The inhibitors include three myelin proteins, myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp), and chondroitin sulfate proteoglycans, which are found on the astrocytic scar and on residual myelin. Axon regeneration inhibitors, on myelin and astrocytes, bind specifically to targets on the axon surface, initiating a signaling cascade that ultimately activates RhoA and halts axon outgrowth by modulating actin dynamics. Detailed knowledge of axon regeneration inhibitors, their axonal ligands, and the signaling pathways leading to changes in the actin cytoskeleton promises to provide opportunities to enhance axon regeneration after CNS injury (e.g. spinal cord injury) and in diseases involving axon loss, such as multiple sclerosis, Parkinson disease, and Alzheimer disease.

MAG has two classes of well-defined axonal targets: sialylated glycans, specifically gangliosides GD1a and GT1b (4), and the glycosphatidylinositol-linked proteins of the NgR family, NgR1 (5, 6) and NgR2 (7), which may also be axonal ligands for Nogo and OMgp (8). There are conflicting data concerning the sialidase sensitivity of MAG-NgR binding (5–7).

Although the relative roles of gangliosides and NgRs as MAG ligands have yet to be resolved, in some systems MAG inhibition is completely reversed by sialidase, suggesting that, at least in those systems, MAG uses sialylated glycans as its major axonal ligands. In those systems, or in combination with blockers of NgR (9), it is anticipated that potent glycan inhibitors of MAG may be valuable tools to enhance axon regeneration.

MAG (Siglec-4), a member of the Siglec (sialic acid-binding immunoglobulin-like lectin) family (10), binds preferentially to the terminal sequence "NeuAc2-3Galβ1-3GalNAc" (11), especially in the context of larger glycan structures bearing additional anionic groups (see Fig. 1). In the brain, this structure is most abundant as the terminus of gangliosides GD1a and GT1b, which are high-affinity MAG ligands and mediate MAG inhibition of axon outgrowth in vitro (4, 12). Glycan structures with an additional sialic acid 6-linked to the GalNAc residue, "NeuAc2-3Galβ1-3(NeuAc2-6)GalNAc," bind to MAG with an order of magnitude higher affinity than the corresponding structure without the 6-linked NeuAc (13–15). This terminus occurs on the quantitatively minor "α-series" gangliosides (16–18) and on O-linked glycoprotein glycans (19). Because they were discovered as extensions of the disaccharide "Galβ1–3GalNAc," which is called core 1 or the T antigen on glycoproteins, we denote the above structures as 3-sialyl T and disialyl T, respectively (see Fig. 1). Ganglioside nomenclature is that of Svennerholm (52).

A screen of synthetic sialoglycans for their ability to inhibit MAG-sialic acid binding in vitro (15) revealed that threonine methyl ester glycosides of disialyl T, 3-sialyl T, and 6-sialyl T (Galβ1-3(NeuAc2-6)GalNAc) were potent inhibitors of MAG-sialoside binding, with IC50 values of 0.3, 1.6, and 10 μM,
mine mixture (Invitrogen). Cells were cultured on 96-well poly-L-lysine-coated microwells at a density of 60,000 cells/well in 100 μl of medium or in 100-mm tissue culture plates at a density of 9 × 10^5 cells/plate in 9 ml of medium. As indicated, sialosides (1 μM to 1 mm), benzyl-a-GalNAc (2.5 μM), sialidase (from Vibrio cholerae; Calbiochem), or anti-MAG mAb 513 (22) was added 45 min after cells were plated.

Neurite Outgrowth Inhibition Assays—MAG-containing substrata were prepared using a detergent extract of rat brain myelin as described previously (4). Briefly, myelin membranes (1 mg of protein) were extracted in 1 ml of extraction buffer (0.2 M sodium phosphate buffer, pH 6.8, containing 0.1 M Na_2SO_4, 1 M EDTA, 1 mm dithiothreitol, protease inhibitor mixture (Sigma), and 1% octylglucoside) at 4 °C for 16 h and then centrifuged at 100,000 × g for 1 h at 4 °C. The resulting supernatant was diluted 2-fold with detergent-free extraction buffer and adsorbed on poly-L-lysine-coated tissue culture wells at a concentration of 75 μg/cm^2 (based on the original myelin protein content). After 4 h at ambient temperature, the plates were washed extensively with Dulbecco’s phosphate-buffered saline (PBS) and then washed with culture medium. Cells were plated on control and experimental surfaces and cultured for 48 h, after which the wells were washed with PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with methanol (−20 °C, 2 min) and immunostained with GAP-43 mAb (1:1000; Sigma) followed by Cy3-conjugated anti-mouse IgG (1:120; Jackson ImmunoResearch Laboratories) mixed with 300 nM 4’-diamidino-2-phenylindole (Molecular Probes). The cells were washed, and multiple random fields from replicate wells were captured for morphometric analysis (see below) using a Nikon TE300 epifluorescent microscope fitted with a Sony charge-coupled device camera.

Cells plated on control surfaces show little or no clumping and have long fine axons that form a lacy network (e.g. Fig. 2A), whereas on substrata adsorbed with myelin proteins, the cells clumped together and extended highly fasciculated axons (e.g. Fig. 2B), leaving large areas of the substratum bare. Axon outgrowth inhibition was determined using an objective semi-automated morphometric analysis method using Metamorph image analysis software (Universal Imaging Systems, Downington, PA) as described previously (4). Briefly, the pixels of each image collected were classified according to their intensity, and the number of pixels (relative image area) having the intensity of independent single axons was quantified and compared between control and experimental cultures in the same experiment. Results are expressed as percentage of inhibition. To ensure analyses were from comparable cell numbers, cell densities on control and inhibitory substrata were determined by 4’,6-diamidino-2-phenylindole staining of nuclei and found to be statistically indistinguishable (data not shown).

On average, for each experimental condition, 24 images were analyzed (four random images from each of six independent wells from three different experiments). Statistical p values were obtained using Student’s t test.

Selective Disruption of O-Linked Glycer Sialylation—Neurons were treated with benzyl-a-GalNAc to selectively disrupt O-linked glycan sialylation (23, 24). Neurons were treated with 2.5 mM benzyl-a-GalNAc diluted from a 0.4 M stock (in Me_2SO) for 48 h.

To test the efficacy of benzyl-a-GalNAc treatment, cells grown on 100-mm dishes (with and without inhibitor) were washed with PBS, suspended in PBS using a rubber policeman, and centrifuged (500 × g). The pellet was washed with PBS and lysed in boiling 2× Laemmli SDS-PAGE sample buffer (25). Lysate proteins were resolved by SDS-PAGE using precoated 10% polyacrylamide gels (G Durham, Frenchs Forest, Australia) according to manufacturer’s instructions. The proteins were transferred onto polyvinylidene difluoride membranes using a semi-dry transfer cell (Bio-Rad) and then blotted with biotinylated peanut agglutinin as described previously (26), except that membranes were blocked for 2 h at ambient temperature, incubated with peanut agglutinin (10 μg/ml) for 16 h at 4 °C and then with streptavidin-conjugated alkaline phosphatase (1:1500; Jackson ImmunoResearch Laboratories) for 1 h at ambient temperature, and finally developed using nitro blue tetrazolium-bromochloroindolyl phosphate (Sigma).

To test the specificity of benzyl-a-GalNAc treatment, gangliosides were extracted from inhibitor-treated and control cells in chloroform-methanol-water (4:8.3), resolved by silica gel TLC using chloroform-methanol-0.25% aqueous KCl (60:35:8) as developing solvent, and then stained with resorcinol reagent as described previously (27, 28).

RESULTS

Glycoside Enhancement of Axon Outgrowth on Myelin-adsorbed Surfaces Correlates with MAG Binding Affinity—Neo-

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**FIG. 1.** Sialosides used in this study and related ganglioside structures. R_1, OCH_2CH_2N_3; R_2, OThr(NAc,OCH_3); R_3, ceramide.

Glycosides of 3-sialyl T, 6-sialyl T, and disialyl T, each was further purified to remove a minor cytotoxic component by sequential adsorption to DEAE-Sepharose (Cl^−; 0.25 ml) elution with 0.5 ml each of 10, 25, 50, 100, and 250 mM ammonium acetate; lyophilization of fractions containing sialoside; and P4-gel filtration chromatography (10 ml column; 5 mM sodium phosphate, 0.15 M NaCl). β-Azidoethyl 3-sialyllactoside was prepared as described previously (21). Benzyl-a-GalNAc and GAP-43 monoclonal antibody (mAb) were purchased from Sigma. Peanut agglutinin was from Vector Laboratories (Burlingame, CA). Streptavidin-conjugated alkaline phosphatase was from Jackson ImmunoResearch Laboratories (West Grove, PA). Other reagents were of the highest purity commercially available.

**Cerebellar Granule Neurons**—Neurons were isolated from postnatal day 5–7 Sprague-Dawley rats. Cerebella were isolated and thoroughly cleared of meninges under a dissecting microscope and dissociated enzymatically using a papain dissociation kit (Worthington Biochemical, Lakewood, NJ). Dissociated cells were collected, washed, and resuspended in culture medium containing minimal essential medium (MEM; Invitrogen) supplemented with 25 mM Hepes, 25 mM KCl, 1.5 g/liter NaHCO_3, 10% heat-inactivated equine serum, 5% fetal bovine serum (Hyclone, Logan, UT), and 1:100 penicillin/streptomycin/gluta-
nata l rat cerebellar granule neurons extend axons when plated on polylysine-coated wells (29), forming a dense axon meshwork within 48 h (Fig. 2A). When the same cells are plated on a substratum adsorbed with extracted myelin proteins, they clump together, extend fasciculated axon bundles between clumps, and leave much of the substratum bare (Fig. 2B). Addition of 10 μM disialyl T glycoside (for glycoside structures, see Fig. 1) had no effect on neurons plated on control surfaces (Fig. 2C) but largely reversed the effect of adsorbed myelin proteins, restoring an extensive axon meshwork (Fig. 2D). Addition of the same concentration of 6-sialyl T glycoside had little effect, whereas addition of 3-sialyl T glycoside resulted in a significant reversal (Fig. 3). None of the glycosides had a significant effect on axon outgrowth on control surfaces, and addition of lactose at up to 100-fold higher concentrations had no effect on axon outgrowth on control or inhibitory substrata (data not shown).

Quantification of axon outgrowth on control and myelin-adsorbed substrata via image analysis revealed that monovalent sialoglycans reversed myelin-mediated inhibition in proportion to their affinity for MAG (Fig. 4, Table 1). Whereas 3-sialyl lactose did not cause significant reversal of inhibition at any concentration tested (up to 100 μM), 6-sialyl T caused statistically significant, although quantitatively modest, reversal only at the highest concentration tested (100 μM). The highest affinity MAG-binding glycans, 3-sialyl T and disialyl T (15), caused statistically significant reversal of inhibition at all concentrations tested (1–100 μM), with disialyl T having ~10-fold higher potency (Table 1).

Axon Outgrowth Inhibition Is MAG- and Sialic Acid-dependent—Multiple axon regeneration inhibitors have been established, including MAG, Nogo, OMgp, and chondroitin sulfate proteoglycans (1). In the experimental system used in this study (cerebellar granule neurons plated on detergent-extracted myelin proteins), axon outgrowth inhibition is primarily MAG-mediated (4), in that addition of an anti-MAG mAb reverses inhibition ~80% (Fig. 5). Likewise, MAG has multiple ligands, including sialoglycoconjugates and the glycosylphosphatidylinositol-anchored NgR proteins. In the experimental system used in this study (4), inhibition is dependent on sialo-glycoconjugates, in that sialidase treatment results in complete reversal of inhibition (Fig. 5). Anti-MAG antibody and sialidase had no effect on axon outgrowth on control substrata (data not shown). Although other axon regeneration inhibitors and other MAG ligands function in vivo and in other experimental systems, the system used in this study isolates MAG and sialo-glycoconjugates as the functional receptor-ligand pair inhibiting axon regeneration, consistent with the finding that the most potent MAG-binding sialoglycan, disialyl T, reverses inhibition >75% (Fig. 4).

O-Linked Glycoprotein Glycans Are Not Functional MAG Ligands on Cerebellar Granule Neurons—In the brain, the 3-sialyl T sequence, NeuAcα2–3Galβ1–3GalNAc, is found most abundantly as the terminal trisaccharide of gangliosides GD1a and GT1b. However, it is also found as a trisaccharide linked to Ser or Thr on O-linked glycans of glycoproteins (19). Likewise, the disialyl T sequence, NeuAcα2–3Galβ1–[NeuAcα2–6]GalNAc, is found as the terminus of “α-series” gangliosides (14, 17) and as a tetrasaccharide on O-linked glycans of glycoproteins (19). Previous studies indicated that gangliosides are functional ligands for MAG-mediated inhibition of axon outgrowth from cerebellar granule neurons and hippocampal neurons (4, 12). The finding that the sialoglycides bind MAG with high affinity (15) led us to test the role of O-linked sialoglycans on glycoproteins of cerebellar granule cells as functional MAG ligands. Benzyl-α-GalNAc was used to disturb O-linked sialoglycan biosynthesis (24). Axon outgrowth on control substrata was unaffected by the presence of 2.5 mM benzyl-α-GalNAc, and axon outgrowth inhibition by myelin proteins was also unaffected (Fig. 6). The efficacy of benzyl-α-GalNAc treatment was confirmed by noting a characteristic increase in peanut agglutinin binding (>4-fold) to glycoproteins resolved by SDS-PAGE and blotted to polyvinylidene difluoride mem-

![Fig. 2. Monovalent sialoside reverses MAG-mediated inhibition of axon outgrowth in rat cerebellar granule neurons in culture. Cells were plated on control substrata (A and C) and on the same substrata adsorbed with a mild detergent extract of myelin, containing MAG (B and D). After 48 h in control medium (A and B) or in the same medium containing 10 μM disialyl T glycoside (C and D), cells were fixed and immunostained with anti-GAP-43 mAb to detect axons. Fluorescent micrographs are presented as reverse gray scale images to enhance clarity. Bar, 100 μM.](image)

![Fig. 3. Sialoside 3-sialyl T reverses MAG-mediated neurite outgrowth. Rat cerebellar granule neurons were plated on surfaces adsorbed with a mild detergent extract of myelin, containing MAG. After 48 h in control medium (A) or in the same medium containing 10 μM 6-sialyl T glycoside (B) or 10 μM 3-sialyl T glycoside (C), cells were fixed and stained with anti-GAP-43 mAb to detect axons. Fluorescent micrographs are presented as reverse gray scale images to enhance clarity. Bar, 100 μM.](image)
branes (data not shown), indicative of a decrease in α2–3 sialylation of O-linked glycans of glycoproteins as reported previously (23). Treatment with benzyl-α-GalNAc had no effect on the quantity or expression pattern of cerebellar granule neuron gangliosides, as determined by TLC (data not shown). The lack of effect of benzyl-α-GalNAc, combined with prior data implicating gangliosides as functional MAG ligands (4), implies that gangliosides, rather than O-linked glycoprotein glycans, are functional MAG ligands on cerebellar granule neurons.

**DISCUSSION**

The key finding reported here is that low molecular weight monovalent glycans enhance axon regeneration in vitro by reversing MAG-mediated axon outgrowth inhibition. The data presented in Table I further support this finding by demonstrating the IC50 values for different sialoglycans. The inhibition of MAG binding by these sialoglycans is significant, with IC50 values ranging from 0.3 to 521 μM. The lack of effect of benzyl-α-GalNAc on axon outgrowth, combined with the known role of gangliosides as functional MAG ligands, suggests that gangliosides, rather than O-linked glycoprotein glycans, are the primary targets for MAG binding in this system.

**FIG. 4.** Reversal of MAG-mediated axon outgrowth inhibition by monovalent sialosides. Rat cerebellar granule neurons were cultured on control substrata and on the same substrata adsorbed with a mild detergent extract of myelin containing MAG. As indicated, monovalent sialosides were added to control and experimental cultures 45 min after plating. After 48 h in culture, the cells were fixed and stained with anti-GAP-43 mAb. Neurite outgrowth was quantified using Metamorph image analysis software (4), and data were expressed as axon outgrowth inhibition (experimental substratum compared with matched control substratum). Values are the mean from an average of 6–7 independent wells, shown as mean ± S.E. Statistical analyses were performed using Student’s t test: †, p < 0.02; *, p < 0.001.

![Graph showing axon outgrowth inhibition](image)

**TABLE I**

Comparison of MAG binding affinities and reversal of axon outgrowth inhibition by sialoglycans

| Glycan Structure | MAG binding IC50 | Axon outgrowth IC50 |
|------------------|------------------|---------------------|
| Sialyl lactoside  | NeuAcα2–3Galβ1–4Glc-R2 | 521 μM |
| 6-Sialyl T        | Galβ1–3NeuAcα2–6GalNAc-R2 | 10 μM | >100 μM |
| 3-Sialyl T        | NeuAcα2–3Galβ1–3GalNAc-R2 | 1.6 μM | 80 μM |
| Disialyl T        | NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAc-R2 | 0.3 μM | 6.2 μM |

- R1, azidoethyl glycoside; R2, N-acetyl carboxymethyl threonine glycoside.
- IC50, based on inhibition of solid phase binding as published previously (15).
- IC50, based on data in Fig. 4.
- 22% reversal at 100 μM.

**FIG. 5.** Axon outgrowth inhibition from rat cerebellar granule neurons plated on substrata adsorbed with mild detergent extract of myelin is MAG- and sialic acid-dependent. Cells were plated on control surfaces (without myelin) or the same surfaces adsorbed with detergent-extracted myelin proteins (Myelin). Some cultures, as indicated, were also treated with 10 μg/ml anti-MAG mAb or 7.5 milliunits/ml V. cholerae sialidase. After 48 h, the cultures were fixed and stained with anti-GAP-43 mAb, and axon outgrowth was quantified as described in the text. Values (in relative units) are presented as the mean and range from duplicate wells. These data are consistent with prior studies (4). Statistical analyses were performed using Student’s t test: *, p < 0.05.

![Graph showing axon outgrowth inhibition](image)

**FIG. 6.** Benzyl-α-GalNAc treatment does not reverse MAG-mediated inhibition of axon outgrowth. Rat cerebellar granule cells were plated on control and myelin-adsorbed substrata and cultured with and without benzyl-α-GalNAc. The cultures were fixed at 48 h and stained with anti-GAP-43 mAb, and axon outgrowth was quantified as described in the text. Values are the mean ± S.E. of 7 independent wells for each condition. Statistical analyses revealed no significant differences due to the presence of benzyl-α-GalNAc. Whether the inhibitor was present or absent, axon outgrowth of cells cultured on substrata adsorbed with myelin proteins was significantly inhibited compared with cells on matched control substrata (Student’s t test: *, p < 0.001).

![Graph showing axon outgrowth inhibition](image)
reversing axon outgrowth inhibition by MAG and do so in proportion to their MAG binding affinities as previously measured in a competitive assay involving MAG binding to sialoglycans (15). This finding encourages further high-throughput screening of glycans, glycan mimetics, and other small molecules that may provide yet better agents to reverse MAG-mediated axon outgrowth inhibition.

Multiple endogenous axon regeneration inhibitors on myelin and the astrycotic scar combine to limit recovery from CNS injury and disease (1–3, 30, 31). MAG, Nogo, OMgp, and chondroitin sulfate proteoglycans may each contribute to the highly inhibitory environment of the injured CNS. Enzymes, antibodies, and small molecules that block any one axon regeneration inhibitor have been reported to significantly, although often only modestly, enhance recovery from experimental axonal injury (9, 32, 33). Likewise, mice genetically engineered to lack a particular axon regeneration inhibitor (or inhibitor ligand) typically show only limited recovery from experimental CNS injuries (34–39). To overcome axon regeneration inhibitors, two general approaches appear feasible: (i) block the common signaling pathways downstream of axon regeneration inhibitors (40, 41); or (ii) block multiple axon regeneration inhibitors simultaneously. Small molecules that block each of the axon regeneration inhibitors will contribute to the latter approach. In that light, we have initiated a search for low molecular weight inhibitors of MAG.

MAG has two classes of functional axonal ligands, gangliosides (4, 12, 42–44) and the glycosylphosphatidylinositol-linked NgR proteins, NgR1 and NgR2 (5–7). Whereas initial reports concluded that MAG-NgR binding is sialic acid-independent (5, 6), a more recent study indicates that MAG binds to NgR1 and NgR2 in a sialic acid-dependent manner (7). The functional role of sialic acids (if any) in MAG-NgR-mediated axon outgrowth inhibition has yet to be determined. MAG binding to gangliosides is fully dependent on terminal α2-3-linked sialic acid residues (13).

Gangliosides and NgR proteins may both contribute to the inhibitory effects of MAG on different axons. Whereas MAG-binding sialoglycoconjugates are widely distributed on neurons and axons (45, 46), NgR1 and NgR2 are not expressed on all axons that respond to regeneration inhibitors in vitro (1, 7, 47, 48). Genetic ablation of NgR1 does not enhance regeneration in the cortical spinal tract, a major spinal cord pathway, although certain other descending pathways may be positively impacted (37, 39). Genetic ablation of NgR2 has yet to be reported. MAG ligands other than the NgRs are likely to be responsible for inhibition in many important axonal pathways (3), and gangliosides may be those other ligands (4).

Prior studies implicate sialoglycans, particularly the major brain gangliosides GD1a and GT1b, as functional ligands for MAG-mediated inhibition of axon regeneration (4, 12, 49). Sialidase treatment reverses MAG inhibition of axon outgrowth from cerebellar granule neurons (Fig. 5) (4) and dorsal root ganglion neurons (49) in vitro. More limited reversal of MAG inhibition of axon outgrowth from cerebellar granule neurons plated on MAG-expressing cells was reported using 9 mM 3-sialyl lactose (49). The role of sialoglycoconjugates was later questioned when MAG engineered to lack a primary sialic acid-binding amino acid, MAG-R118A, still displayed inhibitory activity (50). However, subsequent studies demonstrated that the residual inhibitory activity of MAG-R118A was also sialic acid-dependent (12). In that latter study, neurite outgrowth inhibition of hippocampal neurons by MAG-R118A was effectively reversed by micromolar concentrations of NeuAc and 3-sialyl lactose. To our knowledge, the current study is the first to demonstrate that monovalent high-affinity MAG-binding sialoglycans reverse axon outgrowth inhibition by immobilized native MAG and the first to correlate reversal of MAG inhibition by sialoglycans with their MAG binding affinities. The results support the conclusion that high-throughput screening of inhibitors of MAG-glycan interactions is warranted to identify molecules with potential for blocking MAG-mediated axon outgrowth inhibition.

The concentrations of 3-sialyl T and disialyl T required to reverse the inhibitory effects of MAG on axon outgrowth in vitro were 20–50-fold higher than the IC50 measured in the sialoside binding inhibition assay (Table I). This suggests that monovalent inhibitors must be present at saturating levels to disrupt the interactions of cell surface sialoglycans with immobilized MAG. This may result from a high sialoside ligand concentration on the axon cell membrane, shifting the inhibition curve to higher concentrations of the monovalent glycan inhibitors to compete. Regardless, a 10 μM concentration of disialyl T antigen was efficacious, and the toxicity of saccharides is characteristically low. Thus, testing of sialoglycans for their ability to enhance axon regeneration in nerve injury models in vivo may be feasible and provides impetus for identifying further structural refinements that may generate yet higher affinity sialoglycan inhibitors (51).

Screening of sialoglycans for high-affinity MAG ligands revealed that the aglycone affects MAG affinity. Notably, aryl glycosides of 3-sialyl T antigen were up to 150-fold less potent in binding MAG than the N-acetyl carboxymethyl threonine glycoside. Because the latter is akin to the 3-sialyl T antigen on O-linked glycoproteins, it raised the possibility that O-linked glycans, in addition to the more abundant gangliosides GD1a and GT1b, may be functional MAG ligands. However, benzyl-α-GalNAc, which inhibits O-linked glycan sialylation (23, 24), failed to affect MAG inhibition of axon outgrowth from cerebellar granule neurons. Given prior evidence that glycosphingolipid biosynthesis inhibitors and anti-ganglioside antibodies reverse the inhibition of axon outgrowth by MAG (4), we conclude that gangliosides rather than O-linked glycoproteins are the major MAG ligands on these cells. Whether other sialoglycans have the same function in other types of neurons has yet to be determined.

The results reported here establish that monovalent sialoglycans with high MAG binding affinity effectively enhance axon outgrowth in vitro by reversing MAG-mediated inhibition. In combination with other small molecules, antibodies, or enzymes that reverse other axon regeneration inhibitors, sialoglycans may eventually provide a means to improve recovery after CNS injury.

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