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High affinity sialoside ligands of myelin associated glycoprotein

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Abstract

Myelin associated glycoprotein (Siglec-4) is a myelin adhesion receptor that is well established for its role as an inhibitor of axonal outgrowth in nerve injury, mediated by binding to sialic acid containing ligands on the axonal membrane. Because disruption of myelin-ligand interactions promotes axon outgrowth, we have sought to develop potent ligand based inhibitors using natural ligands as scaffolds. Although natural ligands of MAG are glycolipids terminating in the sequence NeuAcα2–3Galβ1–3(±NeuAcα2–6)GalNAcβ-R, we previously established that synthetic O-linked glycoprotein glycans with the same sequence α-linked to Thr exhibited ~1000 fold increased affinity (~1 μM). Attempts to increase potency by introducing a benzoylamide substituent at C-9 of the α2–3 sialic acid afforded only a 2-fold increase, instead of increases of >100 fold observed for other sialoside ligands of MAG. Surprisingly, however, introduction of a 9-N-fluoro-benzoyl substituent on the α2–6 sialic acid increased affinity 80 fold, resulting in a potent inhibitor with a K_d of 15 nM. Docking this ligand to a model of MAG based on known crystal structures of other siglecs suggests that the Thr positions the glycan such that aryl substitution of the α2–3 sialic acid produces a steric clash with the GalNAc, while attaching an aryl substituent to the other sialic acid positions the substituent near a hydrophobic pocket that accounts to the increase in affinity.

Keywords

Myelin associated glycoprotein; MAG; siglec; Sialic acid; lectin

Myelin associated glycoprotein (MAG, Siglec-4) is a member of the **s**ialic acid **i**mmuno**g**lobulin **lec**tin (siglec) family, which bind sialic acid containing glycans as ligands. [8] While most of the 15 human siglecs described to date are expressed on various white blood cells of the immune system, [8] MAG is unique in that it is exclusively expression by glial cells that produce the myelin wrapped around the axons of neuronal cells. It's function is well established as a cell adhesion protein that is important in maintaining the integrity of the myelin-axon organization, mediated by the interaction of MAG expressed on the innermost leaflet on the myelin membrane, interacting with sialic acid containing gangliosides as ligands on the axon. [9] Gene knockout mice that are missing either MAG or

its ganglioside ligands exhibit increased demyelination and axon degeneration in the central and peripheral nervous system, with resulting neuronal deficiencies. [9]

In addition to its role in stabilizing axon-myelin interactions, MAG is one of several glial receptors that contribute to inhibition of axon growth following nerve injury. [9–11] Others include neurite outgrowth inhibitor (Nogo) and oligodenrocyte myelin glycoprotein MOgp. These receptors mediate inhibition of the outgrowth of axons in injured nerve by interacting with their respective ligands on the axon. Although MAG is unique in its recognition of axonal gangliosides as ligands, Nogo, MAG and MOgp all interact with the Nogo receptors, NgR1 and NgR2, as ligands. Although there is not consensus on the mode of MAG interaction with NgRs, [9, 12] several reports suggest that the interaction is sialic acid dependent. [13–15]

Numerous studies suggest that disruption of the interaction of MAG, Nogo and MOgp with their ligands can release the inhibition of axonal outgrowth and promote nerve regeneration following nerve injury. [9–11] This has been elegantly demonstrated for MAG in *in vivo* experiments that show improvement of nerve regeneration by administration of sialidase to destroy sialic acid dependent ligands. [16] Demonstration that sialoside ligands can reverse MAG dependent inhibition of axon outgrowth *in vitro* [17] has suggested the possibility that small molecules of sufficient potency could also promote nerve regeneration *in vivo*. This has stimulated our interest in the development of inhibitors of MAG based on its natural sialoside ligands. [1–4, 6, 7, 18–23]

Early investigations into the natural axonal ligands of MAG documented that it binds preferentially to gangliosides that contain the terminal sequence NeuAcα2–3Galβ1–3GalNAcβ-R found in GD1a and GT1b, and the di-sialylated sequence NeuAcα2–3Galβ1–3(NeuAcα2–6)GalNAcβ-R found in rare gangliosides such as GQ1bα. [9, 24] These same sequences are found as O-linked glycans of glycoproteins in α-linkage to threonine. These structures β-linked to synthetic aglycons (**1** and **3**) exhibited K_d values for MAG of 820 μM and 180 μM, respectively, [2] affinities that are typical of glycan binding proteins that mediate cell-to-cell contact through multivalent binding of receptors on one cell and glycan ligands on the other. [25–28] However, in contrast to the β-glycosides, the same sialoside sequences α-linked to threonine exhibit potencies over 100 fold higher (Figure 1).

Modifications of sialic acids with substituents at the C-9, C-5 and C-2 positions have also been documented to afford substantial increases in affinity (Figure 1). Addition of an aromatic substituent at C-9 typically results in affinity increases of 60–250 fold, even for simple sialosides such as α-methyl-NeuAc, and other linear sialosides such as NeuAcα2–3Galβ1–4GlcNAc. [2–4, 6, 7, 18–23, 29–31] Systematic analysis of the additive gains in affinity with substituents added at the C-9, C-5 and C-2 positions of sialic acid have yielded sialic acid analogs with K_d values of 150–500 nM (e.g. **8**), representing affinity gains of over 1000 fold over most natural sialosides (e.g. **1, 3**), [1, 6, 7, 18, 19, 31] with the exception of the synthetic O-linked sialosides with an α-Thr aglycon, which already exhibited affinities in this range (**2, 6**). [1]

Since the sialosides α-linked to Thr already exhibited low/sub μM potencies (**2** and **6**), we sought to achieve significant increased affinity by addition of an aromatic substituent at C-9 of the sialic acid linked α2–3 to Gal. To our surprise, only a modest 2-fold increase in affinity was observed. Thus, the affinity increases afforded by the aromatic substituent at C-9 (**7**) and the α-Thr aglycon (**6**) were not additive, a seemingly counter intuitive finding since the substituents were at the non-reducing and reducing ends of the sialoside, respectively.

To explore this finding further, we evaluated the effect of adding single ring aromatic substituents selected from our previous studies [3, 6, 19, 22] to each sialic acid of the disialyl-T-antigen (**2**). As shown in Scheme 1, T-antigen (**9**) was prepared chemically as previously described, [32] and was enzymatically sialylated with either NeuAc or C-9 substituted analogs of NeuAc using porcine ST3Gal I or chicken/human ST6GalNAc I. For C-9 substituted analogs of the NeuAcα2–3Gal sequence, 9-N substituted NeuAc analogs (**10b–d**) were activated to the corresponding CMP-NeuAc donor substrate and transferred directly to the acceptor glycan using the porcine ST3Gal I. For C-9 substituted analogs of the NeuAcα2–6GalNAc sequence, 9-azido-NeuAc was activated to CMP-9-azido-NeuAc and transferred to T-antigen (**9**) or sialyl-T-antigen (**11a**) using ST6GalNAc I. Once transferred, the 9-azide was reduced to the 9-amine, which was then acylated with the corresponding benzoyl chloride. The strategy of introducing the 9-azido-NeuAc followed by reduction and acylation was used because the chicken ST6GalNAc I did not efficiently transfer the 9-amino or 9-N-substituted sialic acids.

Each of the sialylated products was assessed for their ability to compete the binding of a MAG-Fc chimera to NeuAcα2–3Galβ1–4GlcNAc-R coupled to biotinylated polyacrylamide, which is adsorbed to streptavidin coated 96 well plates (Figure 2). To ensure that day-to-day variation in the assay was not a factor in for comparing their potency, each compound was analyzed three times on separate days, with at least one other compound to ensure that there was no significant 'drift' in the assay. The IC_{50} values in Table 1 represent the mean and standard deviation of the values obtained for the three separate assays. The standard error ranged from 5–15%, demonstrating the reproducibility of the results over course of the analysis of all compounds.

Although the earlier results of ours and others that aryl substituents at C-9 of sialic acid afford ~100 fold increased affinity, 9-N-benzoyl (**11b**) or 9-N-p-chloro-benzoyl (**11c**) substituents in the NeuAcα2–3 linked sialic acid of the 3'-sialyl-T antigen revealed affinity increases of only 2 fold. The one exception in this series was **11d** with the p-fluoro-benzoyl substituent, which gave a 14 fold increase in affinity with an IC_{50} of 80 nM. However, with the same substituent on the NeuAcα2–3 linked sialic acid of the disialyl-T-antigen (**13b**) increased affinity by only 2 fold. In contrast, the p-fluoro-benzoyl substituent on the NeuAcα2–6 sialic acid (**13c**) resulted in an 80-fold increase in potency, with an IC_{50} of 9 nM. This represents a potency at least 10 fold higher than any other synthetic ligand of MAG reported to date.

In order to more directly compare the affinities of these synthetic ligands with others reported by us and other groups using various related assays, we determined the K_d of the disialyl-T antigen (**13a**) by isothermal titration calorimetry. This gave a K_d value of 1.2 μM, in good agreement with the IC_{50} of 0.75 ± 0.17 μM (see Supplementary material). Using this as a reference, we have calculated the K_d apparent for each of the compounds assuming that the IC_{50} values are proportional (Table 1).

The apparent K_d of the most potent compound **13c** is 15 nM, representing at least 10,000 fold higher than the native structure **1**. This is remarkable since **13c** is identical except for αThr as the aglycon, and the 9-N-p-fluoro-benzoyl substituent on the sialic acid linked α2–6 to GalNAc. To better understand the basis for these dramatic affinity increases, we investigated the docking of this ligand to a homology model of MAG that is based on existing crystal structures of several siglecs.

A homology model of the carbohydrate binding domain of MAG was constructed based on the available crystal structures determined for sialoadhesin in complex with a 9-N-biphenyl-carbonyl-NeuAcαCH_3, siglec-5 in complex with α2,3-sialylated lactose and siglec-7 in

complex with an α2,8 linked ganglioside (See Figure S3). Distinct structural features were picked based on careful analysis of the X-ray data in combination with structural sequence alignment and homology modeling tools to enable the interpretation of the binding data. A modular approach was used, considering the unique features of MAG relative to the other siglecs. Although the backbone structure surrounding the conserved sialoside binding site is assumed to be the same for MAG as for the available template structures, we considered the CC'-loop and the GG'-linker as critical variable elements of the homology model. [33] Accordingly, we used the sialoadhesion structure as the basis for the backbone and essential parts of the sialic acid recognition site. This included the position of the essential arginine for the salt bridge with the carboxyl of the Neu5Ac, the 5' site for the N-acetyl group, Trp29 stabilizing the glycerol side chain and the positioning of the C-9 aryl substituents. Sialoadhesin was also used as a template for the GG'-linker, a crucial determinant of the hydrophobic pocket at the C-9 position. This represents a conservative assumption since 9-aryl analogs of α–methyl-NeuAc are documented to dramatically increase affinity for both sialoadhesin and MAG. [29] The second determinant of the carbohydrate recognition is the CC'-loop, which is known to vary in siglecs, and influence receptor specificity. [33, 34] Siglec-5 and -7 have the same number of amino acids in the CC'-loop as MAG and were therefore served as templates.

Homology models using these criteria were further subjected to an automated docking of known 9'substituted trisaccharide inhibitors of MAG (see Supplementary material). When bound ligands violated the conserved features of sialic acid recognition at the C-5 N-acetyl, C7–C9 polyhydroxy side chain or C-1 carboxylic acid sites, the model was excluded from further consideration. The final model comprises all features of siglec/sialic acid recognition conserved in available structures of sialoadhesion, siglec-5 and -7 (Figure 3).

The model accommodated sialosides with α2,3 or α2,6 linked sialic acids (e.g. 3'- or 6'-sialyllactose), consistent with the binding of both linkage types to MAG. [1, 6] Di-sialylated ligands such as disialyl-T-antigen (**2**) invoked the question whether the α2,3 or α2,6 linked sialic acid occupied the conserved site. Taking into account the significant gain in affinity when substituting the reducing end with αThr, we assumed there would be an interaction of this group with the protein. Accordingly, we performed docking of the corresponding monosialylated derivatives **11a** and **12a** to investigate the position of the αThr with the sialic acid bound to the conserved site. With the α2,6-linked Neu5Ac of **12a** fixed to the conserved site, there was no interaction of αThr with the protein. In contrast, positioning the α2,3-linked sialic acid of **11a** in the conserved site allows the αThr to interact favorably with the protein. In the model, the αThr is harbored in a shallow grove formed by the CC'-loop and the G'-strand. This pose anchors the reducing end GalNAc with the *N*-acetyl group pointing towards the GG'-linker (Figure 3). This directs the N-acetyl methyl group into the strongly favored hydrophobic site accessed by C-9 substituents, a site well described for other siglecs. [30] The placement of the N-acetyl group in this hydrophobic site then imposes steric restrictions for the substitution of the α2–3 sialic acid glycerol chain in the C-9 position which would occupy the same site (Figure 3c). This model is entirely consistent with the observation that introducing an aryl substituent at C-9 enhances affinity of α-methyl-NeuAc, but does not enhance the affinity of the sialyl-T antigens (**11a** and **13a**), which are anchored by the αThr. The 14-fold increase in affinity observed for the 9-N-para-flurobenzyl substituent of the α2,3-linked Neu5Ac (**11d**) can be accommodated by the successful competition with the GalNAc and αThr N-acetyl groups pointing into the same space.

With the glycan anchored by the α2–3 linked sialic acid at one end, and the αThr at the other, the α2–6 linked sialic acid has a minimal interaction with the protein. Although alternative positioning of the CC' loop might provide such interactions, in the absence of

evidence that such interactions exist, they were not further explored. However, the 9-N-p-fluorobenzyl substituent that affords 80-fold increase in affinity (**13c**) is in position to engage in protein contacts. In this model, the gain of affinity is attributed to a stacking interaction of the para-fluorobenzyl moiety with the conserved arginine (R118) (Figure 3a,b; and Figure SZ). This is a known mode of interaction of fluoro-aryl inhibitors to proteins as described for the binding of type 2 statins to HMG CoA reductase (see also Figure S2). [35] As an alternative, the fluorobenzyl moiety could interact with a hydrophobic pocket seen in Figure 3 below the phenyl ring, previously suggested to account for the increased affinity of aryl substituents attached at C-6 of the GalNAc in place of sialic acid. [4] However, we believe that stacking interaction of the fluorobenzyl with R118 better accounts for the high affinity observed.

We previously reported that the disialyl-T antigen (**11a**) exhibited activity for reversing MAG inhibition of neurite outgrowth in an in vitro cell culture model. [17] However, it is not optimal for *in vivo* studies due to its relatively low (μM) potency, and the rapid clearance of small oligosaccharides from the blood. Since **13c** exhibits significantly higher potency (K_d=15 nM), and has increased hydrophobicity due to the 9-aryl substituent, it will be of interest to determine if it has suitable phamacokinetic properties to evaluate its ability to promote axonal outgrowth in animal models of nerve injury. [16] This would provide an important proof of concept for the use of small molecule inhibitors of MAG for treatment of nerve injury. Longer term, however, we believe that the approach of minimizing the structural complexity of such inhibitors is ultimately the best route to obtaining pharmaceutically acceptable inhibitors. The detailed understanding of the basis for the potent inhibitory potency of **13c** may aid in the rational design of such sialoside mimic inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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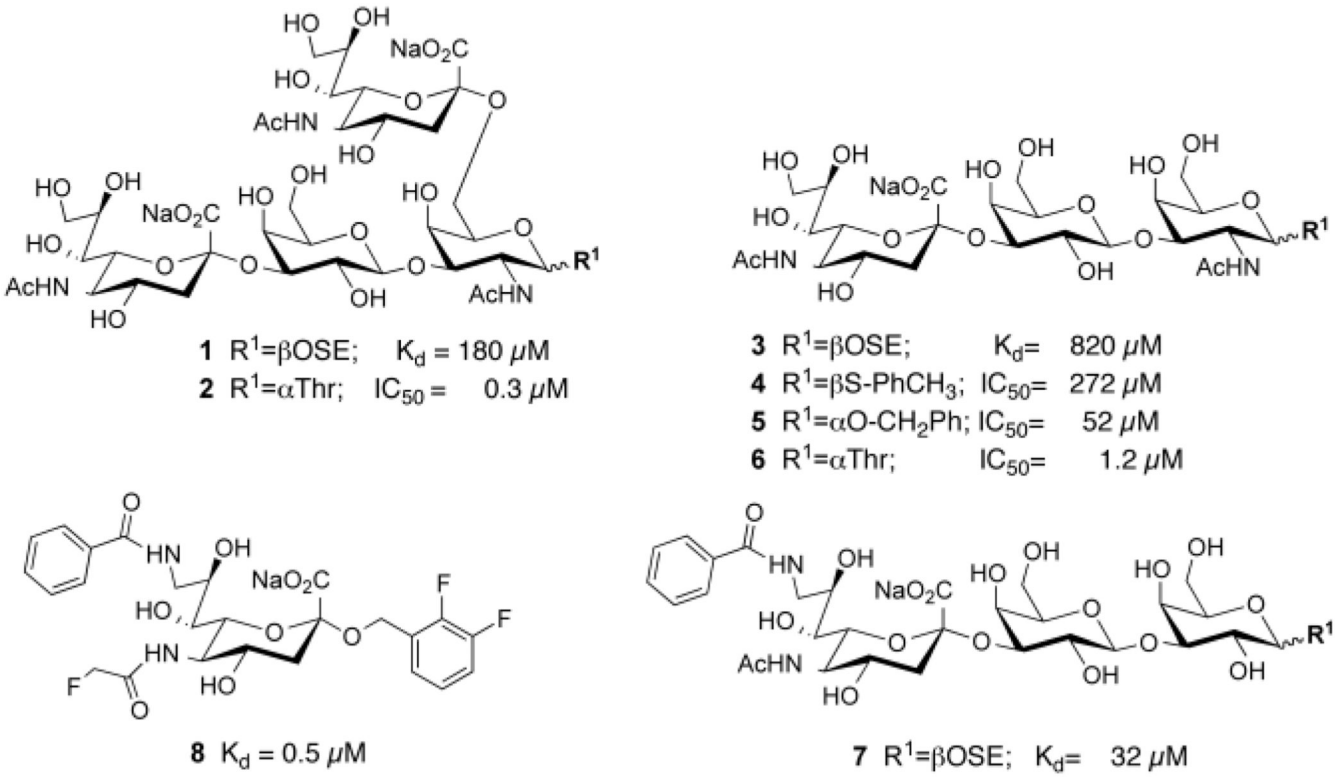


Figure 1.

Relative potencies of representative sialoside ligands of MAG. Natural sialoside sequences (**1–6**) β-linked (**1, 3, 4**) [1, 2] or α-linked (**2, 5, 6**) [1] to synthetic aglycons correspond to MAG ligands found in some gangliosides (e.g. GQ1bα) and O-linked glycans of glycoproteins, respectively. Modification of sialic acid with aryl substituents at C-9 and/or C-2 afford significant gains in affinity (**7,8**) [3–7, 19]

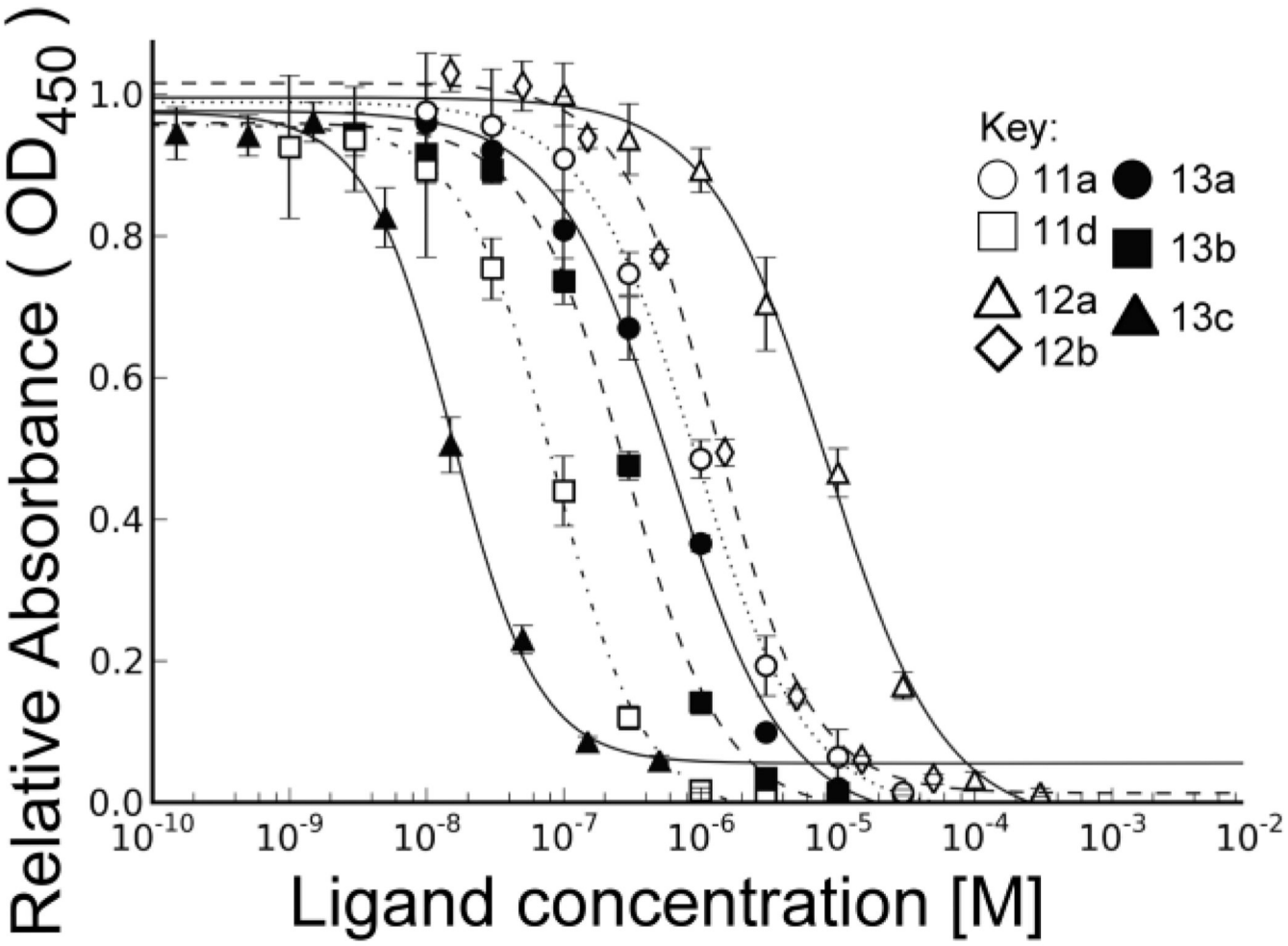


Figure 2.

Analysis of the inhibitory potency of sialoside analogs in MAG binding assay. Each compound is analyzed in triplicate for inhibition of the binding of MAG-Fc chimera to polymeric ligand adsorbed to the wells of 96 well plates as described in Supporting Materials.

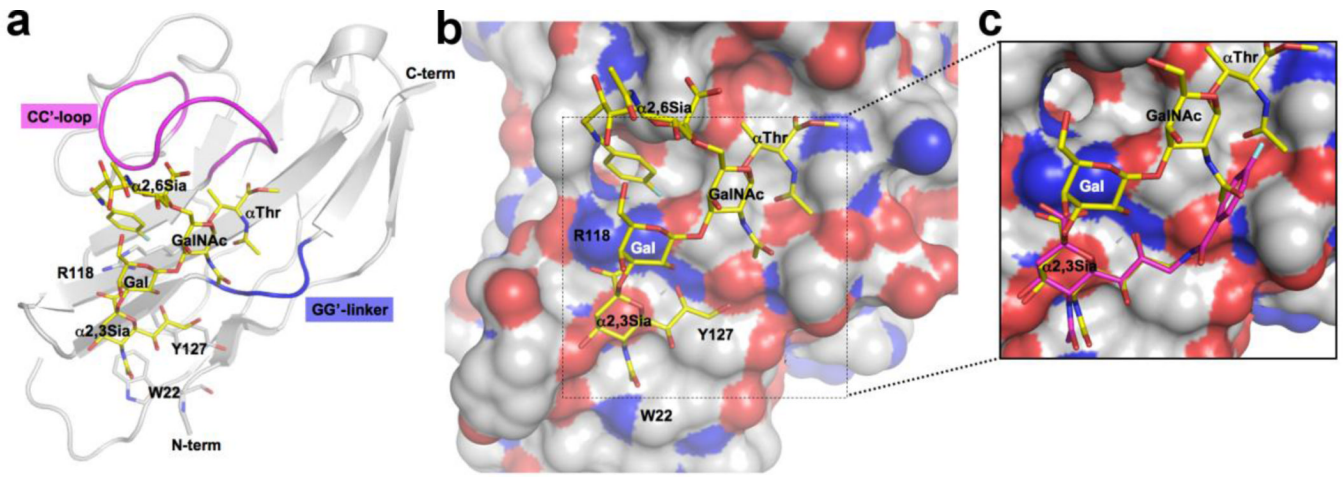
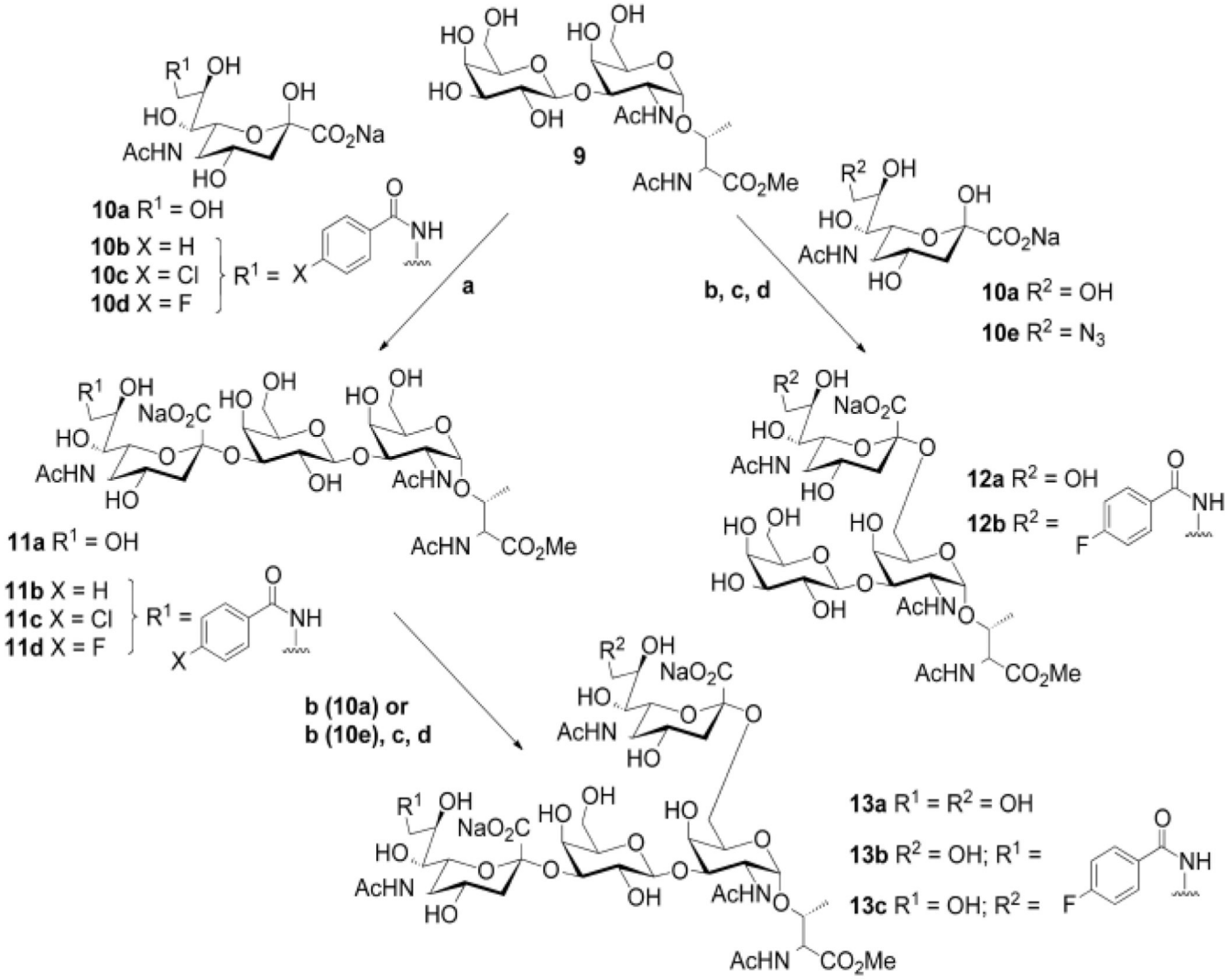


Figure 3.

Proposed binding of sialyl-T antigen analogs to MAG. a) Homology model of MAG constructed from the solved structures siglecs-1, -5 and -7 depicting the binding of an analog of disialyl-T antigen (**13c**). b) Model highlighting the spatial relationship of the α2–3 sialic acid bound to the conserved arginine (R118), the αThr and the 9-N-fluoro-benzolamide on the α2–6 sialic acid (see Supplementary material for analysis of glycan torsion angles; Figure S1). c) Inset showing the steric clash of the 9-N-fluoro-benzolamide on the α2–3 sialic acid with the N-acetyl groups of the GalNAc and αThr.

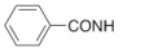
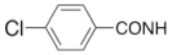
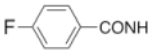
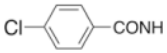
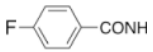
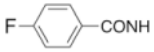
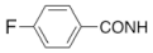


Scheme 1.

Synthesis of the sialosides a) pST3Gal I, buffer, yields 83–94%; b) ChST6GalNAc I, 92%; c) PMe_3, $CH_3OH/THF/H_2O$, 87%, d), RCOCl, DMF, Et_3N, 78–89%

Table 1

Inhibitory potencies of C-9 substituted analogs of sialylated Core 1 O-linked glycans for MAG

Compound[a]	R^1 (NeuAca2–3)	R^2 (NeuAca2–6)	IC_{50}[b] (nM)	rIP[c]	Apparent K_d[d] (μM)
11a	HO-	--	1200 ± 110	1.0	1.9
11b		--	650 ± 73	1.8	1.0
11c		--	610 ± 61	1.9	1.0
11d		--	79 ± 12	14	0.13
12a	--	HO-	7900 ± 420	0.15	13
12b	--		640 ± 54	1.9	1.0
12c	--		1100 ± 230	1.1	1.760
13a	HO-	HO-	750 ± 170	1.6	1.2
13b		HO-	300 ± 53	4	0.48
13c	HO-		9 ± 1	129	0.015

[a] See structures in Scheme 1

[b] IC_{50} values were determined by competition assays as described in Methods

[c] Relative inhibitory potency (rIP) is based on the IC_{50} values relative to **11a**.

[d] Apparant K_d values are calculated from the IC_{50} values with reference to the K_d determined for **13a** by isothermal titration (Figure 2).