Immunoglycotherapy

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C-4 Modified Sialosides Enhance Binding to Siglec-2 (CD22): Towards Potent Siglec Inhibitors for Immunoglycotherapy**

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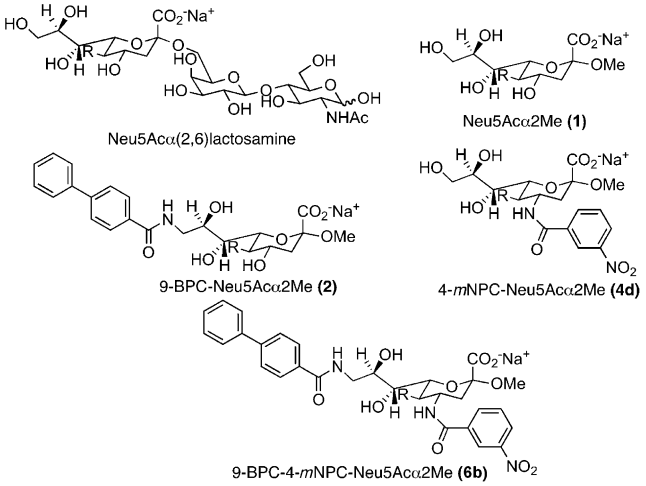
The regulatory functions of Siglecs (sialic acid binding immunoglobulin-like lectins) in the immune system provide opportunities for innovative therapeutic strategies for a wide range of immunological disorders or cancer (immunoglycotherapy).[1] Siglec-2 (CD22), as a consequence of its pivotal role in B cell activation, has become an attractive target for therapies of autoimmune diseases and B cell-derived non-Hodgkin's lymphoma (NHL). NHL is among the ten most common cancers with over 20000 deaths in 2010 for the US alone.[2] Siglec-2 binds with high preference to α(2,6)-linked sialic acids (Sia),[3] such as Neu5Acα(2,6)lactosamine (Scheme 1). Neu5Acα2Me (**1**) interacts with Siglec-2 mainly through 1) the negative charge on its carboxylate group, 2) the C-5 *N*-acetamido substituent, and 3) the glycerol side chain. Furthermore, replacement of the C-9 hydroxy group by an amino group did not interfere with binding to Siglecs.[3] Crystallographic studies on Siglec-1 (sialoadhesin, Sn)[4] demonstrated that acylation of this amino group enhances the overall affinity of the ligand for Siglecs by two to three orders of magnitude.[5] The first breakthrough in the development of potent Siglec-2 inhibitors was the design of 9-biphenylcarboxamido Neu5Acα2Me (9-BPC-Neu5Acα2Me, **2**) which has a more than two orders of magnitude higher affinity to Siglec-2 than **1**,[5d] and **2** has demonstrated potential to modulate signal transduction in B cells. Furthermore, based on **2**, compounds were developed, which kill B cell lymphoma cells.[6] Structural studies[4a,b] and modifications of the C-5 *N*-acyl substituent and the C-2 aglycon moiety of *N*-acetylneuraminic acid (Neu5Ac) have led to further improvement in affinity.[5a–c,e,f]



Scheme 1. *N*-acetylneuraminic acid derivatives (R = NHAc).

Herein we report, for the first time the design, synthesis, and evaluation of a novel class of disubstituted Neu5Ac derivatives that is modified at the C-4 and C-9 positions of **1**. Our structure-based design approach resulted in a promising novel lead compound 9-biphenylcarboxamido-4-*m*-nitrophenylcarboxamido-4,9-dideoxy Neu5Acα2Me (9-BPC-4-*m*NPC-Neu5Acα2Me, **6b**) that has sub-micromolar affinity for Siglec-2 and may provide a pathway for immunoglycotherapy strategies.

An evaluation of our homology model (see Supporting Information) for Siglec-2 and other Siglecs led us to hypothesize that substituents at C-4 may provide additional interactions. To address this hypothesis we posed the following questions:

1) Can C-4 substituents enhance the interaction with Siglecs?
2) Do they interact specifically with the protein?
3) Do C-4 and C-9 modifications act synergistically?
4) Do the C-4 modified Neu5Ac derivatives bind to the same binding site as other Sia, such as **1**?

To see if C-4-modified derivatives of Neu5Acα2Me (**1**) enhance the interaction with Siglec-2 and other siglecs, novel C-4 functionalized compounds of **1** (**4b**–**4g**) were prepared. Thus, per-O-acetylated 4-amino-4-deoxy-Neu5Acα2Me (**3**) was readily synthesized using a literature method,[7] and subsequent treatment of **3** with acyl chlorides or sulfonyl chloride followed by deprotection provided **4b**–**4g** (Table 1, Supporting Information).

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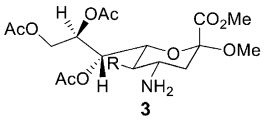

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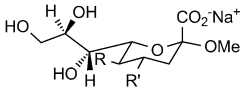
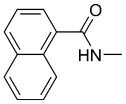
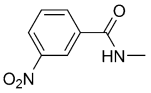
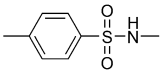
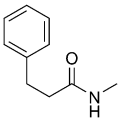
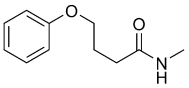
Table 1: Inhibition of Siglec-4 (MAG), Siglec-2 (CD22), and Siglec-9 by C-4-modified Sia.



R = NHAc

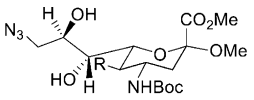
	R′	Siglec-2 (CD22) rIC_{50}[a]	Siglec-4 (MAG)	Siglec-9 IC_{50} [mM][b]
1	OH	1.0	1.0	n.i.[c]
4a	NH_2	0.6	1.7	n.i.[c]
4b		2.2	0.2	n.i.[c]
4c		1.7	0.6	n.i.[c]
4d		15	0.4	6.5
4e		10	0.5	n.i.[d]
4f		0.5	n.i.[d]	n.i.[d]
4g		0.7	n.i.[d]	n.i.[d]

[a] rIC_{50} values were calculated with **1** (IC_{50} 2.0 mM) as reference compound from IC_{50} values determined in at least three titrations, standard deviations were within 15%. [b] rIC_{50} values could not be calculated, since **1** was not inhibitory at up to 20 mM. [c] Not inhibitory at 20 mM. [d] Not inhibitory at 2 mM.

Siglec-2 (CD22), Siglec-4 (myelin-associated glycoprotein, MAG), and Siglec-9, were chosen to evaluate these compounds as potential inhibitors in hapten inhibition assays using immobilized fetuin as the target glycoprotein (Table 1). Compounds **1** and **4a**[7c] that contain a hydroxy or an amino group, respectively, at the C-4 position bind only weakly to all three Siglecs ($IC_{50} \approx 20$ mM or more).

An *N*-acetamido moiety at C-4 (compound **4b**) led to an increase in affinity for Siglec-2 but did not improve the affinity for Siglec-9 and even decreases binding to Siglec-4. The largest increase in binding affinity for Siglec-2 was observed when a *meta*-nitrophenylcarboxamido moiety (*m*NPC) was installed to give **4d** ($IC_{50} = 0.13$ mM; $rIC_{50} = 15$). This result is in contrast to the moderate affinity for Siglec-9 ($IC_{50} = 6.5$ mM) or Siglec-4 ($IC_{50} = 6.3$ mM; $rIC_{50} = 0.4$). In addition the results show that compounds **4d** and **4e** are specific inhibitors of Siglec-2. The presence of an aromatic residue (**4c**, **4f**, or **4g**) was not sufficient to enhance the binding to Siglec-2, suggesting that the C-4 substituents in **4d** and **4e** interact specifically with amino acids positioned adjacent to the Sia binding site. The striking differences in inhibition potencies prompted us to evaluate the interaction of **4d** with Siglec-2, Siglec-4, and Siglec-9 by saturation transfer difference (STD) NMR spectroscopy (Figure 1).[8] STD NMR signals of **4d** were normalized to the methyl protons of the *N*-acetamido group. The relative signal intensity for H*o* proton of the *m*NPC moiety is almost double in Siglec-9:**4d** compared to that in Siglec-2:**4d** and three times that in the Siglec-4:**4d** complex. These results are in full agreement with the hypothesis that the C-4 *m*NPC functionality in **4d** significantly contributes to the compound's inhibitory potential observed in the inhibition assays. The protons of the O-methyl group of **4d** in Siglec-2:**4d** receives 80% saturation, whereas the same protons receive only 36% and 16% in Siglec-4:**4d** and Siglec-9:**4d**, respectively.

A number of bifunctionalized Neu5Acα2Me (**1**) derivatives with modifications at C-4 and C-9 were synthesized to investigate whether such modifications act synergistically. Initial protection of the amino group on C-4 of **3** with butoxycarbonyl (Boc) was undertaken to minimize the number of reaction steps. Thus, introduction of a C-9 azido group provided **5**,[9] which, after reduction to the C-9 amino



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precursor, enabled easy installation of a BPC group. Removal of the C-4 Boc protecting group enabled coupling of substituents to the free amine, providing the C-4/C-9 modified compounds (**6a–d**; Supporting Information).

Table 2 outlines the inhibition potencies of compounds **6a–d** in hapten inhibition assays showing that modifications at C-4 significantly alter binding to Siglec-2. A naphthylamido substituent (**6a**) enhances the binding to Siglec-2 approximately fourfold compared to **2** but the dibenzylamino derivative (**6d**) decreases the binding affinity to Siglec-2 by a factor of 13. The most significant increase in binding affinity was observed for 4-*m*NPC (**6b**; $rIC_{50} = 14$) or 4-toluenesulphonylamido (**6c**; $rIC_{50} = 20$) derivatives. This is in excellent agreement with the rIC_{50} values determined for the Sia derivatives without the C-9 BPC moiety (Table 1) that show the *m*NPC moiety at C-4 (**4d**) increases the binding affinity by a factor of 15. Most striking is the synergistic effect of the C-4 and C-9 modifications resulting in an inhibition 9100 times stronger for **6b** compared to **1**.

Absolute binding affinities were also determined using surface plasmon resonance (SPR) measurements. Dissociation constants (K_D) were obtained for the key compounds **1** ($K_D = 31$ mM), **2**(K_D=7 μM), and **6b** ($K_D = 660$ nM; see Supporting Information). STD NMR competition experiments were undertaken to explore whether C-4 and/or C-9 Sia derivatives bind to the same Siglec-2 Sia binding site as **1** and **2**. Compound **4d** was added incrementally to a mixture of

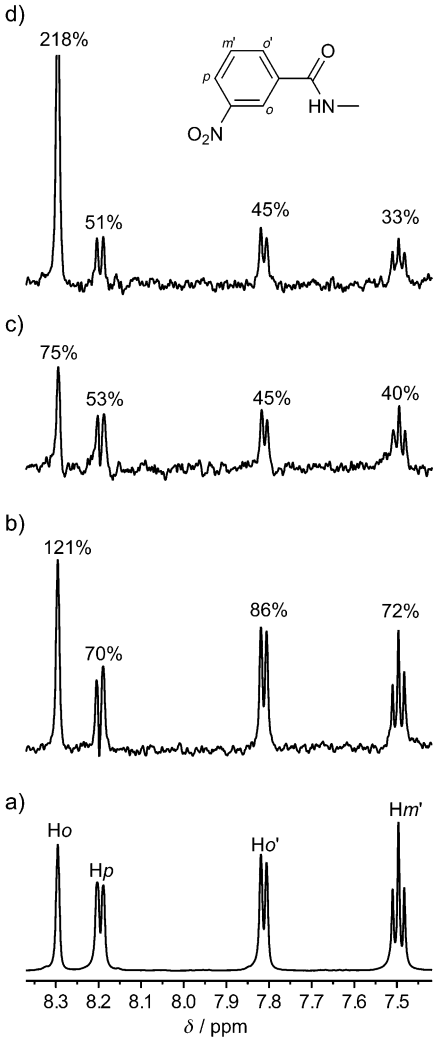
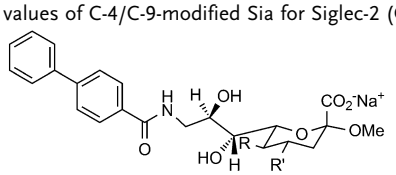
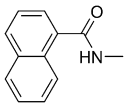
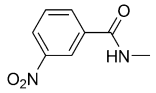
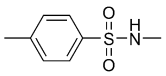
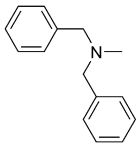


Figure 1. a) ^{1}H NMR spectrum of **4d** and STD NMR spectra of 500 μM **4d** in complex with b) 5 μM Siglec-2, c) Siglec-4, and d) Siglec-9.

Table 2: rIC_{50} values of C-4/C-9-modified Sia for Siglec-2 (CD22).



Compound	R′	rIC_{50}[a] 2	1
2	OH	1.0	667
6a		3.8	2500
6b		14	9100
6c		20	13000
6d		0.1	50

[a] rIC_{50} values were calculated with **2** (IC_{50} = 3.0 μM) or **1** (IC_{50} = 2.0 mM) as the reference compound from IC_{50} values determined in at least three titrations, standard deviations were within 15%.

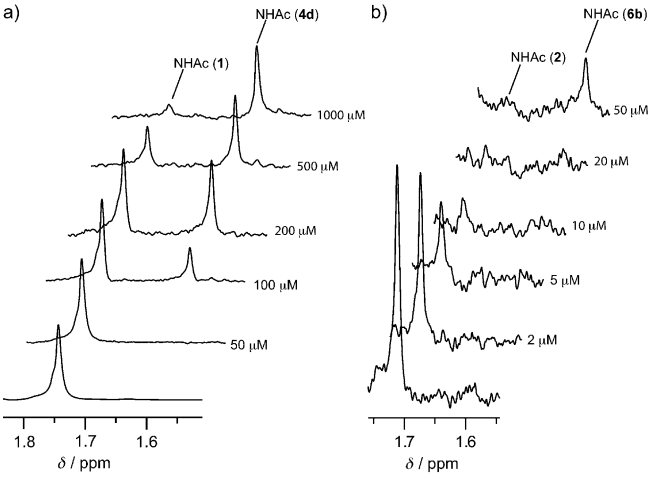


Figure 2. Competition STD NMR experiments of 5 μM Siglec-2 in complex with a) 500 μM **1** and an increasing concentration of **4d**, b) 500 μM **2** and an increasing concentration of **6b**. The ^{1}H NMR spectra of **1** and **2** are shown at the bottom of the respective panels.

5 μM Siglec-2 and 500 μM **1** (Figure 2a). STD NMR signal intensities for the methyl protons of the *N*-acetamido group of **1** reveal that a concentration of 200 μM of **4d** was sufficient to reduce the STD NMR signals of **1** by about 50%. At an equimolar ligand concentration, the STD NMR effects of **1** almost disappeared (Figure 2a). This result is in good agreement with a rIC_{50} value for **4d** of 15 times that of **1** (Table 1).

A second competition experiment was carried out using 5 μM of Siglec-2 and 500 μM of **2**, with a gradual increase in the concentration of **6b**. A very low **6b** concentration (5 μM) was sufficient to reduce the STD NMR signal of **2** by over 50% (Figure 2b). At a concentration of 10 μM of **6b** the STD NMR effects of **2** were decreased that they could hardly be detected, clearly demonstrating the very high affinity of **6b** for Siglec-2. This affinity of **6b** for Siglec-2 became even more clear in a competition experiment with **1** (data not shown). No binding of **1** (500 μM) to Siglec-2 was detected at a very low concentration (5 μM) of **6b**, suggesting that at a concentration equimolar to the protein, **6b** occupies essentially all the available Siglec-2 Sia binding sites.

The binding epitope of **6b** when in complex with Siglec-2 was determined by STD NMR spectroscopy (Figure 3). The STD NMR spectrum showed very strong STD NMR signals for the *m*NPC substituent at C-4. The Ho^A and Hm^A protons revealed a STD NMR effect of 181% and 192%, respectively, suggesting a close contact to the protein. Interestingly, these STD effects are stronger than those determined for **4d**

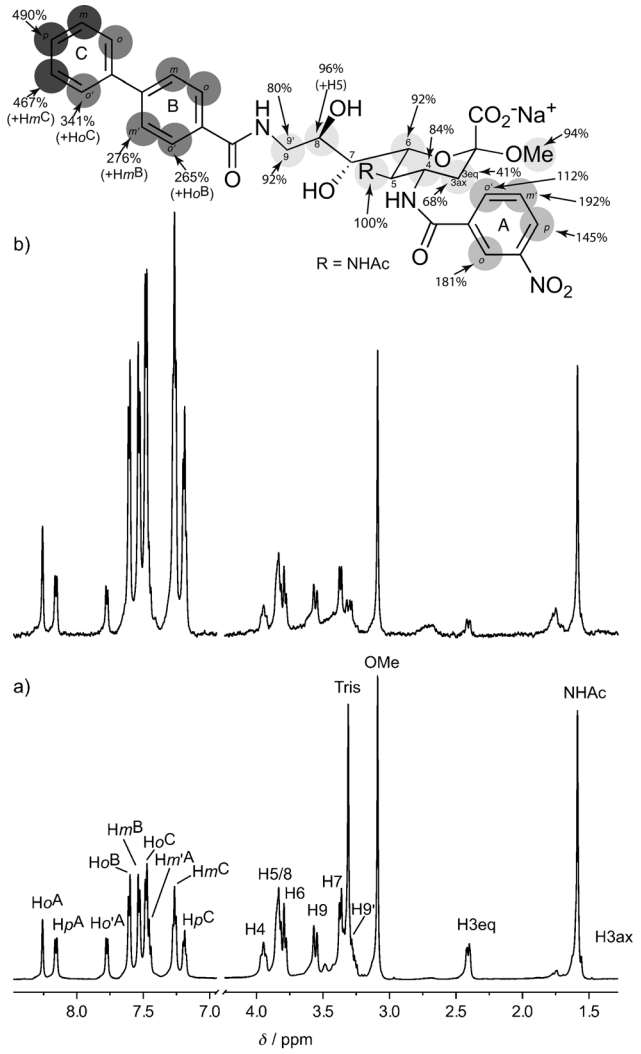


Figure 3. a) ^{1}H NMR and b) STD NMR spectra of 500 μM **6b** in complex with 5 μM Siglec-2; ax = axial, eq = equatorial, Tris = 2-amino-2-hydroxymethylpropane-1,3-diol.

(Figure 1). Similarly, the aromatic rings B and C of the BPC moiety at C-9 receive a significantly higher degree of saturation in **6b** than in compound **2**. This result suggests that both the 4-*m*NPC and the 9-BPC moieties have intimate contact with the protein surface and significantly contribute to the ligands binding affinity. Overall STD NMR effects for the Sia ring and glycerol side-chain protons of **6b** bound to Siglec-2 are clearly higher than those for the Sia ring protons in **1**, **2**, and **4d** bound to Siglec-2. This increase in saturation can be either a consequence of a tighter contact of the Sia scaffold of **6b** with the protein or a result of the intramolecular transfer of saturation received from the aromatic substituents at C-4 and C-9. Overall the epitope maps support the hypothesis that the binding modes of our new bifunctional Sia derivatives are similar, but not necessarily identical, to that of **1** and **2**. This observation is in very good agreement with the result of a computer-based docking experiment (Supporting Information). While all critical interactions between the protein and Sia, through the Sia carboxylate, its *N*-acetamido moiety, and glycerol side chain, are maintained, the BPC substituent is located in a pocket. As targeted in our compound design, the *m*NPC substituent is in close contact with the loop between the F- and G-strands.

In summary, we have successfully developed novel lead structures with sub-micromlolar affinities against Siglec-2. These structures have been functionalized at the C-4 of **1** leading to enhanced interaction with Siglec-2 by at least one order of magnitude as in compound **4d**. Furthermore, these substituents act synergistically with those at C-9 as in compound **6b**. We further conclude that **4d** is likely to bind to the same Sia binding site as **2** and **1**, strongly supporting our initial hypothesis that modifications at C-4 and C-9 act synergistically and that the addition of suitable C-4 substituents significantly increases the binding to Siglec-2. Based on published results[5,9] for Siglec-4 and Siglec-2, it can be expected that the addition of suitable moieties at C-2 will lead to a further increase in affinity as observed for **2**. Similar to antibodies, suitable polymers carrying high-affinity Siglec-2 inhibitors, such as compound **6b**, can induce similar effects[5b,e,f] and will provide even more biologically active compounds than those described for **2**.[6b,10] Thus, our approach provides new lead structures for the design of next generation Siglec inhibitors as potential drugs for immunoglycotherapy.

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