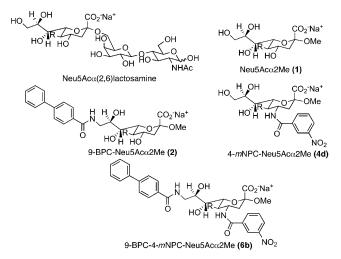
## Immunoglycotherapy

## 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).<sup>[1]</sup> 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.<sup>[2]</sup> Siglec-2 binds with high preference to  $\alpha(2,6)$ -linked sialic acids (Sia),<sup>[3]</sup> such as Neu5Ac $\alpha$ (2,6)lactosamine (Scheme 1). Neu5Aca2Me (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.<sup>[3]</sup> Crystallographic studies on Siglec-1 (sialoadhesin, Sn)<sup>[4]</sup> demonstrated that acylation of this amino group enhances the overall affinity of the ligand for Siglecs by two to three orders of magnitude.<sup>[5]</sup> The first breakthrough in the development of potent Siglec-2 inhibitors was the design of 9biphenylcarboxamido Neu5Aca2Me (9-BPC-Neu5Aca2Me, 2) which has a more than two orders of magnitude higher affinity to Siglec-2 than 1,<sup>[5d]</sup> 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.<sup>[6]</sup> Structural studies<sup>[4a,b]</sup> and modifications of the C-5 N-acyl substituent and the C-2 aglycon moiety of N-

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

acetylneuraminic acid (Neu5Ac) have led to further improvement in affinity.  $^{[5a-c,e,f]}$ 

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 Neu5Ac $\alpha$ 2Me (9-BPC-4*m*NPC-Neu5Ac $\alpha$ 2Me, **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 Neu5Aca2Me (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-Neu5Aca2Me (3) was readily synthesized using a literature method,<sup>[7]</sup> and subsequent treatment of 3 with acyl chlorides or sulfonyl chloride followed by deprotection provided 4b–4g (Table 1, Supporting Information).

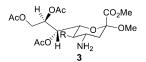


Table 1: Inhibition of Siglec-4 (MAG), Siglec-2 (CD22), and Siglec-9 by C-4-modified Sia.

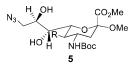
но	H OH CO <sub>2</sub> -Na <sup>+</sup>			
	HO'H R'	Siglec-2 (CD22)	Siglec-4 (MAG)	Siglec-9
	R = NHAc			
	R′	rIC <sub>50</sub> <sup>[a]</sup>		IC <sub>50</sub> [тм] <sup>[b]</sup>
1	ОН	1.0	1.0	n.i. <sup>[c]</sup>
4a	NH <sub>2</sub>	0.6	1.7	n.i. <sup>[c]</sup>
4 b	о — </th <th>2.2</th> <th>0.2</th> <th>n.i.<sup>[c]</sup></th>	2.2	0.2	n.i. <sup>[c]</sup>
4c	HN-	1.7	0.6	n.i. <sup>[c]</sup>
4d	O O <sub>2</sub> N	15	0.4	6.5
4e		10	0.5	n.i. <sup>[d]</sup>
4 f	O H	0.5	n.i. <sup>[d]</sup>	n.i. <sup>[d]</sup>
4g	O O O H	0.7	n.i. <sup>[d]</sup>	n.i. <sup>[d]</sup>

[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**<sup>[7c]</sup> that contain a hydroxy or an amino group, respectively, at the C-4 position bind only weakly to all three Siglecs (IC<sub>50</sub>  $\approx$  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<sub>50</sub>=0.13 mM; *r*IC<sub>50</sub>= 15). This result is in contrast to the moderate affinity for Siglec-9 (IC<sub>50</sub>=6.5 mM) or Siglec-4 (IC<sub>50</sub>=6.3 mM; *r*IC<sub>50</sub>= 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).<sup>[8]</sup> STD NMR signals of 4d were normalized to the methyl protons of the N-acetamido group. The relative signal intensity for Ho proton of the mNPC 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 mNPC 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 Neu5Ac $\alpha$ 2Me (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**,<sup>[9]</sup> which, after reduction to the C-9 amino



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-toluenesul-phonylamido (**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 ( $K_{\rm D}$ ) were obtained for the key compounds **1** ( $K_{\rm D} = 31$  mM), **2**( $K_{\rm D} = 7$   $\mu$ M), and **6b** ( $K_{\rm D} = 660$  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

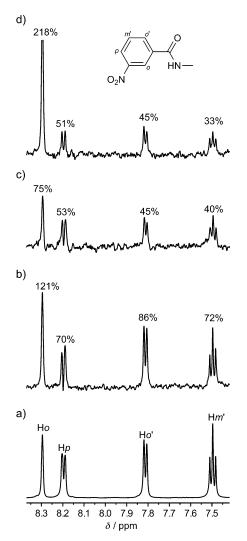
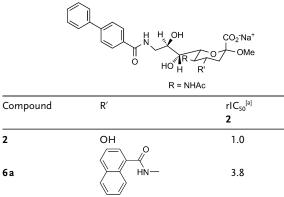


Figure 1. a) <sup>1</sup>H NMR spectrum of 4d and STD NMR spectra of 500 µм 4d in complex with b) 5 µм Siglec-2, c) Siglec-4, and d) Siglec-9

5 µм Siglec-2 and 500 µм 1 (Figure 2a). STD NMR signal intensities for the methyl protons of the N-acetamido group of 1 reveal that a concentration of 200  $\mu$ M 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  $\mu$ M) of **6b**, suggesting that at a concentration Table 2: rIC<sub>50</sub> values of C-4/C-9-modified Sia for Siglec-2 (CD22).



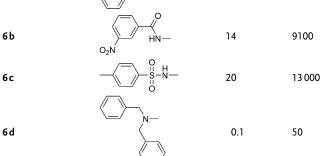
2

6 a

1

667

2500



[a] rIC\_{50} values were calculated with 2 (IC\_{50} = 3.0  $\mu m$ ) 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%.

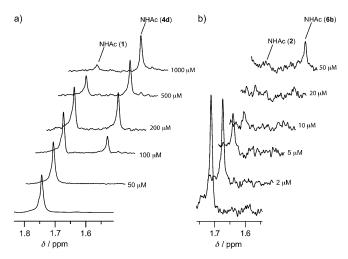
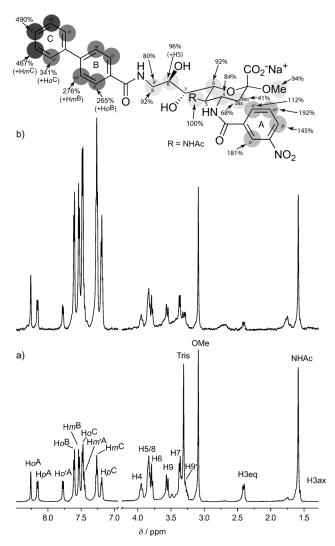


Figure 2. Competition STD NMR experiments of 5 µM Siglec-2 in complex with a) 500  $\mu$ M 1 and an increasing concentration of 4d, b) 500  $\mu$ M **2** and an increasing concentration of **6b**. The <sup>1</sup>H NMR spectra of 1 and 2 are shown at the bottom of the respective panels.

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) <sup>1</sup>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-mNPC 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 mNPC 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<sup>[5,9]</sup> 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<sup>[5-</sup> <sup>b,e,f]</sup> and will provide even more biologically active compounds than those described for 2.<sup>[6b, 10]</sup> Thus, our approach provides new lead structures for the design of next generation Siglec inhibitors as potential drugs for immunoglycotherapy.

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