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Specificity of the binding site of the sialic acid-binding lectin from ovine placenta, deduced from interactions with synthetic analogues

M. Fernanda Troncoso[1], M. Mercedes Iglesias[1], Rainer Isecke[2], Carlota Wolfenstein Todel[1]* and Reinhard Brossmer[2]*

[1]*Instituto de Química y Fisicoquímica Biológicas (IQUIFIB) (UBA-CONICET), Facultad de Farmacia y Bioquímica, Junín 956, 1113 Buenos Aires, Argentina,* [2]*Biochemistry Center, University of Heidelberg, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany*

The specificity of the sialic acid-binding lectin from ovine placenta was examined in detail by haemagglutination inhibition assays applying a panel of 32 synthetic sialic acid analogues. The carboxylic acid group is a prerequisite for the interaction with the lectin, the α-anomer of the methyl glycoside is only a little more effective as an inhibitor than the β-anomer and the most potent inhibitor was 9-deoxy-10-carboxylic acid Neu5Ac, followed by 4-oxo-Neu5Ac. In contrast to the majority of known sialic acid-binding lectins, the N-acetyl group of Neu5Ac is not indispensable for binding, neither is the hydroxyl group at C-9 since substitutions at this carbon atom are well tolerated. Furthermore, all sulfur-containing substituents at C-9 enhanced the affinity of the lectin. This is the first sialic acid-binding lectin found to strongly bind thio derivatives.

Keywords: **lectin specificity, sialic acids, haemagglutination inhibition, ovine placenta**

Introduction

Lectins are multivalent carbohydrate-binding proteins or glycoproteins with the ability to agglutinate cells. Owing to their high degree of specificity towards glycoconjugates, they are useful tools for various biological applications, including blood typing, induction of lymphocyte mitogenesis, diagnosing of microorganisms and differentiation of pathogenic bacteria, purification and subcellular localization of glycoconjugates and discrimination between normal or malignant cells [1–8].

Sialic acids (Sias) are a family of acidic nine-carbon sugars distributed on cell surfaces at exposed positions, generally as terminal components of glycoconjugates but also occurring as part of many glycoproteins of the body fluid e.g. in blood. A particular property of Sias is their structural variability resulting from modifications of hydroxyl and amino groups. This leads to more than 40 natural variants. Sias are known to play important roles in a variety of biological processes, such as: infection of cells by microorganisms, regulation of the immune system, development of the nervous system [9–10] and cancer metastasis [9,11–14]. Most of these lectins have been isolated from arthropods [15–20], from molluscs [21–23], from protozoa [24–26], from plants [27–28]; from bacteria [29–30], from viruses [31] and from human placenta [32].

It is known that malignant transformation of cells is usually associated with a variety of structural alterations on the cell surface carbohydrates. Numerous studies have been carried out comparing binding of lectins to normal and malignant tissues, showing distinct alterations. For instance, human melanoma synthesizes large quantities of gangliosides different from those seen in normal melanocytes and it was found that the lectin from *Cancer antennarius* binds specifically to O-acetylated ganglioside G_{D3} [33]. Besides, the lectin from *Achatina fulica* is being employed as a diagnostic probe to detect 9-OAc-Neu5Ac on the surface of erythrocytes and peripheral blood mononuclear cells of patients suffering acute lymphoblastic leukemia. This lectin is not capable of agglutinating normal human erythrocytes [34–37].

There are many other diseases in which alterations related to O-acetylation of sialic acids are involved. These include visceral leishmaniasis, breast cancer, colon cancer, adenocarcinoma, etc (for review see Ref. 38). As a consequence lectins may be powerful tools to diagnose these diseases.

*To whom correspondence should be addressed.

A sialic acid-binding lectin was isolated from ovine placenta (OPL I) by Iglesias *et al*. [39]. It was purified by affinity chromatography on bovine submaxillary gland mucin-agarose followed by gel filtration, and it showed an apparent molecular weight of 65 000 on SDS-PAGE. The lectin had affinity for sialic acid and haemagglutination activity was not affected by the presence of 100 mM EDTA, showing that Ca^{2+}, Mg^{2+} and Mn^{2+} have no effect on this activity.

Since the molecular features of the protein-carbohydrate interaction of this lectin are not yet precisely established, it is important to clarify its specificity in order to explore its potential use as a diagnostic tool. With this purpose in mind, in the present paper we investigate the detailed specificity of this lectin using a broad range of sialic acid analogues. The results obtained can be expected to reflect the interaction with the lectin-binding site.

Materials and methods

Bovine submaxillary gland mucin (BSM), Sepharose 4B, divinylsulfone, phenylmethylsulfonyl fluoride, aprotinin, bovine albumin, trypsin, N-acetylneuraminic acid, N-glycolylneuraminic acid and 2,3-dehydro-2-deoxy-neuraminic acid were purchased from Sigma Chemical Co., (St. Louis, MO, USA).

Ovine placental tissue was surgically removed from ewes at a slaughterhouse, immediately frozen and stored at −70°C prior to processing.

Sialic acid-binding lectin (OPL I) was obtained as previously described [39]. Briefly, the procedure consisted of affinity chromatography on bovine submaxillary gland mucin-agarose followed by FPLC on a Superose 12 HR column (Pharmacia LKB).

Haemagglutination assays

Assays were carried out in U-well microtiter plates (96 wells). Rabbit erythrocytes were obtained by venipuncture, and treated with trypsin and glutaraldehyde [40]. Serial dilutions in 10 mM Na phosphate buffer, 0.15 M NaCl, pH 7.0, of the protein solution (25 µl) were mixed with equal volume of 1% bovine albumin and of 2.5% suspension of treated erythrocytes in the same buffer. After incubation for 1 h at room temperature, the agglutination was recorded visually. The reciprocal of the highest dilution of protein solution showing visible agglutination was recorded as the titer.

Haemagglutination inhibition assays

Potential inhibitors were dissolved in the same buffer and, when necessary, the pH was readjusted to 7.0. Lectin solution (titer 2) was preincubated with serial two-fold dilutions of these inhibitors in the same buffer for 1 h, and then mixed with 25 µl of 1% bovine albumin and equal volume of 2.5% suspension of treated erythrocytes. In some cases intermediate dilutions of inhibitors were added. The minimum concentration of inhibitor that caused complete inhibition was determined and is presented as means of at least three experiments.

Sialic acid analogs

The following compounds were prepared in part as previously reported.

Neu5AcMeα and Neu5AcMe*β* [41–42]. Neu5AcBnα [43]. Neu5AcMethioα [44]. Meα-Neu5Ac-Me-ester [41–42]. 4-oxo-Neu5Ac [45]. Neu5thioAcMeα and Neu5PropMeα [46]. Reaction of neuraminic acid methyl α-glycoside [46] with nitrophenyl fluoroacetate afforded Neu5FAcMeα. Neu5N-H_2AcMeα [47]. 9-thio-Neu5AcMeα and 9-Acthio-Neu5AcMeα [48]. 9-AcNH-Neu5AcMeα [47]. 9-BzlNH-Neu5AcMeα [47]. 9-deoxy-Neu5AcMeα was prepared by catalytic hydrogenation of 9-I-Neu5AcMeα. Synthesis of the other compounds will be reported in detail elsewhere. All analogues were characterized by NMR spectroscopy and fast atom bombardment MS.

Results

To study the nature of the binding specificity of OPL I, haemagglutination inhibition experiments were carried out with a broad range of sialic acid analogues, the structures of which are schematized in Table 1, and the results of the assays are shown in Table 2.

Our first objective was to determine the importance of the anomeric linkage of sialic acid (Neu5Ac) in the interaction with the lectin. Neu5Ac was compared to both the α- and *β*-anomers of the methyl glycoside. Neu5AcMeα was effective at the same concentration as Neu5Ac, whereas Neu5AcMe*β* was slightly less inhibitory as compared to Neu5Ac.

Benzyl α-glycoside of Neu5Ac, which carries a hydrophobic aglycon and methylthio α-glycoside of Neu5Ac, where a sulfur atom replaces the glycosidic oxygen, showed 2 times less affinity than the α-anomer of the methyl glycoside.

Next we examined the importance of the carboxylic acid group on the interaction with the OPL I. The esterification of sialic acid α-glycoside completely abolished its inhibitory activity.

When an analogue with a modification at C-2 and C-3 (2,3-dehydro-2-deoxy-Neu5Ac) was tested, no inhibition was observed up to a concentration of 50 mM.

The next series of modifications involved the C-4 hydroxyl group. 4-oxo-Neu5Ac was 16 times more effective as an inhibitor than Neu5Ac whereas 4-OAc-Neu5AcBnα was 4 times more active as compared to Neu5AcBnα.

To continue the study on the specificity of OPL I, analogues with modifications at C-5 were tested. Neu5Gc was an inhibitor twice as potent as Neu5Ac while the affinity of Neu5thioAcMeα was 4 times enhanced as compared to the respective sulfer-free Neu5AcMeα. Whereas Neu5PropMeα was active at the same concentration as Neu5AcMeα, the introduction of a fluorine in Meα-5-N-fluoroacetylneuraminic

Table 1. Structures of sialic acid analogues used for haemagglutination inhibition studies. The positions of R_1 to R_5 are in Figure 1

Sialic acid analogue	*R_1*	*R_2*	*R_3*	*R_4*	*R_5*
Neu5Ac	OH	COOH	OH	Ac	$(CHOH)_2CH_2OH$
Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2OH$
Neu5AcBnα	COOH	OBn	OH	Ac	$(CHOH)_2CH_2OH$
Neu5AcMeβ	OCH_3	COOH	OH	Ac	$(CHOH)_2CH_2OH$
Neu5AcMethioα	COOH	SCH_3	OH	Ac	$(CHOH)_2CH_2OH$
Meα-Neu5Ac-Me-ester	$COOCH_3$	OCH_3	OH	Ac	$(CHOH)_2CH_2OH$
2,3-dehydro-2-deoxy-Neu5Ac	COOH		OH	Ac	$(CHOH)_2CH_2OH$
4-oxo-Neu5Ac	OH	COOH	=O	Ac	$(CHOH)_2CH_2OH$
4-OAc-Neu5AcBnα	COOH	OBn	OAc	Ac	$(CHOH)_2CH_2OH$
Neu5Gc	OH	COOH	OH	$COCH_2OH$	$(CHOH)_2CH_2OH$
Neu5thioAcMeα	COOH	OCH_3	OH	$CSCH_3$	$(CHOH)_2CH_2OH$
Neu5PropMeα	COOH	OCH_3	OH	$COCH_2CH_3$	$(CHOH)_2CH_2OH$
Neu5FAcMeα	COOH	OCH_3	OH	$COCH_2F$	$(CHOH)_2CH_2OH$
$Neu5NH_2AcMe\alpha$	COOH	OCH_3	OH	$COCH_2NH_2$	$(CHOH)_2CH_2OH$
9-deoxy-10-carboxy-Neu5Ac	OH	COOH	OH	Ac	$(CHOH)_2CH_2COOH$
9-thio-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2SH$
9-thioPropNH-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2NHCO(CH_2)_2SH$
9-Acthio-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2SAc$
9-Methio-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2SCH_3$
9-MeSO-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2SOCH_3$
9-AcNH-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2NHAc$
9-carboxy-Neu5Ac	OH	COOH	OH	Ac	$(CHOH)_2COOH$
9-carboxy-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2COOH$
9-deoxy-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_3$
$9\text{-}NH_2$-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2NH_2$
9-OAc-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2OAc$
9-I-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2I$
9-diMeN-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2N(CH_3)_2$
9-triMeN^+-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2N(CH_3)_3$
9-succNH-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2NHCO(CH_2)_2COOH$
9-BzlNH-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2NHCOC_6H_5$
9-NH-oxamido-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2NHCOCONH_2$
9-NH-(ethoxycyclobutendion)-Neu5AcMeα	COOH	OCH_3	OH	Ac	$(CHOH)_2CH_2NH$-Ethoxycyclobutendion

[a]Abbreviations used in this table: Ac, acetyl; Bn, benzyl; Bzl, benzoyl; Me, methyl; Neu, neuraminic acid; Prop, propanoyl; Succ, succinyl.

acid decreased 1.5 times the activity. On the other hand, Meα-5-N-aminoacetyl (glycyl) neuraminic acid was ineffective up to a concentration of 50 mM.

Finally, in order to understand the role of the glycerol side chain, we tested compounds with modifications at C-9. Oxidation of the primary hydroxy group at C-9 to a carboxyl, in both free sialic acid and α-anomer of the methyl glycoside did not alter the binding, 9-deoxy-Neu5AcMeα did not cause any difference either. In contrast, 9-deoxy-Neu5Ac-10-carboxylic acid showed a great inhibitory capacity, since it was 32 times more potent than Neu5Ac. Replacement of the primary hydroxyl by thio, methylthio, methylsulfoxido, acetylthio or thiopropionylamino groups increased the inhibitory potency 4 times as compared to Neu5AcMeα. 9-AcNH-Neu5AcMeα was twice as potent as parent Neu5AcMeα. Although the presence of an amino group at C-9 did not alter the interaction, substitution of the hydroxyl at C-9 by iodine slightly decreased the inhibitory potency as compared to Neu5AcMeα. No binding to the OPL I was observed when the haemagglutination inhibition experiments were carried out with analogues substituted with dimethylamino, trimethylammonio, succinylamino, benzoylamino, N-oxamido or ethoxycyclobutendion groups at C-9 of Neu5Ac.

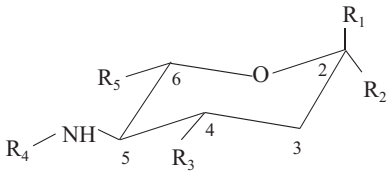

Figure 1. Structure of the sialic acid analogues applied for haemagglutination inhibition studies. Substituents R_1–R_5 are listed in Table 1.

Table 2. Inhibition of ovine placenta lectin (OPL I) haemagglutination by sialic acid analogues. The minimal concentration of the compounds required for total haemagglutination inhibition induced by 2 haemagglutination units of OPL I are shown

Sialic acid analogue	*mM*
Neu5Ac[a]	12.50
Neu5AcMeα	12.50
Neu5AcBnα	25
Neu5AcMeβ	18.70
Neu5AcMethioα	25
Modification of the Carboxyl Group	
Meα-Neu5 Ac-Me-ester	>50
Modification at C-2/C-3	
2,3-dehydro-2-deoxy-Neu5Ac	>50
Modification at C-4	
4-oxo-Neu5Ac	0.78
4-OAc-Neu5AcBnα	6.25
Modification at C-5	
Neu5Gc	6.25
Neu5thioAcMeα	3.12
Neu5PropMeα	12.50
Neu5FacMeα	18.70
$Neu5NH_2AcMe\alpha$	>50
Modification at C-9	
9-deoxy-10-carboxy-Neu5Ac	0.39
9-thio-Neu5AcMeα	3.12
9-thioPropNH-Neu5AcMeα	3.12
9-Acthio-Neu5AcMeα	3.12
9-Methio-Neu5AcMeα	3.12
9-MeSO-Neu5AcMeα	3.12
9-AcNH-Neu5AcMeα	6.25
9-carboxy-Neu5Ac	12.50
9-carboxy-Neu5AcMeα	12.50
9-deoxy-Neu5AcMeα	12.50
$9\text{-}NH_2$-Neu5AcMeα	12.50
9-OAc-Neu5AcMeα	12.50
9-I-Neu5AcMeα	18.70
9-diMeN-Neu5AcMeα	>50
$9\text{-}triMeN^+$-Neu5AcMeα	>50
9-succNH-Neu5AcMeα	>50
9-BzlNH-Neu5AcMeα	>50
9-N-oxamido-Neu5AcMeα	>50
9-N-(ethoxycyclobutendion)-Neu5AcMeα	>50

[a]Abbreviations used in this table: Ac, acetyl; Bn, benzyl; Bzl, benzoyl; Me, methyl; Neu, neuraminic acid; Prop, propanoyl; Succ, succinyl.

Discussion

The haemagglutination inhibition experiments with sialic acid analogues, here described, lead to the conclusion that the α-anomer of the methyl glycoside is a little more effective as an inhibitor than the β-anomer. It follows that the charge of the axially orientated carboxyl group accommodates more efficiently at the lectins binding site than the equatorial one. It is therefore reasonable that the carboxylate anion of the β-anomer is interacting with a negatively charged amino acid of the protein causing repulsion.

The lower activity obtained with benzyl α-glycoside of Neu5Ac and methylthio α-glycoside of Neu5Ac makes evident steric hindrance and a strong influence of the glycosidic bridge, respectively.

The importance of the carboxylic group was demonstrated by the lack of interaction with the lectin observed when sialic acid α-glycoside was esterified. In consequence, it can be assumed that the charge of the carboxyl group is essential for binding to the lectin. It is reasonable to consider an ionic interaction between the carboxylate anion of sialic acid and a positively charged residue of the lectins binding site. Interestingly, Weis and Drickamer [49] analyzing the data of other sialic acid-binding lectins observed that, with the exception of polyoma virus [50], the carboxylic group interacts with main-chain amide groups, polar side chains (especially serine) and ordered water molecules, in which are essentially hydrogen-bond interactions. This leads to the suggestion that in this electrostatic stabilization no charged amino acid side chains are used.

The haemagglutination was not inhibited by 2,3-dehydro-2-deoxy-Neu5Ac, a strong inhibitor of sialidases, showing that the lack of the anomeric oxygen causes the failure of OPL I to recognize the 2,3-dehydro compound.

Experiments with analogues carrying modifications at C-4 suggest that this area of the molecule interacts with the lectin, contributing to binding through the formation of hydrogen bonds. This conclusion is based on the great inhibition strength of 4-oxo-Neu5Ac, which could be due to the fact that the oxo group acts as a good hydrogen bond acceptor or else it could be involved in a Schiff-base formation. Besides, the presence of an O-acetyl group at C-4 enhances the affinity for OPL I.

Introduction of a propanoyl group at C-5 did not cause any difference in inhibitory potency, but substitution of one hydrogen of the N-acetyl group by fluorine produced a small decrease of activity, indicating that the strong electronegativity of the halogen interferes little with the binding to the lectin. The ineffectiveness of Neu5NH2AcMeα can be explained by an intramolecular salt bridge formation, thus reducing its essential negative charge. In fact, a solution of this analogue reacts neutral in contrast to the strongly acidic parent compound Neu5Ac. Conversion of the acetyl at C-5 to hydroxyacetyl (glycolyl) increases the affinity for the lectin, due to the formation of additional hydrogen bonds or Van der Waals interactions. The binding was also enhanced with Neu5thioAcMeα. Replacement of the amide oxygen by sulfur caused a greater inhibition, due to the difference in electronegativity between oxygen and sulfur, leading to a significantly greater capacity of the thio-amide NH to form a hydrogen bond.

Obviously, the hydroxyl group at C-9 does not play a crucial role in the interaction since the inhibitory concentration of 9-deoxy-Neu5AcαMe was the same as that of Neu5AcMeα.

This assumption is supported by the fact that substitution of the hydroxyl by an amino group or by an iodine atom caused no difference or just a slight decrease in the inhibitory potency, respectively. Especially interesting are the results obtained by introduction of a carboxyl to the glycerol side chain of Neu5Ac. Whereas both Neu5Ac-9-carboxylic acid and its α-glycoside show activity comparable to Neu5Ac, the analogue produced by chain elongation, 9-deoxy-Neu5Ac-l0-carboxylic acid, represents by far the strongest inhibitor of the lectin, showing that the position of the carboxyl group is critical. Although the difference between the two compounds is just one methylene group, the negative charge might suitably fit an opposite charged group of the protein, located at just the optimal distance, resulting in a strong electrostatic interaction. No definite conclusions can be drawn without a detailed structural analysis by x-ray.

The hypothesis of a positively charged area at the lectins binding site, opposite to C-10 of 9-deoxy-Neu5Ac-10-carboxylic acid is consistent with the complete abrogation of the inhibitory potency when basic groups such as dimethyl-amino and trimethylammonio are introduced in C-9. On the other hand, although the 9-succinylamino derivative carries a negative charge, the distance is much increased, not allowing interaction. Substitution by bulky groups such as benzoylamino, also abolished the inhibition, probably due to steric constraints.

Worth noting is the finding that the analogues containing either an oxamido or an ethoxycyclobutendion substituent at C-9 did not inhibit at all. Although both are somehow space-filling, the lack of inhibition is more likely caused by the adjacent dione groups as common structural element. The reason is not yet clear.

A most remarkable result obtained was the enhanced activity shown by compounds containing a thiol group at C-9. Furthermore, all sulfur-containing substituents at this carbon produced the same increased affinity, probably caused by its lower electronegativity and greater hydrophobicity with respect to oxygen.

In conclusion, we have delineated the specificity of ovine placenta lectin for the different regions of the sialic acid molecule employing suitable analogues. The knowledge gained may allow to prepare derivatives suited for structural studies of the lectins complementary binding site employing photo-affinity labeling.

Comparison with other sialic acid-binding lectins

Comparison with the human placental lectin [32] showed that N-glycolylneuraminic acid was a more potent inhibitor than neuraminic acid for both lectins, and that bovine submaxillary mucin showed a greater inhibitory capacity, compared on the basis of sialic acid concentration, than other sialoproteins. With respect to the inhibitory potency of gangliosides [29], there was no apparent relation with sialic acid linkage since GT_{1b} ganglioside exhibited the highest inhibitory capacity on OPL 1. In contrast, human placental lectin [32], showed the highest affinity for GD_{1b} ganglioside, consistent with a preference for α-2,8 or α-2,6-linked sialic acid.

Contrary to the majority of sialic acid-binding lectins the specificity of which have been studied in detail (those from *Limulus polyphemus* (limulin), *Thachypleus tridentatus* (TTA), *Limax flavus* (LFA), *Cepaea hortensis* (CHA), *Tritrichomonas mobilensis* and wheat germ (WGA) [15,20,21,25,27,51]), the N-acetyl group of Neu5Ac is not indispensable for binding to OPL I, since the 5-propanoyl derivative was equally potent. Introduction of a hydroxyl group in N-glycolylneuraminic acid increased the affinity for the OPL I, similar to the lectin from human placenta [32] and from *Scilla serrata* [18]. In contrast, this substitution did not affect the affinity for limulin [15] and it diminished the affinity for LFA and CHA [21,51,52]. Interestingly, these sialic acid-binding lectins do not interact with the N-thioacetyl analogue, which however was shown to bind to OPL I with high affinity. The only other lectin that shares this characteristic is limulin [52].

The charged carboxyl group plays an important role in the interaction of Neu5Ac to OPL I, since its esterification abolished the binding activity. The same effect was reported for the binding to limulin, while the methyl ester retained binding activity to LFA, CHA and TTA [52]. Besides, while the α-anomer of the methyl glycoside is only a little more effective as an inhibitor of OPL I than the *β*-anomer, the axially orientated carboxyl group in the sialic acid α-glycoside is a prerequisite for binding to limulin, CHA and LFA [52].

The 4-hydroxyl group contributes to the binding to OPL I, in contrast with the results reported for CHA and LFA.

The presence of the hydroxyl at C-9 does not seem to be essential for the binding to OPL I and it tolerates substituents at this position. Furthermore, presence of a sulfur atom increases the affinity to the lectin. OPL I is the first sialic acid-binding lectin found to strongly bind these thio-derivatives.

The siglecs, formerly called sialoadhesins, are a family of I-type lectins binding to sialic acids on the cell surface. In contrast to OPL I, all siglecs require the hydroxy group at C-9 for binding and some of them (sialoadhesin, myelin-associated glycoprotein and Schwann cell myelin protein) do not interact with N-glycolylneuraminic acid [53]. These differences are not surprising since they represent a different group of proteins belonging to the immunoglobulin superfamily.

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