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The specificity of the binding site of Achatinin$_H$, a sialic acid-binding lectin from *Achatina fulica* ☆

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Abstract

A sialic acid-binding lectin, Achatinin$_H$ (ATN$_H$), having unique specificity towards 9-*O*-acetylneuraminic acid, has been purified and characterized. The specificity of this lectin for *O*-acetylsialic acids was studied in detail, using various sialic acid derivatives and sialoglycoproteins. The potent inhibition of hemagglutination by bovine submaxillary mucin (BSM), which contains 9(7,8)-*O*-acetylsialic acid and by free 9-*O*-acetylneuraminic acid confirms the preferential affinity towards this sugar. Further support for the role of *O*-acetylsialic acid was obtained by sialidase treatment of BSM. *O*-Deacetylation of the sialic acid residue abolished its inhibitory potency. Moreover, when the trihydroxypropyl side chain of the sialic acid molecule was modified by periodate–borohydride treatment, the truncated C_7-sialic acid was unable to bind ATN$_H$. This result suggests that the glycerol side chain of Neu5Ac, especially the C-8 and/or C-9 portion is an important determinant for ATN$_H$. The hemagglutination–inhibition results using several mono-, di-, and tri-saccharides containing terminal sialic acid and various sialoglycoproteins reveals that ATN$_H$ preferentially binds the α-(2 → 6)-linked sialic acid. Furthermore, β-D-GlcNAc-(1 → 3)-[α-NeuGc-(2 → 6)]-GalNAc-ol was found to be the best ligand for ATN$_H$.

Keywords: Achatina fulica; Lectin, sialic acid-binding

☆ Abbreviations used: ATN$_H$, Achatinin$_H$. BSM, Bovine submaxillary mucin. OSM, Sheep submaxillary mucin. ESM, Equine submaxillary mucin. SNA, *Sambucus nigra* agglutinin. WGA, Wheat germ agglutinin. Gal, D-Galactose. Glc, D-Glucose. GalNAc, 2-Acetamido-2-deoxy-D-glactopyranose. GlcNAc, 2-Acetamido-2-deoxy-D-glucopyranose. Neu5Ac, *N*-Acetylneuraminic acid. Neu5Gc, *N*-Glycolylneuraminic acid. Neu59Ac$_2$, 9-*O*-Acetyl *N*-acetylneuraminic acid. Neu45Ac$_2$, 4-*O*-Acetyl *N*-acetylneuraminic acid. GalNAc-ol, 2-Acetamido-2-deoxy-D-galactitol. Me 9-thioacetamido-α-Neu5Ac, Methyl *N*-acetyl-9-deoxy-9-thioacetamidoneuraminic acid. Benzyl α-Neu5Ac9Pr, Benzyl *N*-acetyl-9-*O*-propanoyl-α-neuraminidic acid. Me 9-acetamido-α-Neu5Ac, 2-α-Methyl 9-acetamido-*N*-acetyl-9-deoxy-α-neuraminidic acid. C_7-NeuAc, 5-Acetamido-3,5-dideoxy-L-*lyxo*-heptulopyranosonic acid.

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1. Introduction

Lectins are multivalent carbohydrate-binding proteins, which are generally assayed as hemagglutinins. These, owing to their high degree of specificity towards glycoconjugates, are employed as important discriminating probes in studies of membranes of normal and transformed cells, in blood typing, in the purification and characterization of carbohydrate-containing biopolymers, and in studies of lymphocyte mitogenesis [1,2]. Sialic acids are known to play an important role in certain biological processes, including malignancy. Such sugar binding-lectins are of current interest [3,4]. The combining sites of a few sialic acid-binding lectins have been identified [5–8], and the site of influenza virus agglutinin has been extensively studied by X-ray crystallography [9]. We have purified and characterized [10–14] a unique sialic acid-binding lectin, ATN_H, from the hemolymph of *Achatina fulica* snail, which exhibits a remarkable preference for $Neu59Ac_2$ [15]. The binding parameters of a few sialic acid derivatives, including di- and tri-saccharides, have been reported [12]. It has been observed that the lectin has a strong affinity towards $Neu59Ac_2$, whereas $Neu45Ac_2$ was completely non-inhibitory [15]. The lectin can selectively agglutinate leukemia patients' erythrocytes but not the erythrocytes from normal human individuals [16]. This study extends our earlier reports on the *O*-acetylsialic acid specificity of ATN_H. The precise specificity of this lectin was measured by using various sialic acid derivatives, sialoglycoproteins, and modified sialoglycoproteins. A direct comparison of the activity of eight oligosaccharides has enabled us to determine a relationship between the structures and their ability to bind ATN_H. Comparison with other sialic acid-specific lectins regarding their affinity towards sialic acid is also described. Our results confirm and provide further evidence that ATN_H utilizes $Neu59Ac_2$ as the primary determinant for attachment to cell-surface receptors. However, the trihydroxypropyl side chain, linking Neu5Ac α-(2 → 6) to GalNAc as well as the *N*-acetyl or *N*-glycolyl group at C-5 of sialic acid also contribute to the lectin–sugar interaction.

2. Materials and methods

General analytical procedures.—Protein was determined by the method of Lowry et al. [17]. Neutral hexose was determined by the phenol–H_2SO_4 method [18]. Sialic acid was estimated by the resorcinol [19] or thiobarbituric acid assay [20,21].

Sugars and glycoproteins.—Fetuin type III, bovine submaxillary mucin type 1S, bovine serum albumin, human chorionic gonadotropin, colominic acid, *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and *C. perfringens* neuraminidase were from Sigma Chemical Co., USA. α_1-Acid glycoprotein, human serotransferrin, human lactotransferrin, *N*-acetyl-α-neuraminyl-(2 → 3)-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose were gifts from Dr. H. Debray. $Neu59Ac_2$ and $Neu45Ac_2$ were provided by Professors R. Schauer and James C. Paulson. Benzyl α-Neu5Ac9Pr, Me 9-acetamido-α-Neu5Ac and Me 9-thioacetamido-α-Neu5Ac were generous gifts from Professor Reinard Brossmer. Compounds listed in Table 4 were

provided by Dr. Angela Savage (compounds **1–4**) and by Professor Dirk H. Van den Eijnden (compounds **5–8**).

Purification of ATN_H.—ATN_H was purified from the hemolymph of *Achatina fulica* snail by affinity chromatography on BSM-Sepharose 4B following the same procedure as previously described [10].

Hemagglutination–inhibition assay (HIA).—Hemagglutination tests [22] were performed by the serial dilution technique in Takatsy microtiter plates with 2% (v/v) rabbit erythrocyte suspension in saline. The inhibition of hemagglutination by various inhibitors was studied as follows. Aliquots of the various sugars (25 μL), were serially diluted with 25 μL of saline in microtiter trays. A constant amount of Ca^{2+} (25 μL, 0.12 M) and 25 μL of ATN_H containing 16 minimum hemagglutination units were then added to each well. After incubation for 1 h at 25°C, 25 μL of rabbit erythrocyte suspension (2% in saline) was added and the wells were examined after 1 h. Results were expressed as the minimum concentration (mM) of each sugar required for 50% inhibition of hemagglutination. Results are presented as the mean of at least three experiments.

O-*Deacetylation of BSM.*—*O*-Deacetylation of sialic acid of BSM was performed following the procedures of Sarris and Palade [23] and Schauer [24]. A solution of BSM (0.75 mL, 5 mg/mL) was added to 0.4 M NaOH (0.25 mL), incubated on ice for 45 min, and then neutralized with 0.1 M HCl. Sialic acid was measured after acid hydrolysis (0.05 M H_2SO_4, 1 h at 80°C) or neuraminidase treatment of glycoproteins.

Periodate oxidation of glycoproteins.—Modified glycoproteins carrying the 7-Neu5Ac residue were prepared from BSM and OSM [25]. After *O*-deacetylation of the sialic acid moiety of BSM, BSM, and OSM (4 mg/mL each) were oxidized with 10 mM $NaIO_4$ in 0.05 M sodium acetate buffer (pH 5.0) at 4°C for 32 min. Excess periodate was decomposed with 20% glycerol and the mixture was dialyzed against distilled water. The oxidation product was reduced with excess $NaBH_4$ (10 mg in 0.2 M sodium borate buffer, pH 8.2) at 4°C for 3 h and dialyzed against distilled water.

Sialidase treatment of glycoproteins.—Sialoglycoproteins (BSM/OSM, 2 mg) were treated with *C. perfringens* neuraminidase (0.1 Unit) in 5 mM cacodylate buffer (pH 5.5, 0.4 mL) for 20 h at 37°C [26]. This experiment was also carried out at different time intervals ranging from 0–120 min. As a control, BSM/OSM were treated similarly but without enzyme.

3. Results

To understand the nature of the binding specificity of ATN_H, hemagglutination–inhibition experiments were carried out with a variety of sialoglycoproteins, as shown in Table 1. BSM was the only glycoprotein that showed strong inhibition of ATN_H. Other sialoglycoproteins showed very weak inhibition, although they have been used at very high concentrations. Specifically, this was very interesting for OSM, since OSM has a similar sialic acid content to that of BSM. Both BSM and OSM have the common sequence, α-Neu5Ac-(2 → 6)-GalNAc-Thr/Ser as their predominant oligosaccharide chain. However the major difference between the two mucins is the type of sialic acid

Table 1
Inhibition [a] of ATN_H hemagglutinin by various sialoglycoproteins

Sialoglycoproteins (MW)	Type of glycosidic linkages	Sialic acid concentration (mM)	Relative inhibitory potency (%)	Nature of linkages
BSM (1960000–2750000)	O	0.0002	100	α-Neu5Ac-(2 → 6)-β-D-GalNAc
Asialo-BSM			Nil	
OSM (1300000)	O	0.02	1	α-Neu5Ac-(2 → 6)-β-D-GalNAc
Asialo-OSM			Nil	
Fetuin (48000)	O and N	0.005	4	α-Neu5Ac-(2 → 6)-β-D-Gal
Asialo-fetuin			Nil	
α_1-Acid glycoprotein (44000)	N	0.09	< 1	α-Neu5Ac-(2 → 6)-β-D-Gal α-Neu5Ac-(2 → 3)-β-D-Gal
Human choriogonadotropin (33000)	O and N	3.91	< 1	α-Neu5Ac-(2 → 3)-β-D-Gal
Serotransferrin (80000)	N	0.42	< 1	α-Neu5Ac-(2 → 6)-β-D-Gal
Lactotransferrin (76000)	N	0.46	< 1	α-Neu5Ac-(2 → 6)-β-D-Gal

[a] The minimal concentration of sialoglycoproteins required for total inhibition induced by 16 hemagglutination units of ATN_H are shown. Values are expressed in mM of sialic acid. Considering the inhibitory potency of BSM as 100, the relative inhibitory potency of different sialoglycoproteins and their type and nature of linkages are listed.

Table 2
Inhibition [a] of ATN_H by different modified and native BSM and OSM

Glycoprotein treatment	HIA titer
BSM	256
BSM (periodate treated)	4
BSM (periodate and borohydride treated)	4
OSM	4
OSM (periodate and borohydride treated)	Nil
BSM (NaOH treated)	Nil

[a] Details of treatment are given in Materials and Methods. Titers are expressed as the highest dilution of sialoglycoproteins (1 mg/mL) required for complete inhibition of 16 hemagglutination units of ATN_H.

present. BSM contains mainly 9-*O*-acetyl and 8,9-di-*O*-acetyl-Neu5Ac, whereas OSM contains only Neu5Ac. Among the sialoglycoproteins tested in Table 1, only BSM is known to contain *O*-acetylated sialic acids. Since BSM is 100-fold more inhibitory than OSM, the inhibitory activity is solely dependent on the presence of $Neu59Ac_2$ and Neu5Gc9Ac (25 and 5%) in BSM. It could be stated that BSM is ca. 330-fold more inhibitory than OSM based on the 9-OAc concentration. BSM and OSM were enzymatically as well as chemically modified to determine the possible role of OAc-Neu5Ac in their potent inhibition of hemagglutination. BSM was treated with sialidase at different time intervals (0–60 min) in cacodylate buffer. After 15 and 30 min, a 4-fold and 64-fold decrease in inhibitory potency was observed, respectively. The 60-min treatment completely destroyed its inhibitory activity (data not shown). When BSM was treated with base, it lost its inhibitory property (Table 2) because of specific hydrolysis of the *O*-acetyl groups of sialic acid without cleavage of the peptide bond [24]. These observations suggest that OAc-Neu5Ac probably plays a very important role in the interaction of BSM with ATN_H.

For further confirmation, a hemagglutination–inhibiton study was carried out with free Neu5Ac, Neu5Gc, $Neu59Ac_2$, and $Neu45Ac_2$ (Table 3). It was found that $Neu59Ac_2$ displayed the highest inhibitory potency whereas $Neu45Ac_2$ was non-inhibitory up to a concentration of 100 mM. These observations suggest that binding was mediated by $Neu59Ac_2$ and not through $Neu45Ac_2$. Neu5Gc was 1.58 times stronger than Neu5Ac ($p = 0.01$). However, both compounds are respectively 23 and 15 times less potent than $Neu59Ac_2$. The presence of an acetamido or thioacetamido or propanoyl group at C-9 of the sialic acid molecule decreased their inhibitory potency by 17, 33, and 13 times respectively as compared to $Neu59Ac_2$. 9-Acetamido and 9-propanoyl Neu5Ac were found to be 1.4 and 1.8 times less active as compared to Neu5Ac.

Following periodate and borohydride treatment, the truncated C_7-Neu5Ac did not inhibit ATN_H. Likewise, such sugars as D-galactose, D-glucose, and D-glucosamine did not inhibit hemagglutination at concentrations upto 100 mM. All of these results suggest that ATN_H was sialic acid-specific, with high affinity for the *O*-acetylated derivative. Colominic acid, a linear homopolymer of α-(2 → 8)-linked Neu5Ac also inhibited the hemagglutination activity of ATN_H.

Selective modification of the glyceryl side chain (C-7–C-9) of Neu5Ac residues in BSM and OSM was performed to determine the contribution of this portion of the

Table 3
Inhibition [a] of ATN_H by simple monosaccharides

Monosaccharide		I_{50} (mM)
1	Neu5Ac	30.48
2	Neu5Gc	19.20
3	$Neu59Ac_2$	1.3
4	Me 9-thioacetamido-α-Neu5Ac	43.70
5	Benzyl α-Neu5Ac9Pr	17.06
6	Me 9-acetamido-α-Neu5Ac	22.00
7	Colominic acid (mg/mL)	2.5
8	$Neu45Ac_2$	NI [b]
9	C_7-Neu5Ac	NI
10	D-Galactose	NI
11	D-Glucose	NI
12	D-Galactosamine	NI

[a] The minimal concentration of the monosaccharides required for 50% inhibition of 16 hemagglutination units of ATN_H are shown. The abbreviations used are described elsewhere.
[b] Not inhibited up to a concentration of 100 mM.

molecule to the binding affinity of ATN_H. The glycol group between C-7–C-8 and C-8–C-9 of the Neu5Ac residue in BSM and OSM were selectively cleaved using mild periodate oxidation followed by reduction of the C-7 aldehyde group to a primary alcohol. Both of these modified sialoglycoproteins lost their inhibitory activity, as shown in Table 2. Inhibitory potency was reduced 4-fold with C_7-Neu5Ac-containing BSM as compared with native BSM. However, oxidation of BSM in the presence of 920 mM periodate for a longer time (3 h), resulted in failure to bind to ATN_H. This was further confirmed by the fact that free C_7-Neu5Ac (Table 3) was completely inactive towards ATN_H binding. Both of these results strongly suggest that the glycerol moiety of the Neu5Ac unit plays an important role in the interaction with ATN_H. The cleavage of this portion of the molecule drastically decreased the affinity of the entire sugar moiety with ATN_H.

The inhibition data of various oligosaccharides having different sialic acid derivatives are presented in Table 4. The inhibitory activity decreased in the order β-D-GlcNAc-

Table 4
Inhibition [a] of ATN_H by oligosaccharides containing terminal sialic acid

Oligosaccharide		I_{50} (mM)
1	α-Neu5Ac-(2 → 6)-GalNAc-ol	4.88
2	α-Neu5Gc-(2 → 6)-GalNAc-ol	3.88
3	β-D-GlcNAc-(1 → 3)-[α-Neu5Ac-(2 → 6)]-GalNAc-ol	5.86
4	β-D-GlcNAc-(1 → 3)-[α-Neu5Gc-(2 → 6)]-GalNAc-ol	0.452
5	β-Gal-(1 → 3)-[α-Neu5Gc-(2 → 6)]-GalNAc-ol	0.58
6	α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3)-GalNAc-ol	20.70
7	α-Neu5Ac-(2 → 3)-β-Gal-(1 → 4)-Glc	25.00
8	α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3)-[α-Neu5Ac-(2 → 6)]-GalNAc-ol	3.88

[a] The mimimal concentration of the compounds required for 50% inhibition induced by 16 hemagglutination units of ATN_H are shown. Details of the assay are described in Materials and Methods.

(1 → 3)-[α-Neu5Gc-(2 → 6)]-GalNAc-ol > β-Gal-(1 → 3)-[α-Neu5Gc-(2 → 6)]-GalNAc-ol > α-Neu5Gc-(2 → 6)-GalNAc-ol = α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3)-[α-Neu5Ac-(2 → 6)]-GalNAc-ol > α-Neu5Ac-(2 → 6)-GalNAc-ol > β-D-GlcNAc-(1 → 3)-[α-Neu5Ac-(2 → 6)]-GalNAc-ol > α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3)-GalNAc-ol > α-Neu5Ac-(2 → 3)-β-Gal-(1 → 4)-Glc. Of the disaccharides tested, α-Neu5Gc-(2 → 6)-GalNAc-ol was 1.25 times more potent than α-Neu5Ac-(2 → 6)-GalNAc-ol. Both oligosaccharides **4** and **5** are 8 and 7 times more potent inhibitors than oligosaccharide **2**. Among the trisaccharides tested, β-D-GlcNAc-(1 → 3)-[α-Neu5Gc-(2 → 6)]-GalNAc-ol (compound **4**) was the best inhibitor, which was found to be 13 times more potent than β-D-GlcNAc-(1 → 3)-[α-Neu5Ac-(2 → 6)]-GalNAc-ol (compound **3**). Replacement of GlcNAc by Gal decreased the potency 1.30 fold, as shown by β-Gal-(1 → 3)-[α-Neu5Gc-(2 → 6)]-GalNAc-ol (compound **5**) ($p = 0.008$). α-Neu5Ac-(2 → 3)-β-Gal-(1 → 3)-GalNAc-ol (compound **6**) was 45 times and α-Neu5Ac-(2 → 3)-β-Gal-(1-4)-Glc (compound **7**) was 55 times weaker inhibitors than β-D-GlcNAc-(1 → 3)-[α-Neu5Gc-(2 → 6)]-GalNAc-ol (compound **4**). Moreover the presence of a second sialic acid molecule (compound **8**) decreased its inhibitory potency 7 fold as compared to β-Gal-(1 → 3)-[α-Neu5Ac-(2 → 6)]-GalNAc-ol, which may sterically hinder the above trisaccharide.

4. Discussion

The most important feature in the carbohydrate-binding property of ATN_H is the strong preferential affinity of the lectin for the 9-*O*-acetyl group on Neu5Ac. This finding is supported by the decrease in inhibition pattern when the hydroxyl group at C-9 is replaced by an acetamido (compound **6**), thioacetamido (compound **4**), or propanoyl (compound **5**) group, or the presence of a simple hydroxyl group (compound **1**) (Table 3). It is also confirmed by the profound inhibitory potency of BSM (Table 1) having 9,(7,8)-*O*-acetyl-Neu5Ac, which is completely lost following its *O*-deacetylation (Table 2). This strongly suggests that the *O*-acetyl group may play an important role in the lectin–sugar interaction. The unique property of this lectin was further shown by its non-reactivity towards $Neu45Ac_2$. This corroborates our previous findings that ATN_H can agglutinate only those erythrocytes (rabbit, guinea pig, hamster, and rat) which contain a $Neu59Ac_2$ residue, but does not react with horse erythrocytes containing $Neu45Ac_2$. Moreover, ATN_H is incapable of agglutinating sheep, goat, or human erythrocytes that contain only Neu5Ac but not $Neu59Ac_2$. This restricted specificity of binding is further confirmed by the inhibition with gangliosides that are known to contain OAc-Neu5Ac [15]. Collectively, all this evidence strongly suggest that an acetyl group at C-9 contributes positively towards binding.

Free Neu5Ac occurs principally as the β anomer in solution. Other inhibitors (Table 3) including compounds **4**, **5**, and **6** are in the α-anomeric form. In all these monosaccharides (compounds **1**, **4**, **5**, and **6**), the sialic acid is not *O*-acetylated. Most probably the *O*-acetyl function is more important than the anomeric specificity of the sugar. However, interpretation of the results with monosaccharides is somewhat restricted because of the non-availability of Me α-Neu5Ac and Me α-$Neu59Ac_2$, which would

have served as ideal reference compounds for the inhibition study. The lower potency of 9-acetamido-Neu5Ac as compared with $Neu59Ac_2$ may be due to the extra lone pair of electrons on the oxygen and nitrogen, which are sp^3 hybridized. Free Neu5Ac exists largely in the β-anomeric form (95%), and most probably $Neu59Ac_2$ is also in the β-anomeric form (it is less likely that further acetylation at C-9 will influence its anomeric preference). Therefore, C-2 does not contribute to the binding and derivatization at C-9 is very sensitive. Acetylation at O-9 plays a crucial role, and not its anomeric specificity.

Oxidative cleavage of the C-7–C-8 and C-8–C-9 of the Neu5Ac residue in BSM and OSM, followed by reduction with sodium borohydride, completely destroyed their ability to bind with ATN_H (Table 2). Free C_7-Neu5Ac was also unable to bind ATN_H (Table 3). These observations strongly suggest that the glycerol side chain of Neu5Ac is directly involved in binding with ATN_H.

In addition to the $Neu59Ac_2$ binding-specificity demonstrated earlier, there are other aspects of ATN_H specificity. As may be seen in Table 3, $Neu59Ac_2$ is a 23- and 15-fold better inhibitor for ATN_H than Neu5Ac and Neu5Gc. However Neu5Gc is only 1.6 times better than Neu5Ac. These results suggest that the Neu5Gc9Ac derivative might have been a better inhibitor than $Neu59Ac_2$. Unfortunately, this derivative was not available for our study. These data indicate that both the glycolyl group at C-5, and the acetyl group at C-9, play an important role in lectin–sugar interaction, presumably because of additional Van der Waals interactions or hydrogen bonding between the combining site of ATN_H and the acetyl/glycolyl group, or potentiation of the existing one.

Another aspect of binding becomes most significant when sialic acid is attached in an α-(2 → 6)-linkage to GalNAc in the appropriate disaccharide. It has been observed that extension of Neu5Ac and Neu5Gc with α-(2 → 6)-GalNAc-ol (in compounds **1** and **2**) increased their binding potency 6- and 5-fold, respectively (Table 4). This may be due to the extended binding-pocket of the lectin. Unfortunately we were unable to obtain α-$Neu59Ac_2$-(2 → 6)-GalNAc, which would have allowed us to determine the level of potency. However BSM, having the α-Neu5Ac-(2 → 6)-β-GalNAc sequence showed 24 400 times more inhibition than compound **1** in our binding assay (Table 4). However this reult may be explained by the fact that glycoproteins in general, because of their multiplicity of interaction, are strong inhibitors. A 23-fold increase in inhibitory potency due to the introduction of a 9-*O*-acetyl group to Neu5Ac may not be extrapolated to the case of BSM vs. OSM (Table 1). A 330-fold higher potency of BSM over OSM is not unexpected even though the affinity arises mainly due to presence of 9-*O*-acetyl groups. Therefore, it is apparent from other results (Table 4) that the binding affinity may not be exclusively dependent on the 9-OAc derivative. Further addition of β-D-GlcNAc-(1 → 3) or β-Gal-(1 → 3) (compounds **4** and **5**) to the reducing end of the GalNAc molecule improved the potency by 8.6 and 6.7 fold, respectively, indicating the presence of an extended binding-pocket rather than a disaccharide, which accommodates the trisaccharide. It has been observed for many other lectins that disaccharides and trisaccharides bind more strongly than monosaccharides [6,7].

Additional important information comes from compounds **6** and **7** that have α-(2 → 3)-linkages. Compounds **6** and **7** are 45 and 55 times less potent than compound **4** that

possesses an α-(2 → 6)-linkage (Table 4). In fact, all of the oligosaccharides (compounds **1–5**) are better inhibitors than compounds **6** and **7**. This suggests that the α-(2 → 3)-linked sialic acid is less favorable than the corresponding α-(2 → 6)-linkage. All of these observations indicate that, although none of the oligosaccharides listed in Table 4 are substituted at either C-4 or C-9 of sialic acid, compounds **4** and **5** are better inhibitors than $Neu59Ac_2$. This observation clearly indicates the presence of a more extended oligosaccharide having a α-Neu5Ac-(2 → 6)-Gal linkage, with which it interacts. The marked preference of ATN_H for the α-Neu5Ac-(2 → 6)-Gal sequence as compared to α-Neu5Ac-(2 → 3)-Gal may be explained in two ways. First, substitution at the 3-hydroxyl group of the galactosyl residue by Neu5Ac in the α-(2 → 3)-linked isomer may decrease the affinity of this compound significantly [6]. Second, an NMR study revealed that there are significant differences among the three-dimensional arrangements of the sialic acid and galactosyl residues in the oligosaccharides containing (2 → 3)- or (2 → 6)-linkages [27]. It is possible that the (2 → 6)-linked isomer can assume a conformation that enables better contact with the binding site of ATN_H.

When compared to other sialic acid-binding lectins, the carbohydrate binding behavior of ATN_H resembles that of the influenza C virus [9,28,29], which is specific for $Neu59Ac_2$. ATN_H partially resembles marine crab lectin (*Cancer antennaris*) [26], which is specific for both 4-OAc and 9-OAc-Neu5Ac [26]. 4-*O*-Acetylation of Neu5Ac has no effect on ATN_H binding. On the other hand, ATN_H differs totally from limulin, the American horseshoe (*Limulus polyphemus*) crab (because it has an obligatory need for a free 4-hydroxyl group of sialic acid). 4-*O*-Acetylation interferes with the limulin–sialic acid interaction [5]. ATN_H also differs from influenza A and B viruses in that these viruses do not bind to $Neu59Ac_2$ [9]. Lectins from *Escherichia coli* [30] and Indian scorpion [31] (*Heterometrus granulomanus*) show a higher affinity with $Neu45Ac_2$ that is markedly reduced by 7 or 9-*O*-acetylation.

The possible role of *O*-acetylsialic acid in ATN_H binding was further confirmed by inhibition with sialoglycoproteins. ATN_H showed strongest inhibition with BSM, as has been observed with influenza C virus [9], marine crab lectin [26], the American [5] and Japanese horseshoe crab [32] (*Tachypleus tridentatus*) lectin, lobster [33] (*Homorus americanas*), and slug lectins [34] (*Limux flavus*). However, this is not the case for Indian scorpion lectin. On the contrary, both ATN_H and influenza C virus are not inhibited by equine submaxillary mucin (ESM, containing predominantly $Neu45Ac_2$), whereas marine crab lectin, limulin, and carcinoscorpin [35] (*Carcinoscorpius rotunda-cauda*) are strongly inhibited by ESM.

The proposed specificity of ATN_H correlates well with its agglutinating behavior with erythrocytes of different species. ATN_H, like influenza C virus and marine crab lectin, can agglutinate only those erythrocytes (rabbit, guinea pig, hamster, and rat) which are known to contain mainly $Neu59Ac_2$. Unlike crab lectin, both ATN_H and influenza C virus do not bind to horse erythrocytes. In contrast, other reported sialic acid-binding lectins (e.g., limulin [5], as well as lectins isolated from Indian scorpion [31], Japanese horseshoe crab [32], lobster [33], sea slug [34] and influenza A and B viruses [9]) can agglutinate erythrocytes from many different species.

The exocyclic portion of Neu5Ac is absolutely essential for ATN_H binding. In this respect ATN_H resembles *Sambucus nigra L.* agglutinin [6] (SNA), *Cepaea hortensis*

lectin [8], and *Pila globosa* lectin [36]. Base treatment of BSM, abolished their inhibitory activity but had no effect for limulin [5], sea slug [34], or marine crab [26] lectin. Considering the influence of the *O*-acetyl group on the binding affinity of crab lectin, the binding mechanism appears complex. The binding of wheat-germ agglutinin (WGA, *Triticum vulgaris*) is hindered by the presence of the exocyclic part of Neu5Ac [37]. In fact, increased binding of WGA to the C_7 analogue of Neu5Ac has been reported, which is in contrast to ATN_H, SNA, and influenza virus agglutinin, which were completely inhibitory to this analogue.

ATN_H, like limulin, carcinoscorpion, and *Pila globosa* lectin has a higher binding-affinity with Neu5Gc than with Neu5Ac. In contrast to marine crab and *Cepaea hortensis* lectin, ATN_H and influenza C virus [38] showed lower reactivity with 9-acetamido-Neu5Ac compared to $Neu59Ac_2$. ATN_H as SNA [6], limulin [5], and carcinoscorpin [35] has a greater affinity with sialic acids linked to α-(2 → 6)-Gal/GalNAc as compared to the α-(2 → 3)-linked isomer. In contrast to ATN_H, the *Maackia amurensis* lectin [7] is strictly specific for the α-(2 → 3)-linked isomer. Influenza A and B virus [28] do not bind to α-(2 → 6)-linked GalNAc.

A chemical-modification study [11] indicates that tryptophan, histidine, lysine, aspartic acid, and glutamic acid residues of ATN_H are involved in hydrogen bonding with sialic acid, and this stabilizes the binding with ATN_H. Similar results are reported for influenza virus with its receptor molecule [9].

These comparisons lead to two very important suggestions. One is that influenza C virus and ATN_H can complement each other in the study of sialoglycoproteins. The second is that ATN_H and SNA can complement each other in the studing of the nature of sialic acid on mucin-type glycan chains. As ATN_H is useful in recognition of the expression of OAc sialoglycoproteins on the erythrocyte surface of leukemic patients, it could be used as a specific reagent in screening patients erythrocytes.

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