Chapter 2
RM2 Antigen: Structural Characterization and Determination of $K_{D,Surf}$ for Multivalent Carbohydrate–Protein Interaction

2.1 Introduction

2.1.1 A Novel Ganglioside (RM2) Isolated from Renal Cell Carcinoma

Gangliosides are glycosphingolipids (GSL) containing sialic acids and are present in the outer leaflet of plasma membranes. GSL expression is a complex phenomenon, and pathological or abnormal expression at the tumor cell surface must be defined as the ability of the tumor cell to interact with specific target cell. This interaction may thus promote metastasis through the lymphatic or blood circulatory system.

Renal cell carcinoma (RCC) has been characterized by high expression of disialogangliosides when enhanced expression of higher gangliosides is correlated with the metastatic potential. Two RCC cell lines, TOS-1 and ACNH, derived from its metastatic deposits are characterized by high expression of disialogliosides. Initially, disialosylgalactosylgloboside (DSGG) was identified previously as one of the three types of major disialogangliosides from RCC tissues. However, further studies indicated that DSGG is the major disialoganglioside of ACHN cell lines, but absent from TOS-1 cell lines [1].

The proposed structure of RM2 can be easily confused with DSGG antigen because (1) RM2 and DSGG are indistinguishable on high performance thin-layer chromatography (TLC) with similar and overlapping relative retention factor (Rf); and (2) the RCC expresses many types of disialogangliosides and other globo-series monosialogangliosides, leading to varying proportions of globo-series and ganglio/lacto-series antigens (Fig. 2.1) [2]. Above all, mAb RM2 was observed to react strongly only with the novel TOS-1 heptaglycosylceramide RM2 but not significantly with DSGG. Besides, the DSGG was purified from metastatic renal carcinoma cell line, ACNH. ACNH is the immunogen used to generate mAb 5F3, which was specific for DSGG and not cross-reactive with the RM2 antigen.

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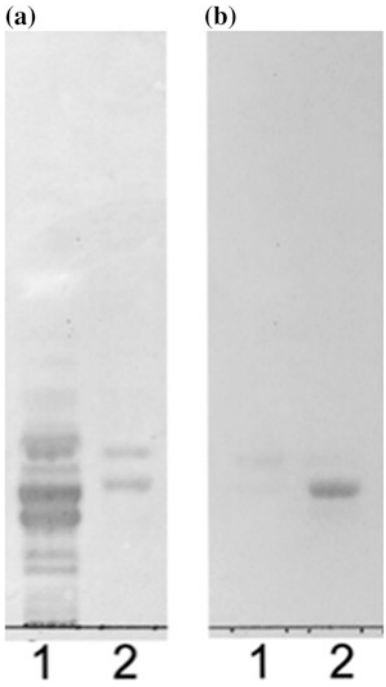


Fig. 2.1 TLC immunostaining of TOS-1 and ACHN disialogangliosides. Lane 1, disialosyl fractions of TOS-1 gangliosides. Lane 2, disialosyl fractions of ACHN gangliosides. *Panel A* mAb RM2 immunostaining. *Panel B* mAb 5F3 immunostaining. Adapted from [2], copyright 2015, with permission from Springer Science+Business Media

On the other hand, this internal hexasaccharide RM2 without the GlaNAc residue is identical to α(2,3)/α(2,6)-disialyl lactotetraose (DSLc4), which was observed as a colon cancer-associated antigen by using a monoclonal antibody (FH9) [3] for siglec-7, which is expressed in natural killer (NK) cells and is able to affect NK activity through binding [4]. To further verify the proposed structure of the RM2 antigen, we chemically synthesized the proposed hexasaccharide **1-1** and evaluated its recognition by monoclonal antibody RM2.

2.2 Characterize the Structure of RM2 Antigen by Monoclonal RM2 Antibody and Further Determine the $K_{D,Surf}$ by Carbohydrate–Protein Interaction

2.2.1 Structural Characterization of RM2 Antigen

To confirm that the synthesized hexasaccharide **1-1** was indeed the RM2 antigen and to further understand the antibody binding specificity, we prepared an expanded glycan array containing the synthesized hexasaccharide **1-1** and its analogs along with the other 90 glycans prepared in our laboratory to form a glycan array for testing (Fig. 2.6). All glycans were directly immobilized onto NHS-coated glass

slides by taking an aliquot from a stock solution of sugar at a fixed concentration (100 μM).

The assay involved an initial treatment with RM2 (a mouse IgM anti-RM2 monoclonal antibody, a kind gift from Prof. Saito), followed by incubation with a fluorescein-tagged anti-mouse IgM secondary antibody against its primary antibody. After 1 h incubation, the slides were washed with ddH_2O twice and scanned to show the binding specificity of the antibody to printed oligosaccharides spots. The resulted images showed that monoclonal RM2 antibody recognize hexasaccharide **1-1** specifically, indicating that the synthetic hexasaccharide **1-1** contains the same antigenic epitope with which RM2 antibody reacts on prostate cancer cells (Fig. 2.2); therefore, we concluded that the synthesized hexasaccharide **1-1** was indeed the RM2 antigen. Moreover, the structure assignment of the synthesized hexasaccharide **1-1** is confirmed by both 1D and 2D-NMR (Fig. 2.3 and Table 2.1).

2.2.2 *Determination of* $K_{D,Surf}$ *for Multivalent Carbohydrate–Protein Interaction on the Surface*

To determine the dissociation constants of RM2 and truncated analogs on the surface interacting with antibodies in a multivalent manner, we utilized the direct measurement method developed in our lab [6], adopting the different concentrations of antibodies and printed sugars. The binding curves were analyzed as Langmuir isotherms, assuming that the system reached equilibrium during incubation,

$$F_{\text{obs}} = F_{\max}[P]/\left(K_{\text{D,surf}} + [P]\right)$$

where $F_{\max}$ is the maximum fluorescence intensity, which is a measure of the amount of active carbohydrate on the surface, $[P]$ is the total antibody concentration, and $K_{\text{D,surf}}$ is the equilibrium dissociation constant for surface carbohydrate and antibody. Each array was printed at five different concentrations with sugars from 100 to 6.25 μM (100, 50, 25, 12.5, and 6.25 μM). The arrays were incubated with 12 concentrations of diluted anti-RM2 antibody, ranging from 25 to 0.06 nM, in combination with the Cy3-labeled goat anti-mouse IgM secondary antibody. After washing, the fluorescence intensity generated the saturated curve for dissociation constant ($K_{\text{D,surf}}$) determination. The results showed that when the printing concentration was below 6.25 μM, the average distance between printed sugars was too far to have enough multivalent effect with antibody RM2. At printing concentrations from 100 to 6.25 μM, however, we observed that the $K_{\text{D,surf}}$ values measured were narrowly distributed from the individual curves (Fig. 2.4 and Table 2.2). Using the same method, the $K_{\text{D,surf}}$ values were determined for the truncated RM2 analog **1-53** interacting with antibodies (Fig. 2.5 and Table 2.3). Overall, the relative binding specificity of RM2 for the epitopes was RM2 > pentasaccharide **1-53** (Table 2.4).

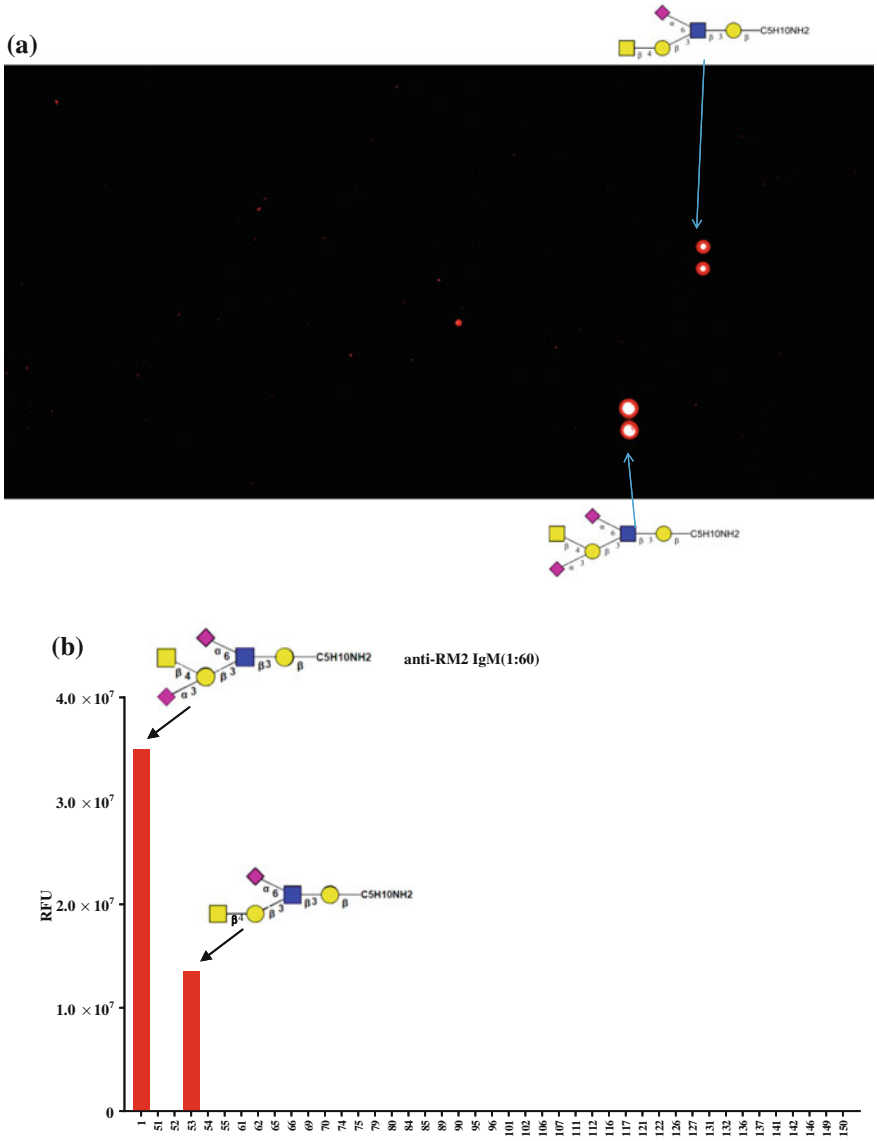


Fig. 2.2 Binding of monoclonal antibodies to RM2 and truncated sequences**. a** Slide image obtained from fluorescence scan after antibody incubation assay with RM2. **b** Binding specificity of monoclonal antibodies RM2 to RM2 antigen and its fragments. Adapted from [5], copyright 2015, with permission from American Chemical Society

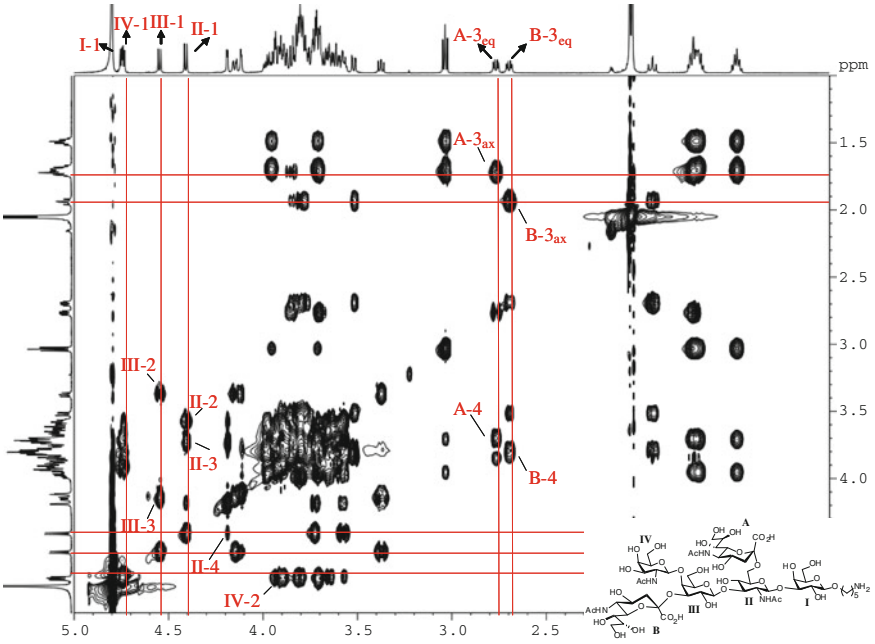


Fig. 2.3 Two-dimensional TOCSY NMR spectra analysis of synthetic RM2 antigen in D_2O at 298 K. Adapted from [5], copyright 2015, with permission from American Chemical Society

Table 2.1 Proton chemical shift, $^3J_{1,2}$ coupling constant (Hz), and ^3J(C-1,H-3$_{ax}$) (Hz) for synthetic RM2 in D_2O at 298 K. Adapted from [5], copyright 2015, with permission from American Chemical Society

Synthetic RM2 NeuAcα2 → 6
GalNAcβ1 → 4 GlcNAcβ1 → 3Galβ1 → linker
NeuAcα2 → 3Galβ1 → 3

H-1 ($^3J_{1,2}$)			4.74 (8.4)	4.54 (7.9)	4.40 (8.0)	4.75 (8.5)
H-2			3.909	3.375	3.576	4.119
H-3	2.69(eq) 1.93(ax)	2.76(eq) 1.72(ax)	3.826	4.150	3.726	
H-4	3.799	3.699			4.191	
H-5						
H-6						
NAc	2.058	2.057	2.045		2.045	
^3J(C-1,H-3$_{ax}$) [Hz]	**5.60**	**10.60**				

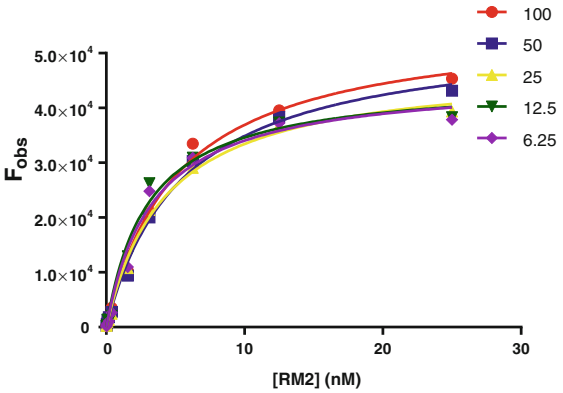


Fig. 2.4 Binding curves for RM2 printed at different concentrations (100, 50, 25, 12.5, and 6.25 μM). The *curves* were obtained using Cy3-labeled goat anti-mouse IgM secondary antibody. Adapted from [5], copyright 2015, with permission from American Chemical Society

Table 2.2 Surface dissociation constants ($K_{D,surf}$) of antibody RM2 and RM2 on microarray

Printing concentration RM2, μM	F_{max}	$K_{D,surf}$, nM
100	55,756	5.104
50	53,982	5.479
25	47,714	4.277
12.5	45,028	3.140
6.25	45,483	4.724

Adapted from [5], copyright 2015, with permission from American Chemical Society

$K_{D,surf}$ values (nM) for RM2 concentrations against fluorescent intensity at different concentrations of printed sugar

Fig. 2.5 Binding curve for RM2 antibody for **1-53** printed at different concentrations. Adapted from [5], copyright 2015, with permission from American Chemical Society

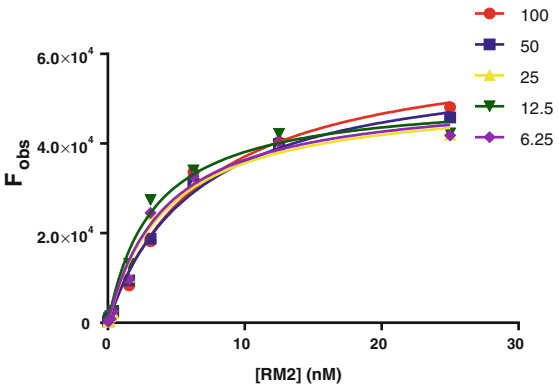


Table 2.3 Surface dissociation constants ($K_{D,surf}$) of antibody RM2 and **1-53** on microarray

Printing concentration RM2, μM	F_{max}	$K_{D,surf}$, nM
100	62,859	6.962
50	59,045	6.440
25	50,970	4.496
12.5	50,900	4.047
6.25	51,597	5.127

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$K_{D,surf}$ values (nM) for RM2 concentrations against fluorescent intensity at different concentrations of printed sugar

Table 2.4 $K_{D,surf}$ (nM) values of different antibodies and different RM2 analogs

	$K_{D,surf}$ (nM) ± SD (nM)	
Antibody	RM2 (**1**)	Pentasaccharide, **1-53**
RM2	4.545 ± 0.903	5.756 ± 1.140

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2.3 Summary

With these synthetic carbohydrates in hand, we used these glycans combined with other related glycans to form a 96-member glycan microarray to study the specificity of monoclonal RM2 antibody and confirmed the structure of our synthetic RM2 hexasaccharide antigen. Moreover, we also used the glycan array to determine the surface dissociation constant with monoclonal antibody RM2. The $K_{D,surf}$ values determined for the RM2 antigen interacting with antibodies was 4.5 nM. Overall, the relative binding specificity of RM2 for the epitopes was RM2 > pentasaccharide **1-53**.

2.4 Experimental Section

Glycan Microarray Fabrication Microarrays were printed (BioDot; Cartesian Technologies) by robotic pin (SMP3; TeleChem International Inc.) deposition of ~0.7 nL 100 μM amine-containing glycans in printing buffer (300 mM phosphate buffer, pH 8.5, containing 0.01 % TritonX-100) from a 14-well microtiter plate onto N−Hydroxysuccinimide (NHS)-coated glass slides. Subsequently, a 96-glycan microarray (Fig. 2.6) was used to determine the polyclonal Ab spectrum of DT-RM series–induced antiserum. Printed slides were allowed to react in an atmosphere of 80 % humidity for 1 h followed by desiccation overnight. These slides were stored at room temperature in a desiccator prior to use.

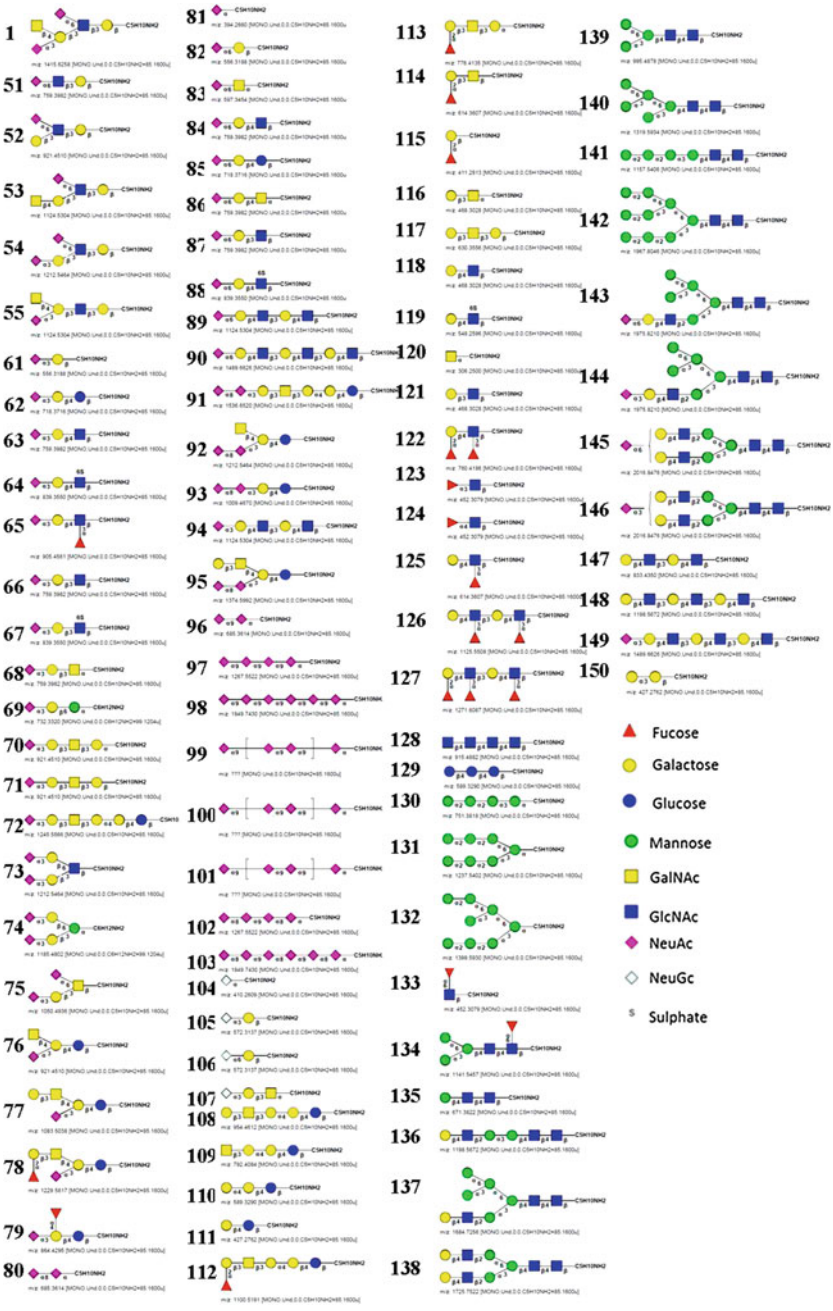


Fig. 2.6 Chemical structures of 96 various oligosaccharides for glycan array chip

Determination of Surface Dissociation Constant The slides were spotted with solutions of RM2 **1-1** and **1-53** with concentrations 100, 50, 25, 12.5, and 6.25 μM two rows from top to bottom with five replicates horizontally placed in each grid. The glycan microarray was blocked with 50 mM ethanolamine for 1 h at 0 °C and washed thrice with PBST buffer before use. The monoclonal RM2 antibody was diluted with 3 % BSA/PBST buffer (PBST buffer: PBS and 0.05 % Tween-20, pH 7.4). Next, Cy3-conjugated goat anti-mouse IgM antibody was added into the diluted solution to precomplexed with primary antibody. The precomplexed solution was applied to each well and incubated at 0 °C for 1 h in the dark. Finally, the slides were washed by PBST washing buffer and ddH_2O in sequence. The slides were spin-dried for 5 min before scanning at 635 nm with a microarray fluorescence chip reader (GenePix 4300A; Molecular Devices Corporation). Scanned images were analyzed with GenePix Pro-6.0 analysis software (Axon Instruments, Union City, CA, USA).

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