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Viable Strategy for Screening the Effects of Glycan Heterogeneity on Target Organ Adhesion and Biodistribution in Live Mice

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This work represents the first broad study of testing diverse heterogenous glycoconjugates (7 different glycoalbumins) for their differential in vivo binding (11 different cancer cell types) in both cell- and animal-based studies. As a result, various changes to biodistribution, excretion, and even tumor adhesion was identified.

On the surface of most cells is a carbohydrate-enriched coating known as the glycocalyx.[1] Due to the wide variance in glycoprotein/glycolipid/proteoglycan assemblies among differing cell types, the glycocalyx is known to play crucial roles for cell-to-cell interactions, differentiation, intracellular trafficking, and immune modulation.[2] One key component that facilitates these interactions are lectins, a class of carbohydrate-binding proteins. Individually, lectin-glycan interactions are poor (millimolar K_D level), such that their one-to-one interactions have little biological selectivity. However, due to the enormous presence of lectin isoforms,[3] the combined interactions of clustered (two or more) sugars allows for strong and selective cell binding in nature; a phenomena referred in this paper as a "glycan pattern recognition" (Fig 1).

Exploiting the glycocalyx as an alternative path for cancer-targeting has been the research focus for many groups, whom have synthesized and/or tested various neoglycoconjugates (synthetic glycan-linked biomolecules) with templates based on dendrimers,[4] nanoparticles,[5] liposomes,[6] etc. In general, these studies broadly fall into two categories with different focuses. One field of study prioritizes the comprehensive *in vitro* screening of different glycan assemblies, for example using microarrays.[7] Another field of study prioritizes biological cell- and animal-based assays. Possibly due to synthetic challenges, the majority of these studies do not test a diverse assembly of glycoconjugates. Furthermore, many artificial templates often face biological complications, such as the formation of serum protein coronas.[8] And although the concept is currently under debate,[9] another issue is the potential for false positives caused by the intrinsic EPR effects of templates.

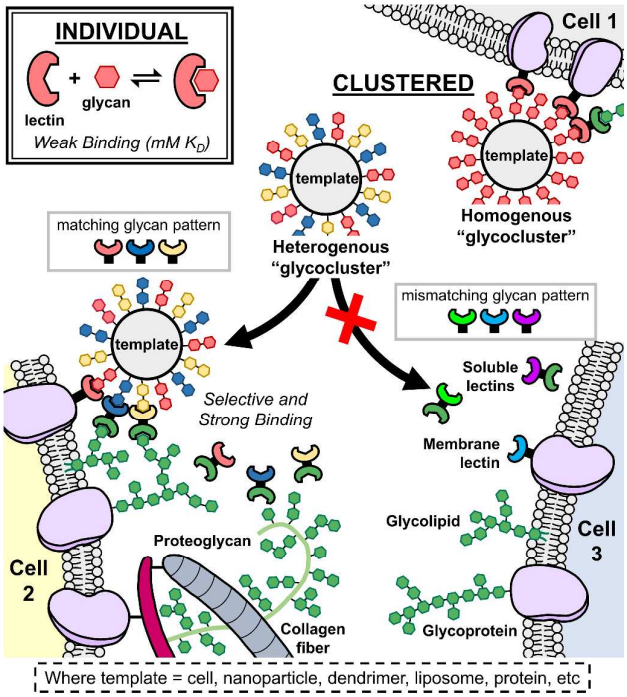

Fig. 1 Concept of glycan pattern recognition. Individual lectin-glycan interactions are generally poor and reversible (mM KD level). However, in the presence of matching glycosylation patterns and lectin expression, glycoclusters can exhibit strong and selective cell binding. In the above figure, the heterogenous glycocluster would be selective for Cell 2 (matching glycan pattern) over Cell 3 (mismatching glycan pattern).

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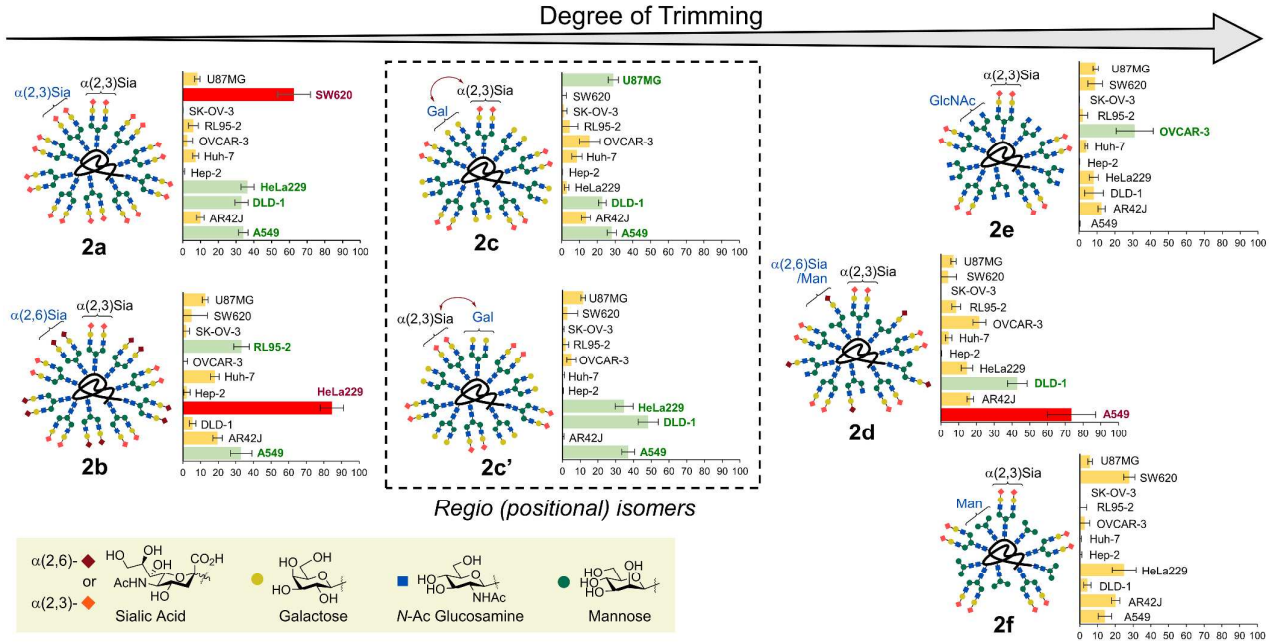

Fig. 2 Heterogenous-clustered glycoalbumins **2a-f** (100 nM) were incubated with 11 different cancer cells lines and measured for preferential cell binding. Fluorescence was averaged and normalized from 10,000 cells (n=10). Red bars = strong binding. Green bars = moderate binding. Orange bars = poor/negligible binding.

Considering the literature, there are a lack of studies that combine both principles of 1) screening diverse glycan assemblies, with 2) *in vivo* testing in live animals to account for biological complexity. This paper describes our efforts to develop the first study that combines these two approaches to further our understanding of the molecular basis that governs *in vivo* glycan pattern recognition. This breakthrough was largely possible due to the use of albumin as a template.

Serum proteins, like albumin, are the ideal delivery template of choice by our group. Due to its natural occurrence and *in vivo* stability, glycoalbumins should experience little biological interference in the blood. In addition, albumin has no known intrinsic tumor accumulation properties. To synthesize clustered glycan-linked albumins with ease, 6π-azaelectrocyclization (RIKEN click reaction) was used,[10] which has also been used for the previous synthesis of homogenous-,[11] and heterogenous-clustered glycoalbumins.[12]

In this study, seven different heterogeneous glycoalbumins **2a-f** (Fig 2, Scheme S1 in SI) were prepared from combinations of 5 different glycan assemblies [α(2,3)Sia-, α(2,6)Sia-, Gal-, Man-, and GlcNAc-terminated glycans]. As a note, heterogeneous glycoalbumin **2c'** exists as the "regioisomer" of the glycoalbumin **2c**, where the positioning of conjugated α(2,3)Sia- and Gal-terminated glycans are switched.

To begin, heterogeneous glycoalbumins **2a-f** were incubated with eleven different cell lines, and then imaged to determine the extent of cell binding via fluorescence (Fig 2). These results show three major trends in relation to cancer cell binding. First, by comparing homogenous glycoalbumin **2a** with heterogenous glycoalbumin **2b**, it can be stated that even small changes to introduce heterogeneity can lead to significant changes in pattern recognition-based binding. This is best exemplified with SW620 cells, where observations show significant binding with **2a**, but negligible levels with **2b**. On the other hand, **2b** showed the strongest binding with HeLa229 cells, whereas much lower levels were monitored with **2a**. These substantial differences between **2a** and **2b** persist in spite of their only structural difference being the linkage position (2,3→2,6) between one of the terminal sialic acid moieties.

A second observed trend is the effect on binding caused by the total negative charge of sialic acid-containing glycoalbumins. For example, 4 out of 4 terminal glycan moieties of **2a** and **2b** were designed with sialic acids, making these the most negatively charged glycoalbumins tested in this study. In addition to 2-3 moderate interactions, **2a** and **2b** were observed to have strong cancer cell binding with SW620 and HeLa229, respectively. However, by progressively removing terminal sialic acids, binding interactions gradually weakened. Glycoalbumin **2d**, which has 3 out of 4 terminal sialic acid moieties, showed strong binding with A549 and only 1 moderate interaction. In the case where there are only 2 out of 4 terminal sialic acid moieties (ex/ **2c**, **2c'**, **2e**, **2f**), binding with cancer cells weakened to the point where no strong interactions were observed.

Finally, the third observed trend is that positioning of the glycan units is also an important factor for pattern recognition. For example, although the glycan constitution of both **2c** and **2c'** is made of α(2,3)Sia- and Gal-terminated glycans, HeLa cells interacted stronger with **2c'** than its regioisomer **2c**. For U87MG cells, this trend was reversed. These observations are a perfect example where glycan positioning can cause substantial cell binding changes, likely driven by

Table 1. Literature survey of cancer cell lectin expression and their glycan specificity.

Cell Lines	Lectin expression and their known ligands				
	2,3-Sia	2,6-Sia	Man	Gal	GlcNAc
A549	galectin-1[13]	siglec-10[14]	SP-D[15]	-	-
AR42J	RNase A[16]	-	-	-	-
DLD-1	galectin-8[17]	-	-	-	-
HeLa229	galectin-1[13b, 18]	siglec-3[19]	-	-	-
Hep-2	-	-	-		-
HuH-7	-	AGCR[20]	-	AGCR[20]	-
OVCAR-3	-	-	-	-	vimentin[21]
RL95-2	L-selectin[22]	galectin-3[23]	-	-	-
SK-OV-3	-	-	-	-	-
SW620	galectin-8[17]	-	-	-	-
U87MG	galectin-1[13b, 24]	-	-	galectin-1[13b]	-

matching/mismatching the spatial arrangement of lectins unique to different cell types.

To consolidate experimental data, a literature survey was done for the 11 tested cancer cell lines to gather information on lectin expression and their known natural glycan ligands. Reviewed in Table 1, the following analysis aims to rationalize the preferential binding observed in cell-based experiments.

For strong glycoalbumin-cell binding interactions (shown by red bars in Fig 2), literature analysis validates these results, which include interactions like SW620 cells (galectin-8 expressing) to **2a** [α(2,3)Sia-rich]; HeLa229 cells (galectin-1 and siglec-3 expressing) to **2b** [α(2,3)Sia- and α(2,6)Sia-rich]; and A549 cells (galectin-1, siglec-10 and SP-D lectin expressing) to **2d** [α(2,3)Sia, α(2,6)Sia, and Man]. For moderate cell binding interactions (shown by green bars in Fig 2), literature validation can be made with interactions like RL95-2 cells (L-Selectin and Galactin-3 expressing) to **2b** [α(2,3)Sia- and α(2,6)Sia-rich]; U87MG cells (Galectin-1 expressing) to **2c** [α(2,3)Sia and Gal-rich]; and OVCAR-3 cells (vimentin expressing) to **2e** [GlcNAc-rich]. Finally, some degree of explanation can also be provided for poor glycoalbumin-cell binding interactions. Glycoalbumins **2a-f** were all constructed with half of α(2,3)Sia-terminated moieties. As such, the lack of α(2,3)Sia-binding lectin expression in Hep2, HuH-7, and SK-OV-3 cancer cells likely accounts for their poor observed glycoalbumin binding in this study.

Overall, from the combination of known lectin expression and experimental binding trends, these cell-based studies have provided a raw understanding of the molecular basis for glycan pattern recognition. However, the influence of other biological factors (ex/ glycan-glycan, hydrogen-bonding, hydrophobic, and hydrophilic interactions) also needs to be taken into account. As such, animal-based studies to screen glycoalbumins **2a-f** for *in vivo* tumor tissue targeting was next performed.

For these studies, three different operationally accessible tumor tissues were implanted into the shoulder and groin regions of BALB/cAJcl-nu/nu mouse models (HeLa229 at right shoulder, U87MG at right groin, and DLD-1 at left groin). Glycoalbumins labelled with the near-infrared fluorophore HiLyte Fluor 750® were injected into mice via the tail vein. Monitored at specific time intervals, *in vivo* fluorescence imaging of mice was then performed (Fig 3). Further imaging results are also shown in the Supporting Information to highlight the consistency among replicates (Fig S33), as well as dosage-dependent effects on accumulation (Fig S34).

On the whole, all glycoalbumins were subject to the conventional excretion pathway typical for proteins such as serum albumin. Following distribution around the body, glycoalbumins were digested in the liver, trafficked to the kidneys and then urinary bladder to be excreted from the body. In this experiment, the principle aim is to identify whether or not different glycan assemblies can be used to influence glycoalbumins to also accumulate onto tumor tissues for detectable amounts of time.

Starting with glycoalbumin **2e**, given its low levels of binding to HeLa, DLD-1, and U87MG cancer cell lines in cell-based experiments, the lack of any significant accumulation to these tumors types within mice was expected and observed (Fig 3A). At both low and high dosages of **2e**, no discernible accumulation to implanted tumors could be seen.

For glycoalbumin **2c**, cell-based experiments showed moderate binding to both U87MG and DLD-1 cells, but not with HeLa229 cells. Initially at lower dosages, *in vivo* accumulation in mice can be observed with implanted U87MG and DLD-1 tumors, thereby consolidating cell-based studies (Figure S34B in SI). However, at higher dosages of **2c** in line with general imaging protocols, no significant accumulation could be detected (Fig 3B). This result helps to emphasize that moderate binding interactions in cell-based assays may likely only translate to weak interactions *in vivo*, likely due to the added complexity of biological systems.

For glycoalbumin **2b**, cell-based assays show strong binding with HeLa229 cells. As such, accumulation in HeLa tumors is expected. However, previous data obtained by our group found that glycoalbumins containing terminal α(2,6)sialic acid moieties were often rapidly excreted *in vivo*, likely caused by asialoglycoprotein receptor (ASGPR) triggered internalization into liver parenchymal cells.[11b] As such, rapid excretion from mice (<30 min) observed with low dosages of **2b** is attributed to this effect (Figure S34C in SI). In contrast, with higher dosages of **2b**, ASGPR receptors are likely to be oversaturated. This rational should explain why higher dosages of **2b** were shown to lead to clear accumulation towards HeLa tumors (Fig 3C).

In conclusion, this work is the first study that successfully combines the screening of a diverse set of heterogeneous glycoconjugates against a wide range of tumors done in cell- and animal-based assays. From these results, some interesting trends related to glycan binding interactions were identified in cell-based assays (glycan heterogeneity, positioning, and charge), as well as in animal-based assays. More importantly, glycoalbumin **2b** was successfully identified for its selective and clear adhesion to HeLa229 tumors within live mice. Naturally, this work will evolve towards larger glycocluster libraries to uncover more diversity among cell-specific binding, and to further our understanding of *in vivo* glycan pattern recognition.

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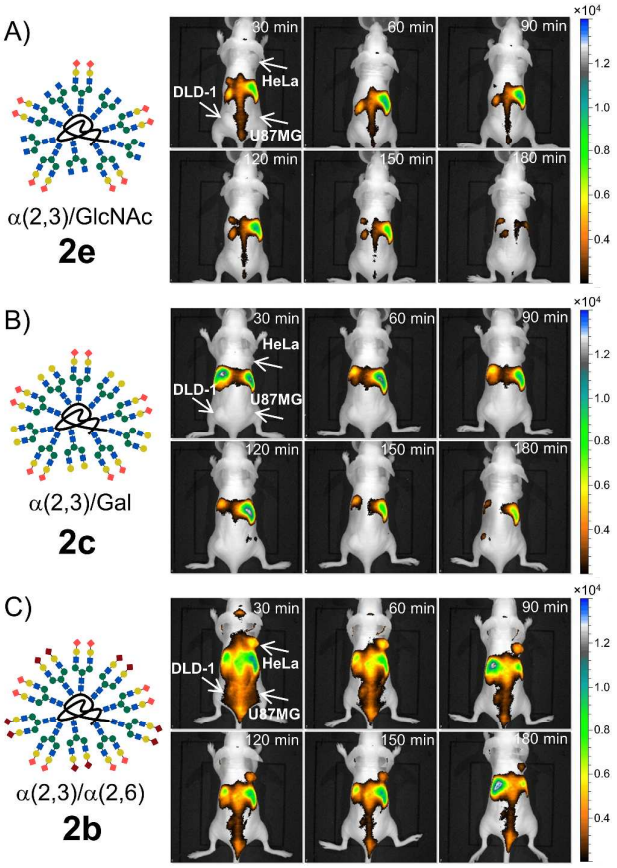

Fig. 3 Noninvasive fluorescence imaging of BALB/c nude mice (dorsal view) following the intravenous injection of B) α(2,3)Sia/GlcNAc terminated glycoalbumin **2e**, B) α(2,3)Sia/Gal terminated glycoalbumin **2c**, and C) α(2,3)Sia/α(2,6)Sia terminated glycoalbumin **2b**.

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