A Possible Role for Metallic Ions in the Carbohydrate Cluster Recognition Displayed by a Lewis Y Specific Antibody

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Abstract

Background: Lewis Y (Le⁰) is a blood group-related carbohydrate that is expressed at high surface densities on the majority of epithelial carcinomas and is a promising target for antibody-based immunotherapy. A humanized Le⁰-specific antibody (hu3S193) has shown encouraging safety, pharmacokinetic and tumor-targeting properties in recently completed Phase I clinical trials.

Methodology/Principal Findings: We report the three-dimensional structures for both the free (unliganded) and bound (Le⁰ tetrasaccharide) hu3S193 Fab from the same crystal grown in the presence of divalent zinc ions. There is no evidence of significant conformational changes occurring in either the Le⁰ carbohydrate antigen or the hu3S193 binding site, which suggests a rigid fit binding mechanism. In the crystal, the hu3S193 Fab molecules are coordinated at their protein-protein interface by two zinc ions and in solution aggregation of Fab can be initiated by zinc, but not magnesium ions. Dynamic light scattering revealed that zinc ions could initiate a sharp transition from hu3S193 Fab monomers to large multimeric aggregates in solution.

Conclusions/Significance: Zinc ions can mediate interactions between hu3S193 Fab in crystals and in solution. Whether metallic ion mediated aggregation of antibody occurs in vivo is not known, but the present results suggest that similar clustering mechanisms could occur when hu3S193 binds to Le⁰ on cells, particularly given the high surface densities of antigen on the target tumor cells.

Introduction

Recent studies of the normal biological functions of the Lewis Y (Le³ or CD174) carbohydrate antigen have revealed new insights into its role in cellular function [1–3]. This type 2 histo-blood group related carbohydrate antigen is expressed at high surface densities on 60% to 90% of carcinomas of the breast, ovary, colon, lung and prostate [4–6]. Together with its frequent overexpression on primary and metastatic tumors, its low abundance in normal tissues, and restricted distribution on normal tissues, Le³ represents a promising target for antibody-based immunotherapeutic approaches [7,8].

During human development, Le³ is expressed on tissues of the fetus, placenta [9] and newborn [10,11]. However, in adults Le³ is either intracellular or at low surface densities on a few tissues including: hematopoietic precursors, vascular endothelial cells, and epithelial surfaces of the gastrointestinal tract [7,12–14]. Fucosylated type 2 determinants (Le⁰ and Le³) have also been demonstrated as the major free oligosaccharides in human seminal plasma [15]. Recently, N-linked Le³ oligosaccharides have been shown to be present at high levels in the acrosome (a large intracellular compartment similar to a lysosome) of human sperm, but are not present on the plasma membrane [3]. Defective or malformed sperm were shown to display Le³ on the plasma membrane. Given that both Le³ and Le⁰ have been shown to interact with human dendritic cells via DC-SIGN to induce T-cell tolerance [16], these oligosaccharides may play a role in the immune privilege of the male reproductive tract [3]. Similarly, tumors may promote T-cell tolerance by expressing high surface levels of type 2 Lewis antigens including Le³. More recently, the low level expression of Le³ on ICAM-2 of human vascular endothelial cells has been shown to support adhesion and rolling of immature dendritic cells and is involved in the initial cell-cell contacts during angiogenesis [1,2]. The involvement of Le³ in cell adhesion and angiogenic events, together with the high surface densities on Le³-positive cancers, suggest the involvement of this carbohydrate antigen in tumor migration (ie., metastasis) and neoangiogenesis [2]. A corollary of these observations is that the
mechanism of action of a Le^+/-specific therapeutic antibody may not solely be through antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytotoxicity (CDC), but may additionally involve direct inhibition of tumor cell migration and neoangiogenesis.

Early clinical trials with Le^+/-specific murine monoclonal antibodies and antibody-toxin conjugates were limited by immunogenicity, dose limiting toxicity [17] and unexpected side-effects like vascular leakage syndrome (LBMB-1, murine B3 antibody linked to Pseudomonas exotoxin) [18]. Phase I trials have now been conducted with Le^+-specific humanized IgG1 monoclonals, IGN311 [19] and hu3S193 [20,21], and have shown encouraging safety, pharmacokinetic and tumor targeting properties. Trials have also been conducted with a chimeric BR96-doxorubicin conjugate (SGN-15) in a range of cancer patients with some modest clinical activity, but some immune responses towards the BR96-doxorubicin conjugates were noted [22]. Clinical studies with hu3S193 in a variety of Le^+-expressing cancer patients have demonstrated that this antibody does not induce human anti-humanized antibody (HAHA) responses, selectively targets and accumulates in Le^+-expressing tumors at high concentrations, retains immune effector function in vivo, and does not show saturable binding to any normal tissue compartment [20,21]. Against Le^+-expressing tumor cells, hu3S193 has potent in vitro immune effector functions, including complement-dependent cytotoxicity (CDC, IC_{50} = 1.0 μg/ml) and antibody-dependent cellular cytotoxicity (ADCC, IC_{50} = 0.5 μg/ml) [23]. Furthermore, hu3S193 is rapidly internalized through the lysosomal/endoosomal pathway in the Le^+-expressing MCF-7 tumor cells [24]. The preferential binding of hu3S193 to tumor cells with high surface densities of Le^+ and lack of binding to normal tissues expressing lower levels of Le^+ suggests that this antibody is involved in carbohydrate cluster recognition as a first step in tumor cell killing.

Previously, we determined the crystal structure of hu3S193 Fab in complex with Le^+ at a resolution of 1.9 Å [25]. Since hu3S193 binding of Le^+ was almost identical to that of the BR96 antibody, we proposed that the antibody response to this tumor-associated antigen was structurally conserved. In a subsequent analysis of all reported free and bound Lewis system carbohydrates (Le^a, Le^b, Le^a and Le^b), we confirmed the overall structural similarity and rigid nature of these carbohydrate antigens [26]. Thus, the free Le^+ conformation closely resembles the biologically active or antibody-bound state. Herein we report three-dimensional structures of both the free (unliganded) and bound (Le^+ tetrasaccharide) hu3S193 Fab in the same orthorhombic P2_12_1 crystals, which were grown in the presence of divalent zinc ions. Comparison of the free and bound Fab shows that the binding site of hu3S193 does not undergo significant changes during complex formation. Furthermore, the findings of a zinc-dependent Fab dimer in crystals and aggregation in solution induced by Zn^{2+} ions, may point towards a role of divalent metallic ions in antibody-based carbohydrate clustering.

Methods

Preparation and Co-Crystallization of hu3S193 Fab with the Le^+ Tetrasaccharide

The hu3S193 IgG1(κ) antibody was produced and purified as described [23]. The hu3S193 Fab was obtained by plasmin digestion and purified to homogeneity following protocols reported earlier [25]. Crystals of hu3S193 Fab were produced in 2 μl sitting drops in the presence of Le^+ by vapor diffusion in a 96-well, round bottom, sitting-drop plate (Corning, Acton, MA, USA) using the
Results

Presence of the Free and Leβ-Bound hu3S193 Fab Molecules in the Same Crystal

Orthorhombic P2₁2₁2₁ crystals of hu3S193 Fab were grown in the presence of the Leβ tetrasaccharide and the crystallographic structure was determined to 2.5 Å resolution (Table 1). The asymmetric unit contained two hu3S193 Fab molecules (Fig. 1), one binding site was in complex with Leβ (Fab1) and the second binding site contained only a loosely associated glycerol molecule and a few solvent molecules (Fab2). Thus, the same crystal binding site contained only a loosely associated glycerol molecule hu3S193 antibody. Four Zn²⁺ ions were associated with cross-pairing of the hu3S193 Fab molecules (136–146) and the crystallographic structure was determined to 2.5 Å resolution (Table 1). The presence of the Leβ tetrasaccharide and the crystallographic structure was nearly identical (Fig. 2 A and C). The only notable difference is the absence of a single hydrogen bond between Asn 28L (ND2 atom) and the Leβ-specific Fuc (O4 atom) saccharide unit (ic., the α1–2 linked Fuc). The Asn 28L to Fuc (ND2 to O4) distance in the Fab1-Leβ complex is 3.6 Å, compared to 3.0 Å in the 1S3K complex. Combined with other distances around the Leβ-specific Fuc, which are consistently slightly larger in the Fab1-Leβ complex compared to 1S3K, it appears that Leβ is more loosely bound in crystals grown in the presence of zinc ions. This observation is supported by the absence of a Leβ ligand in the binding site of Fab2 (Fig. 2 B). The Fab2 binding site contains a few solvent molecules and a glycerol (GOL) molecule, which is weakly held in place by a single water-mediated hydrogen bond. No binding site residues of Fab2 interact directly with the GOL molecule, which originated from the cryoprotectant solution used to flash-cool the crystal for X-ray diffraction data collection. The Leβ ligand either was not bound or was eluted from Fab2 during transfer to the cryoprotectant, but the Fab2 binding site represents the unliganded or free hu3S193 Fab.

Comparison of the Binding Sites of the Free and Leβ-Bound hu3S193 Fab Molecules

In the two independent hu3S193 Fab-Leβ complexes (Fab1 and 1S3K), the interactions between binding site residues and Leβ are nearly identical (Fig. 2 A and C). The only notable difference is the absence of a single hydrogen bond between Asn 28L (ND2 atom) and the Leβ-specific Fuc (O4 atom) saccharide unit (ic., the α1–2 linked Fuc). The Asn 28L to Fuc (ND2 to O4) distance in the Fab1-Leβ complex is 3.6 Å, compared to 3.0 Å in the 1S3K complex. Combined with other distances around the Leβ-specific Fuc, which are consistently slightly larger in the Fab1-Leβ complex compared to 1S3K, it appears that Leβ is more loosely bound in crystals grown in the presence of zinc ions. This observation is supported by the absence of a Leβ ligand in the binding site of Fab2 (Fig. 2 B). The Fab2 binding site contains a few solvent molecules and a glycerol (GOL) molecule, which is weakly held in place by a single water-mediated hydrogen bond. No binding site residues of Fab2 interact directly with the GOL molecule, which originated from the cryoprotectant solution used to flash-cool the crystal for X-ray diffraction data collection. The Leβ ligand either was not bound or was eluted from Fab2 during transfer to the cryoprotectant, but the Fab2 binding site represents the unliganded or free hu3S193 Fab.

Overlays of the hu3S193 Fab structures (two in complex with Leβ and one unliganded Fab) demonstrate almost identical structures reported here revealed very similar structures of the VL:VH and CL:CH1 domain modules, with carbon-alpha (Cα) root-mean-square deviations (RMSD) of 0.37–0.40 Å for VL:VH (232 residues) and 0.49–0.80 Å for CL:CH1 (196 residues) in pairwise comparisons. However, the “elbow bend” angles differed between the 1S3K Fab (136°) and the two Fab molecules in the asymmetric unit (Fab1, 143°; Fab2, 146°), indicating a modest degree of flexibility for the hu3S193 Fab in the V-C “switch” regions (ic., short regions of polypeptide connecting the V and C domains).

Table 1. Data collection and crystallographic refinement statistics.

| Parameter              | Value |
|------------------------|-------|
| Data collection        | P2₁2₁2₁ |
| Space group            | P2₁2₁2₁ |
| Unit cell variables    | a, b, c (Å) |
|                        | 78.8, 101.5, 115.0 |
| Resolution range (Å)   | 100–2.50 (2.59–2.5) |
| Number of unique reflections | 31623 (3074) |
| Percent data completeness | 95.8 (94.7) |
| Average multiplicity    | 5.5 (5.4) |
| Rsym                   | 0.073 (0.38) |
| Mean I/σ(I)            | 23.8 (4.5) |
| Crystallographic refinement | Rmerge |
|                        | 0.212 |
| Rfree                  | 0.263 |
| Ramachandran plot values (%) | |
| Most favored regions   | 86.6 |
| Additional allowed regions | 12.3 |
| Generously allowed regions | 0.8 |
| Disallowed regions     | 0.3 |

*Values in parentheses refer to the highest resolution shell, 2.59–2.5 Å, in the data. Refinement and stereochemical parameters were compiled from the CNS program suite, version 1.0 [30] or PROCHECK version 3.3 [59].

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for the hu3S193 Fab in the V-C “switch” regions (ic., short regions of polypeptide connecting the V and C domains). The elbow bend angles of the hu3S193 Fab molecules (136°–146°) are well within the ranges previously observed for Fab and represent frequently occurring values for Fab containing κ-type light chains [32].
positions for all binding site residues in contact with the Le^y tetrasaccharide (Fig. 2 D). Thus, the binding site of hu3S193 does not appear to have undergone conformational changes during complex formation with Ley. We previously reported that the hu3S193-bound conformation of Ley was fundamentally the same as the known free and bound conformations of Le^y and other Lewis system carbohydrates [26]. Similarly, the bound conformations of Le^y in 1S3K and Fab1 were essentially the same (Table 2), which further supports the idea of the rigid character of the Le^y carbohydrate determinant. Note that the slight variation in the Fuc1-2Gal glycosidic linkage (Table 2) is compatible with the small differences observed in the interactions between the Le^y-specific Fuc and the hu3S193 binding site for the 1S3K and Fab1 structures (Fig. 2 A and C).

At 1.9 Å resolution an extensive solvent network of 13 ordered water molecules surrounds the Le^y tetrasaccharide in complex with hu3S193 Fab [25]. The ordered solvent was involved in hydrogen bonding to protein and carbohydrate residues (7 water molecules) and in forming more extended hydrogen bonding networks (6 water molecules), which led to greater complementarity between Le^y and the hu3S193 binding site [26]. In the vicinity of the Fab1-Le^y interaction, two ordered solvent molecules were observed in the 2.5 Å resolution electron density maps (Fig. 2 A) and both occupied the same locations as in the 1S3K structure (Fig. 2 C). Without the Le^y ligand, the hu3S193 binding site contained 4 ordered water molecules and a glycerol. One water molecule in the unliganded hu3S193 binding site occupied a location (near tyrosines 32H and 33H) where the N-acetyl group of the Le^y tetrasaccharide binds and could have been displaced during complex formation (Fig. 2 A and B). However, none of the water molecules in the unliganded binding site occupied comparable locations to solvent molecules in the hu3S193 complexes with Le^y. Thus, it is likely that the water molecules...
were associated with the Le\(^{a}\) carbohydrate or are recruited to the interaction rather than being already present in the hu3S193 binding site.

**Zinc-Dependent Crystallographic Dimers of hu3S193 Fab Suggest a Possible Role of Divalent Metal Ions in Carbohydrate Cluster Recognition**

The hu3S193 Fab molecules contain four Zn\(^{2+}\) ions bound in two types of environment. Firstly, protein interface zinc-binding sites are formed between the Fab1 and Fab2 molecules and involve the tetravalent coordination by Fab residues (Fig. 3 A and C). Asparagines 142 and 143 of the light chain (A or L) and His 170 of the heavy chain (B or H) of one Fab form a pocket in the CL:CH1 domain interface that binds a Zn\(^{2+}\) ion, which accepts Glu 1 from the heavy chain of the second Fab. In one site, a water molecule acts as a fifth ligand for the Zn\(^{2+}\) ion (Fig. 3 A), but this was not observed in the second protein interface zinc-binding site (Fig. 3 C). Access for the water ligand is possible since the coordination of zinc is distorted from tetrahedral geometry. The two zinc ions form part of a larger protein-protein interface between the hu3S193 Fab molecules in the asymmetric unit. Secondly, surface-located zinc sites were identified in the hu3S193 k\(^{-}\)-type light chains where Zn\(^{2+}\) ions interacted bivalently with His 194L and Asp190L (Fab1; Fig. 3 B) or monovalently with His 194L (Fab2; Fig. 3 D). Rather than being buried at a protein-protein interface the surface-located zinc ions line solvent channels in the orthorhombic \(P_{2_{1}}2_{1}2_{1}\) crystals and are at least 4 Å from any symmetry related Fab.

The presence in crystals of zinc-stabilized hu3S193 Fab homodimers led us to test in solution by DLS the effect of zinc ions on the Fab. In solution, hu3S193 Fab (\(\sim 15 \mu\)M) aggregated in the presence of 40 and 50 \(\mu\)M ZnCl\(_{2}\), but not at lower concentrations or at any concentration of MgCl\(_{2}\) tested (Fig. 4). Interestingly, zinc-induced aggregation of the hu3S193 Fab required at least two molar equivalents of Zn\(^{2+}\) ions, which is similar to the four Zn\(^{2+}\) ions found, associated with the two hu3S193 Fab molecules in the asymmetric unit of the crystals.

The zinc ion induced changes in solution of hu3S193 Fab were further characterized by DLS by monitoring the z-average hydrodynamic diameters (\(D_{H}\)) in the presence of divalent metallic ions (Fig. 5). Without any metallic ions hu3S193 Fab behaved as a monomer with \(D_{H}\) in the range of 5.3 nm to 7.1 nm. Similar \(D_{H}\) values were obtained at all MgCl\(_{2}\) concentrations and when ZnCl\(_{2}\) was between 10 \(\mu\)M and 30 \(\mu\)M. A sharp transition occurred at 40 \(\mu\)M ZnCl\(_{2}\) where the hu3S193 Fab formed large aggregates with a mean \(D_{H}\) value of 211.9 nm (range of 92.8 nm to 333.9 nm). When the ZnCl\(_{2}\) was raised to 50 \(\mu\)M the hu3S193 Fab aggregated.
MgCl₂. Final concentrations of the divalent metal ions were 0 μM of ZnCl₂ (black) or MgCl₂ (white). Mean values (n = 3) are shown and for hu3S193 Fab samples in the presence of increasing concentrations of ZnCl₂.

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Figure 4. Divalent zinc ion mediated aggregation of the hu3S193 Fab. Dynamic light scattering was used to monitor time-dependent fluctuations (correlograms) in scattered light intensity for samples of hu3S193 Fab (~ 15 μM) in the presence of: A) ZnCl₂ and, B) MgCl₂. Final concentrations of the divalent metal ions were 0 μM (short dash), 10 μM (dash-dot), 20 μM (dots), 40 μM (dash) and 50 μM (solid). doi:10.1371/journal.pone.0007777.g004

Fab aggregates had a mean $D_H$ of 511.3 nm (range of 458.5 nm to 547.8 nm). The zinc-mediated aggregates of hu3S193 Fab are substantially larger than polymeric IgM (molecular mass >950 kDa or 19 S), which we have previously shown to have a $z$-average $D_H$ of between 34 and 37 nm [33,34]. Thus, in solution zinc ions can mediate the formation of multimeric aggregates of hu3S193 Fab, but does not appear to produce smaller ordered multimers such as the dimers observed in crystals.

Figure 5. Changes in size of hu3S193 Fab in the presence of zinc ions monitored by DLS. The $z$-average $D_H$ (nm) was determined for hu3S193 Fab samples in the presence of increasing concentrations of ZnCl₂ (black) or MgCl₂ (white). Mean values (n = 3) are shown and error bars represent three standard deviations. doi:10.1371/journal.pone.0007777.g005

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While the physiological levels of Zn²⁺ in blood plasma is around 20 μM, certain tissues and cellular compartments have been shown to have significantly higher levels of Zn²⁺ [35–39]. Furthermore, intracellular Zn²⁺ fluxes have been recently shown to be involved in lipopolysaccharide-induced signals in human monocytes [40], which supports the concept that the local concentration of zinc ions can rapidly change in biological microenvironments. Thus, the 40 μM ZnCl₂ required for aggregation of hu3S193 Fab in solution indicates that in vivo aggregation should not occur in the blood plasma, but would possibly be induced in certain tissue or cellular compartments where Zn²⁺ ions can be at significantly higher concentrations.

Discussion

In the current work, we were presented with an opportunity to examine the three-dimensional structures of both the free and Le⁰-bound hu3S193 binding sites from the same crystal. Apart from the subtle differences in solvent structure and minor adjustments of the Le⁰-specific Fuc residue, the binding sites for the three hu3S193 structures were identical (see Fig. 2 D). Taken together with the established rigid nature of the Le⁰ carbohydrate [26], the available data strongly supports the binding of the Le⁰ antigen by the hu3S193 antibody to resemble the fit of an unbendable key into a rigid lock. This finding is in contrast to the now commonly held view that both antigen and antibody frequently undergo conformational changes or induced fit upon binding [41–46]. Similarly, carbohydrates have traditionally been viewed as more flexible and mobile in solution when compared to globular proteins. Thus, the interaction between the hu3S193 antibody and Le⁰ carbohydrate determinant has made us revisit the lock-and-key concept as a possible mechanism for antibody recognition of carbohydrate antigens, particularly in Le⁰-expressing tumors.

Reynolds and colleagues recently examined the hydration features of free type 2 Lewis antigens (Le⁰ and Leⁱ) by molecular dynamics (MD) simulations [47]. In this study, the solvated carbohydrate determinants remained relatively inflexible or rigid during extensive MD simulations, confirming earlier observations that the free conformation is representative of the biologically active state for Lewis system antigens (reviewed in [26]). Interestingly, the water molecule bridging events or solvent structure around free Le⁰ observed by the MD simulations were mostly represented by water molecules and a few binding site residues in the hu3S193 Fab-Le⁰ complex at 1.9 Å resolution [25,47]. For the present complex (Fab1-Le⁰) that was determined at a resolution of 2.5 Å, we identified two of the same bridging water molecules and these were buried in the carbohydrate-antibody interface. Since most of the water molecules surrounding Le⁰ are not buried in the binding site, it is possible that these were simply not observed in crystals at a resolution of 2.5 Å. However, the finding of key water molecules involved in the hu3S193 interaction with Le⁰ supports the conclusion that solvent mediates the rigid conformational properties of this carbohydrate epitope and is important for antibody recognition. Furthermore, the hydrated structure of Le⁰ antigens when presented on the surface of tumor cells is likely to snugly fit the hu3S193 binding site without the need for displacement of the majority of the water molecules from the carbohydrate.

The in vivo specificity of hu3S193 for Le⁰ on tumor cells and no saturable binding to any normal tissue compartment [20], indicates that the presentation of Le⁰ determinants by tumor cells is different from normal tissues. Clearly, Le⁰ epitopes are both expressed in tumors at relatively high surface densities and are aberrantly expressed on various membrane-bound glycoproteins.
including epidermal growth factor receptors, which are also candidate antigens for tumor immunotherapy [40–53]. Our findings of a zinc-dependent homodimer of hu3S193 Fab in crystals and the corresponding aggregation of Fab in solution, provides the first evidence for a possible new mechanism of carbohydrate antibody cluster recognition. We propose that binding of hu3S193 to dense clusters of Le\(^+\) on tumor cells could be further stabilized by Zn\(^{2+}\) or other diveral metallic ions resulting in an increase in the avidity of the interaction. Similarly the role of zinc in protein-protein interactions has been described for several other biological systems [54–57]. The low surface densities of Le\(^+\) carbohydrates on normal tissues would not be suitable for lateral zinc-mediated interactions to occur between neighboring antibodies, which could explain the evident lack of binding by hu3S193 to normal Le\(^+\)-expressing tissues.

Another immunological solution for specific antibody binding to dense clusters has been reported for the 2G12 IgG, which is highly specific for the complex oligomannose glycans that decorate the “silent face” HIV-1 gp120 [58]. Both the 2G12 Fab and the intact antibody has been shown to contain domain-swapped Fab “silent face” HIV-1 gp120 [58]. Both the 2G12 Fab and the intact antibody has been shown to contain domain-swapped Fab homodimers, where VH domains from each Fab associate with the corresponding VL in the adjacent Fab to form an extended surface for multivalent carbohydrate binding. While domain-swapped antibodies represent an elegant mechanism for carbohydrate antibody cluster recognition, these are likely to be rare and difficult to elicit by standard immunization strategies [58]. Our proposed metallic ion mediated mechanism for Le\(^+\) carbohydrate cluster recognition by hu3S193 may be more general since most IgG/k antibodies have the residues involved in coordinating Zn\(^{2+}\) by hu3S193 Fab.

Understanding the mechanism of action of a candidate therapeutic antibody against solid tumors requires a detailed physicochemical understanding of specificity and immune effector functions as well as the in vivo pharmacokinetics and biological activity in normal and tumor sites. We have shown that the binding specificity for Le\(^+\) by hu3S193 does not involve conformational changes and the interaction mimics the hydration patterns of free Le\(^+\) antigens. Additionally, the structural results presented here indicate a new potential mechanism for hu3S193 selective recognition of Le\(^+\) on tumor cells as opposed to normal tissues, which is based on metallic ion mediated carbohydrate cluster recognition.

Author Contributions
Conceived and designed the experiments: WF AMS PR. Performed the experiments: WF PR. Analyzed the data: WF PR. Contributed reagents/materials/analysis tools: AMS. Wrote the paper: WF PR.

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