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Mimicry of Tandem Repeat Peptides against Cell Surface Carbohydrates

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Specialized carbohydrates, located on the surfaces of cancer, endothelial, and immune cells, interact with specific proteins as part of cell-to-cell communication processes.[1] For example, the sialyl Lewis X (sLeX) and Lewis X (LeX) carbohydrates, found on the surfaces of cancer cells,[2] are specific cell markers, and, consequently, they can be used as therapeutic or diagnostic targets. However, owing to the conformational flexibility of pyranosides, it is often difficult to design substances that bind in a complimentary manner with carbohydrate recognition sites on cell surfaces. Typically, only weakly bound complexes are obtained.[3] Members of the naturally occurring protein families known as selectins and lectins are known to bind to cell surface carbohydrates. Although binding is weak in these cases, it is highly specific, and it facilitates strong integrin interactions which result in carbohydrate-mediated, cell-to-cell adhesion.[4] To optimize the carbohydrate-binding affinity of proteins, nature has developed a strategy based on multivalency.[5] Thus, by using an array of repeating weak binding sites, high avidity is created. Strong evidence has been gained to support the proposal that tandem repeat peptide sequences in lectins are responsible for binding specific carbohydrates.[6]

On the basis of nature's design of carbohydrate-binding proteins, we reasoned that substances which contain tandem repeat carbohydrate-binding peptide units would serve as strong and specific carbohydrate-binding agents. Instead of simply selecting targets with sequences that mimic those in known tandem repeat peptides, we elected to utilize a broader strategy for the design of high carbohydrate-binding affinity peptides, in which phage-displayed peptide libraries are constructed and then biopanned against specific carbohydrates. Numerous efforts are underway to find carbohydrate specific peptides[7] and antibodies[8] using the phage display technique. The mimotope approach has also been explored with the aim of uncovering peptide-carbohydrate cross activity, using anti-carbohydrate antibodies and phage-displayed peptide libraries.[9] Yet, until now, no one has attempted to generate short peptides (<12-mer) against specific carbohydrates, probably owing to the weak binding that exists between short peptides and carbohydrates. Importantly, there have been no reports describing the synthesis of a multivalent peptide that is selective against a defined carbohydrate structure.

Our approach to multivalent peptide construction relies on tentacle peptides, also known as a multiple antigenic peptides, which contain two and four repeats of a selected peptide.[10] In this communication, we report the results of preliminary studies aimed at (1) the selection of short peptides against the carbohydrate, sLeX, (2) the synthesis of tentacle dimers and tetramers of the selected peptides, and (3) the determination of affinities and specificities of the peptides to several related carbohydrates by using the surface plasmon resonance (SPR) and the equilibrium dialysis techniques. Binding affinity studies, as well as assays of in vitro binding of the peptides to a sLeX-specific cell line, have shown that the tetrameric peptides bind to the cell surface sugars.

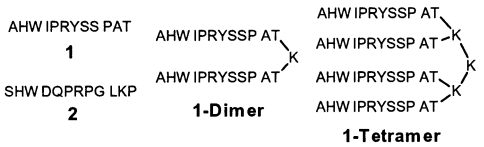

Figure 1. Structures of peptide **1**, **2**, and the tentacle type peptides, **1-dimer** and **1-tetramer**.

Selection was performed by using sLeX-conjugated bovine serum albumin and a 12-mer peptide library, employing standard biopanning techniques.[11] Ten selected phage sequences afforded five peptides,[12] including hexaplicated peptide **1** and four other peptides (**2**−**5**), which showed no sequence homology. However, a BLAST search showed that peptides **1** and **2** have ca. 50% sequence homology with the respective tandem repeat peptides of trans-sialidase from *Trypanosoma cruzi*[13] and P30 adhesin from *Mycoplasma pneumoniae*.[14] Although the natural tandem repeat peptides are not homologous, they do possess common characteristics. The tandem repeats in each are located at C-termini far from the functional sites of the proteins.[15] These proteins seem to be involved in host cell invasion processes.

Encouraged by the similarities of the sequences of peptides **1** and **2** with regions in known carbohydrate-binding proteins, we measured the binding affinities of the cognate sugars to the prepared peptides. To validate binding affinities of the selected phages against the target, an ELISA experiment was performed by using the horseradish peroxidase conjugated, anti-M13 antibody. Because only the peptides **1** and **2** displayed phages giving positive signals, these peptides were selected and synthesized[11] for further study. Synthetic peptides **1** and **2** were then used in conjunction with SPR techniques to measure binding affinities to confirm the results of the initial ELISA studies.[11] This technique gave respective K_d values of >1.0 mM for peptides **1** and **2**. Because peptide **1** shows the best binding affinity, dimeric and tetrameric (**1-dimer** and **1-tetramer** in Figure 1) homologues were prepared. Indeed, 14-fold (K_d = 70 μM) and 10 000-fold (K_d = 0.10 μM) enhancements in binding affinities were observed for **1-dimer** and **1-tetramer**, as compared to the corresponding monomeric peptide **1**. The affinity constant of **1-tetramer** was measured again using the equilibrium dialysis technique,[11] affording almost the same value (K_d = 85 ± 29 nM). Thus, by engineering multiple binding sites, we are able to construct a high-affinity tentacle peptide that binds a flexible sugar molecule.

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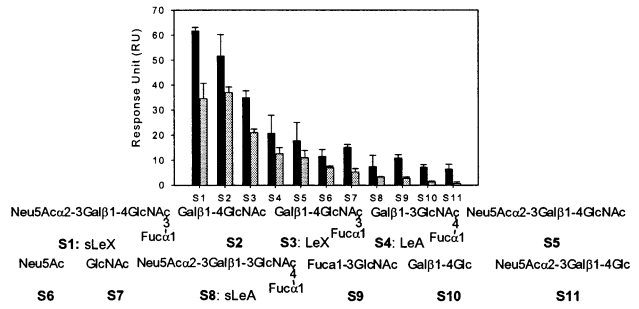

Figure 2. Binding affinities of various sugar-PAA-biotin versus immobilized **1-tetramer** peptide. A solution of sugar (0.3 mg/mL of HBS-EP buffer) was injected over the surface for 120 s and dissociated for 240 s. Black bar, response level (RU) after 115 s from injection start (nearly steady state); gray bar, response level after 245 s from dissociation start.

The specificity of the **1-tetramer** for binding different carbohydrates was evaluated next. For this purpose, **1-tetramer** was injected into three flow cells individually containing the immobilized carbohydrates, sLeX, LeX, and 3′-sialyl-3-fucosyllactose.[11] The binding affinities of **1-tetramer** to sLeX, LeX, and 3′-sialylfucosyllactose were found to be 1.0×10^{-7}, 6.4×10^{-6}, and 5.5×10^{-5} M, respectively. The binding affinities of anti-sLeX antibody (KM93) to these carbohydrates were also measured and found to be 1.0×10^{-10}, 4.3×10^{-8}, and 2.8×10^{-10} M, respectively. Even though KM93 displays a stronger and better specificity against sLeX, **1-tetramer** has comparable discriminating capability against the *N*-acetyllacosamine moiety in sLeX. For example, **1-tetramer** binding to sLeX (cognate sugar) and LeX is high, while it only weakly binds to 3′-sialyl-3-fucosyllactose. The results suggest that 2-positioned *N*-acetyl and hydroxyl groups in the glucopyranoside are key functionality leading to the carbohydrate selective binding (>500-fold affinity difference) by **1-tetramer**.

To more broadly probe carbohydrate-binding selectivity of **1-tetramer**, 11 different types of carbohydrates were eluted through a peptide-immobilized flow cell.[11] The data (Figure 2) demonstrate that binding affinities for sLeX, *N*-acetyllactosamine, and LeX are much higher than those for sLeA, LeA, and other sugars. Consequently, these studies suggest that the *N*-acetylglucosamine moiety is an important structural feature leading to peptide recognition. Although other portions of the carbohydrates, such as Gal and Fuc, affect the specificity of peptide binding, carbohydrates lacking *N*-acetyl group in Glc are the weakest binders. This emphasizes the importance of this functionality in recognition by the peptide.

The findings also suggest that tentacle peptides might serve as inhibitors of anti-sLeX antibody or selectin binding to sLeX specific cells. To test this proposal, the anti-sLeX antibody was incubated with immobilized HL60 cell lines in the presence and absence of **1-tetramer**. A dose-dependent reduction of cell population is observed for the cells that are treated with both the anti-sLeX antibody and the **1-tetramer**, as compared with cells incubated with the anti-sLeX antibody in the absence of the peptide. The observations indicate that **1-tetramer** competitively inhibits anti-sLeX antibody binding to the sLeX-rich cell surface (Figure 3). The effective concentration (EC) of **1-tetramer** is found to be in the range of 1.0 μM when 1.0 nM of the anti-sLeX antibody is used as the competitive binder. The differences in the EC values are well correlated with K_d value differences between **1-tetramer** and KM93 as measured by the SPR method (0.1 μM and 0.1 nM, respectively). The results of the SPR also show that binding by E-selectin to HL60 cells is inhibited by **1-tetramer**.[15] The number of cells bound to immobilized **1-tetramer** is reduced by addition

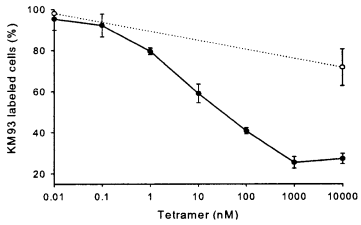

Figure 3. Competitive inhibitory effect of **1-tetramer** using HL60 cells and anti-sLeX antibody. **1-Tetramer** (●) showed dose-dependent inhibition of binding between anti-sLeX antibody and HL60 cells. A scrambled peptide (YPTSHIRPAWSA) was used as the control (○).

of E-selectin in a dose-dependent manner, suggesting that **1-tetramer** and E-selectin share the same cell-surface carbohydrate epitope.

In conclusion, by using the biopanning method, we have been able to successfully construct synthetic repeat peptides, which mimic natural peptides in binding to carbohydrates. Although the exact role(s) of tandem repeat peptides in nature is (are) not fully understood, observations made in this effort suggest that one of these must certainly be that of carbohydrate recognition. The results of this investigation also suggest that it should be possible to use tentacle peptides, derived by mimicry of tandem repeat peptides, to increase the avidity of carbohydrate specific peptides.

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Supporting Information Available: Experimental procedures for the selection, synthesis, and binding affinity studies of peptides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) See the Supporting Information for experimental details.
(12) Peptide sequences are as follows: AHWIPRYSSPAT (**1**), SHWDQP-RPGLKP (**2**), GTHLIAGGASHL (**3**), QTFSAPPSLLQL (**4**), QISQRSLL-DPLL (**5**).
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