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Targeting cancer-specific glycans by cyclic peptide lectinomimics

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Abstract

The transformation from normal to malignant phenotype in human cancers is associated with aberrant cell-surface glycosylation. Thus, targeting glycosylation changes in cancer is likely to provide not only better insight into the roles of carbohydrates in biological systems, but also facilitate the development of new molecular probes for bioanalytical and biomedical applications. In the reported study, we have synthesized lectinomimics based on odorranalectin **1**; the smallest lectin-like cyclic peptide isolated from the frog *Odorrana grahami* skin, and assessed the ability of these peptides to bind specific carbohydrates on molecular and cellular levels. In addition, we have shown that the disulfide bond found in **1** can be replaced with a lactam bridge. However, the orientation of the lactam bridge, peptides **2** and **3**, influenced cyclic peptide's conformation and thus these peptides ability to bind carbohydrates. Naturally occurring **1** and its analog **3** that adopt similar conformation in water bind preferentially L-fucose, and to a lesser degree D-galactose and N-acetyl-D-galactosamine, typically found within the mucin O-glycan core structures. In cell-based assays, peptides **1** and **3** showed a similar binding profile to *Aleuria aurantia* lectin and these two peptides inhibited the migration of metastatic breast cancer cell lines in a Transwell assay. Altogether, the reported data demonstrate the feasibility of designing lectinomimics based on cyclic peptides.

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Compliance with ethical standards

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any other authors.

Keywords

cyclic peptide; lectinomimics; carbohydrate-binding protein; glycosylation; tumor metastasis; cell migration

Introduction

Aberrant surface glycosylation has emerged as a new hallmark of cancer (Munkley and Elliott 2016). These changes often involve the presence of incomplete or truncated glycan structures, the expression of novel carbohydrate moieties that do not occur on healthy cells, and an increased presence of sialic acid on proteins and glycolipids (Hakomori and Kannagi 1983; Drake 2015). Recent evidence suggests that the presence of certain glycan structures is not just an indicator of cancer progression and metastatic potential of tumor (Pinho and Reis 2015). These glycans are also key mediators of several processes involved in tumor cell proliferation, adhesion, and metastasis (Häuselmann and Borsig 2014; Hakomori and Cummings 2012; Pinho and Reis 2015). Thus, establishing connections between glycan structures and their functions has great potential in developing novel cancer prevention, detection and treatment strategies (Dalziel et al. 2014; Fuster and Esko 2005).

Due to the complexity of a cell's glycosylation machinery, and inherently low affinity of glycan interactions, targeting tumor-associated glycans remains a challenge in carbohydrate research. Significant efforts have been undertaken to produce glycan-specific antibodies (Dalziel et al. 2014; Fuster and Esko 2005). However, these have been difficult, as glycans are poor immunogens often resulting in weak affinity IgM antibodies with limited clinical value and low specificity of existing antibodies (Pinho and Reis 2015; Sterner et al. 2016). One of the most promising and the most widely explored tumor-associated glycan structures in many diagnostic and (immuno)therapeutic approaches are truncated mucin-type *O*-glycans such as Tn (α GalNAc-Thr/Ser), sTn (α Neu5Ac-(2,6)- α GalNAc-Thr/Ser), and T ($\text{Gal}\beta 1\text{-3GalNAc}\alpha 1\text{-O-Ser/Thr}$) (Cazet et al. 2010). The major protein carrier of these tumor-associated glycans is the MUC1 glycoprotein, highly expressed in a variety of epithelial cancers, but absent from normal tissues (Nath and Mukherjee 2014). Despite the great potential, the generation of clinically relevant human antibodies with good affinity and specificity for tumor-associated glycans of MUC1 proves to be a challenging task (Loureiro et al. 2015; Feng et al. 2016). Identification of MUC1 glycan/peptide epitopes within a MUC1 tandem repeat, which are able to overcome immunological self-tolerance and yet induce stronger and long-lasting immune response is the current focus of immunotherapy approaches (McDonald et al. 2015; Hossain and Wall 2016). An excellent example of such approaches is the PankoMab-GEX, a humanized monoclonal antibody that binds to a novel carbohydrate-induced conformational epitope on MUC1 (glycopeptide epitope) with a high affinity, which is currently undergoing clinical trials for ovarian cancer (Fiedler et al. 2016).

Besides antibodies, lectins, carbohydrate-binding proteins from animal or plant sources, have been widely used to detect tumor-associated glycan structures, and/or to isolate targeted glycoproteins out of a complex mixture (Tang et al. 2015). The development of microarray-based approaches, with either immobilized lectins or glycans, further improved

glycosylation profiling studies by enabling rapid and sensitive analysis of glycans on glycoproteins or glycolipids in high-throughput fashion (Song et al. 2012) The glycosylated form of α -fetoprotein (AFP), a broadly validated protein for diagnosis of liver diseases, has been approved by FDA as a marker of early detection of hepatocellular carcinoma (HCC) (Zhao et al. 2013) While serum levels of AFP suffer from low sensitivity and specificity, AFP with core fucosylation (AFP-L3) can be detected by the *Lens agglutinin* (LCA) lectin, and is a very specific marker for HCC, used to distinguish HCC from benign liver disease. The core fucosylation (α 1–6), in addition to terminal fucosylation (Lewis blood-group antigens, such as Le^{x/y} and Le^{a/b}) is another example of widely explored cancer-associated changes in glycosylation (Miyoshi et al. 2012) The full potential of the glycomic profiling in glyco-biomarker discovery is still hampered by the structural diversity of the human glycome, lectins' cross reactivity, and the lack of commercial availability of lectins that diversely recognize unique glycan structures (Ambrosi et al. 2005)

To address the aforementioned issues with antibodies and lectins, in particular the specificity, affinity, and accessibility, we have focused on cyclic peptide lectinomimics in the design of artificial carbohydrate receptors. Cyclic peptides offer several advantages over existing antibody/lectin-based glycan targeting approaches including the relatively small size of the binder molecule, stability (through cyclic structure), increased half-life, ease of synthesis, low cost, and a possibility for combinatorial chemistry modifications to improve selectivity and affinity toward certain glycan structures (Loffet 2002) Recently, it has been reported that a 17-mer cyclic peptide isolated from frog skin, named odorranalectin **1**, exhibits lectin-like properties with preferential affinity toward L-fucose (Li et al. 2008) Motivated by the carbohydrate binding properties and the simple structure of **1**, herein we report the design, synthesis, and binding study of lectinomimics based on this natural product. We have demonstrated that substitution of the disulfide bond found in the natural product **1** with a length equivalent and more stable mimic, lactam bridge, does not affect the cyclic peptide's conformation and its lectin-like properties. In addition, we have shown that the binding of lectinomimic analog **3** affected the migration ability of metastatic breast cancer cell lines in a Transwell assay, in a similar manner to the naturally occurring **1**, and in agreement with the essential role of fucosylation in tumor progression and metastasis.

Experimental Section

Reagents

TentaGel XV RAM resin was obtained from Rapp Polymer (Tuebingen, Germany). Fmoc-protected amino acids, 5(6)-carboxyfluorescein (5,6-FAM) and coupling reagents (HOBt, HBTU, PyBOP) were purchased from Chem-Impex (Wood Dale, IL) or Novabiochem (Gibbstown, NJ, USA). DIC was purchased from Acros Organics (Thermo Fisher Scientific, Waltham, MA). Fmoc-NH-(PEG)₂-COOH (20 atoms) was purchased from EMD Millipore (Billerica, MA). Kaiser test was purchased from AnaSpec (Fremont, CA). All solvents were purchased from Fisher Scientific (Atlanta, GA) or Sigma-Aldrich (St. Louis, MO), and were high-performance liquid chromatography (HPLC) grade. Fetuin, ASF, BME and iodine were purchased from Sigma-Aldrich (St. Louis, MO). Human cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). Control antibiotics (penicillin

and streptomycin) and enzymes (neuraminidase from *Clostridium perfringens*) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media and PBS buffers were purchased from Fisher Scientific (Pittsburg, PA). FITC-labeled lectins (AAL, UEA-I, and SNA) and BSA-conjugated monosaccharides (L-fucose, D-glucose, D-galactose and L-galactose) were purchased from Vector Labs (Burlingame, CA). Cell Titer-Glo Luminescent Cell Viability Assay was purchased from Promega (Madison, WI).

Peptide Synthesis

All linear peptidyl-resin precursors for naturally occurring cyclic peptide **1** and its analogs were synthesized by Fmoc-SPPS on TentaGel XV RAM resin (substitution 0.2–0.4 mmol/g, 0.25 mmol scale) using an automated peptide synthesizer. Amino acid couplings were done by using four-fold excess of amino acids and coupling reagents (HBTU/HOBt) in the presence of 0.4M NMM in DMF. Fmoc-deprotection cycles were carried out using 20% piperidine in DMF solution. The *N*-terminal Tyr was coupled as Boc-Tyr(*t*Bu)-OH. The solid-phase cyclization was achieved by either disulfide bridge formation for **1** or amide bond formation in case of amide analogs **2** and **3**. All peptides were cleaved from the resin, and all acid sensitive side-chain protecting groups were simultaneously removed using TFA/thioanisole/H₂O (95:2.5:2.5, v/v/v). The crude peptides were precipitated with cold methyl *tert*-butyl ether.

Synthesis of 1—In the case of **1**, Trt groups were removed from the Cys at position 6 and 16 *in situ* during final cyclization (disulfide bridge formation) with iodine (10 eq) and 2% anisole in CH₂Cl₂. This step was repeated twice for 30 min, and resin washed with DMF. (Zhang et al. 2007)

Synthesis of amide analogs 2 and 3—Amide analogs were prepared by coupling Fmoc-Dap(Mtt) and/or Fmoc-Asp(OAllyl)-OH instead of Fmoc-Cys(Trt)-OH at positions 6 and 16. For amide analog **2**, after assembly of the complete linear peptide sequence, the *N*^α-Boc protected resin was subjected to Pd(0) treatment with Me₂N•BH₃ (6 eq), Pd(PPh₃)₄ (0.1 eq) in CH₂Cl₂ under argon atmosphere for 30 min. (Gomez-Martinez et al. 1999) Following allyl removal, the Mtt protecting group was removed by stirring a solution of TFA:thioanisole:CH₂Cl₂ (2:1:97, v/v/v) for 30 min. Once the side-chains of Dap and Asp were exposed, linear cyclization was carried out overnight by using PyBOP/HOBt/DIEA (2 eq each), followed by subsequent washings with DMF and CH₂Cl₂. In the case of analog **3**, the synthetic scheme was modified due to the Asi formation observed during peptide chain assembly. Upon coupling of Fmoc-Asp(OAllyl)-OH, the resin was treated with Pd(0) with Me₂N•BH₃ (6 eq), Pd(PPh₃)₄ (0.1 eq) in CH₂Cl₂ under argon atmosphere for 30 min. (Gomez-Martinez et al. 1999) Following allyl removal, the Mtt protecting group was removed by stirring a solution of TFA:thioanisole:CH₂Cl₂ (2:1:97, v/v/v) for 30 min. Once the side-chains of Dap and Asp were exposed, cyclization was carried out overnight by using PyBOP/HOBt/DIEA (2 eq each). The cyclic product was formed, and no aspartimide formation was observed. The rest of the linear peptide backbone was assembled using standard Fmoc-SPPS conditions.

Synthesis of fluorescently labeled cyclic peptides 1-FAM, 2-FAM and 3-FAM—

Fluorescently labeled cyclic peptides were synthesized using standard Fmoc-SPPS methodology as reported previously, except that the last amino acid, Boc-Tyr(tBu)-OH, was replaced by its Fmoc-analog, Fmoc-Tyr(tBu)-OH, to allow for further chain elongation. After cyclization, Fmoc deprotection was done with 20% piperidine and a 20-atom linker, Fmoc-NH-(PEG)₂-COOH, was coupled using standard Fmoc-SPPS methodology, followed by subsequent Fmoc deprotection and coupling of 5(6)-carboxyfluorescein (5,6-FAM) overnight using DIC/HOBt (10 eq of all coupling reagents) in DMF (Alsina et al. 1994; Fischer et al. 2003)

Peptide Purification

Analytical RP-HPLC analyses and peptide purifications were performed on 1260 Infinity (Agilent Technologies, Santa Clara, CA) liquid chromatography systems equipped with a UV/Vis detector. For analytical RP-HPLC analysis, a C₁₈ monomeric column (Grace Vydac, 250 × 4.6 mm, 5 mm, 120 Å), 1 ml/min flow rate, and elution method with a linear gradient of 2→100% B over 45 minutes, where A is 0.1% TFA in H₂O, and B is 0.08% TFA in CH₃CN was used. For peptide purification, a preparative C₁₈ monomeric column (Grace Vydac, 250 × 22 mm, 10 mm, 120 Å) was used. Elution method was identical to the analytical method except for the flow rate, which was 20 ml/min. Mass spectrometry was performed on MALDI-TOF Voyager-DE™ STR (Applied Biosystems, Foster City, CA) in reflector-mode using α-cyano-4-hydroxycinnamic acid as a matrix and in positive mode.

Circular Dichroism Spectroscopy

CD spectra were recorded in water on a Jasco-810 spectropolarimeter (Jasco, Easton, MD) using a quartz cell of 1 mm optical path length. Spectra were measured over a wavelength range 180–250 nm with an instrument scanning speed of 100 nm/min and a response time 1 s. Peptides **1–4** concentrations were 1 mM and the CD spectra are the result of eight averaged scans taken at 25 °C. All CD spectra are baseline-corrected for signal contribution due to the water. CD spectra were modeled using the CDPro suite of programs employing SDP42 database.

Conformational Sampling with Distance-Dependent Dielectrics

Conformational analysis was performed using the natural ligand and its linkage analogs. A file containing 20 NMR solution structures of natural **1** (pdbid: 2JQW) was downloaded from the Research Collaboratory for Structural Bioinformatics web service. One of the models was selected as the starting point for **1**. Maestro v9.3 was used to build the analogs by replacing the disulfide bond of the natural ligand with the corresponding linker of each analog. The geometry of each structure was optimized using MacroModel v9.9, and conformational sampling was performed employing the *Macrocycle Conformational Sampling* option of MacroModel v9.9 with the OPLS 2005 force field. The distance-dependent dielectric constant was used with enhanced sampling mode. For each structure conformers above 5.0 kcal/mol of the identified global minimum were excluded, as were conformers that were less than 1.5 Å RMSD of a previously sampled conformer. The large-scale low-mode method was applied to 10,000 simulation cycles. To further reduce the

number of conformers of each compound sampled by MacroModel, the backbone atoms of its conformers were superimposed and clustering was performed using the trjconv and g_cluster modules of GROMACS, respectively. The centroid of each cluster was selected. To analyze the conformational effects of each linker, a pairwise RMSD distance matrix was computed employing the centroids of all the compounds using a python script in Chimera v1.62.

Docking Studies

Twenty odorranalectin NMR structures were downloaded from PDB (pdbid: 2JQW) and ensemble clustered using UCSF Chimera (Meng et al. 2006) Eight structures were grouped in the first cluster, and model number 10 was selected as the most representative for the average odorranalectin structure. Structures of the four sugar ligands were created using Gaussview (Dennington et al. 2009) and optimized using Gaussain09 (Frisch et al. 2009) with the Hartree-Fock method and 3-21G basis set. The optimized sugar structures were rigidly docked to the lectin peptide using Autodock 4 (Morris et al. 2009) The defined site for the docking search was based on the binding site from the NMR structure (Li et al. 2008) The initial positions and orientations of the ligands were randomized. Of the ten docked structures, only the lowest binding energy values for each ligand are given (Online Resource Table 1), and the corresponding structures were visualized with Maestro (Fig. 3). The lactam bridge modified lectin structures **2** and **3** were created by modifying the disulfide bridge between Cys6 and Cys16 to become a lactam bridge using Gaussview (Dennington et al. 2009), the structures were then minimised. The two lactam bridged peptides and the original lectin also underwent docking with L-fucose using Autodock 4 to produce 50 structures of lectin-ligand complexes (Morris et al. 2009).

Fluorescence-Based Binding Assay with BSA-Monosaccharides

The binding specificity of **1**-FAM was determined in the presence of BSA-conjugated monosaccharides (L-fucose, D-galactose, *N*-acetyl-D-galactosamine, D-glucose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-neuraminic acid). Fluorescence-based assay was performed in black 96-well immuno plates (Thermo Scientific™ 437111). A stock concentration of 1 mg/mL of each BSA-monosaccharide was prepared in DI water, and 50 µL were added into each well in 3 replicates and left to dry overnight overnight to inhibit potential adhesion of the tested lectin and peptide to the plastic surface and to assess the nonspecific interaction with BSA portion of the immobilized BSA-monosaccharides. After washing of wells with PBS (2 x), immobilized BSA-monosaccharides were incubated for 1h with 10 µg/mL of AAL-FITC or **1**-FAM at room temperature in the dark, with gentle mixing on a shaker platform. After incubation, wells were washed with PBS buffer (3x), and then fluorescence signal was monitored using a Cytation 5 (Biotek).

Isothermal Titration Calorimetry (ITC) Measurements

Binding studies were performed at 25 °C in 20 mM HEPES at pH 7.0 by using a titration calorimeter PEAQ-ITC (Malvern, Northampton, MA) with a reaction cell volume of 300 µL. Typically, glycosylated protein (ASF and fetuin) solutions (250 µM) were in the reaction cell and titrated with solutions of cyclic peptide (**1**–**3**) at a concentration 10-fold greater (2500

μM) than the glycoprotein. Cyclic peptides (**1–3**) and the glycoproteins (ASF and fetuin) were dialyzed and prepared in exact the same buffer. At least 18 consecutive injections of 2 μL were applied every 180 s intervals while solution was stirred at a constant speed of 1000 rpm. Dilution heats of the cyclic peptides (**1–3**) were independently measured and subtracted from the heats of binding. Experimental curves were analyzed by using Origin, which was provided with the instrument. In all cases, thermodynamic parameters were derived from at least two independent experiments and then averaged.

Cell Culture

Cell lines were grown in their specified media, as recommended by ATCC in tissue flasks to approximately 80% confluency at 37 °C in a 5% CO₂ atmosphere (with exception of the MDA-MB-231 cell line). T-47D (ATCC® HTB-133™) cells were cultured in RPMI 1640 media, supplemented with 2 mM L-glutamine, 10% (v/v) inactivated FBS, 50 μM 2-mercaptoethanol, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.01 mg/mL insulin. BJ (ATCC® CRL-252™) cells were cultured in Eagle's Minimum Essential Medium (EMEM), supplemented with 2 mM L-glutamine, 10% (v/v) inactivated FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. MCF-7 (ATCC® HTB-22™) cells were cultured in Eagle's Minimum Essential Medium (EMEM), supplemented with 10% (v/v) inactivated FBS, 100 U/mL penicillin, 100 U/mL streptomycin, and 0.01 mg/mL insulin. HEP-G2 (ATCC® HB-8065™) cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 2 mM L-glutamine, 10% (v/v) inactivated FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. MDA-MB-231 (ATCC® HTB-26™) and 231mfp cells were cultured in Leibowitz Medium (L-15) supplemented with 2 mM L-glutamine, 10% (v/v) inactivated FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. 4T1 (ATCC® CRL-2539) cells were cultured in RPMI1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) inactivated FBS.

Fluorescently Labeled *in Vitro* Lectin Cell-Based Assays

Cell lines were grown in their specified media, as recommended by ATCC, in tissue flasks to approximately 80% confluency, gently trypsinized with Accutase, washed with PBS and incubated in 4% paraformaldehyde-Dulbecco's PBS solution to fix cells on 96-well plates (Corning, #353962) in their recommended cell/well concentration (for BJ cell line 10,000 cells/well; all other cell lines 30,000 cells/well). The wells were then washed with PBS, incubated with 3% BSA in PBS buffer for 30 min (blocking), followed by Dulbecco's PBS wash and incubated with FITC-labeled lectins (SNA, AAL and UEA-I) or fluorescently labeled peptides (**1-FAM**, **2-FAM**, and **3-FAM**) at varied concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 $\mu\text{g/mL}$) for 90 min at 25 °C with gentle mixing on a shaker platform, then washed with Dulbecco's PBS, mounted, examined, and pictures were taken with a fluorescent microscope (Olympus, Waltham, MA) and camera (Photometrics, Tucson, AZ). Then fluorescence signal was monitored using a Synergy H4 microplate reader (Biotek). For neuraminidase treatment, fixed cells were washed with reaction buffer (50 mM sodium citrate, pH 6.0), treated with 50 mU of neuraminidase overnight at 37 °C in CO₂-free incubator, blocked with 3% BSA in PBS buffer for 30 min, followed by Dulbecco's PBS wash, and further incubated with FITC-labeled lectins (SNA, AAL and UEA-I) or

fluorescently labeled peptides (**1**-FAM, **2**-FAM, and **3**-FAM) at specified concentrations (60–120 µg/mL).

Fluorescently Labeled *in Vitro* Inhibition Competitive Cell-Based Assays

The inhibition of binding of **1**-FAM was determined in the presence of unlabeled **1** ranging in concentration from 1×10^{-12} M to 1×10^{-3} M. Serial dilutions (1:10) of unlabeled **1** was prepared in Dulbecco's PBS buffer, and the appropriate concentration was added (10 µL) to each well, followed by 80 µL of PBS buffer, and 10 µL of **1**-FAM (60 µg/mL final concentration). Reaction mixtures were incubated at room temperature in the dark for over 2.5 h with gentle mixing on a shaker platform, and then fluorescence was measured as described above. The IC₅₀ values were analyzed by non-linear regression analysis by GraphPad Prism v5.04. A competitive binding assay of **1**-FAM was also determined in the presence of monosaccharides (D-glucose, L-fucose, L-galactose, and D-galactose) ranging in concentration from 1×10^{-5} M to 1 M. Serial dilutions (1:10) of each monosaccharide was prepared in Dulbecco's PBS buffer, and the appropriate concentration was added (10 µL) to each well, followed by 80 µL of PBS buffer, and 10 µL of **1**-FAM (60 µg/mL final concentration). Reaction mixtures were incubated at room temperature in the dark for over 2.5 h with gentle mixing on a shaker platform. After incubation, wells were washed with PBS buffer (3x), and then fluorescence signal was monitored using a Synergy H1 microplate reader (Biotek). The IC₅₀ values were analyzed by non-linear regression analysis by GraphPad Prism v5.04.

Cell Toxicity Assay

Viability of peptides was determined after 72 h exposure, and assessed using Cell Titer-Glo Luminescent Cell Viability Assay (Promega), a homogeneous method of determining the number of viable cells in culture based on quantitation of the ATP present using the Cell Titer Glo colorimetric assay. Assays were set up in flat-bottom polystyrene 96-well plates with 10,000 BJ (ATCC® CRL-2522™) cells per well grown in EMEM containing 10% FBS, and 5% penicillin/streptomycin (v/v). After an overnight incubation at 37 °C under a humidified atmosphere with 5% CO₂, media was removed, and fresh media with 2% FBS containing peptides in specified concentrations (0.06, 0.6, and 2 mg/mL) was added. Plates were again incubated at 37°C under a humidified atmosphere with 5% CO₂. As a control, 20% DMSO in EMEM media was used. After incubation for 72 h, media was removed, and 100 µL PBS buffer was added, followed by 100 µL of Cell Titer Glo® Reagent. Plates were incubated for further 10 min at room temperature (protected from light), before luminescence signal was monitored using a Synergy H1 microplate reader (Biotek).

Serum Stability Assay

For stability in 25 % human serum (MilliporeSigma), peptides **1–3** (1 mg each) were dissolved in 200 µL DI water, to which 1300 µL water and 500 µL human serum were added. The solution was incubated at 37°C. After 0 min, 45 min, 2, 4, 8, and 24 h, three samples (3 × 100 µL) of each peptide were taken and precipitated by the addition of 20 µL of 15% aqueous TCA. Samples were quickly vortexed and then centrifuged at 10,000 rpm for 10 min. The supernatant was analyzed by analytical RP-HPLC, main fractions were

collected and analyzed by mass spectroscopy (Thermo Scientific LTQ MS/MS spectrometer equipped with an electrospray source).

Cell Migration Assay

Cell migration assays were performed using modified Boyden chambers with a 6.5 mm diameter, porous (8.0 μm) polycarbonate membrane separating the two compartments of the chambers (Transwell, Corning, Inc., Corning, NY). Cells were harvested by brief exposure to trypsin/EDTA (Invitrogen) followed by soybean trypsin inhibitor (Calbiochem). Cells were washed and re-suspended in fibroblast basal medium (Cambrex Bioscience, Walkersville, MD) containing 0.5% bovine serum albumin, 2 mmol/L CaCl_2 , 1.8 mmol/L MgCl_2 , and 0.2 mmol/L MnCl_2 . Cells (100,000 in 100 μL) were placed in the upper compartment of the migration chamber and 3-fold concentrated NIH/3T3 conditioned medium (CM) was placed in the lower compartment. Lectins were added in the indicated concentrations to both, the upper and lower compartments of the migration chamber. Cells were allowed to migrate for 4 hours at 37°C in 5% CO_2 . At the end of the assay, the upper surface of the membrane was wiped with a cotton tip applicator to remove non-migratory cells. Cells on the lower surface were fixed in 1% paraformaldehyde, stained with 1% crystal violet, and counted.

Results and Discussion

Peptide Design and Synthesis

Odorranalectin **1** (Scheme 1) is a 17-mer cyclic peptide that adopts a β -turn conformation stabilized by one intramolecular disulfide bridge between Cys⁶-Cys¹⁶ and three hydrogen bonds between Phe⁷-Ala¹⁵, Tyr⁹-Val¹³, Tyr⁹-Gly¹² amino acids (Li et al. 2008). In order to increase stability of **1** toward reducing and nucleophilic agents present in biologically relevant conditions, we have explored the possibility of substituting disulfide bridge in this peptide with a length-equivalent lactam bridge and more stable mimics capable of reproducing the peptide conformation (Liskamp et al. 2011; Li et al. 2002). Taking into consideration that solid-phase peptide synthesis (SPPS) is the method of choice for peptide synthesis, our synthetic approach includes assembly of linear precursors using Fmoc solid-phase methodology and on-resin side chain-to-side chain cyclization. To keep the same ring size, the Cys residues at positions 6 and 16 in peptide **1** were replaced with diaminopropionic acid (Dap) and aspartic acid (Asp). In this case, ring closure can be performed in two different ways regarding the amide bond orientation: direct (Dap⁶-Asp¹⁶) following *N* to *C* terminus peptide bond orientation and reversed (Asp⁶-Dap¹⁶) with opposite orientation of the peptide bond.

Linear precursors of cyclic peptides **1–3** and the control linear peptide **4** were assembled by using a standard automated Fmoc-methodology. The PEG-based TentaGel XV RAM resin was used due to its low substitution level (0.21 mmol/g) and extended swelling properties, especially in CH_2Cl_2 , creating a pseudodilution condition required to enhance the intramolecular bond formation (Pipkorn et al. 2013). In the case of **1**, resin-bound peptide oxidation using iodine was chosen over the in-solution DMSO-promoted air oxidation to circumvent the rather tedious work-up of very dilute solutions used to enhance the

intramolecular disulfide formation (Scheme 1) (Pipkorn et al. 2013; Zhang et al. 2007) It has been shown in literature that oxidation of S-trityl peptides with iodine proceeds rapidly in CH_2Cl_2 , thus minimizing the possibility of side-product formation (Kamber et al. 1980) Therefore, 4-methyl trityl (Mtt) protected β -thiol moiety of Cys residues in positions 6 and 16 were chosen for the solid-phase synthesis of **1**. Using this strategy, **1** was obtained in good yields (~85%). The final deprotection and cleavage from the resins for peptides **1** and **4** was carried out with a cleavage cocktail of TFA/thioanisole/ H_2O (95:2.5:2.5, v/v/v). Peptides **1** and **4** were purified by preparative RP-HPLC, and characterized by MALDI-TOF mass spectrometry and analytical RP-HPLC (Online Resource Fig.1, and Fig. 2).

To investigate the effects of the cyclization on proteolytic susceptibility of peptides **1–3**, the disappearance of the intact peptides in 25 % human serum at 37°C was monitored by RP-HPLC and mass spectrometry (Online Resource Fig 10–13). Expectedly, degradation of the linear portion of the peptide sequences was observed for all three peptides (Online Resource Fig. 10A). However, in the case of peptides **1** and **3**, *N*-terminal Tyr¹ and Ala² were cleaved first, resulting in cyclic fragments **1'** and **3'**, respectively, whereas during the same time period the whole linear sequence (Tyr¹-Ala²-Ser³-Pro⁴-Lys⁵) of peptide **2** was cleaved giving fragment **2'**. Further analysis of the degradation of **1'–3'** revealed that the cyclic fragments **1'** and **3'** degrade at much slower rate in comparison to **2'**. After incubation for 24 h in human serum 33 % of a **1'**, 35 % of **3'** and 14 % of **2'** was recovered, respectively (Online Resource Fig. 10B). Peptide conformation and thus accessibility to proteases present in human serum may explain the observed differences in serum stability of peptides **1–3** (Brannon et al. 2015; Cline and Waters 2009).

The Fmoc solid-phase synthesis of lactam analogs **2** and **3** is depicted in Scheme 2. Mtt and allyl groups were chosen for the orthogonal protection strategy that allows for on-resin deprotection and side-chain (Asp) to side-chain (Dap) cyclization by amide bond formation. Mtt was used for the side chain protection of Dap and allyl for the Asp moiety. The Cys residues at positions 6 and 16 were replaced with Fmoc-Dap(Mtt)-OH and/or Fmoc-Asp(OAllyl)-OH ultimately, resulting in lactam analog **2** or **3**, respectively. Following the assembly of a linear precursor on solid-phase, the allyl and Mtt protecting groups were removed by subsequent treatment of the peptidyl resin with $\text{Pd}(\text{Ph}_3\text{P})_4$ as previously described, (Gomez-Martinez et al. 1999) and with 2% TFA in CH_2Cl_2 . On-resin side chain-to-side chain cyclization between residues Dap⁶ and Asp¹⁶ in lactam **2** was achieved by treating the peptidyl resin with PyBOP/HOBt/NMM. Lactam analog **2** was obtained in ~90% yield. The final deprotection and cleavage from the resin for peptide **2** was carried out with a cleavage cocktail of TFA/thioanisole/ H_2O (95:2.5:2.5, v/v/v), purified by preparative RP-HPLC, and characterized by MALDI-TOF mass spectrometry and analytical RP-HPLC (Online Resource Fig.3). However, this particular solid-phase synthetic strategy could not be applied for preparation of lactam analog **3** due to the aspartimide side-product formation observed during the peptide chain elongation (Online Resource Fig. 4). RP-HPLC and MALDI-TOF mass spectrometry analysis of the crude linear peptide precursor of **3** revealed formations of two main products (Online Resource Fig.4, panel A). The product eluting at 15.4 min corresponds to the desired product, the full length Fmoc-protected linear precursor of **3**, linear-**3** ($[\text{M}+\text{H}]^+$, m/z = 1884.61, calculated = 1883.97), whereas product eluting at

16.1 min corresponds to the aspartimide side-product of **3**, Asi-**3** ($[M+H]^+$, found $m/z = 1867.60$, calculated = 1865.96). Aspartimide formation can be rapid, catalyzed by either acids or bases, (Subiros-Funosas et al. 2011) and depends on the Asp side-chain protection and its carboxyl group neighboring residue. In addition, it has been shown that the allyl side-chain-protecting group for aspartyl residues, while having its appeal in being orthogonal to Fmoc or Boc protection strategies, is particularly prone to formation of aspartimide (Flora et al. 2005) Therefore, a different solid-phase synthetic strategy for **3** was designed, Scheme 2B. To minimize the aspartimide formation during the synthesis of **3**, a linear peptide precursor composed of the first 12 amino acids was assembled on solid-phase using standard Fmoc chemistry followed by allyl and Mtt protecting groups removal and side-chain to side-chain cyclization between the residues Asp⁶ and Dap¹⁶ as previously described. Once formation of the lactam bridge was confirmed by RP-HPLC and MALDI-TOF mass spectrometry, the remainder of the peptidyl chain was synthesized by standard Fmoc-SPPS conditions (Scheme 2B). In this case, no aspartimide side-product was observed by RP-HPLC and MALDI-TOF mass spectrometry, and the desired lactam analog **3** was obtained in 86% yield (Online Resource Fig. 4, panel C). The final deprotection and cleavage from the resin for peptide **3** was carried out with a cleavage cocktail of TFA/thioanisole/H₂O (95:2.5:2.5, v/v/v), purified by preparative RP-HPLC, and characterized by MALDI-TOF mass spectrometry and analytical RP-HPLC (Online Resource Fig. 5).

Fluorescein (FAM) labeled analogs **1**-FAM, **2**-FAM and **3**-FAM were synthesized using the same strategies previously described for non-labeled cyclic peptides **1–3** (Scheme 3). To increase the solubility of the labeled peptides and to minimize the potential for aggregation, the Fmoc-NH-(PEG)₂-COOH (20 atoms) was added as a linker between the *N*-terminal Tyr and the fluorescein (FAM) moiety (Veronese and Mero 2008) Following Fmoc protecting group removal by 20% piperidine in DMF, 5(6)-carboxyfluorescein (5,6-FAM) was coupled using DIC/HOBt protocol. The fluorescently labeled peptides **1**-FAM, **2**-FAM and **3**-FAM were purified by preparative RP-HPLC, and characterized by MALDI-TOF mass spectrometry and analytical RP-HPLC (Online Resource Fig. 6–8).

To assess the effect of this substitution on the conformation of peptide **1**, a conformational analysis of the natural product **1** as well as lactam peptide analogs **2** and **3** (Fig. 1) was performed since several studies have pointed out the impact of the disulfide bond surrogate on the cyclic peptide's conformation and biological activities (Hargittai et al. 2000; Fazio et al. 2006; Gazal et al. 2002; Meinander et al. 2014; Gongora-Benitez et al. 2014). Computational methods as well as CD spectroscopy were used to investigate conformational characteristics of peptides **1–3**. The computational analysis involved a conformational sampling, backbone superimposing and clustering of the cyclic peptides **1–3**. It should be noted that only one cluster was found for peptide **1**. To analyze the conformational effects of the lactam bridge orientation, a pairwise RMSD² matrix was computed for peptide analogs **2** and **3**. Interestingly, RMSD values show clear differences. The best overlay was obtained for lactam **3** with RMSD distance of 2.59 Å and a percent population of 80% (Fig. 1B, panel II). In the case of lactam **2**, calculated RMSD was 4.94 Å and a percent population of 93.4% (Fig. 1B, panel I). This data indicated a greater possibility of lactam **3** to adopt a conformation similar to **1** in comparison to lactam **2**. Circular dichroism (CD) spectra of

synthetic peptides **1–3** recorded in aqueous medium, (Fig. 1A), are consistent with the RMSD calculations. The CD spectrum of **1** is characterized by a minimum at 190 nm and a weak maximum at 230 nm, characteristic of an overall β -turn conformation. In the case of lactam **2** the spectral minimum is shifted to 196 nm, indicating significant conformational differences between **1** and **2**. Interestingly, as shown in Fig. 1A, lactam **2** and the control linear peptide **4** exhibit similar CD spectra, suggesting that both peptides adopt rather unordered structures in an aqueous medium. On the other hand, the two minima observed at 190 and 195 nm in the CD spectrum of lactam **3** suggest the presence of multiple conformers in aqueous solution with some adopting a conformation similar to **1**.

Binding Analysis with Simple Monosaccharides and Model Glycoproteins

To verify our hypothesis that the lactam analog **3** that adopts conformations similar to the natural product **1** can also exhibit similar lectin-like properties, we determined, firstly, the affinities of peptide **1** toward simple monosaccharides by using a fluorescence-based binding assay, and secondly, affinities of peptides **1–3** toward model glycoproteins using isothermal titration calorimetry (ITC). Our choice of monosaccharides and glycoproteins was based on the initially reported carbohydrate-binding specificity of naturally occurring **1** suggesting that **1** preferably binds L-fucose amongst simple monosaccharides, and exhibits the strongest affinity for fetuin, and to a lesser extent to two types of mucin, bovine submaxillary (BSM) and porcine stomach (PSM) (Li et al. 2008).

Fluorescence-based binding assay was performed with the following BSA-monosaccharides (L-fucose, D-galactose, D-glucose, *N*-acetyl-D-neuraminic acid, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine) to further assess carbohydrate-binding specificity of **1**. Upon immobilization of BSA-conjugated monosaccharides on the microtiter plate, all wells were incubated overnight with PBS containing 3% of bovine serum albumin (BSA) at room temperature to block potential adhesion to the plastic surface of a multiwell plate and to assess to the nonspecific interaction with BSA portion of the immobilized BSA-monosaccharides. The blocking solution was then removed and fluorescein-labeled fucose-specific lectin *Aleuria aurantia* (AAL-FITC) (10 μ g/mL) or fluorescein-labeled **1**-FAM (10 μ g/mL) was added to each well and allowed to incubate for 1 h. In the next step, wells were washed with PBS (3x) and fluorescence intensities were measured as an indication of lectin's binding to the monosaccharide substrate. As expected, L-fucose was the only monosaccharide showing interaction with AAL-FITC and a strong interaction with **1**-FAM (Fig. 2). Notably, **1**-FAM bound to a lesser degree, to galactose and *N*-acetyl-D-galactosamine. However, no binding was observed with D-glucose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-neuraminic acid.

The binding affinities of two model glycoproteins, fetuin and asialofetuin, for peptides **1–3** were determined by ITC. The measured K_d and thermodynamic parameters for the binding process are summarized in Table 1 (binding curves were provided in Online Resource Fig. 9). As expected, cyclic peptides **1** and **3** revealed similar binding profiles toward fetuin and asialofetuin. Both peptides exhibit approximately one order of magnitude higher affinity toward asialofetuin (K_d 63 and 85 μ M, respectively) than fetuin (K_d 520 and 227 μ M, respectively). The lactam analog **2** that cannot adopt a conformation similar to **1**, did not

show detectable binding affinities toward fetuin or asialofetuin under the applied experimental conditions. Lack of binding for these two proteins was also observed for control peptide **4**, that similarly to lactam **2**, exhibits an unordered structure in aqueous medium (Fig. 1A). Fetuin and asialofetuin were used in this study as model glycoproteins because their oligosaccharide structures have been well characterized (Green et al. 1988). Structurally, the only difference between these two glycoproteins is the presence of the terminal sialic acid moiety in the six glycan chains of fetuin (Spiro and Bhoyroo 1974; Green et al. 1988). The lower affinities of peptides **1** and **3** toward fetuin can possibly be explained by the presence of the sialic acid moiety that blocks the access of **1** and **3** to the terminal glycan moiety present in asialofetuin (resulting from desialylation).

Molecular Docking Analysis

Molecular docking studies with four monosaccharides (L-fucose, D-galactose, *N*-acetyl-D-galactosamine, and D-glucose) were conducted to gain more insights into the binding modes of these monosaccharides with **1**. Energetically favorable interactions were observed for L-fucose, D-galactose and *N*-acetyl-D-galactosamine. The binding pose of D-glucose is distinct in position and higher in energy to that of the other docked sugars causing its binding energy on average to be higher than the other three sugars by 0.98 kcal mol⁻¹ (Online Resource, Table 1). These results are in good agreement with the observed preferential binding of **1** to L-fucose in comparison to the undetectable binding to D-glucose in a fluorescence-based assay. Protein hydrophobic side chains enclose the binding sites of all four sugars. In the case of D-glucose, these are the side chain of Phe⁷ and the aliphatic moiety of Lys⁵; and for the other three sugars, in addition to Phe⁷ and Lys⁵, we have also the sidechain of Tyr⁹. The similar binding orientations of L-fucose, D-galactose and D-galactosamine make it hard to assess the individual van der Waals contributions of the neighboring non-polar residues, to the binding energy. L-fucose, D-galactose and D-galactosamine were found to have the same four hydrogen bonds with Lys⁵, Phe⁷, Arg⁸ and Tyr⁹; whereas D-glucose, was found to form only two hydrogen bonds with the backbone of Cys⁶ (Fig. 3). This may contribute to its relatively higher binding affinity. In addition to the four hydrogen bonds observed in the complexes of D-galactose and D-galactosamine, L-fucose can also form additional weaker hydrogen bond with the backbone carbonyl of Arg⁸. The charge of the ring oxygen of L-fucose is -0.68. Thus L-fucose can interact electrostatically with the positively charged side chain of Lys⁵ and in this way contributing to its strongest binding affinity (Samsonov et al. 2011). The docking of L-fucose to **2** and **3** showed similar docking poses, however slightly more different but in a similar region to the pose of L-fucose to **1** (Online Resource Fig. 14). The main difference in the position of L-fucose in **2** and **3** as opposed to **1** is the loss of the hydrogen bond with the side chain on Lys⁵. Instead the sugar is found to hydrogen bond the sidechain of Asn¹¹, this interaction isn't found in the docking of L-fucose to **1**. The difference in binding is probably due to the conformational changes caused by replacing the disulfide bridge with a lactam bridge. Importantly, the structures of **2** and **3** were minimized structures only, while the structure of **1** is a representative structure from NMR experiment. In order to compare the binding affinities of **1-3**, it is necessary to account for the conformational flexibility of **2** and **3**. Thus more accurate method for free energy calculations such as MMPBSA/GBSA will have to be applied. The proposed alternative binding site for L-fucose interacting with Cys⁶, Cys¹⁶ and

Thr¹⁷, suggested by the NMR titration experiment (Li et al. 2008) was not observed in any of the binding poses of any of the dockings, even after enlargement of the docking area. However, future studies involving the consideration of lectin and glycan flexibility, as well as explicit solvation, are necessary to confirm this observation.

Cell-Based Binding Studies

The cell-based binding studies were performed with fluorescently labeled cyclic peptides **1–3**. The PEG based linker was inserted between the *N*-terminal Tyr and the fluorescein moiety to increase solubility of the fluorescein tagged odorranalectin analogs in aqueous buffer and to reduce potential steric effect of bulky fluorescein moiety on binding. The abilities of the fluorescently labeled cyclic peptides **1–3** to bind cell surface glycosylated epitopes and their binding preferences were assessed by the *in vitro* lectin-cell staining assays using both cancer and normal human cell lines qualitatively by fluorescence microscopy and quantitatively by measurement of the bound compounds' fluorescence intensities (Fig. 4, 5). For these assays we have chosen T-47D (human breast epithelial ductal carcinoma), MCF-7 (human breast epithelial adenocarcinoma), MDA-MB-231 (human breast adenocarcinoma, triple negative-metastatic), and Hep G2 (human liver hepatocellular carcinoma, metastatic) cancer cells known to express different levels of fucosylated and/or sialylated glyco-epitopes on their surfaces (Dall'Olio et al. 2012; Yuan et al. 2008; Muller and Hanisch 2002). As a control, BJ human skin fibroblasts were used. Initial staining of cells (BJ, T-47D, MCF-7) to assess the level of sialylated glyco-epitopes was performed using FITC-labeled lectins, such as *Sambucus nigra* lectin (SNA), known to bind to terminal sialic acid (Shibuya et al. 1987). Since it has been reported in literature that **1** exhibits high affinity and specificity toward L-fucose monosaccharide, (Yuan et al. 2008) the level of fucosylated antigens on the cell surface was assessed using *Aleuria aurantia* lectin (AAL) and *Ulex europaeus* I lectin (UEAI). AAL exhibits broad specificity for $\alpha(1,2)$ -, $\alpha(1,3)$ -, and $\alpha(1,4)$ -, and preferentially binds $\alpha(1,6)$ -fucose-containing oligosaccharides (core fucosylation), (Wimmerova et al. 2003) and UEAI prefers $\alpha(1,2)$ -linked fucose residues (terminal fucosylation) (Baldus et al. 1996). The comparison of the lectin-staining patterns revealed high expression of the terminal sialic acid on the surface of T-47D cells (Fig. 4B), whereas MCF-7 cells exhibited higher expression of fucosylated glycans (Fig. 4C). The control BJ cells show no significant staining by either SNA or fucose specific lectins UEAI and AAL (Fig. 4A), indicating the absence of sialylated and fucosylated antigens on the cell surface. AAL lectin and **1-FAM** exhibit a similar binding profile for these three cell lines. Interestingly, removal of the terminal sialic acid by treatment with sialidase (neuraminidase) did not significantly affect binding of **1-FAM** (Fig. 4) to any of the cell lines used in this study. This is in slight contradiction with the results from the ITC binding studies using fetuin and asialofetuin, and may be explained by the structural differences between the two glycan presentations in a physiological setting on the surface of the cell.

In order to evaluate the binding preferences of lactam analogs **2** and **3**, two highly invasive metastatic cell lines, MDA-MD-231 and Hep G2, were added to the study. As shown in Fig. 5, lactam analog **3-FAM** exhibited similar cell surface binding preferences to those of **1-FAM** and the AAL lectin. The weak binding affinity of AAL lectin for MDA-MD-231 cells, in comparison to **1-FAM** and **3-FAM**, could be attributed to differences in the cell-surface

glycan structures between the two cell lines and the broader carbohydrate specificities of **1**. The fluorescence-based binding assay has revealed that naturally occurring cyclic peptide **1**, besides preferably binding to L-fucose, binds to a lower extent to D-galactose and *N*-acetyl-D-galactosamine typically found within the mucin *O*-glycan core structures. The ability of lactam **2**-FAM to label the cell surface of the tested cells was significantly reduced in comparison to **1**-FAM and **3**-FAM, as expected, based on their conformational differences (Fig. 5). The ability of **1** to label cancer cells expressing fucosylated glycoproteins was further assessed using cell-based assays in a competitive binding configuration (Fig. 6). In the competitive binding mode assay, increase of the concentration of the unlabeled ligand **1** was used to displace the fluorescently labeled **1**-FAM, leading to disruption of the association between **1**-FAM and cell surface epitopes, and thus a decrease in fluorescence signal (Fig. 6A). This assay clearly demonstrated the ability of **1** to inhibit the MCF-7/**1**-FAM interaction with an IC₅₀ value of 3.97 μ M (Fig. 6A). No binding was observed in the assay with the BJ cell line, indicating the absence of non-specific binding (Fig. 6A).

In order to confirm these findings, a different format of the competitive binding assay was performed. In this case an increase in the concentration of monosaccharides (L-fucose, L-galactose, D-galactose and D-glucose) was used to displace **1**-FAM from the cell surface (Fig. 6B). The IC₅₀ values were calculated from the range of increasing concentrations of the monosaccharides (10 μ M to 1 mM). This assay showed that L-fucose and L-galactose, which differs from L-fucose only in the type of substituent at C5, inhibited the MCF-7/**1**-FAM interaction with almost identical IC₅₀ values of 80 and 81 mM, respectively (Fig. 6B). D-glucose and D-galactose inhibited to a much lesser degree with IC₅₀ values of 318 and 252 mM, respectively. These findings suggest that the orientations of the hydroxyl groups at C2, C3 and C4 positions are the key determinants of odorranalectin specificity for fucose.

Cell Toxicity Studies

The *in vitro* toxicity of the OL and its amide analogs towards BJ cells was determined using a Cell Titer-Glo Luminescent Cell Viability Assay (Fig. 7). Cyclic peptide **1** showed some toxicity after 72 h at very high concentrations (2 mg/mL), while the amide analogs **2** and **3** did not show any appreciable cytotoxicity at those high concentrations.

Cell-Based Migration Studies

Given the significant role tumor-associated glycans play in invasion and metastasis, (Stowell et al. 2015) we questioned whether the ability of naturally occurring odorranalectin **1** and its amide analog **3** to bind to the cell surface glycans might affect processes associated with metastasis. Thus, we tested cyclic peptide **1** and its analog **3** at three different concentrations (10, 50, and 100 μ g/mL) in the Transwell migration assay. Three metastatic breast cancer cell lines, MDA-MB-231, 231mfp, a more aggressive sub-line of MDA-MB-231 cells, (Jessani et al. 2005) and 4T1, mouse mammary cancer cells, were chosen for this assay. As shown in Fig. 8, significant differences in inhibition were observed between the three cell lines. The inhibition of migration of 231mfp cells was more pronounced than that of MDA-MB-231 cells (65% in comparison to 50% at 50 μ g/mL), and only a minor inhibition was observed for the mouse 4T1 cell line (30% at 100 μ g/mL concentration). However, there were no observed differences in the ability of lectin **1** and **3** to block migration of these cells

at any concentration used in the assay. Both lectins showed maximum inhibition of MDA-MB-231 and 231mfp cell migration at a concentration of 50 $\mu\text{g/mL}$, and further increase in lectin concentration had no effect on migration. Together these data suggest that odorranalectin **1** is blocking cell-type specific target structures on the cell surface of tumor cells and that there are glycan-dependent and independent mechanisms involved in cell migration.

Conclusions

Odorranalectin's potential application in the medical and/or bioanalytical field may be hampered by its low stability of disulfide bond toward protein disulfide isomerases (PDI) and variety of reducing agents, leading to structural rearrangements and thus, loss of its lectin-like properties. The disulfide bond found in **1** was substituted with a length equivalent lactam bridge formed by on-resin side-chain (Asp) to side-chain (Dap) cyclization. Lactam analogs differing in the orientation of the amide bond (Dap⁶, Asp¹⁶) **2** and (Asp⁶, Dap¹⁶) **3** were prepared and their conformations and lectin-like properties were compared to the natural product **1**.

Conformational analysis using conformational sampling with distance-dependent dielectrics and CD spectroscopy revealed that cyclic peptide **3** is capable of adopting a conformation similar to **1**, whereas this is not the case for analog **2** (Fig. 1). Our binding assay using BSA-conjugated monosaccharides confirmed the binding preference of **1** for L-fucose. However, peptide **1** does not exclusively bind L-fucose, as this peptide also exhibits affinity, although to a lesser degree, toward D-galactose and *N*-acetyl-D-galactosamine. Interestingly, no appreciable affinity was observed for nearly identical D-glucose and *N*-acetyl-D-glucosamine under the applied experimental conditions. The energetically favorable interactions of **1** with L-fucose, D-galactose and *N*-acetyl-D-galactosamine were further confirmed by molecular docking. ITC binding experiments revealed comparable affinities of **1** and **3** toward two fetuin and asialofetuin, with highest affinity for asialofetuin. On the other hand, lactam analog **2** that cannot adopt a conformation similar to **1**, did not show detectable binding affinities toward fetuin or asialofetuin under the applied experimental conditions. Altogether, these data further support our hypothesis that conformation and secondary structure play an important role in the lectin-like properties of this class of cyclic peptides.

In cell-based assays, **1** and **3** exhibited comparable binding profiles, whereas lectin-like properties were not observed for peptide **2**. Based on the similarity with the binding profile of AAL lectin and inhibition binding profile with monosaccharide ligands, we hypothesize that **1** and its amide analog **3** target cancer cells over-expressing L-fucose, and possibly the mucin-type *O*-glycans, both, essential components of malignant and metastatic phenotype of many human cancers. In agreement with our hypothesis, binding of **1** and its amide analog **3** affected the migration ability of metastatic breast cancer cell lines in a transwell assay. Noteworthy, successful substitution of disulfide by an amide bridge opens a possibility for further structural modifications of cyclic peptides similar to odorranalectin, including combinatorial chemistry approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RMSD	root-mean-square deviation
AAL	<i>Aleuria aurantia</i> lectin
ASF	asialofetuin
BME	β -mercaptoethanol
Boc	tert-butyloxycarbonyl
BSA	bovine serum albumin
CM	conditioned medium

CV	coefficient of variation
DIC	diisopropylcarbodiimide
DMF	N,N-dimethylformamide
DMSO	dimethyl sulfoxide
FAM	fluorescein
FITC	fluorescein isothiocyanate
Fmoc	fluorenylmethyloxycarbonyl
HOBt	hydroxybenzotriazole
ITC	isothermal titration calorimetry
MALDI-TOF	matrix assisted laser desorption/ionization time-of-flight
NMM	<i>N</i> -methylmorpholine
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RP-HPLC	reverse-phase high pressure liquid chromatography
RT	room temperature
S/B	signal-to-background
SNA	<i>Sambucus nigra</i> lectin
UEA-I	<i>Ulex europaeus</i> I lectin
SPPS	solid-phase peptide synthesis
TFA	Trifluoroacetic acid
UV-Vis	ultraviolet-visible.

Fig. 1. Conformational analysis of the cyclic peptides **1–3**. A, Circular dichroism (CD) spectra of the cyclic peptides **1–3** and linear control peptide **4**. B, backbone overlays of **1** (purple) with its amide analogs: (I) **2** (blue), and (II) **3** (teal). The RMSDs are shown underneath each overlay.

Fig. 2.

Binding assay of AAL-FITC and peptide **1-FAM** (60 μ g/mL) to BSA-monosaccharides. Monosaccharides at (1 mg/mL): L-fucose, D-galactose, *N*-acetyl-D-galactosamine, D-glucose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-neuraminic acid. No binding of AAL-FITC and **1-FAM** to BSA was observed. Fluorescence signal (RFU) *versus* lectin were plotted as mean of 4 replicate measurements $\pm$ SD.

Fig. 3.

Docking of monosaccharides to **1**. The backbone ribbon of peptide **1** (rainbow tube), the binding residues of the peptide are shown in sticks rendering with C (grey), O (red), N (blue), H (white), and S (yellow). The sugar molecules are shown in CPK rendering with the C atoms (orange), the rest of the atoms are colored the same as the peptide binding residues. Potential hydrogen bonds and the distance between donor and acceptor (light green). A, Highlights the difference in the binding sites for L-fucose and D-glucose; B, L-fucose; C, D-galactose; D, D-galactosamine; E, D-glucose; F, Alternative view of D-glucose.

Fig. 4.

Assessment of lectin selectivity in cell-based assay. Top: A, fixed BJ (10,000 cells/well); B, fixed T-47D (30,000 cells/well). C, fixed MCF-7 cell lines (30,000 cells/well) were treated with fluorescently labeled lectins (40 $\mu\text{g/mL}$) with and without prior treatment with neuraminidase. Pictures show cells stained with: SNA-FITC, UEA1-FITC, AAL-FITC, and 1-FAM. Bottom: fluorescence readout for each cell line with their corresponding fluorescently labeled lectins and neuraminidase treatment.

Fig. 5.

Cell-based lectin studies with fluorescently labeled peptides. Top: fixed BJ (10,000 cells/well), T-47D (30,000 cells/well), MCF-7 (30,000 cells/well), HEP-G2 (30,000 cells/well), and MDA-MB-231 cell lines (30,000 cells/well). Pictures show cells stained with: AAL-FITC (40 $\mu\text{g/mL}$), 1-FAM (60 $\mu\text{g/mL}$), 2-FAM (120 $\mu\text{g/mL}$), and 3-FAM (60 $\mu\text{g/mL}$). Bottom: fluorescence readout for each cell line with their corresponding fluorescently labeled peptides, 1-FAM (60 $\mu\text{g/mL}$), 2-FAM (120 $\mu\text{g/mL}$), and 3-FAM (60 $\mu\text{g/mL}$).

Fig. 6. Competitive binding assays of **1**. A, Competitive binding assay of **1**, with MCF-7 cells (30,000 cells/well) and BJ cells (10,000 cells/well). B, Competitive binding assay of L-fucose, L-galactose, D-galactose and D-glucose to fixed MCF-7 cells (30,000 cells/well). **1** (0.1 μ M to 1 mM), and monosaccharide (1M to 10 μ M) inhibition of **1**-FAM (60 μ g/mL). Curve, Fluorescence signal (RFU) *versus* log [ligand], M, were plotted as mean of 4 replicate measurements $\pm$ SD. IC₅₀ values were obtained by nonlinear regression analysis using GraphPad Prism 5.04.

Fig. 7.

Cell viability assay of **1**. BJ control cell line (10,000 cells/well) with cyclic peptides **1**, **2**, and **3** at different concentrations (0.06, 0.6 and 2 mg/mL).

Fig. 8.

Cell-based migration studies with peptides **1** and **3**. Human (MDA-MB-231 and 231mfp) and mouse (4T1) breast cancer cells (100,000 cells/well) migrated towards fibroblast CM in Transwell chambers. Peptides at different concentrations (10, 50, and 100 µg/mL) were added to the upper and lower compartment of the migration chambers and the number of cells that had migrated to the lower compartment was determined after 4 hours. Each condition was tested in 3 parallel chambers, and 4 representative fields were counted for each chamber.

Scheme 1.

Fmoc-solid phase peptide synthesis of **1** and its linear analog **4**. Reagents and conditions: a) Fmoc-AA-OH, standard Fmoc-SPPS deprotection and coupling protocols; b) I₂ (10 eq), 2% anisole (v/v), CH₂Cl₂; c) TFA:thioanisole:H₂O (95:2.5:2.5, v/v/v).

Scheme 2.

Fmoc-solid phase peptide synthesis of OL amide analogues, **2** and **3**. Reagents and conditions: a) Fmoc-AA-OH, standard Fmoc-SPPS deprotection and coupling protocols; b) $\text{HN}(\text{CH}_3)_2 \cdot \text{BH}_3$ (4 eq), $\text{Pd}(\text{PPh}_3)_4$ (0.1 eq), CH_2Cl_2 , argon atmosphere, r.t., 2×30 min; c) TFA:thioanisole: CH_2Cl_2 (2:3:95, v/v/v), r.t., 2×30 min; d) HOBt (2 eq), PyBOP (2 eq), DIEA (6 eq), DMF, r.t., 18h; e) TFA:thioanisole: H_2O (95:2.5:2.5, v/v/v), r.t. 4 h; f) Fmoc-AA-OH, standard Fmoc-SPPS deprotection and coupling protocols.

Scheme 3.

Fmoc-solid phase peptide synthesis of fluorescently labeled peptides, **1-FAM**, **2-FAM** and **3-FAM**. Reagents and conditions: a) 20% piperidine (v/v), DMF, 3×15min; b) Fmoc-NH-(PEG)₂-COOH (20 atoms) (2 eq), HOBt (2 eq), HBTU (2 eq), DIEA (6 eq), DMF, r.t., 1h; c) 5(6)-carboxyfluorescein (10 eq), DIC (10 eq), and HOBt (10 eq), DMF, r.t., 18h; d) TFA:thioanisole:H₂O (95:2.5:2.5, v/v/v), r.t. 4 h.

Table 1

Thermodynamic parameters for the binding of glycoproteins to cyclic peptides **1–3** obtained by ITC.^{a,b}

	K_a ($\times 10^4$ M)	ΔG (kcal/mol)	ΔH (kcal/mol)	ΔTS (kcal/mol)	n	K_d (μ M)
ASF						
1	1.58	-5.71	-1.51	-4.20	0.85	63
2	no binding	-	-	-	-	-
3	1.17	-5.55	-2.62	-2.93	0.85	85
Fetuin						
1	0.19	-4.54	-1.24	-3.3	1.0	520
2	no binding	-	-	-	-	-
3	0.44	-4.96	-1.38	-3.58	1.1	227

^aThe binding isotherms are presented in Supporting Information Figure S9.

^bValues obtained using the one-site fit model of the binding data with MicroCal analysis software (Origin 7.0) and within experimental error.