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A 'Clicked' Tetrameric Hydroxamic Acid Glycopeptidomimetic Antagonizes Sugar-Lectin Interactions On The Cellular Level

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A tetrameric *N*-acetyl galactosaminyl (GalNAc) peptidomimetic was constructed by *N*-acetylation of repeating proline-based hydroxamic acid units, followed by a convergent 'click chemistry' coupling. This novel glycopeptidomimetic was determined to effectively antagonize the interaction between a transmembrane hepatic lectin and GalNAc on the cellular level.

Transmembrane glycoprotein receptors (TGRs) are calcium (II)-dependent sugar-binding proteins that can recognize and/or endocytose glycoproteins (proteins decorated with a sugar chain) from circulation. These functional receptors including hepatic lectins, selectins, etc., collectively termed the C-type lectins, are critically involved in immunological processes related to inflammation, viral invasion and cancer immunity^{1,2}.

For instance, the galactose/*N*-acetyl-galactosamine (GalNAc)-specific asialoglycoprotein receptor (ASGP-R), which is the first TGR identified on the hepatocyte membrane³, is responsible for endocytosis of asialoglycoproteins (a circulating desialylated glycoprotein)^{4,5}, clearance of IgA⁶ and trapping of activated lymphocytes⁷. However, ASGP-R has also been located as an entry site for hepatotropic viruses⁸, and is overexpressed during some hepatic diseases such as liver inflammation⁹.

As a consequence, molecular tools that can efficiently antagonize the TGRs under various physiological and pathological circumstances may help improve the understanding of the functions of these receptors and facilitate receptor-targeted drug development and delivery^{10,11}.

Given the oligomeric structure of the majority of TGRs that cluster at the cell membrane, a diverse range of multivalent glycoconjugates have been designed and constructed to achieve high binding avidities with these lectins^{12,13}. Of the various molecular backbones used, peptides are the most prevailing due to their modular synthetic pathway and, most importantly, high biocompatibility. Nevertheless, such backbones connected by repeating natural amide bonds are prone to be cleaved in the serum and have, generally, poor water solubility limiting their medicinal and pharmacological values.

Hydroxamic acids are a new class of structural analogs of the amino acids, where a carbon atom of the backbone of the latter is replaced by an oxygen atom¹⁴. Interestingly, peptides that consist of the hydroxamic acids have been reported to adopt more rigid and better pre-organized conformations, leading to improved stability against enzymatic cleavage and enhanced binding affinity with proteins¹⁵. Yang *et al.*^{16,17} also determined that hydroxamic acid-based peptidomimetics can function as anion receptors and channels.

Enlightened by these elegant studies, the incorporation of the hydroxamic acid functionality to sugars was exploited, leading to the formation of a series of sugar hydroxamic acid building blocks^{18–25}. Here, we wish to report the construction of the first functional hydroxamic acid-based oligomeric glycopeptidomimetic that is able to antagonize sugar-TGR interactions on the cellular level.

Results

To begin with, hydroxyl l-proline **1**²⁶ (Fig. 1) was used as a starting material as it features three easily modifiable handles (a carboxylic group, a secondary amino group and a hydroxyl group). Mitsunobu reaction²⁷ of **1** with

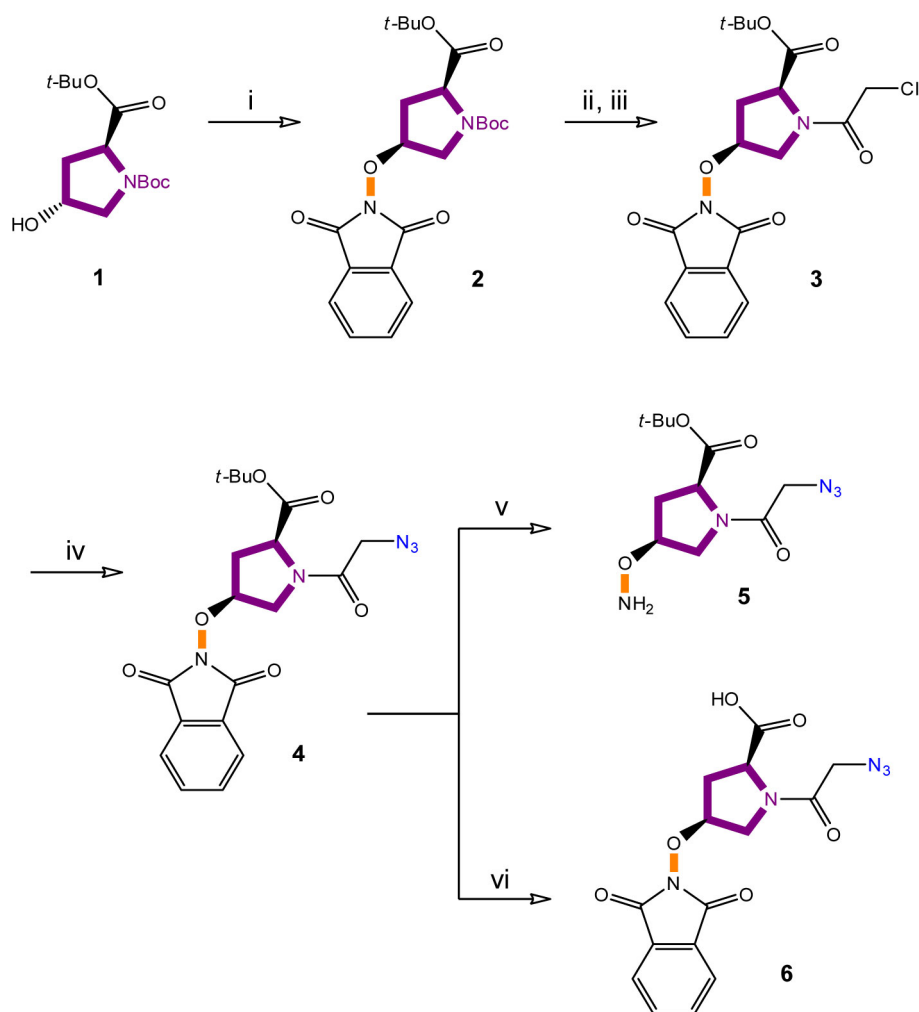


Figure 1 | Synthesis of the proline hydroxamic acid precursor. Reagents and conditions: (i) Ph_3P , DIAD, *N*-hydroxyphthalimide, in CH_2Cl_2 ; (ii) HCl, in EtOAc; (iii) Chloroacetyl chloride; (iv) NaN_3 , NaI, in Acetone; (v) $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$, in EtOH; (vi) TFA, HCl, in CH_2Cl_2 .

N-hydroxyphthalimide gave hydroxamic acid compound **2** in 90% yield. Removal of the Boc group of **2** with HCl, followed directly by addition of chloroacetyl chloride, led to **3** with a chlorine precursor. Treatment of **3** with sodium azide gave the azido hydroxamic acid **4** in a three-step yield of 62%. Then, orthogonal deprotections of the *tert*-butyl and phthalimide groups in the presence of TFA/DCM (1 : 10, V/V, with trace amount of concentrated aqueous HCl) and hydrazine hydrate gave the hydroxamic intermediate **5** and carboxylic acid intermediate **6**, respectively.

As shown in Fig. 2, *N*-acetylation of **5** with **6** in the presence of DCC (dicyclohexylcarbodiimide) and HOBt (hydroxybenzotriazole) gave the azido dipeptidomimetic **7** in 62% yield. Orthogonal deprotection of **7** under the basic and acidic conditions above-employed led to dimeric intermediates **8** (hydroxamic free) and **9** (carboxylic free), respectively. Subsequently, *N*-acetylation of **8** with **9** produced the tetrameric peptidomimetic **10** in 50% yield.

Next, a previously prepared 1- α -*O*-propynyl *N*-acetylgalactosamine (**A**)²⁸ was used to couple with the azido peptides via the Cu(I)-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) reaction (Fig. 3)^{29,30}. Cycloaddition of **A** with the mono-azide **4** in the presence of $[\text{Cu}(\text{CH}_3\text{CN})_4]\text{PF}_6$ as catalyst gave the corresponding triazolyl compound **13** in 80% yield. Likewise, CuAAC of the dimer **7** and tetramer **10** with **A** produced the corresponding triazolyl dimeric (**14**) and tetrameric (**15**) glycopeptidomimetics in 62% and 61% yield, respectively.

As mentioned, the ASGP-R is a transmembrane C-type lectin expressed on hepatocytes, which can recognize GalNAc- or galactose-terminated glycoproteins, thereby leading to endocytosis of the proteins. As a consequence, molecular tools that can efficiently antagonize the ASGP-R-GalNAc/Gal interactions may not only help unravel the biological function of the receptor but also facilitate liver-specific drug delivery^{10,11}.

Against this background, we interrogated the ability of our newly synthesized glycopeptidomimetics to block the ASGP-R-GalNAc interactions on the cellular level. A GalNAc-tailed rhodamine dye (**B**, Fig. 4b)²⁸ that has proven to be capable of fluorescently imaging the ASGP-R expressed on a human hepatoma cell line (Hep-G2)^{31,32} was used as a reagent to stain the receptor. We envisioned that once the interaction was antagonized by the oligomers, the staining would be blocked resulting in suppression of the fluorescence of **B** on cells. By using unmodified GalNAc as a positive control, both the GalNAc peptido-dimer **14** and -tetramer **15** were incubated with the cells before staining with **B**.

As shown in Fig. 4a, the cells were effectively stained by the GalNAc dye (**B**) by taking advantage of the GalNAc-ASGP-R interactions in the absence of an antagonist. Interestingly, whereas the presence of increasing free GalNAc hardly suppressed the fluorescence of **B** (Fig. 4c and Fig. 4d), both the synthesized dimer **14** (Fig. 4e and Fig. 4f) and tetramer **15** (Fig. 4g and Fig. 4h) obviously weakened the staining in a concentration-dependent manner.

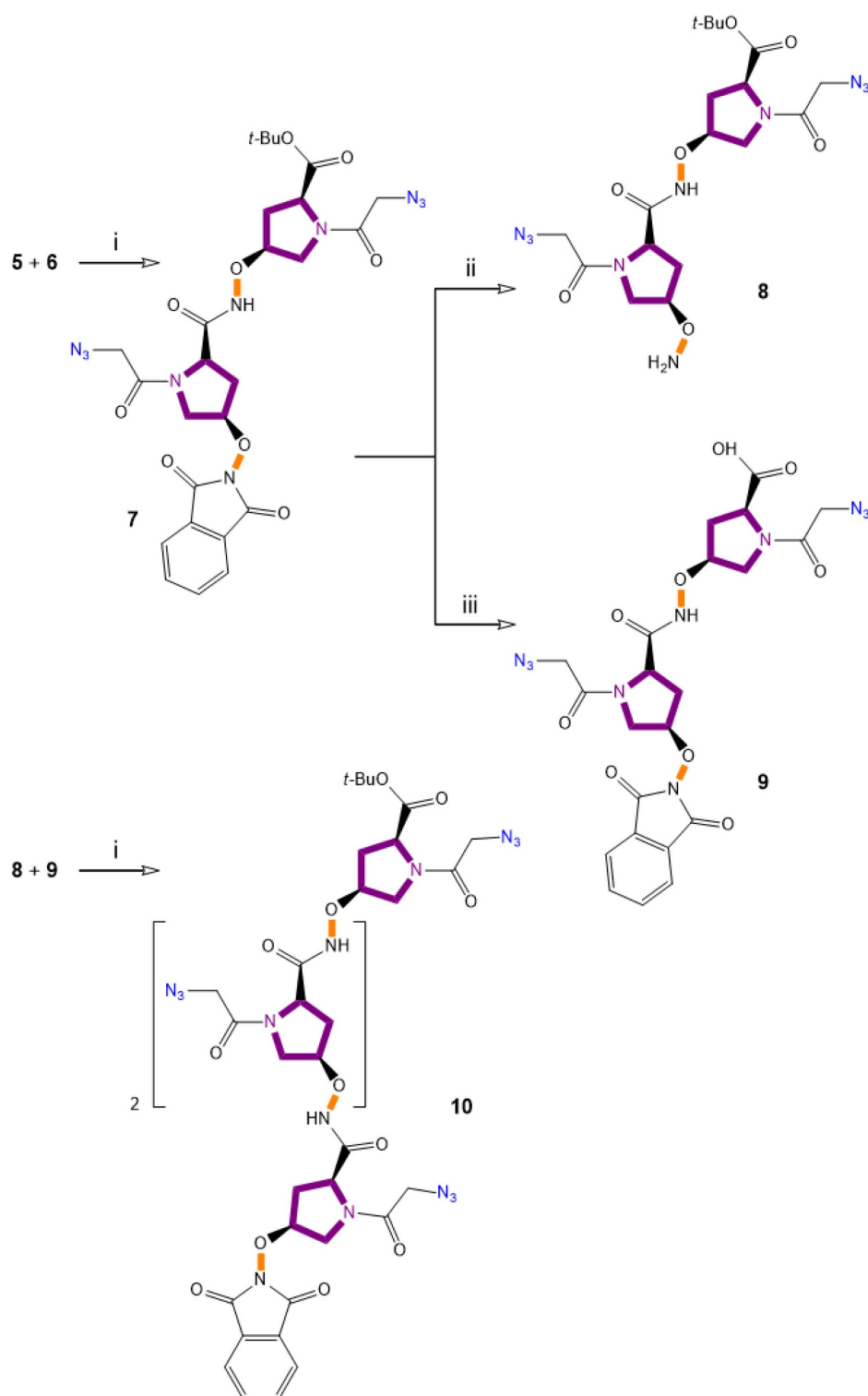


Figure 2 | Synthesis of the hydroxamic acid peptides. Reagents and conditions: (i) DCC, HOBt, in $\text{CH}_2\text{Cl}_2/\text{MeCN}/\text{DMF}$; (ii) $\text{N}_2\text{H}_4 \cdot \text{H}_2\text{O}$, in EtOH; (iii) TFA, HCl, in CH_2Cl_2 .

In addition, the tetramer exerted better suppressive effect than the dimer, meaning that increasing the valency of the oligomer could probably reinforce the ligand-receptor binding. The ability of the synthetic oligomers to suppress the cell staining was much stronger than the unmodified GalNAc. This suggests that the 'clicked' grafting of GalNAc onto the hydroxamic acid-based peptide backbone could enhance the binding affinity (avidity) of the oligomers with the hepatic lectin expressed on the hepatoma cells.

The cytotoxicity of the hydroxamic acid-based glycopeptidomimetics towards Hep-G2 as well as another normal cell line (3T3-L1, mouse embryonic fibroblast) was tested by an MTS assay. Results showed that the 'clicked' peptidomimetics 13, 14 and 15 are not toxic to neither cell lines examined, and the cell viability was independent of compound concentrations (Fig. 4i for Hep-G2 and Fig. 4j for 3T3-L1). All the above-data positively imply that the tetrameric glycopeptidomimetic constructed here could be

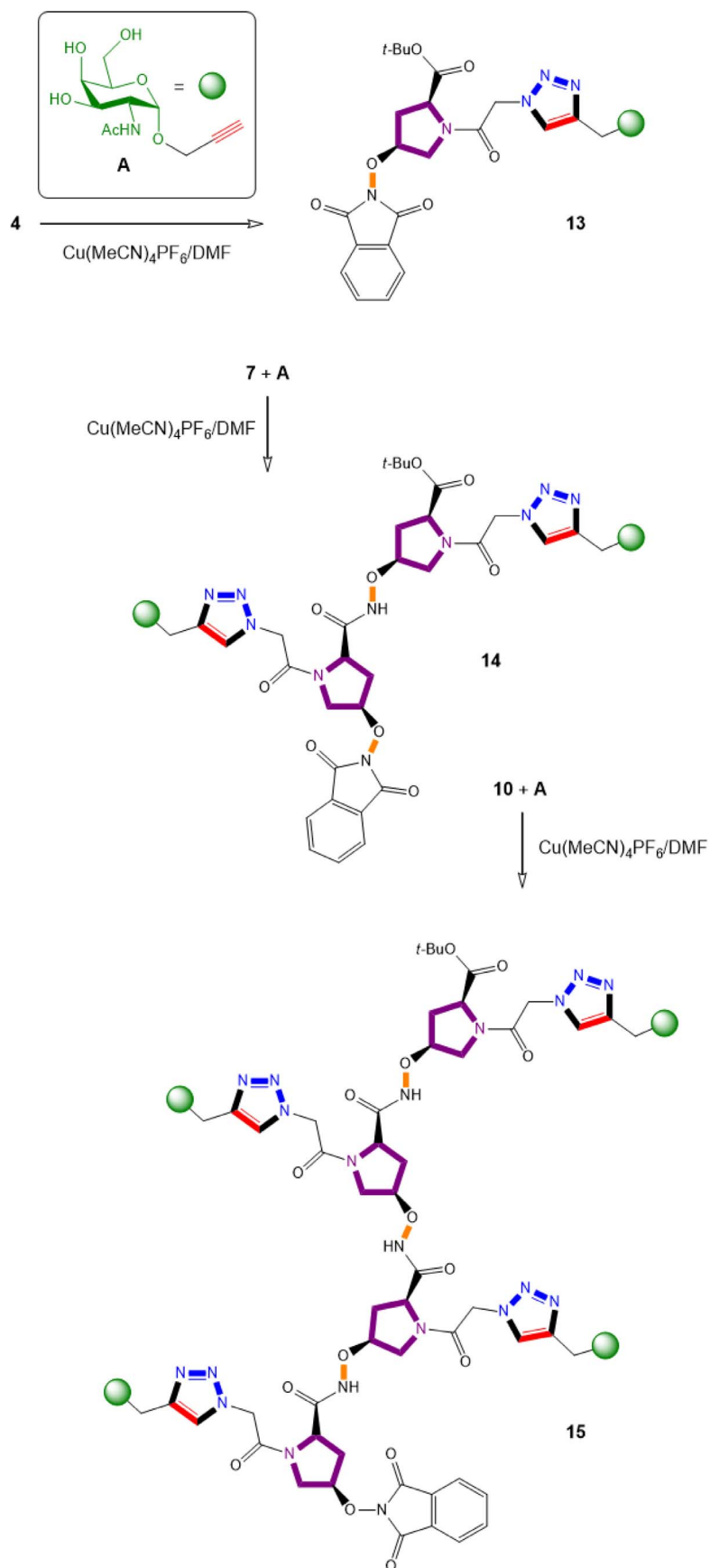


Figure 3 | Construction of the hydroxamic acid-based glycopeptidomimetics by the Cu(I)-catalyzed azide-alkyne cycloaddition reaction.

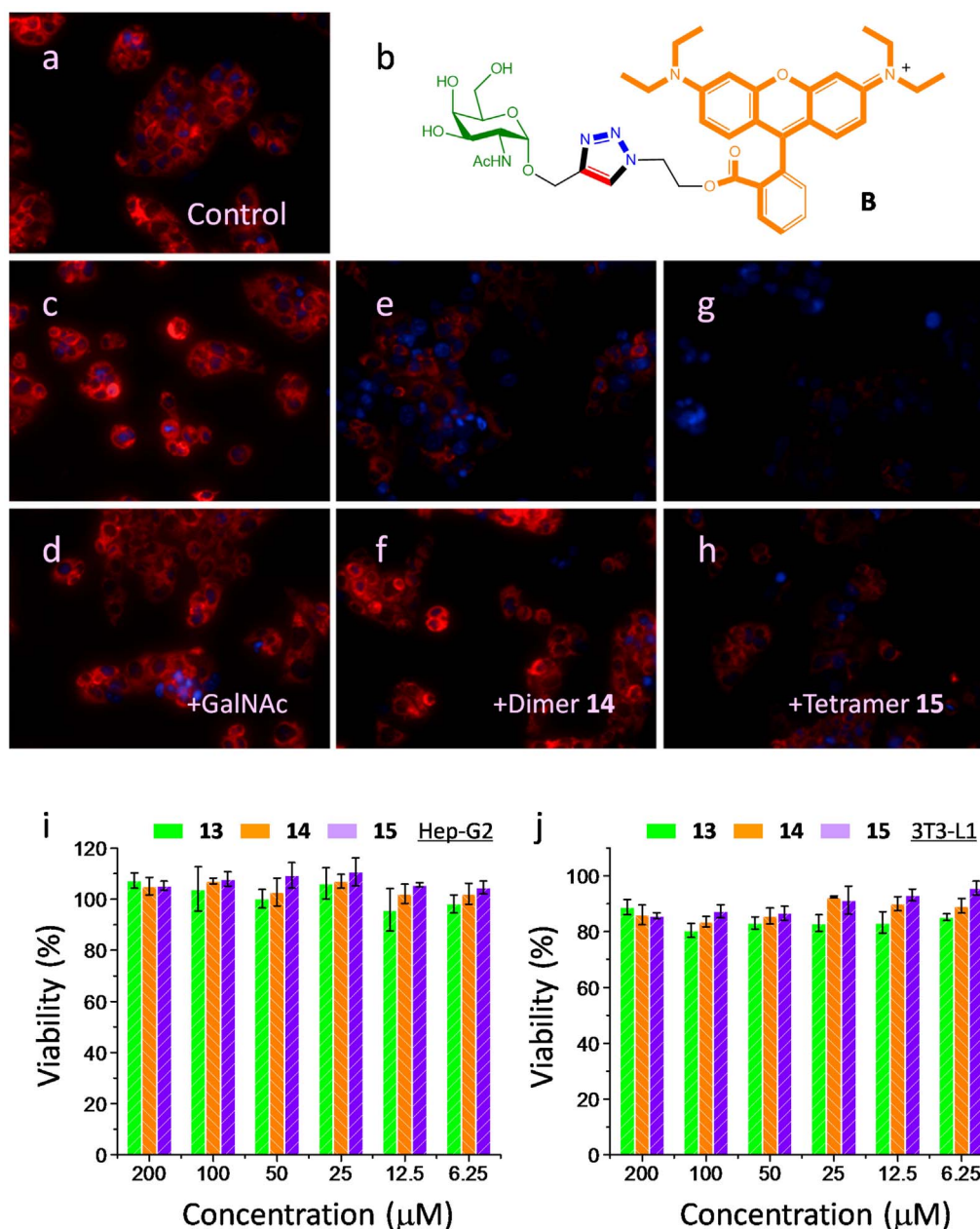


Figure 4 | (a) Fluorescence imaging of the asialoglycoprotein receptors (ASGP-Rs) expressed on Hep-G2 cells by 10 μM of **B** (Cell nuclei were stained by Hoechst in blue); (b) Structure of GalNAc-rhodamine **B**; Fluorescence imaging of the ASGP-Rs expressed on Hep-G2 cells pre-incubated with 500 μM of (c) GalNAc, (e) dimer **14**, (g) tetramer **15**, and 100 μM of (d) GalNAc, (f) dimer **14**, (h) tetramer **15** by 10 μM of **B**; Cell viability of (i) Hep-G2 and (j) 3T3-L1 cells in the presence of increasing glycosyl peptidomimetics **13**, **14** and **15** determined by an MTS cytotoxicity assay.

employed to antagonize ASGP-R-GalNAc interactions on the cellular level.

A GlcNAc (*N*-acetylglucosamine)-clicked dimeric peptidomimetic (**16**) was further synthesized (supporting information) as a negative control as the GlcNAc precursor of which might not have the ability to antagonize the interactions between ASGP-R and GalNAc²⁸. As shown in Fig. S1 (supporting information), **16** did not show any antagonist effect against staining of Hep-G2 by **B** at the identical concentrations used for the counterpart dimer **14**. To preliminarily interrogate the potential serum stability of the neo-glycopeptidomimetics, the tetramer **15** was incubated in a Tris-HCl buffer with trypsin (an enzyme abundantly existed in the serum to cleave peptides) for 1 h and 6 h. By HPLC tracking, we observed that the tetramer was not cleaved by the enzyme even by a co-incubation for 6 h (Fig. S2, supporting information).

Discussion

A number of multivalent glycopolymers and glycoconjugates have been synthesized to target the oligomeric ASGP-R of hepatocyte or hepatoma, achieving efficient target-specific chemotherapy^{33,34}. This study describes the construction of novel hydroxamic acid-based glycopeptidomimetics providing a new class of multivalent glycoconjugates to effectively target the ASGP-R. This class of neo-glycopeptides showed promising properties in terms of low toxicity and stability against trypsin cleavage. Notably, a tetramer could antagonize clearly the GalNAc-ASGP-R interactions on the cellular level. We believe that this unique class of neo-glycopeptidomimetics could serve as new molecular tools for study of the universal intercellular sugar-C-type lectin interactions and as a precursor for fabricating liver-specific drug delivering systems.



Methods

General. All purchased chemicals and reagents are of analytical grade. Solvents were purified by standard procedures. Reactions were monitored by TLC (thin-layer chromatography) using E-Merck aluminum precoated plates of Silica Gel. ¹H and ¹³C NMR spectra were recorded on a Bruker AM-400 spectrometer using tetramethylsilane (TMS) as the internal standard (chemical shifts in parts per million). Optical rotations were measured using a Perkin-Elmer 241 polarimeter at room temperature and a 10 cm length cell of a 1 mL volume. High resolution mass spectra were recorded on a Waters LCT Premier XE spectrometer using standard conditions (ESI, 70 eV). Analytical HPLC was measured using Agilent 1100 Series equipment.

Cell culture and transfection. Hep-G2 cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% Fetal bovine serum (Gibco, Gland Island, NY, USA) and passaged every 3–4 days based on 90% confluency. 3T3-L1 cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% Fetal calf serum (Gibco, Gland Island, NY, USA).

Cellular fluorescence imaging. Hep-G2 cells were cultured in DMEM supplemented with 10% FBS. Cells (15×10^4) were seeded on a black 96-well microplate with optically clear bottom (Greiner bio-one, Germany) overnight. The cells were incubated with GalNAc or the glycopeptidomimetics (100 μM or 500 μM) for 30 min, followed by incubation with B (10 μM) and the nuclear staining reagent Hoechst (5 μg/ml) at 37°C in a humidified atmosphere of 5% CO₂ in air for 5 min. Then, cells were gently washed with PBS three times and fixed using 4% paraformaldehyde. The fluorescence images were recorded using an Operetta high content imaging system (PerkinElmer, US).

MTS cytotoxicity assay. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI, USA). For the MTS assay, cells (5,000 per well) were seeded into 96-well plates. After treatment with compounds of different concentrations for 72 h, cells were added with MTS at a final concentration of 0.5 mg/mL, followed by incubation for another 2 h. The optical density (OD) of each well was determined at 490 nm (background subtraction at 690 nm) by a SpectraMax 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The growth inhibitory ratio was calculated as follows: Growth inhibitory ratio = $(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}$ (where A is the OD value per well).

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Author contributions

G.-R.C., J.X., J.L. and X.-P.H. discussed and conceived the idea. H.-L.Z. synthesized the compounds; Y.Z. performed the biological tests; X.-P.H. and Y.Z. wrote the paper. H.T. G.-R.C. and J.L. supervised the research. All authors commented on the manuscript.

Additional information

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