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Specific Inhibition of Heparanase by Glycopolymer with Well-Defined Sulfation Pattern Prevents Breast Cancer Metastasis in Mice

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Abstract

Heparanase, the heparan sulfate polysaccharide degrading endoglycosidase enzyme, has been correlated to tumor angiogenesis and metastasis and therefore has become a potential target for anticancer drug development. In this systematic study, the sulfation pattern of pendant disaccharide moiety on synthetic glycopolymers was synthetically manipulated to achieve optimal heparanase inhibition. Upon evaluation, glycopolymer with 12 repeating units was determined to be the most potent inhibitor of heparanase ($IC_{50} = 0.10 \pm 0.36$ nM). This glycopolymer was further examined for cross-bioactivity, using a solution based competitive biolayer interferometry assay, with other HS-binding proteins (growth factors, P-selectin, and platelet factor 4) which are responsible for mediating angiogenic activity, cell metastasis, and antibody-induced thrombocytopenia. The synthetic glycopolymer has low affinity for these HS-binding proteins in comparison to natural heparin. In addition, the glycopolymer possessed no proliferative properties towards human umbilical endothelial cells (HUVEC) and a potent antimetastatic effect against 4T1 mammary carcinoma cells. Thus, our study not only establishes a specific inhibitor of heparanase with high affinity, but also illustrates the high effectiveness of this multivalent heparanase inhibitor in inhibiting experimental metastasis *in vivo*.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: Full experimental procedures and characterization data for all new compounds (PDF).

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Keywords

heparanase; glycopolymer; metastasis; biolayer interferometry; cancer therapeutic

INTRODUCTION

Glycosidases, a class of enzymes which catalyze the hydrolysis of glycosidic bonds in complex sugars play a vital role in cellular function.¹ As a result, modulation of the biological activity of glycosidases is a major target for drug discovery.² Heparanase is an endolytic glycosidase that cleaves the internal β -(1,4)-glycosidic bond between glucuronic acid (GlcA) and *N*-sulfated glucosamine (GlcNS) along heparan sulfate (HS) saccharide chains which constitute the extracellular matrix (ECM) and basement membranes.³⁻⁶ Clinical studies have demonstrated that high levels of heparanase expression correlate with increased tumor growth and angiogenesis, enhanced metastasis, and poor patient prognosis for both hematological and solid malignancies. As such, heparanase has become a target for cancer therapeutics.³⁻⁶ Furthermore, these clinical studies emphasize the need for heparanase inhibitors of high specificity.

Several molecules have been developed to target heparanase activity, but only carbohydrate molecules have advanced to clinical trials for cancer patients.^{3,7} Except for compound PG545 (pixatimod, a highly sulfated tetrasaccharide bound to a lipophilic cholestanol aglycone), the carbohydrate-based heparanase inhibitors are heterogeneous in size and sulfation pattern leading to nonspecific binding and unforeseen adverse effects, thus halting their translation into clinical use.^{3,8-13,14,15} Alternatively, saccharide-functionalized glycopolymers,¹⁶ which have been shown to retain the key biological properties of the natural HS polysaccharides, could be an approach for the development of heparanase inhibitors with high specificity and affinity.¹⁷⁻¹⁸ As well, macromolecules including polysaccharides have been utilized in targeted cancer therapies.¹⁹⁻²³ This approach, however, is still met with the challenge of developing an inhibiting epitope (inhibitope) that can gain access to the active site of heparanase.² In comparison to lectins, like many glycosidase enzymes, heparanase is monomeric and possesses a single deep binding groove.^{3,24} These features deterred the use of multivalent scaffolds as glycosidase inhibiting motifs until 2009.²⁵ Soon thereafter, several more examples were developed for the inhibition of a number of glycosidases.^{2,26-30} These studies propose that the valency and relative arrangement of the carbohydrate units are critical parameters for governing the multivalent

effect toward a given glycosidase, therefore, allowing for extension to other glycosidases (including heparanase) if the right inhibotope was selected.³¹⁻³²

Recently, we reported the use of computational studies and the crystal structure of human heparanase to extract the natural HS-heparanase interactions as a template to design HS mimicking glycopolymers containing the disulfated disaccharide component for maximal inhibition and minimal anticoagulant activity (Figure 1).^{24,31-32} Upon evaluation, glycopolymer **1** with 12 repeating units was determined to be the most potent heparanase inhibitor with a picomolar inhibitory concentration and tight-binding characteristics. In addition, this glycopolymer **1** was not hydrolyzed by heparanase as the scissile GlcA β (1,4)GlcN glycosidic bond was removed from the saccharide moiety of **1**.³¹⁻³⁵ Yet, questions still remained on how inhibition of a glycosidase, specifically heparanase, will be affected by changes in the inhibiting epitope (inhibotope) on a multivalent scaffold and how glycopolymer inhibition will translate *in vivo*.

Herein, we report a systematic study on the modulation of multivalent inhibition of heparanase by varying the sulfation pattern of the pendant disaccharide moiety on synthetic glycopolymers. The homogeneity of our approach allows us to dissect the contribution of an individual sulfation to the inhibition of heparanase. Our results indicate that heparanase is capable of recognizing subtle changes on differently sulfated glycopolymers. To ensure heparanase specificity, the most potent glycopolymer inhibitor of heparanase was examined with a solution based competitive biolayer interferometry assay for cross-bioactivity to other HS-binding proteins (growth factors, platelet factor 4, P-selectin) which are responsible for mediating angiogenic activity, antibody-induced thrombocytopenia, and tumor cell metastasis.³⁶⁻³⁸ Compared to heparin, our designed synthetic glycopolymer has a much lower affinity for these proteins. Additionally, the synthetic glycopolymer was shown to have antiproliferative properties when analyzed using a HUVEC cell assay and an anti-metastatic effect in a 4T1 mammary carcinoma model.

EXPERIMENTAL SECTION

Materials.

All commercial chemical reagents used for synthesis were used as received from Sigma Aldrich, Alfa Aesar, TCI, and Combi-Blocks, unless otherwise mentioned. Other reagents and materials were purchased from the following: heparanase, FGF-1, FGF-2, P-selectin, and ATIII were all carrier-free (R&D Systems), HUVECs and their reagents (Lonza), Heparin-biotin (Creative PEGworks), Streptavidin BLI biosensors (fortéBIO), CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Fisher Scientific), TR-FRET heparanase inhibition kit (Cis-bio).

Instrumentation.

All new compounds were analyzed by NMR spectroscopy and High Resolution Mass spectrometry. All ¹H NMR spectra were recorded on either a Bruker 400 or 500 MHz spectrometers. All ¹³C NMR spectra were recorded on either a Bruker 100 or 126 MHz NMR spectrometer. All ¹⁹F NMR spectra were recorded on a Bruker 471 MHz NMR

spectrometer. High resolution (ESI-TOF) mass spectrometry were acquired at Wayne State University. CMC fluorescence measurements were performed on an Aligent Technologies Cary Eclipse Fluorescence Spectrophotometer at the University of Iowa. HTRF emissions were measured using a SpectraMax i3x Microplate Reader (Molecular Devices). Number of cells were determined using a Beckman coulter counter. BLI assays were performed on an Octet Red Instrument (fortéBIO).

Glycopolymer Formation:

³¹⁻³² Synthetic glycomonomer was placed into 10 mL Shlenk flask under inert atmosphere and dissolved in degassed 2,2,2-trifluoroethanol:1,2-dichloroethane solution. A solution of Grubbs 3rd generation catalyst was added, and the mixture heated to 55 °C. After 1 h, reaction was monitored for completion by NMR and then triturated from methanol by diethyl ether. The resulting glycopolymer was then deprotected by LiOH in a water:THF mixture. After 24 h, glycopolymer was dialyzed (3.5K MWCO) against 0.9% NaCl solution (3 buffer changes) and DI water (3 buffer changes).

Computational Docking Study:

³¹⁻³² For the docking studies the *apo* heparanase structure (PDB code: 5E8M) was utilized.²⁴ Global docking with each ligand was performed separately on the heparanase structure using Autodock VINA in the YASARA molecular modelling program.

Biolayer Interferometry Cross-Bioactivity Assay:

BLI assays were performed on an Octet Red Instrument (fortéBIO) at 25 °C. Immobilization and binding analysis were carried out at 1000 rpm using HBS-EP buffer [10 mM HEPES, pH 7.4, 150 mM NaCl, 3.0 mM EDTA, and 0.005% (v/v) surfactant tween20]. A solution affinity assay, used to determine affinities of ligands by SPR analysis was adopted to BLI.³⁹ In this method, protein is mixed with various concentrations of ligand (glycopolymer **20** or heparin, 18 kDa). Free protein in this equilibrium mixture is tested for binding against immobilized heparin (all proteins are carrier-free and purchased from R&D Systems). Heparin-biotin (Creative PEGworks, 18 kDa, 1 biotin per HP polymer), 5 µg /mL was immobilized on to streptavidin biosensors (fortéBio) for 5 min. Binding experiments were carried under conditions of mass transport. Binding was fitted to equation 1 using Graphpad Prism.⁴⁰ BLI response was utilized in place of F and ligand (heparin/glycopolymer **20**) concentration was used in place of [metal]. Binding analysis of P-selectin was carried out with HBS-EP buffer with 2 mM CaCl₂, 2 mM MgCl₂ and 0.5mg/ mL BSA.

HUVEC Culturing:

HUVECs were cultured at 37 °C in a humidified atmosphere of 5% CO₂ using protocols and reagents supplied by Lonza. Endothelial Growth Medium (EGM), supplemented with hydrocortisone, fetal bovine serum (FBS), ascorbic acid, heparin, gentamicin and growth factors such as VEGF, FGF-2, EFG and IGF was used to maintain the cells. The cell cultures were grown to approximately 70-80% confluence. Once at this confluence they treated with 0.025% trypsin in phosphate buffered saline (PBS) and incubated for about 4-5 min until cells detached from the flask surface. EGM (8 ml) was added to the harvested cells and the

cell suspensions were centrifuged at 190Xg for 5 min. The cell pellets were then re-suspended in the growth medium and the number of cells were determined using a Beckman coulter counter. After ensuring uniform suspension, cells were reseeded into new vessel with fresh growth medium at seeding densities around 2500-5000 cells/cm² of vessel surface area.

HUVEC Proliferation Assay:

Endothelial basal medium (EBM-2) containing only 2% FBS and gentamicin was used for cell proliferation. Cells were re-suspended in proliferation medium and 100 μ L was seeded on to 96-well microplate at 3000 cells/well. After incubating for one day, both FGF-2 and glycopolymer **20** in proliferation medium were added to each well maintaining final volume of 200 μ L. Each concentration was done in triplicate. After incubating for 70 h, 20 μ L of the CellTiter 96 Aqueous One Solution Cell Proliferation Assay was added to each well and absorbance at 490 nm was measured 2 h later. The entire assay was repeated three times.

Critical Micelle Concentration (CMC) Protocol:

A stock solution of glycopolymer **20** was serially diluted in 1.5 mL Eppendorf tubes at 16 different concentrations with deionized water from 0 to 1 mg/mL. To each tube containing glycopolymer **20**, pyrene stock solution was added. The resulting mixtures were then covered in aluminum foil and mechanically agitated by an orbital shaker for 2 h and then allowed to equilibrate for 18 h. Fluorescence emission spectra of the glycopolymer solutions containing pyrene were recorded in a 400 μ L microcuvette using an excitation wavelength of 335 nm, and the intensities I_1 and I_3 were measured at the wavelengths corresponding to the first and third vibronic bands located near 373 (I_1) and 384 (I_3) nm.⁴¹

TR-FRET Heparanase Inhibition Assay:

⁴² The glycopolymer inhibitor in Milli-Q water and heparanase (R&D Systems) solution in pH 7.5 triz buffer were added into microtubes and pre-incubated at 37 °C for 10 min. Next, biotin-heparan sulfate-Eu cryptate in pH 5.5 0.2 M NaCH₃CO₂ buffer was added to the microtubes, and the resulting mixture was incubated for 60 min at 37 °C. The reaction mixture was stopped by adding Streptavidin-XLent! solution in pH 7.5 dilution buffer made of 0.1 M NaPO₄, 0.8 M KF, 0.1% BSA. After the mixture had been stirring at room temperature for 15 min, 100 μ L (per well) of the reaction mixture was transferred to a 96 well microplate in triplicate and HTRF emissions at 616 nm and 665 nm were measured by exciting at 340 nm using a SpectraMax i3x Microplate Reader (Molecular Devices).

4T1 Metastasis Assay:

⁴³ Luciferase-labeled 4T1 mammary carcinoma cells (1×10^5 /mouse) were injected i.v. (n=6 mice/group) with vehicle alone (control, PBS), with positive control (natural heparin), or with glycopolymer **20** (100 μ g/mouse) into BALB/c mice (i.p) 20 min prior to cell inoculation and also together with the cells. IVIS bioluminescent imaging was performed on day 7 after cell inoculation. For IVIS imaging, mice were injected intraperitoneally with D-luciferin substrate at 150 mg/kg and anesthetized with continuous exposure to isoflurane (EZAnesthesia, Palmer, PA). The experiment was repeated 3 times with similar results.

Heparanase Enzymatic Activity (ECM Degradation Assay):

⁴⁴ Sulfate [³⁵S] labeled ECM coating the surface of 35 mm tissue culture dishes, was incubated (3–4 h, 37 °C, pH 6.0, 1 mL final volume) with recombinant human heparanase (0.5 ng/mL) in the absence and presence of increasing concentrations of the inhibitory compound (for determination of the IC₅₀ in this assay). The mixture consisted of 50 mM NaCl, 1 mM DTT, 1 mM CaCl₂, and 10 mM buffer phosphate-citrate, pH 6.0. To evaluate the occurrence of proteoglycan degradation, the incubation medium was collected and then applied for gel filtration on Sepharose 6B columns (0.9 × 30 cm). Fractions (0.2 mL) were eluted with PBS and then counted for radioactivity. The excluded volume (V₀) was marked by blue dextran and the total included volume (V_t) by phenol red. Degradation fragments of HS side chains were eluted from Sepharose 6B at 0.5 < K_{av} < 0.8 (peak II). Sulfate labeled material eluted in peak I (fractions 3–10, just after the void volume) represented nearly intact HSPG released from the ECM due to proteolytic activity residing in the ECM. Results were best represented by the actual gel filtration pattern.

RESULTS AND DISCUSSION

Rational Design of Glycopolymers.

In studies with HS oligosaccharides, heparanase has been shown to specifically cleave at an explicit sulfation pattern, GlcAβ(1,4)GlcNS(6S), along the HS polysaccharide chain.⁴⁵ During HS biosynthesis, there is no set blueprint, leaving the epimerization of the uronic acid, sulfation, and acetylation patterns to be randomly generated in domains of heavy sulfation and nonsulfated portions.⁴⁶ The heterogeneity of HS leads to enormous amount of information to be contained within the HS “glyco-code”, allowing HS to bind to a wide variety of proteins.⁴⁷ These proteins are involved in diverse physiological processes, including cell-cell communication, wound healing, immune response, and regulation of cell proliferation.⁴⁷ This promiscuity is what has led to the deleterious cross bioactivity of the previously reported heparanase inhibitors which are heparin/HS derivatives or mimetics.³

Our goal to achieve minimal cross-bioactivity while maintaining strong binding to heparanase is difficult because rational design and predictable efficiency of a neo-glycoconjugate toward a specific lectin and even more so glycosidase remains a challenge.⁴⁸ We have previously illustrated that multivalent glycosidase inhibitors can be rationally designed through computational modeling and looking at previous oligosaccharide cleavage studies and ligand-protein co-crystal structures to extract a high-affinity disaccharide motif.^{31–32} Yet, some ambiguity remains from both the HS oligosaccharide and the crystal structure studies, with most of the uncertainty being with the glucosamine (GlcN) unit in the –2 binding subsite.^{24,45,49} Unfortunately, these questions remain unsolved because the GlcN unit at the +1/–2 subsites cannot be differentiated through enzymatic oligosaccharide synthesis or through the use of isolated heparin oligosaccharide mixtures.⁴⁵ With the ability to systematically synthesize different saccharide motifs from the same building blocks, we rationalized that use of our glycopolymer system was suited for answering these questions. Knowing that our disaccharide moiety had a strong preference for binding to the –2 and –1 subsites,⁵⁴ we designed a disaccharide having the –2 GlcN unit that could be orthogonally

manipulated and then attached to the polymerizable scaffold to be polymerized subsequently.

When designing which disaccharides to place onto the glycopolymers, we took into consideration what previous studies and conclusions about the -2 GlcN unit. The following trends were assessed: (1) Inspection of GlcNS6S at the -2 subsite crystal structure complexes revealed that the electron density for 6-*O*-sulfate is significantly weaker than that for *N*-sulfate, indicating that this subsite was occupied by a mixture of GlcNS and GlcNS6S.²⁴ As such, this data illustrates that heparanase can accommodate a variety of sulfated GlcNX sugars at the -2 position, but it is unknown which has a higher binding affinity; (2) For -2 GlcNS6S, the crystal structure of heparanase-HS trisaccharide ligand indicates that the C(6)-*O*-sulfate participates in electrostatic interactions with the side chain of Lys159. Therefore, preference at the -2 subsite is likely to be GlcNS6S >> GlcNS > GlcNAc because of the formation of additional electrostatic and hydrogen-bonding interactions;²⁴ (3) Structurally, the -2 *N*-sulfate appears to be one of the main determinants for recognition because it is directly in contact with the enzyme through hydrogen bonding networks;²⁴ (4) The -2 C(6)-*O*-sulfate and +1 *N*-sulfate may further stabilize the heparanase-bound trisaccharide through electrostatic interactions with basic residues lining the active site cleft;²⁴ and (5) What effects do addition of a C(3)-*O*-sulfate at the -2 subsite have on the recognition of heparanase.⁵⁰

Synthesis of Designed Glycopolymers.

To resolve the aforementioned questions, we envisaged six disaccharides **2** - **7** with sulfation patterns varying at the C(6)-*O*, C(3)-*O*, and C(2)-*N* positions (Figure 2). Based on the crystal structure of HS substrate-heparanase complex, we hypothesized that *N*-, 3-*O*-, and 6-*O*-SO₃⁻ groups located at -2 subsite of heparanase could be critically important for heparanase-HS interaction. While disaccharides **2** and **3** will examine whether C(6)-*O*-SO₃⁻ located at the -2 subsite is critical for recognition, **2** and **4** will determine whether the sulfate group located at C(6) or C(3) position of the glucosamine unit is more important. On the other hand, disaccharides **5** and **6** will provide a clear picture whether *N*-SO₃⁻ groups located at -2 subsite of heparanase could be critically important for heparanase-HS interaction. Highly sulfated **7** could have a negative or positive impact on HS-heparanase interactions. We anticipate that this study will provide a systematic understanding of substrate binding specificity and sulfate-recognition motifs.

With these intended disaccharides in mind, we developed an orthogonal deprotection and selective sulfation strategy to synthesize the six differently sulfated -2 glucosamine units, starting with a common and properly protected disaccharide building block **8** with a pendant azido linker, under a standard set of reaction conditions. Schematic strategy for the construction of the disaccharide fragments is illustrated in Scheme 1 (for further synthetic details, see SI). Disaccharide **8**, which had been previously synthesized,³² could be quickly diversified by either selective *N*-benzylidene removal under acidic conditions to provide disaccharide **9** or selective C(6)-deacetylation using sodium methoxide in methanol to yield disaccharide **10**. We observed that the selective C(6)-deacetylation can only take place when the *N*-benzylidene group of the glucosamine moiety remains intact (Scheme 1).³²

Disaccharides **9** and **10** would be further functionalized to generate the corresponding six disaccharide intermediates **11** – **16** (Scheme 1). In the first series of disaccharide synthesis, disaccharide **9** could be modified by *N*-acetylation, *N*-CF₃-acetylation, and selective sulfation, followed by removal of the naphthylmethyl (NAP) ether protecting group, to construct the three intermediates **11** – **13** (Scheme 1) in overall good yields. The labile CF₃-acyl group is hydrolyzed after polymerization to reveal the free amine.

On the other hand, disaccharide **10** could be functionalized by *N*-benzylidene removal, followed by simultaneous C(6) and *N*-sulfation, to produce **14**. Furthermore, the C(3)-acetyl group of **10** can be deprotected and then sulfated eventually to generate **15**. In the steps leading to the synthesis of **15**, the following trends were observed. First, for the deacetylation process to proceed smoothly, it was essential for the *N*-sulfate counterions to be sodium cation (Na⁺) as opposed to the triethylammonium (Et₃NH⁺). We discovered that exchange of triethylammonium for sodium reduced the elimination product that forms through deprotonation of the GlcA C(5)-hydrogen. Also, the elimination of the C(5)-hydrogen occurs if there is a free C(2)-amine present during the deacetylation step.⁵¹ For the synthesis of **16**, the primary C(6)-hydroxyl of **10** is first protected as the naphthylmethyl ether, followed by sequential *N*-benzylidene removal and *N*-sulfation. After counterion exchange, the disaccharide intermediate is C(3)-deacetylated and then sulfated. Global NAP-deprotection with DDQ produces the corresponding disaccharide **16**.

With the six differently sulfated deprotected disaccharides **12** - **16** in hand, they could now be individually coupled to the ROMP-capable monomer unit **17** via a CuAAC “click” reaction (Scheme 2, for synthetic details see SI).⁵²⁻⁵³ The newly formed glycomonomers were obtained in moderate yield (27 – 61%) and then underwent polymerization using Grubbs’ third generation catalyst (G3) in a mixture of 1,2-dichloroethane/2,2,2-trifluoroethanol as solvent.⁵⁴⁻⁵⁵ The unique solvent mixture was necessary to ameliorate the solubility of the polar sulfated monomer unit and to prevent the ruthenium catalyst decomposition, which has been reported with utilization of nucleophilic polar solvents such as methanol. The solvent ratio was adjusted according to the number of sulfates and free hydroxyls present on the disaccharide portion (for more details, see SI). Our previous results illustrated that the ideal degree of polymerization (DP) for inhibition of heparanase by a glycopolymer was approximately 11-12 repeating units.^{53,54} As a result, each differently sulfated monomer unit was independently polymerized with 9 mol% Grubbs’ catalyst (G3) to provide high yields of the six differently sulfated glycopolymers within 1 h, all with similar optimal degrees of polymerization.³² Due to their amphiphilic nature, these glycopolymers aggregate to form micelles after polymerization. As such, they cannot be analyzed by gel permeation chromatography (GPC); instead, both the DP and molecular weight (*M_n*) of the six glycopolymers were determined by ¹H-NMR end group analysis. Following polymerization, the resulting glycopolymers were fully deprotected using 0.25 M LiOH in THF/H₂O and then purified by dialysis to remove impurities, affording the corresponding polymers **20-25**.⁵⁶

In Vitro Testing.

Heparanase Inhibition: After purification, the glycopolymers **20** - **25** were evaluated on how their varied sulfation patterns altered their heparanase inhibitory capabilities. Employing a TR-FRET assay against fluorescent labeled-HS, we ultimately found that there is a direct correlation between sulfation pattern of the -2 GlcN and heparanase inhibition (Table 1).⁴² Specifically, we observed that the -2 GlcN must be sulfated at both the C(6) and C(2)-*N* positions in order to induce the highest inhibitory effects on heparanase (**20**, entry 1, $IC_{50} = 0.10 \pm 0.036$ nM). Removal of the C(6)-sulfate (**21**, entry 2) drastically reduced the inhibitory activity against heparanase (IC_{50} to 17.89 ± 0.954 nM). While previous report has demonstrated that heparanase can recognize glucosamine unit (GlcN) carrying either C(6)- or C(3)-*O*-sulfate,^{45,50} we found that glycopolymer **22** bearing C(3)-*O*-sulfate (entry 3, $IC_{50} = 4.041 \pm 0.156$ nM) is less effective at inhibiting heparanase than glycopolymer **20** bearing C(6)-*O*-sulfate (entry 1). The addition of a third sulfate to the GlcNS6S moiety, forming polymer **23** (entry 4, $IC_{50} = 5.48 \pm 0.31$ nM), did not prove to be advantageous. This result suggests that although the interactions are not purely electrostatic, heparanase recognizes the pendant saccharide. Moreover, the utilization of oversulfated saccharide compounds have been reported to increase nonspecific binding leading to unforeseen adverse effects.⁵⁷⁻⁵⁸ Exchanging the *N*-sulfate (entry 4) for the *N*-acetyl (entry 5, **24**; $IC_{50} = 3.40 \pm 0.10$ nM) or ammonium (entry 6, **26**; $IC_{50} = 8.83 \pm 0.52$ nM) did not have a significant impact on the binding affinity. Overall, these results suggest that although -2 *N*-sulfate is important for heparanase recognition, it is not as important as -2 C(6)-*O*-sulfate.

These results obtained with glycopolymers **20** - **25** in Table 1 are in accordance with an *in silico* docking study with the glycomonomer substrates and the *apo* crystal structure of heparanase (PDB code: 5E8M) using the Autodock Vina suite in the YASARA program.^{24,59-60} We initiated the investigation by docking the natural HS substrate, GlcNS(6S) α (1,4)GlcA β (1,4)GlcNS(6S) α (1,4)GlcA tetrasaccharide, into human heparanase to obtain a benchmark for comparison with our synthetically designed compounds (for positioning reference see Figure 3. Also see SI for actual docked structures). Currently, there are no computational programs that could manage the docking of glycopolymers, and so the monomeric precursors were investigated in our computational studies. When both the C(6) and C(2)-*N* positions were sulfated (polymer **20**, Table 1, entry 1), there was a strong network of interactions (ionic and hydrogen bonding) formed.³² The *N*-sulfate interacted with Lys159 and Arg303, while the C(6)-*O*-sulfate from a trivalent network with Asn64, Gly389, and Tyr 391. When the C(3)-*O*-sulfate for the trisulfate saccharide (polymer **23**, Table 1, entry 3) was introduced, it added an additional ionic interaction with Lys98; however, the interaction pulled the C(6)-*O*-sulfate away from Tyr391 and the *N*-sulfate from Arg303. This docking result is consistent with the experimental data wherein polymer **23** ($IC_{50} = 5.48 \pm 0.31$ nM) is less effective at inhibiting heparanase than polymer **20** ($IC_{50} = 0.10 \pm 0.036$ nM).

Finally, the prediction for recognition importance at the C(2)-*N* position (GlcNS6S >> GlcNS > GlcNAc) was partially upheld.²⁴ Heparanase strongly recognized the GlcNS6S motif (Table 1, entry 1), but the preference between GlcNS and GlcNAc (entries 2 and 5) were actually reversed. As previously mentioned, the orientation of the saccharide is vital

and we found that a hydrophobic pocket in the -2 subsite (Gly389, Asp62, Val34, Tyr391) accommodated the methyl of the acetyl group and provided the right orientation for the C(6)-sulfate to potentially interact with Lys232. The GlcNS only made it to the outer periphery of the binding site groove with little interactions. Removal of all substitution at the C(2)-*N* position still yielded fair inhibition (entry 6); however, when looking at the docked compound, the disaccharide unit was found in the +2/+1 subsites with the reducing end directed towards HBD-1, opposite of the natural substrate and the other glycomonomer compounds. This docking result supports the findings of previous studies, that the *N*-sulfate is necessary for recognition in the -2 subsite. Overall, we have concluded that the combinatory effect of having both the C(6)- and C(2)-*N* positions sulfated presents the saccharide in the proper orientation for optimal binding at the -1, -2 subsite of heparanase. Any additional sulfates or changes in the pattern disrupt the positioning of the saccharide, reducing the number of ionic salt bridges and hydrogen bonding interactions.

Cross-bioactivity Studies: After discovering that the GlcNS(6S) α (1,4)GlcA glycopolymer **20** (DP=12) is the most potent inhibitor of heparanase, we next sought to find the specificity of this synthetic glycopolymer since HS polysaccharides are typically promiscuous.⁴⁷ We previously established that glycopolymer **20** presented no anticoagulant activity in the presence of ATIII (Anti-FXa: IC₅₀ > 4500 nM and Anti-FIIa: IC₅₀ > 4500 nM).^{17,32} We next screened the ability of **20** to bind to a variety of HS-binding proteins (Table 2). To achieve this goal, we utilized a solution-based bilayer interferometry (BLI) assay to determine the apparent K_d of our glycopolymer to HS-binding proteins in comparison to biotinylated-heparin (18 kDa) attached to the BLI streptavidin-probe (Table 2).³⁹ The study was initiated by testing the validity of the assay by employing heparin (18 kDa) as the ligand. The apparent K_d found for several HS-binding proteins (Table 2) was similar to previously reported data obtained with a variety of methods.³⁹ Once the binding of heparin to HS-binding proteins has been established, we began the protein screening process by determining the K_d for synthetic glycopolymer **20** to three angiogenic growth factors (FGF-1, FGF-2, and VEGF) which are released during degradation of the ECM's HS by heparanase and are responsible for promoting tumor growth.³ The glycopolymer exhibited very low affinity to these three growth factors with K_d several orders of magnitude greater than the standard 18 kDa heparin utilized in the assay (Table 2). Next, we turned our attention to the binding of **20** to platelet factor-4 (PF4), which is responsible for causing thrombocytopenia, the main reason why clinical trials for other carbohydrate-based heparanase inhibitors were halted.^{3,37} Again, the K_d for the GlcNS(6S) α (1,4)GlcA glycopolymer (45 ± 5.11 nM) was 150 times weaker than that of heparin (0.31 ± 0.028 nM) and three times weaker than that of PI-88 (16.0 ± 1.9 nM), a known heparanase inhibitor.³⁹ Lastly, P-selectin was tested as it plays a vital role in tumor cell metastasis, and the process can be attenuated by heparin.⁶²⁻⁶³ To our excitement, glycopolymer **20** ($K_d = 351.5 \pm 927.6$ nM) presented a similar affinity to that of heparin ($K_d = 124.8 \pm 152.1$ nM). The data obtained with P-selectin suggests that the glycopolymer simultaneously inhibits heparanase and P-selectin, allowing it to suppress both selectin-mediated tumor cell adhesion to endothelial cells and heparanase mediated extravasation through the subendothelial basement membrane.

Interestingly, we found a biphasic behavior in all the binding studies. At lower concentrations of polymer **20** ($< 3 \mu\text{M}$) the binding was linear; however, at concentrations above $3 \mu\text{M}$ there was a drastic change in binding (Figure 4a). These concentrations directly correlate to the previously found $3.3 \mu\text{M}$ critical micelle concentration (CMC) for **20**.³² We reason that at the higher concentrations, glycopolymer **20** exists in its micellar form and begins to tightly sequester the proteins, resulting in that there was no protein available to bind to the heparin attached to the BLI probe.⁶⁴⁻⁶⁵ The biphasic behavior of the GlcNS(6S) α (1,4)GlcA glycopolymer was also observed in the human umbilical vascular endothelial cell (HUVEC) proliferation assay using FGF-2 (Figure 4b). Again, at concentrations below the CMC ($0.0007\text{--}0.75 \mu\text{M}$), there was statistically no cell proliferation compared to the control without glycopolymer. These results support the BLI data for FGF-2 to the glycopolymer, in which very little binding occurred at low concentrations (Figure 4a). It was not until polymer **20** reached $3 \mu\text{M}$ concentration that a small change in HUVEC proliferation was observed (Figure 4b). As previously seen with the BLI data, at concentrations above $3 \mu\text{M}$, there was a strong decrease in cell proliferation, down to the exact same level as that of the control without FGF-2 (Figure 4b). As illustrated in Figure 4c, there is a direct correlation between cell proliferation and the formation of micelle. We hypothesize that sequestering FGF-2 by the newly formed micelles does not allow the protein to bind to the FGF-receptor on the HUVEC surface, either from steric repulsion or improper binding orientation of the ternary complex.³⁶ It is important to note that these concentrations are much greater than the inhibitory concentration of the synthetic GlcNS(6S) α (1,4)GlcA glycopolymer **20** against heparanase.

In Vivo Model.—Metastasis is the leading cause of death of cancer patients.⁶⁶ Although the metastatic cascade is complex, it is well documented that degradation of the ECM's HS by heparanase is a major contributing factor in the dissemination of malignant tumors.³ Breast cancer is the leading cause of female mortality worldwide and accounts for 25% of the total number of cancer cases and 15% of all cancer-associated female mortality.⁶⁷ With the ultimate objective of understanding if the *in vitro* inhibition of heparanase by sulfated glycopolymers would translate *in vivo*, we subjected the GlcNS(6S) α (1,4)GlcA glycopolymer **20** (DP=12) to a 4T1 mammary carcinoma model of experimental metastasis (Figure 5) ⁴³ As a positive control, we also subjected heparin to *in vivo* studies.

Looking at the antimetastatic properties for these two compounds, natural heparin consistently reduced the size of the metastasized lung tumor by about half (Figure 5). When the GlcNS(6S) α (1,4)GlcA glycopolymer **20** (DP=12) was subjected to the same assay, 4 out of the 5 mice presented almost no metastatic spread into the lungs. As demonstrated in Figure 5, GlcNS(6S) α (1,4)GlcA glycopolymer **20** (DP=12) markedly inhibited the extravasation of 4T1 cells and their subsequent colonization in the mouse lungs. This effect was similar to that exerted by Roneparstat, *N*-acetylated, glycol-split heparin, (not shown),^{10,68} a drug that recently finished a Phase I clinical trial in myeloma patients. These results indicate that the synthetic glycopolymer inhibits the ability of blood-borne carcinoma cells to extravasate through the subendothelial basement membrane due to the modulation of heparanase activity, which was further corroborated by a sulfate-[³⁵S] labeled ECM degradation assay (Figure 6).^{6,43-44,69-70} As demonstrated in Figure 6, glycopolymer **20** had

similar inhibitory characteristics to that of natural heparin with complete inhibition at 10 $\mu\text{g/mL}$ and a most impressive inhibition at 1 and 5 $\mu\text{g/mL}$. These studies, together with the data obtained in Table 2, suggest that the impairment of 4T1 cell extravasation is a result of simultaneous inhibition of P-selectin and heparanase activities by glycopolymer **20**.

CONCLUSION

We have described the rational design and synthesis of a powerful multivalent inhibitor of heparanase that translates from *in vitro* inhibition of the enzyme to a potentially effective *in vivo* anticancer agent. We have illustrated that a synthetically designed glycopolymer of 12 repeating units bearing a pendant GlcNS(6S) α (1,4)GlcA saccharide unit affords tight-binding inhibition of the cancer-promoting heparanase. Advantageously, the glycopolymer has minimal cross-bioactivity with serine proteases in the coagulation cascade as well as several HS-binding proteins such as angiogenic growth factors and platelet factor 4. Our studies also illustrate that the synthetic glycopolymer could act against P-selectin, which in combination with heparanase inhibition provides a dual mechanism underlying the potent inhibition of malignant cell dissemination from metastasizing throughout the body. Inhibition of metastasis has been clearly demonstrated in a mouse 4T1 carcinoma cell model, in which the sulfated glycopolymer effectively prohibited the carcinoma cells to extravasate and colonize in the lungs. Overall, we have presented a high affinity, synthetic glycopolymer inhibitor of heparanase that overcomes the limitations associated with the lack of specificity noted with previously developed heparin-based inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Deriving a sulfated glycopolymer inhibitor from natural HS binding to the positively charged binding domains (HBD-1 and HBD-2) of heparanase.

Figure 2.
Rational design of disaccharide motifs bearing the sulfation patterns at the C(6)-O, C(3)-O, and C(2)-N positions of glucosamine unit.

Figure 3. Positioning of the natural HS substrate, GlcNS(6S) α (1,4)GlcA β (1,4)GlcNS(6S) α (1,4)GlcA, in the active site of human heparanase. This tetrasaccharide was docked into the *apo* crystal structure of heparanase (PDB code: 5E8M) using the Autodock Vina suite in YASARA program.^{24,59-60} Figure generating using LigPlot⁺.⁶¹

Figure 4.

a) BLI trace for the binding of various concentrations (0.016-50 μM) of GlcNS(6S) α (1,4)GlcA glycopolymer (DP=12) to FGF-2. **b)** HUVEC cell growth when incubated at 3000 cells/well/100 μL with FGF-2 or FGF-2 plus GlcNS(6S) α (1,4)GlcA glycopolymer (DP=12) at varying concentrations for three days. Absorbance of living cells was measured using CellTiter 96® AQueous One Solution at 490nm. Data were normalized to cells incubated with medium alone (set to 100%). Background absorbance from the polymer at each concentration and medium alone were subtracted from the respective polymer containing samples. Only the medium background absorbance was subtracted from the rest of the samples. The experiment was repeated three times with at least triplicates of each sample per experiment, error bars represent standard deviation. Statistical analysis was done using Welch's t-test. * $p < 0.01$ compared to cells plus FGF-2. **c)** Overlay comparing the critical micelle concentration (CMC) data of GlcNS(6S) α (1,4)GlcA glycopolymer (DP=12) with the HUVEC proliferation data.

Figure 5.

Effect of glycopolymer on 4T1 experimental metastasis. Luciferase-labeled 4T1 breast carcinoma cells (1×10^5 /mouse) were injected i.v (n=6 mice/group) with vehicle alone (control, PBS), with positive control (heparin), or with GlcNS(6S) α (1,4)GlcA glycopolymer (DP=12, 100 μ g/mouse) injected (i.p) 20 min prior to cell inoculation and also together with the cells. IVIS bioluminescent imaging was performed on day 7 after cell inoculation. For IVIS imaging, mice were injected intraperitoneally with D-luciferin substrate at 150 mg/kg and anesthetized with continuous exposure to isoflurane (EZAnesthesia, Palmer, PA). Light emitted from the bioluminescent cells is detected by the IVIS camera system with images quantified for tumor burden using a log-scale color range set at 5×10^4 to 1×10^7 and measurement of total photon counts per second (PPS) using Living Image software (Xenogen). The experiment was repeated 3 times with similar results.

Figure 6.

GlcNS6S glycopolymer **20** prevents ECM degradation by inhibiting heparanase enzymatic activity. Sulfate- ^{35}S labeled ECM, was incubated (5 h, 37 °C, pH 6.0) with recombinant heparanase (200 ng/mL) in the absence or presence of heparin (10 mg/mL) or increasing concentrations (1-10 mg/mL) of glycopolymer **20**. Sulfate labeled material released into the incubation medium was analyzed by gel filtration. Heparan sulfate degradation fragments are eluted in peak II (fractions 20-35). The experiment was repeated twice and the variation did not exceed 10% of the mean.

Scheme 1.
Schematic synthesis of protected disaccharide motifs **12** - **16**

Scheme 2.

Synthesis of HS-mimicking glycopolymers via click chemistry followed by ring-open metathesis polymerization (ROMP).

Table 1.

Inhibition of heparanase by HS mimicking glycopolymers using a TR-FRET assay

Entry	Disaccharide	n (DP) ^a	IC ₅₀ ^b
1	20	12	0.10 ± 0.036 nM
2	21	12	17.89 ± 0.954 nM
3	22	11	4.041 ± 0.156 nM
4	23	10	5.48 ± 0.31 nM
5	24	11	3.40 ± 0.10 nM
6	25	12	8.83 ± 0.52 nM

^aDP and molecular weights (M_n) were determined via ¹H-NMR end group analysis.^bInhibition of heparanase was assessed by *in vitro* TR-FRET assay against fluorescent-tagged heparan sulfate

Table 2.

Binding affinity of GlcNS(6S) α (1,4)GlcA glycopolymer to various HS-binding proteins.

Protein	Apparent K_d (nM) ^a	
	Heparin	Glycopolymer 20
FGF-1	4.6 $\pm$ 3.3	>2000
FGF-2	0.15 $\pm$ 0.11	691 $\pm$ 162
VEGF	4.91 $\pm$ 1.55	281 $\pm$ 162
PF4	0.31 $\pm$ 0.028	45 $\pm$ 5.11
P-Selectin	124.8 $\pm$ 152.1	351.5 $\pm$ 927.6

^aCalculated using Equation 1.⁴⁰