Letter

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Dendrimer Heparan Sulfate Glycomimetics: Potent Heparanase Inhibitors for Anticancer Therapy

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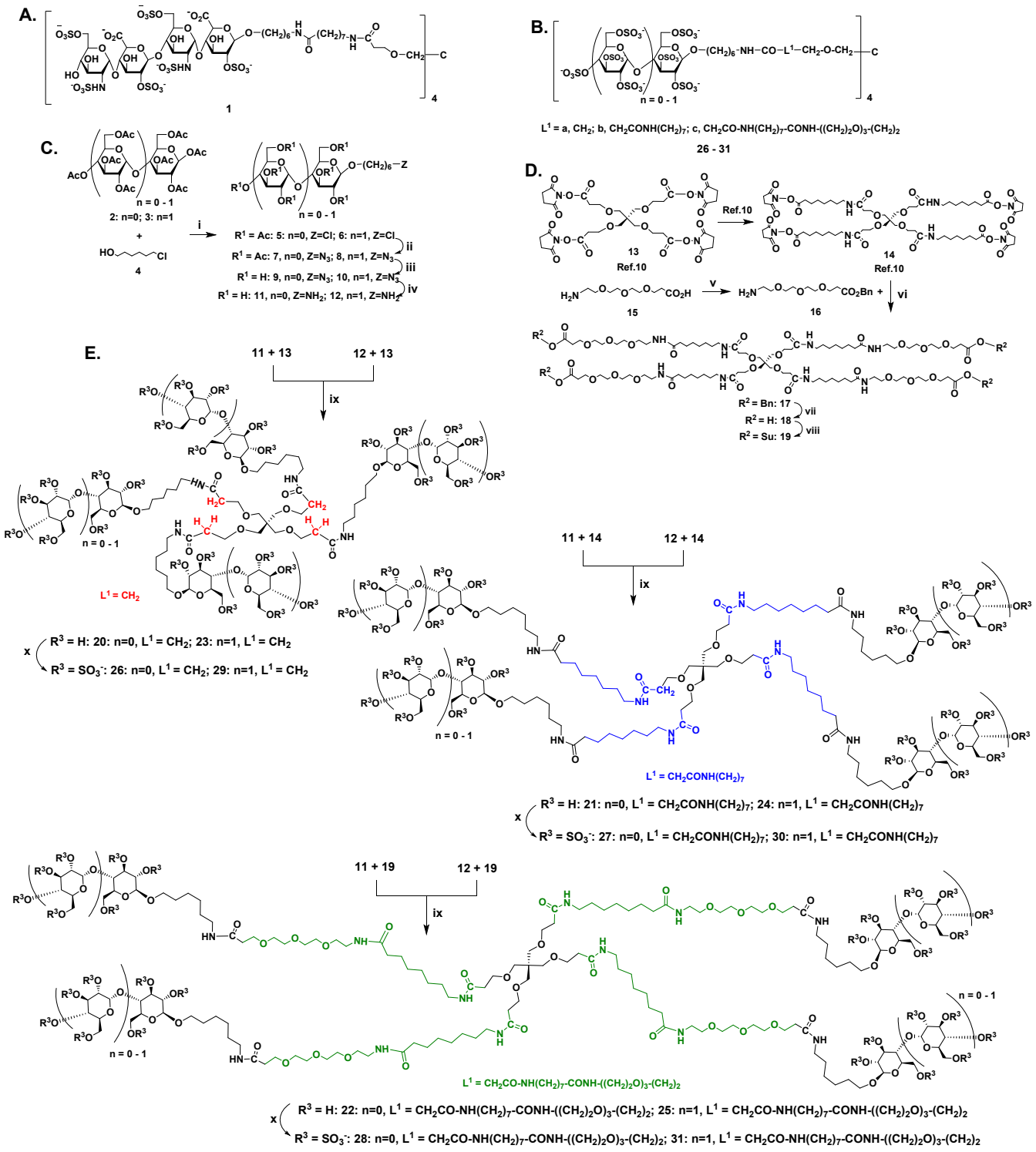
ABSTRACT: Heparanase is a mammalian endoglycosidase that cleaves heparan sulfate (HS) polysaccharides and contributes to remodelling of the extracellular matrix and regulation of HS-binding protein bioavailabilities. Heparanase is upregulated in malignant cancers and inflammation, aiding cell migration and the release of signalling molecules. It is established as a highly druggable extracellular target for anticancer therapy, but current compounds have limitations due to cost, production complexity or off-target effects. Here we report the synthesis of a novel, targeted library of single-entity glycomimetic clusters capped with simple sulfated saccharides. Several dendrimer HS glycomimetics display low nM IC_{50} potency for heparanase inhibition equivalent to comparator compounds in clinical development, and potently inhibit metastasis and growth of human myeloma tumor cells in a mouse xenograft model. Importantly, they lack anticoagulant activity and cytotoxicity, and also inhibit angiogenesis. They provide a new candidate class for anti-cancer and wider therapeutic applications which could benefit from targeted heparanase inhibition.

Heparan sulfate (HS) is a highly sulfated glycosaminoglycan with a variety of critical functions in cell signalling and regulation[1]. HS acts as an extracellular storage depot of growth factors, angiogenic proteins, and chemokines, and also serves to regulate ligand-receptor interactions at the cell surface[2-3]. Heparanase is the only known endo-β-D-glucuronidase capable of cleaving HS side chains of HS proteoglycans (HSPGs) into shorter, active fragments[4-5]. Cleavage of HS by heparanase facilitates structural alterations of the extracellular matrix (ECM) and contributes to releasing various sequestered HS-binding proteins[6]. Heparanase is upregulated in malignant cancers, and its overexpression correlates with angiogenesis, increased tumor size, enhanced metastasis, and poor prognosis[7-8]. The upregulation of heparanase in malignant cancer also correlates closely with increases in new blood vessels around tumors, providing a ready pathway for cancer cell entry into the circulation[9]. This makes heparanase a promising extracellular target for new anti-tumor treatments.

While HS oligosaccharides can mimic or interfere with biological systems, their exploitation has been hindered by the complexity of their synthesis. Recently we developed a novel synthetic approach to more accessible polyvalent HS-mimetic constructs with potential advantages as therapeutics[10]. We employed the clustering effect of dendrimers in which weak interactions of small sugar fragments are significantly enhanced by appending multiple copies of specific sugars on defined chemical scaffolds[11]. Our targeted libraries of single-entity HS oligosaccharides and glycomimetic clusters have already been demonstrated to mimic longer natural HS molecules that have a role in Alzheimer's disease[12-14]. The ability of the target compounds to inhibit the BACE-1 protease was investigated using fluorescence resonance energy transfer (FRET) peptide cleavage assays. All of the glycomimetic clusters inhibited BACE-1 with IC_{50} values in the micromolar to low nanomolar range and showed a lack of off-target anticoagulant activity.

We reasoned that our dendritic cores and associated methodology would provide a novel template for the polyvalent presentation of other HS-mimicking saccharides for other indications. Here we extended our research into anticancer applications and screened a library of HS-glycomimetics for their ability to inhibit heparanase.



Scheme 1. Synthesis of glycomimetics. (A, B) Synthetic targets. (C) Preparation of saccharide fragments: i, $BF_3.OEt_2$, DCM, RT; ii, NaN_3, DMF, 80 ºC; iii, NaOMe, MeOH, RT; iv, Ra-Ni, H_2, aq. MeOH, RT. (D) Preparation of tetramer dendritic cores: v, BnOH, $SOCl_2$, 0 ºC to 100 ºC; vi, Et_3N, DMF, RT; vii, H_2, $Pd(OH)_2/C$, aq. MeOH, RT; viii, NHS, EDC, DMF, DIPEA, RT. (E) Preparation of HS glycomimetics: ix, Et_3N, DMF, RT; x, $SO_3{\cdot}NMe_3$, DMF, 60 ºC.

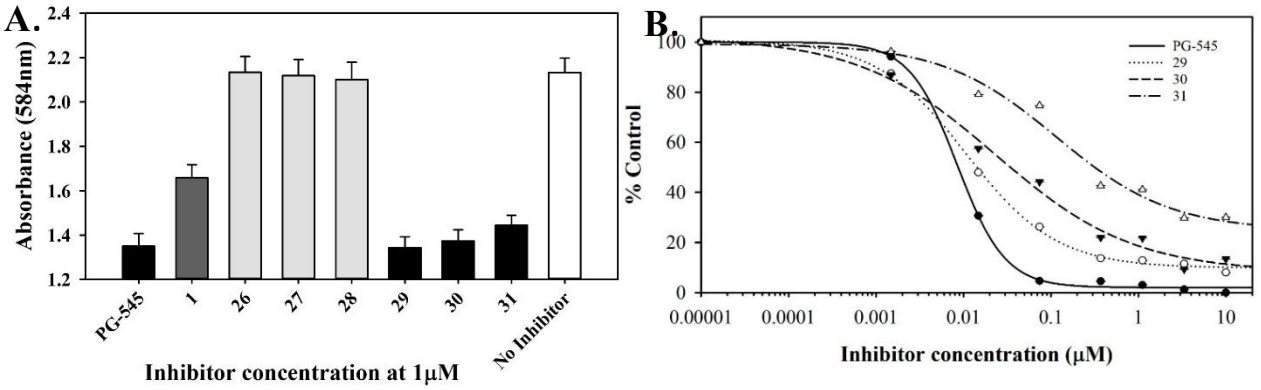


Figure 1. Heparanase inhibitory activity of selected compounds. Panel A: Initial *in vitro* screen of heparanase inhibitory activity of selected compounds at 1 μM concentration in a Fondiparinux substrate assay using 100 nM heparanase. Panel B: dose response curves for heparanase inhibition by selected compounds in the Fondiparinux assay using 10 nM heparanase. IC_{50} values were calculated from the curves (see Methods: PG545, 8 nM; **29**, 11 nM; **30**, 23 nM; **31**, 116 nM).

Our approach was to alter the ECM of tumor cells and their detachment prior to metastasis by preventing heparanase action and thereby altering the oxygen, nutrient and growth factor supply to the tumor. Standard chemotherapeutic anticancer drugs kill rapidly dividing cancer cells but also affect normal cell proliferation, such as occurs in the bone marrow and gut, leading to toxic side effects. In contrast, normal cells express extracellular heparanase at very low levels, and heparanase knock-out animals exhibit no obvious deficits. These characteristics imply that inhibition of heparanase will cause minimal side effects in cancer patients.

Initially we tested the available *N*-acetylated HS mimetics developed for BACE1 inhibition[10]; but, these did not inhibit heparanase with any significant potency. However, one of the *N*-sulfated analogs (compound **1**, Scheme 1, Panel A) did show modest inhibitory activity (60% inhibition at 1 μM concentration; Figure 1, Panel A). Thus, we developed further analogues (**26**-**31**, Scheme 1, Panel B), reasoning that increasing the level of sulfation would lead to more potent inhibitors. As a simplified alternative to complex HS saccharides, we synthesized a novel targeted library of single-entity dendrimer HS glycomimetic clusters capped with simple sulfated saccharides, glucose and maltose, that potentially mimic *gluco*-configured HS. We also varied the length of the dendritic core arms employing PEG linkers to increase the solubility of the glycomimetics. Using affordable starting ingredients, we also significantly shortened the synthesis process, thus markedly reducing manufacturing costs.

Glycosylation of peracetylated sugars[15], glucose (**2**) and maltose (**3**) with 6-chloroethanol (**4**) in dichloromethane in the presence of boron trifluoride etherate at room temperature gave glycosides (**5**) and (**6**). Displacement of the chloro-group with sodium azide in DMF at 80°C followed by Zemplen deacetylation afforded azido-derivatives (**9**) and (**10**). Reduction of the azides at ambient temperature and pressure with Raney Nickel under hydrogen in aqueous methanol furnished amino-glycosides (**11**) and (**12**) in quantitative yields (Scheme 1, Panel C). "Short-armed" tetra-*N*-hydroxysuccinimide activated ester **13** and a "long-armed" tetra-*N*-hydroxysuccinimide activated ester **14** were prepared as described in our earlier work[10]. Treatment of PEG-acid (**15**) with benzyl alcohol and thionyl chloride gave a benzyl ester of PEG amino acid **16**. Coupling the benzyl ester **16** to a "long-armed" core **14** led to the tetravalent dendritic core **17**. Hydrogenolysis of (**17**) gave the tetra-acid **18**, and its treatment with NHS/EDC afforded the PEGylated *N*-hydroxysuccinimide ester **19** (Scheme 1, Panel D).

In our previous work, we observed complex mixtures of starting material, di-, tri- and tetra-substituted clusters, when sulfated HS fragments were attached to the tetrameric dendritic cores. This was likely due to the electrostatic repulsion of anionic sulfo-groups. We improved the coupling yields by first attaching non-charged fragments followed by over-sulfation. Here we followed the same strategy (Scheme 1, Panel E).

Coupling of four equivalents of glycoside fragments **11** and **12** with tetra-*N*-hydroxysuccinimide active esters **13**, **14** and **19** gave tetramer clusters **20**-**25** in 85-95% yield after chromatography. Sulfation of tetramers in dry DMF under argon at 60°C for 72 h afforded the desired *O*-sulfated HS mimetics **26**-**31** in excellent yields of 88-92% after chromatography. All six final products **26**-**31** and their six precursors **20**-**25** were analysed by 1D and 2D NMR spectroscopy in deuterium oxide at 500 MHz. The chemical shift data are presented in the Supporting Information (Tables S1 and S2). Chemical shifts were assigned using the HSQC technique. ^{1}H and ^{13}C NMR spectra indicated that compounds **26**-**31** were fully sulfated since single sets of peaks were observed with resonances shifted downfield.

The ability of the target compounds to inhibit heparanase *in vitro* was investigated using a Fondaparinux substrate heparanase assay[16] (see Methods in the Supporting Information), and demonstrated that HS glycomimetic clusters (**26**-**31**) inhibited heparanase with IC_{50} values in the micromolar to low nanomolar range (Figure 1).

Tetramers **26**-**28** with sulfated monosaccharide (glucose) fragments showed only weak ability to inhibit heparanase in an initial screen at 1 μM dosage (Figure 1, Panel A), with IC50 values >> 1μM; in contrast, tetramers **29**-**31** with larger sulfated disaccharide (maltose) residues were the most active variants (Figure 1, Panel A). Thus at least 2 sulfated monosaccharide sugar units was critical for activity. Dose response experiments indicated that the potency was higher for "long-armed" PET tetramer **30,** (23nM) compared to the "PEGylated long-armed" cluster with sulfated maltose, **31**

(116 nM; Figure 1, Panel B). Interestingly, a "short-armed" tetramer cluster **29** with sulfated maltose fragments showed the highest potency, with an IC_{50} value of 11 nM. These results indicate that the length of the arms is a significant factor in determining potency; this, along with potential activity of dimer constructs will be a useful avenue to explore in further optimisation studies. The potency of the known heparanase inhibitor, PG545, was found to be 8 nM (Figure 1, Panel B), in very good agreement with the literature[28]. Thus, **29** and **30** have potency very similar to the comparator PG545 which is in clinical development[25].

Myeloma tumors express high levels of the HSPGs on their cell surfaces[17]. Heparanase is upregulated in myeloma tumor cells, and the bone marrow plasma of some patients provides a rich source of biologically active fragments that enhance a tumor's growth and metastasis[18]. Previous work has shown that heparanase inhibitors reduce myeloma metastasis and tumor growth in a xenograft mouse model[17,19]. We tested one of our most potent *in vitro* inhibitors **30** in this highly aggressive myeloma model (Figure 2, Panel A), in which cells metastasize to bone marrow with tumors growing predominantly in the bone, closely mimicking these aspects of the human disease[17]. We observed remarkably reduced tumor spread and growth in the mice treated with the inhibitor (dorsal view, 85.4% inhibition, p=0.037 and ventral view, 88.5% inhibition, p=0.024 at 4 weeks; see Methods, Figure S2), indicative of potent heparanase inhibition.

Some cancer cells form precapillary-like tubes or cords when grown in a basement membrane gel extract, a process called vascular mimicry[20]. Both glycomimetics **29** and **30** inhibited vascular mimicry in cultures of B16 melanoma cells at concentrations of 10 and 40 μM (Figure 2, Panel B), without inhibiting cell migration (Supporting Information, Figure S3). Vascular mimicry, a process similar to angiogenesis, generates blood vessels for vascularization of tumors and occurs in the absence of an endothelial cell line in the cultures. Prevention of neovascularization of a tumor would contribute to inhibition of a tumor's growth by the HS glycomimetics.

A notable potential side effect of heparin and related compounds that can limit their wider clinical application is anticoagulant activity. Importantly, the compounds we synthesized displayed a remarkable lack of any significant anticoagulant activity towards Factor Xa at concentrations >100-fold or higher than their IC_{50} for inhibiting heparanase (Figure 2, Panel C), indicating that they differ from some other compounds in this class such as PG545[23] and necuparanib[29]. Importantly, we also established that this class of compounds was not cytotoxic to BaF3 mouse lymphoid cells at 100 μg/mL (~15-32 μM), which is ~ 100-1500 fold higher than their IC_{50} for inhibiting heparanase (see Supporting Information, Figure S4).

A further known activity of heparin is regulation of fibroblast growth factors (FGFs) which are crucial for cell growth and can also promote angiogenesis[21]. We observed no activation of FGF-1 or FGF-2 signalling via FGF receptor FGFR1c with any of the compounds on BaF3 lymphoid cell proliferation (**26**-**31,** Figure 3, Panel A) where viability is dependent on FGF signaling in the absence of Interleukin 3 (IL-3) (see Methods).

We also examined the compounds for inhibitory properties towards FGF signaling in BaF3 cells in which cells are partially activated by addition of 3 μg/mL heparin. Notably, none of the compounds (**1**, **26**-**31**, Figure 3, Panel B) significantly inhibited FGF1 in BaF3 cells transfected with FGFR1c. In contrast, FGF2 was inhibited by some of the compounds, most potently by compounds **30** and **31**. This is significant since inhibition of FGF2 could contribute to the anti-angiogenic properties of these compounds that we observed (Figure 2, Panel B), providing an additional anticancer mode of action. BaF3 cells are a mouse pre-B lymphocytic cell line dependent on IL-3 for survival[22]. They are devoid of heparan sulfate and FGF receptors and do not respond to FGF in the presence or absence of heparin[23]. BaF3 cells transfected with FGFR1c gain the ability to respond to FGF-1 and FGF-2 in the presence of exogenously added heparin or heparin mimetics, and the cells can proliferate normally in the absence of Il-3[23] (Figure 3, Panel A). If no heparin is present or a heparin species is not capable of supporting FGF activity, the cells do not proliferate and eventually die.

There are no therapeutically effective heparanase inhibitors currently available for use in the clinic[24]. Heparin, an antithrombotic drug that also inhibits heparanase, is not clinically useful because of its strong anticoagulant activity. However, the use of HS-related compounds as antitumor agents has been reported in the literature, resulting in clinical trials currently underway. For example, Progen Pharmaceuticals has designed a compound called PG545 that is produced from a very expensive tetrasaccharide maltotetraose[25]. PG545 treatment reduces heparanase activity in primary tumors and is presently undergoing clinical trials[26].

The heparanase inhibitors necuparanib (Momenta Pharmaceuticals)[27] and a glycol-split modified heparin roneparstat (Leadiant Biosciences)[28] are produced from natural heparin by chemical modifications. These compounds display multiple anticancer activities, including heparanase inhibition and anti-angiogenic properties. However, as they are complex mixtures, they may face hurdles in obtaining FDA approval, as the methods required to characterize batch-to-batch variations are very elaborate and costly. Furthermore, both PG545[23] and necuparanib[29] also retain significant anticoagulant activity, and roneparstat requires high dosage levels due to short half-life[27]; these factors that might limit their clinical usage.

Thus, collective data point to heparanase as a good target molecule to interfere with cancer progression, though none of the treatments already under development are ideal. Our HS glycomimetics could provide a solution to these drawbacks since they have distinct advantages, including a unique combination of potency comparable to PG545 (currently the most potent of the described inhibitors with an IC_{50} value of 12 nM)[30], allied with a lack of any significant anticoagulant activity, and also lack of cell toxicity. We have described rapid, convenient, and economical methods for the synthesis of a range of single chemical entity sulfated glycomimetics. Further studies are underway to evaluate their pharmacokinetics, bioavailability, and safety as a prelude to potential clinical testing.

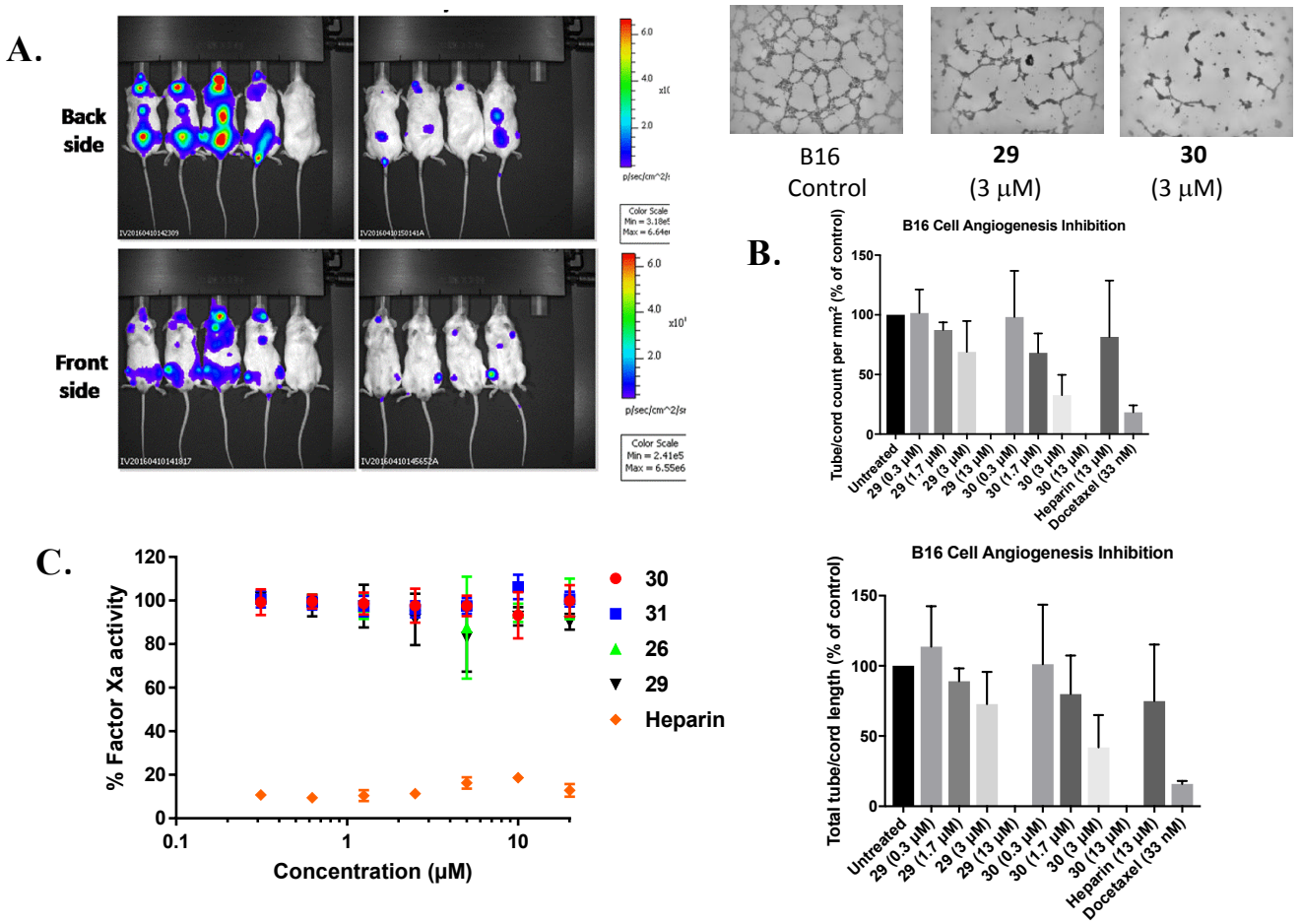


Figure 2. Screen of bioactivities of selected compounds. Panel A: Inhibition of metastasis and tumor growth in an immunodeficient mouse xenograft model with human myeloma cells. See Methods (Figures S1 and S2 in the Supporting Information) section for experimental details. On the left are untreated mice with malignant myeloma tumors containing a luciferase reporter, 4 weeks after injection of tumor cells. A control mouse that was not injected with cells is included (No. 5 from the left). On the right are mice injected with myeloma cells, and treated for 4 weeks with our heparanase inhibitor **30** (600 μg per day). Panel B: Inhibition of angiogenesis. The murine melanoma cell line B16 is capable of vascular mimicry, a process similar to angiogenesis. The effect of the dendrimer HS glycomimetics **29** and **30** were tested to see if they could prevent vascular mimicry in B16 cultures grown in a basement membrane extract (Cultrex BME) (see Methods). Images of epithelial tubes or cords are shown in the upper part of Panel B, and a summary of the results after treatment for 18 h with **29** or **30** is shown on the lower part, presented as total number of tubes/cords and total length of tubes/cords (n=3 experiments; mean±SEM). Heparin was used as a negative control, and docetaxel (DTX) was used as a positive control for inhibition of angiogenesis. Panel C: Inhibition of Factor Xa anticoagulant activity by HS glycomimetics (n=6 replicates; mean±SD).

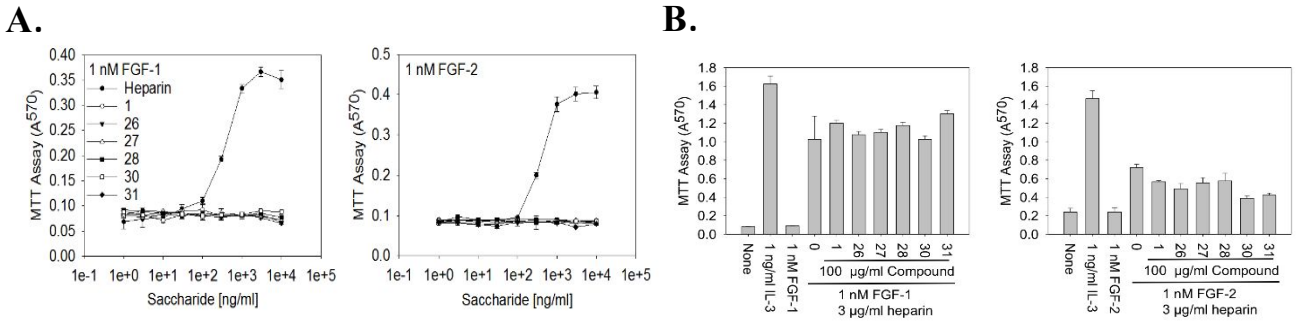


Figure 3. Screen of FGF signalling regulation by selected compounds. Panel A: No HS glycomimetic activation of proliferation of BaF3 cells transfected with FGFR1c by FGF-1 or FGF-2. Left graph, proliferation activity of 1 nM FGF-1 in the presence of increasing concentrations of heparin or compounds; Right, same as left graph but with 1 nM FGF-2. See Methods section for experimental details. Panel B: Inhibition of heparin-induced activation of FGFR1c. FGF2 action is inhibited but

not FGF1 by selected compounds. Left, 100 μg/ml of compounds were added to BaF3 cells transfected with FGFR1c in the presence of 1 nM FGF-1 and 3 μg/ml heparin. Cells exposed to no growth factor, 1 ng/ml IL-3 or FGF-1 alone are also shown. Right, same as left graph but with 1 nM FGF-2. See Methods section for experimental details.

In summary, we have for the first time prepared chemically defined, single-entity polyvalent heparanase inhibitors displaying simple sulfated sugars on dendritic cores. We have shown that these clusters inhibit heparanase with potency equivalent to the comparator PG545, as well as inhibiting angiogenesis and FGF2, and they also completely lack off-target anticoagulant activity and cell toxicity. The compounds reported here are a novel class of anticancer candidates with additional potential applications in diseases where targeting of heparanase inhibition is expected to be beneficial, including inflammatory disorders[31], diabetic nephropathy[32], fibrosis[33] and viral pathogenesis[34-35].

METHODS

All methods are described in the Supporting Information.

ASOOCIATED CONTENT

Supporting Information is available free of charge via the Internet on the ACS Publications website at http://pubs.acs.org. Materials, methods, abbreviations, supplementary figures, detailed description of the synthetic procedures and NMR spectra (PDF).

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Notes

The authors declare no competing financial interest.

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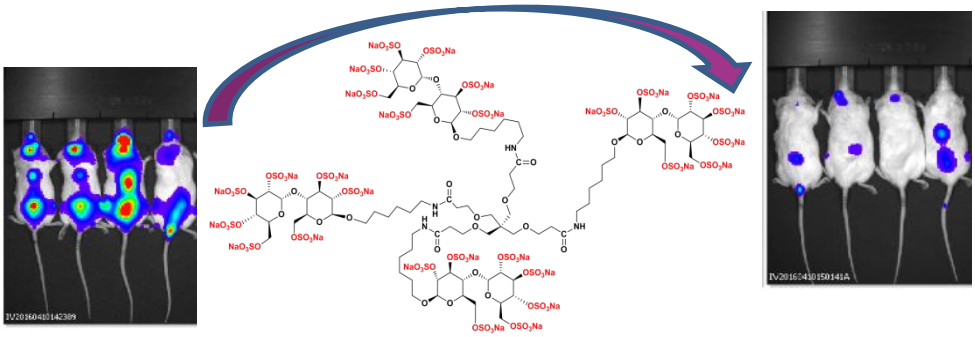

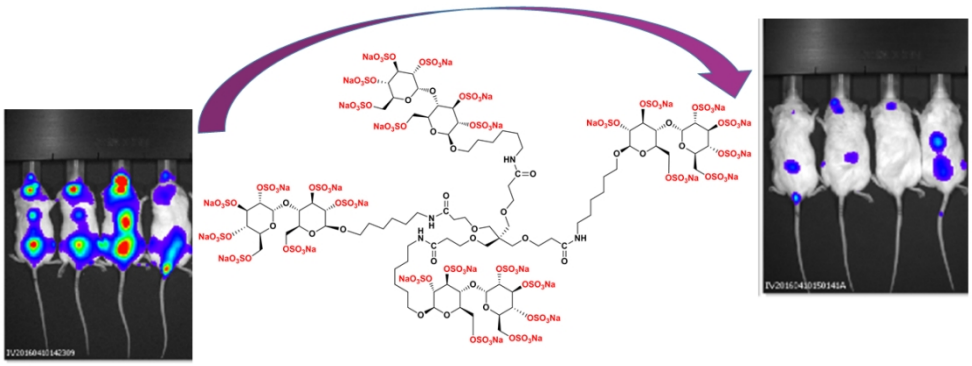

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