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Synthetic Heparan Sulfate Ligands for Vascular Endothelial Growth Factor To Modulate Angiogenesis.

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HS analogs (**HT-0S-NH, HT-0S-NAc, HT-6S-NH, HT- 6S-NAc, HT-2S-NH, HT-2S-NAc, HT-2,6S-NH, HT-2,6S-NAc, HT-2,3S-NH, HT-3S-NH, HT,3,6S-NH, HT-6P, HT-6,2P**) were synthesized according to published procedures.[1]

1. **Glycan array**

Materials. Human $VEGF_{165}$ (AF-100-20), human HB-EGF (100-47), human BMP2 (AF-120-02), biotinylated-rabbit-anti-human $VEGF_{165}$ (500-P10BT), biotinylated-rabbit-anti-human HB-EGF (500-P329BT), anti-human BMP2 (500-P195BT), were purchased from Peprotech. Human FGF2 basic recombinant (RP-8626) and polyclonal biotinylated-anti-human-FGF2 antibody (PA1-25521) were purchased from Thermo Fischer. Cy3-sterptavidin was purchased from Jackson ImmunoResearch.

HS microarray fabrication. HS Arrays were fabricated with NanoPrint LM-60 Microarray Printer (Arrayit) on epoxide-derivatized slides (PolyAn 2D) with four 946MP3 pins (5 µm tip, 0.25 µl sample channel, ~100 µm spot diameter; Arrayit) at 16 sub-array blocks on each slide (array VrHI.01). Glycoconjugates were distributed into 384-well source plates using 4 replicate wells per sample and 7 µl per well. Each glycoconjugate was printed at two different concentration (50 µM and 100 µM) at four replicate spots in an optimized printing buffer (300 mM phosphate buffer, pH 8.4). AlexaFlour-555-hydraside was printed per block (Invitrogen A20501MP, at 1 ng/µl in 178 mM phosphate buffer, pH 5.5) to monitor printing and the humidity level in the arraying chamber was maintained at ~70% during the procedure. Upon printing, slides were left on arrayer deck over-night for allowing humidity to drop to ambient levels (40-45%). subsequent, slides had been packed, vacuum-sealed and stored at room temperature (RT) until used.

HS microarray binding assay. Slides were developed and analyzed as previously described[2] with some adjustments. After rehydrating with dH_2O, slides were incubated for 30 min in a staining

dish containing pre-warmed (50 °C) ethanolamine (0.05 M) in Tris-HCl (0.1 M, pH 9.0) to block the previously unreacted epoxy groups on the slide surface and washed finally with 50 °C pre-warmed dH_2O. Slides were centrifuged at 200×*g* for three min then fitted with ProPlate™ Multi-Array 16-well slide module (Grace Bio-lab) to divide into the sub-arrays (blocks). Slides were washed with PBST (PBS pH 7.3 + 0.1% Tween 20), aspirated and blocked with 200 µl/sub-array of blocking buffer (PBS pH 7.3 + 1% w/v Ovalbumin grade V, Sigma) for 1 hour at RT with gentle shaking. Next, the blocking solution was aspirated and 100 µl/block of primary detection proteins (for each detection, 3 serially decreasing concentrations were used, see Table S1) diluted in blocking buffer, were incubated with gentle shaking for 2 hours at RT. Slides were washed 4 times with PBST, then with PBS for 2 min. Bound antibodies were detected by incubating with biotinylated secondary detections (1 ng/µl, see Table S1) diluted in PBS, at 200 µl/block at RT for 1 hour. Slides were washed 4 times with PBST, then with PBS for 2 min and biotinylated antibodies detected with Cy3-sterptavidin (1.2 µg/ml). Slides were washed 4 times with PBST, then with PBS for 10 min followed by removal from ProPlate™ Multi-Array slide module and immediately dipping in a staining dish with dH_2O for 10 min with shaking. Slide then were centrifuged at 200×*g* for 3 min. and the dry slides immediately scanned.

Supplementary Table S1. Primary detection and secondary biotinylated antibodies used on the array.

Primary Detection	Concentrations used (ng/µl)	Secondary Detection
Human FGF2 (basic recombinant)	20, 10, 5	Biotinylated-FGF2 polyclonal antibody
Human $VEGF_{165}$	5, 2.5, 1.25	Biotinylated-rabbit-anti-human $VEGF_{165}$
Human HB-EGF	20, 10, 5	Biotinylated-rabbit-anti-human HB-EGF
Human BMP2	2, 1, 0.5	Human BMP2 detection antibody

HS microarray slide processing. Genepix 4000B microarray scanner (Molecular Devices) and Genepix Pro 4.0 analysis software (Molecular Devices) was used for the processed slides scanning (using 350 gain at 10 µm) and image analysis respectively. Spots were defined as circular features with a variable radius as determined by the software. RFU from each spot was calculated after subtracting local background. Ranking was done to compare the data between detections (3 dilutions for each detection). For each growth factor and at each dilution, the binding RFU's of the glycans were listed, maximum RFU was determined and set as 100% binding while all the others were calculated compared to the max. Next, the rank for each glycan was averaged between the three dilutions and SEM was calculated.

2. **Surface Plasmon Resonance binding kinetics.**

HS tetrasaccharides were immobilized on CM5 sensor surface using a coupling reaction. Briefly, CM5 chip was activated with EDC (0.2 M) and NHS (0.02 M) at a flow rate of 5 μl min^{-1} for 15 min. Then, activated chip was treated with 50 μL of 0.05 mM of HS tetrasaccharides. At the negative control chip, the activated chip was treated with 0.5 mM of ethanolamine. The binding of ligand was confirmed by positive RU on HS tetrasaccharides CM5 chip. K_D values were analysed by treating the surface with different fraction of $VEGF_{165}$ at a flow rate of 50 μl/min and 25 ° in HBS-EP buffer After 200 S, chip was treated with HBS-EP buffer without $VEGF_{165}$ to measure dissociation. Finally, binding kinetics was analyzed using the BIA evaluation software for T100 by adjusting the fitting curve with 1;1 interaction model. The sensor chip was regenerated by washing with 0.1% SDS and 0.085% H_3PO_4 injected for 3 min at a flow rate of 100 μl/min.

Competitive Binding assay and K_D value calculation

Biotinylated heparin was purchased from Sigma Aldrich was immobilized on strepatavidy chip based on published procedure. Briefly, 0.1 mg/ml of biotinylated heparin was flowed on streptavidin coated ship at a flow rate of 10 μl/min in HBS-EP buffer until a response of 100 RU was obtained. Then unbound heparin was removed by running HBS-EP without biotinylated-heparin. In the negative control cell, streptavidin chip was treated with HBS-EP buffer. Different concentration of $VEGF_{165}$ (0-10 nM) dissolved in HBS-EP buffer was flowed on SPR chip and control chip for 250 secs, followed by dissociated using HBS-EP for 150 secs. Using 1:1 fitting curve binding affinity of heparin and $VEGF_{165}$ was determined (Fig S3).

For competitive binding assay, VEGF165 was mixed with different concentration of HS oligosaccharides (0-1 mM) for 1 hr in HBS-EP buffer. Then the mixture was flowed over heparin-streptavidin and streptavidin (negative control) chip at a flow rate of 50μl/min for 250 sec, followed by dissociation by using HBS-EP buffer. The experiment was repeated trice to get the apparent K_D value using strandard protocol.[3]

3. Cell proliferation assay

HUVECs were plated on 96 well plates in EBM-2 medium in 1% FBS without growth supplements. HS tetrasaccharides (10 and 50 μg/ml) with $VEGF_{165}$ (50 ng/ml) were added to each well. After 48 h of incubation, cells were washed with PBS buffer and fixed with paraformaldehyde. Cells were stained with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium monosodium salt and cell proliferation was quantified by measuring the absorption intensity at 450 nm.

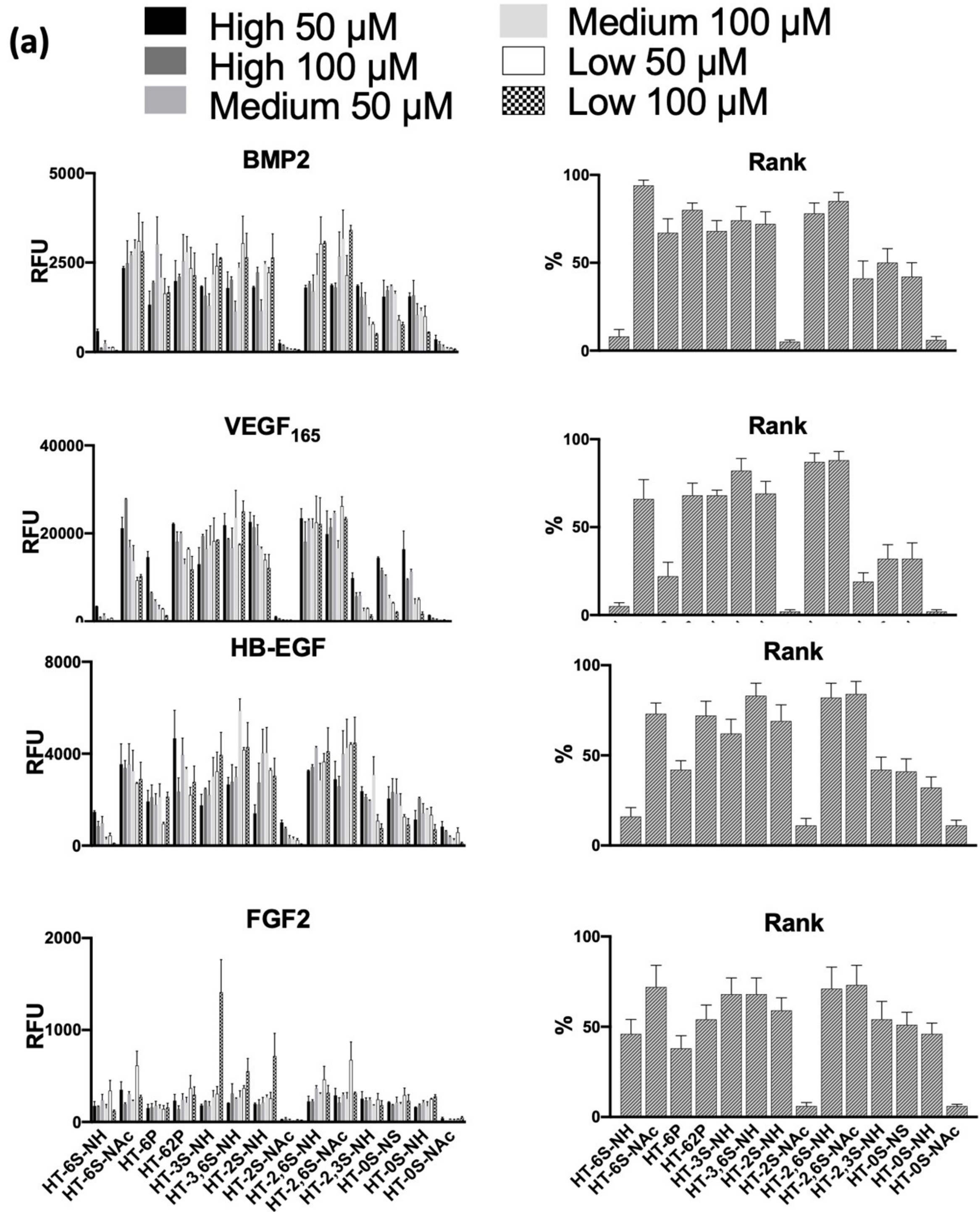
4. Wound healing assay

HUVEC cells were cultured on 24-well plates in EBM-2 media and allowed to grow till monolayer formation. Afterwards, a wound was created by using 1000 μl pipette tip. Cells were then treated with HS analogues (50 μg/ml) and $VEGF_{165}$ or $VEGF_{165}$ alone (50 ng/ml). After 12 h, $VEGF_{165}$ alone treated HUVECs showed complete wound healing, which was considered as 100% healing, and at that point, the percentage of cell migrated in other wells were quantified

5. Tube formation Assay.

Matrigel with a reduced amount of growth factor was coated evenly over an 8-well imaging chamber for 1 h at 37 °C. Approximately 10^4 HUVECs were added on the matrigel coated cells per well in DMEM media with 1% FBS supplemented. For tube formation, $VEGF_{165}$ (50 ng/ml) growth factor with or without **H-1** and **H-2** ligand (50 μg/ml) were added and incubated for 24 h Cells were then fixed and stained with calcein AM for confocal imaging.

6. Supplementary Figures.



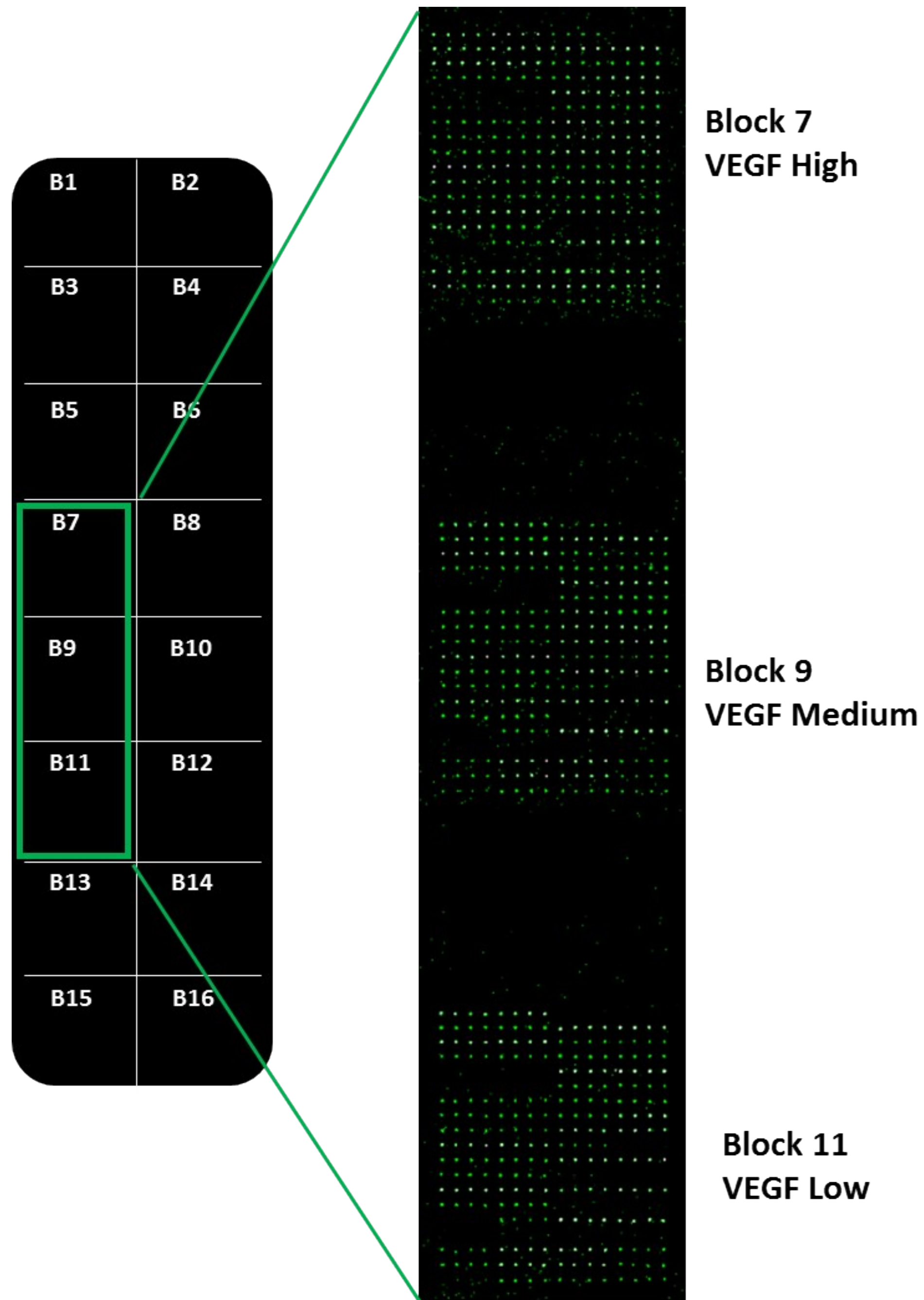


Figure S1. Growth Factors glycan microarray binding assays. (a) Bar graphs of relative fluorescence units (RFUs) of the different growth factors, each examined at three serial dilutions (as specified in Supplementary Table S1). (b) Example of the analyzed arrays, highlighting a representative example of $VEGF_{165}$ developed on three printed arrays (blocks) on one of the processed slides.

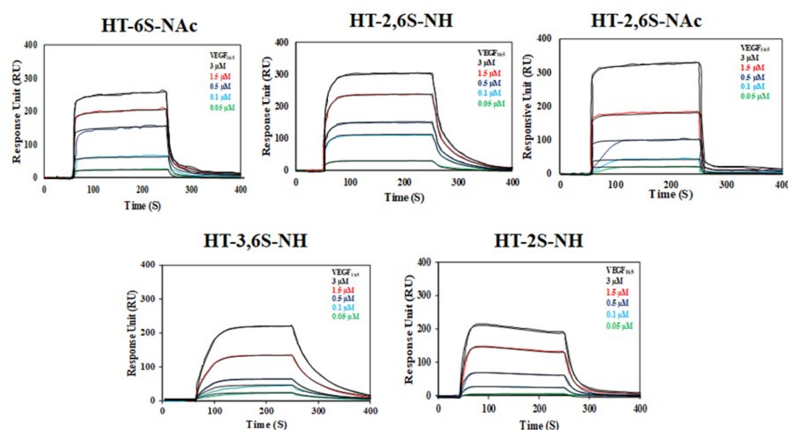


Figure S2: SPR analysis of $VEGF_{165}$ with HS ligands with fitting curves.

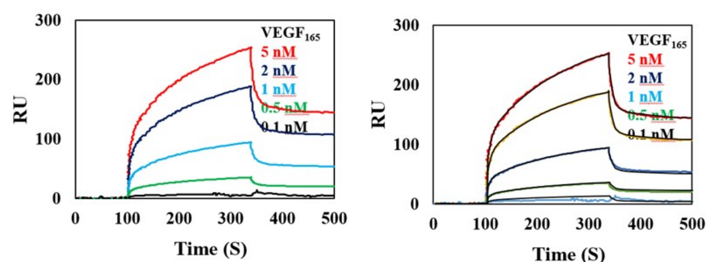


Figure S3. SPR binding affinity of biotyniated-heparin and $VEGF_{165}$ growth factor (a) Kinetics of biotynilated-heparin binding to varying concentration of $VEGF_{165}$. (b) The thin black curves are fitting curves for 1:1 binding model (K_D = 93.4 nM).

7. References:

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