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Synthetic heparan sulfate ligands for vascular endothelial growth factor to modulate angiogenesis†

Prashant Jain,‡[a] Chethan D. Shanthamurthy,‡[a] Shani Leviatan Ben-Arye,[b] Sharon Yehuda,[b] Sharvani S. Nandikol,[c] Hirekodathakallu V Thulasiram,[c] Vered Padler-Karavani *[b] and Raghavendra Kikkeri *[a]

We report the discovery of a potential heparan sulfate (HS) ligand to target several growth factors using 13 unique HS tetrasaccharide ligands. By employing an HS microarray and SPR, we deciphered the crucial structure-binding relationship of these glycans with the growth factors BMP2, $VEGF_{165}$, HB-EGF, and FGF2. Notably, GlcNHAc(6-O-SO_3^-)-IdoA(2-O-SO_3^-) (HT-2,6S-NAc) tetrasaccharide showed strong binding with the $VEGF_{165}$ growth factor. *In vitro* vascular endothelial cell proliferation, migration and angiogenesis was inhibited in the presence of $VEGF_{165}$ and HT-2,6S-NAc or HT-6S-NAc, revealing the potential therapeutic role of these synthetic HS ligands.

Heparan sulfate (HS) is a linear, negatively charged polysaccharide, which is considered to be a member of the glycosaminoglycan family.[1] HS is a major component of the cell surface as well as of the extracellular matrix of all types of cells. Further, HS plays a fundamental role in many cellular events, including cell signalling, cell morphogenesis and pathophysiological functions.[2] However, the structural complexity of HS hinders the identification of its specific domain and its therapeutic significance. HS is known to be associated with two types of structural variations: (i) a variation in the relative composition of the HS disaccharide units (D-glucosamine and uronic acid, with the variation involving either D-glucuronic acid or L-iduronic acid) and (ii) a variation in the sulfation substitutions at 6-OH, 3-OH of the glucosamine residue and 2-OH of the uronic acid residue. In addition, glucosamine also exists in the *N*-acetylated (NA) and *N*-sulfated (NS) forms.[3] The structural diversity exhibited by various sulfation groups located on the HS chain results in interactions with a plethora of proteins,[4] thereby modulating a wide range of biological activities. For example, it has been shown that the 6-*O*-sulfated NS domain of the HS chain plays a pivotal role in fibroblast growth factor-1 (FGF1) activation and signalling.[5] Moreover, HS pentasaccharides featuring a 3-*O*-sulfated NS domain are known to significantly modulate the activity of antithrombin III.[6]

Similarly, the binding of several chemokines are regulated by a highly sulfated NS domain.[7] However, a systematic study of HS analogs with growth factors, particularly with rare *N*-unsubstituted (NU) domain and *N*-acetate domain HS ligands have not been investigated yet, particularly against tetrasaccharide HS compounds. Here, we show that synthetic HS ligands can effectively target growth factors. Moreover, NU and NA domain HS ligands markedly influence growth factors binding and their activities. To further rationalize the ionic interactions between growth factors and HS ligands, we also used phosphate derivative of HS oligosaccharide for comparison.

A library of HS tetrasaccharides (Fig. 1) composed of different sulfation patterns and NU/NA-glucosamine residue were used for growth factors binding studies.[8] These analogs were conjugated to microarray chips at two different concentrations and at four replicates each, and binding of various biotinylated growth factors was examined, each at three different serial concentrations, followed by detection with Cy3-tagged streptavidin. The rationale for selection of growth factors is based on their binding patterns and affinity to native HS.[9] These high-throughput array binding assays were analyzed (Fig. S1a and b, ESI†). Subsequently, to allow direct comparison of binding preferences across the different proteins, in each array all HS ligand-growth factor interactions were ranked, based on their fluorescence intensity with respect to the maximal signal per array block[10] (Fig. 2).

All the growth factors (GFs), BMP2, $VEGF_{165}$, HB-EGF and FGF2 showed a similar binding pattern. For instance, among the 13 HS tetrasaccharides, the non-sulfated ones displayed

[a] *Indian Institute of Science Education and Research, Dr Homi Bhabha Road, Pune-411008, India. E-mail: rkikkeri@iiserpune.ac.in; Fax: +91-20-2590-8207*

[b] *Department of Cell research and Immunology, The Shmunis School of Biomedicine and Cancer Research, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel-Aviv, 69978, Israel. E-mail: vkaravani@tauex.tau.ac.il; Tel: +972-3-640-6737*

[c] *Chemical Biology Unit, Division of Organic Chemistry, CSIR-National Chemical Laboratory, Dr Homi Bhabha Road, Pune 411008, India*

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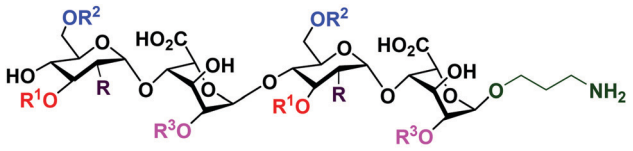
‡ Equal contribution.



HS analogs	R	R^1	R^2	R^3
HT-0S-NH	NH_2	H	H	H
HT-0S-NAc	NHAc	H	H	H
HT-6S-NH	NH_2	H	SO_3^-	H
HT-6S-NAc	NHAc	H	SO_3^-	H
HT-2S-NH	NH_2	H	H	SO_3^-
HT-2S-NAc	NHAc	H	H	SO_3^-
HT-3S-NH	NH_2	SO_3^-	H	H
HT-2,6S-NH	NH_2	H	SO_3^-	SO_3^-
HT-2,6S-NAc	NHAc	H	SO_3^-	SO_3^-
HT-3,6S-NH	NH_2	SO_3^-	SO_3^-	H
HT-2,3S-NH	NH_2	SO_3^-	H	SO_3^-
HT-6P	NHAc	H	PO_3^{2-}	H
HT-6,2P	NHAc	H	PO_3^{2-}	PO_3^{2-}

Fig. 1 Structures of HS tetrasaccharide analogs.

Sulfation/ Phosphation	Substituent	ID	BMP2	$VEGF_{165}$	HB-EGF	FGF2
None	None	HT-0S-NH	42	32	32	46
	None	HT-0S-NAc	6	2	11	6
Di-sulfated	IdoA(2S)	HT-2S-NH	72	69	69	59
	IdoA(2S)	HT-2S-NAc	5	2	11	6
	GlcNH(6S)	HT-6S-NH	8	5	16	46
	GlcNAc(6S)	HT-6S-NAc	94	66	73	72
	GlcNH(3S)	HT-3S-NH	68	68	62	68
Tetra-sulfated	GlcNH(6S)-IdoA(2S)	HT-2,6S-NH	78	87	82	71
	GlcNAc(6S)-IdoA(2S)	HT-2,6S-NAc	85	88	84	73
	GlcNH(3S, 6S)	HT-3,6S-NH	74	82	83	68
	GlcNH(3S)-IdoA(2S)	HT-2,3S-NH	41	19	42	54
Phosphated	GlcNAc(6P)-IdoA(2P)	HT-6,2P	80	68	72	54
	GlcNAc(6P)	HT-6P	67	22	42	38

% Rank: 100, 75, 50, 25, 0

Fig. 2 Microarray analysis of HS ligands with growth factors. Binding was tested at three serial dilutions, then detected with the relevant biotinylated secondary antibody (1 μg ml^{-1}) followed by Cy3-strepavidin (1.5 μg ml^{-1}) (Table S1; ESI,† data file). Arrays were scanned, relative fluorescent units (RFU) were quantified, and maximum RFU determined and set as 100% binding (Fig. S1, ESI†). Then rank binding (per printed glycan per concentration, per each growth factor dilution, per printed block) was determined. Since each glycans was printed at two concentrations, 100% binding was set separately for each concentration. Then, binding to all the other glycans at the same concentration was ranked in comparison to the maximal binding, and the average rank binding and SEM for each glycan across the two glycan concentrations and three examined dilutions of each growth factor was calculated (n = 6; two glycan concentrations across three growth factors dilutions). This analysis allowed to compare the glycan binding profiles of the different growth factors and dissect their binding preferences. The mean rank is shown as a heatmap of all the examined binding assays together (red highest, blue lowest and white 50th percentile of ranking).

weak binding. In contrast, the highly sulfated ligands (**HT-2,6S-NH**, **HT-2,6S-NAc** and **HT-3,6S-NAc**) and the di-sulfated ligands (**HT-2S-NH**, **HT-3S-NH** and **HT-6S-NAc**) displayed strong binding preference (Fig. 2). Furthermore, **HT-2,3S-NH**, **HT-2S-NAc** and **HT-6S-NH** displayed weak binding, illustrating that NU/NA domains with **GlcN(6S)** and **IdoA(2S)** sulfation patterns synergistically improve GFs' binding. Among phosphate HS ligands, **HT-6,2P** displayed moderate to strong binding preference of these growth factors, whereas **HT-6P** displayed weaker binding, with the exception of BMP2, illustrating that sulfate and phosphate groups mimic each other and that heparin phosphate ligands are interesting ligands for further study.

Although most of the GFs showed similar binding patterns with these HS ligands, their fluorescence intensity was found to be three- to six-fold lower than that of $VEGF_{165}$ (Fig S1, ESI†). Hence, a more detailed binding affinity of these HS ligands with $VEGF_{165}$ may establish a sensitive ligand to target $VEGF_{165}$-mediated angiogenesis. To validate the binding affinity of $VEGF_{165}$ with synthetic HS ligands, surface plasmon resonance (SPR) analysis was performed by immobilizing five HS ligands (**HT-6S-NAc**, **HT-2,6S-NH**, **HT-2,6S-NAc**, **HT-3,6S-NH** and **HT-2S-NH**) independently on a CM5 sensor chip and treated with $VEGF_{165}$ at different concentrations (Fig. 3a–e and Fig. S2, ESI†). The K_D value calculated from the binding kinetics revealed that **HT-2,6S-NAc** and **HT-2,6S-NH** (2.76 μM and 3.16 μM, respectively) displayed three-fold stronger binding compared with the other two ligands (**HT-3,6S-NH** [10.14 μM] **HT-2S-NH** [10.91 μM]) (Table 1). There is a 2-fold difference between the pKa of 2^0-amine of glucosamine (~8.5) and the reducing end linker amine (~10.4). In addition, at a given pH the reactivity of primary amine is much higher than that of glucosamine.[11] Nonetheless, the possibility of a reaction between amine group of GlcNH and NHS of CM5 chip cannot be completely ruled out.

To further validate binding affinity, we performed additional competitive binding assays using SPR. In this case, biotinylated heparin was immobilized on streptavidin-coated chip and treated with different concentration of HS oligosaccharides (0 to 1 mM) and $VEGF_{165}$ (5 nM). This analysis showed that the K_D of **HT-2,6S-NAc** and **HT-2,6S-NH** is similar (62 μM and 63 μM respectively), revealing that the NU and NA-domain HS ligands have similar binding strength. Whereas, **HT-2,6S-NAc** displayed a 2-fold stronger binding to **HT-6S-NAc** (112 μM), suggesting that the 2,6-*O*-disulfated HS ligands could be ideal to target $VEGF_{165}$.[12] The disparity in K_D value of direct and competitive binding assay is due to multivalent (natural heparin) *vs.* monovalent (HS ligand) binding interactions with the growth factor.

Active binding between $VEGF_{165}$ and its native receptor VEGFR-2 is known to trigger several cellular events, including vascular cell proliferation, cell migration and angiogenesis.[13] To confirm the inhibitory activity of **HT-2,6S-NAc**, we performed a systematic *in vitro* study using HUVEC cells. First, we performed a cell proliferation assay with $VEGF_{165}$ and HS ligands (**HT-6S-NAc** and **HT-2,6S-NAc**) (Fig. 4a). It was observed that both HS ligands moderately reduced the cell proliferation in the presence of $VEGF_{165}$, illustrating the inhibitory activity of HS analogs.

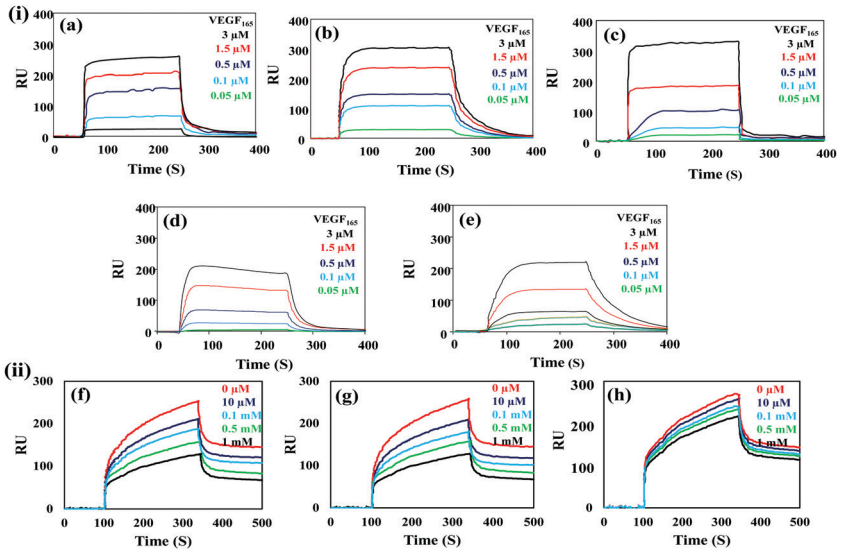


Fig. 3 (i) SPR analysis of $VEGF_{165}$ binding profile on sensor chip having (a) **HT-6S-NAc**, (b) **HT-2,6S-NAc**, (c) **HT-2,6S-NH**, (d) **HT-3.6S-NH** and (e) **HT-2S-NH,** respectively; (ii) SPR competitive binding measurement to heparin and $VEGF_{165}$ (5 nM) in the presence of varying concentration of HS tetrasaccharides (0 to 1 mM) (f) **HT-2,6S-NAc**; (g) **HT-2,6S-NH** and (h) **HT-6S-NAc,** respectively.

Table 1 SPR analysis of kinetic rate constants and equilibrium affinities for HS ligands binding to $VEGF_{165}$ growth factors

Substrate	Growth factors	K_D (μM)	K_{on} (M^{-1} s^{-1})	K_{off} (s^{-1})
Direct binding assay	**HT-6S-NAc**	6.98 ± 0.17	3.17 ± 0.31 × 10^4	2.21 ± 0.28 × 10^{-1}
	HT-2,6S-NH	3.16 ± 0.13	4.89 ± 0.1 × 10^4	1.55 ± 0.1 × 10^{-1}
	HT-2,6S-NAc	2.76 ± 0.32	5.07 ± 0.32 × 10^4	1.45 ± 0.21 × 10^{-1}
	HT-3,6S-NH	10.14 ± 0.23	2.37 ± 0.41 × 10^4	2.40 ± 0.48 × 10^{-1}
	HT-2S-NH	10.91 ± 0.19	2.87 ± 0.18 × 10^4	3.13 ± 0.26 × 10^{-1}
Competitive binding assay	**HT-2,6S-NAc**	62.3 ± 2.1	9.13 ± 0.28 × 10^2	5.67 ± 0.37 × 10^{-2}
	HT-2,6S-NH	63.7 ± 1.9	9.12 ± 0.23 × 10^2	5.81 ± 0.31 × 10^{-2}
	HT-6S-NAc	112.6 ± 3.7	8.35 ± 0.31 × 10^2	9.39 ± 0.61 × 10^{-2}

Next, we studied the potential of HS ligands functional reactivity at inhibiting the cell migration process by using a wound healing assay.[14] To this end, a monolayer of HUVEC cells was cultured and a wound was created. Then, the effect of $VEGF_{165}$ with or without HS ligand or native heparin was monitored. At 50 μg ml^{-1} concentration of HS ligands, we observed a substantial reduction in the rate of cell migration (Fig. 4b).

It was observed that the addition of **HT-2,6S-NAc** induced nearly a 25–28% reduction in the cell migration process, in comparison to 21% of native **HT-6S-NAc**, illustrating the importance of 2,6-*O*-disulfation pattern in $VEGF_{165}$ activation. Finally, we evaluated the influence of the HS ligand on $VEGF_{165}$-dependent tube formation. $VEGF_{165}$ with or without HS ligands (**HT-2,6S-NAc HT-2,6S-NH** and **HT-6S-NAc**) were added to HUVECs cells cultured in matrigel. All three HS analogs showed the strong inhibition of the tube formation of HUVEC cells (Fig. 4c). Among them 2,6-*O*-disulfation ligands showed strong reactivity compared to 6-*O*-sulfted HS ligands, a result which correlates with the cell migration assay. These results clearly showed that **2,6-disulfated HS ligands** are potential ligands for targeting $VEGF_{165}$.

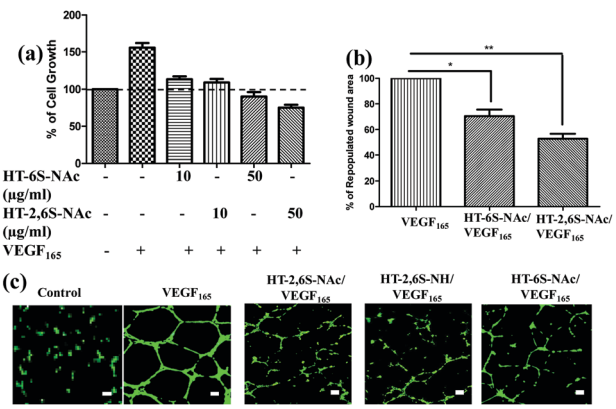


Fig. 4 (a) WST assay was performed to assess HUVEC cell proliferation after 72 h. Concentration of $VEGF_{165}$ concentration is 50 ng ml^{-1}. (b) Wound healing assay with and with $VEGF_{165}$ and HS ligands (conc 50 μg ml^{-1}). The percentage of the effect of growth factors without HS mimics (= 100%) Data expressed as mean ± SD (n = 3; *P <0.01); (c) confocal images of tube formation assay in the presence or absence of HS ligand (50 μg ml^{-1}) and $VEGF_{165}$. After 24 h cells were stained with Calcein AM and imaged; scale bar 30 μm.

In conclusion, we constructed a library of 13 HS ligands and examined them on printed glycan microarrays to generate binding profiles of several growth factors which are implicated in crucial cellular events. The array data provided important structure-binding patterns of glycosaminoglycans (GAGs) that target specific growth factors. In particular, SPR analysis of the $VEGF_{165}$-mediated binding of HS ligands confirmed that **HT-2,6S-NAc** and **HT-6S-NAc** are a potential ligand for targeting $VEGF_{165}$-mediated cellular events. *In vitro* HUVEC cell proliferation, migration and tube formation assays were used to show inhibitory effects of these HS ligands on various cancer biological characteristics.

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Conflicts of interest

There are no conflicts to declare.

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