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Importance of the polarity of the glycosaminoglycan chain on the interaction with FGF-1

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Abstract:

Heparin-like saccharides play an essential role in binding to the FGF-1 and to their membrane receptors FGFR forming a ternary complex that is responsible of the internalization of the signal, via the dimerization of the intracellular regions of the receptor. In this study we report the binding affinities between five synthetic hexasaccharides with human FGF-1 obtained by Surface Resonance Plasmon (SPR) experiments, and compare with the induced mitogenic activity previously obtained. These five oligosaccharides differ in the sulphation pattern and in the sequence. We have previously demonstrated that all the five hexasaccharides have similar 3D structure of the backbone. Consequently, the differences in binding affinity should have their origin in the substitution pattern. Subsequently, the different capacity for induction of mitogenic activity can be, at least partially, explained from these binding affinities. Interestingly, one of the oligosaccharides lacking of axially symmetry (**3**) was biologically inactive whereas the other (**2**) was the most active. The difference between both compounds is the order of the FGF binding motifs along the chain relative to the carbohydrate polarity. We can conclude that the directionality of the GAG chain is essential for the binding and subsequent activation. The relative biological activity of the compounds with regular substitution pattern can be inferred from their values of $IC_{50.}$ Remarkably, the sulphate in position 6 of *D*-Glucosamine was essential for the mitogenic activity but not for the interaction with FGF-1.

Introduction

FGF-1 is a member of the Fibroblast Growth Factor family that interacts with heparin/heparan sulphate (Hep/HS) polysaccharides and the membrane receptors FGFRs. The formation of a ternary complex between FGF, FGFR and Hep/HS is the key step for the activation of the FGF signalling pathway. This is at the origin of different cellular essential functions as regulation of embryonic development, homeostasis and regenerative processes (Bernfield M et al. 1999, Eswarakumar VP et al. 2005, Kreuger J et al. 2006). Dimerization of the receptors and subsequent intracellular autophosphorylation activates a mitogenic response through an enzymatic cascade (Mohammadi M et al. 2005, Pellegrini L et al. 2000, Schlessinger J et al. 2000).

The helical structure of heparin, a highly sulphated form of HS, directs the sulphate groups towards opposite sides of its longitudinal molecular axis (Mulloy B et al. 1993). According to that, the first crystallographic structures of the heparin and human FGF-1 complexes (pdb code: 1amx and 2amx) corresponded to dimeric structures linked by a regular heparin chain in its native helical structure (DiGabriele AD et al. 1998). However, NMR data in solution corresponded to a 1:1 complex (pdb code: 2erm) (Canales A et al. 2006). Remarkably, the dimers have two alternative symmetry relationships: while for 1amx the FGF-1 proteins are related by a centre of symmetry, in the case of 2amx, the symmetry element is a plane along the binding site (DiGabriele AD et al. 1998). In addition, the ternary complexes of heparin and FGF with the extracellular domains of the membrane receptor FGFR are assembled into two different forms (pdb codes 1fq9 and 1e0o respectively) (Schlessinger J et al. 2000)·(Pellegrini L 2001, Pellegrini L et al. 2000)

The analysis of these structures indicates that the Hep/HS binding site corresponds to a swallow depression on the surface of the growth factor that could be considered divided into two sub-binding sites (Digabriele AD et al. 1998). Consequently, as heparin does not much change its helical 3D structure upon binding, in the monomeric case as is the NMR complex (pdb code: 2erm)(Canales A et al. 2006) some of its sulfamate groups will be directed towards the solvent and will not interact with the FGF-1, as the structural studies have shown.

In order to analyse the heparin-FGF-1 binding mode, **2,** a hexasaccharide with axially non-symmetric sulphate groups distribution and unable to form FGF dimers was prepared (Ojeda R et al. 2002). The FGF-1 induced mitogenic activity of hexasaccharide **2** was higher than **1** (de Paz JL et al. 2001), which corresponds to the regular sulphation pattern of heparin (Angulo J et al. 2004) similar to the recently isolated from natural sources as hexamer (Smits N et al. 2010). This result permitted to discard the dimerization of FGF-1 through a chain of bound heparin as a requirement for the FGF-1 mediated bioactivity. Interestingly, **3**, which presents similar symmetry on the sulphate groups distribution than **2**, with respect to the longitudinal axis, was inactive (de Paz JL et al. 2005). The substitution pattern of **3** was designed to fit with the requisites proposed by Pellegrini to maximise the interactions and symmetry in the ternary complex as it was deduced from the analysis of different crystallographic structures (Pellegrini L 2001). Other synthetic oligosaccharides with diverse sulfation patterns prepared in our group lacking of sulphate groups in all the positions 6-*O* of glucosamines (**4**) or in all the 2-*O* of iduronates (**4**) were also inactive (Lucas R et al. 2003). During the revision of this manuscrip a paper describing the synthesis of three hexasaccharides and examining their bioaffinities profiles for was published (Roy S et al. 2014).

Recently performed was an in depth analysis of the three-dimensional structure of **3** using NMR and MD in order to search for any structural differences that might justify the loss of activity (Munoz-Garcia JC et al. 2013). From this analysis it was concluded that **3** exhibits the same main structural features characteristics of heparin than the analogues **1** and **2**, which are known to promote the interaction with FGF-1; a) a well-defined rigid helical backbone with four residues per turn, b) a characteristic chair $^{1}C_{4}$ - skew boat $^{2}S_{O}$ conformational equilibrium for the iduronate residues, and c) a rigid behaviour of the glycosidic linkages. This structural analysis allows to discard any potential difference in the three-dimensional structure that could justify the differences in the observed biological activity between **2** and **3** (e.g. modification of the glycosidic linkages geometry towards an *anti* disposition) (Munoz-Garcia JC et al. 2013). Consequently, the main differences in affinity to FGF-1 among the hexasaccharides **1**-**5** would be due to the capacity of each sulfonate pattern to interact with FGF-1 as a function of its spatial distribution along the chain.

Results

To investigate the ability of the five synthetic oligosaccharides, **1** – **5**, to interact with human FGF-1, an inhibition assay was set up. The growth factor, either alone or coincubated with each of the five molecules to be analysed, was injected over both a heparin-functionalized sensor chip and a streptavidin sensor chip, the latter being used as a control surface, and the interaction was followed by Surface Plasmon Resonance (SPR) spectroscopy (Figure 2). Injection of 8.8 nM of FGF-1 over the heparin surface produced a binding response of 350 response units (RU) at equilibrium whereas a response of 5 RU was observed over the streptavidin surface (data not shown). Analysis of the results showed that these oligosaccharides strongly differ in their ability to prevent FGF-1-Hep binding (Figure 2). The inhibitory activity of **1** was characterized by an IC_{50} of 8.3 10^{-8} M whereas **5** did not display binding activity in the range of concentrations tested indicating that, 2-*O* sulphate groups were essential for the interaction (Angulo J et al. 2004). In contrast, 6-*O* sulphate groups, that are involved in the biological activity (Angulo J et al. 2004), seem to be dispensable for the interaction with FGF-1, with an IC_{50} = 4.7 10^{-7} M for **4**.

Next, to investigate the importance of the presentation of the sulfonate groups along the chain, the same assay was used with **2** and **3** since both have an asymmetric sulphate distribution. Interestingly, it was observed that, **2** features an IC_{50} = 4.6 10^{-7} M, thus similar to **4,** although **2** has five sulphate groups compared to six for the regular oligosaccharide. Finally, **3**, which also displays six sulphate groups with an axially asymmetric distribution, has a much lower binding activity, with an IC_{50} = 1.6 10^{-6} M. Thus, while the mitogenic activity previously obtained was **2** > **1** >> **3**, **4**, **5** (Angulo J et al. 2004), in the case of the binding affinity the order was **1** > **2**, **4** >> **3** >> **5**.

Discussion

Assuming a fundamental role for the electrostatic interactions, and considering the structural differences between the hexasaccharides, the sulphation pattern should be at the origin of the differences in the strength of the interaction and therefore in the activity. Apparent inconsistencies between the larger IC_{50} values for **1** compared with **2**, measured by SPR experiments, and the induction of mitogenic activity, which is larger for **2**, can be explained considering the assembly of the ternary complex. This is essential for the biological activity, and additional hidden requirements may play additional roles (Pellegrini L et al. 2000, Schlessinger J et al. 2000).

Compounds **2** and **3** , as they have their sulfonate groups directed towards one side of the molecular axis, only can interact with FGF using one of their half. On the contrary, as the 3D-structures of **1**, **4** and **5** have an axially symmetric distribution of sulfonate groups, they have the possibility to interact with two molecules of FGF using two opposite sides of the oligosaccharide in a sandwich like fashion, with two simultaneous binding events (Angulo J et al. 2004, de Paz JL et al. 2001, Lucas R et al. 2003). This observation might also explain the observed differences between the mitogenic activity measured by proliferation studies (Angulo J et al. 2004) and the relative binding strength to FGF-1, reported in this study.

The heparin binding site of FGF-1 could be divided into two spatially contiguous sub-sites (DiGabriele AD et al. 1998). The first one binds a trisaccharide GlcNS – IdoA2S – GlcN6S, interacting via three negatively charged sulphate moieties (Saxena K et al. 2010). Such arrangement of charged groups displays the proper number and orientation of sulfonate groups to establish a tight interaction with FGF-1. Recent studies have revealed key differences between FGF-1 and FGF-2 binding to GAG in this subsite. For the case of FGF-2 the trisaccharide that interacts in subsite a is the complementary one, Ido2S – GlcNS – Ido2S (Saxena K et al. 2010). The second sub-site interacts with a disaccharide, GlcN6S – IdoA2S. A central iduronate residue with a non-participating sulfonate group links both motifs. Remarkably, hexasaccharides **2** and **3**, display simultaneously these two decorations for the interaction with FGF-1 but, in reverse order if the polarity of the chain is considered.

In an attempt to find a satisfactory explanation to the lack of activity of **3** with respect to **2**, a molecular modelling docking protocol was employed to analyse the molecular interactions from a structural perspective. Thus, the backbone of the most representative conformation of **3**, taken from 500 ns of unrestrained molecular dynamics trajectory (Munoz-Garcia JC et al. 2013), was manually superimposed to the most representative structure of the NMR complex between FGF-1 and **2** (pdb 2erm). As the distances between the three sulphate groups directed towards the same side of the molecule were similar, two polarities for superimposition were used, from the reducing to non-reducing end and its reversed alternative. We have employed as first criteria, the alignment of the longitudinal axis of both carbohydrates. However, the positions of the sulfonate and sulfamate groups of **3** were not adequate for the complete interaction with the complementary residues of the protein. After that, the non-reducing end trisaccharide of **3** was manually docked into the main sub-site in the "reverse" orientation. In this case, the rest of the oligosaccharide did not fit in the complete binding pocket and pointed towards outside the complex. In addition, a steric clash was observed between the protein side chains and the GlcN – IdoA – GlcN trisulphated trisaccharide of **3** (see Suppl. Info. for description of additional modes). The impossibility to assemble a complex with the complete set of charged interactions between the FGF-1 and the hexasaccharide **3**, lead us to conclude that the correct polarity of the GAG chain is essential for the interaction with the growth factor (FGF-1).

We decided to perform docking calculations in order to get a deeper insight into the possible binding of **3** and FGF-1. We have used Glide, first using the Induced Fit Docking protocol with the standard conditions and then, the results were subjected to a run of Single Precision Docking. In this case, the focus was put into the three saccharides of the triad, leading to a displaced sequence. A remarkable superimposition of the poses for this region was found in the solutions (see supplementary material).

Interestingly, **2** (Figure 3a) has both sulphate clusters in the right disposition to interact simultaneously with both binding sub-sites while **3** is only capable of interacting with the sub-site a (Figure 3) (Canales A et al. 2006). This can explain the value of IC_{50} for **3**, 3.5 fold larger than **2**. This difference can be explained considering that the polarity of the glycosaminoglican chain is essential for the maximum number of interactions take place. While the sub-site a is interacting with **2** through the trisaccharide GlcNS – IdoS – Glc6S starting at glucosamine in position i, the equivalent trisaccharide that binds the main subsite in the case of **3** is shifted to the GlcN at position i+4. The secondary binding sub-site does not establish any interaction with **3**, thus explaining the lower affinity measured by SPR and the absence of mitogenic activity due to the failure to assemble of higher order complexes needed. Therefore, the interaction between FGF-1 and **3** should be weaker than **2.** This should be the cause why **2** and **3** showed such dramatic differences in their binding affinity and bioactivity in spite of bearing the same two binding motifs, but in opposite order.

Additional information can be extracted from the comparison between the affinity experiments and biological activity ones. For instance, the sulphation in position 6 of glucosamine that, according to our previous biological results, is essential for the FGF-1 mitogenic activity (Angulo J et al. 2004), it is not for the interaction with FGF-1. That observation might be exploited in the design of potential inhibitors of the FGF-1 mediated mitogenic activity that being able to interact with the FGF-1, the absence of this key group prevent the assembly of the ternary active complex, and the subsequent biological activity. Another important conclusion that can be extracted from our work is the evidence of the strong influence of the polarity of the GAG chain on the binding. This also can be exploited for the design of inhibitors that interacting with the FGF-1 they do with the opposite polarity and they will not be able to form the active ternary complex.

In summary, we have demonstrated that the polarity of the oligosaccharide chain relative to FGF–1 is a critical factor for the strength of the binary interaction and further assembly of the ternary complex (Brown A et al. 2013).

Materials and Methods

Syntheses of compounds **1** - **5** have been previously described (de Paz JL et al. 2001, de Paz JL and Martin-Lomas M 2005, Lucas R et al. 2003, Ojeda R et al. 2002).

Size defined heparin (Hep; 6 kDa) was immobilized on a Biacore sensorchip. For that purpose, Hep was biotinylated at its reducing end by coincubation with 10 mM biotin/LC-hydrazine for 24 h at room temperature.

The mixture was then extensively dialyzed against H_2O to remove unreacted biotin and freeze-dried. Two flow cell of a CM4 sensorchip were then functionalized with approx. 2500 resonance units (RU) of streptavidin as described (Crublet E et al. 2008) and biotinylated HP (5 µg/ml), in HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4) was injected across one flow cells to obtain an immobilization level of 50 RU. The other flow cell was left untreated and served as negative control. For binding assays, 150 µl of FGF-1 (8.8 nM), co incubated with a range of concentration of the different oligosaccharides, were simultaneously injected, at a flow rate of 50 µl/min, over the control and the HP surfaces. The formed complexes were washed with running buffer for 3 min and the sensorchip surfaces were regenerated with a 3 minute pulse of 2 M NaCl. Control sensorgrams were subtracted on line from HP sensorgrams.

The protein data bank structures 1amx, 2amx and 2erm were used for the preliminary studies of docking described in this paper, performed with GLIDE (Friesner RA et al. 2004). The monomer C from the 1amx complex was isolated from the rest of the aggregates and used to prepare the model of the hexasaccharide **3** with FGF-1 by superimposition of the trisaccharide of its reducing end with the one at the non-reducing end of the 1amx and/or 2erm complexes aligning the sulfate groups. Hydrogens atoms were added to the crystallographic structure when it was necessary using the Maestro protein preparation module. The corresponding hexa- and pentasaccharides were prepared and named consistently and using partial charges from GLYCAM (Kirschner KN et al. 2008), ligand preparation module was run and the structure was minimized. A grid (10 x 10 x 10 Å) centered in the glycosaminoglycan was constructed. We first run an Induced Fit Docking with the standard conditions keeping the GLYCAM charges. The resulting structures were submitted to a Single Precision Docking, with a 10 Å grid using GLYCAM partial charges with an electrostatic cutoff of 2.0. The minimization was performed using OPLS-2005 force field with a dielectric constant of 4r

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Abbreviations

3D, Three-dimentional; FGF, Fibroblast Growth Factor; FGFR, Fibroblast Growth Factor Receptor; Hep, Heparin; HS, Heparan Sulfate; MD, Molecular Dynamics; NMR; Nuclear Magnetic Resonance; RU, Response Units; SPR, Surface Plasmon Resonance

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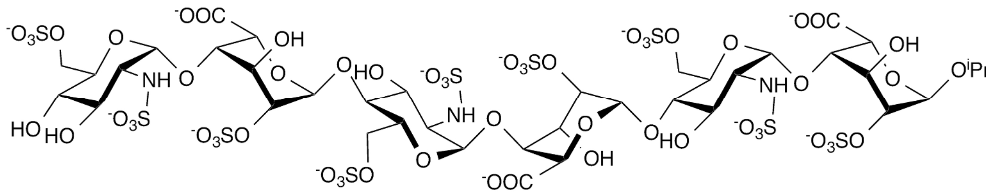
Legends to Figures

Figure 1. Heparin hexasaccharides analysed in this work in planar representation showing the relative disposition of the sulphate groups according to the 3D structure of heparin (PDB code 1hpn).

Figure 2. Inhibition of the FGF-1-HEP interaction by synthetic oligosaccharides. FGF-1 (8.8 nM) was preincubated with a range of concentrations of the different oligosaccharides and injected for 3 min over a HEP-activated sensor chip at 50 µL/min (see Suppl. Info.). The binding responses (in RU) were recorded as a function of time and corresponded to the FGF-1-HEP complexes in presence of **1** (A), **2** (B), **3** (C), **4** (D) and **5** (E). The oligosaccharide concentrations were (from top to bottom curves in each panel) 0, 0.0055, 0.0165, 0.15, 0.5, 1.33 and 4 µM.

Figure 3. Top, representation of the binding modes relatives to the binding subsites on FGF-1 of **2** (red) and **3** (yellow); the non-reducing is at the left. Superimposition of the NMR structure of human FGF-1 with hexasaccharide **2** (red; pdb code 2erm) and model constructed by docking for hexasaccharide **3** (yellow).



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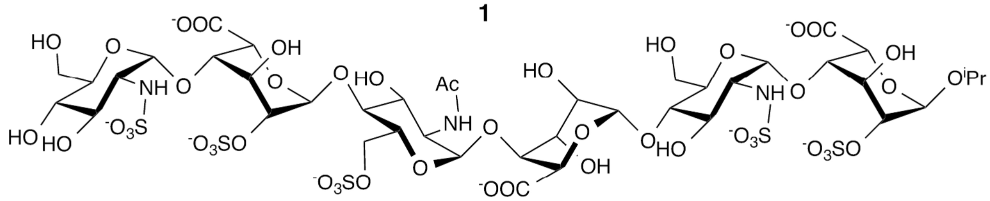

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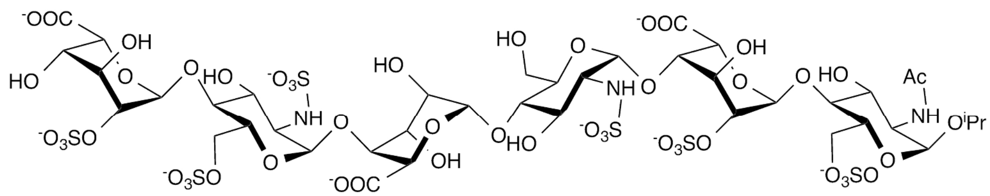

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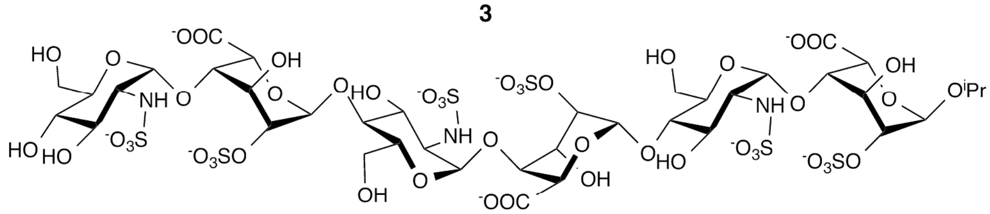

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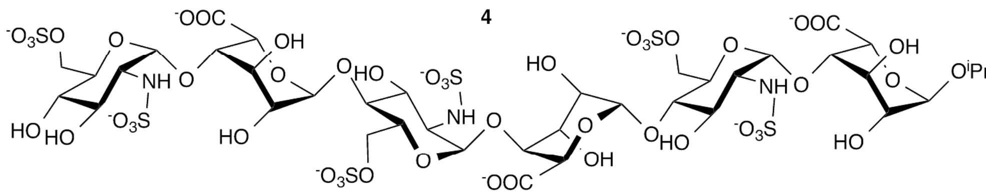

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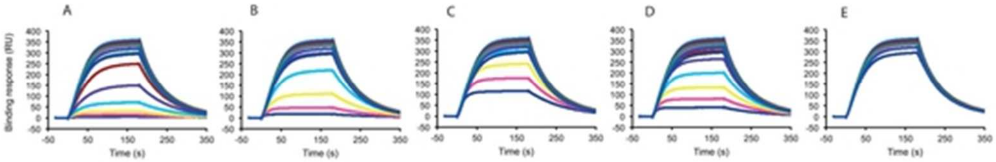

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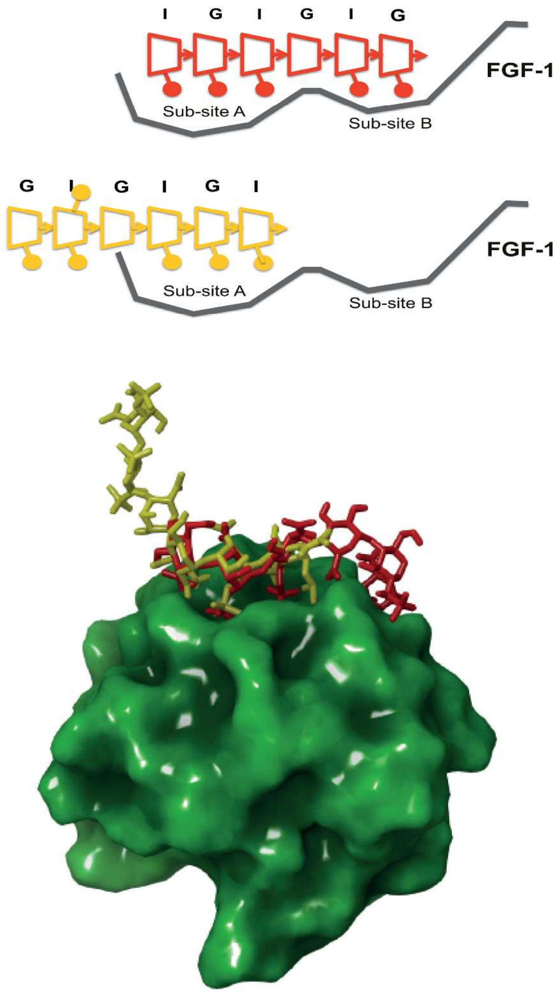

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