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Improvement on binding of chondroitin sulfate derivatives to midkine by increasing hydrophobicity

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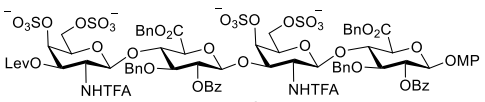
The interactions between chondroitin sulfate (CS) and a wide number of proteins modulate important biological processes. Here, the binding properties to midkine and pleiotrophin of sulfated, fully protected intermediates, typically obtained in the chemical synthesis of CS oligosaccharides, were tested for the first time. Using a fluorescence polarization competition experiment, we discovered that these synthetic precursors strongly bound these two closely related cytokines involved in cancer and inflammation. The relative binding affinities of these intermediates were significantly higher than those displayed by the corresponding fully deprotected oligosaccharides, indicating that the presence of hydrophobic protecting groups strongly enhanced the binding of CS-like derivatives to midkine. These compounds offer novel opportunities for the development of potent inhibitors/activators of CS-protein interactions with potential therapeutic applications.

Glycosaminoglycans (GAGs), a family of linear sulfated polysaccharides that includes heparin and chondroitin sulfate (CS), regulate a wide variety of biological processes through interactions with a large number of proteins.[1-6] One of these proteins is midkine, a cytokine that plays an important role in the early central nervous system development and is involved in inflammation and cancer.[7,8] Midkine is considered as a relevant molecular target for the treatment of various diseases and there is a great interest in the discovery of inhibitors that strongly bind to the protein, blocking its activity in the progression of pathological status, such as tumor invasion and rheumatoid arthritis.[7,9] Midkine has two small independent domains formed by three beta sheets each and stabilized by disulfide bridges, linked by a long hinge region; its structure is completed with two unstructured segments at both ends of the sequence.[7,8] It is known that midkine strongly binds to heparin and chondroitin sulfate chains and these molecular recognition events are essential for protein activity. It has also been demonstrated that the interaction between midkine and CS is mediated by specific oligosaccharide sequences, with a particular sulfation motif, the disulfated disaccharide GlcA-GalNAc(4,6-di-OSO_3), typical of CS-E subtype.[10-12]

The chemical synthesis of well-defined, CS oligosaccharides[13-16] is a valuable tool to determine the structural requirements for CS-protein binding, paving the way for the design and development of CS mimetics[17-20] that can act as more potent inhibitors/activators of those interactions. Typically, the preparation of these molecules involves the use of an orthogonal protecting group strategy that allows the selective introduction of sulfate groups at the desired positions to give protected intermediates such as tetrasaccharide **1** (Figure 1).[21] This type of precursors are finally submitted to deprotection steps, liberating the hydroxyl and carboxyl groups and installing the 2-acetamido moiety present in natural CS sequences. For instance, basic hydrolysis followed by *N*-acetylation of compound **1** afforded the di-benzylated derivative **2** that was finally hydrogenated to give the fully deprotected CS-E tetramer **3** (Figure 1).[21] To the best of our knowledge, the activity of sulfated intermediates, such as **1**, has never been tested.



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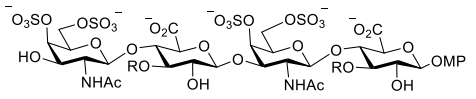

2: R = Bn
3: R = H

Figure 1. Structures of CS-E tetrasaccharide **3** and its synthetic precursors **1** and **2**. Bz = benzoyl; Bn = benzyl; TFA = trifluoroacetyl; Lev = levulinoyl; MP = 4-methoxyphenyl.

Here, we have studied the interactions between midkine and **1** using a fluorescence polarization competition assay, previously developed by us.[21-23] Briefly, the relative binding affinity of the sugar derivative was calculated by measuring its capacity to disrupt the formation of the complex between a

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fluorescent heparin hexamer and midkine, which is characterized by a high polarization (P) value. Thus, we recorded the P of microplate wells that contained increasing concentrations of **1** in the presence of a fixed amount of midkine and fluorescent probe (Figure 2). The concentration dependent decrease of the P value showed that compound **1** interacted with midkine. The curve was fitted to the equation for a one-site competitive interaction and an IC_{50} value of 1.3 μM was obtained. Similar experiments were carried out to determine the IC_{50} values of **2** and **3** (Table 1 and supporting information, Figure S1). We unexpectedly found that the inhibitory potency increased from an IC_{50} value of 254 μM for deprotected tetrasaccharide **3** to a value of 31 μM for **2** and 1.3 μM for **1**, showing that the presence of hydrophobic protecting groups strongly increased the relative binding affinity of CS-like oligosaccharides for midkine. Interestingly, the IC_{50} value of **1**, containing only four sulfate groups, is similar to the IC_{50} value of a heparin hexasaccharide with 13 negative charges (1.1 μM).[21]

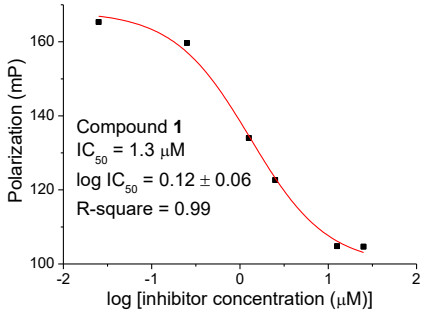

Figure 2. Inhibition curve showing the ability of compound **1** to inhibit the interaction between midkine (63 nM) and fluorescent probe (10 nM). All the P values are the average of three replicate wells.

Table 1. Inhibition of midkine/heparin interactions by synthetic oligosaccharides.

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**
IC_{50} (μM)	1.3	31	254	15	17	20	>250

Fluorescence polarization technique analyses biomolecular interactions in solution. In order to validate our fluorescence polarization findings, we performed an alternative binding assay where we monitored the ability of compounds **1** and **3** to compete with a heparin-coated surface for midkine. We first attached a synthetic heparin hexasaccharide with an amine functionalized linker to Nunc Immobilizer Amino™ microtiter plates, following an experimental protocol previously developed by us.[24] Thus, we created heparin-like coated wells that were incubated with mixtures containing midkine (23 nM) and tetrasaccharides **1** and **3** (Figure 3). We also included in this assay samples incubated with midkine alone, with no potential inhibitor, and with a mixture of midkine and **dp18**, an 18-mer heparin oligosaccharide (from Iduron). The bound protein in each well was detected using a fluorescence microplate reader, after incubations with rabbit anti-midkine antibody and Alexa Fluor 488 labelled anti-rabbit secondary antibody. As shown in Figure 3, fluorescence intensities obtained at positions corresponding to compound **3** indicated that this molecule was not able to block the interaction between the heparin surface and midkine. On the contrary, we did not detect significant fluorescence signals for **1** and **dp18**, pointing out the inhibition of the heparin surface-midkine association. These data were in good agreement with our fluorescence polarization results, confirming that the affinity of protected **1** for midkine is higher than that corresponding to the fully deprotected tetramer **3**.

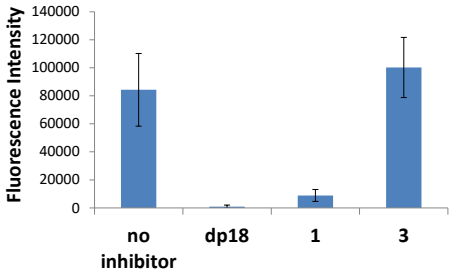

Figure 3. Competition assay employing heparin-functionalized microtiter plates. Heparin-coated wells were incubated with 23 nM midkine alone or in the presence of **dp18** (10 μM), **1** and **3** (100 μM). For each sample, fluorescence signals are the average of six replicate wells and the error bars show the standard deviations for these measurements.

Next, we investigated if a similar trend can be also seen at the disaccharide stage. For this purpose, we carried out the fluorescence polarization competition experiment with derivative **4**, a disulfated protected intermediate in the preparation of a CS-E disaccharide (Figure 4). A sigmoidal decrease in P with increasing disaccharide concentrations was again observed and an IC_{50} value of 15 μM was obtained (Table 1 and Figure S2, supporting information). The competition assay was also performed with disaccharides **5** and **6** that were prepared as shown in Scheme S1 (see supporting information). Compound **5** was considered to assess the influence of substituting the glucuronic acid moiety by a glucose one on the binding affinities, while dimer **6** allowed studying the effect of replacing the cluster of sulfate groups from the reducing galactosamine to the non-reducing glucose unit. IC_{50} values in the range of 17-20 μM were obtained (Table 1 and Figure S2, supporting information). These results were in sharp contrast with that displayed by fully deprotected CS-E disaccharide **7**,[23] which did not inhibit the fluorescent probe-midkine interaction (IC_{50} > 250 μM).[21] Therefore, protected disaccharides **4-6** displayed equivalent relative affinities, much higher than that showed by the naturally occurring CS-E disaccharide.

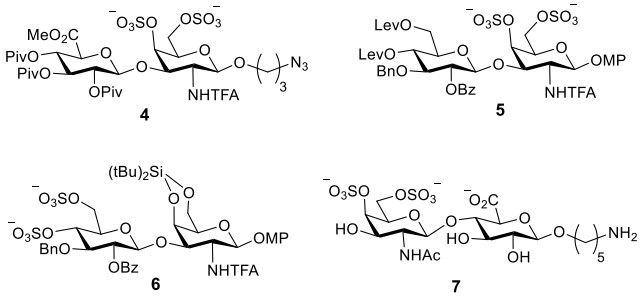

Figure 4. Structures of disaccharides **4-7**. Piv = pivaloyl.

Pleiotrophin is an extracellular heparin-binding protein that shows 45% amino acid sequence identity with midkine.[25] Both growth factors form a two-member family of proteins that share many biological activities and strongly bind to oversulfated CS chains.[26] We decided to investigate if the presence of hydrophobic protecting groups in **1** also increases the binding to pleiotrophin, compared with the fully deprotected tetrasaccharide. First, we measured the direct binding of the fluorescent probe to pleiotrophin, recording the P of microplate wells containing 10 nM concentration of probe and increasing concentrations of protein (Figure S3, supporting information). This initial experiment was required for the correct design of the competition assay. The binding curve was fitted to the equation for a one-site binding model and the dissociation constant (K_D) of the interaction was calculated (K_D = 125 nM). This value is slightly higher than the K_D for the binding of probe to midkine[21] and FGF-2[22] (44 and 117 nM, respectively). We then performed the inhibition assay with compounds **1-3** at 25 µM (Figure 5A). Disaccharide **7** and 5 kDa heparin **dp18** were also included in this experiment as control samples, displaying, as expected, minimum and maximum activities, respectively. Protected tetramer **1** gave ~90% inhibition while **2** and **3** afforded much weaker activities. As in the case of midkine, we found that **1** was the most potent inhibitor of pleiotrophin, suggesting that the increased hydrophobicity of this molecule enhances its protein association. As shown in Figure 5B, the concentration-dependent polarization curve for **1** afforded an IC_{50} value in the low micromolar range (5 µM).

The therapeutic applications of GAG oligosaccharides are seriously hampered by their promiscuous binding to many proteins. The discovery of compounds that selectively bind a limited number of GAG-binding proteins is highly interesting. For this reason, we also evaluated the interactions between compounds **1-3** and basic fibroblast growth factor (FGF-2) (Figure S4, supporting information). The IC_{50} values were 42, 71 and 271 µM for **1**, **2** and **3**, respectively. Although tetrasaccharide **1** was again the most potent ligand, we observed only a ~6 fold inhibition increase for **1**, as compared with the IC_{50} value of deprotected **3**. Interestingly, in the case of midkine, the relative affinity of tetrasaccharide **1** was nearly 200 times higher than that of compound **3**. Our study indicates that compound **1** presents a certain degree of selectivity for midkine and pleiotrophin over FGF-2 (IC_{50} = 1.3-5 µM against 42 µM). Structurally, both midkine and pleiotrophin contain two domains connected by a flexible linker,[27-29] with hydrophobic amino acid clusters exposed on their surface. We hypothesized that this structural feature can explain the higher affinity of **1** for these two proteins.

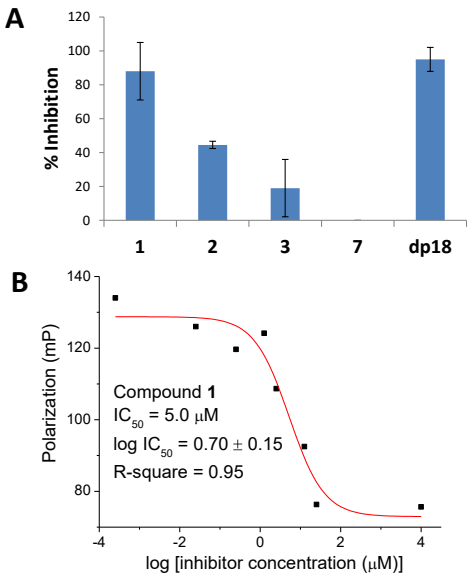

Figure 5. Interaction between pleiotrophin and compounds **1-3**, **7** and **dp18**. A) Competition assay at 25 µM concentration. The inhibition percentages were calculated by using the polarization of reference samples for 100 % and 0% inhibition (see supporting information). The displayed data are the average of two independent experiments, each one in three replicates. B) Inhibition curve showing the ability of compound **1** to block the interaction between pleiotrophin (163 nM) and fluorescent probe (10 nM). All the P values are the average of three replicate wells.

In summary, we have discovered that synthetic sulfated intermediates from the preparation of CS oligosaccharides show a high affinity for midkine, and this binding is much stronger than that displayed by the fully deprotected sequences. Importantly, despite the multiple hydrophobic protecting groups, these derivatives present adequate solubility properties due to the presence of sulfate groups (compounds **1**, **4**, **5** and **6** are soluble in water at 100 µM concentration in the presence of 1% of DMSO). Compared with GAG polyanionic oligosaccharides, these derivatives have less charged groups, an interesting property for potential therapeutic applications.[30] Besides the number and position of sulfate and carboxylate groups, our study indicates that hydrophobicity has also to be considered in the design of high-affinity ligands for CS-binding proteins. In fact, several studies demonstrated that heparin mimetics with increased hydrophobicity exhibit interesting protein binding properties and biological activities.[31-36] Sulfated, fully protected oligosaccharides, typically prepared during CS synthesis, offer an excellent scaffold to introduce chemical modifications and improve binding properties. Therefore, we consider that these compounds are good starting points for further development

of potent inhibitors/activators of CS-protein interactions and the subsequent modulation of important biological processes.

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