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Antiangiogenic heparin-derived heparan sulfate mimics*

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Abstract: Heparan sulfate (HS) is a glycosaminoglycan (GAG) widely distributed as a proteoglycan on the cell surface and in the extracellular matrix of animal tissues. Among other important physiological functions, its polysaccharide chains mediate cell proliferation by binding growth factors [fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF)], which are released in active form through the action of the enzyme heparanase overexpressed by tumor cells. HS is constituted of alternating disaccharide sequences of variously sulfated uronic acid (D-glucuronic, GlcA, or L-iduronic, IdoA) and glucosamine (*N*-acetylated, GlcNAc, or *N*-sulfated, $GlcNSO_3$). HS mimics can be obtained by chemical modification of heparin, a more highly sulfated GAG clinically used as an anticoagulant and antithrombotic drug. With the aim of obtaining antagonists of FGFs as potential inhibitors of tumor neo-vascularization (angiogenesis), arrays of short FGF-binding sequences have been obtained by generating sulfation gaps within the prevalent $(IdoA2SO_3–GlcNSO_36SO_3)_n$ sequences of heparin, by controlled base-catalyzed removal of 2-*O*-sulfate groups of $IdoA2SO_3$ residues. The C(2)–C(3) bond of all nonsulfated uronic acid residues have then been split with periodate, to generate flexible joints along the polysaccharide chain. The novel heparin derivative (poly-PST.sU), chiefly made up of the repeating tetrasaccharide units $–GlcNSO_36SO_3–IdoA2SO_3–GlcNSO_36SO_3–$sU– (where sU is a glycol-split and reduced uronic acid residue) binds FGF2 as strongly as heparin. However, it is a poor inducer of formation of FGF2 dimers and of complexes with FGF receptors, required for triggering mitogenic signals. NMR and molecular modeling studies indicate that formation of these higher-order complexes is prevented by the unfavorable conformation induced by glycol-split residues. In a parallel study, partially *N*-acetylated heparins have been obtained that efficiently inhibit heparanase upon glycol-splitting. It is noteworthy that glycol-splitting involves inactivation of the active site for antithrombin, with consequent loss of anticoagulant activity. In contrast, poly-PST.sU and some of its analogs show potent antiangiogenic activity in in vivo models in which heparin is either proangiogenic or inactive.

INTRODUCTION

Heparan sulfate (HS) is a carbohydrate polymer widely distributed on cell surfaces and extracellular matrix as a proteoglycan (HSPG) [1–3]. Among its multiple functions, HS controls cell adhesion and proliferation through binding to a variety of proteins, among which growth factors of the FGF family [3–5]. Not unexpectedly, HSPGs and their fragments modulate the onset and development of cancer [6]. The enzyme heparanase, overexpressed by tumor cells [7], releases FGFs, which are stored by the HS chains of HSPG in inactive form [8]. As schematized in Fig. 1, fragments of the original polysaccharide

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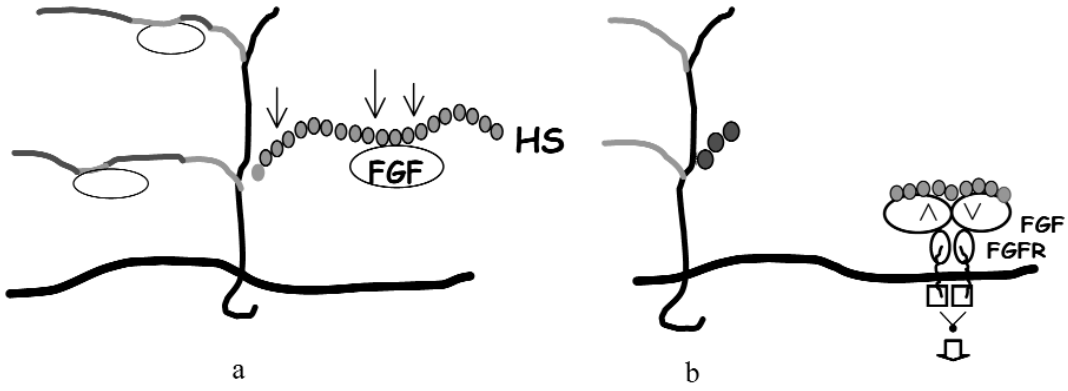

Fig. 1 Simplified representation of an HSPG molecule and its involvement in cellular signaling. HSPGs consist of HS chains linked to a peptide core. Among other proteins, HS stores growth factors such as FGFs in inactive form. The HS chains may be cleaved (arrows) by the enzyme heparanase (a). The released FGFs are activated by HS fragments, which favor their dimerization and formation of a ternary complex with cellular FGFRs (b).

chains induce dimerization of the growth factor and its activation through binding to FGF cellular receptors (FGFRs), a prerequisite for triggering mitogenic signals (Fig. 1).

Like heparin, which is clinically used as an anticoagulant and antithrombotic drug, HS is a GAG constituted of repeating disaccharide sequences of a uronic acid (D-glucuronic, GlcA, and L-iduronic, IdoA) and D-glucosamine (either *N*-acetylated, GlcNAc, or *N*-sulfated, GlcN), with variable degrees of *O*-sulfation on both residues [1]. Though at different locations (heparin is mainly confined in mast cells of connective tissues), heparin and HS are biosynthesized in a similar way, through sequential action of enzymes (*N*-deacetylase/*N*-sulfatase, C5-epimerases, *O*-sulfotransferases) on the common precursor *N*-acetylheparosan, constituted of repeating GlcA–GlcNAc disaccharide sequences. Whereas the biosynthesis of heparin proceeds to reach high degrees of *N*- and *O*-sulfation [their structure being prevalently accounted for by NS domains chiefly constituted by sequences of the trisulfated disaccharide (TD) $IdoA2SO_3$–$GlcNSO_36SO_3$], HS has a much more hybrid structure, with prevalent GlcA- and GlcNAc-containing regions (NA- and NA/NS domains), which are only minor constituents of heparin. Both GAGs contain the minor but important pentasaccharide sequence of the antithrombin-binding domain (AT-bd), essential for the expression of significant anticoagulant properties [1] (Fig. 2).

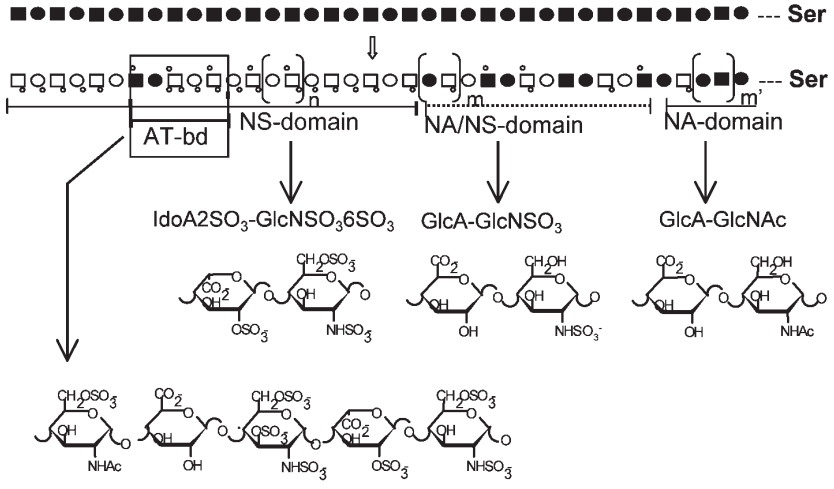

Fig. 2 Biosynthesis and major structural domains of heparin/HS (adapted from [1]).

When compared with polysaccharides with similar degrees of sulfation, heparin has an extraordinary binding versatility [1,4,5]. Such versatility is largely amenable to unusual conformational properties of the iduronic acid residues. Extensive NMR and molecular mechanics studies on heparin fragments and synthetic oligosaccharides have shown that the IdoA (and $IdoA2SO_3$) residues can adopt different equienergetic conformations (mainly the chair $^1C_4$ and the skew-boat $^2S_0$). In solution, these conformations are in rapid dynamic equilibrium, and the population of the different conformers depends on the structure (*N*-acetylation/*N*-sulfation, 6-*O*-sulfation) of aminosugar residues flanking the IdoA residues as well as on intrinsic factors such as type of counterion, ionic strength, and co-solutes [9,10]. Most of the heparin/HS-binding proteins contain clusters of basic amino acids, usually Lys and Arg [4,5]. As illustrated in Fig. 3, the helical conformation of heparin [11] may adapt its shape to better interact with these clusters through "selection" by the GAG of the IdoA form that better facilitates docking to the protein. An impressive experimental evidence of this concept has been provided by the structures of heparin oligosaccharides bound to proteins. In the complex of a specific pentasaccharide bound to antithrombin, the $IdoA2SO_3$ residue settles in the $^2S_0$ conformation in the crystalline state [12] as well as in aqueous solution [13]. In the hexasaccharide-FGF2 crystalline complex (Fig. 4), the protein selected the $^1C_4$ form for one of the two $IdoA2SO_3$ residues of the oligosaccharide, and the $^2S_0$ form for the second one [14]. This "active" conformation of heparin/HS oligosaccharides in the presence of FGF2 has been confirmed in solution [15].

Figure 4 also shows that 1:1 binding of heparin/HS to FGF2 essentially involves only two sulfate groups, i.e., the *N*-sulfate of a $GlcNSO_3$ residue (NS) and the *O*-sulfate of the adjacent $IdoA2SO_3$ residue (2S). Such a minimal binding does not involve 6-*O*-sulfate groups of $GlcNSO_36SO_3$ residues. However, this group is required for binding other growth factors, such as FGF1 and VEGF. As depicted in Fig. 1b, activation of FGFs through dimerization requires longer chains (consisting of at least eight monosaccharide residues) [16–18]. In fact, building-up of ternary complexes with FGFRs ultimately generating mitogenic signals requires even longer chains and involves also some 6-*O*-sulfate groups [1,18]. As shown by recent X-ray crystallographic studies, FGFs/FGFRs assemble in a way to generate "basic canyons", which can accommodate extended heparin chains that stabilize the multimolecular complex [19,20].

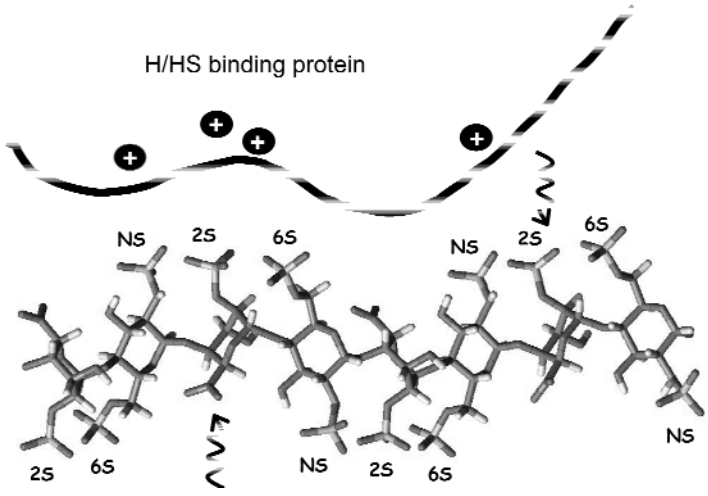

Fig. 3 A heparin helix (featuring triplets of sulfate groups on both sides [11]) in the presence of a heparin/HS-binding protein. The protein induces changes in the conformation of the helix by selecting a form of the "plastic" iduronic acid residues that best facilitates docking to the protein. Further adjustments are favored by the extra flexibility induced by glycol-splitting (see text).

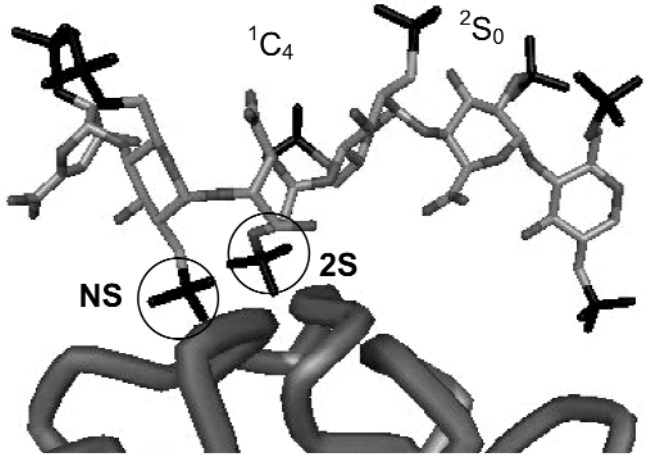

Fig. 4 Conformation of a heparin hexasaccharide in a co-crystal with FGF2 (adapted from [14]), showing that the two IdoA2SO_3 residues are in different conformations. Primary binding involves essentially the two sulfate groups NS and 2S of the hexasaccharide.

FGFs (especially FGF2) and VEGFs are potent inducers of neo-vascularization [6] and are potential targets to develop antitumor drugs. FGFs and VEGFs are heparin-binding proteins [4], and because the NS domains prevalent in heparin are also putative binding domains for these growth factors, heparin represents the starting material of choice to generate angiogenesis inhibitors. Heparin itself is being considered as a potential anticancer drug. In fact, heparin has shown antimetastatic activity in animal models [21]. Moreover, heparin and low-molecular-weight heparin administered as an antithrombotic drug significantly prolong the life of cancer patients [22]. However, the use of unmodified heparin involves hemorrhagic risks. Heparin has also intriguing effects on cells. In fact, it can stimulate or inhibit growth factors in different cellular systems, apparently depending on different types and concentrations of HSPGs [4,23].

Reported strategies to inhibit FGFs with HS mimics are based on the use of heparin fragments or synthetic oligosaccharides able to complex the growth factors, but shorter than necessary to induce their dimerization and activation [24], and (for FGF2) on removal from heparin of the 6-*O*-sulfate groups not required for 1:1 binding, but necessary for activation [25].

TARGETING FIBROBLAST GROWTH FACTORS

Our design of FGF2 inhibitors was based on interruption of the regular TD repeating sequences along the NS region of heparin by selective desulfation, in order to generate arrays of sequences that contain the minimal binding site to the growth factor, but are too short to induce its dimerization and activation. The sulfation gaps were obtained by alkali-induced 2-*O*-desulfation of IdoA2SO_3 residues through intermediate epoxides [26–28]. In addition, the C2–C3 bonds of the nonsulfated uronic acid residues were cleaved with periodate, and the resulting polydialdehydes were stabilized by reduction with borohydride. The glycol-split residues were expected to act as flexible joints along the polysaccharide chains [29], thus facilitating 1:1 binding to FGF2 while disrupting the relatively rigid conformation required to stabilize FGF-FGFR assemblies.

The prevalent structure of heparin (**1**), intermediates **2**, **3**, and of the final product **4** of the desulfation via epoxide under conditions to obtain 2-*O*-desulfation and glycol-splitting of about 50 % of IdoA2SO_3 residues in the NS regions [30], as confirmed/determined by mono- and two-dimensional NMR spectroscopy, are reported in Fig. 5. The spectra of the final product are remarkably clean, with

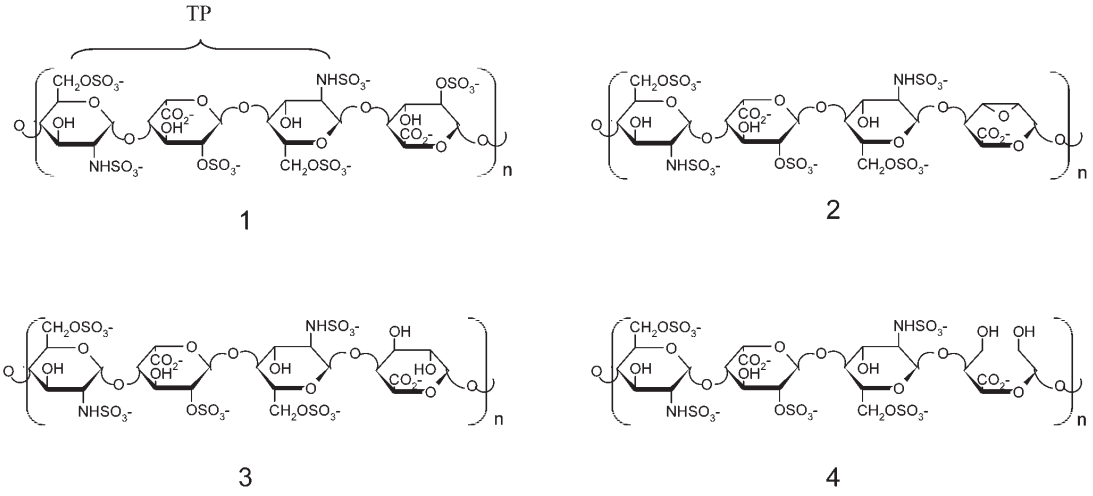

Fig. 5 Prevalent tetrasaccharide units of heparin (**1**), its epoxi derivative **2**, its galacto derivative **3**, and the glycol-split derivative **4** [30].

only weak signals reminiscent of the original, minor NA regions unaffected by the reactions. The structure of **4** was further confirmed by Smith degradation, which cleaved the modified heparin at the level of glycol-split residues and generated the trisaccharide $GlcNSO_36SO_3$–$IdoA2SO_3$–$GlcNSO_36SO_3$–R (where R is the remnant of a glycol-split uronic acid residue) as a major fragment [31].

The 2-*O*-desulfation–glycol-splitting reaction was performed under controlled conditions in order to obtain longer NS sequences separated by glycol-split residues. The prevalent structure of products obtained by splitting a total of about 25, 33, and 50 % uronic acid residues is schematized in Fig. 6, showing that graded modification of heparin yields oligosaccharide sequences of different lengths separated by glycol-split residues.

NMR and molecular mechanics studies on the 50 %-split product (prevalently poly-pentasulfated pentasaccharide, p-PST.sU) confirmed the assumption that glycol-split residues drastically modify the conformation of the heparin chains. As illustrated in Fig. 7 for one of the major conformers of **4** compatible with measured nuclear Overhauser effects (NOEs), the chain of the glycol-split product deviates from the linearity of the heparin helix that can be accommodated in the basic canyon of FGF/FGRF assemblies. It was then expected that, whereas still able to form 1:1 complexes, product **4** (and possibly also some of the heparin derivatives with somewhat longer NS sequences framed between glycol-split residues) is unable to induce formation of higher-order complexes and would accordingly act as an angiogenesis inihibitor.

Indeed, **4** was found to retain the FGF2-binding ability of the original heparin, as shown by its capacity to protect FGF2 from trypsin cleavage and to prevent the formation of HSPS/FGF2/FGFR1 ternary complexes. However, when compared to heparin it showed a reduced capacity to induce FGF2 dimerization, and to favor the interaction of [^{125}I]FGF2 with FGFR1 in HSPG-deficient, FGFR1-transfected CHO cells. Accordingly, it was more effective than heparin in inhibiting the mitogenic activity exerted by FGF2 in cultured endothelial cells. Finally, it inhibited angiogenesis in a chick embryo choroallantoic membrane (CAM) assay in which heparin is inactive. [30]. Such an antiangiogenic activity is retained by corresponding low-molecular-weight derivatives [31]. Although somewhat less active, derivatives prevalently corresponding to poly-OSP.sU (see Fig. 6) also bound FGF2 and inhibited both FGF2-stimulated growth of cultured endothelial cells and capillaries growth in the CAM model [31]. It is worth noting that the presently described derivatization, and especially glycol-splitting, leads to loss of the anticoagulant properties of heparin, which are undesirable owing to hemorrhagic risks. In fact,

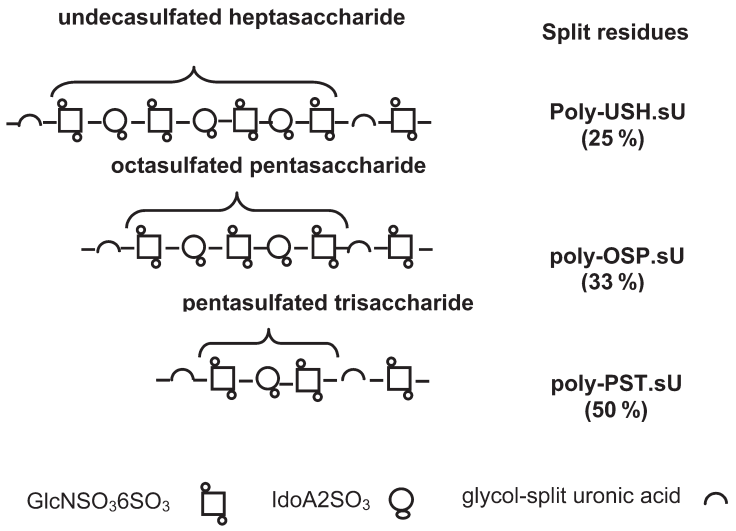

Fig. 6 Schematic representation of prevalent sequences obtained by glycol-splitting of heparin 2-*O*-desulfated to different extents [31].

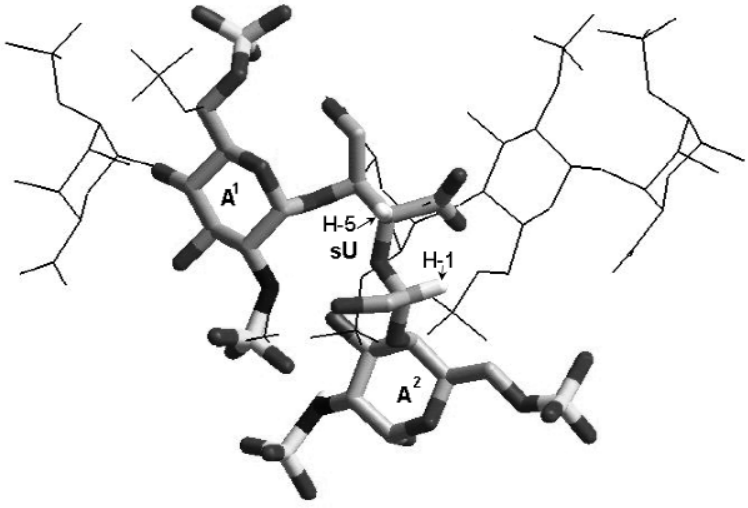

Fig. 7 Molecular model of a trisaccharide segment of p-PST.sU (stick model) compared with unmodified heparin (thin line) [30].

glycol-splitting also involves the GlcA residue of the pentasaccharide sequence of the active site for antithrombin [4] (see Fig. 2).

TARGETING HEPARANASE

Even highly efficient FGF inhibitors may not be sufficient to suppress FGF-induced signaling. It is accordingly desirable to minimize the concentration of growth factors in circulation also by preventing as much as possible their release from HPSPs. In principle, such a release can be prevented by inhibiting heparanase, the β-endoglucosidase that cleaves the HS chains at the level of GlcA residues [32] and

releases FGFs in active form [8]. By preserving the integrity of HSPSs in the extracellular matrix, inhibition of heparanase is expected also to prevent invasion of cancer cells through the connective tissue [7].

X-ray structures of heparanase-substrate complexes are not yet available. However, similarities between the structure of the enzyme and members of several groups of glycosyl hydrolase families have permitted identification of the active-site acid residues and mapping of clusters of basic aminoacids as putative ligand binding domains [33]. HS sequences binding to these clusters most likely belong to the NS (heparin-like) domain of the GAG, and may thus be mimicked by heparin.

Indeed, heparin, as well as *N*-acetyl heparin (i.e., heparin whose *N*-sulfate groups are completely replaced by *N*-acetyl groups) efficiently inhibits heparanase [34,35]. Although different preparations of heparin inhibited the enzyme to significantly different extents [36], suggesting subtle specificities, activities observed for heparin derivatives did not unravel structural features essential for heparanase inhibition. Total substitution of heparin *N*-sulfate groups with *N*-acetyl groups [35] nor complete removal of 2-*O*-sulfate groups [37] were reported to significantly impair the heparanase-inhibition properties of heparin. On the other hand, also extensively sulfated, nonglycosaminoglycan polysaccharides of widely different structures are good heparanase inhibitors [38,39]. Extensive screening of sulfated carbohydrate polymers and their fragments indicated that the enzyme can be strongly inhibited by some persulfated oligosaccharides as small as hexa- and pentasaccharide, such as maltohexaose sulfate and phosphomannopentaose sulfate [38].

Interestingly, reduced oxyheparin (RO-heparin, obtained by glycol-splitting of all the nonsulfated uronic acid residues preexisting in heparin) turned out to be at least as effective an inhibitor of the enzyme as the parent GAG [37]. This observation prompted revisitation of heparins and heparin derivatives as heparanase inhibitors. Whereas removal of 2-*O*-sulfate groups was confirmed to have little effect on the inhibition activity as previously reported [37], the activity increased with increasing content of 6-*O*-sulfate groups [40]. Using a more sensitive test than in previous reports, fully *N*-acetylated heparin was found to be one order of magnitude less active as heparanase inhibitor than heparin. In more detail, while the inhibition activity is barely affected by replacement of about 40 % of the original NSO_3 groups by *N*-acetyl groups, it sharply decreases for higher *N*-acetyl contents, indicating that at least one NSO_3 group per tetrasaccharide unit is needed for binding to (and inhibition of) the enzyme. However, the most dramatic influence of chemical modification on the heparanase-inhibiting activity was observed upon glycol-splitting. As illustrated in Fig. 8, glycol-splitting enhances the activity of *N*-acetylated heparins irrespective of their *N*-acetylation level, the most notable activity increase being observed at about 70 % *N*-acetylation [40]. As earlier observed for lipoprotein lipase [41] and system-

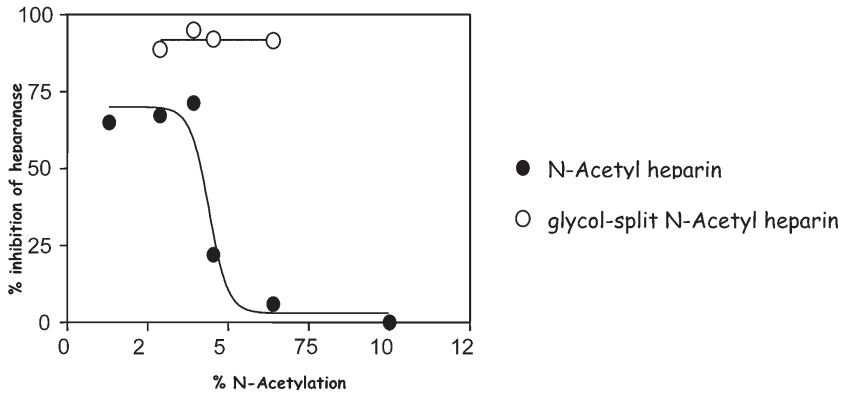

Fig. 8 Heparanase inhibition by heparin at different degrees of *N*-acetylation (●) and of the same products after glycol-splitting of nonsulfated uronic acid residues (○) [40].

atically found for FGF2 in the present studies, the glycol-split residues seem to strengthen the interaction with heparanase by facilitating a deeper penetration of heparin sequences in the active site of the enzyme. However, whereas the strongest inhibition of FGF2 was observed by generating additional glycol-split residues along the heparin chain [30,31], the few glycol-split uronic acid residues of RO–H seem to exert very much the same influence as in the presence of additional glycol-split residues [40].

Further studies will clarify whether the carboxylate group of glycol-split residues are also involved in binding, and whether *N*-acetyl groups participate in the interaction.

CONCLUSIONS AND FURTHER DEVELOPMENTS

The present studies provide remarkable examples of conversion of an anticoagulant, prevalently proangiogenic molecule such as heparin into nonanticoagulant, antiangiogenic derivatives. As expected for products with FGF2- and heparanase-inhibiting activity, some of the described compounds, such as poly-PST.sU and its low-molecular-weight derivatives inhibit angiogenesis also in a number of in vivo tests and are antimetastatic in experimental animal models [42]. They are being further evaluated as potential antitumor drugs. Future studies will be addressed to dissect the contribution of inhibition of FGF2 and heparanase to antiangiogenic and antimetastatic properties. The observation that glycol-splitting enhances both activities does not rule out the possibility of exploiting this strategy to generate arrays of more specific binding sequences spaced by flexible joints, a possibility that will be further explored.

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