Structure and Biological Activity of Heparinoid

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Abstract: Heparin is a biogenic anionic charged sulfated polysaccharide that has a range of desired activities including inhibition of tumor metastasis and inhibition of restenosis. However, its clinical use is limited to treating blood-clotting disorders. Anionic macromolecules called heparinoids have been investigated with the objective of developing heparin-like molecules with reduced anti-coagulant activity and selective anti-metastasis and anti-restenosis activity. This mini-review summarizes the synthesis and biological activity of the main synthetic heparinoids reported in the past three decades.

Keywords: Heparinoid, Heparin, Polyanion, Heparanase inhibitors, Poly(acryl amino acid), FGF release, Heparan sulfate, Anticoagulant.

1. INTRODUCTION

Heparin, a sulfated polysaccharide represents the most commonly used anticoagulant to prevent clotting during cardiac or vascular surgery, whenever blood contacts damaged endothelium in a surgical wound or the synthetic surface of a medical device, the coagulation cascade is activated and a clot is formed, which often require anticoagulation as do extracorporeal therapies, such as heart lung oxygenation and kidney dialysis [1]. Heparin is isolated from either porcine intestine or from beef lung where it is bound to histamine and stored in the mast cell granules. When heparin is isolated, purification leads to a mixture of chains containing sequence of heterogeneity in which, one third of the chains comprising pharmaceutical grade heparin contain a binding site for an important protein in the coagulation cascade called antithrombin III (AT-III) and are termed as high affinity heparin [2]. Like all other polysaccharides, heparin is a polydisperse mixture containing chains having different molecular weights [3] and consists a major trisulfated disaccharide repeating unit (Fig. **1**). It also contains a number of additional disaccharides structures [4, 5], making heparin's structure complex.

Heparin is an acidic polysaccharide with side groups, either sulfates or N-acetyl groups, attached to individual sugar group and is the most highly sulfated member of the glycosaminoglycans family (GAGs) [6]. This involves the α-1, 4-glycosidic linkage of D-glucosamine and D-glucuronic acid to give the disaccharide, and linking the heparin subunits likewise with α-1, 4-glycosidic linkages with one another to form heparin. The position of the sulfo groups may vary; for example, a tetrasaccharide unit contains 4 to 5 sulfate residues. Heparan sulfate (heparitin sulfate) contains fewer O- and N-bonded sulfo groups but also contains N-acetyl groups. Heparin differs from other members of this family in that it is biosynthesized as a proteoglycan (PG) attached to the serglycin protein core, uniquely found intracellularly, in the granules of mast cells. In contrast, heparan sulfate PGs is ubiquitous and found extracellularly in virtually all types of animal tissues [7]. Tissues, from which heparin is prepared, are rich in mast cells but also contain heparan sulfate PGs. All the disaccharide-repeating units found within heparin are also found within heparan sulfate but in different proportions [4, 8]. Their relative proportions of these disaccharides explain why heparin and heparan sulfate differ substantially in their level of sulfation and in their anticoagulant activity [9]. The degree of sulfation and the chain size of the polymer determine the biological activity of heparin [5]. Although heparin has a range of desired activities including inhibition of tumor metastasis and inhibition of restenosis, its clinical use is limited to treating blood-clotting disorders as the anticoagulation activity of heparin may endanger the patient due to the risk of hemorrhage [6].

Heparin can be regarded as an anionic polyelectrolyte and occurs, bound to proteins, specially in the liver and as anticoagulant, prevents coagulation of the blood circulating in the body. Heparan sulfate is found as a constituent of proteoglycans (perlecan) on cell surfaces and in the extracellular matrix of many tissues. Heparin intensifies the inhibitory effect of antithrombin III on thrombin, which blocks the catalysis of the conversion of fibrinogen into fibrin by thrombin, and on various other coagulation factors; for example, the conversion of prothrombin into thrombin is also prevented and breakdown of lipoprotein by lipoprotein lipase is activated.

Heparinoid is a collective term for all substances, which have heparin-like effects and polyanionic in nature. Two major groups of polyanionic substances have been studied during the past three decades. One is polyanionic polysaccharide, both natural and chemically modified [10]. The second group includes synthetic polycarboxylic polymers [11]. These polyanions include pentosan polysulfate, xylan sulfates, dextran sulfates or chitin sulfates, di-, tri-, or oligomers and polymers of iduronic/uronic acids and/or glucosamine, oligo- or polysaccharides composed of

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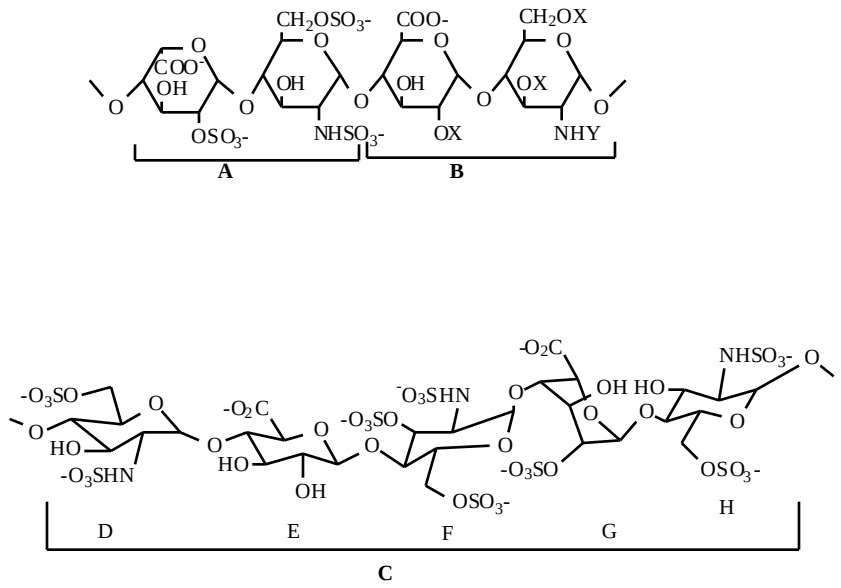


Fig. (1). Structure of heparin: (a) major trisulfated disaccharide repeating unit (X) sulfo or H, (Y) sulfo, Ac or H; (b) undersulfated structural variants; (c) antithrombin III pentasaccharide (DEFGH) binding site (Anionic group in bold are critical (90% loss in binding energy on the removal) and in italic are important (50-60% loss in binding energy on removal) for interaction with ATIII.

pentose and/or hexose units and/or mannitol in random or regular arrangement, heparan sulfates, heparitin sulfates, keratan sulfates or dermatan sulfates, hyaluronic acid, chondroitin sulfate A, B or C, unfractionated heparin and fractionated heparin. Fractionated heparins include enoxaparin, nadroparin (Fraxiparin), dalteparin (Fragmin.RTM.), bemiparin, tinzaparin, ardeparin, low molecular weight heparin (LMWH), and ultra low molecular weight heparin (ULMWH)**.**

STRUCTURE-ACTIVITY RELATIONSHIP

To evaluate the structure activity relationship (SAR), there are three common factors to consider namely: (1) does heparin have defined sequences? (2) Are there defined sequences required for its interaction with specific proteins? (3) With all the proteins that are capable of interacting with heparin, can a truly specific heparin-based drug ever be prepared? The discovery of the ATIII pentasaccharide binding site and the elucidation of its SAR certainly demonstrate that heparin does have defined sequences within its binding domain that can interact with high specificity and high affinity with selected proteins and that this interaction has been exploited in the development of a highly specific anti-factor Xa agent [12].

On the other side of these issues is our seeming inability to completely eliminate "nonspecific" protein interactions from even very well defined heparin oligosaccharides. For example, the ATIII pentasaccharide used as an anti-factor Xa agent can also interact with PF4, causing some undesired side effects [13, 14]. When discussing endogenous activity, however, it is important to remember that heparan sulfate, not heparin, is responsible for normal physiologic roles. Not all heparan sulfates contain highly sulfated domains, such as the repeating trisulfated disaccharide (Fig. **1a**) and the ATIII binding site (Fig. **1c**) commonly associated with protein interactions. These sequences are highly controlled by the selective expression of specific isoforms of the biosynthetic enzymes. Our current lack of understanding of the control mechanisms in biosynthesis severely limits our understanding of specificity. It is clear, however, that expression is spatially (tissue) restricted, temporally (developmentally) restricted, and responsive to environmental signals (pathological insults).

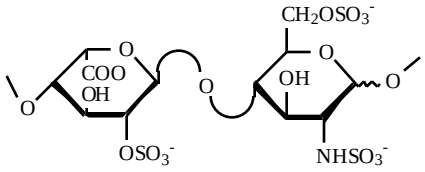
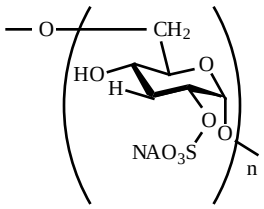
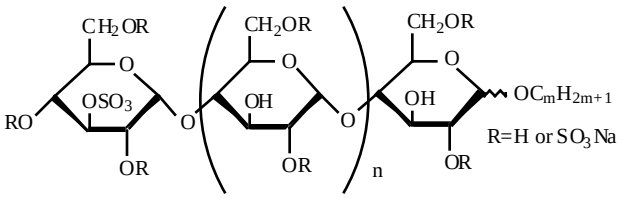
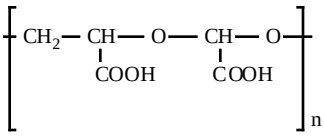
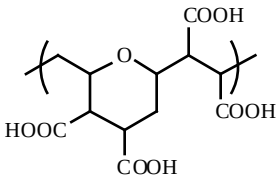
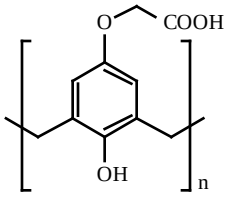
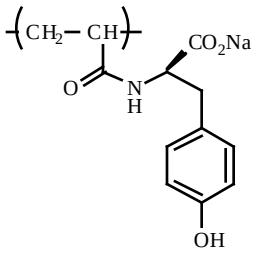
Arguments have been made that the inherent flexibility of the iduronic acid and the flexible positioning of sulfo groups greatly reduce or eliminate heparin's (or heparan sulfate) specificity [2]. These arguments, however, fail to consider the limited spatial and temporal expression of rare sequences in real biological systems. Thus, despite the fact that many proteins can bind to highly sulfated domains, they may not encounter these domains at the time and location where they are present. Finally, many domains within heparan sulfate may be masked *in vivo* by already bound proteins or protein complexes that would need to be released prior to exposing a binding site. This adds yet one more level of specificity and control to these interactions.

Thus, it is clear that there are specific sequences within heparin and heparan sulfate that play specific physiological roles. Furthermore, it is also clear that this specificity is not complete nor it is well understood. Simple *in vitro* assessment of specificity is unlikely to provide a full understanding of specificity. We will certainly need to rely on much more complex biological systems for such an understanding.

SYNTHETIC APPROACH

Sulfation of other polysaccharides such as laminarin yielded very potent new anticoagulants [15, 9, 16]. In the field of synthetic polycarboxylic acids, pyran [11], a copolymer prepared from divinyl ether and maleic anhydride (1:2) is one of the most studied, owing to its very wide

Table 1. Heparinoids Reported in the Literature

Polyanion type	Name	Structure	Biological activity	Reference
Natural polysaccharide	Heparin		Anticoagulant	Casu, B. *Adv. Carbohydr. Chem Biochem.* **1985**, *43*, 51-134.
Chemically Polysulfated polysaccharide	Polysulfated dextran		Anticoagulant	Hatanaka *et al. J. Med. Chem.* **1987**, *30*, 810-814.
Chemically derivatized polysaccharide	Alkyl malto-oligosaccharide		Anti-HIV infection	Katsuraya *et al. Macromolecules* **1995**, *28*, 6697-6700.
Chemically modified polysaccharide	Polyacetal carboxylic acid		Antiviral	Claes, P. *et al. J. Virol.* **1970**, *5*, 313-320.
Synthetic, polymaleic-co-divinyl ether	Pyran		Antitumor antiviral	Ottenbrite R.M. "Biological Activities of Polymers". Carraher, C.E. and Gebelin, C.G. editors. ACS Symposium Series, 186. Washington DC. **1982**.
Synthetic, polyphenol-co-formaldehyde	Poly((4-hydroxy 2-phenoxy) acetic acid-co-formaldehyde)		High b-FGF affinity. SMC proliferation inhibitor	Miao, H.Q. *et al. J. Clin. Invest.* **1997**, *99*, 1565-1575.
Synthetic, poly(N-acryl amino acid)	Poly(N-acryl tyrosine)		Heparanase inhibition. High b-FGF affinity. SMC proliferation inhibitor	Bentolila *et al. J. Med. Chem.* **2000**, *43*, 2591-2600.

biological activity spectrum, including the induction of interferon, antiviral, antibacterial and antifungal activities, stimulation of immune responses, anticoagulant activity, adjuvant disease inhibition and anticancer activity [9]. Galactomannan extracted from *senna macranthera* was sulfated and tested *in vitro* for heparinoid activity [16]. These results suggested that anticoagulant activity of the

sulphated derivative was expressed through binding to AT III and the fraction of sulfated galactomannan, which bind to AT III, showed strong anticoagulant activity. Poly(glucosyloxyethyl methacrylate) sulfate [poly(GEMA) sulfate] and dextran sulfate have been used as heparinoids. Interaction mechanism of heparinoids with fibrinogen was evaluated using surface plasmon resonance technology by Sakamoto *et al*. [17]. The binding ability of each sulfated polymer was estimated by having each polymer-containing buffer interact with the sensor chip surfaces that had immobilized fibrinogen. Dextran sulfate and poly(GEMA) sulfate showed high affinity to the fibrinogen, while the heparin did not. All of the dextran sulfates were desorbed from its surface, while about 30% of the poly(GEMA) sulfate remained on the immobilized fibrinogen upon the addition of NaCl to the buffer which was done in order to analyze the desorption of poly-γ-sulfate or dextran sulfate from the surface of the fibrinogen. Results indicated that the type of binding between fibrinogen-poly-γ-sulfate was different from that of dextran sulfate, which was caused not only by an electrostatic but also by a hydrophobic force and different from that of heparin.

Ottenbrite [18] studied the effect of different molecular weight fractions of the polymers pyran, poly(acrylic acid-co-maleic anhydride), polymaleic acid and poly(acrylic acid-co-3, 6-endoxo-1,2,3,6-tetrahydrophtalic acid) on their activity against Lewis lung carcinoma and encephalomyocarditis virus, in mice. Polymers between 30 and 50kDa retained their activities almost completely, while their toxicities decreased considerably compared with those ofunfractionated polymers. All of the polymers that were tested, pyran showed the most potent activity. It is believed that the antiviral activity is exerted by the longer permanence of higher molecular weight fractions in body fluids before excretion, since low molecular weight fractions (<30kDa) were inactive. In the optimal condition, the polymer should be of sufficient molecular weight to delay body clearance, but smaller than the kidney threshold (40kDa) to avoid accumulation.

There are some other families of synthetic heparinoids namely Poly(N-acryl amino acids) derivatives [19-21]. These polymers have been evaluated for their heparanase activity, SMC proliferation and b-FGF release from ECM by changing their functional group, for example, -NH_2, -COOH, -OH, -SH and phenolic group and their positions on the monomers. The polymer conformation is also important for determining its biological activity. Muck *et al*. [22] reported that isotactic polyacrylic acids are more effective than atactic polyacrylic acids in providing protection against picorna virus infection *in vivo*. The optimal activity was reached at molecular weights ranging from 6 to 15kDa. Bentolila *et al*. [20-22] suggested that macromolecules based on N-acryl - L-amino acids bearing hydrophobic or charged side groups, such as -NH_2, -COOH, -SH, -OH and phenols, arranged into a configuration determined by the chirality of the amino acid α-carbon, may express heparin-like biological activities. The use of pendent amino acids along an acrylate polymer provides specific distribution of charge and of polar and hydrophobic groups arranged in a topology determined by the chirality of the amino acids.

Homo and copolymers of poly(N-acryl amino acids) with different charge densities, nature of the amino acid side group, stereoselectivity and polymeric backbone were tested for their activity as anticoagulants, heparanase inhibition agents, and to basic fibroblast growth factor (b-FGF) release bound to the extracellular matrix (ECM). Some polymers were active either as heparanase inhibitors and/or as b-FGF release agents, none of the polymers show any anticoagulant activity. Polymers bearing hydrophilic side chains that inhibited heparanase, i.e., hydroxyproline, glycine and serine, did not release b-FGF from ECM. The absence of high acidic sulfate-ester groups existing in heparin (hydrophilic) must be compensated by some kind of lipophilic interactions between the polyanion and b-FGF in order to effectively compete with heparan sulfate proteoglycanes, causing its release from ECM. Heparanase inhibitors may have clinical applications in preventing tumor metastasis and inflammatory/autoimmune processes due to the involvement of this enzyme in the extravasation of blood-borne tumor cells and activated cells of the immune system. Molecules that release ECM- bound b-FGF may be applied to accelerate neovascularization and tissue repair [20, 21].

Fibroblast growth factors are a family of structurally related polypeptides that show high affinity to heparin [23]. The dissociation constant of sulfated heparin octasachharide (HS-8) with bFGF have been reported as 4.42 μM [24]. They are highly mitogenic for a variety of cell types and their release from the subendothelial ECM may induce the mesenchyme and new blood vessels formation. They are bound to heparan sulfate proteoglycans in the ECM and cell surfaces, exhibiting a high degree of stability toward proteolytic enzymes and can be released in an active form by heparinoids and heparan sulfate degrading enzymes. Heparanase is an endo-D-glucuronidase that degradesheparan sulfate side chains in basement membranes and ECM. This activity facilitates cell invasion in normal and pathological situations. High levels of heparanase were detected in cells of hematopoietic origin such as neutrophils and inflammatory macrophages and lymphocytes that must invade the blood vessel wall to express their physiological function. Thus, heparanase plays an important role in pathological processes involving cell migration such as tumor metastasis, inflammation and autoimmunity. Polyanionic molecules and some heparinoids are potent inhibitors of this enzyme.

Benezra *et al*. [25] have synthesized a series of nine synthetic polyaromatic compounds by polymerization of aromatic ring monomers with formaldehyde, which yield substantially ordered backbones with different functional anionic groups (hydroxyl and carboxyl) on the phenol ring. They demonstrated that compounds that have two hydroxyl groups para and ortho to the carboxylic group and a carboxylic group at a distance of two carbons from the phenol ring inhibit heparanase activity, inhibit SMC proliferation, and induce an almost complete release of bFGF from ECM [25]. Addition of a methyl group next to the carboxylic group led to a preferential inhibition ofheparanase activity. Similar results were obtained with a compound that contains one hydroxyl group para to the carboxylic group and an ether group near the carboxylic group on the phenol ring. Preferential inhibition of SMC proliferation was best achieved when the position of the hydroxyl group is para and ortho to the carboxylic group and the carboxylic group is at a distance of one carbon from the phenol ring. On the other

hand, for maximal release of bFGF from ECM, the position of the carboxylic group should be three carbons away from the phenol ring. Benezra *et al*. [25] have suggested that these new heparin-mimicking compounds may have a potential use in inhibition of tumor metastasis, arteriosclerosis, and inflammation.

DETERMINATION OF BIOLOGICAL ACTIVITY OF HEPARINOIDS

Synthetic heparinoids have been tested for their heparin-mimicking activity: (1) inhibition of heparanase activity; (2) inhibition of SMC proliferation; and (3) release of bFGF from the ECM.

The ability of cells to degrade heparan sulphate (HS) in the extracellular matrix (ECM) was studied by allowing cells to interact with a naturally-produced sulfate labeled ECM, followed by gel filtration (Sepharose 6B) analysis of degradation products released into the culture medium [26]. Expression of a HS degrading endoglucuronidase (heparanase) was found to correlate with the metastatic potential of mouse lymphoma [26], fibrosarcoma and melanoma [26] and with the ability of activated cells of the immune system to leave the circulation and elicit both inflammatory and autoimmune responses [26, 28]. Several studies have shown that heparanase activity expressed by either normal or neoplastic cells can be effectively inhibited by heparin, modified nonanticoagulant species of heparin, and other sulfated polysaccharides [26, 27].

The effect of the polymers on heparanase activity was tested using $[^{35}S]O_4^{2-}$ labeled ECM produced by cultured endothelial cells. Incubation of partially purified human placental heparanase with this ECM releases into the medium high- and low molecular weight sulfate-labeled degradation products. The high molecular weight material is composed primarily of nearly intact heparan sulfate proteoglycans, serving as a readily accessible heparanase substrate. In the absence of heparanase inhibitors, low-molecular-weight sulfate-labeled degradation fragments of heparan sulfate are released into the incubation medium (Fig. **2**, peak II). Heparin and N-substituted non-anticoagulant species of low-molecular-weight heparin are inhibitors of heparanase, preventing the degradation of heparan sulfate. All polymers were assayed for heparanase inhibition activity (Fig. **3**, Table **1**). Poly(*N*-acrylates) of glycine (**4d**), tyrosine (**4g**), glutamic acid (**4l**), leucine (**4e**), *tert*-leucine (**4f**), and sulfated hydroxyproline (**7a**) yielded 70-80% inhibition of heparanase activity already at a concentration of 1 μg/mL, similar to heparin. The most effective compound was poly(*N*-acrylleucine) (**4e**) (Fig. **3**) [19-21].

Polymers containing *trans*-hydroxyproline (**4a**) (Fig. **2**), proline (**4c**), and phenylalanine (**4h**) inhibited the enzyme only at 5 μg/mL. Polyanions bearing *cis*-hydroxyproline (**4b**) (Fig. **1**), histidine (**4i**), and lysine (**4k**) were inactive. The corresponding decarboxylated poly(acryl amino acids) was inactive even at 25 μg/mL.

The pathogenesis of atherosclerosis involves abnormal migration and proliferation of SMCs that become infiltrated with macrophages and embedded in ECM [29]. Following endothelial cell perturbation due to the activation of atherogenic risk factors, platelets and non-platelet-derived growth factors and cytokines (e.g., PDGF, bFGF, IL-1, thrombin) are released and stimulate monocyte and SMC migration as well as SMC proliferation [29]. Molecules that interfere with the growth-promoting activity of these growth factors may attenuate progression of the atherogenic process.

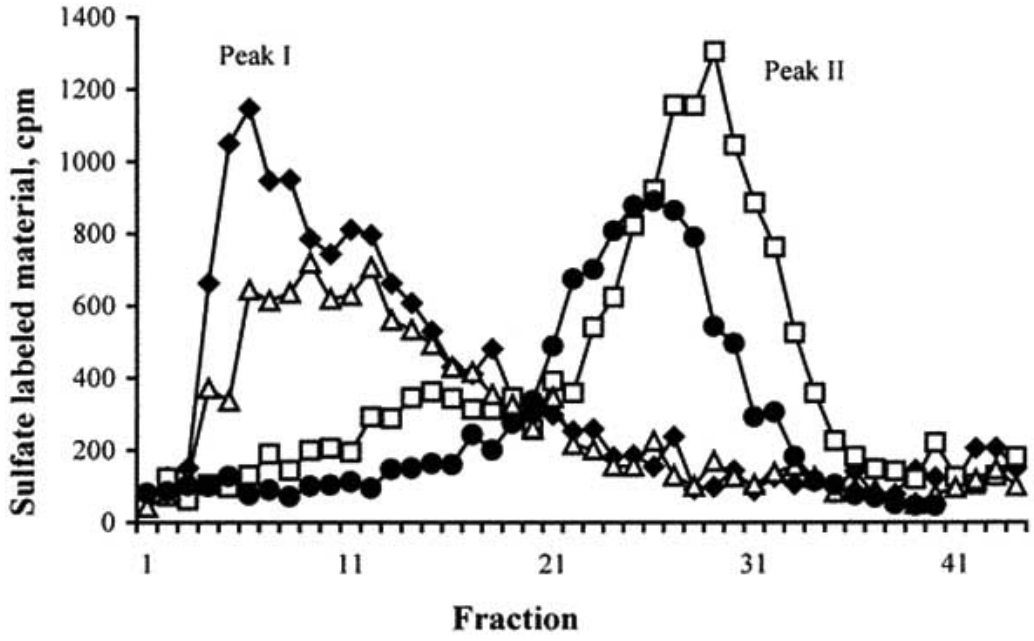


Fig. (2). Effect of heparin and polyanions on heparanase activity. Sulfate-labeled ECM-coated 35-mm dishes were incubated (37 °C, 48 h, pH 6.2) with human placental heparanase in the absence (control) (□) and presence of 5 μg/mL heparin (♦), poly(*N*-acryl-*trans*-4-hydroxy-L-proline) (Δ), or poly(*N*-acryl-*cis*-4-hydroxy-L-proline) (·). Sulfate-labeled material released into the incubation medium was analyzed by gel filtration over Sepharose 6B columns. Heparanase activity is represented by the amount of radioactivity eluted in peak II (fractions 23-35). (adopted from Ref. 18).

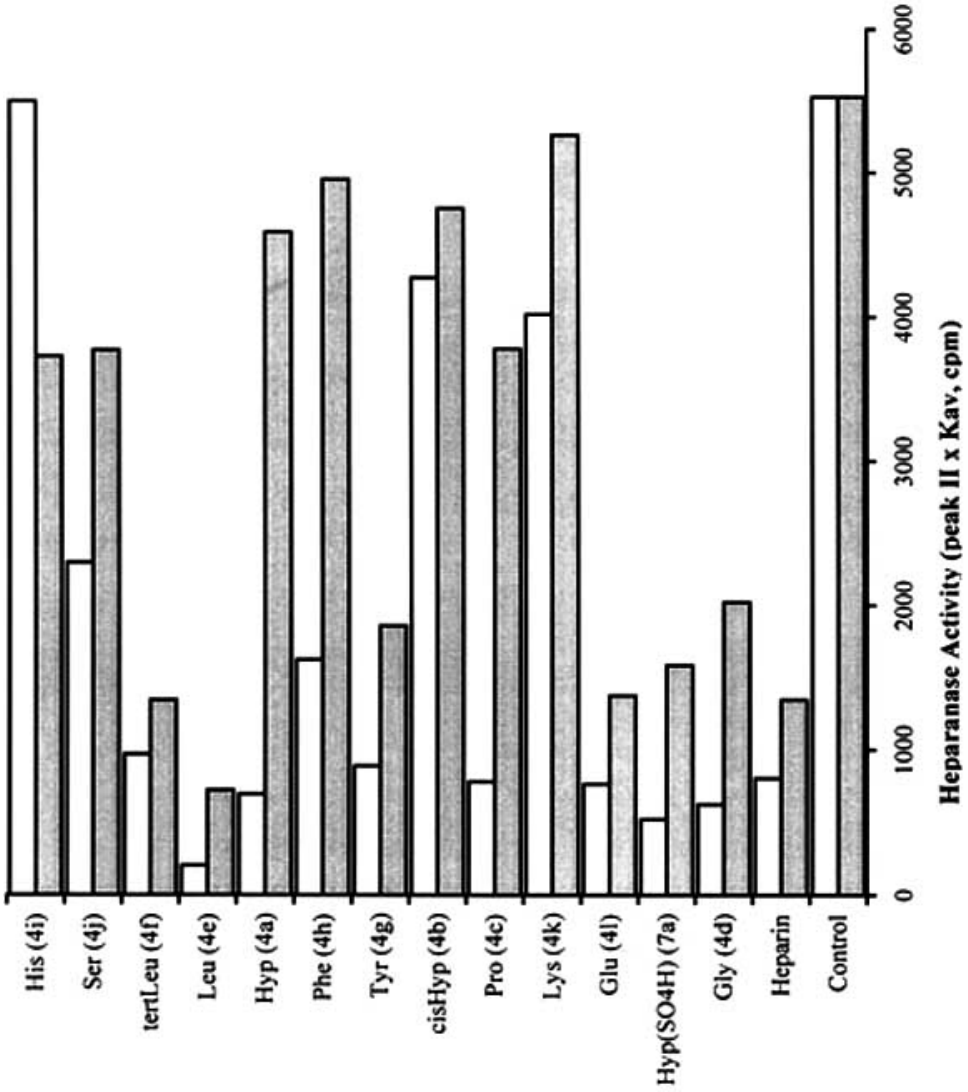


Fig. (3). Inhibition of heparanase by poly(*N*-acryl amino acids). Sulfate-labeled ECM-coated 35-mm dishes were incubated (37 °C, 48 h, pH 6.2) with human placental heparanase in the absence (control) and presence of 1 μ*g*/mL (gray bars) or 5 μ*g*/mL (white bars) polyanionic compounds. Sulfate-labeled material released into the incubation medium was analyzed by gel filtration over Sepharose 6B columns. Heparanase activity is expressed by the amount of radioactivity eluted in peak II (sum of cpm in the highest nine fractions of peak II) multiplied by the K_{av} of this peak (adopted from Ref. 18).

Among these interfering molecules are species of heparin [30-32], and HS [32] that inhibit proliferation of vascular SMCs both *in vitro* and *in vivo*. This may be due to the fact that bFGF and PDGF (the major mitogen in serum) are more potent growth promoting factors for SMCs and heparin derivatives bind these factors and inhibit the proliferation of vascular SMCs.

Proteoglycans have been found to function as the principal binders of growth factors, and cytokines and more than 20 known heparin-binding proteins contain a consensus structural motif that participates in this binding [33-35, 31]. Among these growth factors is bFGF, which was identified as a complex with HS proteoglycans (HSPG) in the subendothelial ECM produced *in vitro* [34], and in basement membranes of diverse tissues and blood vessels [34]. Members of the FGF family exhibit a high affinity to heparin, are highly mitogenic for mesoderm and neuroectoderm-derived cells, and are among the most potent inducers of neovascularization and mesenchyme formation [34, 36]. Release of bFGF from its storage in ECM by heparin-like molecules, HS-degrading enzymes (i.e., heparanase) [26, 37], or proteases (i.e., plasmin) was suggested to elicit a localized neovascularization in processes such as wound healing, inflammation, and tumor development. These studies were undertaken to define the structural requirements for induction of the above-described activities and design the most appropriate ‘‘heparin-mimicking’’ compound for clinical indications.

SUMMARY AND COMMENTS

This mini-review summarizes the trends in heparinoid research. The main motivation for the development of new heparinoids has been the need for heparin like materials that

are more specific than heparin with respect to its potential use for treating metastasis and diseases that are related to the functions that heparin presents such as control of cell proliferation. Currently heparin is used as anticoagulating agent, which is also the main limitation of its use for other indications. The field has been dominated by several groups with expertise in polymer chemistry in collaboration with researchers experienced with heparin research. Most of the work was conducted throughout the past 30 years with a decline in the past 2-3 years.

Although the outcome so far did not result a suitable molecule to be considered for clinical use, efforts should continue, as there is a great opportunity to identify an antimethastatic agent for treating cancer at the advanced stage. Despite the extensive work on identifying an antimethastatic agent such drug does not exist. Several of the compounds described in this review showed promising results, which should be investigated as potential anticancer drugs with high specificity and low toxicity.

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