

Chemistry and Biology of Heparin and Heparan Sulfate  
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## Chapter 18

### Influence of Heparin Chemical Modifications on its Antiproliferative Properties

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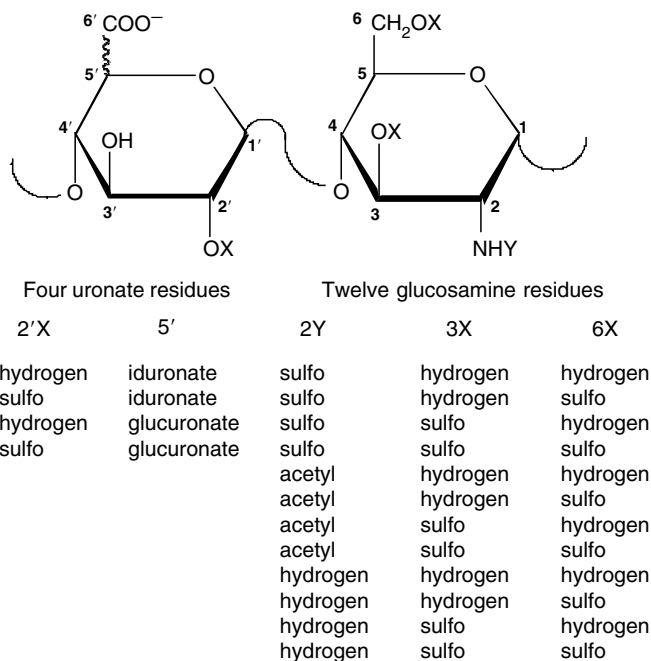
#### I. Introduction

Pulmonary hypertension due to chronic hypoxia is associated with increased quantities of smooth muscle cells (SMCs) in pulmonary arteries, due to thickening of the medial muscle layer in the proximal arteries and extension of the smooth muscle investment into preripheral, normally nonmuscular vessels (1–4). A number of factors are known to cause SMCs migration, such as serum, platelet-derived growth factor (PDGF)-BB (5,6), transforming growth factor  $\beta$  (7), fibrinogen (8), oxidized low-density lipoprotein (9,10), and angiotensin II (11,12). Heparin inhibits hypoxic pulmonary hypertension, possibly by an antiproliferative effect on SMCs (13–15).

Heparin consists of alternating residues of a uronic acid (either  $\beta$ -D-glucuronic acid or  $\alpha$ -L-iduronic acid) with a hexosamine ( $\alpha$ -D-glucosamine) linked by 1  $\rightarrow$  4 glycosidic linkage and covalently bound to serine residues of the core protein. It has various *O*-sulfo, *N*-sulfo, and *N*-acetyl substituents that are usually heterogeneously distributed along the glycosaminoglycan (GAG) chains.

The possible 48 repeating disaccharide sequences in heparin are given in Fig. 1. Many but not all of these sequences have been reported to date (16).

The most common repeating disaccharide sequence ( $70 \pm 16\%$ ) occurring in heparin is the trisulfated disaccharide with a sulfonate group at position 2 of the



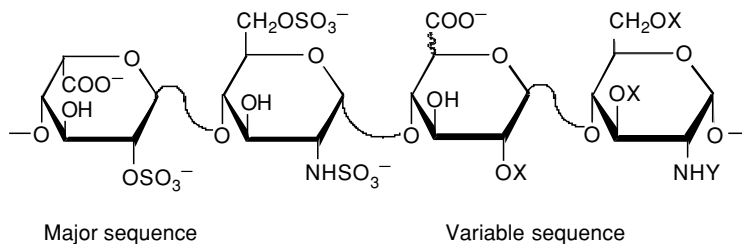
**Figure 1** The possible 48 disaccharide sequences in heparin.

uronate residue and at positions 2 and 6 of the glucosamine residue (17). This structure is shown in Fig. 2 as the major sequence.

However, a number of structural variations of repeating disaccharide units have been observed, leading to the microheterogeneity of heparin (Fig. 2, variable sequence). The amino group at position 2 of the glucosamine residue may be substituted with an acetyl, or sulfo group or unsubstituted. The 3- and 6-positions of the glucosamine residues can either be substituted with an *O*-sulfo group or unsubstituted. The uronic acid, which can either be *L*-iduronic or *D*-glucuronic acid may also contain a 2-*O*-sulfo group (18). An average negative charge in the heparin macromolecule is approximately  $-75$ , (i.e., due to  $\text{SO}_3^-$  and  $\text{COO}^-$ ) (18).

## II. Background and Significance of Chemical Modification of Heparin

Chronic pulmonary hypertension is characterized by structural changes in the pulmonary vasculature, which along with variable degrees of vasoconstriction are responsible for the high pulmonary vascular resistance and associated right heart failure (19,20). The vascular structural changes associated with exposure to chronic hypoxia have been most extensively studied and are characterized by hyperplasia, hypertrophy, and migration of vascular smooth SMC in the media of muscular and



**Figure 2** Major and variable sequence disaccharide repeating units in heparin ( $X = \text{H}$  or  $\text{SO}_3^-$  and  $Y = \text{Ac}[\text{CH}_3\text{CO}]$  or  $\text{SO}_3^-$  or  $\text{H}$ ). AQ6

partially muscular pulmonary arteries (19,20). To develop a potential therapeutic agent to reverse vascular remodeling, which occurs in pulmonary hypertension, we and others have examined the antiproliferative activity of heparin including chemically modified heparin derivatives.

### III. Mechanisms Contributing to Heparin Inhibition of Smooth Muscle Cell Growth

Several laboratories have demonstrated that heparin inhibits cell proliferation (21–28). Although much attention has been focused on the factors that stimulate smooth muscle cell (SMC) proliferation (29), very little is known about the mechanisms maintaining these cells in a quiescent state or about the reestablishment of a quiescent state after their proliferative response has been initiated.

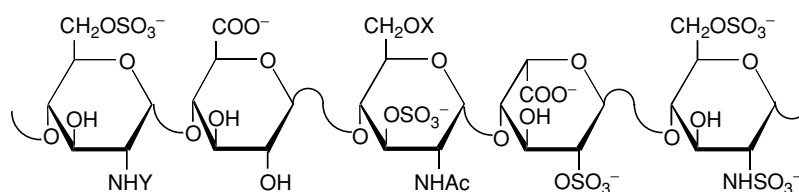
Circulating HP binds to endothelial cells and is taken up by the reticuloendothelial system where it enters a cellular pool to be released at a later stage (30). Furthermore, HP binds to specific binding sites on SMCs and is internalized (24). Some antiproliferative effects are mediated by specific binding, although it is not clear whether internalization is essential. HP blocks the cell cycle at either the  $G_0/G_1$  transition point (22) or at mid to late  $G_1$  progression (24,27,31) and may inhibit such cellular intermediate processes as protein kinase C activation, c-Fos and c-Myc induction (32,33), activator protein-1/Fos-Jun binding activity, and posttranslational modification of Jun B (34–36). HP has also been shown to selectively block the protein kinase C pathway of mitogenic signaling (37) and the phosphorylation of mitogen-activated protein kinase (38). However, these mechanisms responsible for the antiproliferative effects of heparin are not very well understood.

We have demonstrated that PSMC mitogens, such as platelet-derived growth factor and epidermal growth factor, act through the  $\text{Na}^+/\text{H}^+$  antiporter by stimulating a one-for-one exchange of extracellular  $\text{Na}^+$  for intracellular  $\text{H}^+$  to cause intracellular alkalinization, a permissive first step for cell division (39). Dahlberg et al. (40) have demonstrated that antiproliferative HPs block  $\text{Na}^+/\text{H}^+$  exchange in a manner directly related to antiproliferative activity.

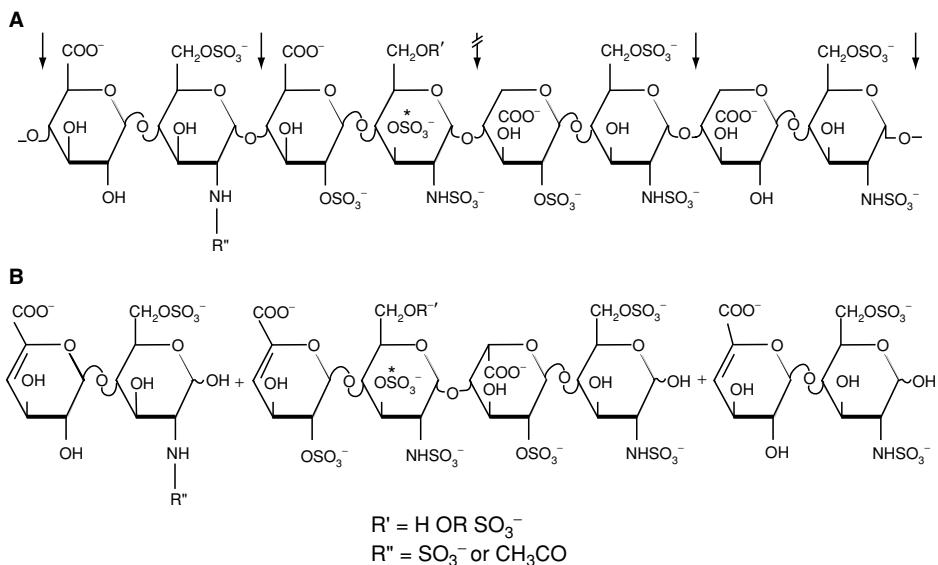
#### IV. Importance of 3-O-Sulfo Group on the Internal Glucosamine Residue of Pentasaccharide for Antiproliferative Activity

Using a synthetically prepared pentasaccharide (Fig. 3), Castellot et al. (25) presented evidence that the 3-O-sulfonate on the internal glucosamine is critical for antiproliferative capacity of the pentasaccharide.

To evaluate whether 3-O-sulfo group containing glucosamine residues in whole commercial HPs are essential for native HP's antiproliferative effect on pulmonary artery smooth muscle cells, we treated three commercial available HPs of varying potency with heparinases I and II (41). These enzymes degrade heparin fragments containing 3-O-sulfo groups to unsaturated  $\Delta$ -tetrasaccharides only (Fig. 4). The above study clearly demonstrated that the 3-O-sulfo group of



**Figure 3** Antiproliferative pentasaccharide demonstrating the structure critical for growth inhibition (10).



**Figure 4** (A) Heparin sequences with different sulfation patterns arbitrarily assigned, and (B) cleavage pattern of heparin containing 3-O-sulfated glucosamine residue by heparinases I and II.

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glucosamine residues is not critical in whole heparin for antiproliferative activity as the most potent HP preparation generated the least amount of  $\Delta$ -tetrasaccharide (Table 1). Different heparin preparations also differ in the contents of the released mono-, di-, and tri-sulfated  $\Delta$ -disaccharides as shown in Table 2.

## V. Minimal Heparin Oligosaccharide Size Necessary for Antiproliferative Activity

Wright et al. prepared oligosaccharides of defined length by nitrous acid cleavage of heparin followed by gel filtration and tested them for their antiproliferative potency in three cell types (42). They reported that the size requirement is similar but not identical for different cell types. Decasaccharides have the same antiproliferative activity as native heparin. Experiments with rat and calf vascular smooth muscle cells growth inhibition showed that dodecasaccharide and larger fragments (Table 3) are essential for the growth inhibitory effect (43). To evaluate the minimum oligosaccharide size necessary to retain full antiproliferative activity, a commercially available heparin preparation under mild enzymatic conditions was cleaved to give oligosaccharides of different sizes. The structure of these oligosaccharides is given in Fig. 5 (44).

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The above oligosaccharides (Fig. 5) were assayed for their antiproliferative activities against bovine pulmonary artery smooth muscle cells and the results are summarized in Fig. 6.

**Table 1** Unsaturated  $\Delta$ -Tetrasaccharide Released after Heparinases I and II Treatments (17)

No.	Heparin preparation	$\Delta$ -Tetrasaccharide released (%)
1	Upjohn #1438	1.7
2	Choay #IC86-1772	17.3
3	Elkins-Sinn #26390	1.9

**Table 2** Disaccharide Composition of Heparins from Different Manufacturers<sup>a</sup>

Disaccharide	X <sup>6</sup>	Y <sup>2</sup>	X <sup>2'</sup>	Upjohn	Elkins-Sinn	Choay
1	H	Ac	H	0.9	3.9	14.5
2	H	SO <sub>3</sub> <sup>-</sup>	H	0.3	2.0	–
3	SO <sub>3</sub>	Ac	H	–	3.9	–
4	H	Ac	SO <sub>3</sub>	–	1.7	–
5	SO <sub>3</sub>	SO <sub>3</sub>	II	5.4	11.5	–
6	H	SO <sub>3</sub>	SO <sub>3</sub>	4.8	6.3	18.0
7	SO <sub>3</sub>	Ac	SO <sub>3</sub>	0.4	1.5	1.5
8	SO <sub>3</sub>	SO <sub>3</sub>	SO <sub>3</sub>	86.8	66.3	66.0

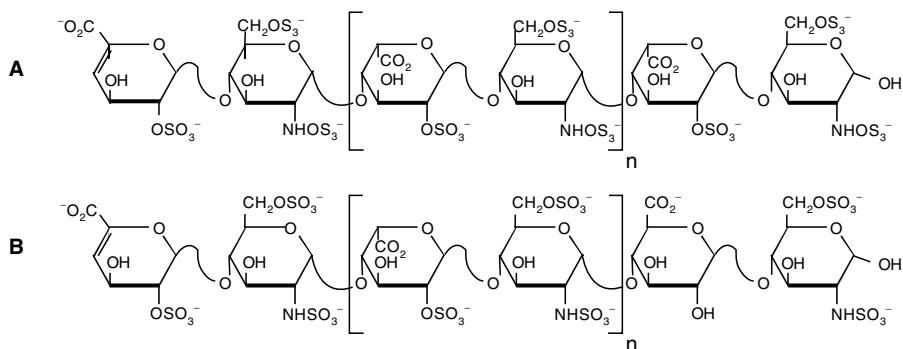
<sup>a</sup>Disaccharides were released by treating exhaustively with heparin lyase I and III. Results are expressed as percentage of total disaccharide released (17).

**Table 3** Comparison of the Antiproliferative Activity and Degree of Sulfation of Heparin Fragments (43)

	Antiproliferative activity <sup>a</sup>		Sulfation degree
	1 µg/ml	100 µg/ml	
Native heparin	36	75	2.7
Disaccharide	0	4	1.8
Tetrasaccharide	2	12	2.3
Hexasaccharide	21	46	2.7
Octasaccharide	29	65	2.7
Decasaccharide	40	71	2.7
Dodecasaccharide	43	82	2.7
~20 saccharide	42	80	2.7

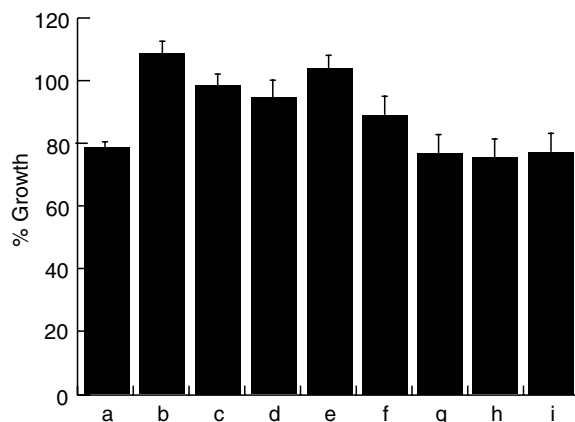
<sup>a</sup>Percent inhibition of rat aortic smooth muscle cells grown in media containing fetal bovine serum (FBS) plus heparin fragment.

<sup>b</sup>Moles sulfate/mole glucosamine (approximate).



**Figure 5** Structure of heparin-derived oligosaccharides. Shown is a fully sulfated structure (A) corresponding to tetrasaccharide (1), decasaccharide (3), dodecasaccharide (7), and the major component in the purified tetradecasaccharide (8), hexadecasaccharide (9), and octadecasaccharide (10) fractions, where  $n = 0, 3, 4, 5, 6,$  and  $7$ . Also shown is undersulfated structure (B) corresponding to decasaccharide (2) and dodecasaccharide (4), where  $n = 3$  and  $4$ , respectively.

These results suggest that the 14-mer (Fig. 5a,  $n = 5$ ) and above, 16- and 18-mers (Fig. 5a,  $n = 6$  and  $7$ ), have as full antiproliferative potency as native heparin. This difference in the size of the oligomer required for antiproliferative activity suggests the origin of vascular smooth muscle cells (i.e., pulmonary or aortic) is important to determine the size of the oligomer.

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**Figure 6** Percent growth of bovine pulmonary artery smooth muscle cells grown in media containing 10% fetal bovine serum (FBS) plus: Column a, native heparin; Column b, 4-mer (Fig. 5a,  $n = 0$ ); Column c, 10-mer (undersulfated) (Fig. 5b,  $n = 3$ ); Column d, 10-mer (Fig. 5a,  $n = 3$ ); Column e, 12-mer (undersulfated) (Fig. 5b,  $n = 4$ ); Column f, 12-mer (undersulfated) (Fig. 5b,  $n = 4$ ); Column g, 14-mer (Fig. 5a,  $n = 5$ ); Column h, 16-mer (Fig. 5a,  $n = 6$ ); and Column i, 18-mer (Fig. 5a,  $n = 6$  and 7).

## VI. Effect of Chemical Modification of Heparin on Its Antiproliferative Activity

To establish which domain of the heparin is especially responsible for antiproliferative activity, the following strategies have been adopted: (a) chemical modification; (b) fractionation of heparin molecule; and (c) preparation of oligosaccharides with a defined chemical structure. The present chapter includes the effects of heparin modifications on the antiproliferative activity.

### A. *N*-Desulfonated, re-*N*-Sulfonated and re-*N*-Acetylated Heparin Derivatives

Castellot et al. (43) examined the antiproliferative activity of chemically modified heparins with native heparin (45). Comparison of the antiproliferative activity and degree of sulfation of chemically modified heparins are summarized in Table 4. All these modifications retained a high degree of antiproliferative activity. Wright et al. (42) found that the presence of 2-*O*-sulfo glucuronic acid was not required for antiproliferative activity.

Tiozzo et al. (46) also determined the role of *N*-linked and *O*-linked sulfo groups on the antiproliferative effect in two different cell types; BHK-21 (hamster fibroblasts) and human arterial smooth muscle cells. In the case of 2-*O*-desulfonated heparins the antiproliferative activity decreased compared to unfractionated

**Table 4** Comparison of the Antiproliferative Activity Against Rat Aorta Smooth Muscle Cells and Degree of Sulfation of Chemically Modified Heparins (43)

	Antiproliferative activity <sup>a</sup>		Sulfation degree <sup>b</sup>	
	1 µg/ml	100 µg/ml	N-sulfo groups	Total sulfo groups
Native heparin	36	75	0.96	2.69
<i>N</i> -desulfonated	10	18	0	1.43
<i>N</i> -desulfonated → <i>N</i> -resulfonated	41	82	0.92	2.31
<i>N</i> -desulfonated → <i>N</i> -reacetylated	24	64	0	1.03
Totally desulfonated	7	10	0	0
Totally desulfonated → <i>N</i> -resulfonated	15	26	0.96	1.03
Totally desulfonated → <i>N</i> -reacetylated	3	12	0	

<sup>a</sup>Per cent inhibition in rat aortic smooth muscle cells grown in media containing fetal bovine serum (FBS) plus individual heparin preparation. Essentially identical results were obtained with calf aortic smooth muscle cells.

<sup>b</sup>Moles SO<sub>4</sub><sup>2-</sup>/moles glucosamine.

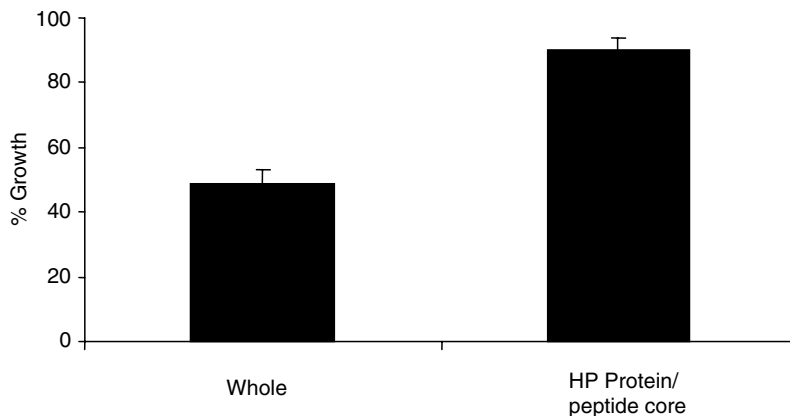
heparin from intestinal mucosa. Although *N*-desulfonation also reduced antiproliferative potency of heparin, the relationship between the degree of sulfonation and the inhibition of cell growth forward is less straightforward. In contrast, these studies suggest that the negative charge, particularly *O*-sulfo group content is important in determining the antiproliferative potency.

## B. Influence of Protein/Peptide Core and Glycosaminoglycan Chains of Heparin

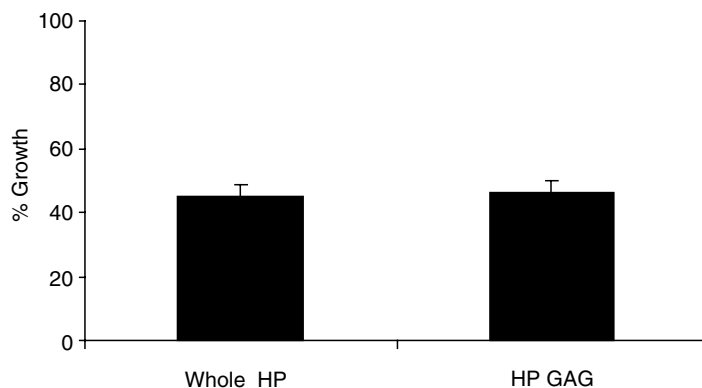
The core protein/peptide of heparin was isolated by digesting with heparinases I and II in sodium acetate buffer (47). The reaction mixture was dialyzed against water and lyophilized to yield core protein/peptide. This process removed the GAG chains, leaving the protein/peptide core with linkage region intact. The released protein/peptide core significantly lost the antiproliferative activity of bovine pulmonary smooth muscle cells in comparison to whole heparin  $88.3 \pm 2.8$  vs  $48.8 \pm 1.9$ , respectively, (Fig. 7) (48).

Glycosaminoglycan chains of heparin were liberated with alkaline borohydride treatment (49). This procedure cleaved the carbohydrate-protein of heparin of HP GAG chains by converting the xylose residue linked with Ser/Thr into xylatol thereby cleaving and degrading the protein core. These peptides were removed by dialyzing against water, leaving the GAG chains. The GAG chains released have similar antiproliferative activity ( $45.5 \pm 3.0\%$ ) as the parent heparin ( $44.6 \pm 2.7\%$ ) (Fig. 8) (48).



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**Figure 7** Percent growth in bovine pulmonary artery SMC grown in media containing 10% FBS plus native heparin or protein/peptide core (10 mg/ml).

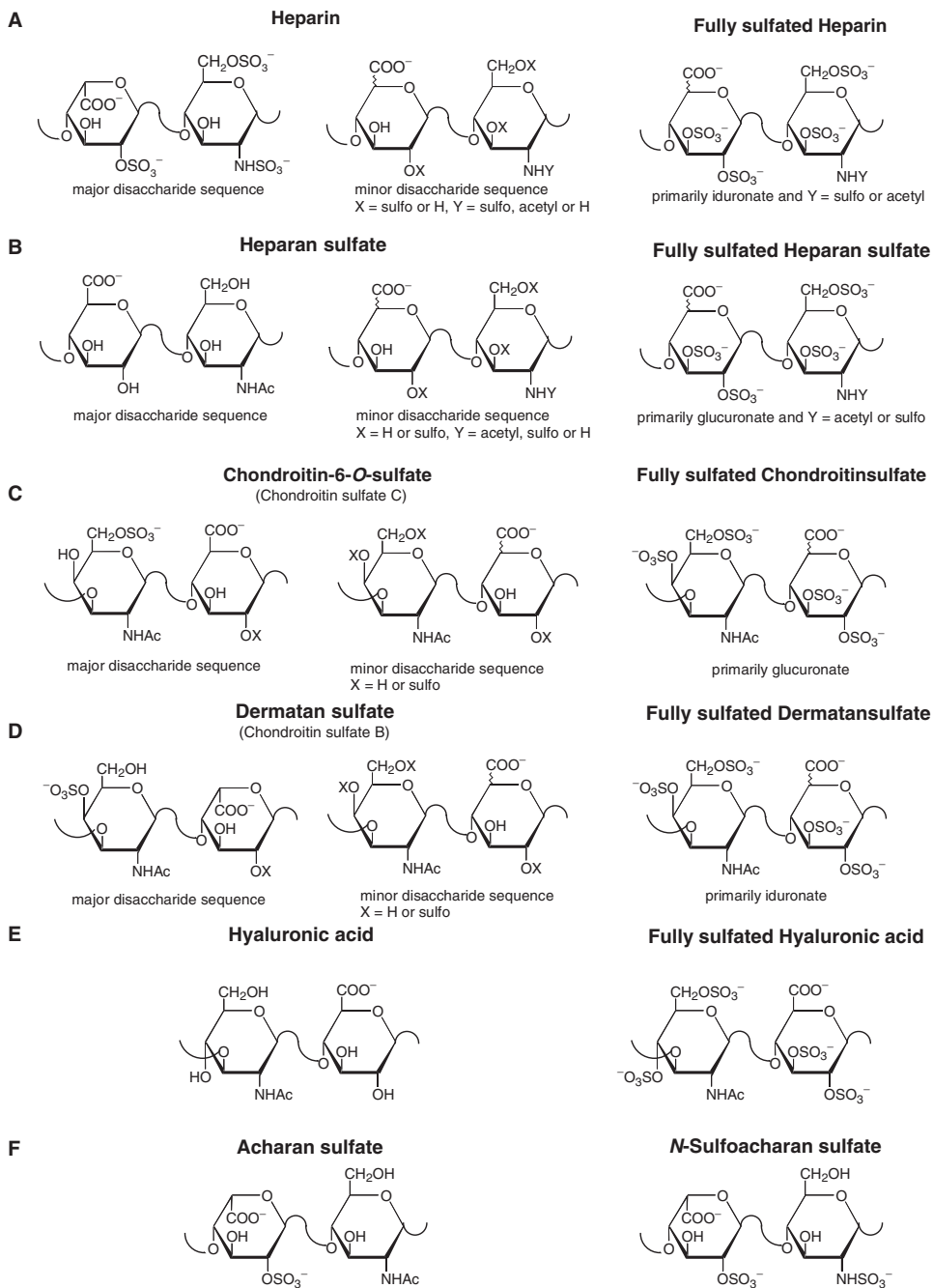


**Figure 8** Percent growth in bovine pulmonary artery SMC in media containing 10% FBS plus native heparin or GAG chains (10 mg/ml).

### C. Influence of Full sulfation of Heparin and Other Glycosaminoglycans

Fully sulfated heparin and other GAGS (Fig. 9) were prepared by treating tributylammonium salt of these with sulfur trioxide (50). Physical properties are summarized in Table 5 (51). All these derivatives were assayed for antiproliferative activity on cultured bovine pulmonary artery SMC (Fig. 10).

No appreciable difference was found between heparin and fully sulfated heparin on the growth of pulmonary artery smooth muscle cells. Chondroitin and dermatan sulfates stimulated the pulmonary artery smooth muscle cells. Hyaluronan was not antiproliferative but full sulfation made HA strongly antiproliferative against pulmonary smooth muscle cells (Fig. 10) (51).



**Figure 9** Major and variable sequences of original and fully sulfated GAGs: (A) heparin; (B) heparan sulfate; (C) chondroitin sulfate; (D) dermatan sulfate; (E), hyaluronan; (F) acharan sulfate and *N*-sulfoacharan sulfate; X = H or SO<sub>3</sub><sup>-</sup>, Y = CH<sub>3</sub>CO or SO<sub>3</sub><sup>-</sup>.

**Table 5** Physical Properties of Heparin and other Glycosaminoglycans, Acharan Sulfate, and Their Fully Sulfated Derivatives (51)

Disaccharide unit				
Compound rotation	Mol $O\text{-SO}_3^-$ groups	Mol $N\text{-SO}_3^-$ groups	Average molecular weight	Optical $[\alpha]_D$
Heparin (HP)	2.0	0.88	16.0	+46.5
Fully sulfated HP	4.0	0.68	20.0	+22.3
Heparan sulfate (HS)	1.0	0.22	14.8	+70.0
Fully sulfated HS	4.0	0.22	24.0	+31.5
Chondroitin sulfate (CS)	1.0	0	15.0	-30.0
Fully sulfated CS	4.0	0	23.8	-8.0
Dermatan sulfate (DS)	1.0	0	30.0	-41.5
Fully sulfated DS	4.0	0	47.5	-10.5
Hyaluronan (HA)	0	0	100	-32.5
Fully sulfated HA	4.0	0	198	-25.0
Acharan sulfate (AS)	1.0	0	29	nd
<i>N</i> -sulfo AS	1.0	1.0	8	nd

Note: nd, not determined.

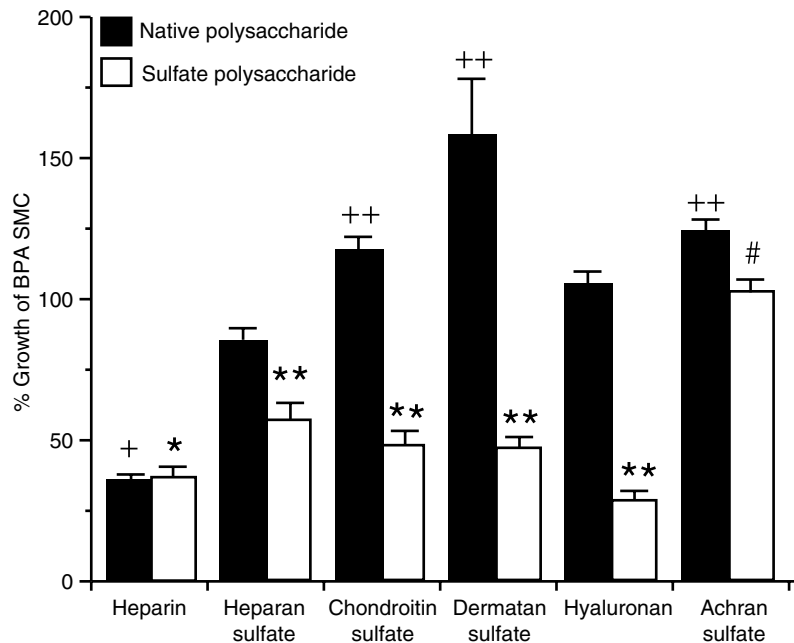
#### D. Effect of Sulfonation Patterns in Heparin and Heparan Sulfate on the Proliferation of SMC

Sulfo groups in HP appear to play an important role in the growth inhibitory effect on smooth muscle cell proliferation. Removal of *N*-sulfo groups from HP reportedly negates its growth inhibitory effect on SMC (46). To understand the significance of *N*- and 6-*O*-sulfo groups in heparin/heparan sulfate for SMC proliferation, six chemically modified HP and HS (Fig. 11) were prepared, which fell into three groups. One group consisted of fully *O*-sulfonated-*N*-acetylated, the second group consisted de-*N*-sulfonated and re-*N*-acetylated, and the third group consisted of 6-*O*-desulfonated HP and HS derivatives (52). Properties of HP and HS derivatives (1–8) are given in Table 6.

These six preparations were assayed for their antiproliferative potency on pulmonary artery SMC (Fig. 12).

The results of this assay showed that (a) full-*O*-sulfonation of both HP and HS increases antiproliferative potency, (b) substitution of hexosamine with *N*-acetyl diminishes antiproliferative activity in both HP and HS, and (c) 6-*O*-desulfonation of HP and HS diminishes antiproliferative potency (52).

Importance of both the *N*-acetylation and *N*-sulfo groups of glucosamine residues in heparin for growth inhibition of SMC is not very well understood. This was studied recently (53) by quantifying the relative *N*-acetylation of three commercial heparins of known antiproliferative activities, using Fourier transform infrared (FTIR) band areas at 1381–1387 and 1320–1317  $\text{cm}^{-1}$ , which combined resulted in 1.0, 1.0, and 1.3  $\text{cm}^2$  for Choay, Elkins-Sinn, and Upjohn HP, respectively. These data show that Upjohn HP, which is at least 44% more antiproliferative than the other two (40), is 30% more *N*-acetylated. Further, Upjohn HP on



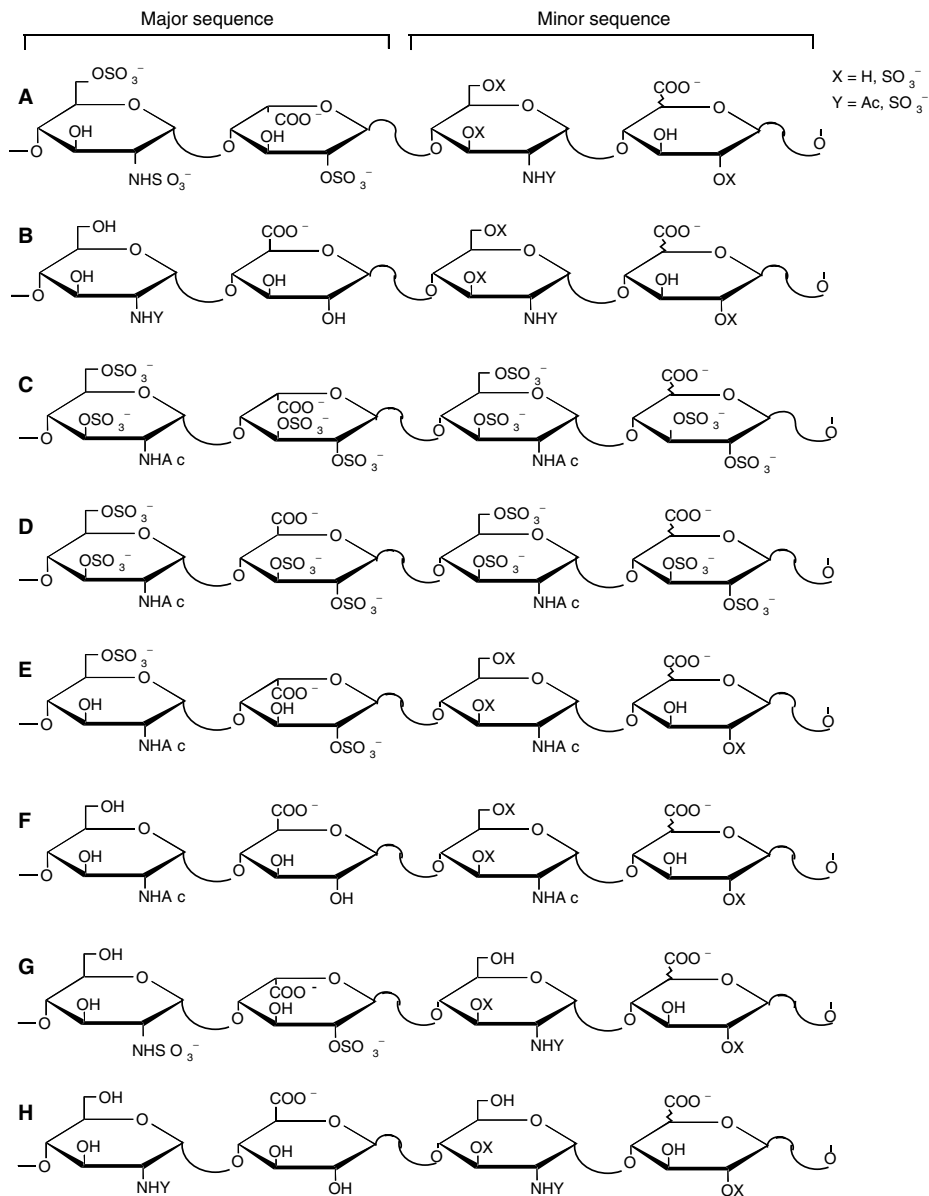
**Figure 10** Effect of various polysaccharides on bovine pulmonary artery smooth muscle cells grown in media containing 10% FBS plus either original (solid bars) or fully sulfated GAG (open bars) or acharan sulfated (solid bar) or *N*-sulfoacharan sulfated (open bar). Samples are standardized to growth in media containing 10% serum without polysaccharide. +, significant reduction in cell growth as compared to the standard ( $p < 0.05$ ); ++, significant increase in cell growth as compared to the standard ( $p < 0.05$ ); \*, significant reduction in cell growth as compared to the standard ( $p < 0.05$ ), but no difference as compared to the parent polysaccharide; \*\*, significant reduction in cell growth as compared to both the standard and parent polysaccharide ( $p < 0.05$ ); #, significant reduction in cell growth as compared to parent acharan sulfate ( $p < 0.05$ ), but not to the standard.

chemically *N*-desulfonation resulted in a 67% decrease in the growth inhibitory potency. The above results show that both *N*-acetyl and *N*-sulfo groups are essential for antiproliferative activity.

## VII. Effect of the Type of Serum on Antiproliferative Activity

Underwood et al. (54) have recently demonstrated that while HP inhibited proliferation of vascular SMC in fetal bovine serum (FBS) as a growth supplement in culture medium, it was ineffective in the presence of human serum. We (55) examined the growth inhibitory effect of our most antiproliferative HP preparation from Upjohn in the presence of FBS as well as human serum on pulmonary artery SMC (Fig. 13) and on aortic SMC (Fig. 14). We also examined the growth

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**Figure 11** Structural formulae of major and variable sequences of repeating disaccharide units of HP and HS preparations. (A) heparin (HP); (B) heparan sulfate (HS); (C) fully *O*-sulfonated and re-*N*-acetylated HP; (D), fully *O*-sulfonated and re-*N*-acetylated HS; (E) de-*N*-sulfonated and re-*N*-acetylated HP; (F) de-*N*-sulfonated and re-*N*-acetylated HS; (G) 6-*O*-desulfonated HP; 6-*O*-desulfonated HS.

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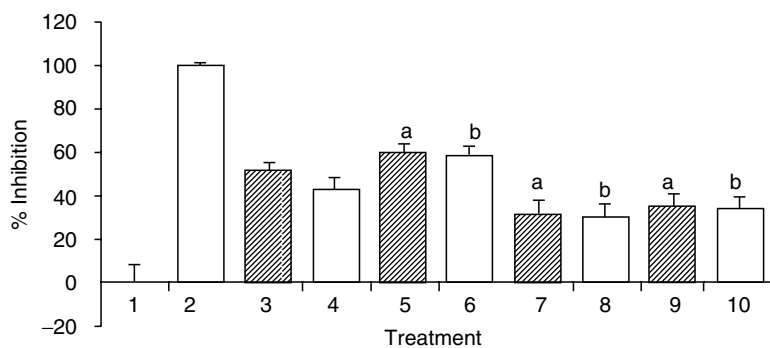
**Table 6** Properties of Heparin and Heparan Sulfate Derivatives (1–8) (52)

Compound	SO <sub>3</sub> <sup>-</sup> /COO <sup>-</sup>	2- <i>O</i> -SO <sub>3</sub> <sup>-</sup> (%)	6- <i>O</i> -SO <sub>3</sub> <sup>-</sup> (%)	<i>N</i> -SO <sub>3</sub> <sup>-</sup> (%)	IdoA/GlcA (%)
HP 1	2.68	86.2	89.7	90.4	92.3/7.7
3	3.88	100	100	<0.5	92.3/7.7
5	1.74	86.2	89.7	<0.5	92.3/7.7
7	1.66	86.2	<0.5	90.4	92.3/7.7
HS 2	0.25	1.6	6.4	17.5	26.7/73.3
4	3.92	100	100	<0.5	26.7/73.3
6	0.10	1.6	6.4	<0.5	26.7/73.3
8	0.19	1.6	<0.5	17.1	26.7/73.3

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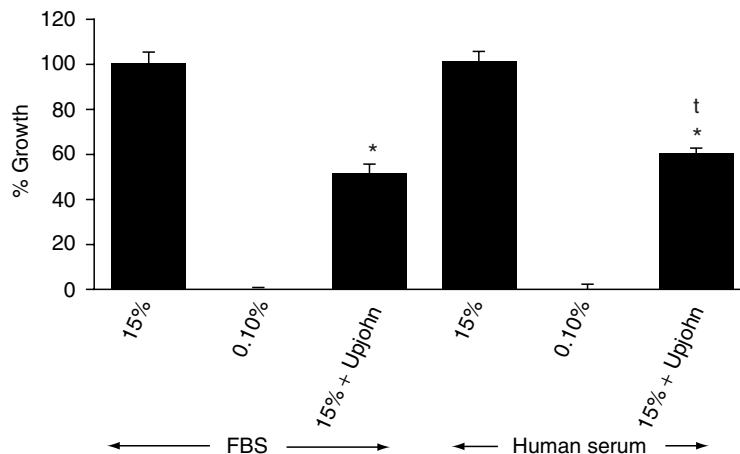
inhibitory effect of the above heparin preparation in the presence of human serum on human pulmonary artery SMC (Fig. 15) (55).

Our results are opposite to those reported by Underwood et al. (54). The variance in resistance to heparin in human serum could be due to the potency of the HP preparations. A potent antiproliferative heparin was effective in either bovine or human serum whether it be for aortic or pulmonary artery SMC.

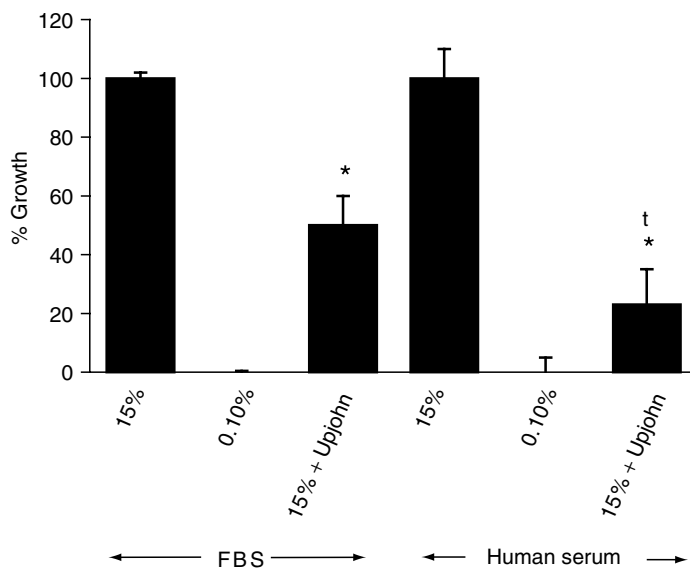


**Figure 12** Inhibition of pulmonary artery SMC proliferation by modified HP, HS preparations including starting HP and HS. Percent inhibition of bovine pulmonary artery smooth muscle cell grown in media containing 10% FBS without HP and HS as negative control, Column (1); 0.1% fetal bovine serum (FBS) without HP or HS positive control (+), Column (2); 10% FBS plus HP (1), Column (3); 10% FBS plus HS (2), Column (4); containing 10% FBS plus fully *O*-sulfonated-re-*N*-acetylated HP (3), Column (5); containing 10% FBS plus fully *O*-sulfonated-re-*N*-acetylated HS (4), Column (6); 10% FBS plus de-*N*-sulfonated-re-*N*-acetylated HP (5), Column (7); containing 10% FBS plus de-*N*-sulfonated-re-*N*-acetylated HS (6), Column (8); 10% FBS plus 6-*O*-desulfonated HP (7), Column (9); 10% FBS plus 6-*O*-desulfonated HS (8), Column (10). Letter “a” represents a significant inhibition in cell growth compared to HP, and “b” represents a significant inhibition in cell growth compared to HS.

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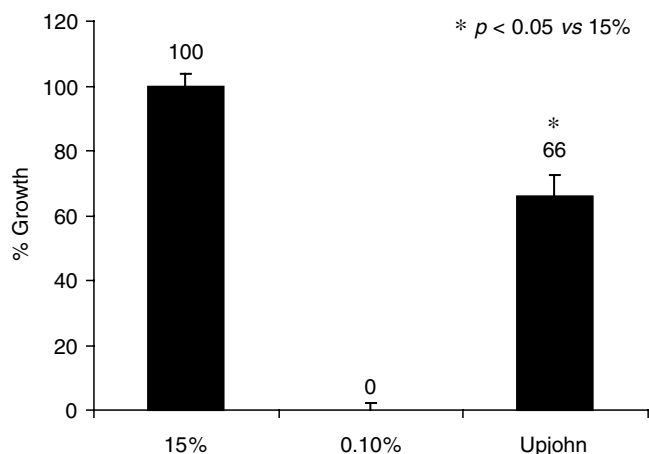


**Figure 13** Bovine pulmonary artery smooth muscle cells (BPASMC) treated with Upjohn heparin in fetal bovine serum (FBS) and human serum. BPASMC were treated with standard medium (RPMI-1640 supplemented with antibiotics) + 15% FBS, standard medium + 15% human serum, standard medium + 0.1% FBS (growth arrest), standard medium + 0.1% human serum (growth arrest), standard medium containing 10  $\mu\text{g}/\text{ml}$  Upjohn heparin + 15% FBS, and standard medium containing 10  $\mu\text{g}/\text{ml}$  Upjohn heparin (Upjohn) + 15% human serum. After 4 days of treatment, cells harvested and the cell number and percent growth were determined; \* $p < 0.0001$  vs 15%; <sup>t</sup> $p < 0.05$  vs 15% human + HP. Values are means  $\pm$  SE;  $n = 15$  in each group. AQ8



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**Figure 14** Bovine aortic smooth muscle cells (BAOSMC) treated with Upjohn heparin in fetal bovine serum (FBS) and human serum. BPAOMC were treated with standard medium (RPMI-1640 supplemented with antibiotics) + 15% FBS, standard medium + 15% human serum, standard medium + 0.1% FBS (growth arrest), standard medium + 0.1% human serum (growth arrest), standard medium containing 10  $\mu\text{g}/\text{ml}$  Upjohn heparin + 15% FBS, and standard medium containing 10  $\mu\text{g}/\text{ml}$  Upjohn heparin (Upjohn) + 15% human serum. After 4 days of treatment, cells harvested and the cell number and percent growth were determined; \*  $p < 0.0001$  vs 15%;  $^{\dagger}p < 0.05$  vs 15% human + HP. Values are means  $\pm$  SE;  $n = 15$  in each group.



**Figure 15** Human pulmonary artery smooth muscle cells (HPASMc) treated with Upjohn heparin in human serum. HPASMc were treated with standard medium (RPMI-1640 supplemented with antibiotics) + 15% human serum, standard medium + 0.1% human serum (growth arrest), and standard medium containing 10  $\mu\text{g}/\text{ml}$  Upjohn heparin + 15% human serum. After 4 days of treatment, cells were harvested and the cell number and percent growth were determined; \* $p < 0.0001$  vs 15%. Values are means  $\pm$  SE;  $n = 15$  in each group.

## VIII. Conclusions

In conclusion, although the structural requirements for antiproliferative activity of heparin are not fully understood, the above studies demonstrate that: (a) an increase in the charge density affects the antiproliferative activity; (b) the molecular size of the HP does not affect the potency of growth inhibition; (c) the HP protein core prepared by digesting with heparitinases I and II significantly loses the antiproliferative activity; (d) the HP GAG chains are responsible for the antiprolifera-



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tive activity; (e) 3-*O*-sulfo group on the internal glucosamine residues is not critical for native HP's antiproliferative activity; (f) both *N*-acetyl and *N*-sulfo groups in HP are important for antiproliferative properties; (g) 14-mer is the minimum size of oligosaccharide, which is essential for full antiproliferative activity; (h) loss of *N*-sulfo and 6-*O*-sulfo groups in the glucosamine residues of heparin reduces antiproliferative potency. AQ2

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