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Note

Increase in the growth inhibition of bovine pulmonary artery smooth muscle cells by an *O*-hexanoyl low-molecular-weight heparin derivative

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Abstract—Proliferation of pulmonary artery smooth muscle cells (PASMCs) appears to play a significant role in chronic pulmonary hypertension. The proliferation of PASMCs is strongly inhibited by some commercial heparin preparations. Heparin fragments were prepared by periodate treatment, followed by sodium borohydride reduction, to enhance potency. The tributylammonium salt of this fragmented heparin was O-acylated with hexanoic anhydride. Gradient polyacrylamide gel electrophoresis showed that the major heparin fragment contained eight disaccharide units. NMR analysis showed that approximately one hexanoyl group per disaccharide residue was present. The *O*-hexanoyl heparin fragments were assayed for growth inhibitory effect on bovine PASMCs in culture. This derivative was found to be more effective in growth inhibition of bovine PASMCs in culture than the heparin from which it was derived. In the future, it is envisioned that this or similar derivatives may be an effective treatment for pulmonary hypertension.
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Smooth muscle cells (SMCs) play an important role in pulmonary hypertension associated with chronic hypoxia. The proliferation of pulmonary smooth muscle cells (PASMCs) are proposed to be key processes in increasing pulmonary hypertension.[1–4] Heparin inhibits SMCs proliferation both in vitro and in vivo.[5–9] This is probably due to its anionic character, in addition to other as yet not well understood structural features of heparin.

Heparin is a glycosaminoglycan and is a member of the family that includes hyaluronan, heparan sulfate, dermatan sulfate, and chondroitin sulfates. The carbohydrate backbone of heparin is made up of alternating (1→4)-covalently linked hexuronic acid (either L-iduronic acid or D-glucuronic acid) and D-glucosamine residues. Most of the sugar residues are substituted with one or several sulfo groups at various positions.[10,11] We are involved in the study of the structural features of heparin critical to its antiproliferative effects at the cellular level.[12–14] Currently, an in vivo study is also underway. In earlier studies, we found that commercial heparin preparations differ in their antiproliferative

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potencies.[15] In addition, we also observed that: (a) the heparin core protein has no antiproliferative activity; (b) the molecular size of heparin does not effect the antiproliferative potency; (c) heparin's antiproliferative properties reside in the glycosaminoglycan chain; (d) a 3-*O*-sulfo group-containing glucosamine residue in heparin is not critical for the antiproliferative effect in full-length heparin; (e) the (1→4)-positions of the anomeric linkages between the uronic acid and hexosamine residues do not influence the antiproliferative activity; (f) heparin's glucosamine residues are replaceable with galactosamine residues; (g) the *N*-sulfo group on the glucosamine residue is not critical for antiproliferative potency; (h) some *N*-acetyl groups are essential for heparin's antiproliferative potency; (i) the 2-*O*-sulfo groups in heparin's uronic acid residues do not influence the antiproliferative potency; and (j) fully O-sulfonated heparin does not exhibit enhanced antiproliferative activity over that of native heparin. Matsuda and co-workers have prepared a terminally alkylated heparin by treatment of the heparin sodium salt with an oxidizing agent to generate a lactone ring at its terminal end and subsequently reacted it with an alkylamine group, containing 4–18 carbons, to synthesize the alkylated heparin.[16,17] They found that increasing the alkyl group chain length reduced the proliferation rate of vascular smooth muscle cells.

These observations led us to hypothesize that conformational changes, induced by either O-desulfonation or N-deacetylation or by O-sulfonation, play a role in the alterations in antiproliferative potency of modified heparin derivatives. To address this hypothesis, we have prepared a modified heparin derivative substituted with bulky *O*-hexanoyl groups and have assayed this derivative for its antiproliferative activity on bovine PASMC.

1. Experimental

1.1. Materials

Heparin from porcine intestinal mucosa was obtained from Celsus Laboratories (Cincinnati, OH), and fetal bovine serum was obtained from BioWhittaker (Walkersville, MD), respectively. Cell culture medium, RPMI-1640 (Mediatech, Washington, DC), contained streptomycin (Lilly, Indianapolis, IN), penicillin (Pfizer, New York, NY), and amphotericin B (GIBCO, Grand Island, NY), which were purchased commercially. ^2H_2O (99.9%), sodium periodate, 4-dimethylaminopyridine, hexanoic anhydride, and tributylamine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Spectra/Por dialysis tubing [1000–3500 molecular weight cut-off (MWCO)] was from Spectrum (Houston, TX). Microfilters (0.22 μm) were purchased from Millipore (Bedford, MA). Dowex-50WX8 cation-exchange resin came from Supelco (Bellefonte, PA). Fourier-

transform ^1H NMR analysis spectra were recorded on a Bruker DRX-400 and 600 MHz instrument and tetramethylsilane or the residual solvent signal were used as internal standards.

1.2. Methods

1.2.1. Periodate-oxidized heparin fragments. Heparin was fragmented by periodate oxidation[18] based on a modification of an earlier procedure[19] (Fig. 1). Briefly, heparin sodium salt (20 g, 1.43 mmol) was dissolved in 175 mL of distilled water. The pH was adjusted to 5.0 using 1 N HCl. NaIO4 (15 g, 0.070 mol), dissolved in 500 mL of water, was added in a single portion with stirring. The pH was readjusted to 5.0 using 1 N HCl and left for 24 h at 4 °C in the dark. The solution was dialyzed against 4 vol of water (with one change of water) for 15 h at 4 °C.

To the approximately 1.5 L of solution obtained after dialysis, 32 mL of 10 N NaOH was added. The solution was stirred at room temperature for 3 h. To prevent the development of colored products, this step was done in the dark.

NaBH4 (1 g, 0.026 mol) was added in one portion, and approximately 1.5 L of the solution was stirred for 4 h. The pH was then adjusted to 4.0 using 37% HCl, and the solution was stirred for an additional 15 min. The solution was neutralized to pH 7.0 using 1 N NaOH. NaCl (32.8 g, 0.56 mol) was added, followed by 2.54 L of EtOH. The solution was left for 3 h without stirring, and the precipitate was recovered by centrifugation at 15,000 rpm (22,000*g*) for 20 min. The precipitate was recovered by decantation and suspended in 400 mL absolute EtOH. The solution was filtered using a Buchner funnel and the recovered solids were left to dry for 5 h under vacuum yielding 14.2 g of the product.

The product was dissolved in 190 mL of water. NaCl (2.8 g, 0.05 mol) was added, and the pH was adjusted to 3.5 using 1 N HCl. The volume was adjusted to 280 mL using water. Absolute EtOH (240 mL) was added with stirring. The solution was stirred for 15 min and then left without stirring for 10 h at room temperature. After decanting, the precipitate was recovered and dissolved in water. The EtOH was removed by rotary evaporation under reduced pressure, and the aq solution was freeze-dried affording ∼10 g of heparin fragments.

1.2.2. The tributylammonium salt of periodate-oxidized heparin fragments. Periodate-oxidized heparin fragments were converted to the tributylammonium salt using a modification of previously described conditions.[17,18] Briefly, heparin fragments (10 g) were dissolved in water (50 mL) and passed through a column (300 mL) of Dowex 50WX8 (H$^+$) cation-exchange resin. The pH of the eluent (∼500 mL) was adjusted to pH 6.0 using Bu3N (∼25 mL, 0.1 mol). Excess Bu3N was elimi-
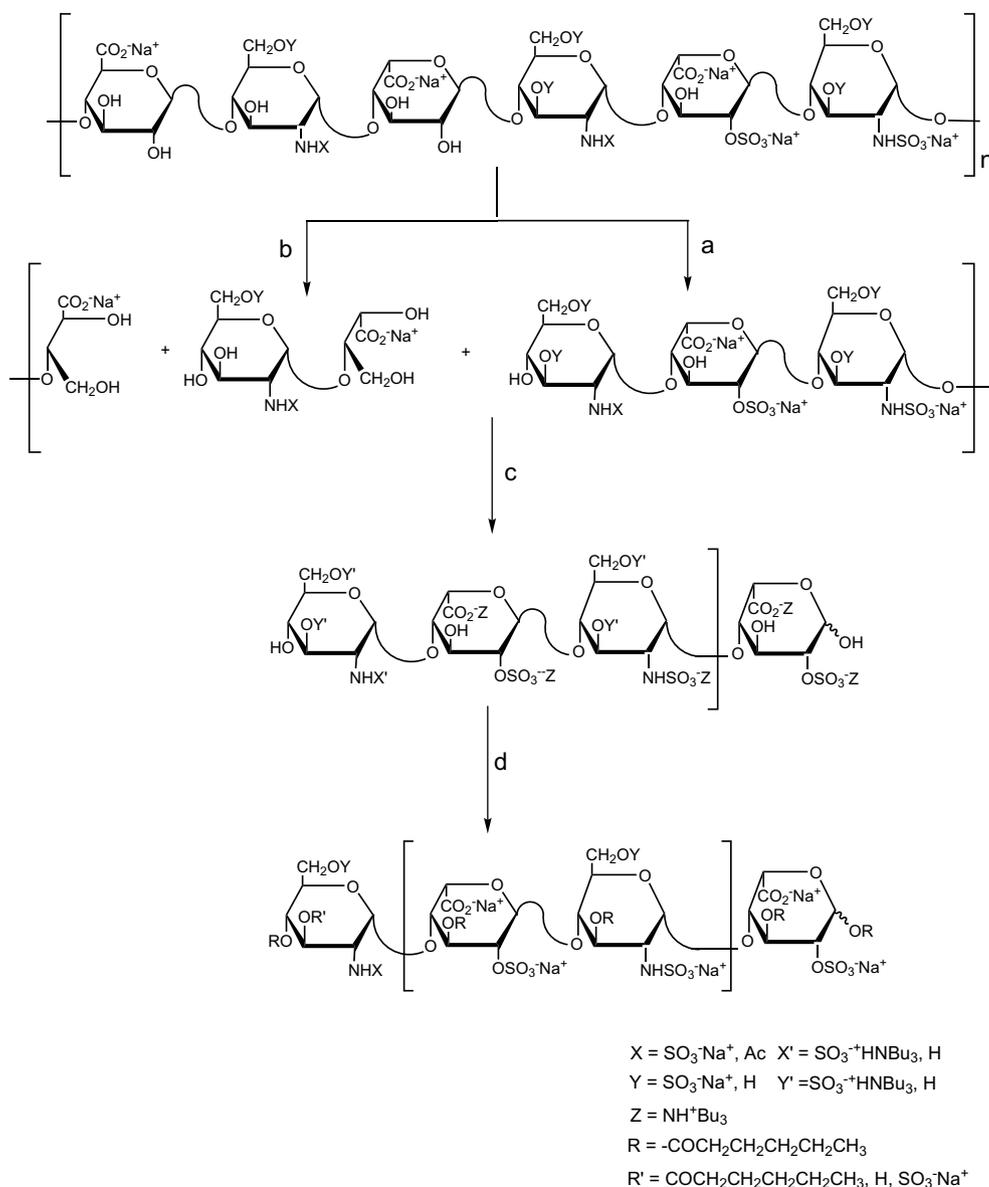
Figure 1. Preparation and structure of an *O*-hexanoyl heparin derivative. Reagents and conditions: (a) periodate oxidation and fragmentation of heparin; (b) removal of the smaller fragments by dialysis; (c) preparation of the tributylammonium salt of heparin fragments; (d) acylation and regeneration of the sodium salt form of the *O*-hexanoyl heparin derivative.

nated by concentrating it to ~100 mL by rotary evaporation under reduced pressure. The concentrate was diluted with ~10 vol of water and lyophilized to afford the tributylammonium salt (13 g).

1.2.3. *O*-Hexanoyl derivative of periodate-oxidized heparin fragments. The derivatives of tributylammonium salt of periodate oxidized heparin fragments were acylated with hexanoic anhydride using a modification of methods previously described.[17,18] Briefly, the dry tributylammonium salt (12 g), dissolved in dry DMF (110 mL) was cooled to 0 °C under an argon atmosphere. 4-Dimethylaminopyridine (0.7 g, 5.7 mmol), hexanoic anhydride (26.2 mL, 0.1 mol), and Bu₃N (27 mL, 0.1 mol) were successively added in single

portions, and the reaction was allowed to proceed under Ar at room temperature for 24 h. After cooling to 0 °C, 5% NaHCO₃ in water (230 mL) was gradually added, and the solution was stirred at room temperature for 48 h. Excess NaHCO₃ was eliminated by slow, dropwise addition of 1 N HCl (~200 mL) until a pH of 4.0 was reached, and then the pH was readjusted to 7.0 with 1 N NaOH (~150 mL). Cold denatured (95%) EtOH (5 L, 5 vol) was added with stirring. The sample was allowed to sit overnight at 4 °C to create a precipitate. The precipitate was recovered by decanting and was dissolved in 0.2 M NaCl (110 mL). The precipitation procedure was repeated by adding absolute EtOH (570 mL). The precipitate was recovered by centrifugation at 15,000 rpm (22,000*g*) for 20 min, dissolved in water

(110 mL), and passed through a column (300 mL) of Dowex 50WX8 (H$^+$) cation-exchange resin and 600 mL was recovered. The acid was neutralized to pH 7.0 with 10 N NaOH, and the solution was filtered through a 0.22-μm Millipore filter. After lyophilization, O-hexanoyl heparin fragments (7.1 g) were obtained as an off-white powder.

1.2.4. NMR sample preparation. For ^{1}H NMR spectroscopy, approximately 10 mg of each sample was exchanged by lyophilization three times from 0.5-mL portions of 99.9% ^{2}H$_2$O before being redissolved in ^{2}H$_2$O for NMR analysis. Chemical shifts were reported relative to TMS at 0.00 ppm. The degree of substitution (O-acylation) was determined from the ratio of the integrated area of the peaks assigned to the aliphatic methyl protons of the hexanoyl group (0.753 ppm) to the anomeric proton of IdoA2S (5.092 ppm) (Table 1).

1.2.5. Gradient PAGE analysis. Gradient polyacrylamide gel electrophoresis (PAGE) was performed on a 32-cm vertical slab gel unit PROTEAN II equipped with Model 1000 power source from Bio-Rad (Richmond, CA). Polyacrylamide linear gradient resolving gels (14 × 28 cm) (12–22% total acrylamide) were prepared and run as previously described.[19] The molecular sizes of the oligosaccharide samples were determined by comparison with a banding ladder of heparin oligosaccharide standards prepared from bovine lung heparin.[20] Oligosaccharides were visualized by Alcian Blue staining.

1.2.6. Cultured pulmonary artery smooth cells proliferation assay. Pulmonary artery smooth muscle cell proliferation assays were preformed as previously described.[12–14]

Briefly, isolated bovine pulmonary artery smooth muscle cells (PASMC) in passages 4–6 were seeded at 1.5×10^4 cells/well into six well tissue culture plates, grown for 2 days, then growth arrested for 48 h by reducing the serum concentration of the medium from 10% to 0.1%. Medium was then changed for experimental samples to contain either standard medium [RPMI-1640 with 10% fetal bovine serum (FBS)], growth arrest media (0.1% FBS) or standard media containing heparin and heparin derivatives at different dose levels. All media contained streptomycin (10 μg/mL), penicillin (100 U/mL), and amphotericin B (1.25 μg/mL). After 4–5 days,

the bovine PASMCs present in the cell culture wells were rinsed with Hank's balanced salt solution to remove the remaining cell culture medium. No dead cells (Trypan Blue exclusion) were observed in the control preparation, in the preparation with heparin or in the ones treated with a heparin derivative. After detachment of bovine PASMCs with trypsin/EDTA, the cell numbers were determined by a Coulter counter.

The O-hexanoyl heparin derivative was dissolved (1 mg/mL) in distilled sterile water. This solution (14, 140 μL, and 1.4 mL) was added to the culture medium (13.99, 13.86, and 12.6 mL, respectively), and 2 mL of the resulting media was added to each well. The O-hexanoyl heparin derivative and non-acylated heparin derivative were completely soluble, as the culture medium was clear (no turbidity) after addition of the heparin samples.
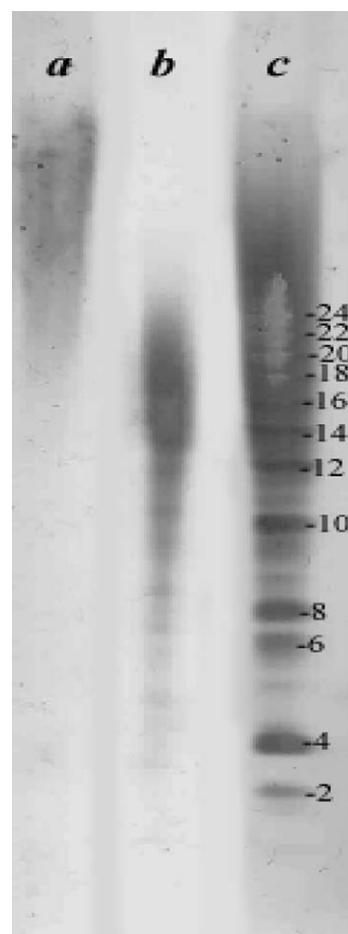

Figure 2. Gradient polyacrylamide gel-electrophoresis analysis of heparin and periodate-fragmented heparin. Lane a corresponds to porcine intestinal heparin starting material. Lanes b shows the heparin treated with sodium periodate, hydrochloric acid, and sodium borohydride. Lane c contains a standard mixture of heparin oligosaccharide prepared from bovine lung heparin using heparin lyase (20). The degree of polymerization (DP) of selected bands within this mixture (determined based on standards) is shown to the right of this lane. For conditions used in these analyses, see the Experimental section.

Table 1. Assignment of selected signals in the ^{1}H NMR spectrum of the O-hexanoyl heparin derivative

Residue	Chemical shift (ppm)				
	H-1	H-2	H-3	H-4	H-5
GlcNS6S	5.302	3.093	3.539	3.629	3.892
IdoA2S	5.092	4.218	4.008	3.920	4.709

In the present study 1, 10, and 100 µg/mL were used for the *O*-hexanoyl heparin derivative as well as for the control Upjohn heparin.

The percent growth was calculated as

$$\% \text{ growth} = \frac{\text{net cell growth in treated medium}}{\text{net cell growth in standard medium}} \times 100$$

where net cell growth = cell growth in standard or treated medium minus cell growth in growth arrest media.

1.3. Statistics

Results are presented as mean ± standard error of the mean. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the Stateview software package (BrianPower, Inc., Calabassas, CA) for Macintosh computers. If ANOVA were significant, multiple comparisons were made using the Fisher protected least significant difference (PSLD) test. In all cases, significance was set as $p < 0.01$.

The structure of the sodium salt of the pharmaceutical heparin was confirmed by ^{1}H NMR spectroscopy (not shown). Porcine mucosal heparin was fragmented into a low-molecular-weight heparin by treatment with sodium periodate, hydrochloric acid, and sodium borohydride (Fig. 1). Periodate oxidatively cleaves the C-2–C-3 bonds of the unsulfated glucuronic and iduronic acid residues, affording a ring-opened residue containing aldehyde groups at C-2 and C-3. This residue is quite labile, even to dilute acid, and thus treatment with 1 N HCl cleaves the product chain into smaller fragments. Reduction of residual aldehyde functional groups to the less reactive alcohol groups was then accomplished with sodium borohydride. ^{1}H NMR analysis (not shown) of the fragmented heparin indicated the H-2 peaks of unsulfated GlcA and IdoA residues, at 3.38 and 3.69 ppm, respectively, were completely lost as would be expected from their periodate cleavage. The *N*-acetyl methyl signals, associated with a loss of antithrombin III binding sites,[19,21] also showed a significant reduction, leading to the conclusion that these chains were devoid of antithrombin III-mediated anticoagulant activity and that virtually all the GlcN in the fragmented heparin contained *N*-sulfo groups. Gradient PAGE analysis[20] showed that the parent porcine mucosal heparin had a MW ∼12,000, while the resulting fragments had an average degree of polymerization (dp) of 16, corresponding to a low-molecular-weight heparin of MW ∼5000 (Fig. 2).

The heparin fragments were passed through a Dowex 50WX8 H$^+$ cation-exchange resin column and converted to the tributylammonium salt by neutralization with tributylamine. Chemical *O*-acylation of the tributylammonium salt of this low-molecular-weight heparin
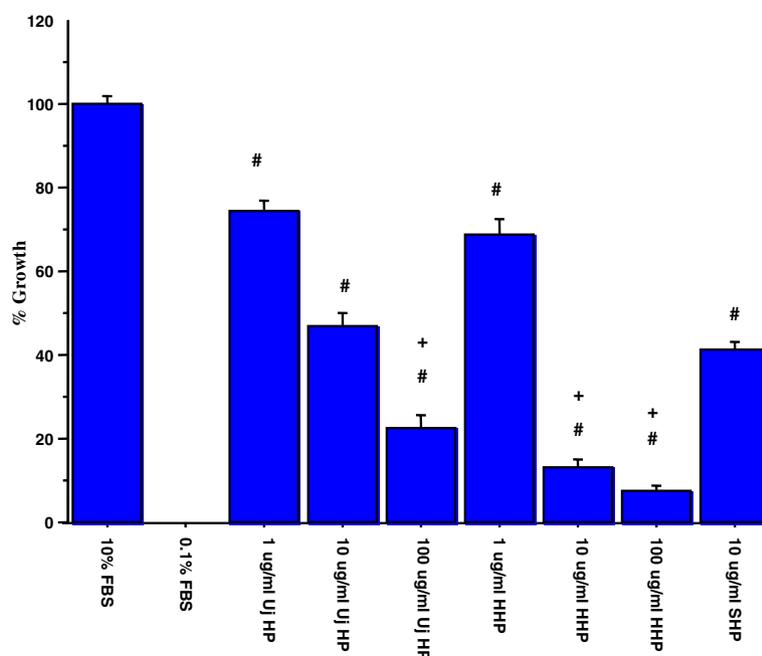

Figure 3. Percentage growth in bovine pulmonary artery smooth muscle cells grown in 10% fetal bovine serum (FBS) plus Upjohn heparin, *O*-hexanoyl heparin fragments or the starting heparin fragments. Standard: Without any heparin derivative (Bar 1), bovine PASMCs grown in 0.1% fetal bovine serum (Bar 2), Upjohn heparin (UjHP) (1 µg/mL) (Bar 3), Upjohn heparin (UjHP) (10 µg/mL) (Bar4), Upjohn heparin (UjHP) (100 µg/mL) (Bar 5), 3-*O*-hexanoyl heparin fragments (HHP)(1 µg/mL) (Bar 6), 3-*O*-hexanoyl heparin fragments (HHP) (10 µg/mL) (Bar 7), 3-*O*-hexanoyl heparin fragments (HHP) (100 µg/mL) (Bar 8); starting heparin fragments (SHP) (10 µg/mL) (Bar 9). (#) Significant inhibition of growth of bovine PASMCs compared to standard, that is, control with10% fetal bovine serum (FBS) without addition of any heparin fragment (100% growth); (+) significant inhibition as compared to starting heparin fragments (SHP).

by treatment with hexanoic acid anhydride in dry DMF afforded *O*-hexanoyl heparin fragments. Ion exchange on the Dowex 50WX8 H$^+$ resin, followed by neutralization with sodium hydroxide, afforded the sodium salt of the *O*-hexanoyl heparin fragments. The degree of substitution of the *O*-hexanoyl heparin fragment derivative was estimated to be one in every repeating unit based on the area of the signals of IdoA2S H-1 and the methyl proton of the hexanoyl group at 5.092 and 0.753 ppm. Theoretically, a higher degree of substitution is possible by acylation of the 3-positions of both the IdoA2S and GlcNS6S residues, but steric constraints may limit the extent of conversion.

Unfractionated heparin (Upjohn) at 1, 10, and 100 µg/mL produced a significant dose response inhibition in the growth of PASMC stimulated with 10% FBS as did the *O*-hexanoyl heparin (Fig. 1), although the derivative was significantly more potent at 10 and 100 µg/mL (Fig. 3). The *O*-hexanoyl derivative was also significantly better at growth inhibition of PASMC than its non-acylated heparin parent (Fig. 3) as reported earlier by Pukac in aortic vascular smooth muscle cells.[22]

In conclusion, we have demonstrated that O-acylation of low-molecular-weight heparin by bulky *O*-hexanoyl groups enhances its antiproliferative effects on bovine PASMCs. These data suggest that conformational changes in glycosaminoglycans induced by bulky O-substituents, such as hexanoyl, are favorable to the antiproliferative effect of heparin. This or similar heparin compounds may be effective candidates for treatment of pulmonary hypertension where excess pulmonary artery smooth muscle cell growth is a problem.

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