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Heparin, carboxyl-reduced sulfated heparin, and Trestatin A sulfate. Antiproliferative and anticoagulant activities*

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ABSTRACT

Human smooth muscle cells were used to investigate the antiproliferative activities of sulfated carbohydrates. The antiproliferative potencies of coarse heparin fractions prepared by ultrafiltration increased with the mean molecular-weight, whereas the anticoagulant activities of a high-molecular-weight fraction had submaximal values. Furthermore, the dependence of antiproliferative activity on sulfate content is discussed. Carboxyl-reduction of heparin abolished both antiproliferative and anticoagulant activities. Sulfation of this compound yielded CRS-heparin with restored antiproliferative potency but devoid of antithrombin III-mediated anticoagulant activity. Sulfation of the pseudo-nonasaccharide, Trestatin A, yielded a compound having the highest antiproliferative activity, so far observed for a low-molecular-weight compound, and having only weak anticoagulant properties.

INTRODUCTION

Smooth muscle cells (SMC) play a pivotal role in the development of arteriosclerotic lesions[1]. These occur in the innermost layer of an affected artery, where SMC have migrated from the media into the subintimal space, and have subsequently proliferated and formed connective tissue. Heparin (**1** represents the regular region of heparin) and heparin-like glycosaminoglycans effectively inhibit the proliferation[2–6] and migration[7] of SMC *in vitro* and in a rat model in which endothelial cells are denuded by balloon catheterization[8]. Fractionation of heparin on an antithrombin III-affinity column established that the antiproliferative potency of heparin was not related to its known anticoagulant function[2,9], suggesting a pharmacological basis for a heparin-like compound as antiproliferative agent after vascular injury.

To date, the exact structural determinant responsible for growth-inhibitory activity of heparin is not known. The O-sulfate groups are essential for antiproliferative activity, wheras N-sulfate groups can be replaced by N-acetyl groups[9]. An investigation by Castellot *et al.*[9] on heparin oligosaccharides, prepared by gel filtration following nitrite degradation, showed that 12 residues were necessary for maximal antiproliferative activity and that a hexasaccharide was the smallest fragment which still had significant antiproliferative activity; other authors[10] also claimed antiproliferative activ-

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ity for heparin di- and tetra-saccharides. The antiproliferative activity of the synthetic heparin pentasaccharide having high anti-Xa activity was comparable to that of a hexasaccharide, the unique 3-sulfate group being critical for the antiproliferative potency of this pentasaccharide[11]. A recent study[12,13] revealed that the antiproliferative activity of heparin oligosaccharides increases with increasing O-sulfation and that D-glucuronic acid 2-sulfate units are not required for growth-inhibitory potency.

RESULTS AND DISCUSSION

For the evaluation of the growth-inhibitory effect of sulfated carbohydrates, human SMC were chosen as a relevant *in vitro* system. Heparin inhibited[14] the growth of this type of SMC. In this system, we investigated the dependence of the antiproliferative activity of heparin on molecular weight. Chemically unchanged heparin fractions have been obtained by gel filtration and other chromatographic methods, or by partition and precipitation techniques[15]. We found it very convenient to prepare heparin fractions by ultrafiltration. The first filtrates of repeated ultrafiltrations contained heparin fractions of lower mean molecular-weights. We exploited this finding to prepare coarse heparin fractions. A typical example is shown in Table I, which demonstrates that more than 60% of heparin passes through the ultrafiltration membrane.

TABLE I

Ultrafiltration of an aqueous heparin solution[a]

Fraction	*Volume of filtrate (mL)*	*Time (h)*	*Mass (%)*
1	150	5	9.5
2	300	64	55.8
3[b]			33.2

[a] Data from a 20-g run, volumes before ultrafiltration were 350 mL. [b] Residue from second ultrafiltration.

TABLE II

Properties of heparin fractions prepared by ultrafiltration

Fraction	*Mol. wt.*	*S content*	*Growth inhibition with heparin*		*Anticoagulant activity* in vitro			
					Chromogenic tests		*Clotting tests*	
		(%)	*10 mg/mL* (%)	*100 mg/mL* (%)	*anti-IIa (IU/mg)*	*anti-Xa (IU/mg)*	*aPTT (IU/mg)*	*anti-Xa (IU/mg)*
1	8400	11.60	7	25	84	109	103	124
2	13 300	11.48	15	51	142	155	185	178
3	31 700	9.72	23	70	124	115	154	124
H[a]	17 900	10.80	20	39	145	149	161	156

[a] Unfractionated heparin.

The properties of the heparin fractions obtained by ultrafiltration are summarized in Table II. The fractions differed in molecular weight as determined by viscometry. The sulfur content of the high-molecular-weight fraction (Fraction 3) was lower than that of the lower-molecular-weight heparin species. The *in vitro* anticoagulant activity of the high-molecular-weight fraction had submaximal values. This feature of high-molecular-weight heparin (in the range of mol.wt. 30 000) has been described by a number of authors[16–23] using different methods of heparin fractionation and diverse biological assays. One group attributed the relatively low anticoagulant activity of a high-molecular-weight fraction to contamination with proteoglycan link-region fragments[18]. Our heparin contained no protein, ruling out this possibility. In contrast to the anticoagulant activity, the antiproliferative activity of heparin steadily increased with increasing molecular weight. This supports the finding that the antiproliferative and anticoagulant properties of heparin depend from different parameters.

The low sulfate content of the fraction having highest antiproliferative potency does not contradict the reported finding[12] that the activity of heparin oligosaccharide fractions increased after oversulfation. It is conceivable that the high-molecular-weight fragments contain regions of high sulfate content. Furthermore, the possibility should be considered that a high degree of sulfation is not mandatory for antiproliferative activity. The identification of a highly active (40 times more than heparin) heparan sulfate from SMC points in this direction, as heparan sulfate is sulfated to a lower degree than heparin[3,24,25].

Owing to our interest in nonanticoagulant sulfated carbohydrates, we modified the uronic acid unit of heparin that is essential for anticoagulant activity[26,27]. Thus, carboxyl-reduced heparin (**2**; CR-heparin)[28] had neither *in vitro* anticoagulant activity,

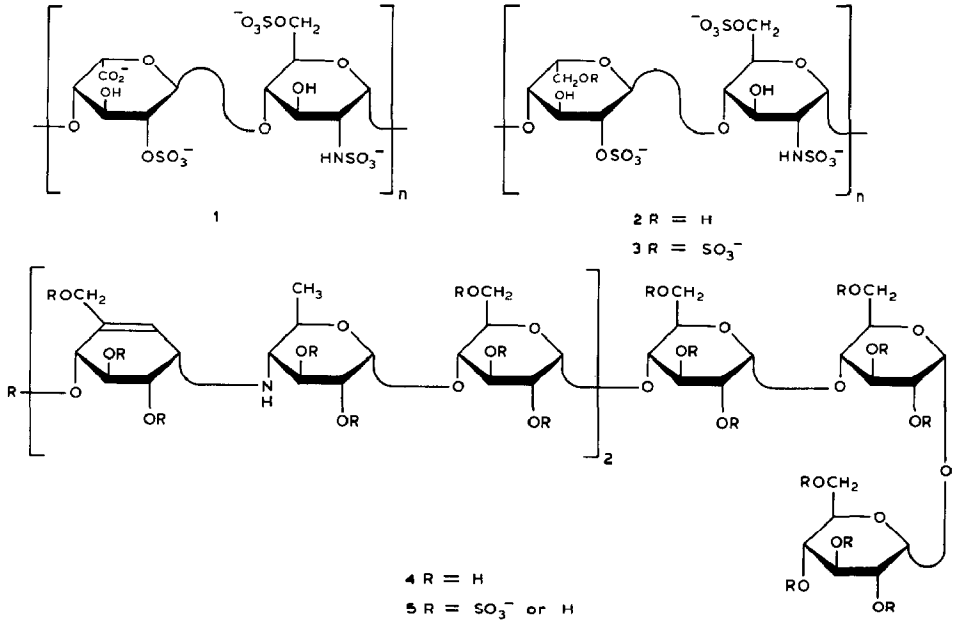

TABLE III

Antiproliferative and anticoagulant activities of heparin (1), Trestatin A (4), and derivatives 2, 3, and 5

Compound	*Antiproliferative activity*			*Anticoagulant activity*			
	Growth inhibition (%) at		IC_{50}	*Chromogenic tests*		*Clotting tests*	
	10 μg/mL	*100 μg/mL*	*(μg/mL)*	*Anti-IIa (μg/mL)*	*Anti-Xa (μg/mL)*	*aPTT (μg/mL)*	*Anti-Xa (μg/mL)*
1	15	50	100	2.2[a]	2.7[a]	1.2[a]	1.3[a]
2	0	4.3	≫100	≫1000	>1000	33	34
3	30	75	28	170	680	3.5	20.5
4	0	0	≫100	[b]	[b]	[b]	[b]
5	45	81	13	≫1000	>1000	8.5	42

[a] Determined for the 4th International Standard of Heparin. [b] Not determined.

nor antiproliferative effect on human SMC (see Table III). The latter result is in contrast to a recent report[12] that carboxyl-reduced heparin inhibited considerably the proliferation of rat aortic SMC (44 ±9% inhibition at 100 μg/mL; compared to 69 ±5% inhibition with heparin at 100 μg/mL). The species difference or the use of different kinds of heparin in these studies (or both) may account for these contrasting findings.

Sulfation of CR-heparin (2) yielded heparin derivative 3 (CRS-heparin) carrying an additional sulfate group per disaccharide unit. This preparation seems to be similar to the one described by Choay and assoc.[12,29]. Compound 3 exhibited significantly higher antiproliferative potency on human SMC than did heparin, whereas the *in vitro* anticoagulant activity was strongly reduced. This means that sulfation of 2 affords only partial recovery of the original anticoagulant activity of heparin. Thus, with CRS-heparin (3), another heparin derivative has been identified that has no antithrombin III-mediated anticoagulant activity. The activated partial thromboplastin time of 3 is reduced *vs.* that of heparin; its residual activity may be due to the interaction with other factors of the coagulation cascade or with Heparin Cofactor II.

These results suggest that carboxyl groups are not a prerequisite for antiproliferative activity, but that they can be replaced by sulfate groups. As a consequence, several nonuronic oligosaccharides were sulfated and tested for antiproliferative potency. Among these was Trestatin A (4). This pseudononasaccharide, obtained from strains of *Streptomyces dimorphogenes*, was developed as an amylase inhibitor[30,31]. Compound 4 itself has no antiproliferative activity. Sulfation of 4 with an excess of sulfur trioxide in *N,N*-dimethylformamide gave Trestatin A sulfate (5). The degree of sulfation, defined as the average number of sulfate groups per monosaccharide unit, was determined to be 2.3±0.3. As shown in Table III, this sulfated carbohydrate had no *in vitro* anticoagulant, but high antiproliferative activity. A comparison of IC_{50}-values revealed an eight-fold higher activity of 5 *vs.* heparin. Thus, Trestatin A sulfate (5) is the first sulfated carbohydrate of low-molecular weight to exhibit a high antiproliferative activity.

EXPERIMENTAL

General. — Hoffmann–La Roche Heparin, lot 93 51 02, was used for ultrafiltration and lot 30 88 12 (mol.wt. 12 500, S content 10.7%) for carboxyl reduction. Since the various samples of sulfated carbohydrates are hygroscopic to different extents, all samples were dried over silica gel for 16 h (aspirator vacuum) before use in biological assays. Sephadex G-50 was used for gel chromatography. Aqueous solutions were concentrated *in vacuo* with bath temperatures $\leq 30°$, and then freeze-dried.

Ultrafiltration. — Ultrafiltration was carried out in an Amicon standard cell using a PM 10 membrane; the nominal molecular-weight cut-off was 10 000 (calibrated with globular proteins). For optimal flow rate an Ar pressure of 0.3 MPa was applied.

Molecular weights. – The viscosity of heparins in 0.5M NaCl solution[32] was determined with an *Ubbelohde* capillary viscometer, and the flow time was the average of 5–7 measurements. The mean molecular-weights were calculated according to the Mark–Houwink equation; the constants had been determined with heparin molecular-weight standards, their molecular weights being measured by quasi-elastic light scattering[33].

CRS-Heparin (**3**). — CR-Heparin (**2**) was prepared according to Taylor and Conrad[28] by use of pH-uncontrolled reaction conditions; the uronic acid content was monitored by two colorimetric assays, a modification of the Dische carbazole assay[34] and a 2-hydroxydiphenyl assay[35]. CR-Heparin exhibited the same h.p.l.c. profile[36] as did the heparin starting material, showing that no degradation had occurred; S content: 12.2%.

CR-Heparin (**2**; 3.0 g) was dissolved in water (12 mL) and acetic acid (0.75 mL), and a 10% solution of benzethonium chloride (45 mL) was added dropwise within 15 min under stirring. After an additional 30 min of stirring, the colorless precipitate was centrifuged off and washed twice with water (50 mL each). Drying in an oil-pump vacuum for 72 h gave a colorless solid (6.07 g). It was dissolved in abs. *N,N*-dimethylformamide (90 mL) and treated with SO_3–triethylamine complex (3.3 g) for 72 h at room temperature under an Ar atmosphere. To the clear, slightly-yellow solution was added anhydrous sodium acetate (60.0 g) in abs. methanol (540 mL). The mixture was stirred for 30 min and centrifuged, and the pellet washed twice with abs. methanol. The solid residue was desalted by diafiltration to give **3** (3.06 g; S content: 15.7%).

Trestatin A sulfate (**5**). — To a solution of dried Trestatin A (**4**; 1.0 g, 697 μmol) in abs. *N,N*-dimethylformamide (30 mL) was added SO_3–pyridine complex (6.8 g, 42.5 mmol). Within a few min, a brownish oil separated. After being stirred for 16 h, the supernatant *N,N*-dimethylformamide solution was decanted, and the residual oil was washed with methanol. An analytical amount of the oil was purified by gel chromatography, and the degree of sulfation (DS) was estimated by spectroscopy. [Integral (I) comparison of pyridinium and methyl protons gave DS 2.4; I (pyridinium, δ 8.67–8.61): I (methyl, δ 1.56–1.46) = 3.6:1]. The rest was treated with 10% aq. sodium acetate solution and evaporated, the residue dissolved again in water, and the solution evaporated until all pyridine was removed. The crude product was desalted by gel chroma-

tography with water as eluent to give **5** as a slightly colored powder (2.28 g, 91%).

Anal. Calc. for $C_{56}H_{73}N_2Na_{21}O_{103}S_{21}$: C, 18.80; H, 2.06; N, 0.75; S 18.82. Found: C, 18.12; H, 2.19; N, 0.73; S 18.86; 21 sulfate groups correspond to DS 2.33.

Cell culture. — Human umbilical cord vein smooth muscle cells (HUVSMC) were isolated, cultured, and characterized as previously described[37]. Briefly, after a first incubation with collagenase to remove the endothelial cells, the umbilical vein was treated again with collagenase for 45 min. Dispersed HUVSMC were then collected by centrifugation and cultured in plastic flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) plus 4mM L-glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cells were kept at 37° in a humidified, 10% CO_2–90% air atmosphere and subcultured by trypsinization. The cells were used between passages 4 to 8. They showed a typical "hill-and-valley" morphology. To growth-arrest HUVSMC, sparsely plated cells were washed and placed in RPMI-1640 medium plus 0.4% FBS for 72–96 h.

Growth-inhibition assay.— To assay for growth-inhibitory activity, $5–10 \cdot 10^3$ HUVSMC were plated into 24-well plates in RPMI-1640 medium plus 10% FBS. After 4 h, aliquots of stock solutions of the appropriate test substance or of the vehicle only (control cultures) were added. Cells were kept for up to 7 d without changing the medium. On the desired days, cell numbers were measured in triplicate samples by washing the cells with phosphate-buffered solution (PBS) and then incubating the cells for several min at 37° in trypsin–ethylenediamine tetraacetate solution, to dislodge the cells. The dislodged cells were then fixed with glutaraldehyde and counted in a Coulter counter. Trypsinized cells were routinely monitored by direct microscopic examination to ascertain complete detachment. In some experiments, 24 h after seeding, the cultures were growth-arrested as described above. The cells were then released from growth arrest by placing them into RPMI-1640 plus 10% FBS, with or without the inhibitors, at the desired concentrations. The net growth of HUVSMC in control cultures was obtained by subtracting the starting cell number (on the day after seeding or at the time the cells were released from G_0) from the cell number on the desired day. The net growth of HUVSMC in inhibitor-containing medium was computed in a similar fashion. The degree of inhibition was determined from the equation, % inhibition = (1 − net growth in inhibitor/net growth in controls) × 100. The IC_{50}-values for the antiproliferative activity of the individual inhibitors were extrapolated from semilogarithmic plots.

Anticoagulant activity of sulfated carbohydrates. — The anticoagulant activity of the saccharides was determined by an aPTT (activated partial thromboplastin time) and an anti-Xa clotting assay.

For the aPTT assay[38], citrated human plasma (100 μL) containing various concentrations of the sulfated carbohydrates (1-4 μg/mL) or heparin (0.5–2 μg/mL) was mixed with Activated Thrombofax (100μL; Ortho Diagnostics, Raritan, NJ, U.S.A.) for 8 min at 37°. Prewarmed 25 mM $CaCl_2$ (100 μL) was added and the clotting time was registered by use of a semiautomatic Fibrometer coagulation timer (Becton Dickinson AG, Basel, Switzerland).

For the anti-Xa clotting assay[39], citrated plasma (25μL) containing various

concentrations of heparin fractions was mixed with 75 μL of factor Xa (Diagnostic Reagents, Thame, Oxon, Great Britain), diluted 1:100 in buffer (0.63% trisodium citrate, 41mM imidazole, 82 mM NaCl, and 0.1% bovine serum albumin, pH 7.3). After prewarming for 2 min at 37°, a 1:1 mixture of factor X–deficient plasma and platelet substitute (both from Diagnostic Reagents) (200 μL) was added and incubated for 20 s. Clotting time was determined in the Fibrometer upon adding prewarmed 50 mM $CaCl_2$ (100 μL).

The activities (IU/mg) of the heparin fractions and of the unfractioned heparin were read from standard curves obtained with nine concentrations (0–0.4 IU/mL, 0–2.3 μg/mL) of the 3rd International Standard for Heparin (173.4 IU/mg) in the aPTT and anti-Xa assays. The values obtained with four different concentrations of each fraction were averaged and assigned a potency in IU/mg. The values reported in Table II are the means of potencies found from two independent determinations.

Modified heparins and Trestatin sulfate showed nonparallel dose response curves with the International Standard for Heparin. Therefore, the activities of these carbohydrates are not expressed in IU of heparin, but characterized by the IC_{50} indicating the concentration of the compound leading to a clotting time of twice the control.

Inhibition of thrombin or of factor Xa determined by chromogenic substrate assays. — Direct inhibition by the heparin fractions of thrombin and of factor Xa was measured also by use of chromogenic substrates. The compounds, dissolved in plasma with a surplus of antithrombin III, were incubated with the enzyme. Residual amidolytic activity for the chromogenic substrates HD-Phe-Pip-Arg-NH·pNa (S-2238) (Kabi Diagnostica, Möndal, Sweden) and BzlCO-Ile-Glu-Gly-Arg-NH·pNa (S-2222) was determined according to Teien *et al.*[40] in a Cobas-Bio centrifugal automatic spectrophotometer (Roche Diagnostica, Basel, Switzerland).

The buffer used consisted of 50mM Tris, 175mM NaCl, 7.5mM EDTA·Na_2, and 0.1% bovine serum albumin, pH 8.4. The sample consisted of buffer (50 μL), antithrombin III (30 μL, 1 unit/mL; Kabi Diagnostica), and plasma containing various cencentrations of the sulfated carbohydrates (20 μL). To measure the inhibition of thrombin, the Cobas-Bio analyzer pipetted automatically into the test cuvette 30 μL of the sample and 20 μL of water, and mixed it with 180 μL of thrombin (1 unit/mL in buffer, Thrombin reagent Roche Diagnostica). After an incubation for 240 s at 37°, 0.75mM S-2238 in water (60 μL) plus water (20 μL) were added as starting reagent. The release of 4-nitroaniline was followed at 405 nm at 10-s intervals for 60 min in comparison to a water blank. The inhibition of factor Xa was measured likewise with bovine factor Xa (Boehringer Mannheim AG, FRG, at 2.8 units/mL) and S-2222 (Kabi Diagnostica at 2mM in water), respectively.

The thrombin and factor Xa inhibitory potencies of the sulfated carbohydrates were read from the nonlinear standard curves obtained with six concentrations (0–0.5 unit/mL) of the 3rd International Standard for Heparin by use of the computerized DENS1-Program (Data Evaluation of Nonlinear Standard curves) of the Cobas-Bio analyzer similar to the clotting assays mentioned above.

Again, owing to the nonparallelism to the heparin standard, the inhibitory

potencies of modified heparins and Trestatin sulfate were expressed as the IC_{50} (μg/mL), *i.e*, the concentration reducing the amidolytic activity of thrombin or factor Xa by 50% as compared to the plasma control sample.

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