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Chemical modifications of heparin that diminish its anticoagulant but preserve its heparanase-inhibitory, angiostatic, anti-tumor and anti-metastatic properties

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Structural features of heparin potentially important for heparanase-inhibitory activity were examined by measuring the ability of heparin derivatives to affect the degradation of [^{3}H]acetylated heparan sulphate by tumor cell heparanases. IC_{50} values were determined using an assay which distinguished degraded from undegraded substrate by precipitation of the latter with cetylpyridinium chloride (CPC). Removal of heparin's 2-O-sulphate and 3-O-sulphate groups enhanced heparanase-inhibitory activity (50%). Removal of its carboxyl groups slightly lowered the activity (18%), while combining the treatments abolished the activity. At least one negative charge on the iduronic acid/idose moiety, therefore, is necessary for heparanase-inhibitory activity. Replacing heparin's N-sulphate groups with N-acetyl groups reduced its activity (37%). Comparing this heparin derivative with 2,3-O-desulphated heparin, the placement of sulphate groups appears important for activity since the two structures have similar nominal linear charge density. In addition, unsubstituted uronic acids are nonessential for inhibition since their modification (periodate-oxidation/borohydride-reduction) enhanced rather than reduced heparanase-inhibitory activity. The most effective heparanase inhibitors (2,3-O-desulphated heparin, and [periodate-oxidized, borohydride-reduced] heparin) were tested in the chick chorioallantoic membrane (CAM) bioassay for anti-angiogenic activity and found to be at least as efficacious as heparin. 2,3-O-desulphated heparin also significantly decreased the tumor growth of a subcutaneous human pancreatic (Ca-Pan-2) adenocarcinoma in nude mice and prolonged the survival times of C57BL/6N mice in a B16-F10 melanoma experimental lung metastasis assay.

Key words: angiogenesis/chemically-modified heparins/endoglycosidase/heparan sulphate/cancer

Introduction

Heparin and heparan sulphate (HS) constitute a class of glycosaminoglycans (GAGs) which can modulate a plethora of cellular functions (e.g., growth, morphology, migration, etc.) by interacting with extracellular matrix (ECM) proteins (Kjellén and Lindahl, 1991), growth factors (Bobik and Campbell, 1993), growth factor high-affinity receptors (Spivak-Kroizman *et al.*, 1994), enzymes (including leukocyte proteases) (Redini *et al.*, 1988) and proinflammatory mediators (Miller and Krangel, 1992). These complex carbohydrates are most often found attached to serine residues of proteins to form proteoglycans. Heparan sulphate proteoglycans (HSPGs) are distributed in basement membranes and stromal matrices, and they are associated with almost all cell surfaces. Through their HS chains, they interact with numerous factors to regulate cell and tissue-specific events such as blood coagulation (Marcum and Rosenberg, 1989), leukocyte activation (Tanaka *et al.*, 1993), cell motility (Makabe *et al.*, 1990) and cell proliferation (Rapraeger *et al.*, 1991).

The heparanases, a family of endoglycosidases which hydrolyze internal glycosidic linkages of HS and heparin chains, can be found in a variety of tissues, and in normal and malignant blood-borne cells (Nakajima *et al.*, 1988; Vlodavsky *et al.*, 1992a). These enzymes have been postulated to participate in new blood vessel formation (angiogenesis) by releasing heparin-binding growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) from the ECM (Bashkin *et al.*, 1989) and to complement the activities of serine proteases and matrix metalloproteases in remodeling subendothelial basement membrane and ECM (Blood and Zetter, 1990). It has also been postulated that metastatic tumor cells egress from the vasculature by utilizing matrix-degrading enzymes (endoglycosidases and proteases) to invade subendothelial basement membrane, ECM and interstitial stroma (Liotta *et al.*, 1991). This invasive phenotype has been shown to correlate with increased levels of heparanase activity in a number of metastatic tumor cell variants (Nakajima *et al.*, 1988).

Heparin has been shown to exhibit heparanase-inhibitory activity *in vitro* (Irimura *et al.*, 1986), to inhibit endothelial cell (EC) ECM HSPG degradation (Bar-Ner *et al.*, 1987; Parish *et al.*, 1987), and to block the dissemination of metastatic tumor cells to the lungs (Irimura *et al.*, 1986). Heparin can be administered only at relatively low concentration *in vivo* due to its anticoagulant potency and potential for inducing hemorrhagic complications (Levine *et al.*, 1989). To enable the administration of drug to higher concentrations without risking excessive bleeding, a number of heparin derivatives with low anticoagulant potency were previously generated and evaluated for heparanase-inhibitory activity. These heparin derivatives, i.e., carboxyl-reduced heparin, [N-O-desulphated, N-resulphated] heparin, and [N-desulphated, N-reacetylated] heparin were shown to inhibit heparanase activity *in vitro* (Irimura *et al.*, 1986), to prevent degradation of ECM HSPG from EC monolayers (Bar-Ner *et al.*, 1987), and to significantly reduce lung colonization by metastatic B16-BL6 melanoma cells (Irimura *et al.*, 1986).

Table I. Molecular weights and disaccharide compositions of heparin derivatives

Heparin derivative	Weight Average MW[a] (kDa)	GM + IM[b]	GMS + GSM[b]	IMS[b]	ISM[b]	GMS_2[b]	ISMS[b]
Heparin	12.5	6.6	11.5	8.8	9.9	4.5	58.7
[Periodate-oxidized, borohydride-reduced] heparin	11.5	0	<1.0	<1.0	9.0	0	61 0
2,3-O-desulfated heparin	10.2	15.5	12.7	66.6	0	0	<1.0
[N-deacetylated, N-resulfated] 2.3-O-desulfated heparin	10.5	16.1	17.8	64.1	0	<1.0	<1.0
[N-desulfated, N-reacetylated] 2,3-O-desulfated heparin	10.5	15.0	16.0	66.1	0	<1.0	1.4
[N-desulfated, N-reacetylated] heparin	12.2	8.0	11.7	9.6	9.3	<3.5	57.9
Selectively 6-O-desulfated heparin	11.5	33 0	6.0	7.0	48.0	0	2.0

[a]Weight average molecular weights were determined as described in 'Materials and methods'.
[b]The heparin derivatives were depolymerized to their constituent disaccharides (Guo and Conrad, 1989), reduced with [^{3}H]sodium borohydride (Shively and Conrad, 1976) and the disaccharide composition determined by reversed-phase ion-pairing high-performance liquid chromatography (Guo and Conrad, 1988). Values represent the percent of the total disaccharide produced after depolymerization of the heparin derivative. GM, GlcUAβ1-4aMan$_R$; IM, IdoAα1-4aMan$_R$; GMS, GlcUAβ1-4aMan$_R$ (6-OSO_3); GSM, GlcUA(2-OSO_3)β1-4aMan$_R$, IMS, IdoAα1-4aMan$_R$(6-OSO_3), ISM, IdoA(2-OSO_3)α1-4aMan$_R$, GMS_2, GlcUAβ1-4aMan$_R$(3,6,-di-OSO_3); ISMS, IdoA(2-OSO_3)α1-4aMan$_R$(6-OSO_3); aMan$_R$, 2,5-anhydro-D-mannitol.

In this study a new series of chemically-modified heparins were evaluated for heparanase-inhibitory activity using size-exclusion HPLC and a CPC precipitation assay. These six heparin derivatives included: (1) 2,3-O-desulphated heparin, (2) [N-deacetylated, N-resulphated] 2,3-O desulphated heparin, (3) [N-desulphated, N-reacetylated] 2,3-O desulphated heparin, (4) carboxyl-reduced 2,3-O-desulphated heparin, (5) preferentially 6-O-desulphated heparin, and (6) [periodate-oxidized, borohydride-reduced] heparin. They were prepared and evaluated to further elucidate the functionalities and domains in heparin that are needed for activity. The most effective heparanase inhibitors from these assays were also evaluated in an *in vivo* angiogenesis assay, the subcutaneous (s.c.) human pancreatic tumor xenograft assay, and the B16-F10 melanoma experimental lung metastasis assay. All of the heparin derivatives were also assayed for their anticoagulant potency relative to heparin.

Results

Hepatoma heparanase hydrolysis of pancreatic HS

The rate of heparanase hydrolysis of [^{3}H]acetylated pancreatic HS substrate measured using the CPC precipitation assay remained constant for 25 min (i.e., the assay is linear over this time period) and was unaffected by lysosomal exohydrolase inhibitors for α-iduronidase and iduronidase-2-sulphatase (data not shown). The apparent absence of these exohydrolase activities might be attributed to the assay pH of 5.2 (optimum pH for enzyme activity, data not shown) and/or the short incubation time. To demonstrate that the time-dependent increase in soluble CPM was enzymatically-generated, hepatoma soluble extracts were boiled and assayed for heparanase activity. This treatment inhibited greater than 95% of substrate hydrolysis (data not shown).

With the CPC precipitation assay, the proportion of HS substrate hydrolyzed to ~ 7 kDa by the endoglycosidase(s) can be determined, but the size of the enzymatically-cleaved HS structures or the number of heparanase-cleavage sites in HS is not ascertained. To obtain this information, size-exclusion HPLC was utilized. Size-exclusion HPLC analysis of HS structures generated when [^{3}H]acetylated pancreatic HS was incubated with soluble hepatoma extracts for 0, 10, 15 or 20 min are shown in Figure 1. The HS chains are typically cleaved in half; going from a weight average MW of 13 kDa to 7 kDa over the 20 min incubation period. This result is consistent with a 'halving' of HS structures by heparanases from other sources (Kjellén *et al.* 1985; Yanagishita and Hascall, 1992). There was very little generation of smaller-sized oligosaccharides formed even at the later time points (see disaccharide std. in Figure 1). Under these conditions, most of the substrate remains uncleaved as seen in Figure 1. (i.e., less than 16% of the total CPM after a 20 min incubation is shifted).

Comparison of heparanase activity measurements: CPC precipitation assay versus size-exclusion HPLC

Heparanase-inhibitory activity of heparin was determined both by the CPC precipitation assay and by size-exclusion HPLC as described in 'Materials and methods.' In the CPC precipitation assay, 4 μg/mL of heparin reduced the non-precipitable CPM by 50% (see Figure 2A), which is comparable to the 61% decrease in the amount of heparanase-cleaved HS structures as measured by size-exclusion HPLC (see Figure 2B). Subsequent measurements of heparanase-inhibitory activity of test compounds were determined with the heparanase CPC precipitation assay.

Evaluation of heparin derivatives and chondroitin sulphates as 'inhibitors' of heparanase activity

Heparin derivatives and chondroitin sulphates assayed for heparanase-inhibitory activity (i.e., IC_{50} values for each test compound and its associated heparin reference) are listed along with their anticoagulant potencies in Table II. In addition, the percent of heparanase inhibition by the test compound with respect to heparin is included. The most

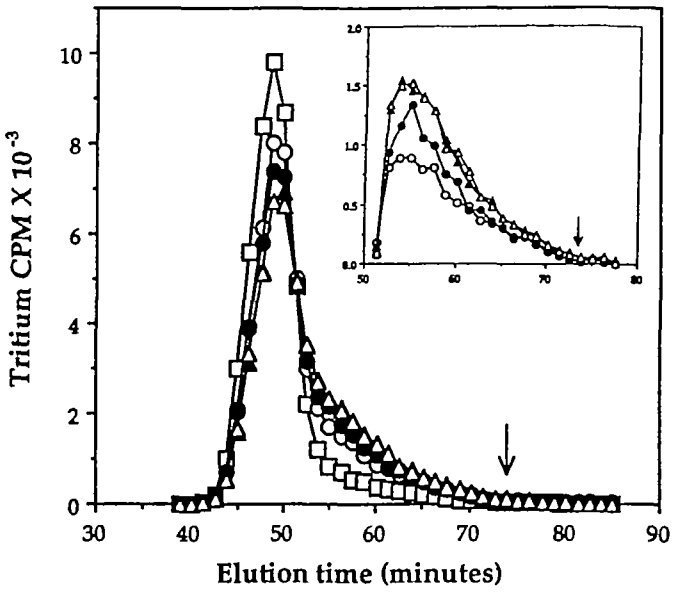

Fig. 1. Size-exclusion HPLC profiles of heparanase-cleaved [^{3}H]acetylated pancreatic HS. Hepatoma soluble extracts (10 μg protein as measured by the Macro BCA assay) were incubated with [^{3}H]acetylated pancreatic HS (250 ng, 80,000 CPM) for 0, 10, 15 or 20 min with lysosomal exohydrolase inhibitors ISMS and GlcNAc (final concentrations of 200 μg/mL and 600 μg/mL, respectively) or for 20 min without these inhibitors in a 37°C water-bath. Size-exclusion HPLC profiles of samples taken at 0 (□), 10 (○), 15 (●), and 20 min (▲) with inhibitors and at 20 min (△) in the absence of inhibitors are shown. Inset: the profiles for 10, 15 and 20 min with inhibitors and 20 min without inhibitors were subtracted from the 0 min profile with inhibitor. The 0 min profiles with and without inhibitors were indistinguishable (data not shown). The peaks were integrated and the areas (total CPM) of the 10, 15, and 20 min profiles from 51 to 70 min were determined to be 7.80, 9.45 and 12.45 × 10^3 CPM (or 780, 630 and 625 CPM released per min), respectively. The total CPM for the 20 min profiles with and without exohydrolase inhibitors were identical. The arrows indicate the elution time of nitrous acid-depolymerized heparin disaccharides.

efficacious heparanase inhibitors examined were [periodate-oxidized, borohydride-reduced] heparin (I) and the 2,3-O-desulphated heparins (III and IV). These chemically-modified heparins were ≥ 1.5-times more potent than heparin in heparanase-inhibitory activity. The data in Table II also indicate that the size of the 2,3-O-desulphated heparin can be decreased from 10.2 to 5 kDa without adversely influencing the inhibitory activity (i.e., III vs. IV). But, reduction of the carboxylic acids of heparin to alcohols increased the IC_{50} value by 18%. N-desulphation of heparin followed by N-reacetylation decreased the heparanase-inhibitory activity by 37%. Preferential desulphation of the 6-O-sulphate groups on heparin caused no measurable change in activity with respect to heparin. Removal of the N-acetyl groups on 2,3-O-desulphated heparin and replacement with N-sulphated groups resulted in a slight loss of activity. A more dramatic decrease in the activity of the 2,3-O-desulphated heparin was measured after N-desulphation of the polymer followed by N-reacetylation. The most intriguing result from the selective chemical modifications involved the carboxyl-reduced 2,3-O-desulphated heparin which completely lacked heparanase-inhibitory activity. Neither chondroitin 4-O-sulphate nor chondroitin 6-O-sulphate were effective as heparanase inhibitors.

Evaluation of heparin and 2,3-O-desulphated heparin as competing substrates for heparanase

To establish by which means the heparin derivative 2,3-O-desulphated heparin (which is > 1.5 times more potent than heparin as a heparanase inhibitor) was affecting heparanase activity, similarly-sized [^{3}H]heparin, [^{3}H]2,3-O-desulphated heparin and [^{3}H]pancreatic HS were examined as substrates for the hepatoma heparanase by reducing newly-formed reducing ends with B^3H_4. [^{3}H]heparin and [^{3}H]2,3-O-desulphated heparin are hydrolyzed by heparanase, but at initial rates approximately one-half that of [^{3}H]pancreatic HS (see Figure 3).

Determination of the angiostatic activity of heparin derivatives in the CAM bioassay

The most active heparanase inhibitors, [periodate-oxidized, borohydride-reduced] heparin and 2,3-O-desulphated heparin, were evaluated in the CAM assay to assess their ability to inhibit angiogenesis. As shown in Table III, in combination with hydrocortisone, both chemically-modified heparins were as effective as heparin in inhibiting neovascularization.

Determination of the anti-tumor potential of 2,3-O-desulphated heparin in a subcutaneous human CaPan-2 adenocarcinoma in nude mice

As shown in Figure 4, 2,3-O-desulphated heparin significantly reduced the tumor growth of a multidrug-resistant human pancreatic (CaPan-2) adenocarcinoma in nude mice ($p < 0.05$). The heparin derivative at 100 μg/mL did not inhibit *in vitro* proliferation of CaPan-2 cells during a 96 h incubation at 37°C (data not shown, see 'Materials and methods').

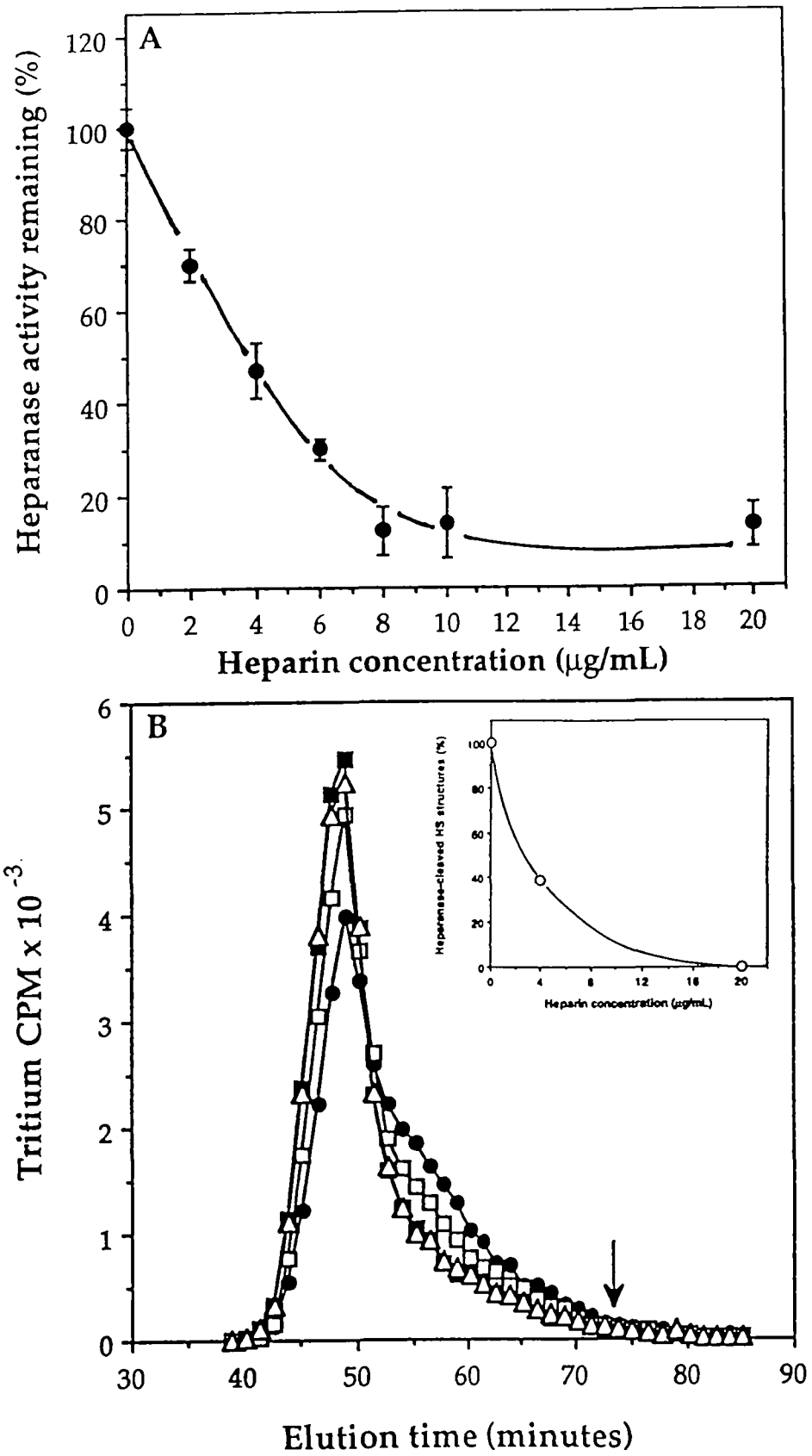

Fig. 2. Inhibition of heparanase activity with heparin. Hepatoma soluble extracts (30 μL, 10 μg protein as measured by the Macro BCA assay), [^{3}H]acetylated pancreatic HS (10 μL, 250 ng, 80,000 CPM) and heparin (10 μL, 0–100 μg/mL) were incubated for 20 min at 37°C. Background CPM (0 μg/mL heparin at 0°C) was determined. Samples were processed using the CPC precipitation assay (panel A) or characterized by size-exclusion HPLC (panel B) as described in 'Materials and methods.' Panel A, values represent the difference of the means of two populations with sample size 3 ± the square root of the sum of their variances. For each concentration of heparin examined, the difference of the means of soluble CPM measured at 20 min and background CPM was calculated (n = 3) as well as the square root of the sum of their variances. The data were converted to % heparanase activity remaining ± the standard deviation, with the control containing no heparin being used as 100% heparanase activity. Panel B, heparin at 0 (●), 4 (□) and 20 (△) μg/mL at 37°C or 0 μg/mL at 0°C (■). Inset: the profiles from 51 to 70 min for 0, 4 and 20 μg/mL heparin at 37°C were subtracted from the background profile and the resulting peaks were integrated. The areas (total CPM) for the 0, 4 and 20 μg/mL heparin samples were 6039, 2341 and 0 CPM, respectively. The data were converted to % heparanase-cleaved HS structures with the control containing no heparin being used as 100% heparanase activity.

Table II. Heparanase-inhibitory activities of heparin derivatives and chondroitin sulfates

	Glycosaminoglycan	Weight Average MW[a] (kDa)	Anticoagulant Potency[a] (% relative to heparin)	Heparin IC_{50}[b] (μg/mL)	GAG IC_{50}[b] (μg/mL)	Heparanase inhibition[c] (%)
I	[Periodate-oxidized, borohydride-reduced] heparin	11.5	10–14	7.3 ± 0.6	5.0 ± 0.9 (4)	146
II	Carboxyl-reduced heparin	11.5	5	7.8 ± 0.7	9.5 ± 0.7 (3)	82
III	2,3-O-desulfated heparin	10.2	8–12	6.4 ± 0.3	3.9 ± 0.4 (8)	164
IV	2,3-O-desulfated heparin	5.0	2–4	7.4 ± 0.6	4.8 ± 0.5 (5)	154
V	[N-deacetylated, N-resulfated] 2,3-O-desulfated heparin	10.5	55	6.6 ± 0.5	6.7 ± 0.1 (3)	98
VI	[N-desulfated, N-reacetylated] 2,3-O-desulfated heparin	10.5	<1	7.0 ± 0.2	23.5 ± 2.5 (4)	30
VII	[N-desulfated, N-reacetylated] heparin	12.2	3–4	7.1 ± 0.2	11.2 ± 1.0 (3)	63
VIII	Carboxyl-reduced, 2,3-O-desulfated heparin	10.0	<1	8.0 ± 0.8	104.3 ± 10.7 (3)	8
IX	Selectively 6-O-desulfated heparin	11.5	5–15	5.5 ± 0.7	6.0 ± 0.6 (4)	92
X	Chondroitin 4-O-sulfate	15.3	<1	5.8 ± 0.6	138.3 ± 27.2 (3)	4
XI	Chondroitin 6-O-sulfate	18.5	<1	5.8 ± 0.6	56.0 ± 9.2 (3)	10

[a]Weight average molecular weights and anticoagulant potencies were determined as described in 'Materials and methods'.
[b]Soluble hepatoma extracts were incubated with [^{3}H]acetylated pancreatic HS and increasing concentrations of heparin derivative or chondroitin sulfate. Heparin (M_w = 12.5 kDa) was also assayed with each compound as a positive reference. Samples were processed using the CPC precipitation assay as described in 'Materials and methods'. Number of experiments performed for each compound is shown in the parantheses. Values were determined from plots of % heparanase activity remaining versus concentration of compound and are expressed as the mean ± standard error of the mean.
[c]Heparanase inhibition = (Heparin IC_{50}/GAG IC_{50} * 100).

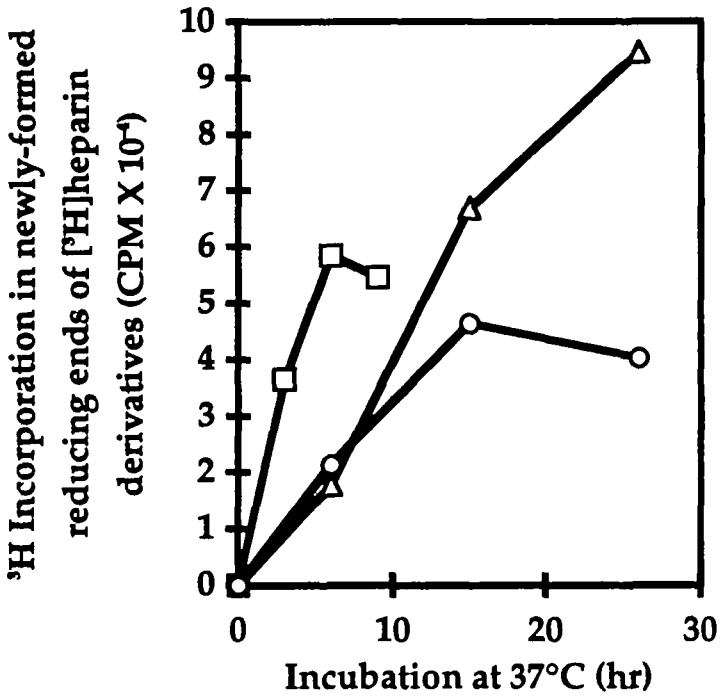

Fig. 3. Time course of heparanase hydrolysis of [^{3}H]pancreatic HS, [^{3}H] heparin, and [^{3}H]2,3-O-desulphated heparin. Soluble hepatoma extracts (50 μg protein as measured by the Macro BCA assay) were incubated with [^{3}H]heparin derivatives (5.0 X 10^4 CPM, ~ 5 μg) and lysosomal exohydrolase inhibitors [D-saccharic acid 1,4-lactone (1921 μg/mL) and ISMS (200 μg/mL)] for the indicated times at 37°C. The amount of radioactivity incorporated into [^{3}H]pancreatic HS (□), [^{3}H]heparin (△), and [^{3}H]2,3-O-desulphated heparin (○) by [^{3}H]sodium borohydride-reduction of the newly-formed reducing ends is shown. The CPM determined for [^{3}H]pancreatic HS, [^{3}H]heparin, and [^{3}H]2,3-O-desulphated heparin incubated with hepatoma heparanase at time = 0 and following [^{3}H]sodium borohydride reduction and subsequent steps described in 'Materials and methods' was 4.8, 4.8 and 5.2 × 10^4 CPM, respectively.

Table III. Angiostatic activities of heparin derivatives in the CAM bioassay[a]

Sample	No Effect	+	++
Buffer control	61 (100)	0	0
Hydrocortisone (50 μg/pellet)	47 (94)	3 (6)	0
Hydrocortisone + heparin (μg/pellet)			
50	5 (15)	11 (33)	17 (52)
25	9 (36)	8 (32)	8 (32)
12.5	13 (52)	10 (40)	2 (8)
6	18 (78)	5 (22)	0
3	23 (92)	2 (8)	0
Hydrocortisone + [periodate-oxidized, borohydride-reduced] heparin (μg/pellet)			
50	7 (8)	27 (30)	56 (62)
25	11 (24)	19 (41)	16 (35)
12.5	10 (23)	21 (49)	12 (28)
6	21 (58)	13 (36)	2 (6)
3	21 (78)	5 (18)	1 (4)
Hydrocortisone + 2,3-O-desulfated heparin (μg/pellet)			
50	12 (40)	13 (43)	5 (17)
25	14 (41)	10 (29)	10 (29)
12.5	9 (36)	10 (40)	6 (24)
6	16 (59)	9 (33)	2 (7)
3	20 (83)	4 (17)	0

[a]The CAM assay for evaluating angiostatic activity was described previously (Castellot *et al.*, 1986). The CAMs were scored at 24 h after placement of pellets, using a dissecting microscope to examine the microvasculature surrounding each pellet. If a completely avascular zone was observed more than 3/4 of the way around the pellet, it was scored a ++. A + score indicates that either the overall vascularity was significantly reduced, or that an avascular zone existed around 1/4–3/4 of the pellet. No effect indicates that no change in vascularity of the area immediately surrounding the pellet was observed. Heparin, [periodate-oxidized, borohydride-reduced] heparin and 2,3-O-desulfated heparin are inactive in the absence of hydrocortisone (50 μg/pellet). Numbers in parentheses represent percent of total score for compound at designated concentration (μg/pellet).

Determination of the anti-metastatic activity of chemically-modified heparins in a murine experimental lung metastasis assay

Mice challenged with B16-F10 melanoma cells and treated with 2,3-O-desulphated heparin showed a significant increase in survival times at the higher doses (i.e., 50 and 100 mg/kg, $p < 0.05$) with respect to the vehicle control (Figure 5). Few lung metastases (3 to 10 tumor foci < 3 mm in diameter) were detected in one-third of the animals from the treatment groups which were sacrificed on the last day of the study, while numerous lung metastases were present in all animals of the vehicle control group. As observed with the CaPan-2 cells, 2,3-O-desulphated heparin (100 μg/mL, 96 h) had no inhibitory effect on the *in vitro* proliferation of B16-F10 melanoma cells (data not shown). Heparanase activity expressed by this B16-F10 melanoma subline also was inhibited with 2,3-O-desulphated heparin as effectively as observed for hepatoma heparanase (data not shown).

Bleeding problems with [periodate-oxidized, borohydride-reduced] heparin prohibited administration of this compound at doses that might have shown significant efficacy in either the s.c. CaPan-2 tumor xenograft and/or the B16-F10 melanoma experimental lung metastasis assays. With respect to heparin, this heparin derivative possesses elevated antiplatelet and equivalent heparin cofactor II activities (data not shown). This might account for the hemorrhagic complications observed in the animals.

Discussion

Heparanases have been implicated in tumor cell proliferation, metastasis, and tumor neovascularization. A quantitative assay for heparanase was developed to assess which chemical groups in heparin, a well-documented 'inhibitor' of heparanases (Nakajima *et al.*, 1988), might contribute to heparanase-inhibitory activity. Such information might lead to the development of better heparanase inhibitors.

Two chemically modified heparins were shown to have better heparanase-inhibitory activity than heparin: 2,3-O-desulphated heparin and [periodate-oxidized, borohydride-reduced] heparin. Both these heparinoids exhibited low anticoagulant potency indicating, as in prior studies (Irimura *et al.*, 1986; Bar-Ner *et al.*, 1987), that anticoagulant activity is not required for heparanase-inhibitory activity. In the case of the former, it retains heparanase-inhibitory activity even with the substantial loss of sulphate groups (i.e., 2- and 3–0 sulphates), suggesting that a specific, not simply a polyanionic, heparin sequence or set of sequences is required for enzyme inhibition.

Results from this study also indicate that 2,3-O-desulphated heparin still retains the structural epitopes of heparin that are required for it to interact with the catalytic site of heparanase and function as a competitive inhibitor/substrate. In fact, it should be noted that the removal of the 2- and 3-O-sulphates in heparin should increase the number of potential tetrasaccharide sequences proposed as the backbone 'recognition sequence' for the hepatocyte and platelet heparanases: $GlcNSO_3 \rightarrow$ uronic acid $\rightarrow$ GlcNAc $\rightarrow$ GlcUA (Oldberg *et al.*, 1980; Oosta *et al.*, 1982; Kjellén *et al.*, 1985). However, similar to heparin this heparin derivative is hydrolyzed slower (~ 50%) than the HS substrate, suggesting that additional structural epitopes that favor catalysis must be present in HS. In addition, 2,3-O-desulphated heparin might also function as a noncompetitive inhibitor similar to the tight-binding hyperbolic non-competitive inhibition described previously for heparin's inhibition of leukocyte elastase (Redini *et al.*, 1988). However, characterization of the type of 'inhibition' exerted on heparanase by heparin and other heparin derivatives must await examination with a purified enzyme.

A lower MW 2,3-O-desulphated heparin (5 kDa) exhibited activity comparable to the 10.2 kDa molecule, indicating that the required sequence(s) for heparanase-inhibitory activity is likely to span less than 5 kDa. Recently, researchers reported heparanase-inhibitory activity in heparin-derived oligosaccharides of this MW range (Bitan *et al.*, 1995). Lower MW heparins have also been shown to be comparable to heparin as inhibitors of neutrophil heparanase (Bar-Ner *et al.*, 1987).

Chemical modifications of heparin in which 6-O-sulphate or 2,3-O-sulphate groups are removed does not cause any deleterious effects on heparanase-inhibitory activity. However, the loss of 2,3-O-sulphate groups in combination with reduction of the carboxylate groups produce an inactive inhibitor. Since the most noteworthy result of these combined chemical treatments is the formation of idose,

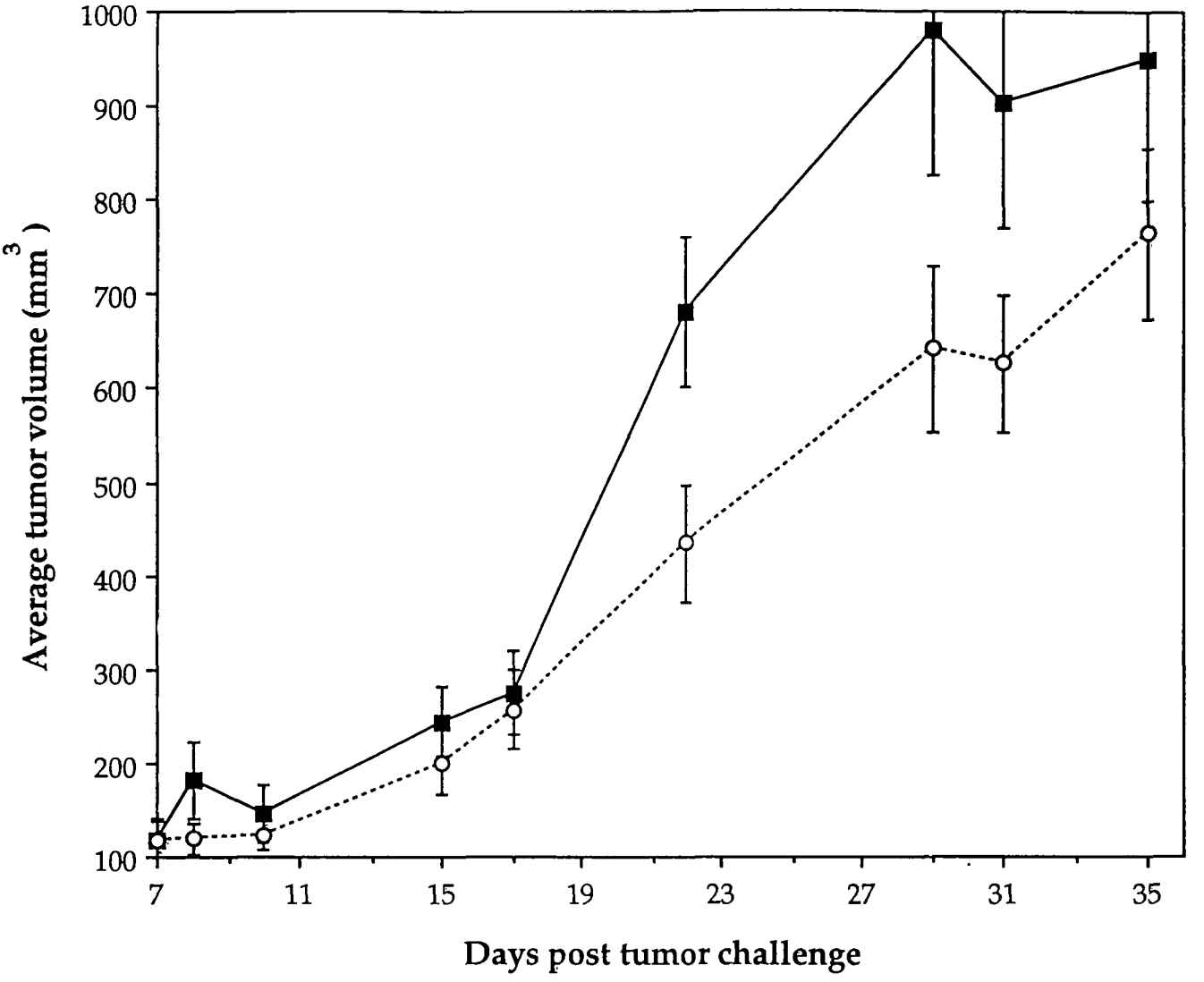

Fig. 4. Effects of 2,3-O-desulphated heparin on subcutaneous tumor growth of the human pancreatic (CaPan-2) adenocarcinoma in nude mice Male Balb/c athymic mice (10 animals per treatment group) were challenged with 3 × 10^6/0.1 mL of CaPan-2 cells s c. After 24 h, the mice were dosed each day for the duration of the experiment with either PBS vehicle control (■) or 2,3-O-desulphated heparin (○) administered as a single dose of 60 mg/kg s.c.. Mean tumor volumes are shown ± SE An Analysis of Variance SAS JMP Version 3 was used to analyze the data. When compared to the vehicle, the heparin derivative significantly inhibited the growth of the CaPan-2 tumor ($p < 0.05$).

it would appear that having at least one negative charge on the Idose/IdoA moieties is a structural requirement for heparanase inhibition.

In comparing [N-desulphated, N-reacetylated] heparin and 2,3-O-desulphated heparin as heparanase inhibitors, the latter is much more effective at inhibiting heparanase activity, even though the linear sulphate densities for the two heparin derivatives are roughly equivalent. These results suggest that the placement or orientation of sulphate groups is important, in particular, N-sulphate groups on glucosamines appear more critical than O-sulphate groups on IdoA.

The loss of the 2- and 3-O-sufate groups in combination with N-desulphation/N-reacetylation of GlcN residues in heparin generates a 6-O-sulphated heparin derivative exhibiting low heparanase-inhibitory activity (compare the heparanase-inhibitory activities of [N-desulphated, N-reacetylated] 2,3-O-desulphated heparin (30%) with [N-desulphated, N-reacetylated] heparin (63%) versus heparin (100%)). In contrast, the activity of [N-deacetylated, N-resulphated] 2,3-O-desulphated heparin was comparable to heparin. Based on these data, the activity loss following reacetylation of free amino groups on 2,3-O-desulphated heparin might be explained by a simple decrease in overall sulphation of the heparin derivative or might suggest that an important heparanase-inhibitory structural feature of heparin is N-sulphation of GlcN.

The formation of blood vessels is essential for tumor growth and also facilitates the dissemination of tumor cells to distant sites in the host. Heparanases can contribute to tumor angiogenesis by participating in hydrolysis of the basement membrane of endothelial cells and the ECM (Nakajima *et al.*, 1988; Blood and Zetter, 1990). In this report, heparin derivatives (2,3-O-desulphated heparin and [periodate-oxidized, borohydride-reduced] heparin) exhibited angiostatic activity in the CAM bioassay compa-

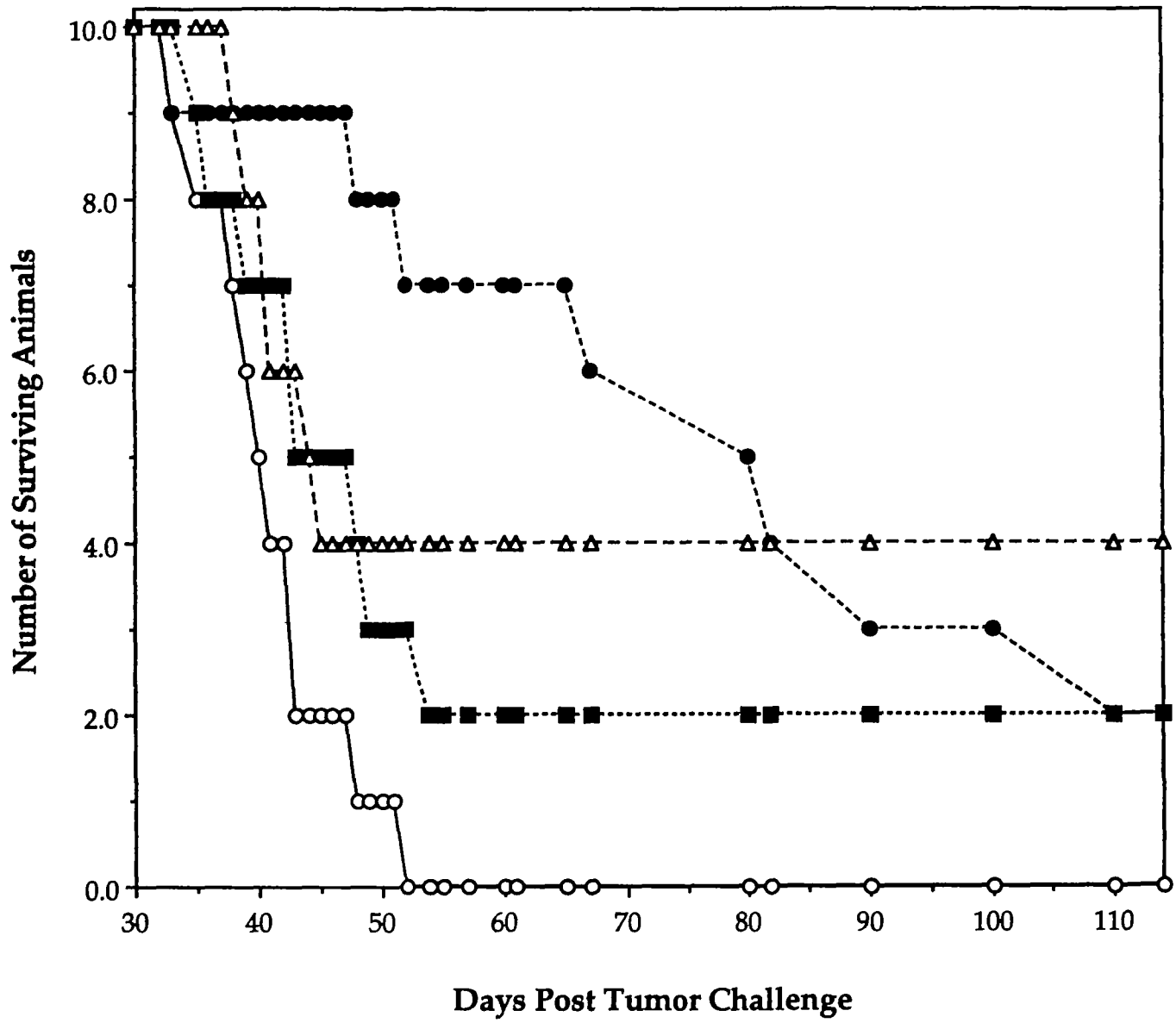

Fig. 5. Effects of escalating doses of 2,3-O-desulphated heparin on the rate of survival of C57BL/6N mice after i.v. challenge with B16-F10 melanoma cells. Female C57BL/6N mice were challenged with 5×10^4/0.1 mL of B16-F10 melanoma cells i.v.. The saline vehicle control or heparin derivative were administered s.c. at a single dose (0.05 mL) from days 0–4 with the first dose occurring one h prior to tumor challenge. Survival times were recorded for animals treated with saline vehicle control (○) and animals dosed with 10 (■), 50 (●), and 100 (△) mg/kg of 2,3-O-desulphated heparin. Results were evaluated with Chi-squared statistical analysis. Doses at 50 and 100 mg/kg significantly increased the survival rate of the mice following challenge with B16-F10 melanoma cells when compared to the vehicle ($p < 0.05$).

rable to heparin (in the presence of hydrocortisone). Both heparin derivatives have lower anticoagulant potency than heparin and 2,3-O-desulphated heparin shows no measurable binding to bFGF (Ishihara *et al.*, 1994), indicating that the mechanism of action of this heparin derivative in attenuating new vessel formation does not require its interaction with this angiogenic factor. However, a heparanase inhibitor might indirectly affect growth factor-stimulated angiogenesis by inhibiting the release of HS-bound angiogenic growth factors sequestered in the ECM (Bashkin *et al.*, 1989).

Additionally, 2,3-O-desulphated heparin significantly reduced the rate of tumor growth of a s.c. CaPan-2 adenocarcinoma in nude mice. This heparin derivative did not affect the growth of CaPan-2 cells in culture, indicating that the compound's anti-tumor activity is not due to a cytotoxic effect on the tumor cells. This result is consistent with the predicted anti-tumor activity profile of this class of compounds. Hence, 2,3-O-desulphated heparin is not expected to cause complete ablation of the tumor, but is expected to decrease the rate of s.c. tumor growth by inhibition of tumor angiogenesis (Folkman and Klagsbrun, 1987), which might at least in part be due to its ability to inhibit heparanase. Heparin fragments with low sulphate content have been shown to inhibit angiogenesis (Hahnenberger *et al.*, 1993) At present, the heparanase-inhibitory activities of such heparin fragments have not been determined, nor have their anti-angiogenic activities been

compared with that of 2,3-O-desulphated heparin. The relationship between heparanase-inhibitory activity and the attenuation of tumor growth by these anti-angiogenic compounds [heparin fragments (Hahnenberger *et al.*, 1993) and 2,3-O-desulphated heparin] might best be delineated through a comparison of their heparanase-inhibitory activities and their effectiveness in decreasing tumor growth in the s.c. CaPan-2 and other s.c. human tumor xenograft assays.

Heparanases also are believed to promote the migration of tumor cells from the vasculature to selective organ parenchyma (Nakajima *et al.*, 1988). In this study, the low anticoagulant, heparin derivative 2,3-O-desulphated heparin, in addition to its presumed inhibition of tumor angiogenesis, also functions as an effective anti-metastatic agent in the B16-F10 melanoma experimental lung metastasis assay. Taken together, these data (i.e., the CAM bioassay, the s.c. CaPan-2 tumor xenograft assay and the B16-F10 melanoma experimental lung metastasis assay) suggest that the heparanase inhibitor 2,3-O-desulphated heparin might have therapeutic benefit for treating certain human cancers.

The high heparanase-inhibitory activity of certain chemically-modified heparins described here and elsewhere (Irimura *et al.*, 1986; Nakajima *et al.*, 1988; Vlodavsky *et al.*, 1992b) has been associated with the ability of these molecules to inhibit angiogenesis, tumor cell growth and tumor cell metastasis. With the synthesis of specific heparanase inhibitors, the association currently noted can be tested and the significance of heparanase function in these pathophysiologies can be more accurately assessed.

Materials and methods

Cells and reagents

The rat hepatoma cell line (Gershenson *et al.*, 1970), was provided by Dr. H. Edward Conrad, University of Illinois. The murine B16-F10 melanoma subline was a generous gift from Dr. Brian Brandley (Department of Pharmacology, Rush-Presbyterian-St. Luke's Medical Center). The multidrug-resistant, human pancreatic adenocarcinoma line (CaPan-2) was obtained from the American Type Tissue Culture Collection. Pancreatic HS was kindly provided by Drs. G. Mascellani and P. Bianchini, Opocrin S.p.a. Laboratoria Farmacobiologico, Corlo (Modena), Italy. Porcine mucosa heparin was obtained from the Ming Han Company (Oakland, CA). Chondroitin sulphate A (bovine trachea) and chondroitin sulphate C (shark cartilage) were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]Sodium borohydride (468.7 mCi/mmol) and [^{3}H]acetic anhydride (500 mCi/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA).

Disaccharide composition of pancreatic HS

Pancreatic HS was depolymerized to its constituent disaccharides (Guo and Conrad, 1989), reduced with [^{3}H]sodium borohydride (Shively and Conrad, 1976) and the disaccharide composition determined as described previously (Conrad, 1980; Shaklee and Conrad, 1984; Guo and Conrad, 1988). The disaccharide composition of pancreatic HS substrate used in this study was 6.2% IM, 62.9% GM, 12.3%, ISM, 8.6% GMS/GSM, 7.5% IMS, and 2.5% ISMS.

Activated partial thromboplastin time (APTT) of heparin derivatives

Clotting times were determined using an ELECTRA® 800 automated coagulation timer (Medical Control, Level I, lyophilized normal human plasma (Baxter Diagnostics Inc., Deerfield, IL), and Dade® Actin® FS Activated PTT Reagent (Baxter Diagnostics). The APTT-200 protocol described in the MLA ELECTRA® 800 operator's manual was closely followed except that 10 μL of heparin derivative in water was added to cuvettes containing 100 μL of plasma just prior to initiation of assay.

Average molecular weight of heparin derivatives and HS chains

Heparin derivatives and HS chains were fractionated according to hydrodynamic size and molecular weight distributions were analyzed using a high-performance size-exclusion chromatography method similar to the one described previously (van Dedem and Nielsen, 1991). Molecular weight assignments were made on the basis of retention time relative to a series of heparin oligosaccharides of known molecular weight obtained from partial depolymerization of heparin with nitrous acid. Collected data were analyzed and the weight average molecular weight (M_w) calculated using the formula $M_w = \Sigma(h_i M_i)/\Sigma h_i$, where h_i is either the refractive index detector response or the ^{3}H radioactivity eluting at retention time i and M_i is the corresponding molecular weight obtained from the calibration curve.

Preparation of [^{3}H]acetylated pancreatic HS

Pancreatic HS was N-deacetylated (Guo and Conrad, 1989) and reacetylated with [^{3}H]acetic anhydride (500 mCi/mmole, 25 mCi) as described previously (Höök *et al.*, 1982). Fractions corresponding to HS structures of 11.5–16 kDa were pooled and recharacterized by size-exclusion chromatography as described above. [^{3}H]Acetylated pancreatic HS preparations with M_w of ~ 13 kDa were used as substrate to quantify heparanase activity.

Preparation of the heparin derivatives

A modified procedure of Jaseja *et al.* was employed to prepare 2,3-O-desulphated heparin (Jaseja *et al.*, 1989). Briefly, 1 g of porcine mucosa heparin was dissolved in 100 mL of 0.2 N NaOH. The solution was then frozen and lyophilized to dryness. The crusty yellow residue was dissolved in 100 mL of deionized water and adjusted to pH 7 with 20% acetic acid. The solution was then dialyzed and lyophilized to give the final product (0.8 g). Carboxyl-reduced heparin was prepared as recently described (Shaklee *et al.*, 1993) using a modification of an earlier procedure (Taylor *et al.*, 1976). It was necessary to carry out the reduction at 0°C for 48 h, with all other steps from the original procedure retained (Taylor *et al.*, 1976). Carboxyl-reduced 2,3-O-desulphated heparin was prepared by combining in sequence the procedures for carboxyl reduction and 2,3-O-desulphation of heparin as described above. Preferentially 6-O-desulphated heparin was prepared by incubating pyridinium heparin in DMSO: water (9:1) at 80°C for 24 h (Nagasawa *et al.*, 1977) to produce an intermediate product lacking N-sulphate groups on GlcN residues and with reduced O-sulphation at the 6-O-positions of glucosamines. The intermediate was converted to the final product by N-resulphation, with trimethylamine-sulphur trioxide complex in alkaline aqueous media (pH 9) (Lloyd *et al.*, 1971). A modified procedure of Rej *et al.* was used to N-acetylate N-desulphated heparin (Rej *et al.*, 1990). This involved treating N-desulphated heparin [prepared by solvolytic treatment of pyridinium heparin in DMSO:water (9:1) at 50–60°C for 1–1.5 h (Inoue and Nagasawa, 1976)] in sodium bicarbonate solution, with multiple aliquots of acetic anhydride and DMF over a period of 24 h. The pH was maintained at neutrality by adding solid sodium bicarbonate as necessary. [N-desulphated, N-reacetylated] 2,3-O desulphated heparin was typically prepared by solvolytic N-desulphation of 2,3-O-desulphated heparin as described for N-desulphation of heparin. N-reacetylation of N-desulphated 2,3-O-desulphated heparin was performed as described above (Rej *et al.*, 1990). [N-deacetylated, N-resulphated] 2,3-O desulphated heparin was prepared by producing 2,3-O-desulphated heparin by alkaline desulphation according to the conditions described earlier. This compound was N-deacetylated (Guo and Conrad, 1989) and then subjected to mild and selective conditions (Lloyd *et al.*, 1971) to sulphate the free amine groups as previously reported for heparin (Ayotte and Perlin, 1986). [Periodate-oxidized, borohydride-reduced] heparin was prepared essentially as described previously (Fransson and Lewis, 1979) with some modifications (Conrad and Guo, 1992). Weight average molecular weights and disaccharide compositions of the heparin derivatives are shown in Table I.

Preparation of soluble extracts from rat hepatoma cells

Rat hepatoma cells in Higuchi's medium were grown to confluence. After removing the conditioned medium, flasks were washed three times with ice-cold 50 mM HEPES, 0.25 M sucrose, 0.14 M NaCl, pH 7.4. To each flask, 1 mL of buffer containing 50 mM MES, pH 5.2, 15 mM D-saccharic acid 1,4-lactone (exoglucuronidase inhibitor), 2 mM EDTA (sodium salt), 1 mM PMSF, 100 μg/mL soybean trypsin inhibitor, 0.5 μg/mL leupeptin,

0.2 μg/mL aprotinin, 0.14 M NaCl and 6 mM NaN_3 (Buffer 1) was added and the cells were dislodged from the flask using a disposable cell scraper. Cells resuspended in Buffer 1 were freezed/thawed three times in an ethanol/dry ice bath, homogenized with fifteen strokes using a tight pestle, transferred to 2 mL microcentrifuge tubes and then spun at 16,000 × g at 4°C for 30 min. The supernatants were pooled and the protein concentration was determined using the Macro BCA assay using BSA as the standard (Pierce, Rockford, IL).

Quantitation of heparanase activity in soluble extracts of rat hepatoma cells using a CPC precipitation assay

The CPC precipitation assay was developed from the observation that heparanase-cleaved HS chains (derived from [^{3}H]pancreatic HS substrate) can be distinguished from uncleaved chains by selectively precipitating the latter with CPC (Oldberg *et al.*, 1980; Bame, 1993) Briefly, this assay is done by combining [^{3}H]acetylated pancreatic HS (10 μL, 250 ng, 80,000 CPM) in 200 mM MES, 0.14 M NaCl pH 5.2, with test compound (10 μL) prepared at concentrations of 0–1000 μg/mL in water. All assays were run in triplicate along with heparin as a positive control. Hepatoma soluble extracts (30 μL) diluted to 333 μg/mL in freshly prepared Buffer 1 were added to each tube (with the exception of those used to measure background CPM, i.e., no test compound) at 0°C. The samples were incubated for 20 min in a 37°C water bath, after which heparin (150 μL, 333 μg/mL) was added to terminate the reaction. The 20 min incubation time was selected after observing that the rate of HS hydrolysis in this system had begun to decrease by 30 min. It was determined that substrate depletion rather than enzyme inactivation caused the decrease in rate since no increase in the rate of substrate cleavage was observed after the addition of fresh enzyme. Furthermore, preincubating the enzyme at 37°C did not affect the rate of HS hydrolysis. Soluble hepatoma extracts were added to each of the background tubes at this time, after which heparin was added. A 100 mM solution of sodium acetate, pH 5 5 (200 μL), was added to each tube followed by a solution of 0.6% CPC w/v in water (100 μL). The tubes were vortexed and incubated for 1 h at ambient temperature, and then centrifuged for 10 min at 4,000 × g in an Eppendorf 5415C microcentrifuge. Supernatant (400 μL) was carefully removed and assayed for ^{3}H by liquid scintillation counting. To verify that the CPC reagent was not depleted at the highest concentration of heparin or test compound used in the assay, 200 μg/mL of compound was incubated for 20 min with hepatoma extracts and [^{3}H]pancreatic HS substrate at 0°C. There was no detectable difference between the soluble CPM from these reaction tubes and background CPM. To examine the effects of other lysosomal exohydrolase inhibitors on heparanase activity in this assay, 10 μL of ISMS (1000 μg/mL) and GlcNAc (3000 μg/mL) in water were added to the assay tubes containing the radiolabeled HS substrate and soluble hepatoma extracts (50 μL total volume). Inhibitor concentrations were chosen to inhibit > 99% of iduronate 2-sulphatase and α-iduronidase activities based upon K_i values for the inhibitors and K_m values of liver iduronate 2-sulphatase for the substrate ISMS, and of liver α-iduronidase for the substrate IMS (Hopwood, 1989; Freeman and Hopwood, 1992).

Characterization of heparanase-cleaved [^{3}H]acetylated pancreatic HS by size-exclusion HPLC

[^{3}H]acetylated pancreatic HS samples were incubated with hepatoma soluble extract for 0, 10, 15 and 20 min, and then the reaction was quenched by adding heparin (150 μL, 333 μg/mL) followed by boiling for 10 min. Samples were filtered through 0.2 mm microcentrifuge filter inserts, spun for 10 min at 16,000 × g, evaporated to dryness under nitrogen, and then redissolved in filtered water (20 μL). Sample aliquots (17 uL) were injected onto the HPLC size-exclusion system described previously. Fractions (375 μL) were collected and assayed for [^{3}H] by liquid scintillation counting. [^{3}H]-labeled heparin hexasaccharide standard was chromatographed to determine the time lag between observing the oligosaccharide using the refractive index detector, and elution of the oligosaccharide as measured by liquid scintillation counting. The CPM for each sample time point was normalized to the sample time point with the highest total CPM in order to compare profiles. Total CPM refers to the sum of the CPM measured from 39–84 min (see Figures 1 and 2B).

Average molecular weight of [^{3}H]pancreatic HS, [^{3}H]heparin and [^{3}H]2,3-O-desulphated heparin

The weight average molecular weights for [^{3}H] sodium borohydride-reduced pancreatic HS, heparin and 2,3-O-desulphated heparin were, respectively: 13.1, 11.3 and 10.9 kDa, based on size-exclusion HPLC using refractive index detection.

Evaluation of heparin and 2,3-O-desulphated heparin as competing heparanase substrates by monitoring the generation of new reducing ends

Soluble hepatoma extract (100 μL, 500 μg/mL protein by Macro BCA assay) in Buffer 1 was combined with ISMS (25 μl of 1200 μg/mL in 200 mM MES, pH 5.2, 140 mM NaCl) to minimize substrate degradation by the lysosomal exohydrolase, iduronate 2-sulphatase (molar equivalents of ISMS to substrate is ~ 100:1). To a series of reaction tubes, aliquots of either [^{3}H]pancreatic HS, [^{3}H]heparin or [^{3}H]2,3-O-desulphated heparin (25 μL, 5.0 × 10^4 CPM, ~ 5 μg) were added, and individual tubes were incubated at 37°C for increasing times. The amount of [^{3}H]heparin derivative added is calculated on the basis of the specific activity of [^{3}H]sodium borohydride (468.7 mCi/mmol) and the average molecular weight of the [^{3}H]-labeled substrate, which should be equivalent on a molar basis. The reactions were quenched by placing the sample tubes in a boiling water bath for 10 min. Samples were filtered through 0.2 μm microcentrifuge filter inserts to remove precipitated proteins. Supernatants (130 μL) were collected. Newly-generated reducing ends of the [^{3}H]heparin derivatives were [^{3}H]-labeled with [^{3}H]sodium borohydride at 50°C for 2.5 h as described previously (Shively and Conrad, 1976). The total volume of each sample was recorded. To remove unincorporated [^{3}H], samples were chromatographed on PD-10 columns equilibrated with 0.5 M ammonium bicarbonate, pH 7.8, and eluted with the same buffer. Fractions of 250 uL were collected and a 50 μL aliquot of each fraction was assayed for ^{3}H by liquid scintillation counting. The ^{3}H counts voided by the PD-10 columns, with the appropriate mathematical adjustments, represent the total of the counts from the pre-labeled substrate plus the ^{3}H counts introduced at newly-generated reducing ends.

Subcutaneous tumor growth of a human pancreatic (CaPan-2) adenocarcinoma in nude mice

CaPan-2 tumor cells at 3 × 10^6 cells/mL were injected in a volume of 0 1 mL s.c. in the anterior dorsal region of male Balb/c athymic nude mice (Simonsen Laboratories, Gilroy, CA). Animals (10 animals/treatment group) received a daily s.c administration of 60 mg/kg of 2,3-O-desulphated heparin or PBS vehicle on day 1 to day 35. Tumor measurements were performed three times a week and data were evaluated with Analysis of Variance SAS JMP Version 3.

B16-F10 melanoma experimental lung metastasis assay

Female C57BL/6N mice, 4–6 weeks old (Charles River, Raleigh, NC), were injected with 5 × 10^4 B16-F10 melanoma cells in a volume of 0.1 mL intravenously (i.v.) in the tail. Animals were randomly distributed prior to assignment of saline vehicle control and treatment groups. Animals (10 animals/treatment group) received 2,3-O-desulphated heparin at 100 mg/kg, 50 mg/kg, and 10 mg/kg s.c. at a single dose (0.05 mL) or 0.9% saline vehicle on days 0–4 with the first dose occurring one h prior to tumor challenge. Survival times were recorded for animals in the vehicle control and treatment groups. Results were evaluated with Chi-squared statistical analysis.

Effects of heparin derivatives on proliferation of human CaPan-2 and B16-F10 melanoma cells

In 48-well plastic tissue culture plates (Corning), CaPan-2 cells were plated at 20,000 cells per well in McCoy's 5A media containing 10 % fetal bovine serum and L-glutamine while B16-F10 melanoma cells were plated at 2,000 cells per well in DMEM with 10% fetal bovine serum. The media also contained 100 μg/mL of heparin derivative dissolved in the appropriate vehicle (0.9% saline or PBS) or vehicle alone. Using a Coulter counter, the total number of cells in each well was determined after 24, 48 and 96 h incubation at 37°C. Six replicates were done for each condition used and the means of total cell counts were determined.

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Abbreviations

PBS, phosphate-buffered saline; DMF, dimethylformamide; DMSO, dimethylsulphoxide; HPLC, high-performance liquid chromatography; HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; CPC, cetylpyridinium chloride; GlcUA, D-glucuronic acid; GlcUA (2-OSO_3), D-glucuronic acid 2-O-sulphate; IdoA, L-Iduronic acid; IdoA(2-OSO_3), L-Iduronic acid 2-O-sulphate; GlcN, D-glucosamine; $GlcNSO_3$, D-glucosamine-N-sulphate; $GlcNSO_3$ (6-OSO_3), D-glucosamine-N-,6-O-sulphate; $GlcNSO_3$ (3-OSO_3), D-glucosamine-N-,3-O-sulphate; $GlcNSO_3$ (3,6,-di-OSO_3), D-glucosamine-N-,3-O, 6-O-sulphate; $aMan_R$, 2,5-anhydro-D-mannitol; GM, GlcUAβ1–4aMan_R; IM, IdoAα1–4aMan_R; GMS, GlcUAβ1–4aMan_R(6-OSO_3); GSM, GlcUA(2-OSO_3)β1–4aMan_R; IMS, IdoAα1–4aMan_R(6-OSO3): ISM, IdoA(2-OSO_3)α1–4aMan_R; GMS_2, GlcUAβ1–4aMan_R(3,6,-di-OSO_3); ISMS, IdoA(2-OSO_3)α1–4aMan_R(6-OSO_3).

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