Suramin

A POTENT INHIBITOR OF MELANOMA HEPARANASE AND INVASION*

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Suramin, a polysulfonated naphthylurea, has anti-reverse transcriptase and anti-proliferative activities and inhibits the binding of various growth factors to their cell surface receptors. This drug is used in the treatment of acquired immunodeficiency syndrome and several types of cancers. Increased levels of circulating glycosaminoglycans have been observed in suramin-treated cancer patients, suggesting that it may inhibit glycosaminoglycan catabolism. Melanoma-derived heparanase, a heparan sulfate-specific endo-

β-D-glucuronidase that plays an important role in metastatic melanoma cell invasion through basement membranes, is inhibited by suramin in a dose-dependent manner: 100% inhibition was observed at a concentration of ~100 μM. Structurally related polysulfonated compounds, such as trypsin blue and Evans blue, had lower heparanase inhibitory activities: the concentrations required for 50% heparanase inhibition (ID₅₀) were 310–320 μM and six times higher than for suramin (ID₅₀ = 46 μM). Oversulfated heparin tetrasaccharide, whose average molecular size is similar to suramin, had also much lower heparanase inhibitory activity than suramin. The inhibition constants (Kᵢ) for suramin and oversulfated heparin tetrasaccharide were 48 and 290 μM, respectively. Suramin had a remarkable inhibitory activity against B16 melanoma cell invasion through reconstituted basement membranes (ID₅₀ <10 μM). The inhibitory effects of suramin on melanoma heparanase and cell invasion appeared to be completely independent of its antiproliferative activity, because significant effects on melanoma cell growth were not observed at the concentrations of suramin used in this study. The results suggest that the antmetastatic effects of suramin may be due to its anti-invasive rather than antiproliferative activities.

Suramin (M, 1429), a polysulfonated naphthylurea, has been widely used for the treatment of onchocerciasis and trypanosomiasis. It is a potent competitive inhibitor of reverse transcriptase (1) and blocks in vitro the infectivity and cytopathic effects of human T-lymphotropic virus type III (HTLV-III) (2). Following these observations, clinical trials were initiated to treat patients with acquired immunodeficiency syndrome (3, 4). Suramin has also been tested for antitumor activity and found to be an active agent in the treatment of metastatic cancers (5).

Suramin blocks the cell surface binding of various growth factors, such as platelet-derived growth factor, epidermal growth factor, and transforming growth factor-β, and prevents cell proliferation (6-9). It has also been found to inhibit many enzymes including phosphoinositidase and diacylglycerol kinases (9) and DNA polymerases (10), suggesting that its antitumor effects may be mediated through its antiproliferative activities.

In animals and patients high doses of suramin dramatically increase tissue glycosaminoglycans and, through elevations in the concentration of circulating heparan sulfate (HS)¹ and dermatan sulfate (DS), diminishes blood coagulation (11, 12). The HS and DS increases appear to be due, in part, to inhibition of iduronate sulfatase, one of the lysosomal enzymes responsible for HS and DS intracellular degradation (11).

We investigated the effect of suramin on glycosaminoglycan degrading endoglycosidases produced by malignant tumor cells and the ability of suramin to inhibit heparanase, an endo-

β-D-glucuronidase that specifically degrades HS (13) and participates in the degradation of basement membranes by invasive tumor and normal cells (14). Indeed, heparanase activities in malignant cells, including melanoma (15), T-

lymphoma (16), fibrosarcoma (17), rhabdomyosarcoma (18), and colon carcinoma (19), correlates with the metastatic potential of these tumors. Furthermore, natural and synthetic inhibitors of heparanase are inhibitors of lung colonization of B16 melanoma cells in their syngenic host (20-23).

Here we demonstrate that suramin is one of the most potent inhibitors of melanoma heparanase and that suramin can inhibit melanoma cell-mediated degradation of subendothelial extracellular matrix (ECM). We also report that suramin greatly inhibits melanoma cell invasion through reconstituted basement membranes and that this effect is independent of suramin's antiproliferative activity.

EXPERIMENTAL PROCEDURES

Chemicals—Suramin (M, 1429) was a gift from FBA, Bayer AG, Leverkusen, Federal Republic of Germany, and related polysulfonated reagents, trypsin blue (C.I. 23850; Direct Blue 14, M, 961) and Evans

¹ The abbreviations used are: HS, heparan sulfate; DS, dermatan sulfate; RLE, rat lung endothelial; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's medium; F-12, Ham's F-12 nutrient medium.
blue (C. L. 23860; Direct blue 53, M, 961) were purchased from Sigma (structures shown in Fig. 1). Heparin tetrasaccharide and its oversulfated derivative were kind gifts of Dr. Jean Choay, Laboratoire Choay, Paris, France.

**Cells and Cell Culture**—The highly invasive and lung metastatic murine B16 melanoma subline (B16-BL6) was obtained from Dr. I. J. Fidler (Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX) and grown in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient medium (DMEM/F-12, GIBCO) supplemented with 5% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT). In some experiments B16-BL6 cells were grown in DMEM/F-12 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 25 mM sodium selenite (Sigma) or only with 0.1% bovine serum albumin (RI grade, Sigma). Rat lung endothelial clone 8 (RLE cl.8) cells were isolated as described previously (24) and grown in DMEM/F-12 supplemented with 10% plasma-derived horse serum and 100 μg/ml endothelial mitogen (Biomedical Technologies, Inc., Cambridge, MA) and 5 μg/ml porcine intestinal heparin (Sigma).

**Heparanase Assay**—Heparanase was assayed as described previously (19, 22, 25). Briefly, HS purified from bovine lung (10 mg) was labeled with 5 μCi of [3H]acetic anhydride (100 μCi/mmol, ICN Radiochemicals) in 0.4 M sodium acetate, pH 6.5, and [3H]HS with a Mr, ~30,000 was purified using a Sephacryl S-200 column (Pharmacia LKB Biotechnology Inc.) equilibrated with 0.2 M pyridine acetate, pH 5.0. B16 melanoma heparanase was prepared as described previously (26). Briefly, B16-BL6 cells were extracted at 4°C in 50 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride, 5 mM N-ethylmaleimide, 0.5% Triton X-100, and 0.05% sodium azide. The cell extracts were subjected to heparanase purification by sequential treatments with 0.1% Triton X-100 and 10 mM NH$_4$OH as described previously (22, 24). The RLE-ECM was incubated in medium containing 10% fetal bovine serum for 2 h.

B16-BL6 melanoma cells were harvested from subconfluent cultures and suspended in 5% fetal bovine serum in DMEM/F-12. Cell suspensions (5 × 10$^7$ cells/900 μl) and 100-μl aliquots of DMEM/F-12 containing suramin at various concentrations were plated on isolated RLE-ECM and incubated over 90 h at 37°C in a CO$_2$ incubator. Culture supernatants were withdrawn and centrifuged at 18,000 × g for 10 min, and the [3H]sulfate radioactivity released into 400 μl supernatant was determined.

Degradation products of the RLE-ECM were analyzed by size exclusion chromatography using a Bio-Gel TSK-40 XL column. Elution was performed at 23°C with 12.5 mM Tris-HCl, 150 mM NaCl, pH 7.5, at a flow rate of 1 ml/min, the eluents collected every 30 s, and radiolabel determined on a liquid scintillation counter. Various degradation products were tested for their susceptibility to Flavobacterium heparitinsae (Seikagaku Amerika, St. Petersburg, FL) digestion.

**Invasion Assay**—Invasion assay was performed as described previously (23, 26), using Costar 6.5-mm Transwell™ chambers equipped with 8.0-μm pore size polycarbonate membranes. The upper surface of the membrane was coated with Matrigel™ (200 μg of protein, Collaborative Research, Bedford, MA). The bottom chamber was filled with 600 μl of a solution containing laminin (100 μg/ml, Collaborative Research) and fibronectin (50 μg/ml, Sigma) in DMEM/F-12 supplemented with 5 μg/ml insulin, 5 μg/ml transferrin, and 25 mM sodium selenite. B16-BL6 melanoma cells grown as subconfluent cultures were harvested by a brief treatment with 2.5 mM EDTA and suspended in DMEM/F-12 plus 5 μg/ml insulin, 5 μg/ml transferrin, and 25 mM sodium selenite. Melanoma cells (5 × 10$^6$ to 2 × 10$^7$) were seeded on the reconstituted Matrigel™ in the upper Transwell™ chamber. After a 60-h incubation, cells that penetrated through the polycarbonate membrane were harvested from the bottom chamber by trypsin-EDTA treatment and counted.

**RESULTS**

**Suramin Inhibition of Melanoma Heparanase**—When [3H]HS (5 mg) was incubated at 37°C with 25 mg of the partially purified melanoma heparanase (specific activity, 60 mg HS/mg protein/h), the intact [3H]HS peak decreased with time, and degradation fragments of characteristic molecular sizes (Mr, ~8000 and ~5400) appeared on the chromatogram (13). This degradation was almost completely inhibited by 100 μM suramin (Fig. 2), but it was not affected by 100 μM concentration of related polysulfonated compounds, such as trypan blue and Evans blue. The concentration of suramin required for 50% inhibition (ID$_{50}$) of the melanoma heparanase was 46 μM under the conditions used, and the ID$_{50}$ of trypan blue and Evans blue for heparanase activity were 310–320 μM, or six times higher than that of suramin.

We have reported that heparin and its nonanticoagulant derivatives are potent competitive inhibitors of melanoma heparanase (20, 21). Here we employed a heparin tetrasaccharide and its oversulfated derivative with molecular sizes similar to suramin and compared their effects on heparanase. Heparin tetrasaccharide had no significant effect on heparanase activity even at the high concentration of 500 μM, whereas the oversulfated tetrasaccharide inhibited heparanase in a dose-dependent manner (Fig. 3). The ID$_{50}$ of oversulfated heparin tetrasaccharide for heparanase activity was 435 μM, almost 10 times higher than that of suramin.

The Dixon plots (27) of the data from inhibition experiments using different concentrations of the substrate demonstrated that the heparanase inhibition by suramin is primarily noncompetitive, whereas the heparanase inhibition by oversulfated heparin tetrasaccharide is a mixed type of com-
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**Fig. 2.** Heparanase inhibition by suramin. N-\(^3\)H-acetylated heparan sulfate (M, ~34,000, 5 mg) was incubated at 37°C for 6 h with 25 μg of partially purified murine melanoma heparanase (specific activity, 60 μg HS/mg protein/h) in 100 μl of 0.2 M sodium acetate, pH 5.0, in the presence or absence of suramin. Incubation products were analyzed by size exclusion chromatography using a Bio-Gel TSK-30 XL column. Percent inhibition was determined by measuring the decrease in area of the first one-half of the peak of intact \(^3\)HHS on the chromatogram.

**Fig. 3.** Heparanase inhibition by heparin tetrasaccharide. Heparin tetrasaccharide and oversulfated tetrasaccharide were examined for heparanase inhibition by the assay described in Fig. 2.

**Fig. 4.** Kinetics of heparanase inhibition by suramin and oversulfated heparin tetrasaccharide. A, Cornish-Bowden plot of S/V (min/μl) against suramin concentration (micromolar); B, Dixon plot of 1/V (min/μg HS) against oversulfated heparin tetrasaccharide concentration (micromolar).

Competitive and uncompetitive inhibition (Fig. 4). The inhibition constant (K\(_i\)) was determined by the methods of Dixon (27) and Cornish-Bowden (28). The K\(_i\) values for suramin and oversulfated heparin tetrasaccharide were 48 and 290 μM, respectively (Fig. 4, A and B).

**Suramin Inhibition of Endothelial Cell ECM Degradation by B16 Melanoma Cells—**Highly metastatic B16 melanoma cells are capable of degrading basement membrane-like endothelial ECM in vitro and producing characteristic size fragments of HS proteoglycans (15, 29). When B16-BL6 cells were seeded on \(^{35}\)Sulfate-labeled RLE-ECM, \(^{35}\)S radioactivity in the culture medium increased with time (Fig. 5). During the first 24 h of incubation, concentrations of suramin between 1 and 100 μM slightly increased the spontaneous release of \(^{35}\)Sulfate-labeled materials from RLE-ECM. However, over a 90-h incubation period suramin effectively inhibited B16-BL6 cell-mediated degradation of \(^{35}\)Sulfate-labeled ECM components in a dose-dependent manner. The ID\(_{50}\) of suramin for melanoma cell-mediated ECM degradation was approximately 80 μM. The released \(^{35}\)Sulfate-labeled materials were analyzed by size exclusion chromatography using a Bio-Gel TSK-40 XL column. Large molecular size HS proteoglycans (M, ~200,000) were found in the first peak, and HS degradation fragments of M, ~10,000 and ~5,000 were identified in the second and third peaks (Fig. 6) by their susceptibility to Flavobacterium heparitinase digestion. Suramin at a concentration of 100 μM markedly reduced the production of small HS degradation fragments (Fig. 6).
transferrin, the growth of B16-BL6 melanoma cells was not significantly affected by suramin of up to 100 pM. Only a slight reduction (13%) in cell growth was observed in the presence of 100 pM suramin (Fig. 7). Other compounds used in this study such as trypsin blue, Evans blue, heparin tetrasaccharide, and oversulfated heparin tetrasaccharide at concentrations as high as 100 pM had no significant effect on B16-BL6 cell growth over a 72-h incubation period.

**Suramin Inhibition of Melanoma Cell Invasion**—We showed previously that some of the natural and chemically modified heparanase inhibitors inhibit B16 melanoma cell experimental pulmonary metastasis and invasion through reconstituted basement membranes (20-23). When B16-BL6 cells were seeded onto Matrigel™, the cells migrated into this matrix but did not penetrate through the matrix layer. Fibronectin and laminin (50 and 100 µg/ml, respectively) were added to the bottom chamber to enhance melanoma cell invasion. The number of cells penetrating through the Matrigel™ and polycarbonate membrane was dependent on the total number of cells seeded as well as the quality of the Matrigel™ and conditions used. In general, approximately 0.5% of the total cells penetrated through both the Matrigel™ and underlying polycarbonate membrane during a 60-h incubation (Table I). This invasion was effectively inhibited by suramin: the ID₅₀ of suramin for melanoma cell invasion in four independent experiments varied from 0.8 to 9.9 pM. The results from two such experiments are shown in Table I.

### DISCUSSION

Suramin has been employed in the treatment of malignant diseases, primarily because it inhibits tumor cell growth (3-9). It has been reported that suramin is useful for the treat-
ment of some metastatic cancers, such as adrenal (5) and prostate carcinomas (30) and lymphomas (31), that are unresponsive to conventional cytotoxic chemotherapy. Here we examined the effect of suramin on metastatic murine melanoma cell heparanase, an HS-specific endo-β-D-glucuronidase, whose activity correlates with metastatic potential of melanoma cells and is involved in their invasive degradation of subendothelial ECM. The effect of suramin was compared with those of related polysulfonated compounds as well as oversulfated and unmodified heparin tetrasaccharides of similar size. Suramin inhibited heparanase activity in a dose-dependent manner, and its ID₅₀ was significantly lower than that of the other compounds tested.

Jentsch et al. (32) reported that molecular size and steric hindrance seemed to be more important for the reverse transcriptase inhibitory activity of the suramin-related compounds and that the inhibitory activity of these compounds on reverse transcriptase did not correlate with its effect against filariae or trypanosomes. In our study the effect of suramin on melanoma heparanase appeared to be specific, and both the molecular size and the number of sulfonic acid groups seemed to be important for the heparanase inhibitory activity. However, these factors do not account for the difference in heparanase inhibitory activity between suramin (Mᵦ, 1429) and oversulfated heparin tetrasaccharide (Mᵦ, 1530). Analysis of the kinetic data using the Dixon plot (27) and the Cornish-Bowden plot (28) indicated that heparanase inhibition by suramin is primarily noncompetitive. This interpretation is supported by the report (11) that both lysosomal iduronate sulfatase and β-glucuronidase are noncompetitively inhibited by suramin. In contrast, heparanase inhibition by heparin is a mixed type of competitive and uncompetitive inhibition. Nonetheless, we conclude that suramin is one of the most potent inhibitors of heparanase.

Suramin inhibits a large number of enzymes, including urease, hexokinase, acid phosphatase, serine proteases in the complement system, DNA polymerases, and kinases involved in phosphoinositide metabolism (9, 10, 11, 33–35). The enzymes involved in the catabolism of glycans, such as ganglioside neuraminidase, β-glucuronidase, hyaluronidase, and idurionate sulfatase, have also been found to be inhibited by suramin, perhaps accounting for the aberrant accumulation of sialoglycosides and glycosaminoglycans, including HS, DS, and hyaluronic acid, in various organs of suramin-treated patients and animals (11, 12, 36). Since normal and tumor cell heparanases are the major endoglycosidases that initiate the sequential cleavage of HS linked to HS-proteoglycan core peptides (13, 37, 38), heparanase inhibition is much more critical to the inhibition of total HS degradation than the inhibition of exoglycosidases and sulfatases. Thus, the inhibition of heparanase in the liver and blood vasculature is thought to be a major cause of increased levels of plasma HS in suramin-treated patients. In fact, the suramin levels in treated patients’ plasma are generally two to three times higher than the concentration required for 100% inhibition of heparanase activity (5).

Suramin was highly inhibitory against B16 melanoma cell invasion through reconstituted basement membranes (ID₅₀ <10 μM). The antivasive effects of suramin appeared to be completely independent of its antiproliferative activity, because no significant effect on cell growth was observed at the concentrations used in this study. Zabrenetzky et al. (39) have recently reported that suramin inhibits human melanoma cell adhesion and chemotaxis to laminin and thrombospondin without affecting cell growth. Inhibition of B16 melanoma cell invasion through reconstituted basement membranes by suramin is probably due to inhibition of both ECM degradation and cell migration. Suramin did not alter the production of heparanase in B16-BL6 cells (data not shown). It is therefore plausible that the direct inhibition of heparanase activity could be a major cause of ECM degradation inhibition. Suramin may also modify other tumor-secreted enzyme activities. It is noteworthy that one of the heparanase inhibitors, 6-O-sulfated carboxymethyl chitin, effectively reduced type IV collagenolysis by B16-BL6 cells (23). We are currently studying the effect of suramin on other enzymes and endogenous inhibitors secreted by melanoma cells.

In conclusion, this study has documented that suramin is a potent inhibitor of melanoma cell heparanase and invasion. The ID₅₀ for these activities are very low compared to the suramin levels detected in plasma of patients during the treatment of trypanosomiasis, onchocerciasis, acquired immunodeficiency syndrome, or malignant diseases (5). These results support further investigation of the possible usefulness of suramin as a therapeutic agent for metastatic disease.

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