NEOPLASIA
www.neoplasia.com

Volume 13 Number 5 May 2011 pp. 445–452 445

Sulfated Hexasaccharides Attenuate Metastasis by Inhibition of P-selectin and Heparanase[1]

Lubor Borsig*, Israel Vlodavsky†, Rivka Ishai-Michaeli‡, Giangiacomo Torri§ and Elena Vismara¶

*Zürich Center for Integrative Human Physiology, Institute of Physiology, University of Zürich, Zürich, Switzerland; †Cancer and Vascular Biology Research Center, Bruce Rappaport Faculty of Medicine, Technion, Haifa, Israel; ‡Sharett Institute, Hadassah-Hebrew University Medical Center, Jerusalem, Israel; §Istituto di Ricerche Chimiche e Biochimiche "G. Ronzoni" via Giuseppe Colombo, Milan, Italy; ¶Dipartimento di Chimica, Materiali e Ing. Chimica "G. Natta" Politecnico, Via Mancinelli, Milan, Italy

Abstract

Development of compounds that target both heparanase and selectins is emerging as a promising approach for cancer therapy. Selectins are vascular cell adhesion molecules that mediate tumor cell interactions with platelets, leukocytes, and the vascular endothelium. Heparanase is an endoglycosidase that degrades heparan sulfate in the tumor microenvironment, cell surfaces, and vessel wall. Acting together, these molecules facilitate tumor cell arrest, extravasation, and metastasis. Here, we report the preparation of novel semisynthetic sulfated tri mannose C-C–linked dimers (STMCs) endowed with heparanase and selectin inhibitory activity. The P-selectin specificity of the STMC was defined by the anomeric linkage of the C-C bond. This STMC hexasaccharide is an effective inhibitor of P-selectin *in vivo*. We show that selective inhibition of heparanase attenuates metastasis in B16-BL6 melanoma cells, expressing high levels of this endoglycosidase, but has no effect on the metastasis of MC-38 carcinoma cells that express little or no heparanase activity. P-selectin–specific STMC attenuated metastasis in both animal models, indicating that inhibition of tumor cell interaction with the vascular endothelium is critical for cancer dissemination. Thus, the small size, the stability of the C-C bond, and the chemically defined structure of the newly generated STMCs make them superior to heparin derivatives and signify STMCs as valuable candidates for further evaluation.

Neoplasia (2011) 13, 445–452

Introduction

The control of cancer metastasis remains the major obstacle in treatment of cancer patients. Metastasis is a multiple-step process encompassing tumor cell release from primary sites, their survival in the circulation, and extravasation in distant tissues [1]. Metastasis is determined by the cellular origin, intrinsic features of the tumor, and the adhesive properties of tumor cells.

Heparanase, the only mammalian endoglycosidase that cleaves heparan sulfate (HS), is upregulated in essentially all human tumors examined [2–7]. A causal involvement of heparanase in tumor metastasis was demonstrated by increased lung, liver, and bone colonization of cancer cells after overexpression of the heparanase gene and by a marked decrease in metastatic potential of cells subjected to

Abbreviations: STMC, sulfated tri mannose C-C–linked dimer

Address all correspondence to: Lubor Borsig, PhD, Institute of Physiology University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.
E-mail: lborsig@access.uzh.ch

[1]This work was supported by grants from the Swiss National Foundation (3100A0-116295 to L.B.); the Israel Science Foundation (grant 593/10); the National Cancer Institute, National Institutes of Health (grant RO1-CA106456 and CA138535); the Ministry of Science & Technology of the State of Israel and the German Cancer Research Center (to I.V.); and the Fondazione Umberto Veronesi, Milan, Italy (http://www.fondazioneveronesi.it; to E.V.). I. Vlodavsky is a research professor of the Israel Cancer Research Fund.
Received 15 December 2010; Revised 23 February 2011; Accepted 24 February 2011

DOI 10.1593/neo.101734

heparanase gene silencing [8,9]. Moreover, inhibition of heparanase resulted in attenuation of metastasis in a number of animal models [8–12]. A significant role of heparanase in tumor angiogenesis and lymphangiogenesis was demonstrated, applying similar experimental approaches [2,7,13]. Clinically, increased heparanase levels are most often associated with increased tumor metastasis, high microvessel density, and reduced patients' survival time after operation [2–4,6,14,15].

In another set of studies, the adhesive properties of tumor cells to vascular cell adhesion molecules, selectins, have been shown to mediate tumor cell interaction with platelets, endothelium, and leukocytes. Inhibition of selectin-mediated tumor cell interaction with blood constituents resulted in attenuation of tumor metastasis in a number of animal models [16–20].

Several laboratories, including ours, are developing heparin-mimicking compounds that compete with HS and thereby inhibit heparanase and selectin prometastatic activities [10,21–23]. Heparin has long been known to possess antiheparanase activity [24] and to effectively inhibit P- and L-selectins [25,26]. Results from several clinical trials using unfractionated heparin and low-molecular-weight heparin (LMWH) in preventing thromboembolic complication in advanced stage cancer patients indicated that heparin prolonged survival [27] probably owing to a direct effect on the tumor, potentially through inhibition of heparanase enzymatic activity [28]. However, the use of heparin or LMWH as anticancer agents is limited because of the risk of inducing adverse bleeding complications. Moreover, heparin exhibits a number of biologic activities including inhibition of thrombin generation [29], release of tissue factor pathway inhibitor from endothelial surfaces [30], modulation of growth factors' receptor binding and activity [31], affecting angiogenesis [32], heparanase enzymatic activity [33], and selectin-mediated cell interactions [34,35]. Despite a significant progress in the analysis of heparin activities affecting cancer progression, there is a need for a synthetic small molecule inhibitor of heparanase and/or selectins to be tested as a potential antimetastatic treatment.

In the present study, we tested novel semisynthetic hexasaccharide compounds for their capacity to inhibit heparanase and/or P-selectin activities *in vitro* as well as experimental metastasis *in vivo*. For this purpose, we have synthesized hexasaccharide mimics of maltohexaose sulfate in which a central glycosidic bond was substituted by a hydrolase-resistant C-C bond. Maltohexaose sulfate was chosen because of its bioequivalence with phosphomannopentaose sulfate (PI-88), a potent carbohydrate-based heparanase inhibitor currently

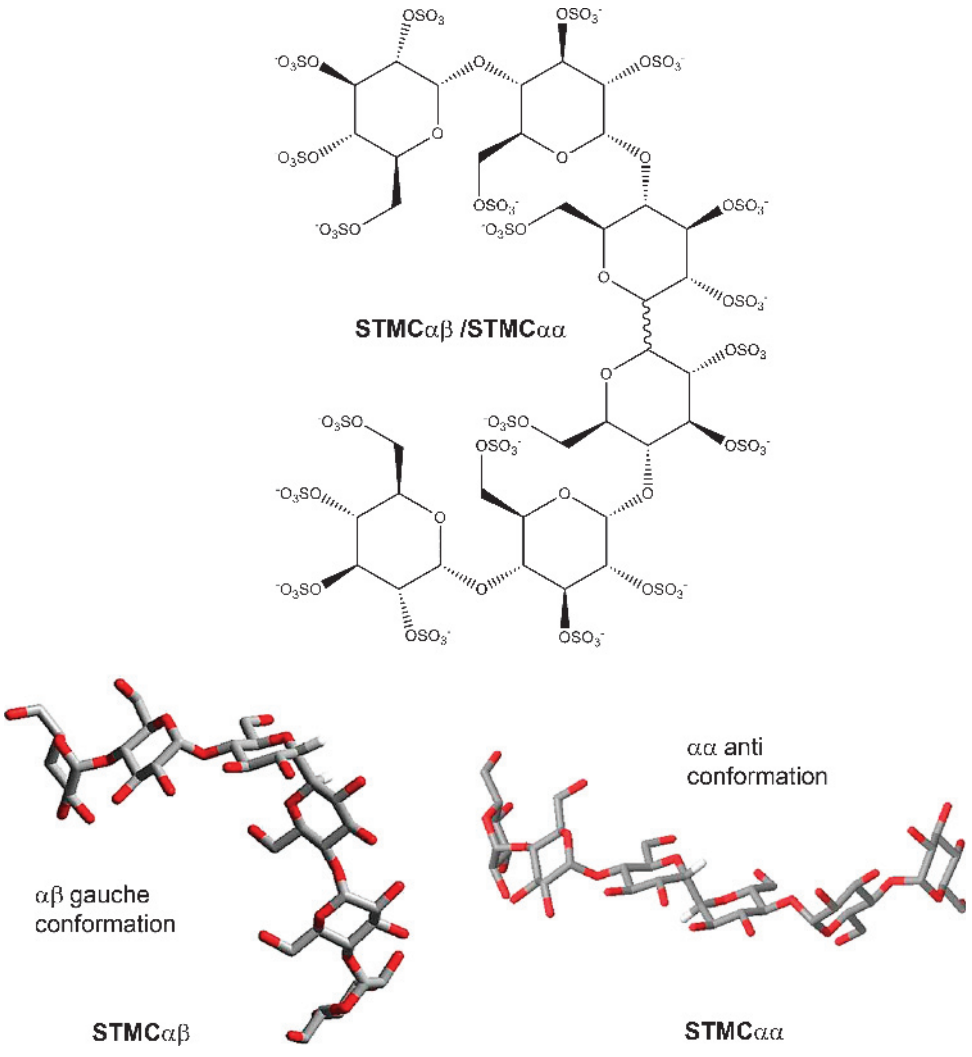

Figure 1. Structures and preferred conformation of STMCα,α and STMCα,β.

being subjected to phase 2/3 clinical trials in cancer patients [36]. The sugar chains of these compounds are characterized by the presence of an interglycosidic C-C bond expected to confer chemical and metabolic stability compared with malto-oligosaccharides.

Materials and Methods

Preparation of Compounds

Halo-sugars' electroreduction on silver cathode has been used to prepare double sugar units cancelled through the formation of stable interglycosidic C-C bonds. This procedure is accompanied by the loss of a halide anion from an electrochemically reduced halo-sugar, and the radical reactivity dictates the statistic distribution of products with formation of α,α: α,β: β,β C-C bonds (1:2:1) [37,38]. Tri-maltose C-C–linked dimers TMCα,α and TMCα,β were prepared by acetobromomaltotriose electrochemical reduction followed by deacetylation [39]. Sulfation of TMCα,α and TMCα,β was performed by addition of 590 mg of sulfur trioxide pyridine complex (3.68 mmol, 10 Eq/Eq –OH) to a pyridine solution of TMC (0.0185 mmol, 4×10^{-3} M), resulting in STMCα,α and STMCα,β, respectively (Figure 1). The reaction mixture was warmed to 80°C and stirred for 6 hours, avoiding moisture with a $CaCl_2$ trap. The mixture was cooled down to room temperature, neutralized with a saturated solution of $NaHCO_3$, and exhaustively evaporated under vacuum. The solid residue was dissolved in water and separated on TSK column. STMCα,α and STMCα,β were fully characterized by NMR analysis (E. Vismara et al., unpublished data).

Cell Lines and Reagents

Mouse colon carcinoma cell line MC-38 stably expressing GFP, MC-38GFP [17], and mouse melanoma cell line B16-BL6 [8] were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). Human colon carcinoma cells LS180 (ATCC, Manassas, VA) were grown in α-minimum essential medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). All reagents were from Sigma (St. Louis, MO) unless otherwise stated.

Mice

Wild-type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of the Zürich Center for Integrative Human Physiology.

Inhibition of P-selectin

Ability of STMCs to inhibit adhesion of LS180 cells to immobilized P-selectin was examined as described previously [34]. Briefly, an ELISA plate (Nunc, Rochester, NY), coated overnight with soluble Protein A, was blocked with 1% bovine serum albumin in Hank's balanced salt solution for 30 minutes at room temperature and incubated with mouse P-selectin chimera (400 ng/well) for 3 hours at room temperature. Calcein AM–labeled LS180 tumor cells were added to the plate in the presence or absence of serially diluted STMCs at concentrations ranging from 0.58 to 500 μg/ml. After 1 hour of incubation while rotating at 4°C, adherent cells were quantified by measuring the fluorescence with a GENios ELISA reader (Tecan, Männedorf, Switzerland). The half-maximal inhibitory concentration (IC_{50}) values were calculated from three independent experiments.

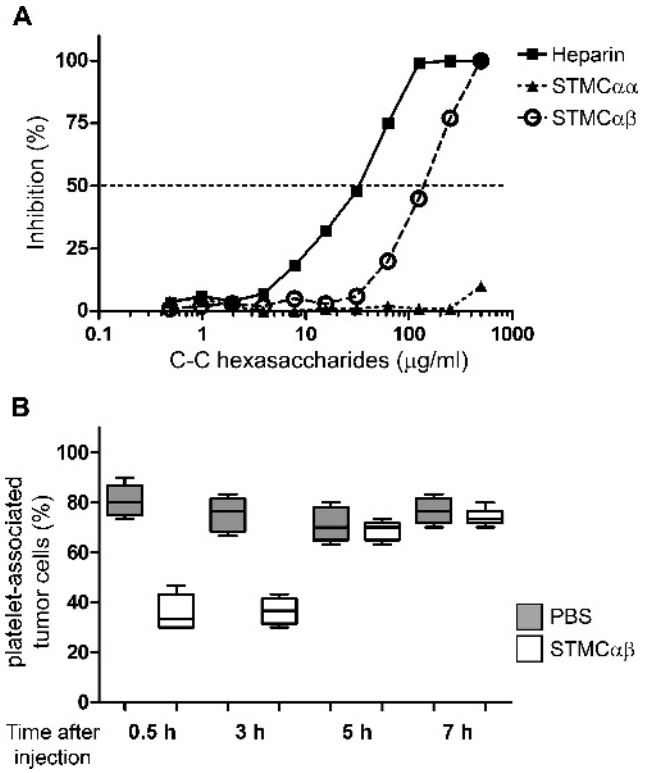

Figure 2. (A) Selectin inhibitory activity of C-C hexasaccharides. The ability of STMCα,α and STMCα,β hexasaccharides to inhibit P-selectin–mediated adhesion of LS-180 human colon carcinoma cells was compared to that of unfractionated heparin as described in Materials and Methods. Data are representative of three independent experiments. (B) Bioavailability of STMCα,β *in vivo*. Platelet adhesion to intravenously injected MC-38GFP cells was analyzed in mice receiving STMCα,β and compared to PBS-injected mice. The number of tumor cells positive for platelet staining was determined in lungs from mice killed at 30 minutes, 3 hours, or 7 hours after injection as described in Materials and Methods. The difference in platelet/tumor cell association between control-injected mice (PBS) and STMCα,β-injected mice was found to be statistically significant at 30 minutes and 3 hours by one-way ANOVA ($P < .001$).

Coagulation Assays

Automatic determination of partial thromboplastin time (PTT) of samples was performed in duplicates by mixing pooled normal plasma with PTT reagent (FSL actin; Dade Behring, Deerfield, IL). After 10 minutes, the clotting time was determined on a Sysmex CA-1500 analyzer (Diamond Diagnostics, Holliston, MA). Photometric determination of anti-Xa activity was performed to evaluate the activity of STMCs in human plasma on Sysmex CA-1500 analyzer, using the LMWH Kit (Chromogenics, MöIndal, Sweden).

Heparanase Activity Assay

Preparation of sulfate-labeled extracellular matrix (ECM)–coated dishes and determination of heparanase enzymatic activity were performed as described in detail elsewhere [40,41]. STMCs were tested for their ability to inhibit heparanase, as previously described [33].

Briefly, sulfate-labeled ECM coating the surface of 35-mm culture dishes was incubated (4 hours, 37°C, pH 6.0) with constitutively active (GS3) [42] recombinant human heparanase (120 ng/ml) in the absence or presence of 5 μg/ml of each STMCs. The incubation medium containing sulfate-labeled degradation fragments was subjected to gel filtration on a Sepharose CL-6B column. Fractions (0.2 ml) were eluted with PBS, and their radioactivity was counted in a β-scintillation counter. Degradation fragments of HS side chains were eluted at 0.5 < Kav < 0.8 (peak II, fractions 15-35). Nearly intact heparan sulfate proteoglycan (HSP) was eluted just after the Vo (Kav < 0.2, peak I, fractions 3-12). We have previously demonstrated that labeled fragments eluted in peak II are degradation products of HS because they were 1) fivefold to sixfold smaller than intact HS side chains, 2) resistant to further digestion with papain and chondroitinase ABC, and 3) susceptible to deamination by nitrous acid [40].

Platelet–Tumor Cell Aggregation In Vivo

C57BL/J6 mice were killed at various time points after intravenous injection of tumor cells, and frozen lungs sections were incubated with anti-CD41 antibody (Becton Dickinson, Mountain View, CA), followed by detection with goat antirat antibody conjugated with Alexa568 (Invitrogen) as described [34]. The extent of platelet–tumor cell association was quantified in 20 view fields (40× magnifications) by immunofluorescence microscopy.

Experimental Metastasis

C57BL/J6 wild-type mice were intravenously injected with 300,000 MC-38GFP cells or B16-BL6 melanoma cells [43]. Some mice received 150 μg of STMCs 10 minutes before tumor cell injection. Mice injected with B16-BL6 melanoma cells were killed after 14 days, and the number of lung metastatic foci was counted. Mice injected with MC-38GFP cells were killed after 28 days and macroscopically evaluated. Metastatic burden in the lungs was determined by measurements of GFP in the lung homogenate [17].

Results

Sulfated Hexasaccharides as Potential Inhibitors of Metastasis

Modified heparins that block P-selectin and/or heparanase have been tested as potential inhibitors of metastasis [10,33]. We aimed at synthesizing small oligosaccharide structures endowed with inhibitory activity of P-selectin and/or heparanase and testing their therapeutic potential in mouse models of metastasis.

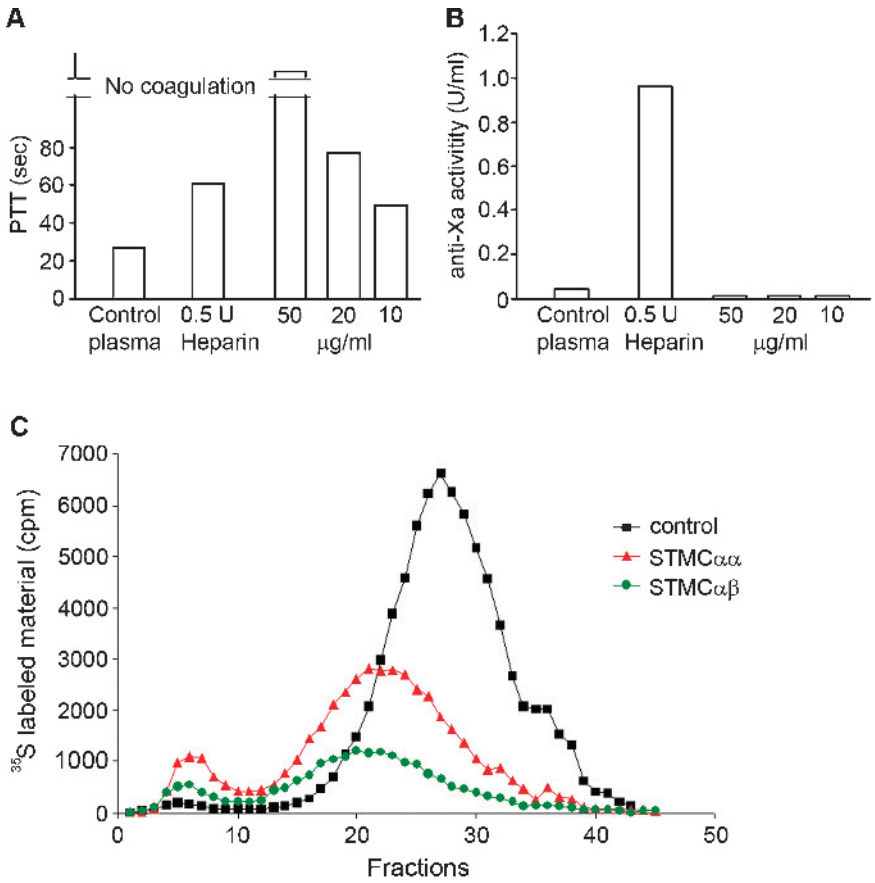

Figure 3. Heparanase and anticoagulant activity of STMCs. (A and B) Anticoagulant activity. STMCs were tested for effect on PTT (A) and anti-Xa activity (B) as described in Materials and Methods. Shown are the results obtained with STMCα,β. Same results were obtained with STMCα,α. Data are representative of three independent determinations, and the variation did not exceed ±10% of the mean. (C) Heparanase inhibitory activity. The ability of STMCα,α and STMCα,β hexasaccharides to inhibit recombinant heparanase enzymatic activity was determined as described in Materials and Methods. Data are representative of three independent experiments.

Halo-monosaccharides electroreduction on silver cathode allows dimerization of a carbon-centered radical and affords C-disaccharide mimics [38]. By extending this technique to oligosaccharides, we succeeded in preparing sulfated tri-maltose C-C–linked dimers STMCα,α and STMCα,β (Figure 1). A detailed description of the synthetic procedure is provided elsewhere [38].

STMCs Inhibit P-selectin Binding in a Linkage-Specific Manner

Attenuation of metastasis in the absence of P-selectin or its inhibition by native and chemically modified heparins has been documented in a number of animal models [10,16,34,44]. To investigate C-C–linked hexasaccharides as potential inhibitors of P-selectin, we tested the ability of both STMCα,α and STMCα,β to inhibit adhesion of LS-180 colon carcinoma cells to immobilized P-selectin [10]. STMCα,β showed a good inhibitory activity of P-selectin (IC_{50} 150 μg/ml), although less effective than unfractionated heparin (IC_{50} 40 μg/ml) (Figure 2*A*). In contrast, STMCα,α exerted no P-selectin inhibitory activity, indicating that change of the C-C linkage from αα to αβ is critical for binding to P-selectin.

To determine an effective dose of STMCα,β for the metastasis experiments, we first analyzed its bioavailability *in vivo*. Tumor cells carrying selectin ligands are known to form tumor cell emboli mediated primarily through platelet P-selectin [16,34]. We intravenously injected 150 μg of STMCα,β 10 minutes before injection of MC-38GFP mouse colon carcinoma cells. Mice were killed at different time points between 30 minutes and 7 hours after injection, and the extent of platelet–tumor cell association was quantified (Figure 2*B*). Approximately 80% of tumor cells in the lung tissue of PBS-injected mice were associated with platelets at all times. Injection of STMCα,β reduced platelet aggregation on tumor cells to approximately 40% during the first 3 hours, but there was no inhibition at later time points (5 and 7 hours). These results indicate that STMCα,β is biologically active for at least 3 hours after tumor injection and is relatively rapidly cleared from the circulation.

Anticoagulant Activity of STMCs

Both compounds STMCα,α and STMCα,β exhibited a high, dose dependent anticoagulant activity (PTT, at 10 μg/ml comparable to that of 0.5 U heparin), but were devoid of anti-Xa activity (Figure 3, *A* and *B*).

STMCs Effectively Inhibit Heparanase Enzymatic Activity

Heparanase activity is associated with cancer progression in a variety of cancers and its inhibition by heparin derivatives attenuates tumor growth and metastasis [2–5,9,12]. Using a naturally produced sulfate-labeled ECM as a substrate [40,41], we tested the ability of both STMCα,α and STMCα,β to inhibit heparanase enzymatic activity. As demonstrated in Figure 3*C*, compound STMCα,β was more effective than compound STMCα,α, yielding 85% and 58% inhibition by 5 μg/ml, respectively.

STMCs Attenuate Metastasis Primarily by Inhibition of P-selectin

To determine the ability of STMCs to attenuate metastasis, we intravenously injected wild-type mice with 150 μg/mouse of STMC followed by injection of B16-BL6 melanoma cells 10 minutes later. B16-BL6 cells were shown to express P-selectin ligands as well as significant amounts of heparanase [10]. Heparanase-specific STMCα,α reduced metastasis by ~65%, whereas the P-selectin–specific STMCα,β, which inhibits both P-selectin and heparanase, was more effective, yielding 82% inhibition (Figure 4). Next, we tested both STMCs in the MC-38GFP colon carcinoma model [10,17]. Intravenous injection of STMCs was followed by injection of MC-38GFP cells, and mice were killed after 28 days (Figure 5). The P-selectin–specific STMCα,β attenuated metastasis to similar levels as observed with P-selectin–deficient mice [10,17], whereas STMCα,α had no effect, in agreement with the little or no heparanase activity observed in MC-38 colon carcinoma cells [10]. Taken together, these findings indicate that the newly synthesized hexasaccharides effectively attenuated experimental metastasis by targeting P-selectin and that inhibition of heparanase enzymatic activity is a valid approach in heparanase-expressing tumor cells.

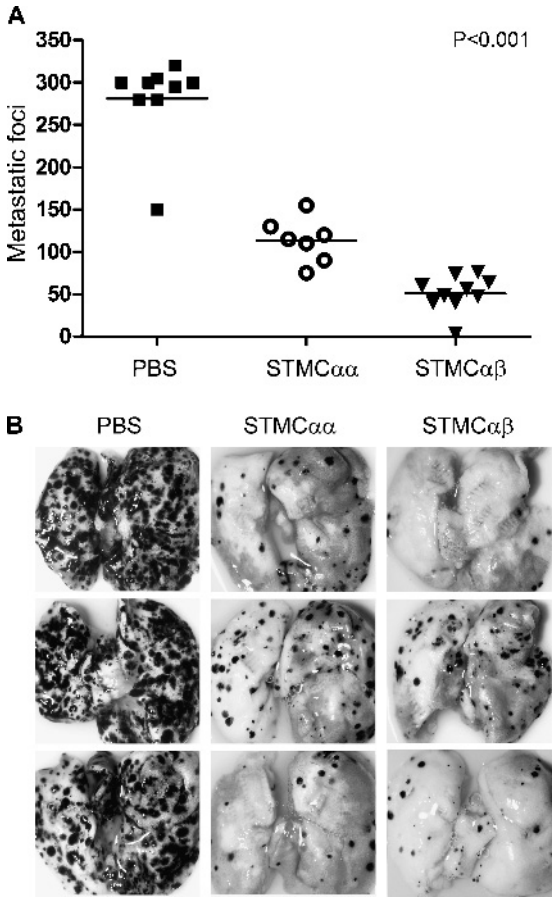

Figure 4. C-C hexasaccharides attenuate metastasis of B16-BL6 melanoma cells. (A) Mice were intravenously injected with 150 μg of STMCα,α or STMCα,β 10 minutes before injection of 3×10^5 B16-BL6 cells. Fourteen days after injection mice were killed, and the lungs were dissected and evaluated for the number of metastatic foci/lung. Statistical significance was determined by one-way ANOVA ($P < .001$). (B) Representative images of lungs derived from mice injected with STMCα,α or STMCα,β *versus* control (PBS).

Discussion

Several studies have shown that the antimetastatic activity of heparin is based on its ability to inhibit heparanase and selectins [23,33,34,45]. The bioactive moieties of natural polysaccharides reside in specific or

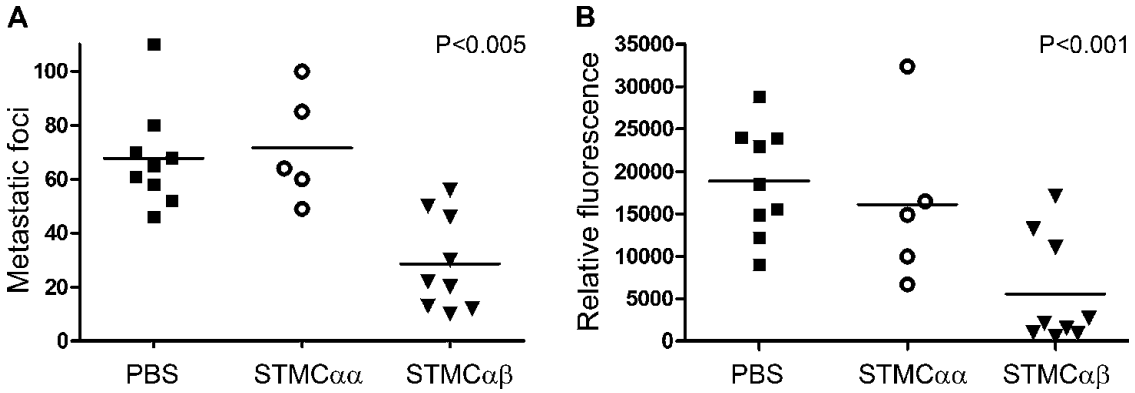

Figure 5. Experimental metastasis of MC-38 colon carcinoma cells is efficiently attenuated by STMCα,β endowed with selectin inhibitory activity. C57BL/J6 mice were intravenously injected with 150 μg of STMCα,β 10 minutes before injection of 3×10^5 MC-38GFP cells and killed 28 days later. (A) The number of metastatic foci/lung representing initial metastatic seeding of tumor cells was counted. (B) The extent of lung colonization was quantified by measurement of GFP fluorescence in lung homogenates. Statistical difference was found only for the STMCα,β compound as determined by *t* test ($P < .001$).

unspecific oligosaccharides mostly consisting of more than four carbohydrate units [46]. Owing to their small molecular size, oligosaccharides are expected to have enhanced bioavailability than their polymeric precursors. These considerations led to isolation of sequences from natural oligosaccharides and development of glycan mimetics having the capacity to inhibit heparanase or selectins [47–49]. Sulfated maltohexaose has been identified to be an efficient inhibitor of tumor growth and metastasis, primarily because of inhibition of heparanase [47]. PI-88, a mixture of highly sulfated oligosaccharides, reached phase 3 clinical development for hepatocellular carcinoma. A new class of heparanase-inhibiting HS mimetics based on anomerically pure, fully sulfated oligosaccharides was recently optimized for anticancer drug development [49]. In the case of selectin inhibitors, the early developments of sialyl Lewis X–based glycan mimetics has been replaced by development of nonglycoside small molecule inhibitors [50]. In the present work, we tested semisynthetic sulfated maltooligosaccharides for their potential to inhibit heparanase and P-selectin. These glycan mimetics are characterized by the presence of an interglycosidic C-C bond, which, similar to other C-glycosides that are less vulnerable to metabolic processing than their O-analogs, is expected to confer improved chemical and metabolic stability relative to malto-oligosaccharides [51]. Molecular modeling and conformational analysis have shown that the interglycosidic C-C bond modifies the geometry of the sugar chains, increasing their conformation rigidity [39]. Whereas STMCα,β was found to be an effective inhibitor of P-selectin, the conformational change to STMCα,α completely eliminated its selectin binding activity (Figure 2*A*), emphasizing the high specificity of this interaction. In contrast, both STMCs inhibited heparanase enzymatic activity, the αβ configuration being more effective (Figure 3*C*), indicating a less restricted specificity for interaction with heparanase compared with selectins. Because the conformational flexibility of oligosaccharides is critical for their binding to proteins and, consequently, for their bioactivity [52], our observations provide evidence that a rigid C-C structures defines the specificity of selectin binding.

STMCs effectively attenuated metastasis in both the B16 melanoma and MC-38 colon carcinoma systems (Figures 4 and 5). Because the heparanase-specific STMCα,α did not affect metastasis of MC-38GFP carcinoma cells expressing no heparanase (Figure 5), tumor-derived heparanase, as opposed to heparanase contributed by other blood-borne cells (i.e., neutrophils, platelets), seems to be critical for metastasis in this experimental setting [10]. STMCα,β that efficiently inhibits P-selectin was a better inhibitor of lung colonization by B16-BL6 melanoma and MC-38GFP carcinoma cells than STMCα,α (Figure 5). Likewise, modified heparins endowed with P-selectin–inhibitory activity effectively attenuated metastasis, to an extent similar to that observed in P-selectin–deficient mice [10,22,23,53]. The rapid cell surface expression of P-selectin on platelets and endothelial cells on activation makes P-selectin one of the earliest molecules mediating cell adhesion [54,55]. Accumulating evidence indicates that P-selectin–mediated interactions contribute to cancer progression (reviewed in Laubli and Borsig [55,56] and Ludwig et al. [57]). Yet, the recently developed specific selectin inhibitors are being tested only in different inflammatory situations such as ischemia-reperfusion injury, atherosclerosis, and deep vein thrombosis [50,58]. Here, we provide evidence that sulfated mannose–based hexasaccharides (STMCα,β) specifically inhibit P-selectin and thereby attenuate experimental metastasis (Figure 5). Thus, STMCα,β represents one of the smallest glycan-based selectin inhibitor that is active *in vivo*.

High heparanase expression by multiple myeloma cells is associated with enhanced bone metastasis [6] and modified non–anticoagulant glycol-split heparins endowed with heparanase inhibitory activity have been shown to effectively attenuate myeloma tumor growth and bone metastasis [10,28]. Similarly, inhibition of heparanase either by modified heparins [10] or by the newly developed STMCs hexasaccharides (Figure 4) attenuated metastasis, further confirming the critical involvement of heparanase in metastasis. Importantly, the heparin-derived compounds effectively inhibited xenograft tumor growth and spontaneous metastasis of human myeloma [9,28] and sarcoma [59]. Synthesis of STMCs is being scaled up to enable their evaluation in the same systems.

Non–anticoagulant heparin–derived inhibitors of metastasis have been developed and tested in a number of laboratories (reviewed in Casu et al. [60], Borsig [61], and Kragh and Loechel [62]). However, heparin-based inhibitors exhibit a limited bioavailability and poor pharmacokinetics *in vivo*. Unlike heparin, the synthetic hexasaccharides (STMCs) presented in this work represent stable, small size

single entity oligosaccharides, expected to be more readily optimized for drug development in terms of target (e.g., selectin *vs* heparanase) specificity and bioavailability. Notably, the therapeutic potential of compounds targeting heparanase and/or selectins is not restricted to cancer, taking into account their involvement in inflammatory diseases [63–65] and renal dysfunction [66].

Acknowledgments

The authors thank Laura Tinè for technical assistance by synthesis of compounds. The authors also thank B. Casu ('Ronzoni' Institute, Milan, Italy) and R. Sanderson (University of Alabama at Birmingham) for their continuous support and collaboration. The authors thank Ben-Zion Katz (Hematology Institute, Tel-Aviv Medical Center, Israel) for his kind help in performing the coagulation studies.

References

[1] Nguyen DX, Bos PD, and Massague J (2009). Metastasis: from dissemination to organ-specific colonization. *Nat Rev Cancer* **9**, 274–284.

[2] Barash U, Cohen-Kaplan V, Dowek I, Sanderson RD, Ilan N, and Vlodavsky I (2010). Proteoglycans in health and disease: new concepts for heparanase function in tumor progression and metastasis. *FEBS J* **277**, 3890–3903.

[3] Levy-Adam F, Ilan N, and Vlodavsky I (2010). Tumorigenic and adhesive properties of heparanase. *Semin Cancer Biol* **20**, 153–160.

[4] Ilan N, Elkin M, and Vlodavsky I (2006). Regulation, function and clinical significance of heparanase in cancer metastasis and angiogenesis. *Int J Biochem Cell Biol* **38**, 2018–2039.

[5] Parish CR, Freeman C, and Hulett MD (2001). Heparanase: a key enzyme involved in cell invasion. *Biochim Biophys Acta* **1471**, M99–M108.

[6] Yang Y, Macleod V, Bendre M, Huang Y, Theus AM, Miao HQ, Kussie P, Yaccoby S, Epstein J, Suva LJ, et al. (2005). Heparanase promotes the spontaneous metastasis of myeloma cells to bone. *Blood* **105**, 1303–1309.

[7] Sanderson RD, Yang Y, Suva LJ, and Kelly T (2004). Heparan sulfate proteoglycans and heparanase—partners in osteolytic tumor growth and metastasis. *Matrix Biol* **23**, 341–352.

[8] Edovitsky E, Elkin M, Zcharia E, Peretz T, and Vlodavsky I (2004). Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. *J Natl Cancer Inst* **96**, 1219–1230.

[9] Yang Y, MacLeod V, Dai Y, Khotskaya-Sample Y, Shriver Z, Venkataraman G, Sasisekharan R, Naggi A, Torri G, Casu B, et al. (2007). The syndecan-1 heparan sulfate proteoglycan is a viable target for myeloma therapy. *Blood* **110**, 2041–2048.

[10] Hostettler N, Naggi A, Torri G, Casu B, Vlodavsky I, and Borsig L (2007). P-selectin– and heparanase-dependent antimetastatic activity of non-anticoagulant heparins. *FASEB J* **21**, 3562–3572.

[11] Lerner I, Baraz L, Pikarsky E, Meirovitz A, Edovitsky E, Peretz T, Vlodavsky I, and Elkin M (2008). Function of heparanase in prostate tumorigenesis: potential for therapy. *Clin Cancer Res* **14**, 668–676.

[12] Ferro V, Hammond E, and Fairweather JK (2004). The development of inhibitors of heparanase, a key enzyme involved in tumour metastasis, angiogenesis and inflammation. *Mini Rev Med Chem* **4**, 693–702.

[13] Cohen-Kaplan V, Naroditsky I, Zetser A, Ilan N, Vlodavsky I, and Doweck I (2008). Heparanase induces VEGF C and facilitates tumor lymphangiogenesis. *Int J Cancer* **123**, 2566–2573.

[14] Gohji K, Hirano H, Okamoto M, Kitazawa S, Toyoshima M, Dong J, Katsuoka Y, and Nakajima M (2001). Expression of three extracellular matrix degradative enzymes in bladder cancer. *Int J Cancer* **95**, 295–301.

[15] Doweck I, Kaplan-Cohen V, Naroditsky I, Sabo E, Ilan N, and Vlodavsky I (2006). Heparanase localization and expression by head and neck cancer: correlation with tumor progression and patient survival. *Neoplasia* **8**, 1055–1061.

[16] Kim YJ, Borsig L, Varki NM, and Varki A (1998). P-selectin deficiency attenuates tumor growth and metastasis. *Proc Natl Acad Sci USA* **95**, 9325–9330.

[17] Borsig L, Wong R, Hynes RO, Varki NM, and Varki A (2002). Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. *Proc Natl Acad Sci USA* **99**, 2193–2198.

[18] Läubli H, Stevenson JL, Varki A, Varki NM, and Borsig L (2006). L-selectin facilitation of metastasis involves temporal induction of fut7-dependent ligands at sites of tumor cell arrest. *Cancer Res* **66**, 1536–1542.

[19] Läubli H and Borsig L (2010). Selectins as mediators of lung metastasis. *Cancer Microenviron* **3**, 97–105.

[20] Biancone L, Araki M, Araki K, Vassalli P, and Stamenkovic I (1996). Redirection of tumor metastasis by expression of E-selectin *in vivo*. *J Exp Med* **183**, 581–587.

[21] Vlodavsky I, Ilan N, Naggi A, and Casu B (2007). Heparanase: structure, biological functions, and inhibition by heparin-derived mimetics of heparan sulfate. *Curr Pharm Des* **13**, 2057–2073.

[22] Ludwig RJ, Alban S, Bistrian R, Boehncke WH, Kaufmann R, Henschler R, and Gille J (2006). The ability of different forms of heparins to suppress P-selectin function *in vitro* correlates to their inhibitory capacity on blood-borne metastasis *in vivo*. *Thromb Haemost* **95**, 535–540.

[23] Stevenson JL, Choi SH, and Varki A (2005). Differential metastasis inhibition by clinically relevant levels of heparins—correlation with selectin inhibition, not antithrombotic activity. *Clin Cancer Res* **11**, 7003–7011.

[24] Bar-Ner M, Eldor A, Wasserman L, Matzner Y, Cohen IR, Fuks Z, and Vlodavsky I (1987). Inhibition of heparanase-mediated degradation of extracellular matrix heparan sulfate by non-anticoagulant heparin species. *Blood* **70**, 551–557.

[25] Nelson RM, Cecconi O, Roberts WG, Aruffo A, Linhardt RJ, and Bevilacqua MP (1993). Heparin oligosaccharides bind L- and P-selectin and inhibit acute inflammation. *Blood* **82**, 3253–3258.

[26] Koenig A, Norgard-Sumnicht K, Linhardt R, and Varki A (1998). Differential interactions of heparin and heparan sulfate glycosaminoglycans with the selectins. Implications for the use of unfractionated and low molecular weight heparins as therapeutic agents. *J Clin Invest* **101**, 877–889.

[27] Lazo-Langner A, Goss GD, Spaans JN, and Rodger MA (2007). The effect of low-molecular-weight heparin on cancer survival. A systematic review and meta-analysis of randomized trials. *J Thromb Haemost* **5**, 729–737.

[28] Casu B, Vlodavsky I, and Sanderson RD (2008). Non-anticoagulant heparins and inhibition of cancer. *Pathophysiol Haemost Thromb* **36**, 195–203.

[29] Rickles FR (2006). Mechanisms of cancer-induced thrombosis in cancer. *Pathophysiol Haemost Thromb* **35**, 103–110.

[30] Mousa SA (2002). Anticoagulants in thrombosis and cancer: the missing link. *Semin Thromb Hemost* **28**, 45–52.

[31] Jayson GC and Gallagher JT (1997). Heparin oligosaccharides: inhibitors of the biological activity of bFGF on Caco-2 cells. *Br J Cancer* **75**, 9–16.

[32] Folkman J and Shing Y (1992). Control of angiogenesis by heparin and other sulfated polysaccharides. *Adv Exp Med Biol* **313**, 355–364.

[33] Vlodavsky I, Mohsen M, Lider O, Svahn CM, Ekre HP, Vigoda M, Ishai-Michaeli R, and Peretz T (1994). Inhibition of tumor metastasis by heparanase inhibiting species of heparin. *Invasion Metastasis* **14**, 290–302.

[34] Borsig L, Wong R, Feramisco J, Nadeau DR, Varki NM, and Varki A (2001). Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. *Proc Natl Acad Sci USA* **98**, 3352–3357.

[35] Stevenson JL, Varki A, and Borsig L (2007). Heparin attenuates metastasis mainly due to inhibition of P- and L-selectin, but non-anticoagulant heparins can have additional effects. *Thromb Res* **120**(suppl 2), S107–S111.

[36] Johnstone KD, Karoli T, Liu L, Dredge K, Copeman E, Li CP, Davis K, Hammond E, Bytheway I, Kostewicz E, et al. (2010). Synthesis and biological evaluation of polysulfated oligosaccharide glycosides as inhibitors of angiogenesis and tumor growth. *J Med Chem* **53**, 1686–1699.

[37] Alberti A, DellaBona MA, Macciantelli D, Pelizzoni F, Sello G, Torri G, and Vismara E (1996). Reactivity of glucosyl radical in the presence of phenols. *Tetrahedron* **52**, 10241–10248.

[38] Guerrini M, Mussini P, Rondinini S, Torri G, and Vismara E (1998). Electrochemical reduction of halogenosugars on silver: a new approach to C-disaccharide–like mimics. *Chem Commun* **15**, 1575–1576.

[39] Guerrini M, Guglieri S, Santarsiero R, and Vismara E (2005). Synthesis and characterisation of hexa- and tetrasaccharide mimics from acetobromomaltotriose and acetobromomaltose, and of C-disaccharide mimics from acetobromoglucose, obtained by electrochemical reduction on silver. *Tetrahedron Asymmetry* **16**, 243–253.

[40] Vlodavsky I, Fuks Z, Bar-Ner M, Ariav Y, and Schirrmacher V (1983). Lymphoma cell–mediated degradation of sulfated proteoglycans in the subendothelial extracellular matrix: relationship to tumor cell metastasis. *Cancer Res* **43**, 2704–2711.

[41] Vlodavsky I (2001). Preparation of extracellular matrices produced by cultured corneal endothelial and PF-HR9 endodermal cells. *Curr Protoc Cell Biol* **Chapter 10**, Unit 10.14.

[42] Nardella C, Lahm A, Pallaoro M, Brunetti M, Vannini A, and Steinkuhler C (2004). Mechanism of activation of human heparanase investigated by protein engineering. *Biochemistry* **43**, 1862–1873.

[43] Elkin M and Vlodavsky I (2001). Tail vein assay of cancer metastasis. *Curr Protoc Cell Biol* **Chapter 19**, Unit 19.12.

[44] Ludwig RJ, Boehme B, Podda M, Henschler R, Jager E, Tandi C, Boehncke WH, Zollner TM, Kaufmann R, and Gille J (2004). Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. *Cancer Res* **64**, 2743–2750.

[45] Kragh M, Binderup L, Vig Hjarnaa PJ, Bramm E, Johansen KB, and Frimundt Petersen C (2005). Non–anti-coagulant heparin inhibits metastasis but not primary tumor growth. *Oncol Rep* **14**, 99–104.

[46] Imberty A and Perez S (2000). Structure, conformation, and dynamics of bioactive oligosaccharides: theoretical approaches and experimental validations. *Chem Rev* **100**, 4567–4588.

[47] Parish CR, Freeman C, Brown KJ, Francis DJ, and Cowden WB (1999). Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel *in vitro* assays for angiogenesis and heparanase activity. *Cancer Res* **59**, 3433–3441.

[48] Wong CH, MorisVara F, Hung SC, Marron TG, Lin CC, Gong KW, and WeitzSchmidt G (1997). Small molecules as structural and functional mimics of sialyl Lewis X tetrasaccharide in selectin inhibition: a remarkable enhancement of inhibition by additional negative charge and/or hydrophobic group. *J Am Chem Soc* **119**, 8152–8158.

[49] Dredge K, Hammond E, Davis K, Li CP, Liu L, Johnstone K, Handley P, Wimmer N, Gonda TJ, Gautam A, et al. (2010). The PG500 series: novel heparan sulfate mimetics as potent angiogenesis and heparanase inhibitors for cancer therapy. *Invest New Drugs* **28**, 276–283.

[50] Bedard PW and Kaila N (2010). Selectin inhibitors: a patent review. *Expert Opin Ther Patents* **20**, 781–793.

[51] Gong HG and Gagne MR (2008). Diastereoselective Ni-catalyzed Negishi cross-coupling approach to saturated, fully oxygenated C-alkyl and C-aryl glycosides. *J Am Chem Soc* **130**, 12177–12183.

[52] Qasba PK (2000). Involvement of sugars in protein-protein interactions. *Carbohydrate Polymers* **41**, 293–309.

[53] Borsig L (2007). Antimetastatic activities of modified heparins: selectin inhibition by heparin attenuates metastasis. *Semin Thromb Hemost* **33**, 540–546.

[54] Ley K (2003). The role of selectins in inflammation and disease. *Trends Mol Med* **9**, 263–268.

[55] Laubli H and Borsig L (2010). Selectins promote tumor metastasis. *Semin Cancer Biol* **20**, 169–177.

[56] Läubli H and Borsig L (2009). Heparins attenuate cancer metastasis: are selectins the link? *Cancer Invest* **27**, 474–481.

[57] Ludwig RJ, Schon MP, and Boehncke WH (2007). P-selectin: a common therapeutic target for cardiovascular disorders, inflammation and tumour metastasis. *Expert Opin Ther Targets* **11**, 1103–1117.

[58] Calvey CR and Toledo-Pereyra LH (2007). Selectin inhibitors and their proposed role in ischemia and reperfusion. *J Invest Surg* **20**, 71–85.

[59] Shafat I, Ben-Arush MW, Issakov J, Meller I, Naroditsky I, Tortoteto M, Cassinelli G, Lanzi C, Pisano C, Ilan N, et al. (in press). Preclinical and clinical significance of heparanase in Ewing's sarcoma. *J Cell Mol Med*, DOI: 10.1111/j.1582-4934.2010.01190.x.

[60] Casu B, Naggi A, and Torri G (2010). Heparin-derived heparan sulfate mimics to modulate heparan sulfate–protein interaction in inflammation and cancer. *Matrix Biol* **29**, 442–452.

[61] Borsig L (2010). Antimetastatic activities of heparins and modified heparins. Experimental evidence. *Thromb Res* **125**(suppl 2), S66–S71.

[62] Kragh M and Loechel F (2005). Non–anti-coagulant heparins: a promising approach for prevention of tumor metastasis [review]. *Int J Oncol* **27**, 1159–1167.

[63] McEver RP (1997). Selectin-carbohydrate interactions during inflammation and metastasis. *Glycoconj J* **14**, 585–591.

[64] Waterman M, Ben-Izhak O, Eliakim R, Groisman G, Vlodavsky I, and Ilan N (2007). Heparanase upregulation by colonic epithelium in inflammatory bowel disease. *Mod Pathol* **20**, 8–14.

[65] Li RW, Freeman C, Yu D, Hindmarsh EJ, Tymms KE, Parish CR, and Smith PN (2008). Dramatic regulation of heparanase activity and angiogenesis gene expression in synovium from patients with rheumatoid arthritis. *Arthritis Rheum* **58**, 1590–1600.

[66] van den Hoven MJ, Rops AL, Vlodavsky I, Levidiotis V, Berden JH, and van der Vlag J (2007). Heparanase in glomerular diseases. *Kidney Int* **72**, 543–548.