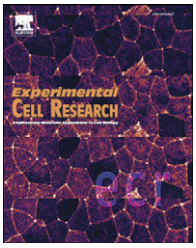
EXPERIMENTAL CELL RESEARCH 316 (2010) 2504–2512

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/yexcr



Research Article

A 4-deoxy analogue of *N*-acetyl-D-glucosamine inhibits heparan sulphate expression and growth factor binding *in vitro*

Xander M.R. van Wijk[a], *Arie Oosterhof*[a], *Sebastiaan A.M.W. van den Broek*[b], *Arjan W. Griffioen*[c], *Gerdy B. ten Dam*[a], *Floris P.J.T. Rutjes*[b], *Floris L. van Delft*[b], *Toin H. van Kuppevelt*[a,*]

[a]*Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Geert Grooteplein 26-28, 6525 GA Nijmegen, The Netherlands*

[b]*Synthetic Organic Chemistry, Institute for Molecules and Materials, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, The Netherlands*

[c]*Angiogenesis Laboratory, Department of Medical Oncology, VU University Medical Centre, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands*

ARTICLE INFORMATION

Article Chronology:
Received 5 March 2010
Revised version received 20 April 2010
Accepted 21 April 2010
Available online 28 April 2010

Keywords:
Glycosaminoglycan
Heparan sulfate
Deoxysugar
N-acetyl-D-glucosamine
Growth Factor Binding
Angiogenesis

ABSTRACT

Heparan sulphate (HS) is a long, linear polysaccharide, which has a basic backbone of -β1-4GlcA-α1-4GlcNAc- units. The involvement of HS in many steps of tumourigenesis, including growth and angiogenesis, makes it an appealing target for cancer therapy. To target the biosynthesis of HS by interfering with its chain elongation, a 4-deoxy analogue of *N*-acetyl-D-glucosamine (4-deoxy-GlcNAc) was synthesized. Using immunocytochemistry and agarose gel electrophoresis it was shown that incubation with the 4-deoxysugar resulted in a dose dependent reduction of HS expression of MV3 melanoma cells, 1 mM resulting in an almost nullified HS expression. The parent sugar GlcNAc had no effect. 4-deoxysugar treated cells were viable and proliferated at the same rate as control cells. Other glycan structures appeared to be only mildly affected, as staining by various lectins was generally not or only modestly inhibited. At 1 mM of the 4-deoxysugar, the capacity of cells to bind the HS-dependent pro-angiogenic growth factors FGF-2 and VEGF was greatly compromised. Using an *in vitro* angiogenesis assay, 4-deoxysugar treated endothelial cells showed a sharp reduction of FGF-2-induced sprout formation. Combined, these data indicate that an inexpensive, easily synthesized, water-soluble monosaccharide analogue can interfere with HS expression and pro-angiogenic growth factor binding.

Introduction

Glycosaminoglycans (GAGs) are long, linear, and strongly negatively charged polysaccharides, generally bound to a core protein (forming proteoglycans) and present at the cell surface and in the extracellular matrix. Different GAG family members are distinguished on the basis of their structural backbone and include heparin/heparan sulphate (HS), chondroitin/dermatan sulphate

* *Corresponding author.* Department of Biochemistry (280), Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, PO. Box 9101, 6500 HB Nijmegen, The Netherlands. Fax: +31 24 3540339.
E-mail address: A.vankuppevelt@ncmls.ru.nl (T.H. van Kuppevelt).

Abbreviations: CS, chondroitin sulphate; DS, dermatan sulphate; FGF, fibroblast growth factor; GAG, glycosaminoglycan; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; GlcA, glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine; HS, heparan sulphate; HSPG, heparan sulphate proteoglycan; IdoA, iduronic acid; IgG, immunoglobulin G; LacNAc, *N*-acetyllactosamine; NDST, *N*-deacetylase/*N*-sulphotransferase; PG, proteoglycan; SD, standard deviation; VEGF, vascular endothelial growth factor; VSV, vesicular stomatitis virus

0014-4827/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2010.04.025

(CS/DS), keratan sulphate and hyaluronan. HS consists of about a hundred repeating disaccharides and its backbone structure is composed of an uronic acid and *N*-acetyl-D-glucosamine (GlcNAc). This backbone is extensively modified by a number of biosynthetic reactions including *N*-deacetylation and *N*-sulphation, epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA), and various O-sulphations [1]. Extracellularly, HS can be further edited by removal of sulphate groups [2]. Specific modifications, especially the degree and position of sulphation, are involved in the biological activity of HS. It is becoming increasingly clear that HS is a regulatory polysaccharide [3], interacting with a variety of proteins, including growth factors and their receptors [4].

HS is involved in many aspects of tumourigenesis including growth, invasion, angiogenesis and metastasis [5]. This involvement is in line with its capacity to bind, store and modulate many effector molecules including pro-angiogenic factors such as fibroblast growth factor-2 (FGF-2) [6] and vascular endothelial growth factor (VEGF) [7]. Tumour cells and tissues are generally associated with altered levels of HS and changed degrees of sulphation. Metastatic melanoma cells, for example, express high levels of the HS proteoglycan (HSPG) perlecan [8], and inhibition of perlecan reduces melanoma formation [9]. Furthermore, the HSPGs syndecan-1 [10,11] and glypican-1 [12] are essential for tumourigenesis in mice. In addition to the quantitative aspect, qualitative aspects play an important role in tumourigenesis, especially the degree and position of sulphation. For example, extracellular sulphatases, which remove 6-O-sulphation from HS, have been reported to be negative [13,14] as well as positive [15] regulators of tumourigenesis. We previously showed upregulation of specific sulphated GAG domains in melanoma [16] and ovarian cancer [17]. Also downregulation of specific domains was noted in metastatic melanoma [18].

The involvement of HS at different steps in tumour formation makes HS an appealing target for cancer therapy [19]. Generally, HS in tumours has been targeted by degradation using specific enzymes (e.g. bacterial heparinases [20]) or by using HS mimetics such as PI-88 [21] and suramin [22]. In this study, we do not focus on interference with existing HS chains, but rather focus on the inhibition of the formation of new HS chains. For this, we used the principle of deoxysugars, as probed by us [23] and by others [24–28]. A 4-deoxy analogue of GlcNAc, 4-deoxy-GlcNAc (Fig. 1A), was synthesized in order to target the biosynthesis of HS. Incorporation of the monosaccharide analogue may result in HS chain truncation (acting as a chain stopper), since the 4-OH group

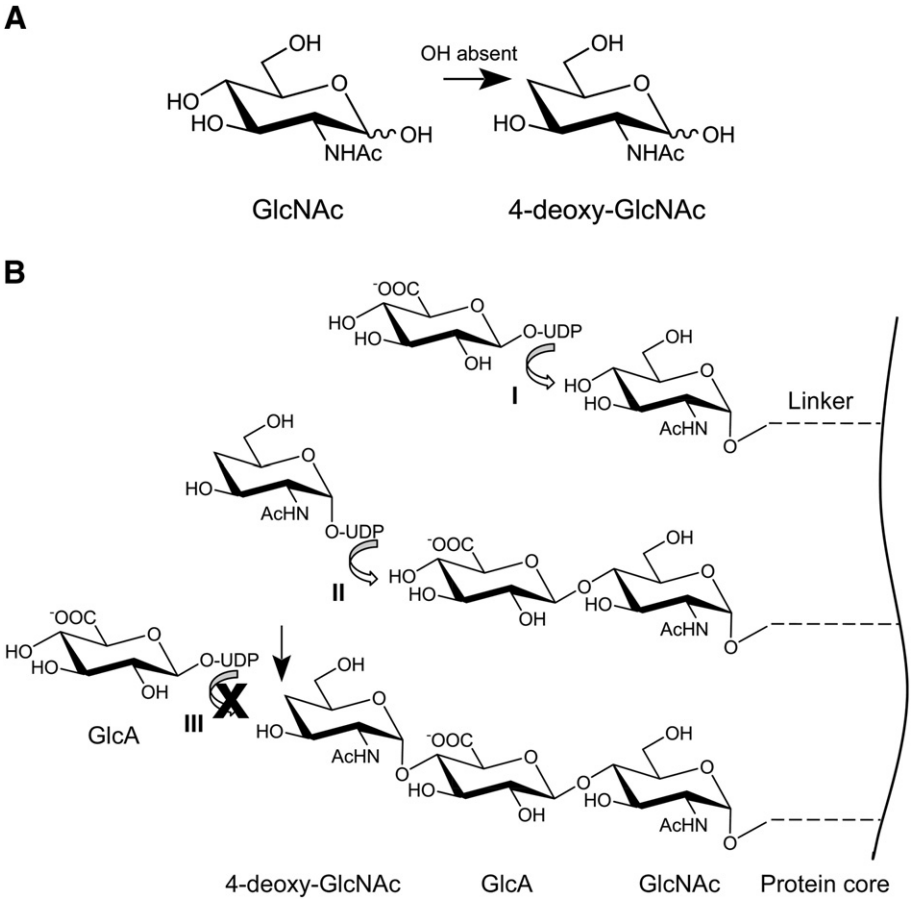


Fig. 1 – Structure and proposed working mechanism of 4-deoxy-GlcNAc. (A) Structure of *N*-acetyl-D-glucosamine (GlcNAc) and its analogue 4-deoxy-GlcNAc. Note that the 4-OH group of GlcNAc is replaced by a hydrogen group in case of 4-deoxy-GlcNAc (indicated by the arrow). (B) Proposed working mechanism of 4-deoxy-GlcNAc. After UDP-activation, glucuronic acid (GlcA) and GlcNAc are incorporated in growing heparan sulphate (HS) chains (step I). If cells are cultured in the presence of 4-deoxy-GlcNAc this analogue can also be incorporated into the growing HS chain (step II). This will result in chain termination, since the 4-deoxysugar lacks the 4-OH group (indicated by the arrow) needed for the subsequent attachment of GlcA (step III).

is required for subsequent attachment of GlcA to continue chain elongation (Fig. 1B). Here, we show that 4-deoxy-GlcNAc dose dependently inhibits HS expression in melanoma cells, prevents growth factor binding, and inhibits sprouting of endothelial cells.

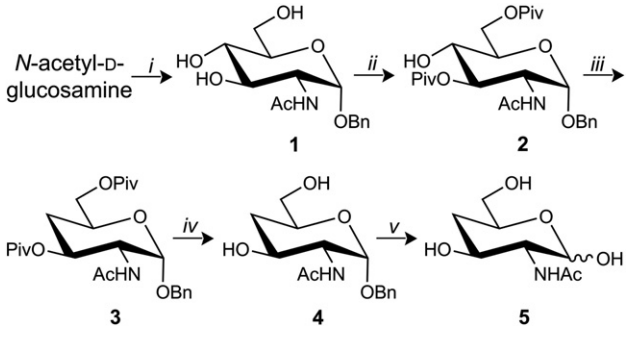
Materials and methods

Antibodies, lectins, enzymes and monosaccharides

N-acetyl-D-glucosamine (A3286), chondroitinase ABC from *Proteus vulgaris* (C3667), papain from *Papaya latex* (P3125), the rabbit polyclonal anti-FGF-2 antibody (F3393; reactive with rat, human and bovine FGF-2) were purchased from Sigma-Aldrich (St Louis, MO, USA). The biotinylated lectins, AAL (*Aleuria aurantia*), ConA (*Canavalia ensiformis*), DSA (*Datura stramonium*), GNA (*Galanthus nivalis*), LCA (*Lens culinaris*), PNA (*Arachis hypogaea*), RCA-I (*Ricinus communis*), SBA (*Glycine max*), VVA (*Vicia villosa*) and WGA (*Triticum vulgaris*) were from Vector Laboratories (Burlingame, CA, USA). Heparinase III from *Flavobacterium heparinum* was purchased from IBEX Pharmaceuticals Inc. (Montreal, Quebec, Canada), goat anti-rat $VEGF_{164}$ (AF564; IgG fraction) from R&D systems (Minneapolis, MN, USA) and mouse mAb anti-HS stub (3G10; IgG2b) from Seikagaku corporation (Tokyo, Japan).

Synthesis of 4-deoxy-GlcNAc

Synthesis of the 4-deoxy analogue of *N*-acetyl-D-glucosamine was achieved in a straightforward route from inexpensive *N*-acetyl-D-glucosamine by modification of a known procedure [24] (Scheme 1). For an overview of the reagents and conditions used, see legend of Scheme 1. First, Fischer glycosidation in benzyl alcohol gave, after crystallization from diethylether, the anomerically pure α-benzyl glycoside **1** [29] (42% yield). Dual esterification of both the 3-OH and 6-OH with a sterically bulky pivaloyl group led to compound **2** in excellent yield (91%), leaving the 4-OH selectively free for deoxygenation. The latter transformation was effected according to the two-step Barton-McCombie protocol [24,30], involving alcohol-to-thionocarbamate conversion, followed by radical treatment in the presence of Bu_3SnH (86% yield for the 2 steps). Subsequent deprotection of esters under Zemplén conditions (**3**→**4**, 68% yield) and debenzylation by hydrogenolysis with 5% Pd-C (100% yield) gave the desired 4-deoxy derivative of GlcNAc **5** (2-acetamido-2,4-dideoxy-D-*xylo*-hexopyranose). A stock solution of 0.5 M in milli-Q of compound **5**, referred to as 4-deoxy-GlcNAc, was made and stored at −80 °C.



Scheme 1 – Synthesis of 4-deoxy-GlcNAc (compound 5). Synthesis according to Berkin et al. [24] with minor changes. Reagents and conditions used: *i*: BnOH, AcCl, 4 h, 75 °C; *ii*: DCM/pyridine (4:3), 2.5 equiv. PivCl, 2 h, 0 °C; *iii*: a) DCE, 2 equiv. TCDI, 18 h, reflux, b) toluene, 2.5 equiv. Bu_3SnH, 1.0 equiv. DMAP, cat. AIBN, 18 h, reflux; *iv*: MeOH, NaO*t*-Bu (0.5 M), 3 h, 60 °C; *v*: MeOH, cat. 10% Pd-C, 3 atm., 3 days, rt, ($\alpha/\beta = 1{:}1$). For further information see text. Atm.: atmosphere, cat.: catalytic, equiv.: equivalent, rt: room temperature.

Cell culture

The highly metastatic human MV3 melanoma cell line [31] was generously provided by Dr. G. van Muijen (Radboud University Nijmegen Medical Centre (RUNMC), Nijmegen, The Netherlands). MV3 cells were cultured in DMEM medium containing glutamax (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (PAA, Pasching, Austria). Cultures were passaged twice a week and incubated at 37 °C in the presence of 5% CO_2. For immunofluorescence analysis, MV3 cells were seeded on 10-well 7 mm microscope slides (1.5×10^3 cells/well; Thermo Scientific, Waltham, MA, USA). For GAG isolation, approximately 0.5×10^6 MV3 cells were seeded in a T175 tissue culture flask. After 24 h (cell confluency around 30%), medium was supplemented with GlcNAc or 4-deoxy-GlcNAc at various concentrations ranging from 0.13 to 10 mM. After 3 days, cells were fixed with cold methanol and air-dried (immunofluorescence) or washed with PBS for GAG isolation.

Immunofluorescence assays

For detection of HS, cells were incubated with the VSV-tag containing, single chain variable fragment (ScFv) anti-HS antibodies AO4B08 [32], HS4C3 [33] and RB4EA12 [32]. Next, cells were incubated with mouse anti-VSV IgG, followed by Alexa488 fluorochrome conjugated anti-mouse IgG antibody (1:200; Invitrogen). For staining of other glycans, biotinylated lectins were used. Cells were first incubated with avidin-biotin blocking solution (Vector Laboratories), followed by lectins (1:200 - 1:1000), and Alexa488 fluorochrome conjugated streptavidin (1:1000; Invitrogen). To establish FGF-2/VEGF binding capacity, cells were incubated with 5 μg recombinant rat (rr) FGF-2/ml or 10 μg $rrVEGF_{164}$/ml [34]. Bound FGF-2/VEGF was detected by anti-FGF-2 (1:250)/anti-$VEGF_{164}$ (1:25) antibodies and Alexa488 fluorochrome conjugated anti-rabbit/anti-goat IgG antibodies (1:200; Invitrogen), respectively. Finally, cells were dehydrated with ethanol and mounted in mowiol. As a control, primary antibodies were omitted. Images were captured using a Leica-CTR6000 microscope.

Glycosaminoglycan isolation

After washing with PBS, cells were detached by 2.5 U papain/ml digestion buffer (50 mM sodium phosphate, 2 mM cysteine, 2 mM EDTA, pH 6.5) for 30 min at 37 °C and further digested for 16 hours at 65 °C. To precipitate proteins, trichloroacetic acid (TCA) was added to a final concentration of 15% (v/v) and samples were put on ice for 30 min. To remove the precipitated proteins, samples were centrifuged at 10,000*g* for 30 min at 4 °C. The supernatant containing the GAGs was diluted five times to reduce the concentration of salt and subjected to diethylaminoethyl (DEAE) sepharose (GE Healthcare, Chalfont St. Giles) columns. GAGs were eluted with 2 M NaCl in 10 mM Tris (pH 6.8) and precipitated by addition of five volumes methanol (incubation overnight -20 °C).

GAGs were pelleted by centrifugation at 10,000*g* for 30 min at 4 °C, air-dried, dissolved in milli-Q and stored at 4 °C.

Agarose gel electrophoresis

Agarose gel electrophoresis was basically performed as described earlier [35]. In short, isolated GAGs were separated using 1% agarose (w/v) in 50 mM $Ba(Ac)_2$ buffer (pH 5.0). After electrophoresis (30 mA/gel, 50 min), gels were fixed and stained with 0.1% (w/v) cationic dye azure A, destained with 10 mM NaAc buffer (pH 5.5) and washed in milli-Q. GAGs were visualized by silver staining.

Enzymatic glycosaminoglycan digestion

Cells or isolated HS, were digested with 0.04 or 0.02 IU heparinase III/ml 50 mM NaAc/50 mM $Ca(Ac)_2$ buffer (pH 7.5), respectively, overnight at 37 °C. Heparinase III digestion on fixed cells was evaluated with antibody 3G10, which recognizes HS stubs resulting after heparinase digestion, and anti-HS antibodies. Chondroitin sulphate and dermatan sulphate were digested overnight at 37 °C with 1 IU chondroitinase ABC/ml 25 mM Tris/2 mM $Mg(Ac)_2$ (pH 8.0). As control, cells or isolated GAGs were incubated with digestion buffer only.

WST-1 cell proliferation assay

To assess cell proliferation 1.5×10^3 cells/well were seeded into 96-wells plates. After 24 h, GlcNAc or 4-deoxy-GlcNAc was added to the medium at a final concentration of 1 mM. WST-1, a tetrazolium salt, was added according to the protocol provided by the manufacturer (Roche Diagnostics, Mannheim, Germany). The absorbance at 450 nm, corresponding to the number of metabolically active cells, was measured 30 min after WST-1 addition by a microtiter plate reader at 1, 2, 3, 4 or 5 days after the addition of GlcNAc or 4-deoxy-GlcNAc.

Endothelial cell sprouting assay

Sprouting of endothelial cells was studied with the use of cytodex-3 beads overgrown with endothelial cells in a three-dimensional gel and performed as described earlier [36]. After gel-formation, medium containing 20 ng FGF-2/ml with or without 1 mM GlcNAc or 1 mM 4-deoxy-GlcNAc was applied on top of the gel. After 24 h, photographs were made.

Statistics

Data are presented as mean with standard deviation. Sprouting assay data were analyzed using a two-tailed Student's *t*-test, with $P<0.05$ considered as significant. All statistical analyses were performed in GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA).

Results

4-deoxy-GlcNAc inhibits HS expression

The melanoma cell line MV3 was used to determine the effect of 4-deoxy-GlcNAc on HS expression. MV3 cells were cultured in the presence of various concentrations of the 4-deoxysugar and HS expression was analysed by agarose gel electrophoresis (Fig. 2A). Without addition of the 4-deoxysugar, HS and CS were the main glycosaminoglycans produced by MV3 melanoma cells. The HS band was sensitive to heparinase III treatment, whereas the CS band was abolished by chondroitinase ABC treatment, indicating the HS and CS nature of the respective bands (Fig. 2B). Addition of the 4-deoxysugar resulted in a dose dependent reduction of HS expression (Fig. 2A). At a concentration of 1 mM, the HS band was not detectable anymore. Therefore, this concentration was used for further studies. In addition to HS, CS band intensity was affected by the 4-deoxysugar, possibly by interference with 4-O-sulphation of CS (see discussion). Addition of 1 mM of the parent sugar GlcNAc had no effect on the amount or type of glycosaminoglycans produced by MV3 melanoma cells.

The reduction of HS by 4-deoxy-GlcNAc was further substantiated by immunocytochemistry using anti-HS antibodies (Fig. 2C). At a concentration of 1 mM 4-deoxy-GlcNAc no staining was observed in cultures of MV3 melanoma cells, in contrast to clear HS staining in cultures without 4-deoxy-GlcNAc or with 1 mM GlcNAc. No major changes in cellular morphology were observed. Similar results were obtained with Mel57 melanoma cells, SKOV-3 ovarian carcinoma cells and HFL-1 lung fibroblasts (data not shown).

Effect of 4-deoxy-GlcNAc on cell proliferation

To determine the effect on cell proliferation and viability, cells were cultured for up to 5 days in the presence of the 4-deoxysugar. On each day, the amount of metabolically active cells was established by the WST-1 assay (Fig. 3). Cells incubated with the 4-deoxysugar remained viable and proliferated at the same rate as untreated or GlcNAc treated cells. Comparable results were found with Mel57 melanoma cells, SKOV-3 ovarian carcinoma cells and HFL-1 lung fibroblasts (data not shown).

Effect of 4-deoxy-GlcNAc on lectin-positive glycans

To assess the effect of 4-deoxy-GlcNAc on other glycan structures, cells were stained with various lectins (Fig. 4 and Table 1). For most of the lectins (6 out of 10), staining remained largely unaffected. Four lectins showed a moderate reduction of staining, and they included the polyLacNAc recognizing lectin DSA and the O-glycan associated lectins PNA, SBA and VVA. Out of these four, staining by DSA seemed most affected. Staining for the latter three was quite similar and was found predominantly intracellular, presumably in the Golgi apparatus. Staining of WGA and the N-glycan associated lectins ConA, GNA and RCA-I appeared not affected by 4-deoxysugar treatment, or seemed slightly elevated in case of LCA and AAL.

4-deoxy-GlcNAc inhibits growth factor binding

HS is able to bind several growth factors, including FGF-2 [6] and VEGF [7]. Therefore, it was tested whether the binding of FGF-2 and VEGF to HS was impaired in 4-deoxysugar treated cells. As endogenously bound FGF-2 and VEGF could not be detected, cells were incubated with FGF-2 or VEGF and subsequently stained for bound growth factors using anti-FGF-2 or anti-VEGF antibodies (Fig. 5). 4-deoxysugar treated cells showed a pronounced decrease in bound FGF-2 and VEGF, compared to control and GlcNAc treated cells. Furthermore, the importance of HS for growth factor binding

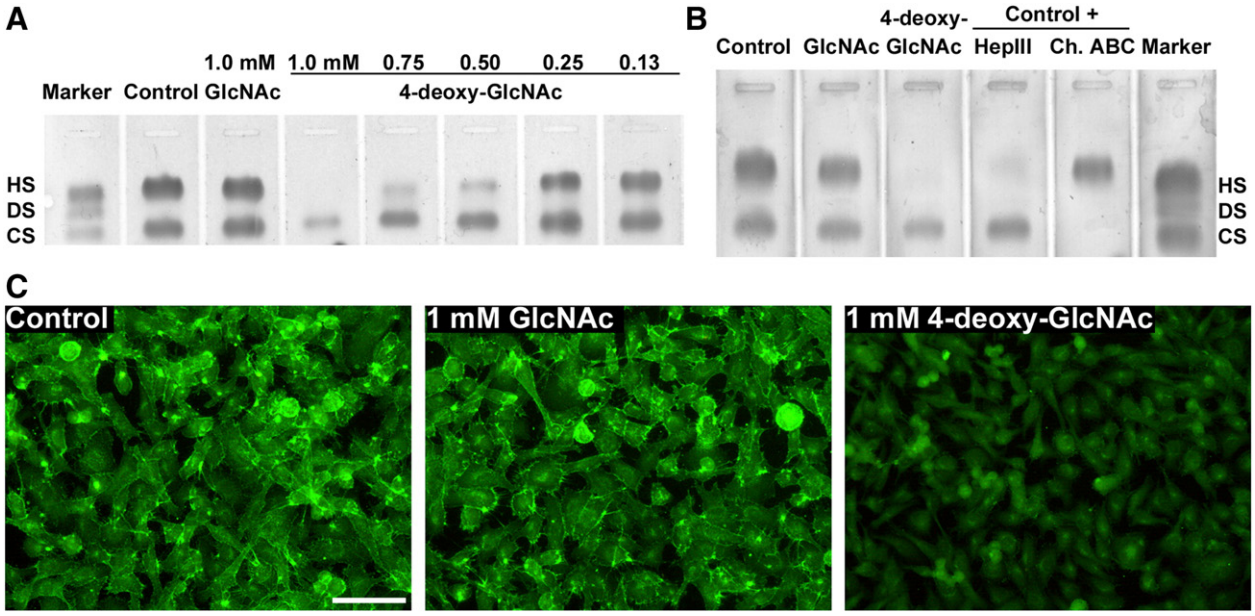


Fig. 2 – 4-deoxy-GlcNAc strongly reduces HS expression. MV3 melanoma cells were cultured for 3 days with or without 0.13–1 mM (A)/1 mM (B and C) 4-deoxy-GlcNAc or with 1 mM GlcNAc. Hereafter, cells were (A and B) analyzed for GAGs using agarose gel electrophoresis and a combined azure A-silver staining procedure; (C) stained for HS using antibody AO4B08 (scale bar = 100 μm); same results were observed for the antibodies HS4C3 and RB4EA12, which recognize different HS epitopes (data not shown). HepIII: heparinase III, Ch.ABC: Chondroitinase ABC, HS: heparan sulphate, CS: chondroitin sulphate, DS: dermatan sulphate.

was confirmed as binding of FGF-2 and VEGF to heparinase III digested control cells was strongly reduced (Fig. 5).

4-deoxy-GlcNAc inhibits endothelial cell sprouting

FGF-2 is an important player in the cascade of angiogenesis [37]. Since HS can bind this growth factor and can regulate its signalling, it

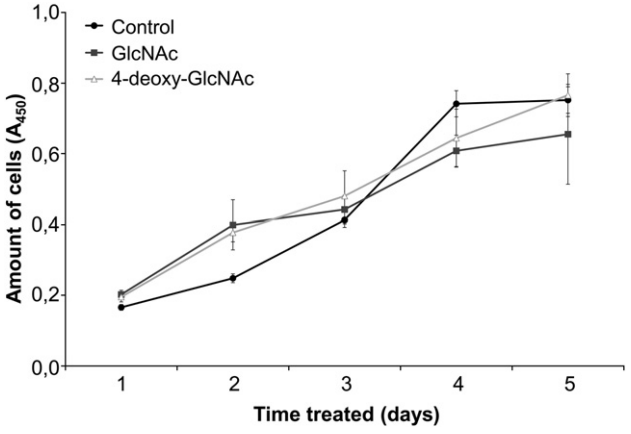


Fig. 3 – The effect of 4-deoxy-GlcNAc on cell proliferation. MV3 melanoma cells were cultured for several days with or without 1 mM GlcNAc or 1 mM 4-deoxy-GlcNAc, and the amount of metabolically active cells was measured on each day by the WST-1 cell proliferation assay. The absorbance of formazan (the cleavage-product of WST-1), which increases proportionally to the number of viable cells, was measured 30 min after addition of WST-1 at 450 nm. Data are expressed as mean ± SD ($n = 5$).

was tested whether the 4-deoxysugar has the ability to inhibit FGF-2 dependent sprouting of endothelial cells in an *in vitro* angiogenesis assay (Fig. 6). Endothelial cells were cultured on the surface of beads and embedded in a three-dimensional collagen gel and sprouting was induced by FGF-2. In the presence of 4-deoxy-GlcNAc sprouting of endothelial cells was strongly inhibited as compared to control and GlcNAc treated cells (Fig. 6A). Quantification of these results showed that the 4-deoxysugar significantly inhibited sprouting by 75% as compared to the control (Fig. 6B). Sprouting of GlcNAc treated cells did not significantly differ from control.

Discussion

It is becoming increasingly clear that the amount and the fine structure of glycosaminoglycans are important determinants in tumourigenesis. Especially HS is a key player by regulating tumour cell proliferation, angiogenesis, invasion and metastasis [5]. A central aspect is the function of HS chains as co-receptors for growth factors, including FGF-2 and VEGF [6,7]. By this involvement, HS serves as a good target for cancer therapy. Most strategies to reduce/eliminate HS in tumours are based on either digestion of the HS by enzymatic treatment, or by interference of the activity of HS by administration of HS mimetics. Some agents interfering with HS function have already entered clinical trials, e.g. PI-88 [38].

An alternative route to decrease HS is by targeting its biosynthesis. There are a number of strategies which can be applied including interference with biosynthetic enzymes such as EXT1/EXT2 polymerizing enzymes [39,40], and modifying enzymes, such as *N*-deacetylase/*N*-sulphotransferase-1 (NDST-1) [41], 6-O-sulphotransferase-1 [42] and 6-O-endosulphatase hSulf-1

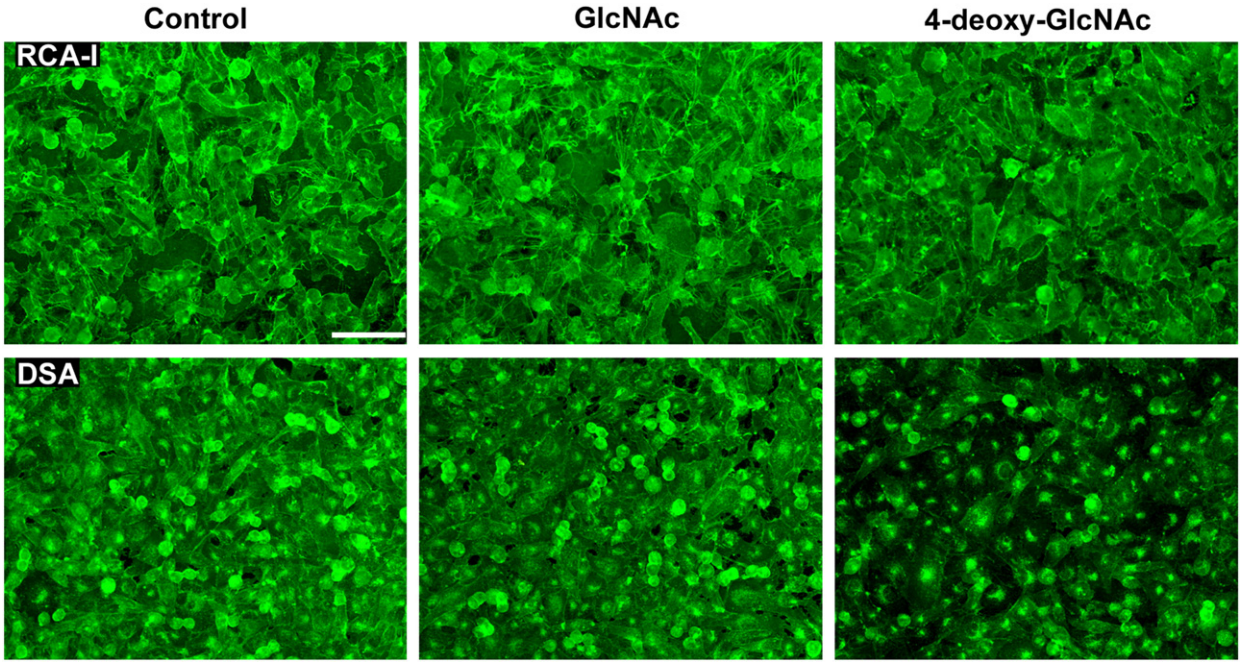


Fig. 4 – The effect of 4-deoxy-GlcNAc on staining of the lectins DSA and RCA-I (also see Table 1). MV3 melanoma cells were cultured for 3 days with or without 1 mM GlcNAc or 1 mM 4-deoxy-GlcNAc, fixed and stained with various lectins. Shown here is the staining by the lectins RCA-I (from *Ricinus communis*) and DSA (from *Datura stramonium*). Staining by RCA-I is predominantly found at the cell surface, whereas staining by DSA is also found intracellular (probably in the Golgi-apparatus). For further information see Table 1. Scale bar = 100 μm.

Table 1 – The effect of 4-deoxy-GlcNAc on staining by lectins of MV3 melanoma cells.

Lectin	Binding specificity[a]	Control	GlcNAc	4-deoxy-GlcNAc
AAL	α1-2/3/6 linked Fuc to Gal/GlcNAc	++ i s	++	++[b]
ConA	αMan (oligomannose, hybrid and biantennary complex N-glycans)	+++ i s	+++	+++
DSA	(-β1-3Galβ1-4GlcNAc-)$_n$ (PolyLacNac; N- and O-glycans)	++ i s	++	↓
GNA	Terminal α1-3Man (oligomannose N-glycans)	+++ i	+++	+++
LCA	Fucosylated core region of bi- and triantennary complex N-glycans	++ i s	++	++[b]
PNA	Galβ1-3GalNAcα-R (O-glycans)	+ i	+	↓
RCA-I	Terminal βGal (N-glycans)	+++ s	+++	+++
SBA	GalNAcα/β-R (O-glycans)	+ i	+	↓
VVA	GalNAcα-R (O-glycans)	++ i	++	↓
WGA	GlcNAcβ-R, Neu5Acα-R (O- and N-glycans)	++ s	++	++

MV3 melanoma cells were cultured for 3 days with or without 1 mM 4-deoxy-GlcNAc, or with 1 mM GlcNAc, and stained with various lectins.
[a] Binding specificities are from [50,51]. Where possible, it is indicated whether these structures are found on N- and/or O-linked glycans. +++ strong; ++ moderate; + weak staining.
[b] Slight increase; ↓ modest decrease compared to staining of control and GlcNAc treated cells. Staining found i: intracellular (presumably Golgi); s: at the cell surface.

[13]. As an alternative to such approaches, we probed a monosaccharide analogue (4-deoxy-GlcNAc) for its capacity to inhibit HS expression. The rationale of this strategy is that when cells incorporate the 4-deoxysugar into HS chains, the formation of a glycosidic bond between the 1-OH group of GlcA and the 4-OH group of GlcNAc will be prevented, thereby stopping chain elongation. A similar approach has been used by us and others [23–28] and the 4-deoxysugar has been reported to inhibit HS biosynthesis of rat fibroblasts (RFL-6) [23], hepatocytes [24–26], islets of Langerhans and ß-TC3 cells [27], and cells of the central nervous system [28]. Kisilevsky et al. [25] have found that incorporation of the 4-deoxysugar resulted in truncated HS chains *in vitro* and inhibition of amyloidogenesis *in vivo*, with no apparent toxicity. In the present study, we found a strongly diminished HS expression as a result of 4-deoxy-GlcNAc treatment, as shown by HS immunocytochemistry and agarose gel electrophoresis. In addition, binding of pro-angiogenic growth factors was inhibited, as was FGF-2 dependent endothelial sprouting.

As GlcNAc is not only incorporated into HS, it is likely that the 4-deoxysugar may also affect other glycosaminoglycans (e.g. hyaluronan, keratan sulphate), and/or N- and O-linked glycan containing glycoproteins. We did observe a reduction in staining for chondroitin sulphate. Since 4-deoxy-GlcNAc lacks the hydroxyl group which distinguishes between GlcNAc and *N*-acetyl-D-galactosamine (GalNAc), it is not unlikely that the 4-deoxysugar also affects the GalNAc containing CS/DS chains. If the 4-deoxysugar is incorporated into CS/DS it may result in an inhibition of 4-O sulphation. Alternatively, it may inhibit epimerase activity required for the formation of UDP-GalNAc out of UDP-GlcNAc, as previously suggested for a peracetylated 4-fluoro analogue of GlcNAc [43]. Unexpectedly, staining with lectins was not or only mildly affected, in sharp contrast to the strong effect on HS. Out of the ten lectins

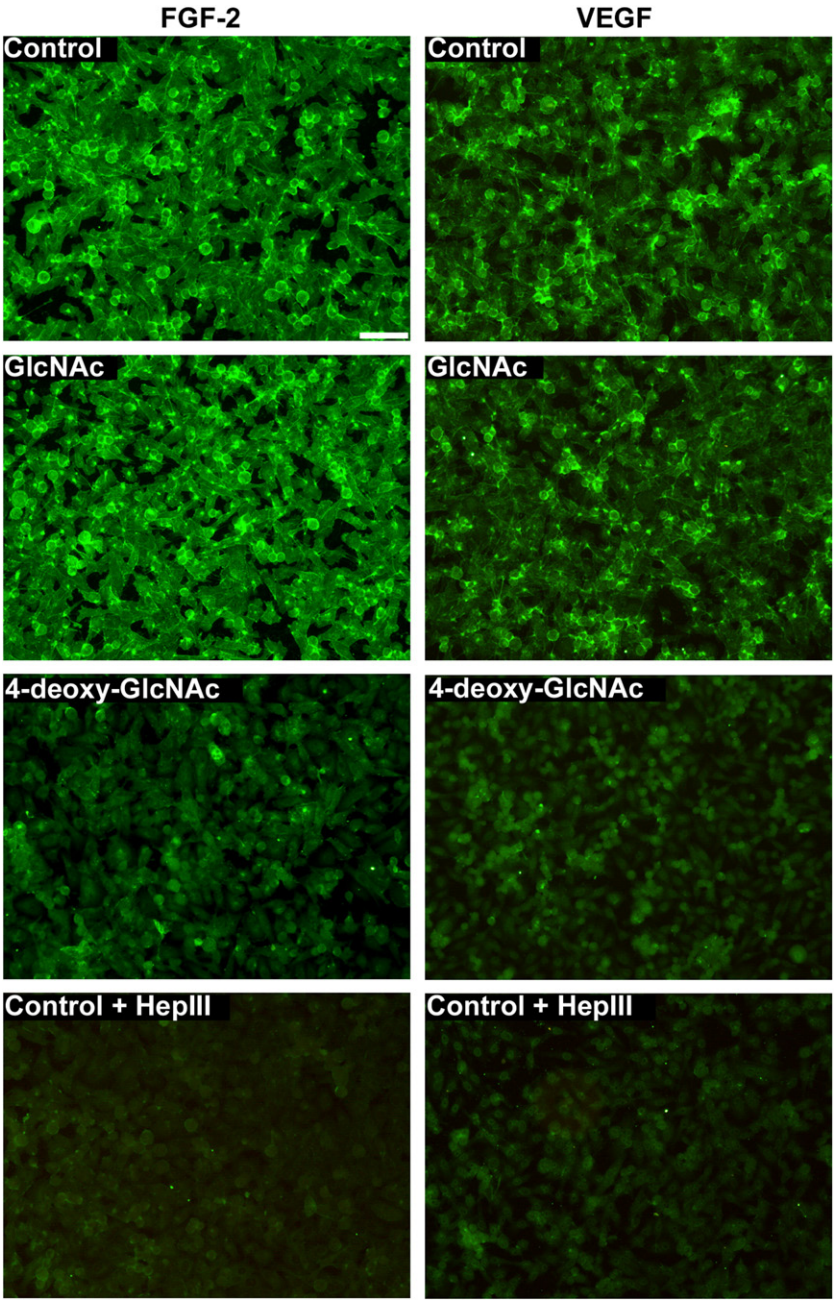


Fig. 5 – 4-deoxy-GlcNAc strongly reduces binding of FGF-2 and VEGF. MV3 cells treated with or without 1 mM GlcNAc or 1 mM 4-deoxy-GlcNAc were fixed, incubated with and stained for FGF-2 or $VEGF_{164}$. In the case of 4-deoxy-GlcNAc treated cells, a strongly reduced staining for both growth factors was observed. Removal of HS by heparinase III (HepIII) also results in strong reduction of growth factor staining. Scale bar = 100 μm.

tested, staining by DSA appeared to be affected the most, which might be explained by the fact that *N*-acetyllactosamine (LacNAc) units (-β1-3Galβ1-4GlcNAc-), as recognized by DSA, contain multiple 4-GlcNAc linkages.

Reduction of HS by the 4-deoxysugar was associated with a reduced capability of cells to bind the HS-dependent growth factors FGF-2 and VEGF. Sprouting of endothelial cells in response to FGF-2 was strongly reduced. For efficient signalling, FGF-2 as well as its cognate receptor have to bind to HS [44,45], and FGF-2 induced sprouting of endothelial cells has been reported to be strongly decreased by heparinase III treatment [41]. Furthermore, angiogenic sprouting in response to VEGF is dependent on HS, as treatment with a combination of heparinase I, II and III markedly decreased the number of angiogenic sprouts induced by VEGF [46]. It was observed that heparinase I and III inhibited neovascularisation in an *in vivo* chicken chorio-allantoic membrane angiogenesis assay [47]. Combined, these data indicate that reduction of HS results in an inhibition of angiogenic events.

Next to the effect of quantitative reduction of HS, changes in HS fine structures may also result in altered growth factor binding. Although not tested here, the deoxysugar approach allows such a strategy e.g. by application of deoxysugars lacking a specific

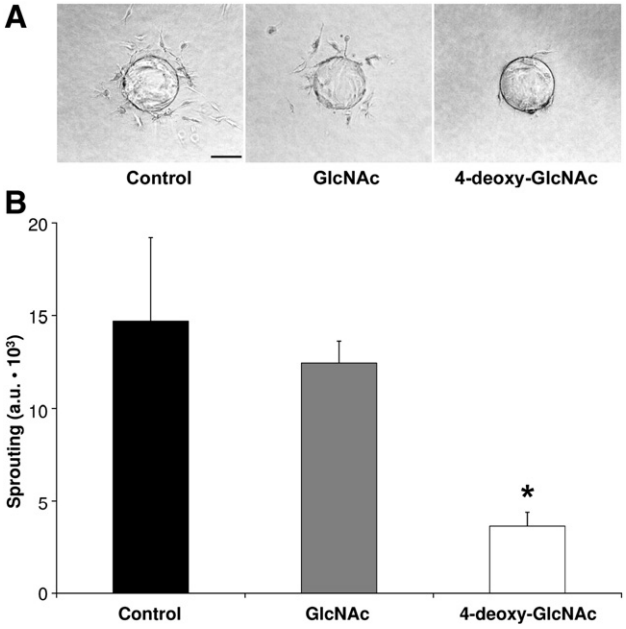


Fig. 6 – 4-deoxy-GlcNAc inhibits the sprouting of endothelial cells. (A) Sprouting of bovine microvascular endothelial cells cultured on gelatin-coated beads in a collagen gel was induced by FGF-2. Sprouting was strongly affected by addition of 1 mM 4-deoxy-GlcNAc. Scale bar = 100 μm. (B) Quantification of sprouting was performed by NIH image software. Data are expressed as arbitrary units (a.u.) and are shown as mean with SD ($n = 3$ or 4). Representative data of one of two separate experiments is shown. $^{*}P < 0.005$ compared to control or GlcNAc, using the Student's *t*-test.

hydroxyl group involved in O-sulphations. Binding of FGF-2 to HS is dependent on *N*-sulphated glucosamine units and one IdoA2S unit [48]. For interaction with its cognate receptor and downstream signalling additional 6-O-sulphation is required [45]. Binding of dimeric VEGF to HS depends on two highly sulphated domains, in which 6-O-sulphation appears particularly important, both domains linked by a less sulphated domain [49]. An example of fine structure alteration resulting in reduced growth factor binding, is the endothelium specific knock out of NDST-1 in mice [41]. Endothelial cells showed a reduced binding capacity for FGF-2 and VEGF, whereas endothelial cell sprouting in response to these growth factors was markedly reduced [41]. The dependency on sulphation motifs provides options for a more specific inhibition of growth factor binding. Current research focuses on novel deoxysugars aimed at blocking specific sulphation sites.

In conclusion, we have shown that an inexpensive, easily synthesized, water-soluble monosaccharide analogue can interfere with HS expression and pro-angiogenic growth factor binding. Further research will focus on the effect of the 4-deoxysugar on tumour formation *in vivo*.

Acknowledgments

We thank Loes van Eijk for performing endothelial cell sprouting assays. Lectins were a generous gift from the department of nephrology (RUNMC, Nijmegen, The Netherlands).

This work was supported by the Dutch Cancer Society [grant number 2008-4058] (to GtD) and the International Human Frontier Science Program Organization [grant RGP0062/2004-C101] (to TvK) The funding sources had no active involvement in the contents of this work.

REFERENCES

[1] J.D. Esko, S.B. Selleck, Order out of chaos: assembly of ligand binding sites in heparan sulfate, Annu. Rev. Biochem. 71 (2002) 435–471.
[2] G.K. Dhoot, M.K. Gustafsson, X. Ai, W. Sun, D.M. Standiford, C.P. Emerson Jr., Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase, Science 293 (2001) 1663–1666.
[3] J.R. Bishop, M. Schuksz, J.D. Esko, Heparan sulphate proteoglycans fine-tune mammalian physiology, Nature 446 (2007) 1030–1037.
[4] M. Bernfield, M. Gotte, P.W. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, M. Zako, Functions of cell surface heparan sulfate proteoglycans, Annu. Rev. Biochem. 68 (1999) 729–777.
[5] R. Sasisekharan, Z. Shriver, G. Venkataraman, U. Narayanasami, Roles of heparan-sulphate glycosaminoglycans in cancer, Nat. Rev. Cancer 2 (2002) 521–528.
[6] A. Yayon, M. Klagsbrun, J.D. Esko, P. Leder, D.M. Ornitz, Cell surface, heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor, Cell 64 (1991) 841–848.
[7] H. Gitay-Goren, S. Soker, I. Vlodavsky, G. Neufeld, The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules, J. Biol. Chem. 267 (1992) 6093–6098.
[8] I.R. Cohen, A.D. Murdoch, M.F. Naso, D. Marchetti, D. Berd, R.V. Iozzo, Abnormal expression of perlecan proteoglycan in metastatic melanomas, Cancer Res. 54 (1994) 5771–5774.
[9] B. Sharma, M. Handler, I. Eichstetter, J.M. Whitelock, M.A. Nugent, R.V. Iozzo, Antisense targeting of perlecan blocks tumor growth and angiogenesis in vivo, J. Clin. Invest. 102 (1998) 1599–1608.
[10] C.M. Alexander, F. Reichsman, M.T. Hinkes, J. Lincecum, K.A. Becker, S. Cumberledge, M. Bernfield, Syndecan-1 is required for Wnt-1-induced mammary tumorigenesis in mice, Nat. Genet. 25 (2000) 329–332.
[11] Y.B. Khotskaya, Y. Dai, J.P. Ritchie, V. Macleod, Y. Yang, K. Zinn, R.D. Sanderson, Syndecan-1 is required for robust growth, vascularization, and metastasis of myeloma tumors in vivo, J. Biol. Chem. 284 (2009) 26085–26095.
[12] T. Aikawa, C.A. Whipple, M.E. Lopez, J. Gunn, A. Young, A.D. Lander, M. Korc, Glypican-1 modulates the angiogenic and metastatic potential of human and mouse cancer cells, J. Clin. Invest. 118 (2008) 89–99.
[13] K. Narita, J. Staub, J. Chien, K. Meyer, M. Bauer, A. Friedl, S. Ramakrishnan, V. Shridhar, HSulf-1 inhibits angiogenesis and tumorigenesis in vivo, Cancer Res. 66 (2006) 6025–6032.
[14] Y. Dai, Y. Yang, V. MacLeod, X. Yue, A.C. Rapraeger, Z. Shriver, G. Venkataraman, R. Sasisekharan, R.D. Sanderson, HSulf-1 and HSulf-2 are potent inhibitors of myeloma tumor growth in vivo, J. Biol. Chem. 280 (2005) 40066–40073.
[15] R. Nawroth, A. van Zante, S. Cervantes, M. McManus, M. Hebrok, S.D. Rosen, Extracellular sulfatases, elements of the Wnt signaling pathway, positively regulate growth and tumorigenicity of human pancreatic cancer cells, PLoS ONE 2 (2007) e392.
[16] T.F. Smetsers, E.M. van de Westerlo, G.B. ten Dam, R. Clarijs, E.M. Versteeg, W.L. van Geloof, J.H. Veerkamp, G.N. van Muijen, T.H. van Kuppevelt, Localization and characterization of melanoma-associated glycosaminoglycans: differential expression of chondroitin and heparan sulfate epitopes in melanoma, Cancer Res. 63 (2003) 2965–2970.
[17] G.B. ten Dam, E.M. van de Westerlo, A. Purushothaman, R.V. Stan, J. Bulten, F.C. Sweep, L.F. Massuger, K. Sugahara, T.H. van

Kuppevelt, Antibody GD3G7 selected against embryonic glycosaminoglycans defines chondroitin sulfate-E domains highly up-regulated in ovarian cancer and involved in vascular endothelial growth factor binding, Am. J. Pathol. 171 (2007) 1324–1333.
[18] G.B. ten Dam, E.M. van de Westerlo, T.F. Smetsers, M. Willemse, G.N. van Muijen, C.L. Merry, J.T. Gallagher, Y.S. Kim, T.H. van Kuppevelt, Detection of 2-O-sulfated iduronate and N-acetylglucosamine units in heparan sulfate by an antibody selected against acharan sulfate (IdoA2S-GlcNAc)n, J. Biol. Chem. 279 (2004) 38346–38352.
[19] M.M. Fuster, J.D. Esko, The sweet and sour of cancer: glycans as novel therapeutic targets, Nat. Rev. Cancer. 5 (2005) 526–542.
[20] D. Liu, Z. Shriver, G. Venkataraman, Y. El Shabrawi, R. Sasisekharan, Tumor cell surface heparan sulfate as cryptic promoters or inhibitors of tumor growth and metastasis, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 568–573.
[21] C.R. Parish, C. Freeman, K.J. Brown, D.J. Francis, W.B. Cowden, Identification of sulfated oligosaccharide-based inhibitors of tumor growth and metastasis using novel in vitro assays for angiogenesis and heparanase activity, Cancer Res. 59 (1999) 3433–3441.
[22] M. Nakajima, A. DeChavigny, C.E. Johnson, J. Hamada, C.A. Stein, G.L. Nicolson, Suramin, A potent inhibitor of melanoma heparanase and invasion, J. Biol. Chem. 266 (1991) 9661–9666.
[23] T.H. van Kuppevelt, S. Buiting, J.H. Veerkamp, Feasibility of sequencing glycosaminoglycans using (di)deoxysugar analogues, abstract C16, XVth Meeting of the Federation of European Connective Tissue Societies (FECTS), Munich, Germany, August 4 - 9 1996.
[24] A. Berkin, M.A. Szarek, J. Plenkiewicz, W.A. Szarek, R. Kisilevsky, Synthesis of 4-deoxy analogues of 2-acetamido-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-xylose and their effects on glycoconjugate biosynthesis, Carbohydr. Res. 325 (2000) 30–45.
[25] R. Kisilevsky, W.A. Szarek, J.B. Ancsin, E. Elimova, S. Marone, S. Bhat, A. Berkin, Inhibition of amyloid A amyloidogenesis in vivo and in tissue culture by 4-deoxy analogues of peracetylated 2-acetamido-2-deoxy-α- and β-D-glucose: implications for the treatment of various amyloidoses, Am. J. Pathol. 164 (2004) 2127–2137.
[26] A. Berkin, W.A. Szarek, R. Kisilevsky, Biological evaluation of a series of 2-acetamido-2-deoxy-D-glucose analogs towards cellular glycosaminoglycan and protein synthesis in vitro, Glycoconj. J. 22 (2005) 443–451.
[27] R.L. Hull, S. Zraika, J. Udayasankar, R. Kisilevsky, W.A. Szarek, T.N. Wight, S.E. Kahn, Inhibition of glycosaminoglycan synthesis and protein glycosylation with WAS-406 and azaserine result in reduced islet amyloid formation in vitro, Am. J. Physiol. Cell Physiol. 293 (2007) C1586–1593.
[28] R. Kisilevsky, J.B. Ancsin, W.A. Szarek, S. Petanceska, Heparan sulfate as a therapeutic target in amyloidogenesis: prospects and possible complications, Amyloid 14 (2007) 21–32.
[29] D.P. Sutherlin, T.M. Stark, R. Hughes, R.W. Armstrong, Generation of C-glycoside peptide ligands for cell surface carbohydrate receptors using a four-component condensation on solid support, J. Org. Chem. 61 (1996) 8350–8354.
[30] D.H.R. Barton, S.W. McCombie, A new method for the deoxygenation of secondary alcohols, J. Chem. Soc. Perkin Trans. 1 (1975) 1574–1585.
[31] G.N. van Muijen, K.F. Jansen, I.M. Cornelissen, D.F. Smeets, J.L. Beck, D.J. Ruiter, Establishment and characterization of a human melanoma cell line (MV3) which is highly metastatic in nude mice, Int. J. Cancer 48 (1991) 85–91.
[32] G.J. Jenniskens, A. Oosterhof, R. Brandwijk, J.H. Veerkamp, T.H. van Kuppevelt, Heparan sulfate heterogeneity in skeletal muscle basal lamina: demonstration by phage display-derived antibodies, J. Neurosci. 20 (2000) 4099–4111.
[33] T.H. van Kuppevelt, M.A. Dennissen, W.J. van Venrooij, R.M. Hoet, J.H. Veerkamp, Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney, J. Biol. Chem. 273 (1998) 12960–12966.
[34] S.T. Nillesen, P.J. Geutjes, R. Wismans, J. Schalkwijk, W.F. Daamen, T.H. van Kuppevelt, Increased angiogenesis and blood vessel maturation in acellular collagen-heparin scaffolds containing both FGF2 and VEGF, Biomaterials 28 (2007) 1123–1131.
[35] C.H. van de Lest, E.M. Versteeg, J.H. Veerkamp, T.H. van Kuppevelt, Quantification and characterization of glycosaminoglycans at the nanogram level by a combined azure A-silver staining in agarose gels, Anal. Biochem. 221 (1994) 356–361.
[36] R.P. Dings, D.W. van der Schaft, B. Hargittai, J. Haseman, A.W. Griffioen, K.H. Mayo, Anti-tumor activity of the novel angiogenesis inhibitor anginex, Cancer Lett. 194 (2003) 55–66.
[37] A.W. Griffioen, G. Molema, Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation, Pharmacol. Rev. 52 (2000) 237–268.
[38] K.D. Lewis, W.A. Robinson, M.J. Millward, A. Powell, T.J. Price, D.B. Thomson, E.T. Walpole, A.M. Haydon, B.R. Creese, K.L. Roberts, J.R. Zalcberg, R. Gonzalez, A phase II study of the heparanase inhibitor PI-88 in patients with advanced melanoma, Invest. New Drugs 26 (2008) 89–94.
[39] X. Lin, G. Wei, Z. Shi, L. Dryer, J.D. Esko, D.E. Wells, M.M. Matzuk, Disruption of gastrulation and heparan sulfate biosynthesis in EXT1-deficient mice, Dev. Biol. 224 (2000) 299–311.
[40] D. Stickens, B.M. Zak, N. Rougier, J.D. Esko, Z. Werb, Mice deficient in Ext2 lack heparan sulfate and develop exostoses, Development 132 (2005) 5055–5068.
[41] M.M. Fuster, L. Wang, J. Castagnola, L. Sikora, K. Reddi, P.H. Lee, K.A. Radek, M. Schuksz, J.R. Bishop, R.L. Gallo, P. Sriramarao, J.D. Esko, Genetic alteration of endothelial heparan sulfate selectively inhibits tumor angiogenesis, J. Cell Biol. 177 (2007) 539–549.
[42] H. Habuchi, N. Nagai, N. Sugaya, F. Atsumi, R.L. Stevens, K. Kimata, Mice deficient in heparan sulfate 6-O-sulfotransferase-1 exhibit defective heparan sulfate biosynthesis, abnormal placentation, and late embryonic lethality, J. Biol. Chem. 282 (2007) 15578–15588.
[43] J. Nigro, A. Wang, D. Mukhopadhyay, M. Lauer, R.J. Midura, R. Sackstein, V.C. Hascall, Regulation of heparan sulfate and chondroitin sulfate glycosaminoglycan biosynthesis by 4-fluoro-glucosamine in murine airway smooth muscle cells, J. Biol. Chem. 284 (2009) 16832–16839.
[44] M. Kan, F. Wang, J. Xu, J.W. Crabb, J. Hou, W.L. McKeehan, An essential heparin-binding domain in the fibroblast growth factor receptor kinase, Science 259 (1993) 1918–1921.
[45] L. Lundin, H. Larsson, J. Kreuger, S. Kanda, U. Lindahl, M. Salmivirta, L. Claesson-Welsh, Selectively desulfated heparin inhibits fibroblast growth factor-induced mitogenicity and angiogenesis, J. Biol. Chem. 275 (2000) 24653–24660.
[46] L. Jakobsson, J. Kreuger, K. Holmborn, L. Lundin, I. Eriksson, L. Kjellen, L. Claesson-Welsh, Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis, Dev. Cell 10 (2006) 625–634.
[47] R. Sasisekharan, M.A. Moses, M.A. Nugent, C.L. Cooney, R. Langer, Heparinase inhibits neovascularization, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 1524–1528.
[48] M. Maccarana, B. Casu, U. Lindahl, Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor, J. Biol. Chem. 268 (1993) 23898–23905.
[49] C.J. Robinson, B. Mulloy, J.T. Gallagher, S.E. Stringer, VEGF165-binding sites within heparan sulfate encompass two highly sulfated domains and can be liberated by K5 lyase, J. Biol. Chem. 281 (2006) 1731–1740.
[50] A. Varki, R.D. Cummings, J.D. Esko, H.H. Freeze, P. Stanley, C.R. Bertozzi, G.W. Hart, M.E. Etzler, Antibodies and lectins in glycan analysis, essentials of glycobiology, Cold Spring Harbor Laboratory Press, New York, USA, 2009 pp. 633–647.
[51] E. Tian, K.G. Ten Hagen, O-linked glycan expression during Drosophila development, Glycobiology 17 (2007) 820–827.