

# Neoglycans, Carbodiimide-modified Glycosaminoglycans: A New Class of Anticancer Agents That Inhibit Cancer Cell Proliferation and Induce Apoptosis<sup>1</sup>

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## ABSTRACT

The soluble form of the syndecan-1 heparan sulfate proteoglycan acts as a tumor suppressor molecule that inhibits growth and induces apoptosis of some cancer cell lines *in vitro*. Analogs of syndecan-1 were produced by carbodiimide (EDAC) conjugation of glycosaminoglycan (GAG) chains to a protein scaffold, thereby generating synthetic proteoglycans that were evaluated for anticancer properties. Surprisingly, when analyzing activities of the controls, we discovered that EDAC modified GAG chains inhibit myeloma cell viability even in the absence of protein. Here, we describe the production and the activities of these novel molecules called neoglycans. The GAG chains heparin and chondroitin sulfate (CS) were exposed to EDAC to generate the neoglycans neoheparin and neoCS, respectively. Heparin and CS in the absence of EDAC modification have no effect or a slight growth promoting effect on cancer and normal cell lines. However, neoheparin and neoCS substantially reduce cell viability by induction of apoptosis of myeloma and breast cancer cells *in vitro*. NeoCS when injected directly into breast tumors growing in nude mice reduces or abolishes their growth without causing apparent toxicity to the adjacent normal tissue. The neoglycans need not be continuously present in cell cultures because a short pulse exposure is sufficient to reduce cell viability. NeoCS fractions purified by size exclusion chromatography reduce myeloma cell viability, confirming the specificity of neoglycan activity. Collectively, the results of this study demonstrate the anticancer activities of this new class of GAG chain-based molecules and provide the foundation for future development of neoglycans as novel therapeutic agents.

## INTRODUCTION

The concept that GAG<sup>3</sup> chains can impact cancer progression either positively or negatively has been investigated for decades. GAG chains, through binding and regulation of a formidable number of ligands, are important mediators of tumor cell and normal cell behaviors such as proliferation, differentiation, migration and adhesion (1–5). The GAG chain heparin can inhibit or stimulate tumor growth and metastasis depending on the type of cancer and the animal model (6, 7). In cancer patients, administration of low molecular weight heparin improves three-month survival possibly by reducing angiogenesis or by inhibiting metastasis (8–12). Another GAG chain, hyaluronic acid (HA), inhibits melanoma tumor growth in an animal model but rescues and enhances proliferation of IL-6-dependent myeloma cells cultured in the absence of exogenous IL-6 (13, 14). The specific structure of GAG chains and the type of cancer likely influ-

ence the effect of GAG chains on tumor cell proliferation and metastasis and therefore, on cancer progression (5).

Most GAG chains are attached to core proteins to form proteoglycans which are found on cell surfaces and in the extracellular matrix. The syndecans and the glypicans are the major families of cell surface heparan sulfate proteoglycans and there is substantial evidence that syndecan-1 acts as a powerful tumor suppressor. Expression of syndecan-1 on myeloma cells reduces disease-related morbidity in a mouse model (15). Syndecan-1 shed from the cell surface by sheddases or secretases (16, 17) retains biological activity and can reduce cell growth and induce apoptosis of myeloma cells *in vitro* (15). The syndecan-1 ectodomain also suppresses the growth of CarB, S115 and MCF-7 mouse and human mammary tumor cells *in vitro* but not NIH3T3, NMuMG or HaCaT normal cell lines (18). There is also an emerging correlation between loss of cell surface heparan sulfate proteoglycan expression and cancer progression. Simpson-Golabi-Behmel syndrome is the result of mutation of the heparan sulfate proteoglycan glypican-3 gene and is characterized by prenatal and postnatal overgrowth and an increased incidence of tumor development (19, 20). Mutations that inhibit heparan sulfate polymerase enzymes cause hereditary multiple exostoses which is characterized by the formation of benign bone tumors (21, 22). Also, loss of syndecan-1 expression on tumor cells correlates with a poor prognosis in patients with head and neck cancer, mesothelioma and gastric carcinoma and is thought to contribute to increased metastatic potential of non-small-cell lung and hepatocellular carcinomas (23–28). In contrast to these cancers, pancreatic tumors and their metastatic lesions express high amounts of syndecan-1 demonstrating that syndecan-1 expression is not always lost with cancer progression (29).

To study the tumor suppressor functions of syndecan-1 and to develop its potential as a therapeutic agent requires the isolation of large quantities of the molecule, a process that is severely hampered by the lack of a suitable system for the production of the protein bearing attached and appropriately modified heparan sulfate chains. To overcome this obstacle, we produced synthetic proteoglycans (neoproteoglycans) by linking GAG chains to human serum albumin by carbodiimide (EDAC) conjugation. Although these preparations significantly reduced cell viability, surprisingly, EDAC modified GAG chains prepared in the absence of protein also reduce cell viability *in vitro* and *in vivo*. This is in sharp contrast to unmodified heparin and CS which do not reduce cell viability. These novel compounds, called neoglycans, constitute a new class of anticancer agents.

## MATERIALS AND METHODS

**Neoglycan Production.** Neoglycans were prepared from porcine intestinal heparin and chondroitin sulfate C from shark cartilage (Sigma, St. Louis, MO). A 5 mg/ml solution of GAG chains in deionized H<sub>2</sub>O was brought to a 0.1 M concentration of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC). The mixture was rocked overnight at 4°C followed by centrifugation at 20,000 × g for 10 min to pellet any precipitant. The supernatant was collected and exhaustively buffer exchanged into H<sub>2</sub>O and concentrated using 5,000 kDa

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<sup>3</sup> The abbreviations used are: GAG, glycosaminoglycan; EDAC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; CS, chondroitin sulfate; IC<sub>50</sub>, 50% inhibitory concentration; PBMC, peripheral blood mononuclear cell; MTT, 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium; PI, propidium iodide.

molecular weight cutoff spin columns (Millipore, Bedford, MA). Neoglycan preparations were stored at 4°C. Before *in vivo* studies, neoCS was brought to 0.15 M NaCl.

**Neoglycan Concentration Determination by Carbazole Assay.** Carbazole assays were performed according to the protocols of Blumenkrantz and Asboe-Hansen (30) and Filisetti-Cozzi and Carpita (31). Known concentrations of heparin and CS ranging from 0 to 60 µg were aliquoted into glass test tubes. One µl of each neoglycan, approximately 50 µg, was aliquoted into glass test tubes in duplicate. The volume of each tube was brought to 200 µl with H<sub>2</sub>O. One ml of 0°C 125 mM sodium tetraborate (Sigma) in sulfuric acid was added to each tube followed by the addition of 35 µl of a carbazole reagent composed of 0.125% carbazole (Sigma) w/v in ETOH. The tubes were mixed carefully and heated to 100°C for 20 min. Cooled aliquots were transferred to a 96-well plate and the absorbance at OD<sub>540</sub> was measured on a microplate reader. Known GAG chain concentrations served as a standard curve for the extrapolation of the unknown neoglycan concentrations.

**Cells and Cell Culture.** ARK, ARP-1 and CAG cells were established at the Arkansas Cancer Research Center from bone marrow aspirates of multiple myeloma patients (32, 33). U266 myeloma cells and ARH-77 cells [an EBV-transformed cell line established from a patient with plasma cell leukemia, (34)] were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Peripheral blood mononuclear cells (PBMCs) were collected from healthy donors by Ficol-Histopaque separation of whole blood and were mitogen activated for 3 days with 2.5 µg/ml PHA (Sigma) before neoglycan treatment. The cell lines and PBMCs were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin sulfate. HBL-100, Hs578t, MCF-7, MDA-MB-231, MDA-MB-435 and MDA-MB-436 breast cancer cell lines were obtained from the ATCC and were maintained in culture media as suggested by the ATCC. Normal cell lines including CHO-K1 chinese hamster ovary cells, NIH3T3 Swiss murine embryo fibroblast cells, NMuNG murine mammary cells, MDCK canine kidney cells and MCF-10A and MCF-10F human mammary cell lines were all purchased from the ATCC and grown in media specified by the ATCC. All of the cells were cultured at 37°C in an atmosphere of 5% CO<sub>2</sub> and adherent cell lines were harvested using standard trypsinization procedures.

**Determination of Cell Viability by MTT Assay.** Cells were plated in the appropriate media on 96-well plates in a 100 µl total volume. Nonadherent cells were plated at a density of  $2 \times 10^4$  cells/well. Adherent cell density varied based on the growth characteristics of each cell line and these cells were plated one day before neoglycan addition to allow the cells to attach. Triplicate wells were treated with media, 10 µM dexamethasone, 50 µg heparin or CS, and 3.2 µg, 1.6 µg, and 0.32 µg of neoheparin or neoCS. The plates were incubated at 37°C in 5% CO<sub>2</sub> for 72 h. Cell viability was determined based on mitochondrial conversion of 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) to formazan. The amount of MTT converted to formazan is indicative of the number of viable cells (35). Each well was supplemented with 50 µl of a 2 mg/ml solution of MTT in complete media. The plates were returned to 37°C, 5% CO<sub>2</sub> for 4–5 h. The media was carefully removed from each well and 150 µl of 37°C DMSO was added. The plates were gently agitated until the color reaction was uniform and the OD<sub>540</sub> was determined using a microplate reader. SigmaPlot 2000 software was used for data analysis. Media only treated cells served as the indicator of 100% cell viability.

To determine whether continuous exposure to neoglycans is required for growth inhibition, ARP-1 cells were exposed to 320 µg/ml and 32 µg/ml neoglycans for 5, 15, 30 and 60 min. The cells were washed twice in complete media, plated in triplicate and incubated at 37°C, 5% CO<sub>2</sub> for 72 h. A control was included in which the cells were treated with the neoglycans for the usual 72 h. Cell viability was determined by MTT assay as described above.

**Apoptosis Determination.** ARP-1 cells and MDA-MB-231 cells were treated with media only, 32 µg/ml GAG chains and 32 µg/ml neoglycans. The ARP-1 cells were harvested after a 48 h incubation period and the MDA-MB-231 cells were harvested after a 96 h incubation period. The induction of apoptosis was determined by staining with fluorescein isothiocyanate-labeled Annexin V (Annexin V-FITC, CALTAG Laboratories, Burlingame, CA) following the manufacturer's instructions. Just before analysis 0.5 mg/ml propidium iodide (PI, Sigma) in PBS, pH 7.4 was added. Flow cytometry was performed with a Becton-Dickinson (Mountain View, CA) FACScan using

Cellquest 1.2 software. Annexin V-FITC positive, PI negative cells were considered apoptotic.

To evaluate DNA ladder formation, a late event of apoptosis, TUNEL assays were performed after treatment of ARP-1 and MDA-MB-231 cells as described above. ARP-1 and MDA-MB-231 cells were harvested after a 48 h or a 120 h incubation period, respectively. Apoptotic cells were identified by TdT-mediated dUTP nick end labeling (TUNEL) assay using the Roche Molecular Biochemicals Fluorescein In Situ Cell Death Detection Kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's instructions. The cells were analyzed by FACScan using Cellquest 1.2 software.

**Treatment of Established MDA-MB-231 Tumors with CS and neoCS.** BALB/c nu/nu mice were anesthetized by i.p. administration of a mixture of ketamine (42.8 mg/ml), xylazine (8.6 mg/ml) and acepromazine (1.4 mg/ml) at a dose of 1.8–2.0 ml/kg. MDA-MB-231 cells ( $1 \times 10^6$ ) were injected at two s.c. sites on each mouse. The tumors were allowed to grow for two weeks before treatment. Once established, the tumors were injected with a single 1.6 mg dose of CS or neoCS in a 50 µl total volume. Immediately after injection, a caliper electrode was positioned on two sides of the tumor with contact on the skin. An electrical pulse was applied to the tumor through the electrodes with a BTS EC830 power supply (Genetronics, Inc., San Diego, CA). At the indicated time points the tumors were measured with calipers and tumor volumes calculated according to the equation:  $V = (L \times W^2) \times 0.5$ . The results reflect two separate experiments in which one tumor was treated with CS and the second tumor on the same animal was treated with neoCS. In the first experiment  $n = 8$  tumors in each treatment group and in the second experiment  $n = 7$  tumors/group, therefore the results include 15 tumors total in each treatment group.

**SDS-PAGE Analysis of Neoglycans.** GAG chains and neoglycans were separated by SDS-PAGE through 14% gels. The gels were first stained for the presence of protein with 0.25% Coomassie Blue for 3 h and destained in methanol:H<sub>2</sub>O (1:1 v/v) and 10% glacial acetic acid overnight. The gels were subsequently stained with 0.1% alcian blue, 3% glacial acetic acid for 15 min and destained in H<sub>2</sub>O. Alcian blue specifically stains GAG chains and not protein. Rainbow molecular weight markers (Amersham Pharmacia Biotech, Piscataway, NJ) serve as size standards on each gel. However, because of the inherent differences in protein and GAG chain structures, the markers indicate only an estimation of GAG chain size.

**Polysaccharide Lyase Digestion and Superdex 200 Column Chromatography of Neoglycans.** CS and neoCS (500 µg each) were speed vacuumed to dryness and resuspended in H<sub>2</sub>O, 20 mM Tris-HCL, pH 8.0 (lyase buffer), or 150 µU chondroitin ABC lyase (Segagiku, Tokyo, Japan). Digestions were performed at 37°C for 4 h.

Superdex 200 prep grade gel filtration media (Amersham Pharmacia Biotech), which fractionates proteins by size in a range from 10–600 kDa, was equilibrated in 6 M guanidine, 50 mM sodium acetate, pH 5.8 column running buffer. A 95 cm  $\times$  2 cm Superdex 200 column was poured and packed at a flow rate of 6 ml/hour. The column was washed with one bed volume of running buffer. A 100 mg preparation of neoCS was digested with 0.3 U of chondroitin ABC lyase in 20 mM Tris, pH 8.0 for 4 h. The digested neoCS was brought 1:1 with column running buffer, boiled for 10 min, cooled to room temperature, loaded on the Superdex 200 column and fractions were collected. Numerous fractions were buffer exchanged into H<sub>2</sub>O by 5,000 molecular weight cutoff spin column filtration (Millipore) and stored at 4°C.

**Statistical Analysis.** The 50% inhibitory concentration (IC50) is defined as the concentration at which 50% of treated cells die within 72 h. Data from triplicate wells and replicate experiments were used jointly to estimate a common IC50 through use of a compound nonlinear degradation model based on the three-parameter logistic. Data from the triplicate wells at each neoglycan concentration were combined by computing means, denoted by  $Y_{ij}$  for concentration  $i$  ( $i = 1,5$ ) and replicate  $j$  ( $j = 1,2$ ), and these were used as the dose levels in the modeling. The model was defined as

$$Y_{ij} = \beta_{1j}/[1 + (x/\beta_3)]^{\beta_2j}$$

where the dose levels were transformed as:

$$x = 2 + \log_{10}(\text{dose}), \text{ if dose} > 0, \text{ else } x = 0.$$

In this model, the estimate of IC50 is given by  $10^{(b_3 - 2)}$ , where  $b_3$  represents the estimate of the parameter  $\beta_3$  obtained by using the NLIN procedure of SAS (The SAS System, Version 8, SAS Institute Inc., Cary NC). Ninety-five

percent confidence intervals were based on asymptotic limits for the estimated parameter which were inverse transformed to obtain limits for IC50.

## RESULTS

**Neoglycans Reduce Cell Viability *in Vitro*.** In previous studies, we demonstrated that the growth inhibitory effect of soluble syndecan-1 on cancer cells *in vitro* requires the presence of an intact proteoglycan ectodomain composed of a core protein and attached heparan sulfate chains (15). The initial goal of the present study was to synthesize neoproteoglycans and test their potential for killing tumor cells. The neoproteoglycans were produced by coupling human serum albumin to heparin or CS by EDAC conjugation. Controls composed of heparin or CS alone treated with EDAC were also prepared. Surprisingly, both neoproteoglycans and neoglycan controls reduced myeloma cell viability (data not shown). In contrast, native, unmodified heparin and CS and protein coupled to protein had no inhibitory effect on cell viability. Because of the advantage of producing a therapeutic agent lacking a protein component, we were compelled to further assess the activity of neoglycans. The effect of GAG chains and neoglycans on cell viability was tested on several cancer cell lines including myeloma cell lines (ARK, ARP-1, CAG and U266), a plasma cell leukemia cell line (ARH-77) and breast cancer cell lines (Hs578t, MCF-7, MDA-MB-231, MDA-MB-435 and MDA-MB-436). Dexamethasone, a known inducer of apoptosis in some myeloma cell lines (36), was included as a control. Results of MTT assays demonstrate a remarkable dose-dependent reduction in cancer cell viability in response to treatment with either neoheparin or neoCS (Fig. 1A). For example, after 72 h of treatment, neoheparin and neoCS (32  $\mu\text{g/ml}$ ) produce a 79% and 96% reduction in ARP-1 cell viability, respectively. In comparison, unmodified heparin treatment may slightly enhance cell proliferation and CS has no effect. The 50% inhibitory concentration (IC50) values for neoheparin and neoCS on ARP-1 cells are 21.94  $\mu\text{g/ml}$  (95% confidence interval (CI) of 18.18 to 26.48  $\mu\text{g/ml}$ ) and 14.79  $\mu\text{g/ml}$  (95% CI of 12.13 to 18.06  $\mu\text{g/ml}$ ), respectively (Table 1). In comparison to ARP-1 cells, U266 myeloma cells exhibit a similar sensitivity to neoCS but are less susceptible to treatment with neoheparin. All of the breast cancer cell lines tested including MDA-MB-231 and Hs578t are also sensitive to treatment with the neoglycans (Fig. 1A and Table 1).

Identical experiments were performed to evaluate the effect of the neoglycans on nontransformed cell lines including CHO-K1, MDCK, NIH3T3, NMuMG, MCF-10A, MCF-10F and HBL-100. HBL-100 is a human breast cell line which is not tumorigenic at low passage numbers but is tumorigenic at high passage numbers. The results demonstrate that all of the normal cell lines tested are sensitive to treatment with either neoglycan (Fig. 1A and Table 1). Mitogen activated peripheral blood mononuclear cells (PBMCs) also show a similar sensitivity to both neoglycans (Fig. 1A and Table 1). Therefore, the viability of myeloma, breast cancer and normal cell lines and activated PBMCs is reduced by exposure to either neoglycan.

To determine whether the continuous presence of the neoglycan is required for its activity, ARP-1 cells were pulse-treated with neoheparin or neoCS for 5, 15, 30 or 60 min at 37°C, 5% CO<sub>2</sub>. Cells were washed several times to remove neoglycans, placed in fresh media and, after a 72 h incubation, cell viability was determined by MTT assay. Pulse treatment of ARP-1 cells with 320  $\mu\text{g/ml}$  of neoheparin and neoCS for as short a period as 15 min reduces ARP-1 cell viability by 44% and 92%, respectively (Fig. 1B). A 5 min treatment of ARP-1 cells with neoCS reduces cell viability by 42%. At the lower dose of 32  $\mu\text{g/ml}$ , a 60 min pulse with the neoheparin produces a 44% reduction in cell proliferation whereas the same length pulse with neoCS produces a 23% reduction in cell viability (Fig. 1B). These

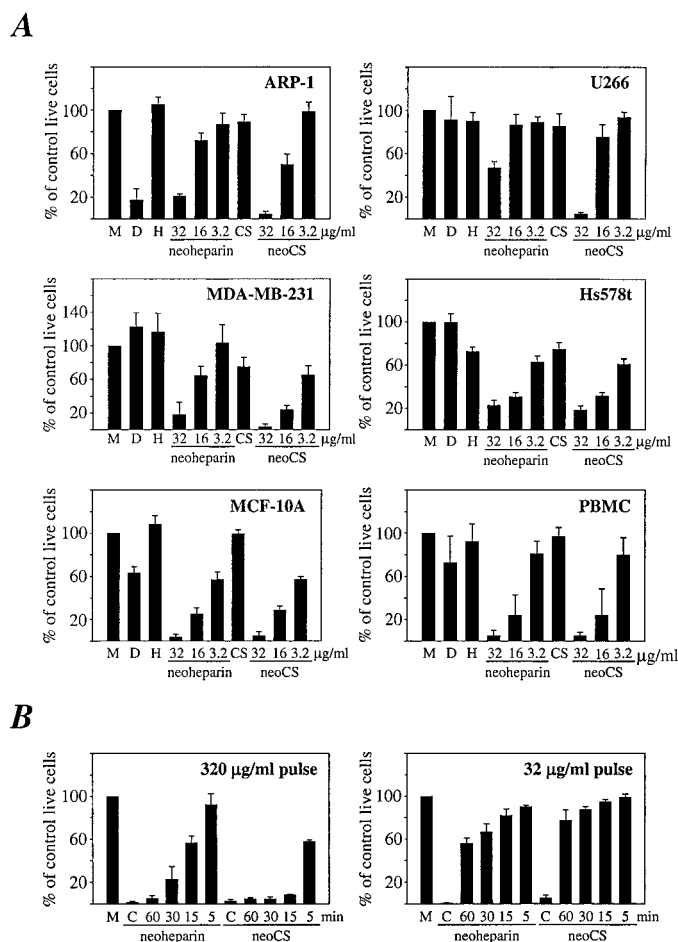


Fig. 1. Neoglycans reduce cell viability in a dose-dependent manner. A, The indicated cell lines were cultured for 72 h in the continuous presence of 32, 16 or 3.2  $\mu\text{g/ml}$  concentrations of neoglycan (neoheparin or neoCS), media only (M), 10  $\mu\text{M}$  dexamethasone (D) or 500  $\mu\text{g/ml}$  of either heparin (H) or CS. Cell viability was determined by MTT assay and the results show means of triplicate wells for each condition  $\pm$  SD of duplicate experiments. B, ARP-1 cells were treated with media only (M) or neoglycans for 72 h as controls (C) or with 320  $\mu\text{g/ml}$  or 32  $\mu\text{g/ml}$  neoglycans for 60, 30, 15 or 5 min. Cells were washed to remove neoglycans and placed in fresh culture medium for 72 h. Results of MTT assays are shown as means  $\pm$  SD of triplicate wells and duplicate experiments. In each experiment the media only treatment indicates 100% cell viability.

results indicate that the neoglycans need not be present continually for a reduction in cancer cell viability, which suggests that the neoglycans are affecting cells directly and not simply neutralizing nutrients within the culture media. Moreover, this suggests that even a transient high concentration of neoglycan at the tumor site *in vivo* could be effective in eradicating tumor burden.

**Neoglycans Induce Apoptosis in Cancer Cells.** To determine whether neoglycan treatment of cells promotes apoptosis, ARP-1 cells and MDA-MB-231 cells were treated with media only or media supplemented with GAG chains or neoglycans. Apoptosis was evaluated by two methods, Annexin V-FITC staining and TUNEL assay. Treatment of the ARP-1 cells for 48 h or MDA-MB-231 cells for 96 h with media only or 32  $\mu\text{g/ml}$  unmodified GAG chains results in no induction of apoptosis. However, when either cell line was treated with neoglycan for the same period of time, cells underwent extensive apoptosis as demonstrated by Annexin V-FITC staining on the cell surface (Fig. 2A). For TUNEL assays, ARP-1 and MDA-MB-231 cells were treated with 32  $\mu\text{g/ml}$  of the neoglycans and GAG chains and incubated for 48 and 120 h, respectively. Media only treatment results in no apoptosis, whereas treatment of either cell line with either neoglycan results in apoptosis as shown by the presence of a signif-

Table 1  $IC_{50}$  of neoglycans

Cell line	Tumorigenic	Neoheparin $IC_{50}^a$ ( $\mu\text{g/ml}$ )	Neoheparin $CI^b$ ( $\mu\text{g/ml}$ )	neoCS $IC_{50}^a$ ( $\mu\text{g/ml}$ )	neoCS $CI^b$ ( $\mu\text{g/ml}$ )
ARK	+	27.42	21.96–34.24	24.92	18.03–34.45
ARP-1	+	21.94	18.18–26.48	14.79	12.13–18.06
CAG	+	16.02	14.09–18.21	13.65	11.88–15.68
U266	+	32.00	26.44–38.73	19.05	16.83–21.55
ARH-77	+	19.74	12.18–31.99	21.87	9.06–52.80
HBL-100	+/-	8.28	5.89–11.65	6.81	2.92–15.90
Hs578t	+	2.34	1.67–3.28	3.65	3.20–4.16
MCF-7	+	11.49	3.52–37.49	6.55	4.26–10.06
MDA-MB-231	+	17.70	14.59–21.28	5.00	3.28–7.61
MDA-MB-435	+	17.10	12.25–23.86	6.82	4.51–10.29
MDA-MB-436	+	4.21	2.66–6.68	3.86	2.80–5.33
MCF-10A	-	4.16	2.76–6.29	4.29	2.67–6.89
MCF-10F	-	14.20	10.10–19.96	21.94	18.18–26.48
CHO-K1	-	3.38	2.23–5.13	3.33	3.13–3.53
MDCK	-	15.98	4.97–51.36	10.64	7.63–14.83
NIH3T3	-	7.73	3.42–17.50	15.74	12.22–20.29
NMuNG	-	6.29	4.26–9.29	5.87	4.60–7.50
PBMC	-	3.94	3.22–4.82	4.21	3.64–4.86

<sup>a</sup> Estimated  $IC_{50}$  values were determined after cell viability assays by use of a compound nonlinear degradation model based on the three-parameter logistic. Calculations are based on triplicate wells from duplicate experiments.

<sup>b</sup> 95% CI.

icant shift in fluorescence (ARP-1 cells) or the presence of a population of brightly stained cells (MDA-MB-231 cells; Fig. 2B). A slight shift in fluorescence intensity is noted after treatment of the MDA-MB-231 cells with the native GAG chains however, a brightly stained second population of cells that is indicative of apoptosis is not present. Collectively, the results of two separate assay systems demonstrate that the neoglycans, but not unmodified GAG chains, reduce cell viability by inducing apoptosis.

**A Single neoCS Dose Eliminates MDA-MB-231 Tumors *in Vivo*.** Because neoCS is more potent than neoheparin *in vitro* (Fig. 1 and Table 1) and because neoheparin may exhibit the anticoagulant properties of native heparin, we evaluated neoCS for *in vivo* activity. MDA-MB-231 breast cancer tumors were established in female BALB/c nu/nu mice by s.c. injection of  $1 \times 10^6$  cells at two sites on each animal. A single injection of 1.6 mg of CS or neoCS was administered to separate tumors on the same animal. After injections, tumors were electroporated to enhance permeability of the tumor cells. The size of each tumor was determined 48 h later and at several subsequent time points. Results from duplicate experiments demonstrate that a single dose of neoCS completely eradicated the MDA-MB-231 breast tumors in every animal by day 5 (Fig. 3). In both experiments only one animal had a neoCS-treated tumor reemerge. The neoCS-treated tumors on the other animals did not recur during the one month experimental period (data not shown).

**Isolation of an Active neoCS Fraction.** The *in vitro* and *in vivo* studies demonstrate that EDAC modified GAG chains inhibit cell viability and that neoCS but not native CS reduces tumor burden. It is possible that a contaminant such as protein found in the GAG chain preparations because of isolation procedures or residual EDAC remaining after modification of the GAG chains may contribute to activity. Analysis of the native GAG chains and the corresponding neoglycans by SDS-PAGE followed by coomassie staining or silver staining for protein content reveal no protein contamination (data not shown). To confirm that the GAG component of the neoglycans is required for activity and therefore eliminate EDAC contamination as a contributing factor, neoCS was digested with chondroitin ABC lyase in an attempt to eliminate the GAG chains of the neoglycan. CS and neoCS (500  $\mu\text{g}$  each) were digested with 150  $\mu\text{U}$  of lyase and a portion of each along with controls was visualized on SDS-PAGE gels after Alcian blue staining (Fig. 4A). As expected, CS (CS-W and CS-B) is very heterogeneous in size and appears as a large smear covering the entire lane. The CS-L lane and the neoCS-L lane show high molecular weight smears that remain after lyase digestion. Fur-

ther lyase treatment failed to digest the remaining CS or neoCS (data not shown). Because most of the neoCS was digested, cell viability experiments were performed to determine whether killing activity had been reduced. ARP-1 cells were treated with digested CS and neoCS at a concentration equivalent to 32  $\mu\text{g/ml}$  nondigested material. Interestingly, the neoCS activity is not blocked by lyase digestion (Fig. 4B, neoCS-L) and the digested neoCS is as active as the undigested controls including neoCS in  $\text{H}_2\text{O}$  (neoCS-W) and neoCS in lyase buffer (neoCS-B). The chondroitin ABC lyase used in these experiments did not affect ARP-1 cell proliferation because cells treated with lyase-digested CS grew as well as media only treated cells (Fig. 4B, compare CS-L and M). These results indicate no detectible protein contamination of the neoglycan preparations and suggest either the high molecular weight neoCS remaining after lyase digestion is active, the small fragments of the neoCS remaining after lyase digestion are active or residual EDAC is present and active.

To eliminate possible residual EDAC in the neoglycan preparations and to separate high molecular weight neoCS from low molecular weight digested fragments, neoCS was digested with chondroitin ABC lyase and separated by size exclusion chromatography over a Superdex 200 column in buffer containing 6 M guanidine. At this concentration of guanidine, everything is denatured and separates based solely on molecular size. Pure fractions of high molecular weight neoCS were collected, buffer-exchanged into  $\text{H}_2\text{O}$ , evaluated by SDS-PAGE and the effect on cell viability was determined on ARP-1 cells. Alcian blue staining of the SDS-PAGE gels confirms the separation of high molecular weight components of lyase-digested neoCS ranging in MW from  $>220\text{kDa}$  to approximately 97kDa (Fig. 5A). The 66 kDa band that is evident in fractions 35–41 is BSA that is included as a carrier in the lyase reagent. The GAG concentration of each of these fractions is too low to determine by carbazole reaction although SDS-PAGE analysis of equal volumes of the fractions suggests similar concentrations in the GAG chain-containing fractions. To examine the activity of the individual fractions, ARP-1 cells were treated with equal volumes of fractions 25–42, incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  for 72 h and cell viability was determined by MTT assay. The results indicate that the fractions vary greatly in their activity, with the most activity contained within fractions 32–37 (Fig. 5B). Interestingly, a series of fractions very high in activity are clustered around fraction 33. Because free EDAC is less than 0.2 kDa in size, it would not be present within the high molecular weight fractions where the activity resides. Thus, these results demonstrate that a contaminant is not producing activity and confirm that the chondroitin ABC lyase-

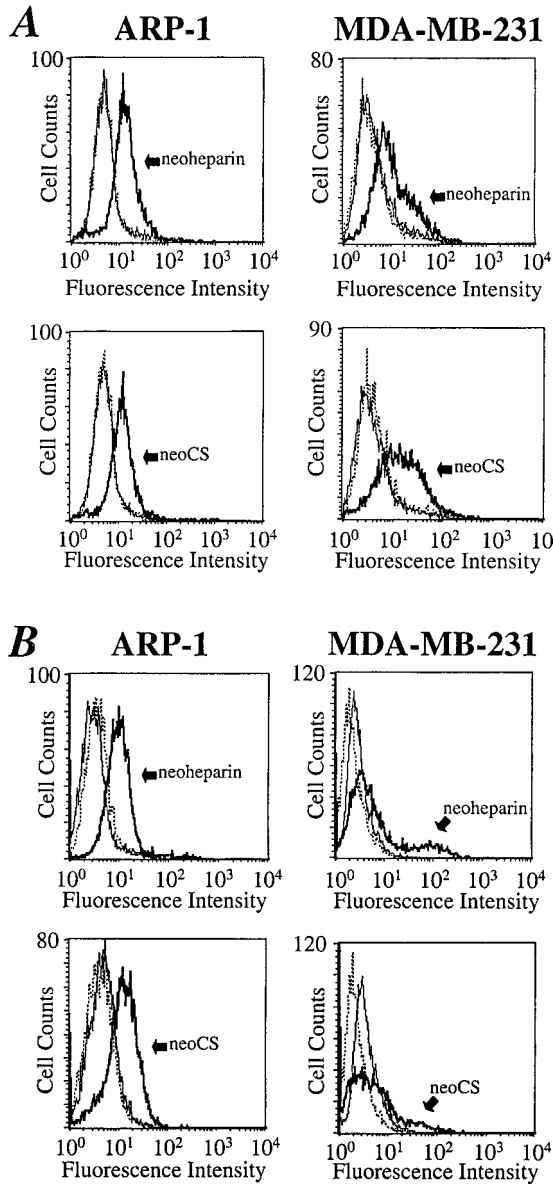


Fig. 2. Neoglycans induce apoptosis. A, ARP-1 myeloma and MDA-MB-231 breast cancer cells were incubated with 32  $\mu\text{g}/\text{ml}$  of either heparin or CS (thin lines), neoheparin or neoCS (thick lines) or media only (dashed lines). Apoptotic cells were detected by flow cytometry based on AnnexinV-FITC staining and PI exclusion. B, ARP-1 and MDA-MB-231 cell lines were treated with media (dashed line), 32  $\mu\text{g}/\text{ml}$  heparin or CS (thin line) or 32  $\mu\text{g}/\text{ml}$  neoglycans (thick lines). DNA laddering, indicative of the late stages of apoptosis, was detected by TUNEL assay. Arrows indicate apoptotic cell populations.

resistant high molecular weight neoCS specifically reduces cell viability.

**DISCUSSION**

In this study we show that modification of heparin or CS with EDAC produces reagents that inhibit cancer cell proliferation and induce apoptosis. Induction of apoptosis by neoglycans is concentration-dependent and occurs after a short exposure to the cells. Fractionation by Superdex 200 gel filtration chromatography yields a narrow range of GAG chain-containing fractions having high activity against cancer cells. These neoglycans are produced in the absence of any exogenous protein suggesting that their activity occurs through modification of the GAG chain structure. Therefore, the neoglycans,

unlike syndecan-1, do not require a protein component for cell killing activity.

EDAC conjugation is a two-step chemical reaction that is widely used to conjugate molecules containing carboxyl and amino groups (37, 38). In the first step of the reaction, EDAC couples to a carboxyl group forming an activated *O*-acylisourea derivative. Subsequent attack by an amino group produces an amide linkage between the carboxyl and amino group-containing molecules and displaces a urea derivative of EDAC. Heparin and CS contain a free carboxyl group on the number 6 carbon of the glucuronic acid component of the GAG chains. Heparin that has been isolated from porcine intestinal mucosa

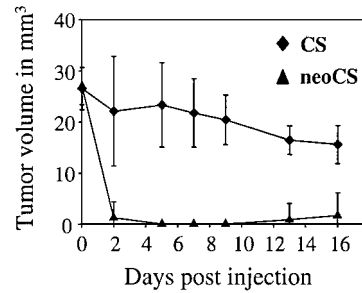


Fig. 3. NeoCS eliminates MDA-MB-231 tumors in mice. Established MDA-MB-231 tumors were injected with a single dose of either 1.6 mg CS ( $\blacklozenge$ ) or 1.6 mg neoCS ( $\blacktriangle$ ) followed by electroporation of the tumors. On the indicated days after treatment, tumor size was measured and tumor volume calculated. Bars indicate means  $\pm$  SD of two experiments. The results represent a total of 15 tumors treated with neoCS. However, for the CS control group  $n = 15$  on days 0, 2 and 5,  $n = 14$  on days 7 and 9 and  $n = 13$  beginning on day 13. The reduction in control-treated tumor numbers is because of mouse morbidity.

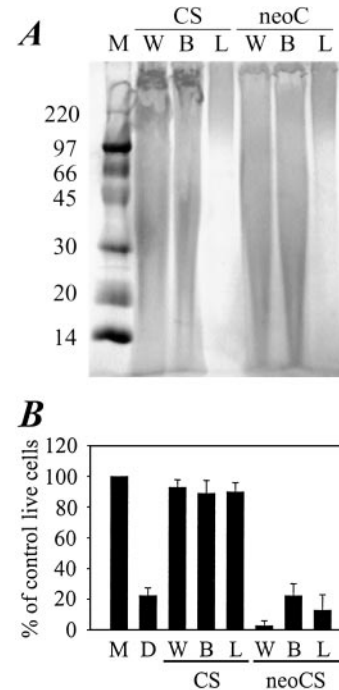


Fig. 4. Chondroitin ABC lyase digested neoCS retains activity. CS and neoCS were suspended in water, chondroitin ABC lyase buffer or chondroitin ABC lyase for 4 h and aliquots were either separated by SDS-PAGE and visualized after alcian blue staining or tested in a cell viability assay. A, SDS-PAGE analysis of CS and neoCS after incubation is water (W), chondroitin ABC lyase buffer (B) or chondroitin ABC lyase (L). Molecular weight markers are shown in lane M and the size of each is indicated in kDa. B, Triplicate wells of ARP-1 cells were exposed to media only (M) or media supplemented with 10  $\mu\text{M}$  dexamethasone (D), 32  $\mu\text{g}/\text{ml}$  CS or neoCS in water (W) or in lyase buffer (B) and the predigestion equivalent of 32  $\mu\text{g}/\text{ml}$  CS or neoCS in chondroitin ABC lyase (L). Cells were placed in a culture incubator for 72 h and cell viability was determined by MTT assay. The results are expressed as the mean of percent viable cells relative to media only treated cells and the SD for each condition is shown.

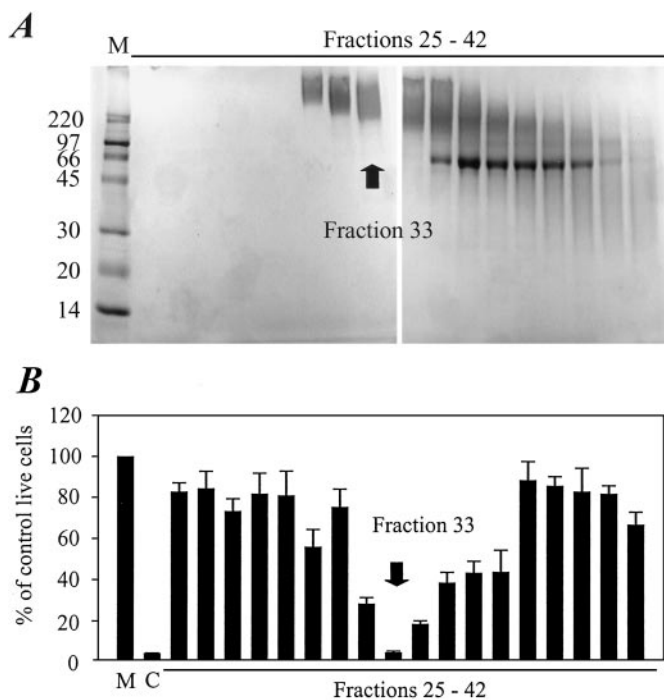


Fig. 5. Identification of active neoCS fractions. NeoCS was digested with chondroitin ABC lyase, fractionated by size exclusion chromatography and the guanidine removed from fractions by buffer exchange using spin column filtration. A, Equivalent amounts of each fraction were visualized on SDS-PAGE gels by alcian blue staining. The approximately 66 kDa band in fractions 35–41 is BSA that is included as a carrier in the lyase reagent. B, Equivalent amounts of each fraction were added to triplicate wells of ARP-1 cells in culture, incubated for 72 h and the percent cell viability determined by MTT assay. Media only treatment (M) indicates 100% cell viability and neoCS treatment (C) demonstrates the usual reduction in cell viability. Cell viability after treatment with the neoCS fractions 25–42 is shown and the means  $\pm$  SD are reported.

contains a limited number of N-nonsubstituted glucosamine residues and therefore a limited number of free amino groups (39). Heparin and CS used for the production of neoglycans may contain free amino groups on covalently bound amino acids remaining from conventional isolation procedures (40, 41). Therefore, EDAC modification of heparin and CS could produce molecules that are composed of GAG chains coupled together producing GAG complexes, perhaps similar to the multimeric array of heparan sulfate found on syndecan-1. However, because there are abundant carboxyl groups and limited amino groups present on GAG chains, the EDAC reaction may not go to completion. The result of the incomplete EDAC reaction would be the *O*-acylisourea derivative produced in the first step of the reaction (42). Indeed, preliminary  $^1\text{H-NMR}$  spectra analysis of neoCS compared with native CS or EDAC  $^1\text{H-NMR}$  spectra reveals the presence of EDAC bound to the GAG chains at approximately a 1:1 ratio of EDAC to GAG disaccharide suggesting the presence of an *O*-acylisourea derivative. Other signals are present but have not been identified however, contamination with free EDAC or free GAG chains has been excluded (data not shown). The  $^1\text{H-NMR}$  analysis also supports the conclusion that there is little if any protein contamination and there are probably few unsubstituted amino groups present on the GAG chains because GAG bound to GAG was not detected as a major product. On the basis of the chemistry of the EDAC reaction, one would expect the production of GAG bound to GAG if contaminating amino acids were bound to the GAG chains or if unsubstituted amino groups on the GAG chains were available. Analysis of the active neoCS fractions isolated after lyase digestion and size exclusion chromatography should reveal the specific structure of the active neoCS. Interestingly, *O*-acylisourea derivatives of highly sulfated GAG chains such as heparin and CS are reminiscent of suramin, a

polysulfated naphthylurea compound that also exhibits anticancer properties (43).

EDAC modification of GAG chains produces molecules with strikingly different activities as compared with the native GAG chains. Treatment of cell lines with native heparin and CS does not reduce cell growth and in fact enhances cell growth in some cases (Fig. 1). Heparin and CS do not induce apoptosis in ARP-1 and MDA-MB-231 cancer cell lines (Fig. 2). In great contrast to heparin and CS, neoheparin and neoCS severely reduce cell viability in normal and cancer cell lines and induce apoptosis in the ARP-1 myeloma cell line and the MDA-MB-231 breast cancer cell line. Interestingly, the activity of the neoglycans is not inhibited by the addition of excessive amounts of GAG chains. Treating ARP-1 cells with 10-fold greater concentrations of native GAG chains does not block the killing affect of the neoglycans (data not shown), a result that highlights the different activities of GAG chains and neoglycans. The contrasting functions of the native CS and the neoCS are also evident in the results of *in vivo* experiments. NeoCS eradicates breast cancer tumor burden in nude mice whereas CS has no detectable effect (Fig. 3).

An important characteristic of any therapeutic agent is effectiveness at an achievable and safe dosage. The cell culture studies reported here demonstrate that varying but high concentrations of neoglycan are required for a 50% reduction in cell viability *in vitro* (Table 1). However, chondroitin ABC lyase-digested neoCS reduces cell viability as effectively as undigested neoCS (Fig. 4B) despite the obvious reduction in total GAG content (Fig. 4A). This result suggests that neoCS is a mixture of active and nonactive molecules. Evaluation of the Superdex 200-purified neoCS fractions confirms this finding. Some neoCS fractions did not reduce cell viability whereas others did even at concentrations too low to determine. Therefore, the neoglycan preparations contain nonactive components and future studies using purified active neoglycan should demonstrate improved IC50 values and lower therapeutic dose requirements. In terms of safety, the neoglycans inhibit the growth of several normal cell lines and activated primary PBMCs *in vitro* (Fig. 1A). However, treatment of established breast cancer tumors in nude mice abolishes tumors without obvious toxicity to surrounding tissue or the whole animal. Therefore, the effect of neoglycans on normal cells growing *in vitro* may be an artifact of rapidly dividing laboratory cultures. Moreover, a safe and effective dose of neoCS is achievable in nude mice.

Importantly, neoglycans act on the target cells in an expeditious manner. Pulse treatment of cells with neoglycans (32  $\mu\text{g/ml}$ ) for 1 h resulted in a reduction in ARP-1 cell viability whereas at a higher concentration (320  $\mu\text{g/ml}$ ) a pulse as short as 5–15 min resulted in a 42–92% reduction in cell viability (Fig. 1B). This finding suggests that the mechanism of activity of the neoglycans involves direct interaction with the cells as opposed to an indirect means of killing such as depletion of essential components in the media. The ability to produce an effect quickly and irreversibly is beneficial because *in vivo* the neoglycans may be cleared rapidly or metabolized.

In conclusion, we report the initial description of novel anticancer therapeutic agents called neoglycans. The mechanism of antitumor activity is wholly unknown, although it is likely that the neoglycans interact with the cells because a short exposure to neoheparin or neoCS irreversibly kills cells. The isolation of active neoCS fractions provides the foundation for future studies of the structure and function of neoglycans and for the optimization of neoglycan activities. Exploitation of the structural and functional diversity of GAG chains and the neoglycans made from them represents an opportunity for development of a new class of anticancer agents.

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## REFERENCES

- Turnbull, J., Powell, A., and Guimond, S. Heparan sulfate: decoding a dynamic multifunctional cell regulator. *Trends Cell Biol.*, *11*: 75–82, 2001.
- Park, P. W., Reizes, O., and Bernfield, M. Cell surface heparan sulfate proteoglycans: selective regulators of ligand-receptor encounters. *J. Biol. Chem.*, *275*: 29923–29926, 2000.
- Lindahl, U., Lidholt, K., Spillmann, D., and Kjellen, L. More to “heparin” than anticoagulation. *Thromb. Res.*, *75*: 1–32, 1994.
- Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.*, *68*: 729–777, 1999.
- Sanderson, R. D. Heparan sulfate proteoglycans in invasion and metastasis. *Semin. Cell Dev. Biol.*, *12*: 89–98, 2001.
- Hejna, M., Raderer, M., and Zielinski, C. C. Inhibition of metastases by anticoagulants. *J. Natl. Cancer Inst. (Bethesda)*, *91*: 22–36, 1999.
- Smorenburg, S. M., and Van Noorden, C. J. The complex effects of heparins on cancer progression and metastasis in experimental studies. *Pharmacol. Rev.*, *53*: 93–105, 2001.
- Siragusa, S., Cosmi, B., Piovella, F., Hirsh, J., and Ginsberg, J. S. Low-molecular-weight heparins and unfractionated heparin in the treatment of patients with acute venous thromboembolism: results of a meta-analysis. *Am. J. Med.*, *100*: 269–277, 1996.
- Bijsterveld, N. R., Hettiarachchi, R., Peters, R., Prins, M. H., Levi, M., and Buller, H. R. Low-molecular weight heparins in venous and arterial thrombotic disease. *Thromb. Haemost.*, *82 (Suppl)*: 139–147, 1999.
- Folkman, J., and Shing, Y. Control of angiogenesis by heparin and other sulfated polysaccharides. *Adv. Exp. Med. Biol.*, *313*: 355–364, 1992.
- Collen, A., Smorenburg, S. M., Peters, E., Lupu, F., Koolwijk, P., Van Noorden, C., and van Hinsbergh, V. W. Unfractionated and low molecular weight heparin affect fibrin structure and angiogenesis *in vitro*. *Cancer Res.*, *60*: 6196–6200, 2000.
- Borsig, L., Wong, R., Feramisco, J., Nadeau, D. R., Varki, N. M., and Varki, A. Heparin and cancer revisited: mechanistic connections involving platelets, P-selectin, carcinoma mucins, and tumor metastasis. *Proc. Natl. Acad. Sci. USA*, *98*: 3352–3357, 2001.
- Zeng, C., Toole, B. P., Kinney, S. D., Kuo, J. W., and Stamenkovic, I. Inhibition of tumor growth *in vivo* by hyaluronan oligomers. *Int. J. Cancer*, *77*: 396–401, 1998.
- Vincent, T., Jourdan, M., Sy, M. S., Klein, B., and Mechti, N. Hyaluronic acid induces survival and proliferation of human myeloma cells through an interleukin-6-mediated pathway involving the phosphorylation of retinoblastoma protein. *J. Biol. Chem.*, *276*: 14728–14736, 2001.
- Dhodapkar, M. V., Abe, E., Theus, A., Lacy, M., Langford, J. K., Barlogie, B., and Sanderson, R. D. Syndecan-1 is a multifunctional regulator of myeloma pathobiology: control of tumor cell survival, growth, and bone cell differentiation. *Blood*, *91*: 2679–2688, 1998.
- Arribas, J., Coodly, L., Vollmer, P., Kishimoto, T. K., Rose-John, S., and Massague, J. Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. *J. Biol. Chem.*, *271*: 11376–11382, 1996.
- Hooper, N. M., Karran, E. H., and Turner, A. J. Membrane protein secretases. *Biochem. J.*, *321*: 265–279, 1997.
- Mali, M., Andtfolk, H., Miettinen, H. M., and Jalkanen, M. Suppression of tumor cell growth by syndecan-1 ectodomain. *J. Biol. Chem.*, *269*: 27795–27798, 1994.
- Pilia, G., Hughes-Benzie, R. M., MacKenzie, A., Baybayan, P., Chen, E. Y., Huber, R., Neri, G., Cao, A., Forabosco, A., and Schlessinger, D. Mutations in *GPC3*, a glypican gene, cause the Simpson-Golabi-Behmel overgrowth syndrome. *Nat. Genet.*, *12*: 241–247, 1996.
- Neri, G., Gurrieri, F., Zanni, G., and Lin, A. Clinical and molecular aspects of the Simpson-Golabi-Behmel syndrome. *Am. J. Med. Genet.*, *79*: 279–283, 1998.
- Lind, T., Tufaro, F., McCormick, C., Lindahl, U., and Lidholt, K. The putative tumor suppressors EXT1 and EXT2 are glycosyltransferases required for the biosynthesis of heparan sulfate. *J. Biol. Chem.*, *273*: 26265–26268, 1998.
- McCormick, C., Leduc, Y., Martindale, D., Mattison, K., Esford, L. E., Dyer, A. P., and Tufaro, F. The putative tumour suppressor EXT1 alters the expression of cell-surface heparan sulfate. *Nat. Genet.*, *19*: 158–161, 1998.
- Inki, P., Joensuu, H., Grenman, R., Klemi, P., and Jalkanen, M. Association between syndecan-1 expression and clinical outcome in squamous cell carcinoma of the head and neck. *Br. J. Cancer*, *70*: 319–323, 1994.
- Pulkkinen, J. O., Penttinen, M., Jalkanen, M., Klemi, P., and Grenman, R. Syndecan-1: a new prognostic marker in laryngeal cancer. *Acta Otolaryngol.*, *117*: 312–315, 1997.
- Kumar-Singh, S., Jacobs, W., Dhaene, K., Weyn, B., Bogers, J., Weyler, J., and Van Marck, E. Syndecan-1 expression in malignant mesothelioma: correlation with cell differentiation, WT1 expression, and clinical outcome. *J. Pathol.*, *186*: 300–305, 1998.
- Wiksten, J. P., Lundin, J., Nordling, S., Lundin, M., Kokkola, A., von Boguslawski, K., and Haglund, C. Epithelial and stromal syndecan-1 expression as predictor of outcome in patients with gastric cancer. *Int. J. Cancer*, *95*: 1–6, 2001.
- Nackaerts, K., Verbeken, E., Deneffe, G., Vanderschueren, B., Demedts, M., and David, G. Heparan sulfate proteoglycan expression in human lung-cancer cells. *Int. J. Cancer*, *74*: 335–345, 1997.
- Matsumoto, A., Ono, M., Fujimoto, Y., Gallo, R. L., Bernfield, M., and Kohgo, Y. Reduced expression of syndecan-1 in human hepatocellular carcinoma with high metastatic potential. *Int. J. Cancer*, *74*: 482–491, 1997.
- Conejo, J. R., Kleeff, J., Koliopoulos, A., Matsuda, K., Zhu, Z. W., Goecke, H., Bicheng, N., Zimmermann, A., Korc, M., Friess, H., and Buchler, M. W. Syndecan-1 expression is up-regulated in pancreatic but not in other gastrointestinal cancers. *Int. J. Cancer*, *88*: 12–20, 2000.
- Blumenkrantz, N., and Asboe-Hansen, G. New method for quantitative determination of uronic acids. *Anal. Biochem.*, *54*: 484–489, 1973.
- Filiseti-Coizz, T. M., and Carpita, N. C. Measurement of uronic acids without interference from neutral sugars. *Anal. Biochem.*, *197*: 157–162, 1991.
- Ridley, R. C., Xiao, H., Hata, H., Woodliff, J., Epstein, J., and Sanderson, R. D. Expression of syndecan regulates human myeloma plasma cell adhesion to type I collagen. *Blood*, *81*: 767–774, 1993.
- Borsat, M., Hjertner, O., Yaccoby, S., Epstein, J., and Sanderson, R. D. Syndecan-1 is targeted to the uropods of polarized myeloma cells where it promotes adhesion and sequesters heparin-binding proteins. *Blood*, *96*: 2528–2536, 2000.
- Burk, K. H., Drewinko, B., Turjillo, J. M., and Ahearn, M. J. Establishment of a human plasma cell line *in vitro*. *Cancer Res.*, *38*: 2508–2513, 1978.
- Pieters, R., Huismans, D. R., Leyva, A., and Veerman, A. J. Adaptation of the rapid automated tetrazolium dye based (MTT) assay for chemosensitivity testing in childhood leukemia. *Cancer Lett.*, *41*: 323–332, 1988.
- Alexanian, R., Barlogie, B., and Dixon, D. High-dose glucocorticoid treatment of resistant myeloma. *Ann. Intern. Med.*, *105*: 8–11, 1986.
- Sehgal, D., and Vijay, I. K. A method for the high efficiency of water-soluble carbodiimide-mediated amidation. *Anal. Biochem.*, *218*: 87–91, 1994.
- Bauminger, S., and Wilchek, M. The use of carbodiimides in the preparation of immunizing conjugates. *Methods Enzymol.*, *70*: 151–159, 1980.
- Hook, M., Riesenfeld, J., and Lindahl, U. N-[<sup>3</sup>H]Acetyl-labeling, a convenient method for radiolabeling of glycosaminoglycans. *Anal. Biochem.*, *119*: 236–245, 1982.
- Lindahl, U., and Roden, L. The linkage of heparin to protein. *Biochem. Biophys. Res. Commun.*, *17*: 254–259, 1964.
- Lindahl, U., and Roden, L. The chondroitin 4-sulfate-protein linkage. *J. Biol. Chem.*, *241*: 2113–2119, 1966.
- Inoue, Y., and Nagasawa, K. On the reaction of *N*-acetylchondrosine. *N*-acetylchondrosine 6-sulfate, chondroitin 6-sulfate, and heparin with 1-(2-dimethylaminopropyl)-3-ethylcarbodiimide. *Carbohydr. Res.*, *111*: 113–125, 1982.
- Stein, C. A. Suramin: a novel antineoplastic agent with multiple potential mechanisms of action. *Cancer Res.*, *53*: 2239–2248, 1993.