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13. ABSTRACT ***(Maximum 200 Words)***
Heparan sulfate proteoglycans (HSPGs) are a new class of tumor suppressors. The focus of this project is to test novel proteoglycan based therapies for the treatment of breast cancer. In the first objective, the ability of neoproteoglycans (nPGs) to mimic the anti-tumor activities of naturally occurring proteoglycans is evaluated. Surprisingly, we found that molecules composed of carbodiimide modified GAG chains that differ from nPGs and native proteoglycans in that they are devoid of a protein component inhibit cancer cell viability. These molecules we call neoglycans, inhibit breast cancer cell viability *in vitro* through the induction of apoptosis. Treatment of established MDA-MB-231 tumors in nude mice with the neoglycan produced from chondroitin sulfate reduced or abolished tumors following a single dose without any apparent toxicity. In the second objective, a gene therapy approach is tested utilizing the HSPG gene syndecan-1. Tagged full length and truncated human syndecan-1 genes have been constructed and subcloned cell lines stably expressing the trans-genes have been established and are being evaluated. This project is the first attempt to use HSPG genes therapeutically for anti-cancer therapy. Also as a result of this project, a new class of anti-cancer therapeutics called neoglycans have and continue to be developed.

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INTRODUCTION:

Current data strongly support the idea that heparan sulfate proteoglycans (HSPGs) represent a new class of tumor suppressors. When syndecan-1, a HSPG, is lost from the surface of mammary epithelia, the cells lose their epithelial morphology, invade collagen gels and exhibit characteristics of neoplastic growth. When transfected with the cDNA for syndecan-1, transformed mammary epithelial cells regain the epithelial morphology and lose neoplastic growth characteristics. Remarkably, addition of purified intact syndecan-1 ectodomain to tumor cells inhibits growth in culture and induces apoptosis. These growth inhibitory activities are not exclusive to syndecan-1 because the HSPGs glypican-1 and betaglycan have similar affects on tumors. The work of this proposal focuses on the development of novel proteoglycan-based therapies that will stop the growth and perhaps kill breast tumor cells. Because the anti-tumor effects are not specific to any one HSPG we propose in the first objective to construct neoproteoglycans (nPGs) that will potentially mimic the activities of naturally occurring HSPGs. nPGs will be produced by coupling heparin or other glycosaminoglycan chains to human serum albumin (HSA). These molecules will be characterized and analyzed for anti-tumor effects when added to cells in culture or injected directly into established tumors growing in the mammary fat pad of nude mice. The second objective of this project is to test the potential of the syndecan-1 gene as an anticancer therapy. Syndecan-1 gene cassettes will be transfected into breast cancer cells *in vitro* and tumors *in vivo* and the affects on growth will be evaluated. This project represents the first attempt to use HSPGs as anti-cancer therapy.

BODY:

Task 1: To produce and characterize neoproteoglycans (nPGs) and test their efficacy in inhibiting tumor growth in vitro and in vivo (months 1-36).

- Prepare and characterize nPGs using various glycosaminoglycans (heparin, heparan sulfate, chondroitin sulfate) and HSA (1-6 months).
- Test nPGs in cell culture for their ability to inhibit tumor cell growth (months 7-18).
- Test efficacy of nPGs in inhibiting tumor growth in nude mice (months 12-24).

During the first year of funding nPGs were prepared and characterized and their ability to inhibit tumor cell growth was evaluated *in vitro*. This evaluation of the affects of nPGs on cell viability yielded the surprising observation that modified glycosaminoglycan (GAG) chains such as heparin and chondroitin sulfate reduce tumor cell viability even though no protein component is present. These data were presented last year in the first Annual Report. Subsequently, we have continued our evaluation of these modified GAG chains that we call neoglycans. The neoglycans are produced by carbodiimide

(EDAC) modification of GAG chains. Native heparin and chondroitin sulfate have little effect on normal or breast cancer cells however, the neoglycans produced from either GAG chain substantially reduce cell viability through the induction of apoptosis. Injection of the chondroitin sulfate neoglycan into established MDA-MB-231 breast cancer tumors in nude mice reduces or abolishes tumor growth without apparent toxicity (see appended manuscript entitled: Neoglycans, carbodiimide modified-glycosaminoglycans: a new class of anticancer agents that inhibit cancer cell proliferation and induce apoptosis, submitted to Cancer Research, July 2001).

Task 2: To determine if transfer of the syndecan-1 gene into breast cancer tumors is a viable therapeutic approach (months 1-36)

- Prepare liposomal and retroviral vectors for transfer of the syndecan-1 gene (months 1-12).

- Test vectors for their ability to confer high levels of syndecan-1 expression on tumor cell growing in culture (months 6-24).

In the first year of funding we found that the MCF-7, MDA-MB-231, MDA-MB-435, MDA-MB-436, and Hs578t breast cancer cell lines express syndecan-1 on the cell surface. Therefore, during this past year, for the purpose of distinguishing native syndecan-1 from syndecan-1 expressed from transferred gene cassettes, a c-myc tag was incorporated into the human syndecan-1 gene construct by site directed mutagenesis. Primers for the production of two syndecan-1 gene cassettes including a full-length cassette and a truncated construct were engineered and used to PCR amplify the appropriate DNA fragments (Figure 1). Expression of the truncated cassette will generate syndecan-1 that will be secreted instead of found on the cell surface.

The integrity of both modified gene cassettes was confirmed by DNA sequence analysis. Plasmid vector without an inserted gene, or plasmid vector containing either the full length, c-myc tagged syndecan-1 gene or the truncated, c-myc tagged syndecan-1 gene were attached to liposomes and transferred into MDA-MB-231 breast cancer cells. Following G418 selection, the batch transfectants were evaluated for trans-gene expression using antibody specific for human syndecan-1 (BB4 antibody) or antibody specific for the c-myc tag. Flow cytometric analysis revealed a population of the full length syndecan-1 transfected cells that overexpressed syndecan-1 and a population of cells that have the c-myc tag on the cell surface indicating that some cells do expressed the trans-gene. Vector only transfected cells do not overexpress syndecan-1 and do not stain positive for the c-myc tag. Dot blot analysis of media conditioned by the vector only, full length syndecan-1, and truncated syndecan-1 gene transfected cells indicates that the cells transfected with the truncated gene secrete syndecan-1 into the media at high levels whereas the other cell lines do not. Therefore each of the three batch transfected cells were subcloned by limiting dilution to isolate cells lines expressing the

trans-genes. Subclones from each of the three transfecatants were evaluated by flow cytometry and their conditioned media analyzed by dot blot. MDA-MB-231 cells transected with vector only (neo) stain positively for human syndecan-1 on the cell surface and do not stain with the c-myc antibody (Figure 2). B4 and C4 subclone cells transfected with the human syndecan-1, c-myc tagged trans-gene overexpress syndecan-1 and express c-myc on their cell surface. As expected, subclones of the cells transfected with the truncated syndecan-1 do not overexpress cell surface syndecan-1 and do not stain with the c-myc antibody. Dot blot analysis of the conditioned medias from cells transfected with the truncated syndecan-1 gene cassette were used to identify subclones that secrete syndecan-1 into the media (Figure 3).

KEY RESEARCH ACCOMPLISHMENTS:

Task 1.

- Neoglycans have been prepared by carbodiimide condensation reaction modification of GAG chains.
- Neoglycans reduce breast cancer and normal breast cell viability *in vitro*.
- Neoglycans reduce breast cancer cell viability through the induction of apoptosis.
- Neoglycan treatment eliminates MDA-MB-231 breast tumors *in vivo*.
- An active fraction of the chondroitin sulfate neoglycan has been isolated by size exclusion chromatography.

Task 2.

- DNA sequence analysis confirmed the integrity of the c-myc tagged human syndecan-1 gene cassettes.
- Plasmid vector only or plasmid vector containing gene cassettes were attached to liposomes and transferred into MDA-MB-231 breast cancer cells.
- Bulk cells lines expressing the human c-myc tagged syndecan-1 gene cassettes were established.
- Subclones of each bulk cell line were isolated by limiting dilution.

- Subcloned cell lines were evaluated and found to appropriately express high levels of their respective trans-genes.

REPORTABLE OUTCOMES:

- A manuscript entitled: "Neoglycans, carbodiimide modified-glycosaminoglycans: a new class of anticancer agents that inhibit cancer cell proliferation and induce apoptosis," has been submitted to *Cancer Research*.

- An abstract entitled: "Neoglycans, a new class of anticancer therapeutic agents, induce apoptosis in cancer cells and reduce tumor burden," has been accepted for presentation at the 41st Annual Meeting of The American Society for Cell Biology.

- A provisional patent entitled "Synthetic, Highly Charged Molecules and Uses Thereof" filed on January 8, 1999 and the patent application filed January 7, 2000 have been amended and are in review.

- A continuation in part of the original patent application has been filed to cover the neoglycan technology.

CONCLUSIONS:

The first objective to construct nPGs and test their efficacy in inhibiting the growth of breast cancer cells has yielded a very interesting result. When we analyzed the activities of control molecules produced by carbodiimide modification of GAG chains in the absence of a protein scaffold, we found that these molecules inhibit cell viability. Production and evaluation of these molecules that we call neoglycans, demonstrates that these molecules reduce breast cancer cell viability whereas native glycosaminoglycan chains do not. Neoglycans were found to reduce cell viability through the induction of apoptosis. The neoglycan produced from chondroitin sulfate reduced or abolished MDA-MB-231 tumors growing in nude mice following a single treatment without apparent toxicity. These results are very exiting and demonstrate the anti-breast cancer activities of this new class of GAG chain-based molecules. Evaluation and optimization of neoglycan structure to enhance anticancer activity is ongoing.

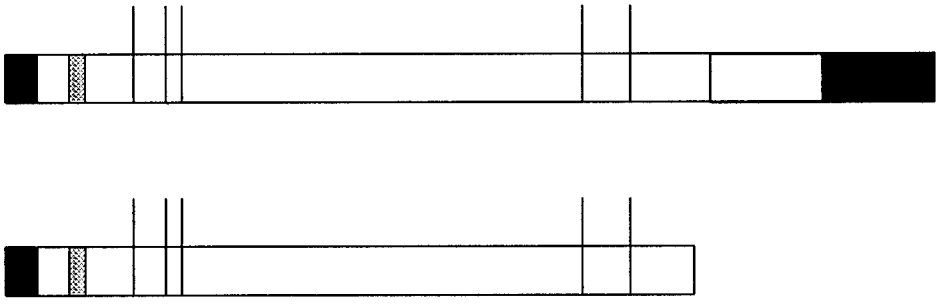
The second objective of the proposal is to transfer syndecan-1 gene constructs into breast cancer cells lines and tumors growing in mice to test the ability of this tumor suppressor gene to slow growth. Full length and truncated syndecan-1 gene cassettes containing a c-myc tag have been engineered and transferred into breast cancer cells

using liposomal carriers. Evaluation of the cell lines and subcloned cell lines produced indicates that the cells support the overexpression of syndecan-1 or the production of a truncated and therefore secreted syndecan-1. Evaluation of the morphological changes and growth changes *in vitro* and *in vivo* due to trans-gene expression are ongoing. This work represents the first attempt to test the syndecan-1 gene as an anticancer therapy and has produced a novel class of therapeutic agents called neoglycans.

APPENDIX:

Please see the attached manuscript entitled: "Neoglycans, carbodiimide modified-glycosaminoglycans: a new class of anticancer agents that inhibit cancer cell proliferation and induce apoptosis."

A.



B.

Full length syndecan-1 forward primer:
5′ cgcggatcctccgggcagc**atg**aggcg

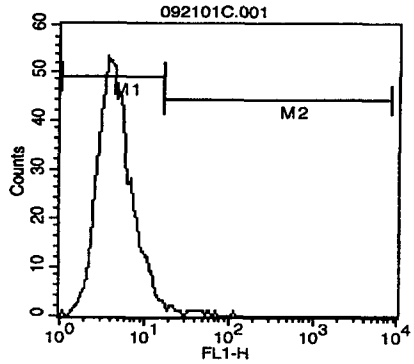
c-myc tag insertion primer:
5′ctgcagctggccctg**gaacagaaactcatctctgaagaggatctg**ccgcaaattgtggct

Full length syndecan-1 reverse primer:
5′cccaagcttgcgtcaggcatagaattcctc

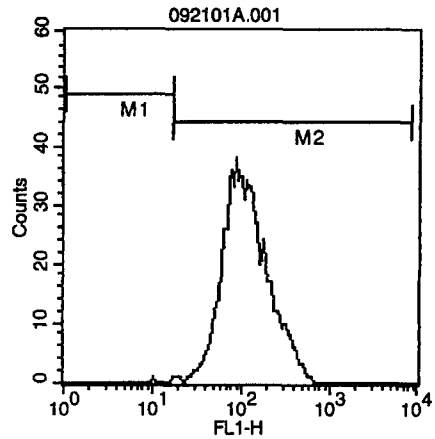
Truncated syndecan-1 reverse primer:
5′cccaagctttcactgatccactggggactg

Figure 1. Full length and truncated syndecan-1 protein structure and primers for gene construction. Panel A. illustrates the c-myc tagged full length syndecan-1 and truncated forms. The full length syndecan-1 is composed of an N-terminal signal sequence (gray box), an ectodomain (open box) containing glycosaminoglycan attachment sites (lines), a transmembrane domain (hatched box), and a cytoplasmic domain (black box). The c-myc tag (dotted box) was inserted in the ectodomain between the signal sequence and glycosaminoglycan attachment sites. The truncated form of syndecan-1 lacks the transmembrane and cytoplasmic domains. Panel B. list the primers used to insert the c-myc tag into the syndecan-1 gene and the primers used for the production of full length and truncated syndecan-1 gene cassettes. The syndecan-1 start codon and the c-myc tag DNA sequences are in bold.

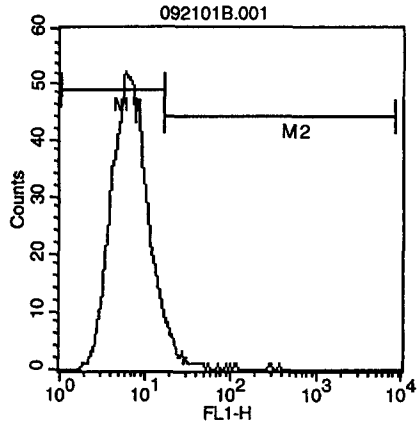
231 neo
Negative control



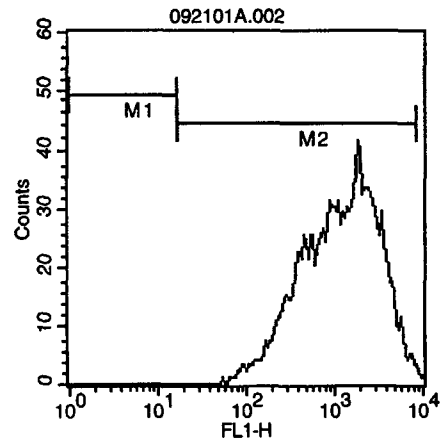
231 neo/BB4



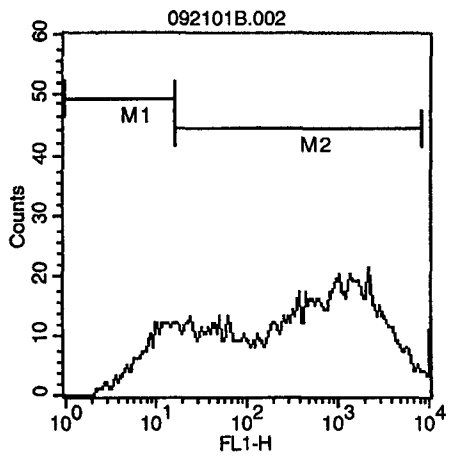
231 neo/c-myc



231 B4/BB4



231 B4/c-myc



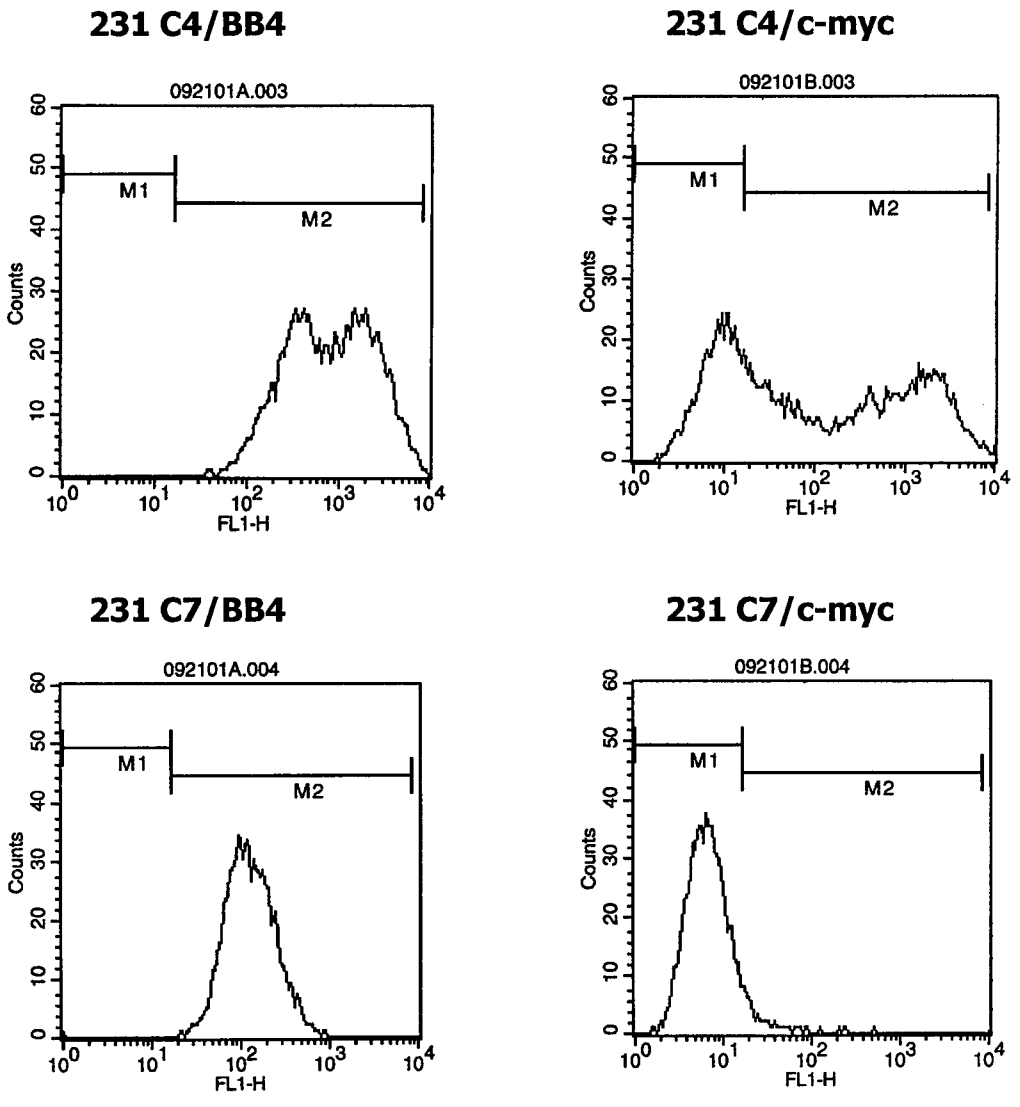


Figure 2. Cell surface expression of c-myc tagged human syndecan-1 on MDA-MB-231 subclones. MDA-MB-231 cells were transfected with vector only (231 neo), vector containing either a c-myc tagged human syndecan-1 gene cassette (231 B4 and 231 C4 subclones) or vector containing a truncated c-myc tagged human syndecan-1 gene (231 C7 subclone). Cell surfaces were stained with a secondary antibody (neo negative control), BB4 anti-human syndecan-1 antibody or a c-myc specific antibody and analyzed by flow cytometry.

Figure 3. MDA-MB-231 cells transfected with a truncated human syndecan-1 gene cassette secrete syndecan-1 into the culture media. The media conditioned by subclones C7 and D4 contains syndecan-1 as determined by dot blot analysis using BB4 antibody. Cells transfected with the vector only (neo) do not secrete syndecan-1.

Neoglycans, carbodiimide modified-glycosaminoglycans: a new class of anticancer agents that inhibit cancer cell proliferation and induce apoptosis[1]

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Running Title: Neoglycans as anticancer agents

Keywords: glycosaminoglycan, neoglycan, anticancer therapy, apoptosis

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[3]The abbreviations used are: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; GAG, glycosaminoglycan; CS, chondroitin sulfate; PBMC, peripheral blood mononuclear cell; MTT, 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium; PI, propidium iodide; IC50, 50% inhibitory concentration.

ABSTRACT

The soluble form of the syndecan-1 heparan sulfate proteoglycan acts as a tumor suppressor molecule that inhibits growth and induces apoptosis of several 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

Glycosaminoglycan (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). Therefore, the concept that GAG chains can impact cancer progression either positively or negatively has been investigated for decades. 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-dependant myeloma cells cultured in the absence of exogenous IL-6 (13, 14). The specific structure of GAG chains and the type of cancer likely influence 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 pre- and postnatal overgrowth and an increased incidence of tumor development (19, 20). Mutations that inhibit HS 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 HS 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_2O 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 x g for 10 minutes to pellet any precipitant. The supernatant was collected and exhaustively buffer exchanged into H_2O and concentrated using 5,000 kDa molecular weight cut off spin columns (Millipore, Bedford, MA). Neoglycan preparations were stored at 4°C. Prior to *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_2O. 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 minutes. Cooled aliquots were transferred to a 96-well plate and the absorbance at OD_{540} 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) prior to

neoglycan treatment. The cell lines and PBMCs were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/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_2 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. Non-adherent cells were plated at a density of 2 x 10^4 cells/well. Adherent cell density varied based on the growth characteristics of each cell line and these cells were plated one day prior to 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_2 for 72 hours. 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_2 for 4-5 hours. 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_{540} 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 if 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 minutes. The cells were washed twice in complete media, plated in triplicate and incubated at 37°C, 5% CO_2 for 72 hours. A control was included in which the cells were treated with the neoglycans for the usual 72 hours. 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 following a 48 hour incubation period and the MDA-MB-231 cells were harvested after a 96 hour 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 prior to 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 following treatment of ARP-1 and MDA-MB-231 cells as described above. ARP-1 and MDA-MB-231 cells were harvested following a 48 hour or a 120 hour 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 intraperitoneal 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 (1×10^6) were injected at two subcutaneous sites on each mouse. Once established, the tumors were injected with a single 1.6 mg dose of CS or neoCS in a 50 µl total volume. Immediately following 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 per 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 with 0.25% coomassie blue for several hours and destained in methanol:H_2O (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 minutes and destained in H_2O. Rainbow molecular weight markers (Amersham Pharmacia Biotech, Piscataway, NJ) served as size standards on each gel.

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_2O, 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 hours.

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 x 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 hours. The digested neoCS was brought 1:1 with column running buffer, boiled for 10 minutes, cooled to room temperature, loaded on the Superdex 200 column and fractions were collected. Numerous fractions were buffer exchanged into H_2O by 5,000 molecular weight cut off 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 hours. 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

$x = 2 + \log10\ (\text{dose})$, if dose>0, 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 β_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-dependant reduction in cancer cell viability in response to treatment with either neoheparin or neoCS (Fig. 1*A*). For example, after 48 hours of treatment, neoheparin and neoCS (32 μg/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 μg/ml (95% confidence interval (CI) of 18.18 to 26.48 μg/ml) and 14.79 μg/ml (95% CI of 12.13 to 18.06 μg/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. Breast cancer cell lines MDA-MB-231 and Hs578t are also sensitive to treatment with the neoglycans.

Identical experiments were performed to evaluate the effect of the neoglycans on non-transformed 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 the normal cell lines tested are sensitive to treatment with either neoglycan (Fig. 1*A*, Table 1). Mitogen activated peripheral blood mononuclear cells (PBMCs) also show a similar sensitivity to both neoglycans (Fig. 1*A* 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 if 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 minutes at 37°C, 5% CO_2. Cells were washed several times to remove neoglycans, placed in fresh media and, following a 72 hour incubation, cell viability was determined by MTT assay. Pulse treatment of ARP-1 cells with 320 μg/ml of neoheparin and neoCS for as short a period as 15 minutes reduces ARP-1 cell viability by 44% and 92%, respectively (Fig. 1*B*). A 5 minute treatment of ARP-1 cells with neoCS reduces cell viability by 42%. At the lower dose of 32 μg/ml, a 60 minute pulse with the neoheparin produces a 44% reduction in cell proliferation while the same length pulse with neoCS produces a 23% reduction in cell viability (Fig. 1*B*). These 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 if 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 hours or MDA-MB-231 cells for 96 hours with media only or 32 μg/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. 2*A*). For TUNEL assays, ARP-1 and MDA-MB-231 cells were treated with 32 μg/ml of the neoglycans and GAG chains and incubated for 48 and 120 hours, respectively. GAG chain or media only treatment results in no apoptosis, while treatment of either cell line with either neoglycan results in apoptosis as shown by DNA fragmentation (Fig. 2*B*). 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 establish in female BALB/c nu/nu mice by subcutaneous injection of 1×10^6 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. Following injections, tumors were electroporated to enhance permeability of the

tumor cells. The size of each tumor was determined 48 hours 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. EDAC is used routinely to covalently couple amino groups to carboxyl groups thereby combining molecules intra- or intermolecularly depending on the availability of the reactive groups (37). Heparin and CS contain free carboxyl groups on D-glucuronic acid residues and heparin has limited free amino groups found on N-unsubstituted glucosamine residues (38). Moreover, previous studies have shown that CS and heparin each have free amino groups on covalently bound amino acids left from isolation procedures (39, 40). This indicates that EDAC modification of these GAG chains could produce multimeric mixtures of GAG coupled to GAG.

To analyze the EDAC-treated GAG chains, equivalent amounts of heparin, neoheparin, CS and neoCS were separated by SDS-PAGE and visualized following alcian blue staining (Fig. 4). As expected, native heparin and CS are very heterogeneous in size (Fig. 4, Lanes H and CS). Comparison of the native GAG chains to their neoglycans shows no obvious differences suggesting that the GAG chains are not coupled or that coupling of the GAG chains is masked. For example, native heparin is a large smear ranging in size from less than 14 kDa to greater than 97 kDa. Therefore, even if several low molecular weight chains combined in the EDAC reaction, the resulting multimeric complex could be less than 97 kDa and not distinguishable from native heparin. Coupling of different numbers and sizes of GAG chains would not yield single-sized products but molecules that vary considerably in size and specific structure.

To ensure that contaminating EDAC does not contribute to the neoglycan-induced cell killing, neoCS was digested with chondroitin ABC lyase in an attempt to block activity and demonstrate that neoCS specifically kills cancer cells. CS and neoCS (500 μg each) were digested with 150 μU of lyase and a portion of each along with controls was visualized on SDS-PAGE gels following alcian blue staining (Fig. 5*A*). The CS-L lane and the neoCS-L lane show high molecular weight smears that remain following lyase digestion. Further 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 if killing activity had been abolished or reduced. ARP-1 cells were treated with digested CS and neoCS at a concentration equivalent to 32 μg/ml nondigested material. Interestingly, the neoCS activity is not blocked by lyase digestion (Fig. 5*B*, neoCS-L) and the digested neoCS is as active as the undigested controls including neoCS in H_2O (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 (CS-L) grew as well as media only treated cells (Fig. 5*B*). These results suggest either the high molecular weight neoCS remaining following lyase digestion is active, the small fragments of the neoCS produced by enzyme digestion are active or residual EDAC is present and active.

To eliminate the possibility that neoglycan preparations contain residual EDAC 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 H_2O, evaluated by SDS-PAGE and the effect on cell viability was

determined on ARP-1 cells. SDS-PAGE analysis confirms the separation of high molecular weight components of lyase-digested neoCS ranging in MW from >220kDa to approximately 97kDa (Fig. 6*A*). The 66 kDa band that is evident in fractions 35-41 is bovine serum albumin that is included as a carrier in the lyase reagent. The 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°C, 5% CO_2 for 72 hours 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. 6*B*). 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-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 following 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 or conjugation of GAG

chains to each other. Therefore, the neoglycans, unlike syndecan-1, do not require a protein component for cell killing activity.

The structure of neoheparin and neoCS is unknown. At least two configurations are possible including coupled GAG chains that form a multimeric array and/or a urea derivative composed of GAG chains and attached EDAC. Coupling via EDAC is widely used to conjugate molecules containing carboxyl and amino groups (41). Heparin and CS are composed of long, variable length, unbranched chains of a repeating disaccharide unit of D-glucosamine and D-glucuronic acid or L-iduronic acid (heparin), or D-galactosamine and a D-glucuronic acid residue (CS (4)). The number 6 carbon of each glucuronic acid component of both GAG chains contains free carboxyl groups. Heparin that has been isolated from porcine intestinal mucosa contains a limited number of N-nonsubstituted glucosamine residues and therefore a limited number of free amino groups (38). Heparin and CS used for the production of neoglycans may contain free amino groups on covalently bound amino acids remaining from conventional isolation procedures (39, 40). Therefore, EDAC modification of heparin and CS likely results in at least a fraction of 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. A second possible structure produced from EDAC modification of GAG chains is an O-acylisourea derivative of heparin and CS (42). The initial reaction of EDAC with a carboxyl group forms an O-acylisourea intermediate which is subsequently attacked by an amino group yielding a coupled product and displaced urea derivative of EDAC. Because there are abundant carboxyl groups and limited amino groups present on GAG chains, the EDAC reaction may not go to completion at every residue and therefore O-acylisourea derivatives could remain. 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 to 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 which highlights the different functions 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. 5*B*) despite the obvious reduction in total GAG content (Fig. 5*A*). 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 while others did even at concentrations too low to determine. Therefore, the neoglycan preparations contain

nonactive components and future studies utilizing 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. 1 *A*). 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 μg/ml) for one hour resulted in a reduction in ARP-1 cell viability while at a higher concentration (320 μg/ml) a pulse as short as 5-15 minutes resulted in a 42-92% reduction in cell viability (Fig. 1*B*). 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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Table 1. Fifty percent inhibitory concentration of neoglycans

Cell line	Tumorigenic	neoheparin IC50[a] (μg/ml)	neoheparin CI[b] (μg/ml)	neoCS IC50[a] (μg/ml)	neoCS CI[b] (μg/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

[a]Estimated IC50 values were determined following cell viability assays by use of a compound nonlinear degradation model based on the 3-parameter logistic. Calculations are based on triplicate wells from duplicate experiments.
[b]95% confidence interval.

FIGURE LEGENDS

Fig. 1. Neoglycans reduce cell viability in a dose-dependent manner. *A*, The indicated cell lines were cultured for 72 hours in the continuous presence of 32, 16 or 3.2 μg/ml concentrations of neoglycan (neoheparin or neoCS), media only (M), 10 μM dexamethasone (D) or 500 μg/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 ± standard deviation of duplicate experiments. *B*, ARP-1 cells were treated with media only (M) or neoglycans for 72 hours as controls (C) or with 320 μg/ml or 32 μg/ml neoglycans for 60, 30, 15 or 5 minutes. Cells were washed to remove neoglycans and placed in fresh culture medium for 72 hours. Results of MTT assays are shown as means ± standard deviation of triplicate wells and duplicate experiments. In each experiment the media only treatment indicates 100% cell viability.

Fig. 2. Neoglycans induce apoptosis. *A*, ARP-1 myeloma and MDA-MB-231 breast cancer cells were incubated with 32 μg/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 μg/ml heparin or CS (thin line) or 32 μg/ml neoglycans (thick lines). DNA laddering, indicative of the late stages of apoptosis, was detected by TUNEL assay. Arrows indicate apoptotic cell populations.

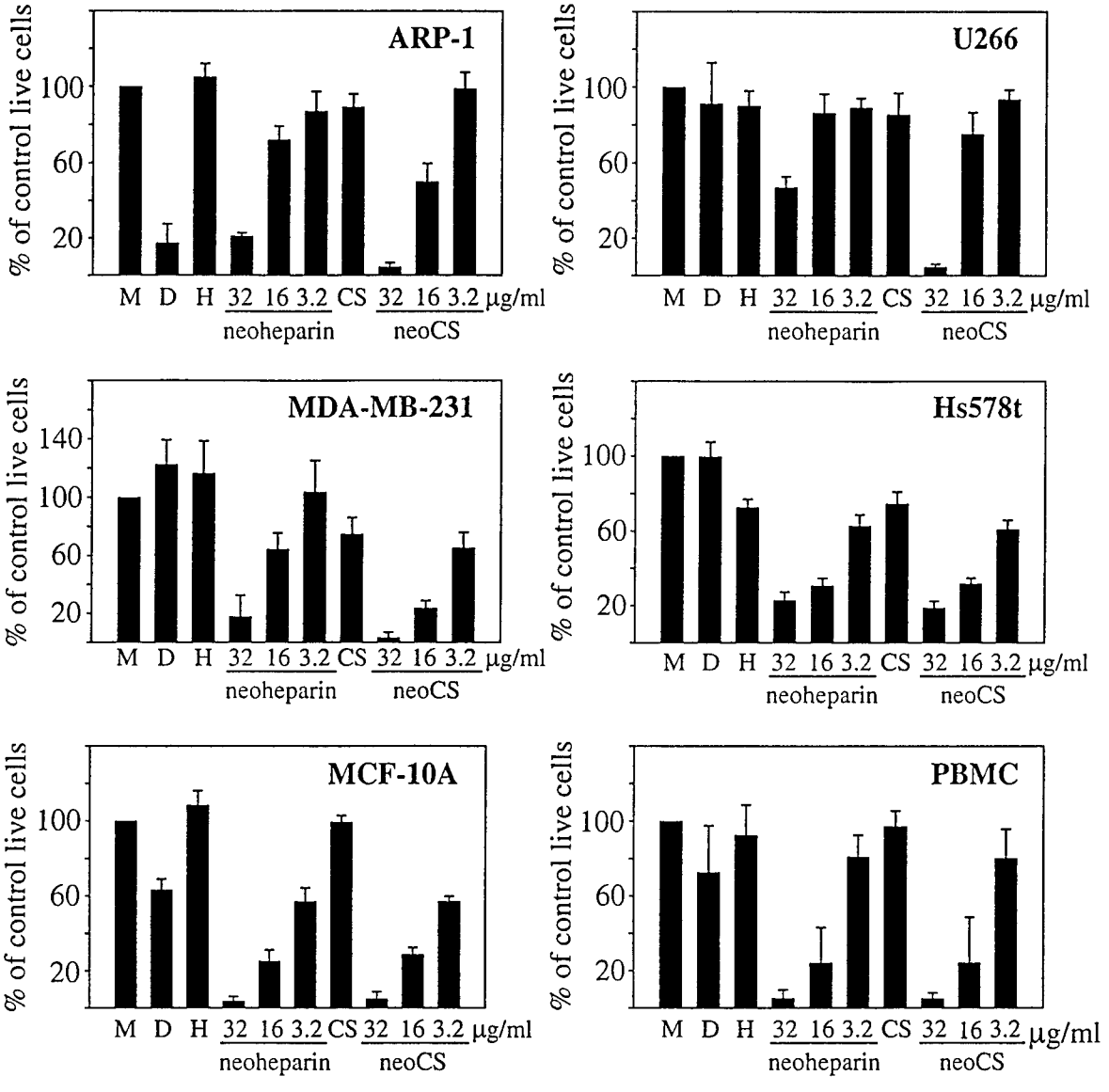
Fig. 3. NeoCS eliminates MDA-MB-231 tumors in mice. Established MDA-MB-231 tumors were injected with a single dose of either 1.6 mg CS (♦) or 1.6 mg neoCS (▲) followed by electroporation of the tumors. On the indicated days following treatment, tumor size was measured and tumor volume calculated. Bars indicate means ± standard deviation 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 due to mouse mortality.

Fig. 4. Visualization of neoglycans and GAG chains. Neoglycans (neoheparin (neoH) and neoCS) and the GAG chains heparin (H) and CS were separated by SDS-PAGE under reducing conditions and visualized following alcian blue staining. Molecular weight markers are shown in lane M. Numeric values indicate molecular weights in kDa.

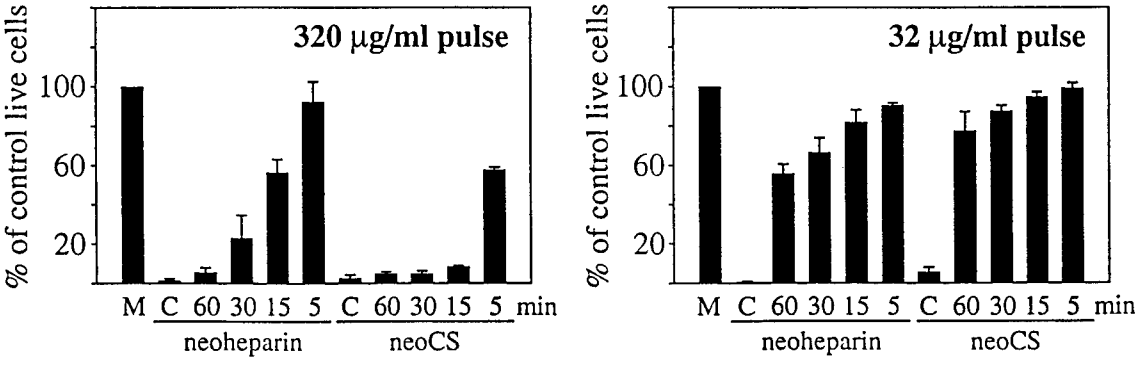
Fig. 5. Chondroitin ABC lyase digested neoCS retains activity. *A*, CS and neoCS were suspended in water (W), chondroitin ABC lyase buffer (B) or chondroitin ABC lyase (L) for several hours and a portion was separated by SDS-PAGE and visualized following alcian blue staining. Molecular weight markers 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), 10 μM dexamethasone (D), 32 μg/ml CS or neoCS in water (W) or in lyase buffer (B) and the predigestion equivalent of 32 μg/ml CS or neoCS in chondroitin ABC lyase (L). Following a 72 hour incubation, 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 standard deviation for each condition is shown.

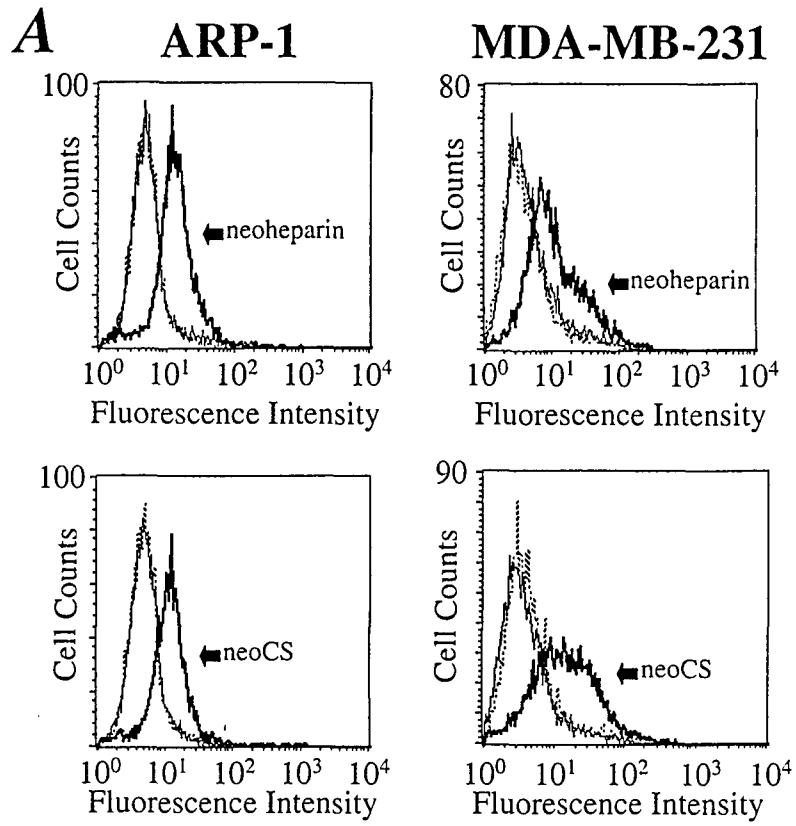
Fig. 6. Identification of active neoCS fractions. NeoCS was digested with chondroitin ABC lyase, fractionated by size exclusion chromatography and the guanidine removed from fractions by buffer exchange using spin column filtration. *A*, Equivalent amounts of each fraction were visualized on SDS-PAGE gels by alcian blue staining. The approximately 66 kDa band in fractions 35-41 is bovine serum albumin 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 hours 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 following treatment with the neoCS fractions 25-42 is shown and the means $\pm$ standard deviation are reported.

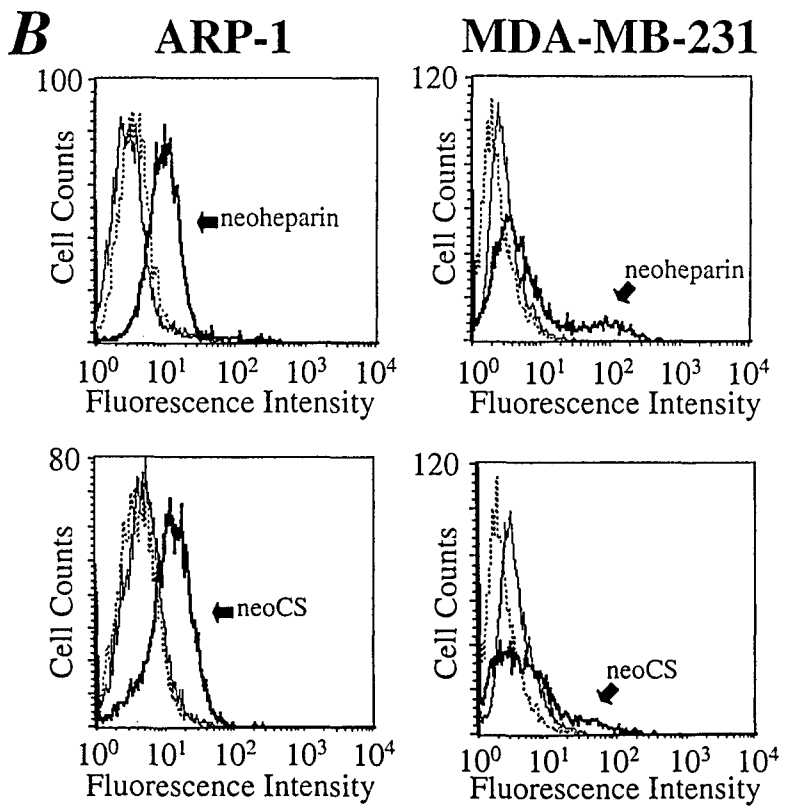
A

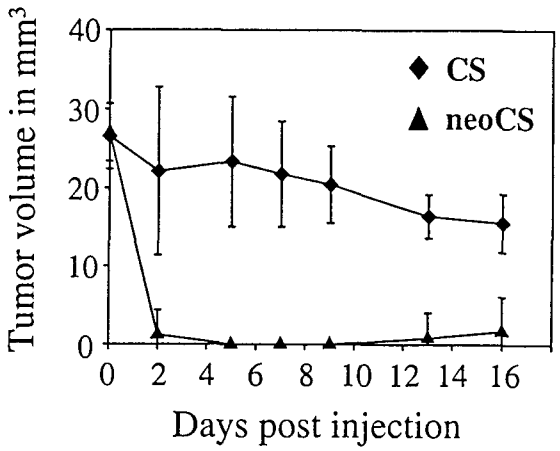

ARP-1
U266
MDA-MB-231
Hs578t
MCF-10A
PBMC
% of control live cells
M D H 32 16 3.2 CS 32 16 3.2 µg/ml
neoheparin
neoCS

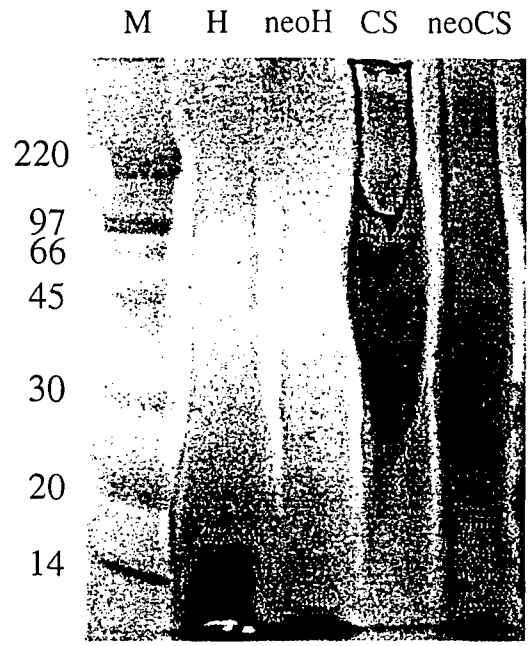
B

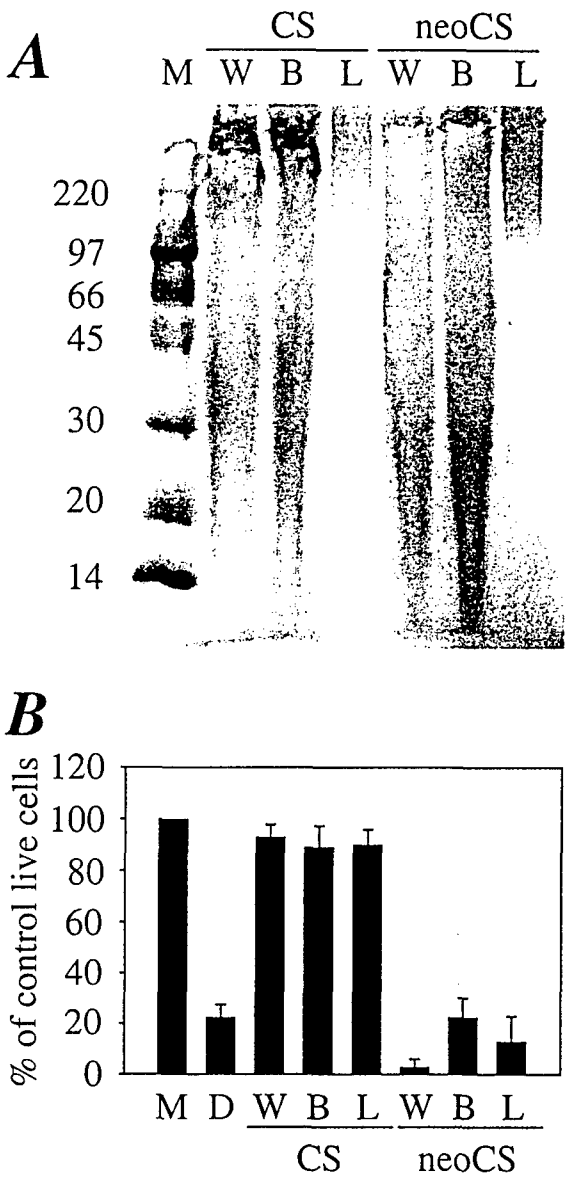

320 µg/ml pulse
32 µg/ml pulse
% of control live cells
M C 60 30 15 5 C 60 30 15 5 min
neoheparin
neoCS

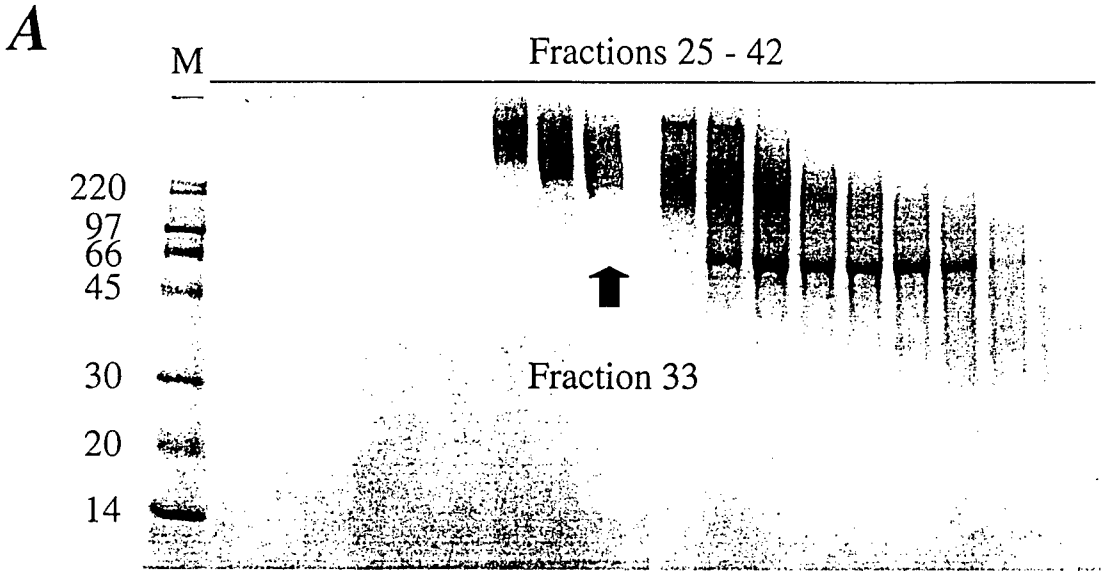

A
ARP-1
MDA-MB-231
Cell Counts
neoheparin
neoCS
Fluorescence Intensity
100
80
90
10^0 10^1 10^2 10^3 10^4

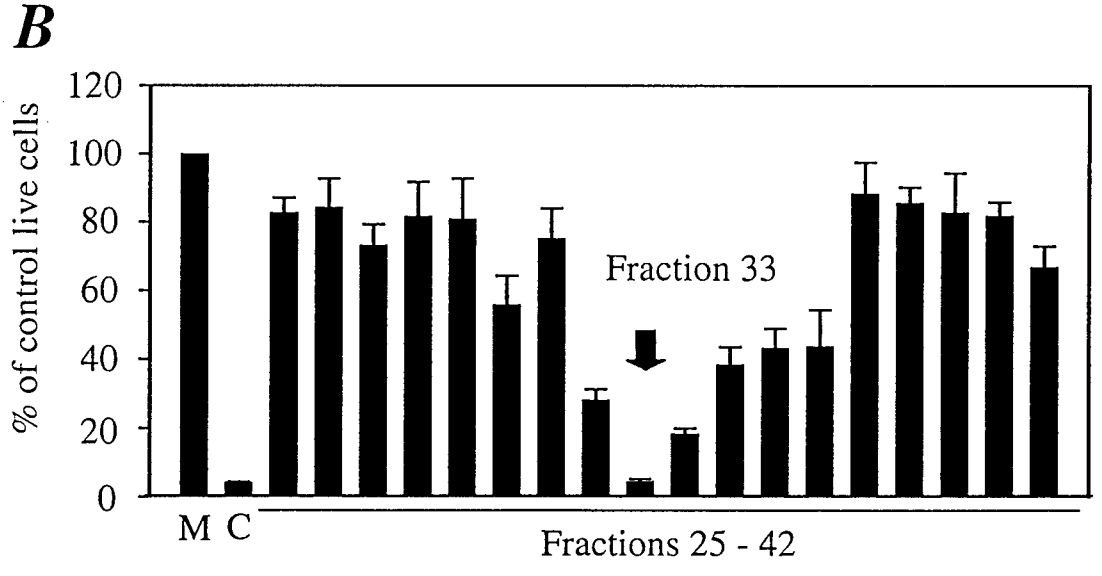

B
ARP-1
MDA-MB-231
Cell Counts
neoheparin
neoCS
Fluorescence Intensity
100
80
120
10^0 10^1 10^2 10^3 10^4


Tumor volume in mm3
40
30
20
10
0
0 2 4 6 8 10 12 14 16
Days post injection
CS
neoCS


M
H
neoH
CS
neoCS
220
97
66
45
30
20
14


A
CS
neoCS
M W B L W B L
220
97
66
45
30
20
14
B
% of control live cells
120
100
80
60
40
20
0
M D W B L W B L
CS
neoCS


A
M
Fractions 25 - 42
220
97
66
45
30
20
14
Fraction 33


B
% of control live cells
120
100
80
60
40
20
0
Fraction 33
M C
Fractions 25 - 42

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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