**Title:** SYNTHETIC, HIGHLY CHARGED MOLECULES AND USES THEREOF

The present invention discloses a synthetic molecule consisting of a charged unit covalently linked to a moiety and methods of producing the same. Specifically, a glycosaminoglycan chain(s) is covalently linked to a moiety to synthesize proteoglycan. Examples of the moiety are human serum albumin, β2-microglobulin, avidin, a peptide and a lipid. Also provided are various therapeutic applications of the synthetic molecules.
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SYNTHETIC, HIGHLY CHARGED MOLECULES
AND USES THEREOF

BACKGROUND OF THE INVENTION

Cross-Reference to Related Applications
This application claims benefit of provisional application
U.S. Serial Number 60,115,053, filed January 8, 1999, now
abandoned.

Field of the Invention
The present invention relates generally to the field of
carbohydrate biochemistry. More specifically, the present invention
relates to synthetic, highly charged molecules and uses thereof.

Description of the Related Art
Cell surface heparan sulfate proteoglycans regulate cell
behaviors by binding various growth factors, proteases, cell-matrix
and cell-cell adhesion molecules. Through these interactions they
mediate cell growth, survival, differentiation, adhesion and migration.
In most cells, the major source of cell surface heparan sulfate is
syndecans. Syndecans are a family of cell surface proteoglycans
consisting of a core protein to which long unbranched carbohydrate
polymers, called glycosaminoglycans (GAGs), are covalently attached
(Carey, 1997). All four known members of the syndecan family (designated syndecan-1, -2, -3 and -4) are type I transmembrane proteins, with highly conserved transmembrane and cytoplasmic domains and an ectodomain that contains at least three sites for heparan sulfate attachment. Most of the syndecans’ known functions occur via interaction of their heparan sulfate chains with extracellular ligands and recent studies indicate that at least some syndecan cytoplasmic domains may be directly involved in signaling (Oh et al., 1997; Reiland et al., 1996). Moreover, heparan sulfates are obligate partners for the high affinity signaling mediated by some growth factors (e.g. basic fibroblast growth factor, chemokines) (Rapraeger, 1993; Tanaka et al., 1998). Because they function in both growth control and cell adhesion, syndecans (and other heparan sulfate proteoglycans) play important roles in regulating the behavior of tumor cells (Inki and Jalkanen, 1996).

Myeloma cells that are normally highly invasive in type I collagen gels lose their ability to invade following transfection with syndecan-1 (Liebersbach and Sanderson, 1994). Normal mammary epithelial cells which are non-invasive on collagen gels become invasive when normal levels of cell surface syndecan-1 are lost (Kato et al., 1995). Upon hormone-induced transformation of S115 mammary epithelia, these cells down-regulate their expression of syndecan-1, lose their epithelial morphology and become fusiform (Leppa et al., 1991). Following their transfection with the cDNA for syndecan-1, the transformed S115 cells regain their epithelial morphology and growth characteristics (Leppa et al., 1992). Similarly, when normal mammary epithelial cells are made deficient in syndecan-1 they lose their epithelial morphology and exhibit characteristics of neoplastically transformed cells including a fibroblastic appearance, the presence of elongated filopodial
extensions and an absence of stress fibers and focal adhesions (Kato, et al., 1995).

In non-neoplastic lesions and metaplastic squamous cells of human uterine cervix, staining for syndecan was similar to that in normal uterine cervix (Inki et al., 1994). However, progression of cervical intraepithelial neoplasia from grade I to grade III was associated with a reduction in syndecan-1, and syndecan-1 was absent from poorly differentiated squamous cell carcinomas. In primary squamous cell carcinomas of the head and neck, tumors positive for syndecan-1 were associated with a more favorable patient prognosis. This prognosis included both a higher two year survival and a higher recurrence-free survival than those patients with tumors expressing little or no syndecan-1 (Inki et al., 1994). And in a more recent study of 100 patients with laryngeal squamous cell carcinoma, intermediate or strong staining for syndecan-1 was associated with higher overall survival than those tumors with little or no syndecan-1 expression (Pulkkinen et al., 1997).

E-cadherin is known to suppress the invasion of numerous tumors including breast carcinoma (Mbalaviele et al., 1996). In mammary epithelia, loss of syndecan-1 is accompanied by a dramatic loss of E-cadherin expression (Kato, et al., 1995). In NM-c-ras-MAC1 mammary tumor cells, downregulation of E-cadherin with antisense RNA suppresses syndecan-1 expression and cells become invasive. Conversely, NM-f-ras-TD cells transfected with E-cadherin cDNA upregulate syndecan-1 expression and become non-invasive (Leppa et al., 1996).

Expression of syndecan-1 inhibits the growth of mammary epithelial tumor cells and loss of syndecan-1 on normal mammary epithelial cells promotes anchorage-independent growth, a hallmark of the neoplastic phenotype (Kato, et al., 1995). In myeloma cells,
expression of syndecan-1 inhibits G1-S progression with downregulation of cyclin D1 (Dhodapkar, et al., 1998). When injected into SCID mice, myeloma tumorigenesis is dramatically inhibited in cells transfected with the syndecan-1 cDNA as compared to vector-only transfected cells (Dhodapkar, et al., 1998). Addition of 1-4 nM of exogenous, purified syndecan-1 ectodomain inhibits the growth of both carcinoma (including S115 and MCF-7 breast carcinoma) and myeloma cell lines (Dhodapkar, et al., 1998; Mali et al., 1994). Moreover, exogenous purified syndecan-1 induces a striking increase in apoptosis suggesting its potential usefulness for cancer therapy (Dhodapkar, et al., 1998). These effects on growth require the intact proteoglycan because addition of either isolated heparan sulfate chains or core protein without heparan sulfate chains does not alter tumor cell growth.

These studies from three different laboratories using different cell lines and assay systems provide strong experimental evidence that syndecan-1 expressed on the cell surface or added exogenously can control tumor cell growth and motility. However, these growth inhibitory activities are not exclusive to syndecan-1 because glypican-1 and betaglycan, two other cell surface heparan sulfate proteoglycans, have similar effects on tumors (Lesoon-Wood, et al., 1995). However, heparan sulfate proteoglycans are not present in large amounts on cells, it is difficult to harvest enough from natural sources to use for treatments. Therefore, it is important to make “synthetic” proteoglycans, called neoproteoglycans (nPGs).

The prior art is deficient in the lack of “synthetic” molecules such as proteoglycans useful for a variety of biological and medical purposes. The present invention fulfills this long-standing need and desire in the art.
SUMMARY OF THE INVENTION

The present invention discloses that addition of exogenous heparan sulfate proteoglycans can inhibit growth and induce apoptosis of human myeloma cell lines. Also disclosed is that expression of heparan sulfate proteoglycans by myeloma cells inhibits their growth in SCID mice. These effects on cell behavior required an intact proteoglycan as heparin or heparan sulfate alone (glycosaminoglycan chains not attached to a core protein) did not alter growth of these cells. However, further studies indicated that the biological activity was not determined by the core protein. Rather, the determining factor was heparin or heparan sulfate presented to cells in a multi-valent format. A synthesized proteoglycan consisting of heparin chains covalently linked to bovine serum albumin was tested and found to induce apoptosis of the human myeloma cell lines. Therefore, heparin, heparan sulfate or other highly sulfated or highly negatively charged substances presented in a multi-array, multi-valent format by linking it to any natural or synthetic moiety such as avidin, albumin, peptides, lipids, can be used as biological modifiers to regulate the behavior of cells.

In one embodiment of the present invention, there is provided a pharmaceutical compound, i.e., a synthetic molecule, consisting of a charged unit covalently linked to a moiety. Preferably, a glycosaminoglycan, such as heparin, heparan sulfate chondroitin sulfate, dermatan sulfate, keratin sulfate or dextran sulfate, is linked to avidin, albumin, β2-microglobulin or a peptide to generate a synthetic proteoglycan.

In one embodiment of the present invention, there is provided a method of producing the synthetic molecule of the
present invention by using carbodiimide condensation to link the charged unit to the moiety or by using biotin-avidin interaction to link biotinylated charged unit to the avidin protein.

In another embodiment of the present invention, there is provided a method of inhibiting tumor growth or further killing the tumor by exposing the tumor with the synthetic molecule. Preferably, the tumor is a myeloma or a tumor of hematopoietic origin. Still preferably, the tumor is a solid tumor.

In still another embodiment of the present invention, there is provided a DNA molecule coding for the synthetic molecule. Such DNA molecule can be used to transduce or lipofect a cancer cell in order to treat the cancer. Representative examples of cancer are breast cancer, prostate cancer, lung cancer and a cancer of hematopoietic origin.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

So that the matter in which the above-recited features, advantages and objects of the invention, as well as others which will become clear, are attained and can be understood in detail, more particular descriptions of the invention briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting in their scope.
Figure 1 shows the effect of neoproteoglycan (nPG) on myeloma cell viability. Heparin or chondroitin sulfate was conjugated to human serum albumin (HSA) by carbodiimide condensation to generated nPGH and nPGC, respectively. Figure 1A shows the effect of nPGH and nPGC on myeloma cell line ARH-77. Figure 1B shows the effect of nPGH and nPGC on myeloma cell line ARP-1. Cell viability was determined by MTT analysis. Cells, 2 x 10^4, were treated with media only (lane 1) or 10 µg of the following: HSA (lane 2), heparin (lane 3), heparin mixed with HSA (lane 4), chondroitin sulfate (lane 5), chondroitin sulfate mixed with HSA (lane 6), the product of carbodiimide condensation of HSA only (lane 7), nPGH (lane 8), and nPGC (lane 9). Cell viability was determined following a 72-hour incubation at 37°C. The mean OD_{540} readings and standard deviation are shown for triplicate wells.

Figure 2 shows the effect of biotinylated heparin coupled to avidin on myeloma cell viability. Myeloma cell lines ARH-77 (Figure 2A) and ARP-1 (Figure 2B) were treated with media only (lane 1), 10 µg of biotinylated heparin only (lane 2), and 10 µg avidin:biotinylated heparin nPG (lane 3). Cell viability was determined by MTT analysis following a 72-hour incubation at 37°C. The mean OD_{540} readings and standard deviation are shown for triplicate wells.

DETAILED DESCRIPTION OF THE INVENTION

Heparan sulfate proteoglycans on the cell surface act as tumor suppressors. When the syndecan-1 proteoglycan is lost from the surface of normal 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 their epithelial morphology and lose their neoplastic growth characteristics. Remarkably, addition of purified intact syndecan-1 ectodomain to tumor cells also inhibits their growth in culture and can induce extensive apoptosis. These effects require the intact proteoglycan because addition of either isolated heparan sulfate chains or core protein without heparan sulfate chains does not alter tumor cell growth. However, anti-tumor effects are not specific for syndecan-1, because other cell surface heparan sulfate proteoglycans (e.g. glypican-1 and betaglycan) also inhibit tumor cell growth.

One object of the present invention is to construct and purify neoproteoglycans and test their efficacy in inhibiting growth of cancer cells. Because anti-tumor effects are not specific for a single core protein, heparin (or heparan sulfate) attached to a carrier will mimic naturally occurring proteoglycans and exert growth inhibitory effects on cells.

Use of neoglycoprotein -molecules constructed by attaching carbohydrate derivatives to proteins – is widespread. These synthesized molecules are utilized for both biological and therapeutic applications because it allows for large-scale preparation of molecules of known composition (Lee and Lee, 1994). This technology has been exploited for attaching glycosaminoglycan chains to carriers to form neoproteoglycans (nPGs) (Yamagata et al., 1989). However, cross-linked polymers could occur, which might cause problems. In the present invention, neoproteoglycans were produced by conjugating heparin or specific fractions of heparin to human serum albumin and modification of Yamagata's procedure is under way to maximize neoproteoglycan yield and activity on tumor cells. Following biochemical characterization, these neoproteoglycan-like
molecules were analyzed for their anti-tumor effects when added to cells in culture.

Another object of the present invention is to transfer the gene for syndecan-1 directly into breast tumors growing in nude mice. For these gene therapy studies, both liposome and retroviral delivery systems containing the syndecan-1 gene under control of a breast-specific promoter are used. Following gene transfer, expression of syndecan-1 in cell lines or tumors are confirmed and the effects of expression on tumor growth analyzed. These studies represent the first attempt to use heparan sulfate proteoglycans in anti-cancer therapy.

In one embodiment of the present invention, there is provided a pharmaceutical compound, i.e., a synthetic molecule, consisting of a charged unit covalently linked to a moiety. Preferably, a glycosaminoglycan, such as heparin, heparan sulfate, chondroitin sulfate, dermatin sulfate, keratin sulfate or dextran sulfate, is linked to avidin, albumin, β₂-microglobulin or a peptide to generate a synthetic proteoglycan.

In one preferred embodiment, there is provided a method of producing the synthetic molecule of the present invention by using carbodiimide condensation to link the charged unit to the moiety or by using biotin-avidin interaction to link biotinylated charged unit to the avidin protein.

In another preferred embodiment, there is provided a method of inhibiting tumor growth or further killing the tumor by exposing the tumor with the synthetic molecule. Preferably, the tumor is a myeloma or a tumor of hematopoietic origin. Still preferably, the tumor can be a solid tumor.

In still another preferred embodiment, there is provided a DNA molecule coding for the synthetic molecule. Such a DNA
molecule can be used to transduce or lipofect a cancer cell in order to treat the cancer. Representative examples of cancer are breast cancer, prostate cancer, lung cancer and a cancer of hematopoietic origin.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

**EXAMPLE 1**

**Neoproteoglycan (nPG) Production**

Glycosaminoglycans (GAG, Sigma), heparin or chondroitin sulfate, were coupled to human serum albumin (HSA, Sigma) by either carbodiimide condensation or through the noncovalent interaction of biotin and avidin. For the carbodiimide condensation reaction, an aqueous solution of human serum albumin alone or human serum albumin plus glycosaminoglycans (1:50 ratio w/w) was prepared. Aqueous N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma) was added to a 5-fold excess over glycosaminoglycan concentration and the pH of the solution was adjusted to 5.0. The mixtures were rocked at 4°C for 12 hours, centrifuged at 20,000 x g for 10 minutes, and the supernatants exhaustively dialyzed against dH₂O. Products were collected by lyophilization and reconstituted in RPMI without serum.

Avidin:biotinylated heparin neoglycosaminoglycans was produced through the strong coupling of avidin to biotin. To biotinylate heparin, 10 mM heparin in 2 M NaOH was combined with 30 mM NaBH₄ and 1.9 M epichlorohydrin and incubated at room temperature for 24 hours. The solution was dialyzed against dH₂O for 24 hours. 1,6-Diaminohexane was added to 0.1 M from a 0.5 M
stock in 0.1 M NaHCO₃ followed by another 24 hour incubation period at room temperature and subsequent dialysis against 0.1 M NaHCO₃, pH 8.5. Biotin-X-NHS (Calbiochem) was added from a 10 mg/ml stock in DMSO to give a 1:1 w/w ratio of biotin to heparin. The solution was incubated at 37°C for 3 hours, dialyzed against dH₂O, and stored at 4°C. A neoglycosaminoglycan was produced by combining avidin-FITC (Vector) and biotinylated heparin at a ratio of 1:50 w/w.

**EXAMPLE 2**

Isolation and Characterization of Neoproteoglycans (nPGs)

Following conjugations, the material was brought to 6 M urea, 50 mM NaAc, pH 4.5 for nPGH or pH 6.0 for nPGC and passed over a Pharmacia HiTrap Q ion exchange column. At this pH, neoproteoglycans bound to the column and unconjugated human serum albumin did not. Following elution of the column with 4 M GdnHCl, the sample was subjected to size separation by FPLC. This facilitates the removal of free heparin from nPGs and produces fractions containing neoproteoglycans of different sizes, due predominantly to the number of heparin chains attached to the human serum albumin. Protein amounts per fraction were determined by BCA assay, and nPGs were visualized by SDS-PAGE separation and coomassie and alcian blue staining.

**EXAMPLE 3**

Functional Assays of Neoproteoglycans

*In Vitro Studies:* Breast cancer cells [MCF-7, MA-MB-231, BT-20, BT-474, MB157 (ATCC)], normal breast epithelial cells [MCF-
10A and MCF-10F (ATCC) and myeloma cell lines ARH and ARP were used for these studies. These lines differ in the amount of syndecan-1 they express (e.g. normal lines express high levels, MA-MB-231 express low levels). FPLC-generated fractions of neoproteoglycans were exchanged into complete media and added to cells at varying concentrations (1–100 μg/ml protein). As controls, purified syndecan-1 ectodomain was added at 1 nM, a concentration known to inhibit cell growth by more than 75%. Negative controls included pretreatment of neoproteoglycans with heparinase or chondroitinase which should abolish growth inhibitory effects. Assays were performed on cells growing in 96 well plates and cell numbers present after 4 days were assessed spectrophotometrically as described (Mali, et al., 1994). Neoproteoglycan reduced myeloma cell viability (Figure 1). Apoptosis was analyzed using antibody to Apo 2.7 (Coulter), a mitochondrial protein expressed early in committed apoptosis pathways. These experiments determine if the neoproteoglycans inhibit growth and induce apoptosis and, if so, what neoproteoglycan size (i.e., number of chains/core protein) is optimal for these functions. Further adjustments of cross-linking conditions are to be made to optimize the amount of neoproteoglycan produced having the maximal biological effect.

In Vivo Studies: Choice of cell lines for use in in vivo studies is determined by results of in vitro studies. 3 x 10⁶ exponential phase cells are to be injected into the mammary fat pad of female athymic nude mice. Once large enough to palpate, tumors will be injected daily with 25 μl of sterile PBS containing neoproteoglycan (or PBS only as a control) at the concentration proven effective in in vitro studies. Xenograft size will be measured externally on a daily basis using a caliper, and tumor volume
determined from the equation: \( V = (L \times W^2) \times 0.5 \) where \( L \) is length and \( W \) is width. Regression will be defined as a decrease of at least 40% from the peak volume of the tumor size. If no size difference is seen between control and experimental animals, the frequency of injection and/or the concentration of neoproteoglycan will be increased. Upon termination of the experiment, tumors will be formalin-fixed for histological examination and apoptosis will be determined using Apotag (Oncor).

**EXAMPLE 4**

**Alternative Approaches**

Although little information is available on the production and use of neoproteoglycans, the carbodiimide reaction has been proven successful (Yamagata, et al., 1989). However, this reaction can result in formation of cross-linked polymers which could be problematic. Fractions containing large aggregates are eliminated by HPLC size separation. If polymers or aggregates comprise a large portion of the product, one can minimize this by altering the time of the condensation reaction and the ratios of heparin, human serum albumin and carbodiimide. If further manipulation is necessary, standard chemical blocking reagents specific for carboxylic acids and/or amines are used to increase the specificity of cross-linking to the protein.

The use of human serum albumin as a core protein has the advantage of being inexpensive, readily available and non-immunogenic. If it proves unsuitable, other proteins such as human \( \beta_2 \)-microglobulin may be used. This protein may not be as likely to form aggregates because of its size (11.6 kDa). However, it is
considerably more expensive than human serum albumin. Another alternative is to prepare heparin-avidin probes. Heparin can be easily biotinylated (Pankhurst et al., 1998; Stearns et al., 1997) and bound with high affinity to avidin thereby forming multivalent molecules. Manipulation of biotinylation conditions and titration of ratios of biotinylated heparin to avidin will allow production of probes having a single avidin complexed with multiple heparins in the absence of extensive polymerization into aggregates. When biotinylated heparin is coupled to avidin, avidin:biotinylated heparin neoproteoglycan reduces myeloma cell viability (Figure 2). Although not a problem in nude mice studies, a potential drawback of this approach is that avidin will likely produce an immune response if these complexes were used to treat patients. However increased immune activity within the tumor may be advantageous if the response promotes immune-mediated killing of tumor cells.

The variability in the composition and biological activity of the heparins may be a problem. Therefore, if initial neoproteoglycans lack biological activity, one can test commercially available heparins isolated from various sources such as kidney and lung, as well as purified heparan sulfate. It is also possible that a certain fraction of the heparin/HS within a commercial lot will contain the most biologically active chains. To isolate this fraction, heparin is subjected to size and/or charge density (ion exchange) chromatography, fractions conjugated to human serum albumin, and specific activity of the nPGs tested.

In regard to in vivo studies, it is not known how long the neoproteoglycans will remain localized within the tumor and how well they will diffuse throughout the tumor mass. Therefore, multiple, frequent injections may be necessary. Neoproteoglycans can be biotinylated so they can be localized histochemically on tumor
sections and their distribution assessed. Approaches to address diffusion problems include encapsulation of neoproteoglycans into beads or discs and implantation into tumors at multiple sites. Another problem could occur due to the anti-coagulant activity of neoproteoglycans. If this occurs, heparins are fractionated and those with high anti-coagulant activity removed prior to conjugation to human serum albumin.

**EXAMPLE 5**

**Vector Production and Characterization**

A number of studies have shown that reintroducing a wild-type tumor suppressor gene into cancer cells can slow or halt their growth (Baker et al., 1990; Bookstein and Allred, 1993; Lesoon-Wood et al., 1995). Two methods (retroviral transduction and lipofection) were designed and tested for transfer of the syndecan-1 gene into breast cancer cell lines and tumors growing in nude mice, and to determine the effects of the expressed transgene on tumor growth and behavior.

The human syndecan-1 cDNA is modified by insertion of DNA sequence encoding the HA1 epitope at a position just 3' to the syndecan-1 amino-terminal signal sequence. This fragment is cloned into the XM6 retroviral vector containing the mouse mammary tumor virus (MMTV) long terminal repeat which promotes selective expression in breast cells (Arteaga and Holt, 1996). *In vivo* human gene therapy trials are currently underway using this retrovirus (Holt et al., 1996). Because shed syndecan-1 also can affect tumor growth (Dhodapkar, et al., 1998; Mali, et al., 1994), vectors containing syndecan-1 cDNA having an internal stop codon beginning at nucleotide position 993 are also constructed. These truncated
syndecan-1 molecules are secreted from the cell rather than intercalated into the plasma membrane. Following DNA sequencing, vectors are transfected into the amphotropic packaging cell line PA317 (ATCC) by calcium-phosphate precipitation and subclones established by G418 selection and limiting dilution. Retroviral stocks are titered on NIH3T3 (ATCC) cells based on a G418 resistant colony assay (Byun et al., 1996). For DNA delivery via lipofection, cationic liposomes composed of dioleoylphosphatidylethanolamine (DOPE, Avanti Polar Lipids) and 3-beta-[N-(N',N'-dimethylaminoethane)carbamoyl] cholesterol (DC-chol, Life Technologies) at 1:1 (w/w) are synthesized and complexed with the XM6-syndecan vector.

**EXAMPLE 6**

**Functional Assays of Transgene Syndecan-1**

Breast cancer cell lines [MCF-7, MA-MB-231, BT-20, BT-474, MB157 (ATCC)] and normal breast epithelial cells [MCF-10A and MCF-10F (ATCC)] are lipofected or transduced and G418 resistant cells are stained with the HA-tag specific monoclonal antibody HA1.1 (Babco) and isolated by flow cytometry. Clones are isolated by limiting dilution, levels of syndecan-1 expression determined by immunostaining and proteoglycan characterized by Western blot analysis of whole cell lysates or partially purified conditioned medium (Dhodapkar, et al., 1998; Sanderson, et al., 1994). The goal is to isolate clones with different levels of syndecan-1 expression and analyze growth by quantifying \(^3\)H-thymidine uptake (Dhodapkar, et al., 1998), colony-forming potential and extent of apoptosis using antibody Apo 2.7 (Coulter). In addition, the potential for syndecan-1-transduced cells exerting a bystander effect by effecting the growth of
neighboring untransduced cells is examined. Transduced breast cancer cell lines are cocultured with nontransduced breast cancer and normal cell lines and growth and apoptosis analyzed. These assays address the important issue of whether syndecan-1 expressing cells produce a tumor suppressor effect on neighboring nontransduced cells without damaging normal cells.

Once optimal conditions for gene transfer and growth inhibition have been determined, one can evaluate therapy in vivo using cells shown to be responsive in vitro. Mice are divided into control, vector only and syndecan-1 treatment groups and injected with $3 \times 10^6$ cells into the mammary fat pad of female athymic nude mice. Once tumors are established, mice are injected with liposomes or retroviral vectors directly into the tumor or via the tail vein every ten days for a total of four treatments. Tumor size is determined daily and upon termination, tumors are harvested and syndecan-1 expression and extent of apoptosis determined immunohistochemically. Syndecan-1 isolated from tissue extracts is probed by Western blotting to determine the nature and extent of its heparan sulfate glycosylation.

**EXAMPLE 7**

**Alternative Approaches**

A problem of retroviral gene transfer is that cells need to undergo division to allow transgene incorporation into the host genome. Also, when introduced systemically, viral particles are often destroyed by serum components. Because of these difficulties in performing successful gene transfer (Anderson, 1998; Romano et al., 1998), two delivery approaches (liposome and retrovirus) and two
routes (injections into the tumor and via the tail vein) may be used. Although liposome delivery of genes has been used to treat breast tumors (Lesoon-Wood, et al., 1995; Xu et al., 1997), the success of this approach is highly dependent on numerous factors including, among others, DNA-to-liposome ratio (Fasbender et al., 1995), DNA-liposome complexing volume (Staggs et al., 1996) and the lipid components making up the vesicles (Stamatatos et al., 1988). Therefore initial studies may require alterations of numerous conditions to succeed. Other approaches such as fusogenic liposomes (liposomes coated with Sendai virus coat proteins) which have a greater than 95% efficiency for introduction of macromolecules into cultured cells (Kaneda et al., 1995) may also be used. If poor expression of the transgene is detected, one can insert the Alfalfa Mosiac Virus 4 translational enhancer between the promoter and the syndecan-1 gene. This results in the enhancer being positioned in the 5'-untranslated region of the transcribed mRNA and increases expression by as much as 30% (Jobling and Gehrke, 1987). If both liposomal and retroviral delivery fail, the Biolistic PDS-1000/HE Particle Delivery System (gene gun, available in the Arkansas Cancer Research Center) will be employed.

Another possible pitfall could occur because some tumor cells produce proteoglycans with undersulfated heparan sulfate chains which may not be as biologically active as heparan sulfates from normal cells. If this occurs in certain breast tumors growing in vivo, the problem of undersulfation may be overcome by overexpression and shedding of properly sulfated syndecan-1 from surrounding normal cells that are also transduced (a bystander effect). This is entirely possible because shed syndecan-1 does have a dramatic effect on tumor growth (Dhodapkar, et al., 1998; Mali et al., 1994). Moreover, no adverse side-effects are expected from
overexpression by normal cells. This is very important because it suggests that if i.v. delivery of the syndecan-1 gene is successful, it may be possible to treat breast tumor metastases without damage to normal tissue.

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Discussion

Syndecan-1 is a tumor suppressor that can regulate tumor growth when expressed on the cell surface or when added exogenously to cells. However, tumor growth inhibitory activities are not exclusive to syndecan-1. Other cell surface heparan sulfate proteoglycans such as glypican-1 and betaglycan have similar effect on tumors. Although it has been believed for several years that heparan sulfate proteoglycans play important tumor regulatory roles, no studies have directly addressed their potential for therapies. The idea to use neoproteoglycans as therapeutic agents is novel. This strategy has the potential to produce a relatively simple, inexpensive product that will effectively control tumor growth with little if any negative side effects. These experiments represent a logical next-step for proteoglycan research and, hold promise for the development of new therapies that alone or in combination with other cytostatic agents will arrest or eradicate tumors.

The molecular constructions of the present invention possess the advantage of being relatively easy to construct by simple chemical or other linkage of charged substances to a carrier moiety that results in a multivalent array. Another advantage is that these can be assembled from abundant, commercially available products such as heparin and serum albumin. Also, these constructions can be designed to lack immunogenicity in humans (e.g. linkage of heparin to human serum albumin). Constructed molecules are an advantage over use of naturally occurring heparan sulfate proteoglycans
because the latter are not abundant enough for use therapeutically. Also, recombinant production of proteoglycans is not feasible because they are not properly glycosylated (with heparan sulfate chains). Constructed sulfated arrays also have a decided advantage over the use of heparin alone. For example, heparin alone does not control growth of myeloma tumor cells. Heparin alone also has adverse effects – when injected into humans, it is an anti-coagulant and it stimulates osteoclastogenesis which results in bone loss. However, *in vitro* experiments suggest that heparan sulfate proteoglycans do not stimulate osteoclastogenesis.

The novel sulfated array constructs of the present invention possess many uses. For example, the constructs may be introduced into humans as anti-tumor therapy. The constructs may be particularly useful against tumors easily assessable via the blood, e.g., multiple myeloma, which could be treated by intravenous injection. Solid tumors may be treated with direct injection of constructs into the tumor. Following high dose chemotherapy or other purging techniques, sulfated constructs could be used to kill residual tumor cells. These constructs may also bind numerous heparin-binding growth factors or other ligands and regulate their behavior – this could have effects on the pathobiology of numerous diseases (e.g. inflammatory diseases such as rheumatoid arthritis). The constructs may regulate angiogenesis by binding to angiogenic factors such as bFGF and VEGF, both heparin-binding molecules. It is possible that these constructs could be either pro- or anti-angiogenic *in vivo* depending on their localization and concentration. Because many viruses and microbes bind to host cells via heparan sulfate, these sulfated arrays could block their entry into human tissue (e.g. certain HHV, chlamydia). Depending on their concentration *in vivo*, they could promote or inhibit cell adhesion.
There are possible gene therapy applications of the constructs of the present invention. It has been shown that heparan sulfate at the cell surface promotes internalization of cationic liposomes into cells. If array constructs when introduced in vivo will bind to cells, this could enhance liposome mediated delivery of genes or other substances into cells.

The constructs of the present invention could be used as a drug delivery tool. Arrays containing attached drug may bind to heparan binding receptors on the cell surface thereby delivering them to the cell. cDNA constructs coding for small peptides that will be substituted with heparan sulfate chains could be prepared. Such genes could be transferred to cells in vivo, resulting in cells that secrete therapeutic amounts of multi-valent heparan sulfate molecules. Another potential use of arrays of various compositions is for screening libraries for heparin-binding molecules.

It is possible that the molecules of the present invention will have anti-coagulant effects in vivo. If so, they can be fractionated and fractions analyzed for their anti-coagulant effect. The anti-coagulant effects of heparin and heparan sulfate are mediated by a very specific carbohydrate sequence, therefore it is likely that other biological effects of heparin will not be lost by removing the anti-coagulant fraction. The effects of heparin constructs on normal cells are unknown, but could possibly be detrimental.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.
One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.
WHAT IS CLAIMED IS:

1. A pharmaceutical compound, wherein said compound is a synthetic molecule, consisting of a charged unit covalently linked to a moiety.

2. The pharmaceutical compound of claim 1, wherein said synthetic molecule is a synthetic proteoglycan.

3. The pharmaceutical compound of claim 1, wherein said charged unit is a glycosaminoglycan chain(s).

4. The pharmaceutical compound of claim 3, wherein said glycosaminoglycan is selected from the group consisting of heparin, heparin sulfate, chondroitin sulfate, dermatin sulfate, keratin sulfate and dextran sulfate.

5. The pharmaceutical compound of claim 1, wherein said moiety is either natural or synthetic.

6. The pharmaceutical compound of claim 5, wherein said moiety is selected from the group consisting of avidin, albumin, β2-microglobulin, a peptide and a lipid.

7. The pharmaceutical compound of claim 6, wherein said albumin is human serum albumin.
8. A method of producing the pharmaceutical compound of claim 1, comprising the step of:
linking said charged unit to said moiety using carbodiimide condensation.

9. A method of producing the pharmaceutical compound of claim 1, comprising the steps of:
biotinylating said charged unit; and
linking said biotinylated charged unit to avidin.

10. A method of inhibiting tumor growth, comprising the step of:
exposing said tumor to the pharmaceutical compound of claim 1.

11. The method of claim 10, wherein said tumor is a myeloma or a tumor of hematopoietic origin.

12. The method of claim 10, wherein said tumor is a solid tumor.

13. A method of killing a cancer cell, comprising the step of:
exposing said cancer cell to the pharmaceutical compound of claim 1.

14. The method of claim 13, wherein said cancer cell is a myeloma cell or a tumor cell of hematopoietic origin.
15. The method of claim 13, wherein said cancer cell is a solid tumor cell.

16. A DNA molecule coding for the pharmaceutical compound of claim 1.

17. A method of treating a cancer cell, comprising the step of:
   transducing said cell with the DNA molecule of claim 16.

18. The method of claim 17, wherein said cancer is selected from the group consisting of breast cancer, prostate cancer, lung cancer and a cancer of hematopoietic origin.

19. A method of treating a cancer cell, comprising the step of:
   lipofecting said cell with the DNA molecule of claim 16.

20. The method of claim 19, wherein said cancer is selected from the group consisting of breast cancer, prostate cancer, lung cancer and a cancer of hematopoietic origin.
HSA:GAG nPG

Cell Viability (ARH)

FIG. 1A
Avidin: Biotinylated Heparin nPG

Cell Viability (ARH)

FIG. 2A
Avidin:Biotinylated Heparin nPG

Cell Viability (ARP)

FIG. 2B
INTERNATIONAL SEARCH REPORT

INTERNATIONAL APPLICATION NO. PCT/US00/00338

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/1, 2, 44, 54; 530/395; 435/69.1, 320.1, 377

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<tr>
<th>Category</th>
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<th>Relevant to claim No.</th>
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<tr>
<td>X</td>
<td>US 5,464,942 A (SAKURAI et. al.) 07 November 1995 (07/11/95), see entire document.</td>
<td>1-6, 8, 10</td>
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<td></td>
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<td>7, 11, 12</td>
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<td>Y</td>
<td>MALI et al. Suppression of tumor cell growth by syndecan-1 ectodomain. J. Biol. Chem. 11 November 1994, Vol. 269, No. 45, pages 27795-27798, see entire document.</td>
<td>1-6-16-20</td>
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<tr>
<td>X</td>
<td>DHODAPKAR et al. Syndecan-1 is a multifunctional regulator of myeloma pathobiology: control of tumor cell survival, growth, and bone cell differentiation. Blood. 15 April 1998, Vol. 91, No. 8, pages 2679-2688, see entire document, especially abstract.</td>
<td>1-6, 10, 11, 13, 14</td>
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Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search 08 APRIL 2000
Date of mailing of the international search report 08 MAY 2000

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks
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PETER BRUNOVSKIS, PH.D.

Telephone No. (703) 308-0196
<table>
<thead>
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<td>X</td>
<td>WO 95/34316 A1 (ORION CORPORATION) 21 December 1995 (21/12/95), see entire document.</td>
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<td>X</td>
<td>EP 0 466 966 A1 (SEIKAGAKU CORPORATION) 22 January 1992 (22/01/92), see entire document.</td>
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**INTERNATIONAL SEARCH REPORT**

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. **X** As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

□ The additional search fees were accompanied by the applicant's protest.

**X** No protest accompanied the payment of additional search fees.
A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):
A61K 31/726, 31/727, 31/737, 31/738, 38/02, 38/38, 48/00; C12N 15/63, 15/85; 15/88

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :
514/1, 2, 44, 54; 530/395; 435/69.1, 320.1, 377; 536/23.1, 23.5

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):
STN, MEDLINE, CAPLUS, BIOSIS, EMBASE, SCISEARCH, WEST, USPT
search terms: neoproteoglycan, glycosaminoglycan, synthetic, heparin sulfate, chondroitin sulfate, 
dermatin sulfate, keratin sulfate, dextran sulfate, avidin, albumin, lipid, carbodiimide, condensation, proteoglycan, ectodomain, syndecan, 
decorin, cancer, tumor, gene therapy, apoptosis, killing, treatment

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single 
inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees 
must be paid.

Group I, claims 1-7, drawn to a pharmaceutical compound.
Group II, claim 8, drawn to a method of producing a pharmaceutical compound.
Group III, claim 9, drawn to a method of producing a biotinylated pharmaceutical compound.
Group IV, claims 10-15, drawn to methods for treating cancer cells with a pharmaceutical compound.
Group V, claim 16, drawn to a nucleic acid encoding a pharmaceutical product.
Group VI, claims 17-20, drawn to methods for treating cancer cells with a nucleic acid encoding a pharmaceutical 
product.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under 
PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: There is no 
special technical feature in the first invention of independent claim 1 shared by the inventions of Groups II-VI, 
considered as a whole, which defines a contribution over the prior art. Claim 1 is broadly drawn to a pharmaceutical 
compound, wherein said compound is a synthetic molecule, consisting of a charged unit covalently linked to a moiety. 
Sakurai et al. (US 5,464,942; see attached) discloses a pharmaceutical compound comprising a charged unit, 
glycosaminoglycan, linked to a lipid moiety (see abstract). Thus there is no special technical feature shared by any of 
the groups that depend on an invention clearly anticipated by the prior art. Consequently, the claimed invention is drawn 
to a set of independent, patentably distinct inventions (as described above) which lack any special technical feature over 
the prior art. These inventions represent multiple products, multiple distinct methods of making them, and multiple 
distinct methods of using them. It is further noted that claims 16-20 improperly recite a DNA molecule coding for the 
pharmaceutical compound of claim 1 to which it can not properly depend on, since claim 1 does not recite any protein.