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(54) Title: PREBLOCKING WITH NON-HA GAGS INCREASES EFFECTIVENESS OF HA CONJUGATED ANTICANCER AGENTS

(57) Abstract: A cell-targeted polymeric drug delivery system was designed based on the specific interaction between hyaluronic acid (HA) and its cell surface receptors over-expressed on cancer cell surface. Compounds composed of a carrier molecule, wherein the carrier molecule contains at least one residue of an anti-cancer agent and at least one residue of a hyaluronic acid, are described. Also described are methods comprising pre-administering a non-HA GAG blocking agent before administering the HA conjugate. Also described are methods of making and using the compounds thereof.

PREBLOCKING WITH NON-HA GAGS INCREASES EFFECTIVENESS OF HA CONJUGATED ANTICANCER AGENTS

I. CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation in part of international application PCT/US02/14402 filed May 6, 2002, and claims the benefit of provisional application Serial No. 60/378,529, filed on May 6, 2002, which applications are both herein incorporated by reference in their entirety.

II. ACKNOWLEDGEMENTS

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III.BACKGROUND OF THE INVENTION

A major challenge in cancer therapy is to selectively deliver small molecule anti-cancer agents to tumor cells. One of the most promising methods involves the combination or covalent attachment of the cytotoxin with a macromolecular carrier [1]. Many kinds of drug carriers, including soluble synthetic and natural polymers [2], liposomes [3], microspheres [4], and nanospheres [5,6] have been employed to increase drug concentration in target cells. By altering the pharmacokinetic distribution of drugs, a sustained therapeutic concentration can be maintained at tolerable doses. Water-soluble polymer-anti-cancer drug conjugates seem to offer great potential because they can traverse compartmental barriers in the body [7] and therefore gain access to a greater number of cell-types. A variety of water-soluble polymers, such as human serum albumin (HSA) [2], dextran [8], lectins [9], poly(ethylene glycol) (PEG) [10], poly(styrene-co-maleic anhydride) (SMA) [11], poly(*N*-hydroxylpropylmethacrylamide) (HPMA) [12], and poly(divinylether-co-maleic anhydride) (DIVEMA) [13] have been used to prepare polymeric anti-cancer prodrugs for cancer treatment. Such drug-polymer conjugates have demonstrated good solubility in water, increased half-life in the body, and high anti-tumor effects. For example, poly (styrene-co-maleic acid)-neocarzinostain conjugate (SMANCS) was approved for the treatment of liver cancer in Japan [11,14]. The linking of doxorubicin to HPMA (HPMA-DOX) gives a new prodrug with improved *in vitro* tumor retention, a higher therapeutic

ratio, and avoidance of multi-drug resistance [12]. This system has passed the Phase I clinical trial and is currently in Phase II trials against ovarian cancer [15]. The conjugate of HPMA copolymer-camptothecin was also pre-clinically evaluated and is now in Phase I [16,17].

Anti-cancer polymer-drug conjugates can be divided into two targeting modalities: passive and active. The biological activity of the passive targeting drug delivery systems is based on the anatomical characteristics of tumor tissue, and allows polymeric prodrugs to more easily permeate tumor tissues and accumulate over time. This is one of the chief reasons for the success of polymeric drugs, it is often referred to as the enhanced permeability and retention (EPR) effect. Maeda proved that macromolecules can accumulate more efficiently in solid tumors than free drugs [11]. Active targeting drug delivery systems can be achieved using specific interactions between receptors on the cell surface and the introduction of targeting moieties conjugated to the polymer backbone. In this way, active therapeutic agents conjugated to polymers can be selectively transported to tumor tissues. The active approach therefore takes advantage of the EPR effect, but further increases therapeutic index through receptor-mediated uptake by target cancer cells. Previous studies showed that *N*-acylated galactosamine [18] and monoclonal antibodiy fragments [19] were valuable targeting moieties for HPMA-DOX conjugates, selectively increasing the cytotoxicity of the polymer-drug conjugates to tumor cells.

Hyaluronic acid (HA, also known as hyaluronan, Figure 1), a linear polysaccharide of alternating *D*-glucuronic acid (GlcUA) and *N*-acetyl-D-glucosamine (GlcNAc) units, is present in the extracellular matrix, the synovial fluid of joints, and the scaffolding that comprises cartilage [20]. It is an immunoneutral building block for preparing biocompatible and biodegradable biomaterials [21-25], and has been employed as both a vehicle and an angiostatic agent in cancer therapy [26-28]. Mitomycin C and epirubicin were coupled to HA by carbodiimide chemistry and the HA-mitomycin adduct was selectively toxic to a lung carcinoma xenograft [29]. Recently, the use of mild hydrazide chemistry to prepare an HA-Taxol® bioconjugate [30,31] has been described, which showed good selectivity in cell culture studies. It is evident that directly correlates uptake with cytotoxicity using a fluorescently-labeled HA-Taxol® derivative, and it was demonstrated that toxicity is due to hydrolytic release of the parent drug.

HA serves a variety of functions within the extracellular matrix, including direct receptor-mediated effects on cell behavior. These effects occur *via* intracellular signaling pathways in which HA binds to, and is internalized by, cell surface receptors. Several cell

membrane-localized receptors (HA binding proteins) have been identified including: CD44, RHAMM, IVd4, and the liver endothelial cell clearance receptor [32-35]. HA-protein interactions play crucial roles in cell adhesion, growth and migration [36-38], and HA acts as a signaling molecule in cell motility, inflammation, wound healing, and cancer metastasis [39]. The structure and regulation of HA receptors [40] is a growing area of structural and cellular biology that is critical to understanding how HA-protein interactions enhance metastasis.

Most malignant solid tumors contain elevated levels of HA [41], and these high levels of HA production provide a matrix that facilitates invasion [42]. Clinically, high HA levels correlate with poor differentiation and decreased survival rate in some human carcinomas. HA is an important signal for activating kinase pathways [43,44] and regulating angiogenesis in tumors [45]. HA internalization is mediated *via* matrix receptors, including CD44, which is a transmembrane receptor that can communicate cell-matrix interactions into cells and can alter the matrix in response to intracellular signals. The pathological enrichment of HA in tumor tissues suggests that manipulation of the interactions between HA and its receptors could lead to dramatic inhibition of growth or metastasis of several types of tumor. Antibodies to CD44, soluble forms of CD44 or RHAMM, HAse, and oligomers of HA have all been used effectively to inhibit tumor growth or metastasis in animal models.

In addition to elevated HA in the environment surrounding tumors, most malignant cell-types overexpress CD44 and RHAMM. As a result, malignant cells with the highest metastatic potential often show enhanced binding and internalization of HA [46]. Apparently, such cells can effectively breach the tumor-associated HA barrier by binding, internalizing, and degrading this glycosaminoglycan. Cell culture experiments suggest that CD44-HA interactions occur *in vivo* and are likely to be responsible for retention of HA-enriched matrices. Thus, HA can bind to the cell surface *via* interactions with CD44, and a portion subsequently undergoes endocytosis. In addition, internalization of ^{3}H-labeled HA revealed that intracellular degradation of HA occurs within a low pH environment, such as that of lysosome. Targeting of anti-cancer agents to tumor cells and tumor metastases can be accomplished by receptor-mediated uptake of bioconjugates of anti-cancer agents conjugated to HA [29-31], followed by the release of free drugs through the degradation of HA in cell compartments. Isoforms of HA receptors, CD44 and RHAMM are over-expressed in transformed human breast epithelial cells [47], human ovarian tumor cells [48], and other cancers [49,50].

Targeting of drug delivery, such as chemotherapy, aims to increase the

concentration of drugs in specific tissues such as tumor sites, and to reduce the drug distribution in other tissues, such as the normal organs. Effective targeting can enhance the therapeutic effect and minimize the toxicity of delivered therapeutics. Several approaches have been utilized for this purpose. For example, the antibodies against erbB2, vesicular endothelium growth factor receptor or transferrin have been used to conjugate molecules, such as anti-tumor agents, to target the molecules to cells and tissue expressing the cognate receptors. In addition, anti-tumor agents have also been conjugated with biopolymers or lipid to provide a longer circulation time, controlled release and high retention in specific target tissues, such as tumor sites, because the permeability of vessels is higher in tumor than in normal tissues.

Disclosed are methods, compositions, and compounds for increasing the delivery and specificity of Hyaluronic acid (HA) conjugated and containing molecules. For example, disclosed are methods for increasing the delivery and/or specificity of anti-cancer compositions and compounds comprising HA to tumors.

IV. SUMMARY OF THE INVENTION

As embodied and broadly described herein, the following, in one aspect, relates to compounds comprising an anti-cancer agent, a carrier molecule, and hyaluronic acid or a derivative thereof, wherein the anti-cancer agent, the carrier molecule, and the hyaluronic acid or a derivative thereof are attached to one another via a covalent bond. In another apsect, the following relates to compounds comprising hyaluronic acid and methods of delivery of these compounds related to a blocking step with non-HA GAGs, such as chondroitin sulfate. The following also relates to methods of making and using these compounds.

Additional advantages of the following will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the described. The advantages will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the described.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art.

It will be apparent to those skilled in the art that various modifications and variations can be made to the described embodiments without departing from the scope or spirit of that described. Other embodiments will be apparent to those skilled in the art from consideration of the specification and practices disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit being indicated by the following claims.

V. BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification together with the description serve to explain the principles described.

Figure 1 shows a tetrasaccharide fragment of HA with the repeating disaccharide units.

Figure 2 shows the possible attachments of the anti-cancer agent, the carrier molecule, and the hyaluronic acid or derivative thereof to one another.

Figure 3 shows a synthesis of HA-DOX conjugates.

Figure 4 shows a structure of HPMA-HA-DOX conjugates.

Figure 5 shows data for an *In vitro* cytotoxicity of HPMA-HA-DOX conjugates against HBL-100 human breast cancer cells. Cell viability of HBL-100 cells as function of DOX equivalent concentration. The cytotoxicity of polymer conjugates (targeted and non-targeted) were determined using MTT assay.

Figure 6 shows a binding of targeted HPMA-HA-DOX conjugate on human ovarian cancer SK-OV-3 cells surface, (a) transmission image; (b) fluorescence (50 μg/ml HA equivalent of HPMA-HA-DOX at 0°C for 2hr).

Figure 7 shows a time course of internalization of targeted HPMA-HA-DOX conjugates (50 μg/ml HA equivalent) on human ovarian cancer SK-OV-3 cells in comparison with non-targeted HPMA-DOX conjugate.

Figure 8 shows *in vitro* cytotoxicity of DOX, non-targeted HPMA-DOX conjugate, targeted HPMA-HA-DOX with 17% and 36% HA loading against human prostate cancer cell-line DU-145.

Figure 9 shows that HA-Taxol effectively reduce the growth of tumors in mice model. The 4T1 mouse breast cancer cells (10^6/site) were subcutaneously injected into either BABL/c mice and allowed to grow for 2 days for the tumor to be established. Then,

the mice bearing with tumor were randomly divided into three groups (5 mice/group) and i.p. injected with 0.4 ml of : 1) saline alone as vehicle control; 2) 4 mg/kg of Taxol; or 3)HA-Taxol containing Taxol equal to 4 mg/kg, respectively. The injection was carried out every other day for two to four weeks. The tumor sizes were measured twice a week. At the end of experiment, the mice were sacrificed and the tumor were harvested, photographed and weighted.

Figure 10 shows results with other tumor models. The human TSU bladder cancer cells were subcutaneously injected into flank of nude mice and the treatment procedures were similar to the experiment carried out with 4T1 tumor model. Results from the TSU tumor model were similar to that obtained from 4T1 tumor model showing that mice treated with HA-Taxol had slower tumor growth than those treated with vehicle or Taxol alone. These data suggest that the anti-tumor effect of HA-Taxol is reproducible and is universal, not particular to a tumor type.

Figure 11 shows that chondroitin sulfate reduces the organ up-take of HA and enhances the tumor up-take of HA. Figure 3A shows the difference in the up-take of HA between the tumors and major organs (liver, lung, heart, brain, spleen, kidney and muscle), the mice bearing tumors were intravenously injected with 0.2 ml of biosynthesized ^{3}H-HA (4×10^{5}cpm/μg HA/ml) and sacrificed 24 hours later. The tumors and organs were collected, weighted, and homogenized with ultrasound to make tissue homogenates. The protein concentration was normalized to 0.1 mg/ml. Then, 300 μl of tissue homogenates were mixed with 2.5 ml of scintillation solution and counted for the radioactivity. The result showed that the tumor, liver, spleen and kidney had the high up-take of HA. Figure 3B shows two groups (5 mice/group) and intravenously injected with 0.3 ml of saline (as control) or chondroitin sulfate (100 mg/ml) to block the binding sites of HA in the major organs. Two hours later, 0.2 ml of biosynthesized ^{3}H-HA (4×10^{5}cpm/μg HA/ml) was intravenously injected into mice. Twenty-four hours later, the mice were sacrificed and the homogenates of tumors and organs were counted for the radioactivity. The results indicated that the pretreatment with chondroitin sulfate blocked the HA binding sites in major organ and reduce their up-take of HA, but the tumors accumulated high level of HA. Figure 3 C shows that the ^{3}H-HA in tumor to major organs in mice pretreatment with chondroitin sulfate was higher than that of untreated mice. Figure 3D shows the results of monitoring the liver for HA up-take for two days the amount of ^{3}H-HA in the liver of mice treated with chondroitin sulfate was much lower than that in mice treated with vehicle alone. Figure 3E

shows the ratio of tumor to liver uptake for treatment of animals with or without a pretreatment of chondroitin sulfate.

Figure 12 shows that the pre-treatment with chondroitin sulfate enhances the therapeutic effect of HA-Taxol. Mice bearing 4T1 tumors received i.p injections of 0.4 ml of either saline (as control) or chondroitin sulfate (100 mg/ml) followed by HA-Taxol (8 mg/ml) two hours later. This procedure was carried out every other day for 20 days, and mice received a total of ten injections. The mice were recorded for their survival days and the survival rate was calculated.

Figure 13 shows HA conjugated uptake of TSU and 4T1 cells.

Figure 14 shows the interaction of HA with tumor cells CD44, including expression of DC44 in 4 T1 cells, binding to H-HA, and degradation of H-HA. Also shown is that the CD44 mediated degradation of H-HA could be inhibited by excess cold HA, anti-CD44, neutralization antibody (KM201) and lysosomal inhibitor chloroquinone.

Figure 15 shows the *in vivo* distribution of HA. Both tumor and lymph nodes contain the highest amount of HA as compared to other organs.

Figure 16 shows 4T1 primary tumor and lymph node metastases. Both the popliteal lymph node and inguinal lymph node metastases are shown.

Figure 17 shows the results from pathohistological analysis, showing that while the lymph nodes from the control group had spontaneous metastases, there was no tumor cells detected in the HA-Taxol treated group.

VI. DETAILED DESCRIPTION

Reference to the following detailed description of embodiments and the Examples included therein and to the Figures and their previous and following description will allow for better understanding.

Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that specific synthetic methods, specific compositions, or to particular formulations, as such may, of course, vary, and are not limited by their description. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used in the specification and the appended claims, the singular forms "a," "an"

and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a pharmaceutical carrier" includes mixtures of two or more such carriers, and the like.

Ranges can be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that when a value is disclosed that "less than or equal to" the value, "greater than or equal to the value" and possible ranges between values are also disclosed, as appropriately understood by the skilled artisan. For example, if the value "10" is disclosed the "less than or equal to 10"as well as "greater than or equal to 10" is also disclosed. It is also understood that the throughout the application, data is provided in a number of different formats, and that this data, represents endpoints and starting points, and ranges for any combination of the data points. For example, if a particular data point "10" and a particular data point 15 are disclosed, it is understood that greater than, greater than or equal to, less than, less than or equal to, and equal to 10 and 15 are considered disclosed as well as between 10 and 15.

In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not.

Reference will now be made in detail to the present preferred embodiments, examples of which are illustrated in the accompanying drawings. Wherever possible, the same reference numbers are used throughout the drawings to refer to the same or like parts.

Disclosed are the components to be used to prepare the disclosed compositions as well as the compositions themselves to be used within the methods disclosed herein. These

and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a particular blocking agent is disclosed and discussed and a number of modifications that can be made to a number of molecules including the blocking agent are discussed, specifically contemplated is each and every combination and permutation of blocking agent and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods.

A. Compounds

Free anti-cancer agents typically enter cells via passive, or non-energy-requiring, mechanisms. This can lead to loss of drug efficacy as a result of the action of the evolution of the multidrug resistance gene (MDR) due to the P-glycoprotein product, which pumps free drugs out of the cell. Polymeric drugs enter cells by pinocytosis or endocytosis rather than membrane fusion, and polymeric drugs are less susceptible to inducing MDR. Polymeric drugs also exhibit enhanced permeability and retention (EPR), e.g., the leaky vasculature of tumors allows macromolecular drugs to "concentrate" in the tumor tissues. The EPR effect improves targeting to malignant cells over normal cells; however, the macromolecular drugs have reduced overall cytotoxicity to all cells relative to the free drug. Thus, polymeric (macromolecular) drugs have reduced systemic side effects relative to the free drug. Furthermore, the cytotoxicity to cancer cells can be enhanced, without increasing toxicity to normal cells, by using a targeting agent, e.g., an antibody to a tumor antigen. These compounds possess these attributes, increasing the delivery of anticancer agents. In

addition the disclosed compositions enhances both the targeting to a specific cell as well as the uptake by the targeted cancer cells relative to other targeting strategies for small molecule or macromolecular anticancer drugs.

Disclosed are compounds that can be used, for example, in anti-cancer therapies. These compounds typically increase or alter the targeted delivery of anticancer compounds or other therapeutic compounds. Typically these compounds will comprise an anti-cancer agent, some other type of carrier molecule, and a molecule, such as HA. Disclosed are methods that use these and other compounds, wherein the methods comprise a preblocking step of administering to the subject, a non-HA molecule to aid in preventing non-specific HA interactions and uptake.

Disclosed are compounds comprising an anti-cancer agent, a carrier molecule, and hyaluronic acid or a derivative thereof, wherein the anti-cancer agent, the carrier molecule, and the hyaluronic acid or a derivative thereof are attached to one another via a covalent bond.

There are a number of different ways the anti-cancer agent, the carrier molecule, and the hyaluronic acid or derivative thereof can be attached to one another by a covalent bond. A non-limiting set of exemplary linkages are depicted in Figure 2. In Figure 2, X is the tethered moiety of the anti-cancer agent , Y is the tethered moiety of the carrier molecule, and Z is the tethered moiety of hyaluronic acid or the derivative thereof. A "tethered moiety "can be any portion of a starting molecule that becomes a portion of a molecule produced in a reaction with the starting molecule. For example, hyaluronic acid could be depicted as Z-COOH. If Z-COOH was reacted with another molecule, such as A, and the product formed from this reaction was Z-A, then Z would be considered a tethered moiety . Likewise, if a subpart of Z was considered Z' and the reaction of Z-COOH and A produced Z'-A, then Z' would also be considered a tethered moiety . When Z-COOH reacts with a dihydrazide to produce a derivative of hyaluronic acid, Z remains the same and is part of the derivatized hyaluronic acid. In other words, Z is the tethered moiety of the original hyaluronic acid. In one embodiment, the anti-cancer agent, the carrier molecule, and the hyaluronic acid or derivative thereof can be directly attached to one another. For example, the anti-cancer agent and/or hyaluronic acid or derivative thereof are directly attached to the carrier molecule via a covalent bond (Figures 2(a) and (b), respectively). In another embodiment, the anti-cancer agent is directly attached to the carrier molecule via a covalent bond, and hyaluronic acid or derivative thereof is directly attached to the anti-cancer agent

residue. Alternatively, hyaluronic acid or a derivative thereof is directly attached to the carrier molecule via a covalent bond, and the anti-cancer agent is directly attached to the hyaluronic acid or derivative thereof. These embodiments are depicted in Figures 2(c) and (d), respectively.

In another embodiment, the anti-cancer agent, carrier molecule, and the hyaluronic acid or a derivative thereof can be indirectly attached to one another by a linker. These embodiments are depicted in Figures 2(e)-(j). For example, in Figure 2(e), the anti-cancer agent is indirectly attached to the carrier molecule by a linker (L denotes the residue of the linker), wherein the anti-cancer agent and the carrier molecule are individually and directly attached to the linker via a covalent bond. Examples of linkers include, but are not limited to, succinates, disulfide-containing compounds, and diol-containing compounds. The linkers may also include short peptides with specific targeting sequences for lysosomes and for lysosomal degradation, such as Gly-Phe-Leu-Gly. Other examples include, for prostate cancer, linkages targeted to prostate cells and to a prostate-specific antigen (PSA), which has sequence-specific proteolytic capabilities. In this example, PSA hydrolyzes His-Ser-Ser-Lys-Leu-Gln and glutaryl-4-hydroxyprolyl-Ala-Ser-cyclohexaglycyl-Gln-Ser-Leu.

The linkers are typically cleavable so that the anti-cancer agent can be released, for example, under reducing conditions, oxidizing conditions, or by hydrolysis of an ester, amide, hydrazide, or similar linkage forms the covalent bond between the linker and the anti-cancer agent. Additionally, the type of linker may augment the selective cytotoxicity (and thus improve the therapeutic index) aspect by permitting selective release of the anti-cancer agent inside the cells targeted by the targeting moiety (carrier molecule or HA).

Also contemplated is further attaching an anti-cancer agent to hyaluronic acid or a derivative thereof that is indirectly attached to the carrier molecule via a linker. Additionally, it is possible to attach hyaluronic acid or a derivative thereof to an anti-cancer agent that is indirectly attached to the carrier molecule via a linker. These embodiments are depicted in Figures 2(g) and (h), respectively.

In another embodiment, the anti-cancer agent and hyaluronic acid or a derivative thereof can be attached to one another via a linker molecule. These embodiments are depicted in Figures 2(i) and (j). In Figures 2(i) and (j), the anti-cancer agent and the hyaluronic acid or derivative thereof, respectively, are directly attached to the carrier molecule.

The anti-cancer agent, the carrier molecule, and the hyaluronic acid or derivatives thereof used to produce the compounds are discussed below. Disclosed herein are methods wherein the HA-anticancer agent-carrier molecule are administered after the or concurrently or before the addition of a non-HA GAG or derivative, which can act as a blocking agent for non-specific HA interactions.

1. Non-HA GAG and Derivatives Thereof

There are many different receptors for HA and their derivatives. Some receptors are specific for HA and other have less specificity for HA than for other glycosaminoglycans (GAGs). Disclosed herein, a preblocking step with GAGs other than HA can aid in the specific uptake of small molecule cancer agents, such as taxol or doxirubicin delivery when attached to HA.

Exemplary non-HA preblocking agents can be CS-A (chondroitin 4-sulfate) and CS-C (chondroitin 6-suflate), heparin, heparin sulfate, dextran sulfate, keratan, or keratan sulfate.

Preadministration of the non-HA GAG, such as CS, by oral or iv dosing to achieve an adequate serum level (approximately 5 to 500 ug/ml) can protect non-targeted organs, especially the liver.

The non-HA agents can be added prior to the therapeutic composition, concurrently with the therapeutic composition, or after the therapeutic composition. It is understood that their effectiveness can vary depending on the amount of time that the blocking step can occur. For example, if the blocking step occurs before the addition of the HA therapeutic composition, then more effective blocking can occur, however, as the therapeutic reagent is taken up over time, some benefit of blocking can be achieved even if the blocking agent is added after the administration of the therapeutic composition.

2. Anti-cancer Agents

Any anti-cancer agent can be directly or indirectly attached to the carrier molecule and the hyaluronic acid or derivatives to be aided in transport across the cellular membranes. There are many anti-cancer agents known in the art. In one embodiment, the anti-cancer agent is any small molecule that targets intracellular function, such as protein kinase inhibitors including but not limited to Gleevac. In another embodiment, radionuclides including, but not limited to, I-131, Y-90. In-111, Tc-99m can be used. In another embodiment, Gd+3 compounds can be used. In yet another embodiment, meso e-

chlorin and cis-platin derivatives can be used as the anti-cancer agent. A partial list of anti-cancer agents that can be used with the disclosed compositions can be found in, for example, United States Patent No. 5,037,883, which is herein incorporated by reference as well as any publications and patents, or patent applications, cited therein which contain anti-cancer agents. Other anti-cancer agents, such as, cytotoxic agent, a chemotherapeutic agent, a cytokine, antitubulin agents, and a radioactive isotope, can also be used in the disclosed compounds. Anticancer agents, such as, vincristine, vinblastine, vinorelbine, and vindesine, calicheamicin, QFA, BCNU, streptozoicin, and 5-fluorouracil, neomycin, podophyllotoxin(s), TNF-alpha, .alpha$_v$beta$_3$ colchicine, taxol, , a combretastatin antagonists, calcium ionophores, calcium-flux inducing agents, and any derivative or prodrug thereof can also be used herein. United States patent Nos. 6,348,209, 6,346,349, and 6,342,221 are also disclosed for agents related to anti-cancer compounds. In certain embodiments, the anti-cancer agent comprises 5-fluorouracil, 9-aminocamptothecin, or amine-modified geldanomycin. In another embodiment, the anti-cancer agent is doxorubicin. In yet another embodiment the anticancer agent can be Taxol®. However, anti cancer agents, such as the anti-growth factor receptor antibodies (e.g., Herceptin), are understood to not typically have a need for transport across a cell membrane, and therefore, would typically be used in combination with the disclosed compounds and compositions.

3. Carrier Molecules

Any carrier molecule can be used. Typically carrier molecules will be polymer molecules. Typically the carrier molecule is a large macromolecule of at least 5,000 daltons. The carrier molecule can range from 2,000 daltons to 25,000 daltons, or from 25,000 daltons to 100,000 daltons, or from 100,000 daltons to 1,000,000 daltons. It is preferred that the carrier molecule be in the range of 10,000 to 25,000 daltons. The carrier molecule typically aids in the transport of anti-cancer agent across the cell membrane. Thus, when the anti-cancer agent is directly or indirectly attached to the carrier molecule it typically crosses a cell membrane better than the anti-cancer agent alone. There are numerous carriers and macromolecular carriers known in the art that will function as the carrier molecule. Examples of carrier molecules are also described in, for example, United States Patent Nos: 5,415,864 for "Colonic-targeted oral drug-dosage forms based on crosslinked hydrogels containing azobonds and exhibiting PH-dependent swelling;" 5,258,453 for "Drug delivery system for the simultaneous delivery of drugs activatable by enzymes and light;" 5,037,883 for "Synthetic polymeric drugs;" 4,074,039 for "Hydrophilic

N,N-diethyl acrylamide copolymers;" 4,062,831 for "Copolymers based on N-substituted acrylamides, N-substituted methacrylamides and N,N-disubstituted acrylamides and the method of their manufacturing;" 3,997,660 for "Soluble hydrophilic polymers and process for producing the same;" 3,931,123 for "Hydrophilic nitrite copolymers;" and 3,931,111 for "Soluble hydrophilic polymers and process for processing the same" each of which is individually and specifically herein incorporated by reference at least for material related to carriers. It is understood that in certain embodiments, the carrier does not include HA or derivatives thereof.

In one embodiment, the carrier molecule comprises a polymer produced by the polymerization of an ethylenically unsaturated monomer. Examples of monomers include, but are not limited to, acrylates and methacrylates. In one embodiment, the carrier molecule is a polymer produced from the polymerization of *N*-(2-hydroxypropoyl)methacrylamide, which is referred to herein as HPMA.

4. Hyaluronic Acid and Derivatives Thereof

There are many uses and derivatives of Hyaluronic acid (HA), which is a macromolecule. HA and derivatives of HA are conjugated to molecules, such as anticancer agents. HA, derivatives of HA, their uses and synthesis are disclosed in, for example, see United States Patent Nos: 6,096,727 for "Method for treating wounds using modified hyaluronic acid crosslinked with biscarbodiimide," 6,013,679 for "Water-insoluble derivatives of hyaluronic acid and their methods of preparation and use," 5,874,417 for "Functionalized derivatives of hyaluronic acid," 5,652,347 "Method for making functionalized derivatives of hyaluronic acid," 5,616,568 "Functionalized derivatives of hyaluronic acid" 5,502,081 "Water-insoluble derivatives of hyaluronic acid and their methods of preparation and use, " as well as United States Provisional Application Nos. 60/116,021 and 60/218,725, all of which are herein incorporated by reference as well as the publications, patents, and patent applications cited therein at least for material related to hyaluronic acids. The hyaluronic acid is modified with a dihydrazide compound such as adipic dihydrazide.

Hyaluronic acid is a polysaccharide of at least 4 disaccharide repeat units of HA, e.g., at least 1,000 daltons. HA and derivatives thereof can range from 1,000 daltons to 10,000 daltons, or from 10,000 daltons to 100,000 daltons, or from 100,000 daltons to 1,000,000 daltons. It is preferred that HA and its derivatives be at least 1,000 daltons. In one embodiment, the lower limit of the molecular weight is, 1000, 2000, 3000, 4000, 5000,

6000, 7000, 8000, 9000, or 10,000, and the upper limit is 100,000, 200,000, 300,000, 400,000, 500,000, 600,000, 700,000, 800,000, 900,000, or 1,000,000, where any lower limit can be combined with any upper limit. Hyaluronic acid typically aids in the transport of the anti-cancer agent across the cell membranes through an active mode of transport.

Hyaluronic acid typically aids in the transport of the anti-cancer agent across the cell membranes through an active mode of transport.

HA is a linear polysaccharide with alternating repeats of D-glucuronic acid and N-acetyl-D-glucosamine. The conjugation of small anti-tumor drug with HA will avoid the quick clearness by kidney and confer a long circulating time to new derivative. CD44, one of hyaluronan (HA) surface receptor, is highly expressed in variety of tumors which will facilitate the taking up of HA-drugs. Furthermore, once HA enters the peri-tissue, it would re-enter only the lymph path and be highly concentrated in lymph nodes, which are the most important sites for targeting metastatic tumors.

5. Efficiency and Specificity of Uptake by the Cells

The disclosed compounds can be characterized in that they allow for the uptake of anti-cancer agents by cells using typically different mechanisms than used by the anti-cancer agent alone. This efficiency can be measured in a number of ways. There are many ways to determine whether the efficiency and/or specificity of the uptake is increased by hyaluronic acid and/or the carrier molecule. For example, one can block the HA mediated transport and look at the change in saturation of the cells. One can do this by performing the assays with saturating HA present, using HA specific antibodies which block the HA function, using cells without HA receptors, and using cells that over express HA receptors like cancer cells. Typical increases of efficiency and/or specificity can be greater than or equal to at least 2 fold, 5 fold, 10 fold, 25 fold, 50 fold, 100 fold, 500 fold, 1000, fold 5000 fold or 10,000 fold.

The compounds have greater specificity for uptake and retention in the targeted cancer cells. This increased specificity is consistent with the specific hyaluronic acid receptors which import hyaluronic acid into cells. Typically disclosed compounds have a 5 to 100 fold greater specificity than either the anti-cancer-carrier molecule or anti-cancer-hyaluronic acid systems. This specificity can be assayed in a number of ways. For example, the intrinsic fluorescence of the anti-cancer agent doxorubicin may be observed directly by fluorescence microscopy in anti-cancer agent-carrier molecule systems and the

disclosed compounds. The presence of hyaluronic acid in the disclosed compounds results in increases of 5 to 50 fold of the anti-cancer agent present inside prostate, ovarian, colon, or breast cells as well as other cells, for example, (among others). melanoma, bladder, lung, and gastrointestinal tumors. have also been described .

B. Method of Making Compounds

The compounds can be prepared using techniques known in the art. As described, there are three components used to produce the compounds: the anti-cancer agent, the carrier molecule, and hyaluronic acid or a derivative thereof. Any of the components previously described can be reacted with one another in any possible combination to produce the compounds. Also contemplated is the use of two or more anti-cancer agents, carrier molecules, or hyaluronic acid or its derivatives thereof when producing the compounds. In addition, it is sometimes preferred to couple (*i.e.*, react) two of the three components together to produce a new reaction product or intermediate, then chemically connect the intermediate with the third component. For example, the anti-cancer agent can react with the carrier molecule to produce an anti-cancer/carrier molecule. Similarly, the anti-cancer agent can react with hyaluronic acid or a derivative thereof to produce an anti-cancer/hyaluronic acid molecule, and hyaluronic acid or a derivative thereof can react with the carrier molecule to produce a hyaluronic acid/ carrier molecule. Each of these intermediates can be reacted with an individual component (*e.g.*, the reaction of anti-cancer/hyaluronic acid molecule with carrier molecule) or, alternatively, each of the intermediates can react with one another to produce the compound (*e.g.*, reaction of anti-cancer/hyaluronic acid molecule with the anti-cancer/carrier molecule). In one embodiment, the compound can be produced by (1) reacting the anti-cancer agent with the carrier molecule to produce a carrier/anti-cancer molecule and (2) reacting the carrier/anti-cancer molecule with hyaluronic acid or the derivative thereof. For example, the carrier molecule HPMA is reacted with doxorubicin (DOX) to produce HPMA-DOX, then HPMA-DOX is reacted with hyaluronic acid modified with adipic dihydrazide to produce HPMA-DOX-HA. It should be noted that the reaction requires compatible reactive functionalities and generally includes a linker connecting the two tetherable moieties.

In another embodiment, the compound can be produced by (1) reacting the anti-cancer agent with hyaluronic acid or the derivative thereof to produce an anti-cancer/hyaluronic acid molecule; (2) reacting the anti-cancer agent with the carrier molecule to produce a carrier/anti-cancer molecule; and (3) reacting the anti-

cancer/hyaluronic acid molecule with the carrier molecule/anti-cancer molecule. For example, hyaluronic acid is reacted with doxorubicin to produce HA-DOX, then HA-DOX is subsequently reacted with HPMA-DOX to produce HA-DOX-HPMA.

As described above, the anti-cancer agent, carrier molecule, and hyaluronic acid can be attached to one another directly or indirectly via a linker. In addition, the attachment of each component to one another can vary depending upon the types of components selected and the order in which the components are permitted to react with one another.

Also contemplated is that two or more compounds can be produced simultaneously when the anti-cancer agent, the carrier molecule, and the hyaluronic acid or a derivative thereof are reacted with one another. Thus, it is possible to produce compositions or mixtures of compounds depending upon the type and amount of starting materials that are used. In one embodiment, the molecular weight of the carrier molecule and/or the hyaluronic acid or its derivatives will vary for each compound in the composition. In another embodiment, the attachment of the anti-cancer agent, carrier molecule, and hyaluronic acid or its derivatives to one another may vary from one compound to another in the composition. In another embodiment, the anti-cancer agent may be modified once it is attached to the carrier molecule or hyaluronic acid or its derivative thereof. Also contemplated is the formation of compositions composed of one or more compounds and free anti-cancer agent. For example, an excess of anti-cancer agent is used relative to the carrier molecule and/or the hyaluronic acid to produce these compositions.

An exemplary compound for the disclosed methods is an anti-tumor compound. Taxol 2-OH has been linked via a succinate ester to adipic dihydrazide (ADH)-modified HA. Once this HA-Taxol conjugate is internalized by tumor cells, the active form of Taxol could be hydrolytically released via the cleavage of the labile 2' ester linkage. The results of *in vitro* assays demonstrated that HA-Taxol selectively exerted toxicity toward several human cancer cell lines with no toxicity on a mouse fibroblast cell line.

C. Method of Using Compounds

The disclosed compounds can be used for targeted delivery of anti-cancer agents to cells. The disclosed methods also increase the efficiency and specificity of delivery of hyaluronic acid (HA) containing compounds and compositions. These compounds and compositions can be used thus, to treat a variety of disorders that require the delivery of anti-cancer or similar agents. It is understood that any of the compounds disclosed can be

used in this way. Those of skill in the art understand the compounds will be administered in pharmaceutically acceptable forms and in doses wherein delivery occurs. Typically the compounds would be administered to patients in need of delivery of the anti-cancer agent or a similar compound. It is understood that the goal is delivery of the compound and that through delivery affect the cells of the patient in need of the anti-cancer agent or similar agent.

Disclosed herein the conjugated anti-cancer agents can be given to a subject. Any subject in need of receiving an anti-cancer agent can be given the disclosed conjgated anti-cancer agents. The subject can, for example, be a mammal, such as a mouse, rat, rabbit hamster, dog, cat, pig, cow, sheep, goat, horse, or primate, such as monkey, gorilla, orangutan, chimpanzee, or human.

Disclosed herein the conjugated anti-cancer agents can used for inhibiting cancer cell proliferation. Inhibiting cancer cell proliferation means reducing or preventing cancer cell growth. Inhibitors can be determined by using a cancer cell assay. For example, either a cancer cell line can be cultured on 96-well plates in the presence or absence of the conjugated anti-cancer agent or anti-cancer agent alone or anti-cancer agent prepared differently then the disclosed compositions (for example, just anticancer agent and carrier) for any set period of time. The cells can then be assayed. In certain embodiments the conjugated anti-cancer compounds are those that will inhibit 10% or 15% or 20% or 25% or 30% or 35% or 40% or 45% or 50% or 55% or 60% or 65% or 70% or 75% or 80% or 85% or 90% or 95% of the cells growth relative to any of the controls as determined by the assay.

Disclosed are compositions which inhibit metastatic tumor formation in this type of assay disclosed herein, as well as compositions that reduce metastatic tumor formation by at least 10% or 15% or 20% or 25% or 30% or 35% or 40% or 45% or 50% or 55% or 60% or 65% or 70% or 75% or 80% or 85% or 90% or 95% of a control compound.

Disclosed herein the disclosed conjugated anti-cancer agents can be administered to cells and/or cancer cells which have HA receptors.

Disclosed herein is the *in vivo* effect of HA-Taxol. Two highly tumorigenic cancer cell lines, 4T1 mouse breast cancer and TSU human bladder cancer, were examined for their abilities to bind, internalize and degrade HA via their functional CD44. These cells were used to form tumor xenograft models in mice, then treated with HA-Taxol via i.v.

injection. To trace the tissue distribution of HA derivative, the biosynthetically labeled ^{3}H-HA was i.v. injected into mice bearing tumors and 24 hours later, the radioactivity of ^{3}H-HA in the homogenates from tumors and different organs were determined with β-counter. This tracing method was used to show that pre-injection of chondroitin sulfate (CS) could decrease the amount of HA derivative in liver and increase its level in tumors, which lead to a higher efficiency in treatment of tumors with HA-Taxol.

Disclosed are methods comprising administering a blocking agent prior to the addition of an HA conjugated molecule, such as an HA conjugated anti-cancer agent. The blocking agent can be administered to the organism concurrently, at least 10 minutes, 15 minutes, 20 minutes, 30 minutes, 45 minutes, 60 minutes, 90 minutes, 120 minutes, 150minutes, 180 minutes, 240 minutes, 300 minutes, or 360 minutes, 420 minutes, 10 hours, or 15 hours prior to the addition of the HA conjugated molecule. The blocking agent is added such that receptors capable of binding the blocking agent can bind the blocking agent. For example, preadministration of CS by oral or iv dosing to achieve an adequate serum level (approximately 5 to 500 ug/ml) can protect non-targeted organs, for example, the liver, kidney, or the lymph nodes, unless these are tumor containing tissues.

It is understood that the blocking agent can be added concurrently or even after the addition of the HA agent. This is arises because the administration of the HA does not occur immediately, and therefore, some benefit of adding a blocking agent can occur thus, even if the blocking agent has not been added prior to the addition of the HA agent. It is understood, however, that the longer the blocking agent is added after the addition of the HA agent, the less effective the blocking agent will be, however, even significant delay can have some effect as competition for the non-specific sites can occur, releasing bound or inactivated HA-agent. It certain embodiments the blocking agent is added within 1 hour, 2 hours, 4, hours, 8 hours, 12 hours, 24 hours, 2 days, 3 days, 4 days, or 7 days of adding the HA agent.

The disclosed compositions can be used to treat any disease where uncontrolled cellular proliferation occurs such as cancers. A non-limiting list of different types of cancers is as follows: lymphomas (Hodgkins and non-Hodgkins), leukemias, carcinomas, carcinomas of solid tissues, squamous cell carcinomas, adenocarcinomas, sarcomas, gliomas, high grade gliomas, blastomas, neuroblastomas, plasmacytomas, histiocytomas, melanomas, adenomas, hypoxic tumours, myelomas, AIDS-related lymphomas or sarcomas, metastatic cancers, or cancers in general.

A representative but non-limiting list of cancers that the disclosed compositions can be used to treat is the following: lymphoma, B cell lymphoma, T cell lymphoma, mycosis fungoides, Hodgkin's Disease, myeloid leukemia, bladder cancer, brain cancer, nervous system cancer, head and neck cancer, squamous cell carcinoma of head and neck, kidney cancer, lung cancers such as small cell lung cancer and non-small cell lung cancer, neuroblastoma/glioblastoma, ovarian cancer, pancreatic cancer, prostate cancer, skin cancer, liver cancer, melanoma, squamous cell carcinomas of the mouth, throat, larynx, and lung, colon cancer, cervical cancer, cervical carcinoma, breast cancer, and epithelial cancer, renal cancer, genitourinary cancer, pulmonary cancer, esophageal carcinoma, head and neck carcinoma, large bowel cancer, hematopoietic cancers; testicular cancer; colon and rectal cancers, prostatic cancer, or pancreatic cancer.

Compounds disclosed herein may also be used for the treatment of precancer conditions such as cervical and anal dysplasias, other dysplasias, severe dysplasias, hyperplasias, atypical hyperplasias, and neoplasias.

1. Metastatis

Metastasis, the leading death cause of breast cancer, mainly starts from lymph path. The surgical therapy can remove the primary tumor and some metastatic lymph nodes, however, in most cases, the lymphatic metastases are so spread out and so difficult to find that they can not be all removed. Currently, the residual lymphatic metastases of breast cancer are treated mainly by irradiation or/and chemotherapy. However, these two approaches have the disadvantages: 1) they target systemically or a board range of local tissues, not specific in the tumor sites; 2) they cause the adverse side effects, such as systemically impairing the body immunity and the regeneration of blood cells which results in life-threatening infections, and locally destroying the normal tissue structure that results in the permanent damages (such as scaring, arm/hand edema). Furthermore, even if these side effects can be overcome, the current therapy still can not control progression of breast cancer metastasis and the survival rate is not dramatically improved as compared to that of ten years ago.

Due to its large molecular weight, HA mainly enters the lymph path, and is taken up by endothelial cells which express high level of CD44, the native high affinity receptor for HA.

Lymphatic fluid contains a large amount of HA, which appears to serve as a chemo-

attract force for lymphocytes, since they have such a high level of CD44 as their "homing receptor" to guide their way back to the lymph node. Cancer cells also utilize the CD44 to make their way to the lymph node. HA plays a critical role in attracting cells to the lymph node.

Since the lymph path naturally collects HA, this lymph path specific draining property of HA can be used as a carrier to deliver anti-tumor drug specifically to the lymph path, where the metastatic cancer cells have settled.

2. Dosages

The dosage ranges for the administration of the compounds are those large enough to produce the desired effect in which delivery occurs. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary from about 1 mg/kg to 30 mg/kg in one or more dose administrations daily, for one or several days.

The dose, schedule of doses and route of administration may be varied, whether oral, nasal, vaginal, rectal, extraocular, intramuscular, intracutaneous, subcutaneous, or intravenous, to avoid adverse reaction yet still achieve delivery.

3. Pharmaceutically Acceptable Carriers

Any of the compounds can be used therapeutically in combination with a pharmaceutically acceptable carrier.

Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration of compositions to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH. The compositionscould also be administered intramuscularly or subcutaneously. Other compounds will be administered according to standard procedures used by those skilled in the art.

Molecules intended for pharmaceutical delivery may be formulated in a pharmaceutical composition. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally), orally, by inhalation, or parenterally, for example by intravenous drip, subcutaneous, intraperitoneal or intramuscular injection. The disclosed compositions can be administered intravenously, intraperitoneally, intramuscularly, subcutaneously, intracavity, or transdermally.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions which may also contain buffers, diluents and other suitable additives. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Compositions for oral administration may include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets, or tablets. Thickeners, flavorings, diluents, emulsifiers, dispersing aids or binders may be desirable.

The compositions as described herein can also be administered as a pharmaceutically acceptable acid- or base-addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide, potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

C. References

The following references may be referred to in the specification and each one is specifically herein incorporated by reference.

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D. Examples

Disclosed herein the selective delivery of polymeric–antitumor agent conjugate to cancer cells can be markedly enhanced, and that overall doses could be reduced.

Cell-targeted hyaluronic acid (HA)-doxorubicin (DOX) biconjuagtes (HA-DOX), and *N*-(2-hydroxypropoyl)methacrylamide (HPMA) copolymer-DOX conjugates containing HA as a side chain (HPMA-HA-DOX) were synthesized based on the specific interaction between hyaluronic acid (HA) and its receptors overexpressed on cancer cell surface. Selective *in vitro* cell cytotoxicity was studied with three human cell-lines (HCT-116 colon tumor, HBL-100 breast cancer, and SK-OV-3 ovarian cancer). In addition, enhanced uptake of HPMA-HA-DOX conjugate was visualized by confocal fluorescence microscopy in comparison to non-targeted HPMA-HA-DOX system, providing compelling evidence for the uptake of the targeted conjugates through receptor-mediated pathway.

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for.

Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

1. Example 1 Enhanced Targeting of Tumors with Hyaluronan Conjugated Taxol

Disclosed herein it was demonstrated *in vitro* that hyaluronan (HA) conjugated Taxol (HA-Taxol) has a selective toxicity toward several human cancer cell lines. In this study, the *in vivo* anti-tumor effect of HA-Taxol was examined. The human TSU bladder cancer cells and mouse 4T1 breast cancer cells expressed HA receptor (CD44) and were capable to bind, internalize and degrade the HA. When treated the tumor xenograft formed by these cells in mice model, the inhibitory effect of HA-Taxol was greater than Taxol alone. The results of i.v. injection of ^{3}H-HA indicated that several normal tissues, especially liver and kidney also had a high capability for taking up HA conjugates.

To reduce the unspecific retention of HA in normal organs, chondroitin sulfate (CS) that can bind to some HA binding proteins was injected into mice 2 hours prior to the administration of ^{3}H-HA. This approach did reduce the HA taken up by liver and increased the amount of HA accumulated in tumors. When mice bearing tumors were treated with CS and then HA-Taxol, their survival time was longer than those treated with vehicle or HA-Taxol alone.

The results of tissue distribution of mice receiving i.v. injection of ^{3}H-HA showed that tumors and lymph nodes had the highest concentration. The ^{3}H-HA up-taken in the blood-enriched peri-tumors was at least 3 times higher than that in the central portion of tumors.

a) Preparation of ^{3}H-HA

^{3}H-HA was prepared as previously described with some modification of Dr. Underhill's method . Briefly, the rat fibrosarcoma cells were cultured in 10 of 100 mm dishes with 10% fetal calf serum-90% DMEM to 80% confluence and then charged to media 2% fetal calf serum-98% DMEM supplemented with 2 mCi of ^{3}H-acetate for 2 days. The conditional media was digested with proteinase and dialyzed extensively against distilled water. The biosynthetic ^{3}H-HA in dialyzed media was precipitated by cetylpryridinium chloride, washed with alcohol and redissolved in saline. The radioactivity of the preparation was 5.4 X 10^{4} cpm/ μg HA and the ^{3}H-HA was 144 μg/ml. The preparation of ^{3}H-HA was sterilized with 0.2 μM filter and stored in -20°C for use.

b) Preparation of HA-Taxol

The conjugation of HA with Taxol was carried out as described by Drs. Lou and Prestwich (Luo Y, Ziebell MR, Prestwich GD: A hyaluronic acid-taxol antitumor bioconjugate targeted to cancer cells. Biomacromolecules. 2000; 1(2):208-18). Briefly, the fermentation-derived HA (Clear Solutions Biotechnology, Inc. Stony Brook, NY) was partially digested by hyaluronidase to size about 12,000 Dalton and dialyzed with a tubing (*Mw* cutoff 3,500 Da) to get rid of very small Mw of HA. A 5-fold adipic dihydrazide (ADH) was added to 50 mg of HA to make ADH-HA.

The Taxol-NHS ester was synthesized with two steps. First, Taxol®-2'-hemisuccinate was made as following procedure: 38 mg of succinic anhydride was added to 270 mg of Taxol followed by addition of 36 µl of pyridine. The mixture was stirred at room temperature for 3 days and purified on silica gel (wash with hexane; elute with ethyl acetate). Then, 1.51 g Taxol®-hemisuccinate and 0.83 g of SDPP (*N*-hydroxysuccinimido diphenyl phosphate) in 30 mL acetonitrile was added with 0.67 ml of triethylamine. The reaction was stirred for 6 h at room temperature and then concentrated *in vacuo*. The residue was dissolved in 5 ml ethyl acetate and purified on silica gel.

The purified Taxol-NHS ester (345 mg) dissolved in 400 ml acetone was added to 4.0 g HA-ADH dissolved in 250 ml water to give a homogeneous solution at 0°C. The reaction mixture was stirred at room temperature for 12 days. Acetone was removed by rotary evaporation before lyophilization. 1/8 of the residue was dissolved in 20 ml acetone/water=1:1 (v/v), and purified on a Sephadex G-25 column. The purity of HA-Taxol was monitored by GPC analysis. Taxol loading was determined by UV absorbance (λ_{max}=228 nm, $\varepsilon = 2.8 \times 10^4$) in acetonitrile: H_2O (80:20, v/v) to be 1.35 wt %.

c) Western blotting for CD44

4T1 and TSU tumor cells were cultured in 100 mm dishes to 70% confluence and lyzed with 1ml of lysis buffer (1% Triton X-100, 0.5% Na deoxycholate, 0.5 µg/ml leupetin, 1 mM EDTA, 1 µg/ml pepstatin and 0.5 mM phenylmethylsulfonyl fluoride). The protein concentration of the lysate was determined by the BCA method (Pierce, Rockford IL) and 30 µg of protein was loaded onto 10 % PAGE gel, electrophoresed and transferred to a nitrocellulose membrane. The loading and transferring of equal amounts of protein were confirmed by staining of the membrane with a solution of Ponceau S (Sigma, St. Louis MO). The membranes were blocked with 5% fat free milk in phosphate buffer saline (PBS,

pH 7.4) for 30 min and then incubated overnight with 0.2 μg/ml of BU52 monoclonal antibody against standard CD44 After washing, the membrane was incubated with peroxidase labeled anti-mouse IgG for one hour, followed by a chemo-luminicent substrate and exposed to ECL Hyperfilm MP (Amersham, Piscataway, NJ).

d) ^{3}H-HA binding assay

4T1 and TSU tumor cells were cultured in 24 well plate to 80% confluence, washed with PBS and lysed with 1 ml of DOC buffer (0.1 % Na deoxycholate, 0.5 M NaCl, 0.02 M Tris-HCl, pH 8.0). The equal amount of lysate (200 μl) were mixed with or without 100 μg of HA, and then added 20 μl (3 μg) of ^{3}H-HA. After shaken at room temperature for 30 minutes, 300 μl of saturated $(NH_4)_2SO_4$ was added to the reaction tubes, followed by 25 μl of nonfat milk. The tubes were spun at 12000 rpm for 5 min. The pellets in the tubes were washed twice with 50% of $(NH_4)_2SO_4$, dissolved in 0.2 ml H_2O, transferred to scintillation tubes, mixed with 1.2 ml of scintillation solution and counted for the radioactivity with β-counter.

e) HA degradation assay

The assay was carried out as described (CBU) with some modification. The cells 80% confluent in 24 well plate were changed to 1 ml fresh media containing ^{3}H-HA (4 X 10^5 cpm/7.5ug HA/ml) and incubated at 37°C for 48 hours. In some wells, the media contained 200 μg/ml of KM201 (a neutralization antibody against CD44) or 0.1 mM of chloroquine (an inhibitor of lysosomal enzymes). The media were collected, which contained some of the released and degraded ^{3}H-HA. The cells were frozen and thawed three times and then spin at 12,000 rpm for 30 min to obtain the degraded ^{3}H-HA inside cells. The media and the supernatant from cells were centrifuged with Centricon 30 (Amicon, Danvers, MA). The un-degraded high MW ^{3}H-HA was retained in the upper chamber. The 500 μl of degraded low MW ^{3}H-HA passing through the filter membrane was mixed with 6 ml of scintillation solution and counted for the radioactivity.

f) *In Vivo* Anti-tumor Effect of HA-Taxol

One millions tumor cells were subcutaneously injected into either BABL/c mice (for 4T1 cells) or nude mice (for TSU cells) and allowed to grow for 2 days for the tumor to establish. The tumor bearing mice were randomly divided into three groups and then i.p. injected with 0.4 ml of : 1) saline alone as vehicle control; 2) 4 mg/kg of Taxol; or 3)HA-

Taxol containing Taxol equal to 4 mg/kg, respectively. The injection was carried out every other day for two weeks. The tumor sizes were measured twice a week. At the end of experiment, the mice were sacrificed and the tumor were harvested, photographed and weighted.

In the chondroitin sulfate blocking study, the tumor bearing mice received i.p injections of 0.4 ml of either PBS (as control) or chondroitin sulfate (100 mg/ml) followed by HA-Taxol (8 mg/ml) two hours later. This procedure was carried out every other day for 20 days, and mice received a total of ten injections. The mice were recorded for their survival during a 33 day experimental period s and the survival rate was calculated.

g) *In Vivo* Distribution of ^{3}H-HA

To trace the distribution of ^{3}H-HA in both tumor and normal organs after i.v. injection, the mice tumor bearing with tumor were injected 0.2 ml of ^{3}H-HA. One day later, the tumors and organs were collected, weighed, homogenized with ultrasound to make tissue lysate at a protein concentration of 0.1 mg/ml. Then, 300 µl of tissue lysate were mixed with 2.5 ml of scintillation solution and counted for the radioactivity.

h) Statistical Analysis

The mean and standard error were calculated from above raw data and then subjected to *Student's t test*. The P value < 0.05 was regarded as statistically significant.

i) Results

Functional CD44 mediates the binding and degradation of HA by tumor cells. In initial experiments, the expression level of CD44 was examined. CD44 is the HA receptor on the cell surface, and is considered the basic target of HA carried drugs. A significantly high amount of CD 44 was expressed by both TSU and 4T1 tumor cells (Fig 9A).

To determine the binding activity of the CD44, the cells were lysed in DOC buffer and 100 µg of lysate proteins was incubated with 20 µl of ^{3}H-HA with or without HA. The results (Fig 9B) showed that the lysate proteins, including detectable CD44, bound to ^{3}H-HA, which could be competitively reduced by "cold" HA.

It was then determined that HA carried drug could be taken up by CD44 receptor by addition of high MW ^{3}H-HA to the media of culture cells, and allowed the cells to bind, up-take, and degrade the high MW ^{3}H-HA to low MW derivatives. This mixture was then separated by filter membrane with MW cut of 30,000 Dalton. The results indicated that

TSU and 4T1 cells were able to conduct the whole process and degrade the high MW ^{3}H-HA. This process could be blocked by cold ^{3}H-HA and by CD44 neutralization antibody, KM201, suggesting that the process is mediated by CD44-HA interaction. In addition, the functional lysosomes are required for this process, since the blocking of lysosomal enzymes with chloroquine also reduce the degraded ^{3}H-HA.

(1) HA-Taxol effectively reduce the growth of tumors in mice model: Chondroitin sulfate reduces the organ up-take of HA and enhances the tumor up-take of HA:

The results of in vivo animal experiments (Fig 9A) indicated that while the Taxol alone did not reduce the size of 4T1 tumors, the equal amount of Taxol conjugated with HA did exert anti-tumor effect, as evidenced by the fact that the tumors in HA-Taxol group were much smaller than those in the vehicle (saline control) and Taxol alone groups. This difference was statistically significant (Fig 9B).

To examine if this effect is true with other tumor models, the human TSU bladder cancer cells were subcutaneously injected into the flank of nude mice and the treatment procedures were similar to the experiment carried out with 4T1 tumor model. The results from the TSU tumor model were similar to that obtained from the 4T1 tumor model (Fig 10), showing that mice treated with HA-Taxol had slower tumor growth than those treated with vehicle or Taxol alone. These data suggest that the anti-tumor effect of HA-Taxol is reproducible and universal, not particular to one tumor type.

(2) Chondroitin sulfate reduces the organ up-take of HA and enhances the tumor up-take of HA

Injection of tumor bearing mice with labeled HA alone caused a high up-take of HA in the major organs, such as liver, spleen and kidney compared with the tumor (Fig 11A). However, pretreatment with chondroitin sulfate blocked the HA binding sites in the major organs and reduced their up-take of HA while the tumors accumulated high levels of HA (Fig 11B). The ^{3}H-HA in tumors of mice having a pretreatment with chondroitin sulfate was higher than that of untreated mice (Fig 11C). Furthermore, when the liver, a major organ involved in the up-take of HA, was monitored for two days for the level of HA, the amount of ^{3}H-HA in the liver of mice treated with chondroitin sulfate was much lower than that in mice treated with vehicle alone (Fig 11D). This indicated that the pretreatment with chondroitin sulfate has a relatively long effect in blocking the HA taken up by liver.

(3) Pre-treated with chondroitin sulfate enhances the therapeutic effect of HA-Taxol:

The pretreatment of mice with chondroitin sulfate can increase the accumulation of HA in tumors, which can be utilized to enhance the therapeutic effect of HA-Taxol.

Animal experiments demonstrated that the mice pretreated with chondroitin sulfate followed by HA-Taxol could be prevented from death during the experiment period of 33 days, while those treated with vehicle alone had only a 20% survival rate and Taxol alone had only a 60% survival rate. (Figure 12). The data strongly suggested that pre-treatment with chondroitin sulfate enhanced the therapeutic effect of HA-Taxol.

2. Example 2 Doxyrubicin HA-HPMA

a) Methods

(1) Reagents

Fermentation-derived HA (sodium salt, M_r 1.5 MDa) was provided by Clear Solutions Biotechnology, Inc. (Stony Brook, NY). 1-Ethyl-3-(3-(dimethylamino)-propyl)carbodiimide (EDCI), Adipic dihydrazide (ADH), succinic anhydride, anhydrous DMF, and triethylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Testicular hyaluronidase (HAse), Dulbecco's phosphate-buffered saline (DPBS) and cell culture media were purchased from Sigma (St. Louis, MO). Doxorubicin (DOX) was a kind of gift from Dr. A. Suarato, Pharmacia-Upjoin, Milano, Italy. Fluorescence images were recorded on a Bio-Rad (Hercules, CA) MRC 1024 laser scanning confocal imaging system based on a Zeiss (Oberkochen, Germany) Axioplan microscope and a krypton/argon laser.

(2) Cell Lines.

HBL-100, a human breast cancer cell-line, was maintained in culture in high glucose D-MEM (Dulbecco's Modified Eagle Medium), which was supplemented with 10% γ-irradiated fetal bovine serum (FBS) and 1% sodium pyruvate; SK-OV-3, a human ovarian cancer cell-line was cultured in D-MEM/F12 + 10% FBS; HCT-116, a colon tumor cell-line, was maintained in culture in α-MEM (Minimal Essential Medium, Eagle) + 10% FBS. 4T1 mouse breast cancer cells and TSU human bladder cancer cells were cultured with 10% calf serum-90% Dulbecco's modified Eagle's medium (DMEM) at 37°C in 5% CO_2 incubator.

(3) Analytical Instrumentation.

All ^1H NMR spectral data were obtained using an NR-200 FT-NMR spectrometer at 200 MHz (IBM Instruments Inc.). UV-Vis spectra were recorded on a Hewlett Packard 8453 UV-Vis diode array spectrophotometer (Palo Alto, CA). HA was characterized by gel permeation chromatography (GPC) was on the following system: Waters 515 HPLC pump,Waters 410 differential refractometer, and Waters™ 486 tunable absorbance detector. Waters Ultrahydrogel 250 and 2000 columns (7.8 mm ID × 30 cm) (Milford, MA) were used for GPC analysis, the eluent was 150 mM pH 6.5 phosphate buffer/MeOH = 80:20 (v/v), and the flow rate was 0.5 mL/min. The system was calibrated with HA standards supplied by Dr. O. Wik (Pharmacia). HPMA copolymer conjugates were characterized by GPC on a Pharmacia FPLC with Superose analytical column, pH 7.4 PBS buffer was used as eluent with a flow rate of 0.4 ml/min. Cell viability in cell culture was determined by thiazoyl blue (MTT) dye uptake protocols measured at 540 nm, which was recorded on a BIO-RAD M-450 microplate reader (Hercules, CA). Laser scanning confocal microscopy was carried out on a Keller type Bio-Rad MRC 1024 with LASERSHARP acquisition software. Fluorescence images were taken using FITC settings with the 488 nm excitation line and a 522 nm 32 bandpass filter was used to collect the images.

b) Preparation of low molecular weight (LMW) HA and HA hydrazide derivative (HA-ADH).

LMW HA was obtained by the degradation of high molecular weight HA (1.5 MDa) in pH 6.5 phosphate-buffered saline (PBS) buffer (4 mg/mL) with HAse (10 U/mg HA) as previously described, and purified by dialysis against H_2O [30]. Hydrazide-derivatized HA (HA-ADH) was prepared [30,51] using a modified purification method that gives preparations free of small molecules [30]. In a representative example, LMW HA (50 mg) was dissolved in water to give a concentration of 4 mg/mL, and then a fivefold excess of ADH was added into the solution. The pH of the reaction mixture was adjusted to 4.75 by addition of 0.1 N HCl. Next, 1 equiv of EDCI was added in solid form. The pH of the reaction mixture was maintained at 4.75 by addition of 0.1 N HCl. The reaction was quenched by addition of 0.1 N NaOH to adjust the pH of reaction mixture to 7.0 for different reaction time. The reaction mixture was then transferred to pretreated dialysis tubing (Mw cutoff 3,500) and dialyzed exhaustively against 100 mM NaCl, then 25% EtOH/H_2O, and finally H_2O. The purity of HA-ADH was monitored by GPC. The purified polymer solution was then filtered through 0.2 μm cellulose acetate membrane, flash frozen, and lyophilized. The loading of

ADH on the polymer backbone was determined by ^1H NMR in D_2O [51]. 37 mg of HA-ADH was obtained with 9 mol% and 18 mol% loading based on available carboxylates modified respectively, with the reaction time to be 12 min and 20 min.

c) Preparation of HA-DOX conjugates (Figure 3).

First, DOX was derived to be an active ester form (DOX-NHS). Briefly [52], DOX at a 20-mg quality (34 μmol) was dissolved in 1.2 ml of anhydrous DMF, followed by 15 μl triethylamine and 3.8 mg succinic anhydride. The reaction was stirring at room temperature in dark for 24 hrs. DOX-hemisuccinate was purified by C_{18} cartridge (Varian, Harbor City, CA) with methanol as the eluent.

Next, *N*-hydroxysuccinimido diphenyl phosphate (SDPP) was prepared from 10 mmol of diphenylphosphoryl chloride, 10 mmol of *N*-hydroxysuccinimide, and 10 mmol triethylamine in 6 mL of CH_2Cl_2 as previously described [30,53]. Crude SDPP was titrated with ether, dissolved in ethyl acetate, washed (2 × 10 mL H_2O), dried ($MgSO_4$), and concentrated *in vacuo* to give SDPP with mp 89-90°C (85%). To the solution of DOX-hemisuccinate and 18.5 mg (1.5 equiv) of SDPP in 2 ml DMF, was added with 60 μL (10 equiv) triethylamine. The reaction was stirred for 6 h at room temperature, and then concentrated *in vacuo*. The DOX-NHS ester was purified on a LH-20 column with methanol as the eluent.

HA-DOX conjugates were prepared by the conjugation of LMW HA-ADH and DOX-NHS. 50 mg HA-ADH (9 mol% and 18 mol%) was dissolved in 7 ml 3 mM pH 6.0 phosphate buffer, 2 mg DOX-NHS in 15 ml DMF was added to this solution under ice-water bath. The reaction was stirring at room temperature for 3 days. The HA-DOX conjugates were purified on a Sephadex G-25 column using PBS buffer as the eluent, following by dialysis against H_2O to remove the buffer salt. The DOX loading was determined by the absorption of UV spectrum at λ=484 nm.

d) Preparation of HPMA-HA-DOX conjugates (Figure 4).

The HPMA copolymer-bound DOX (HPMA-DOX or P(GFLG)-DOX; P is the HPMA copolymer backbone) was synthesized as previously described [54,55]. A lysosomally degradable glycylphenylalanylleucylglycine (GFLG) spacer was used as the oligopeptide side chain. The conjugate was synthesized using a two step procedure [56]. In the first step, the polymer precursor HPMA-(GFLG)-ONp was prepared by radical precipitation

copolymerization of HPMA and *N*-methacryloylglycylphenylalanylleucylglycine p-nitrophenyl ester [55]. The polymer precursor contained 7.1 mol% active ester groups (Mw=17,800, Mn=14,500). DOX was bound to the polymer precursor by aminolysis [57]. 200 mg HPMA-(GFLG)-ONp and 21.9 mg doxorubicin (DOX) hydrochloride were dissolved in 1.0 ml DMSO, and 50 μl of Et3N was added. The mixture was stirred at room temperature for 1 hr, and precipitated in acetone/ether (3/1) mixture solvent. The red polymer solid was collected and washed with acetone, ether, dried under vacuum to give 210 mg product. The HPMA-(GFLG)-DOX-ONp conjugate contained 1.1 mol% of DOX.

HPMA-HA-DOX conjugates were prepared by the conjugation of HA-ADH (9 mol% and 18 mol% hydrazide modification) to the above HPMA-(GFLG)-DOX-ONp with ONp residue. For example, 90 mg HPMA-(GFLG)-DOX-ONp copolymer-drug conjugate prepared previously was dissolved in 2.0 ml DMSO, and 90 mg HA-ADH of 18 mol% hydrazide modification was dissolved in 1.0 ml water and 2.0 ml DMSO. The two solutions were mixed together and stirred it overnight at room temperature. Aminoethanol (100 μl) was added to destroy unreacted ONp active ester. The HPMA-HA-ADR conjugate was isolated and purified by gel filtration on a Sephadex LH-20 column twice with methanol as eluent. The solvent was removed under vacuum, and the residue was dissolved in distilled water and lyophilized. The DOX loading was determined by the absorption of UV spectrum at λ=484 nm. HA composition was calculated by mass balance.

e) *In Vitro* Cell Culture.

The cytotoxicity of HA-DOX and HPMA-HA-DOX conjugates against HBL-100, SKOV-3 and HCT-116 cells was determined using a 96-well plate format in quadruplicate with increasing doses range from 0.001-10 mg/mL of DOX equivalent. Each well contained approximately 20,000 cells in 200 μL cell culture media. Thus, a 2-μL aliquot of the stock solution was added to each well, and cells were continuously incubated at 37 °C, 5% CO_2 for 3 days with the test substance, and cell viability was determined using MTT dye uptake at 540 nm. Response was graded as percent live cells compared to untreated controls [58]. Dose-response curves were constructed, an d the concentration necessary to inhibit the growth of the cells by 50% relative to the non-treated control cells (IC_{50} dose) was determined.

Internalization of HPMA-HA-DOX conjugates by cancer cells by confocal fluorescence microscopy. SKOV-3 cells were incubated in a cell culture flask, harvested by

trypsinization, and transferred into a 8-well cell culture slide. 20,000 cells were seeded in each well of the slide and cultured for 48 hr. The cultured medium was replaced with medium containing HPMA-HA-DOX conjugates, the concentration was adjusted to 50 μg/ml of HA equivalent. Meanwhile, HPMA-DOX conjugate with equal amount of DOX drug to HPMA-HA-DOX was used as a control. Cells were cultured with the conjugates for various time intervals. Unbound conjugate was removed by washing the cell layer 3 times with DPBS. Cells were fixed with 3% paraformaldehyde for 10 min at room temperature and washed again with DPBS. Internalized HPMA-HA-DOX conjugate was visualized by fluorescence images taken with the confocal microscopy.

In the cell surface binding experiment, cells were incubated with the HPMA-HA-DOX conjugate at 0°C for 2hr (a condition under which no internationalization occurs), followed by the DPBS washing and paraformaldehyde fixing described above. The cell surface binding conjugate was determined by the fluorescence images.

Fluorescence microscopy. Cells were examined by using an inverted microscope (Nikon) and a Bio-Rad (Hercules, CA) MRC 1024 laser scanning confocal microscope. Cell images were collected by using a x 60 oil immersion objective, no postacquisition enhancement of images was performed. DOX fluorescene image acquisition was accumulated *via* the BHS block of filters (excitation 488 nm and emission through a 522 nm 32 bandpass filter). A coverslip was mounted on a microscope slide containing fixed cells with ProLong Antifade Kit (Molecular Probes, Eugene, OR) as the mounting medium. Fluorescence images were scaled to 256 gray levels.

f) Preparation of HA-DOX conjugates

The hydrazide method to make the HA-ADH derivatives [30,51,59] allows attachment of reporter molecules, drugs, crosslinkers, and combinations of these moieties to HA [23,24]. LMW HA was generated in this study for three reasons: (i) proton NMR allowed rapid quantification of the modification, (ii) LMW HA and its derivatives give injectable, non-viscous solution at concentrations up to 10 mg/mL, and (iii) LMW HA has a longer plasma half-life and is readily cleared by renal ultrafiltration. The LMW HA was prepared by partial degradation of high molecular weight HA (1.5 MDa) with testicular HAse [60] in pH 6.5 PBS buffer at 37 °C. The final size of LMW HA was characterized by GPC analysis: M_n = 3,883, M_w = 11,199, and molecular dispersity (DP) = 2.88. Next, HA-ADH with different ADH loadings were prepared by carbodiimide coupling chemistry [30,31], in

which the extent of ADH modification was controlled through use of specific molar ratios of hydrazide, carboxylate equivalents, and carbodiimide. The purity and molecular size distribution of the HA-ADH were measured by GPC, and the substitution degree of ADH was determined by the ratio of methylene hydrogens to acetyl methyl protons as measured by ^{1}H NMR [51]. HA-ADH with ADH loadings of 9 mol% and 18 mol% were obtained and used in preparing the HA-DOX and HPMA-HA-DOX conjugates.

Furthermore, HA-DOX conjugates was synthesized by the conjugation of HA-ADH to the activated DOX-NHS ester to give a non-cleavable hydrazide linkage between the DOX drug and the HA polymer carrier. The HA-DOX conjugates were purified by gel filtration on a Sephadex G-25 column using PBS buffer as the eluent, following by dialysis against H_2O. The DOX loading was determined by the UV spectrum at λ=484 nm. The DOX composition of the HA-DOX conjugates used in the *in vitro* cytotoxicity test were 2.3 wt% and 3.5 wt% which were made from 9 mol% and 18 mol% ADH loading of HA-ADH, respectively.

g) Preparation of HPMA-HA-DOX conjugates.

This cell targeted delivery system was designed with HA on the side chain of the HPMA copolymer serving as a targeting moiety to cancer cell surface, and DOX linked to the polymer carrier through an lysosomal enzyme degradable peptide linkage [12]. HPMA-HA-DOX conjugates were synthesized by the conjugation of HA-DOX with HPMA-DOX copolymer with active ONp residue. HA-ADH with 9 mol% and 18 mol% hydrazide modification were used in the conjugation. The conjugates were purified by gel filtration on a Sephadex LH-20 column. HA loading was determined by mass balance. The DOX loading was determined by the UV spectrum at λ=484 nm. HPMA-HA-DOX conjugates made from 18 mol% HA-ADH gave 36 wt% HA and 3.3 wt% DOX with molecular weight of Mw=35,000 and Mn=19,000. HPMA-HA-DOX conjugates made from 9 mol% HA-ADH gave 17 wt% HA and 3.2 wt% DOX with molecular weight of Mw=18,000 and Mn=14,000.

h) Cytotoxicity assay of HA-DOX and HPMA-HA-DOX conjugates

Free DOX drug and non-targeted HPMA-DOX and targeted HA-DOX, HPMA-HA-DOX conjugates were assessed for their dose-dependent growth inhibitory effect on human breast cancer HBL-100 cells, human ovarian cancer SKOV-3 cells and human colon cancer

HCT-116 cells which have been reported to overexpress HA receptors on the tumor cell surface. Cells were exposed to various DOX concentration (DOX equivalent for polymer-drug conjugates) to determine the concentration necessary to inhibit the tumor cell growth by 50% relative to non-treated control cells (IC_{50} dose). Typical curves describing the dependence of cell viability on the concentration of DOX equivalent covalently bound to the polymer conjugates, were presented in Figure 5. The IC_{50} doses for the free DOX drug and the conjugates were listed in Table 1. From these results it is clear that DOX attached to a non-targeted polymer carrier (HPMA-DOX) markedly decrease the cytotoxicity of DOX drug. For SKOV-3 cells, the IC_{50} doses increase from 0.92 μM for free DOX drug to 58.2 μM for HPMA-DOX. These increases probably reflect the different mechanisms of cell uptake (free diffusion for free DOX drug vs. endocytosis for DOX-polymer conjugates) resulting in different intracellular drug concentration. Targeted HPMA-HA-DOX conjugates which enter cells by receptor-mediated endocytosis, nearly restored the original low IC_{50} dose for DOX drug. The IC_{50} doses against HBL-100 cells were 0.52 μM and 1.67 μM for the targeted HPMA-HA-DOX conjugates with 36 wt% and 17 wt% HA loading, respectively, in comparison of the 18.7 μM for the non-targeted HPMA-DOX conjugate and 0.15 μM for free DOX drug. Against each cell line overexpressed HA receptors on cell surface, the cytotoxicity of targeted HPMA-HA-DOX conjugates had a magnitude increase over the non-targeted HPMA-DOX conjugate.

However, for the HA-DOX conjugate system, the cytotoxicity of the conjugates were even slightly higher than the non-targeted HPMA-DOX conjugate. The IC_{50} doses against SKOV-3 cells were 157 μM and 141 μM for HA-DOX conjugates, comparing to 58.2 μM for non-targeted HPMA-DOX conjugate, and 9.2 μM for targeted HPMA-HA-DOX conjugate (36 wt%). Two possible factors would contribute to the loss of cytotoxicity: the conjugation decreases the activity of DOX drug; the non-cleavable hydrazide linkage between DOX and HA polymer carrier. From our previous study, the cytotoxicity HA-Taxol conjugates with esterase cleavable linkage between Taxol drug and HA polymer carrier had a comparable value to free Taxol drug in cell culture against SKOV-3 cells [30].

Table 1. Cytotoxicity of free DOX drug, HA-DOX conjugates and HPMA-HA-DOX conjugates against SKOV-3 cells *in vitro*.

	IC_{50} (μM) of DOX equivalent

Drugs	HBL-100 cells	SK-OV-3 cells	HCT-116 cells
DOX	0.15	0.92	0.35
HA-DOX (2.3 wt% DOX)	100	157	140
HA-DOX (3.5 wt% DOX)	75.5	141	62.0
HPMA-DOX	18.7	58.2	56.6
HPMA-HA-DOX (36 wt% HA)	0.52	9.2	4.32
HPMA-HA-DOX (17 wt% HA)	1.67	10.3	5.66

i) Cell binding and uptake of HPMA-HA-DOX conjugates

Several different fluorescently-labeled HA derivatives have been prepared in order to study receptor-mediated cellular uptake. Previously, fluorescein-HA was employed to study HA uptake in a variety of systems, e.g., cells expressing CD44 variants [40,41,61-64], uptake by tumor cells for correlation with metastatic potential [50,65], internalization by chondrocytes [46], and as a measure of liver endothelial cell function [66]. Most recently, RHAMM-mediated uptake and trafficking of HA by transformed fibroblasts [67] was observed with Texas Red-HA, and BODIPY-labeled HA was employed to distinguish HA uptake in cancer vs. untransformed cell-lines [30,31].

In order to correlate the receptor-mediated endocytosis of conjugates by cells with their cytotoxicity, the cell binding and uptake of the targeted HPMA-HA-DOX conjugates were followed by the fluorescene microscopy using the intrinsic fluorescence of DOX. Cells were cultured in the presence of HPMA-HA-DOX conjugates of 50 μg/ml HA equivalent for various period of time, afterwards the amount of material internalized and bound to cell surface was visualized by confocal fluorescene microscopy.

SKOV-3 Cells chilled to 0°C was incubated with HPMA-HA-DOX for 2hr. After fixing and washing, a well-developed cluster of cells was chosen for the fluorescence microscope analysis. Cells were sectioned optically using confocal microscopy, fluorescence images were taken *via* the BHS block of filters of excitation 488 nm and emission 522 nm, along with the transmission images. Figure 6 provided a particularly dramatic illustration of the initial binding of the HPMA-HA-DOX conjugate on the SKOV-

3 cells surface where the overexpressed HA binding receptor-CD44 located. The anchoring of the targeted HPMA-HA-DOX on the cell surface prior to the cellular uptake through the specific binding between HA and HA binding proteins, provides the opportunity of the enhanced internalization of the polymer conjugates by receptor-mediated endocytosis.

In addition, the internalization of polymer conjugates directly determined the cytotoxicity of conjugate system. Thus, with the intrinsic fluorescence of DOX, the cellular uptake of the targeted HPMA-HA-DOX conjugates were also followed by the confocal fluorescence microscopy. SKOV-3 cells were incubated with the HPMA-HA-DOX conjugates (36 wt% and 17 wt% HA loading) of 50 μg/ml HA equivalent for various intervals, before the fluorescence images were taken. The non-targeted HPMA-DOX of equal amount of DOX equivalent was used as a control. Confocal fluorescence images of HPMA-HA-DOX uptake by SKOV-3 cells were presented in Figure 7. Initially the 2 hr images, HPMA-HA-DOX polymer conjugates could be seen mainly on the cell membrane; over the course of 8 hr, it was gradually taken up into the cells. 24 hr and 32 hr later, cells showed the polymer conjugates in most subcellular compartments. The uptake of HPMA-HA-DOX conjugate with 36 wt% HA loading was rapid than the conjugate with 17 wt% HA loading, however, no significant difference was observed. In the control of non-targeted cellular uptake of HPMA-DOX, the fluorescence inside cells was gradually increase along with the incubation time of cells with the polymer conjugate. However, very weak fluorescence (polymer conjugate) was observed even after 32 hr incubation, in comparison of the targeted HPMA-HA-DOX system. The uptake of HPMA-HA-DOX into HBL-100 cells and HCT-116 cells occurred with a similar appearance and time course. These images provided a particularly dramatic illustration of the initial binding of the targeted HPMA-HA-DOX conjugates onto the tumor cell surface, following by rapid endocytosis *via* HA receptor-mediated pathways. HA incorporated into HPMA-DOX conjugates significantly increase the efficiency of the endocytosis process by cancer cells. The trafficking of cellular binding and uptake of HPMA-HA-DOX conjugates by confocal fluorescence images is consistent with the cytotoxicity results, and provides the further support for the increase cytotoxicity of targeted HPMA-HA-DOX conjugates of which the enhanced internalization of polymer conjugates mediated through an HA-specific, receptor-mediated process comparing to the non-targeted HPMA-DOX system.

In summary, the data reported herein indicate that the cytotoxicity of HPMA-HA-DOX polymer conjugates requires cellular uptake of the bioconjugate followed by the

release of the active free DOX drug by the lysosomal enzyme cleavage of the GFLG tetra-peptide spacer. Targeting of a variety of anti-cancer agents to tumor cells and tumor metastases could be achieved by receptor-mediated uptake of an HA containing-anti-cancer agent conjugate, followed by the intracellular release of the active drug and subsequent cell death. The ability to "seek and destroy" micrometastases is one of the most compelling and attractive potential outcomes for the disclosed HA containing-anti-tumor bioconjugates.

j) *In vitro* Cytoxicity of HPMA-HA-DOX conjugates

The *in vitro* cytotoxicity of HPMA-HA-DOX with 17% and 36% HA loading against cultured prostate cancer cell line DU-145 was examined. Figure 8 depicts the *in vitro* cytotoxicity results of the HPMA-HA-DOX bioconjugates. The cytotoxicity of targeted HPMA-HA-DOX bioconjugates were dramatically higher than non-targeted HPMA-DOX conjugate (Table 2), and 8- to 12-fold higher than the free DOX drug against this prostate cancer cell-line. These data indicate that HPMA-HA-DOX bioconjugate can be used as a specific prostate cancer macromolecular chemotherapeutic agent.

Table 2. *In vitro* Cytotoxicity of free DOX drug, HA-DOX conjugates and HPMA-HA-DOX conjugates against human prostate cancer cell-line DU-145.

	IC_{50} (μM) of DOX equivalent against DU-145
DOX	31.5
HPMA-DOX	> 100.0
HPMA-HA-DOX (36 wt% HA)	2.4
HPMA-HA-DOX (17 wt% HA)	4.7

3. Example 3 Interaction of HA with Tumor Cells CD44

CD44 was examined to determine if it was expressed by 4T1 cells and if it could interact with HA. First, CD44 was detected by Western blotting. Secondly, the binding activity of CD44 was determined by mixing 30 μg of 4T1 lysate with ^{3}H-HA (as test) or ^{3}H-HA plus 50 folds excess of cold HA (as specificity control). Thirdly, the functional CD44 mediated ^{3}H-HA degradation was determined by incubating 4T1 cells with ^{3}H-HA for 72 hours and then separating degraded small MW ^{3}H-HA from intact HA high MW ^{3}H-HA by MW cut Centricon spin (Culty et al., J Cell Biol. 1992; 116(4): 1055-62.). The results showed that CD44 was expressed in 4T1 cells (Fig. 14A) and it could bind to ^{3}H-HA (Fig. 14B) and degrade ^{3}H-HA (Fig. 14C). The binding was specific as it could be inhibited by excess of cold HA (Fig. 14B).

The CD44 mediated degradation of ^{3}H-HA could be inhibited by excess clod HA, anti-CD44 neutralization antibody (KM201, Akima et al.; J Drug Target 1996; 4(1): 1-8) and lysosomal inhibitor chloroquine (Fig. 14C), showing that CD44 did mediate the uptake and degradation of HA.

4. Example 4 In Vivo Distribution of HA

The ^{3}H-HA was i.v. injected into mice bearing with 200 mm^{3} size of breast cancer xenografts on their mammary fat pat. After 24 hours, the mice were sacrificed, the organs were homogenized and 20 mg of homogenates from different tissues was measured for ^{3}H-HA by β-counter. The results **(Fig 15)** showed that both tumor and lymph node contained the highest amount of HA as compared to other organs.

5. Example 5 Targeting spontaneous metastatic tumors with HA-Drug

The 4T1 breast cancer cells were injected into foot pat of syngenic BABL/c mice. Three days after inoculation, the mice were randomly divided into three different treatment groups: 1) saline alone as vehicle control; 2) 4 mg/kg of Taxol; or 3) HA-Taxol containing Taxol equal to 4 mg/kg. About 0.2 ml of above agents were administrated by subcutaneous (s.c.) injection at the middle of leg three times a week, from where HA-drug could be drained/ absorbed into popliteal and inguinal lymph path, in which the spontaneous metastases were expected to take place.

The s.c. injection was chosen because ^{3}H-HA injected s.c. was mainly drained/absorbed from the injection sites into lymphatic pathway as evidenced by the increased local ^{3}H-HA and reduced plasma ^{3}H-HA when the lymphatic structure was surgically destroyed. The specific distribution of HA to the lymph nodes was also observed with ^{14}C-labelled HA and fluorescent HA by other groups (Akima et al.; J Drug Target 1996; 4(1): 1-8). The subcutaneous administration of HA specially targeting the lymph path shows the effectiveness of using HA as carrier to destroy lymphatic metastases.

After three weeks of s.c. injection of HA-Taxol, the mice were sacrificed and the primary tumors, the popliteal and inguinal lymph nodes (as represented in Fig. 16) were collected. The lymph nodes were carefully dissected out from surrounding fat, measured for the weights and processed for pathology.

The results (Fig. 17) from pathohistological analysis showed that while the lymph

nodes from the control group had spontaneous metastases, there was no tumor cells detected in HA-Taxol treated group.

The sizes of the near distant popliteal lymph nodes were smaller in HA-Taxol group than those in control saline and Taxol alone groups (Table 3).

Table 3. Inhibition of popliteal lymph node metastasis

Treatment	Weight of lymph node	Inhibition ratio (%)
Saline	45.90±5.58	--
Taxol	46.70±5.05	-1.7%
HA-Taxol	29.70±2.99	35.3%**

**: P<0.001

Similarly, the far distant inguinal lymph nodes were also reduced in size by the HA-Taxol treatment. It seems that HA-Taxol could prevent the tumor cells from their settle-down and growth in both the near and the far distant lymph nodes.

Table 4. Inhibition of inguinal lymph node metastasis

Treatment	Weight of lymph node	Inhibition ratio (%)
Saline	13.20±3.37	--
Taxol	9.00±0.79	31.8%
HA-Taxol	4.70±0.40	64.4%**

**: P<0.001

6. Example 6 Conjugation of HA with Mitomycin C:

The conjugation process was carried out according to Akima's method (Akima et al.; J Drug Target 1996; 4(1): 1-8) with some modification. 2 mg mitomycin C powder (Sigma) was added to 4 mg of hyaluronan (Lifecore, MW 1.2 x10^6 kDa) in 35% DMF (dimethyformamide, pH 5.0). After mixing well, 4 mg of water-soluble 1-ethyl-3-(-dimethylaminopropyl) carbodiimide (EDAC) was added and then reacted overnight at room temperature. Then, the unconjugated mitomycin C was separated from HA-mitomycin C by dialysis of the reaction mixture against distilled water.

The result of UV absorbency showed a HA-mitomycin C complex peak, indicating the conjugation was successful. The conjugation ratio of HA-mitomycin C was 4.7%.

What is claimed is:

1. A method of administering a hyaluronic acid (HA) conjugated molecule or a derivative thereof to a subject, comprising administering to the subject a blocking agent and an HA conjugated molecule or a derivative thereof.

2. The method of claim 1, wherein the HA conjugated molecule or derivative thereof comprises an anti-cancer agent.

3. The method of claim 2, wherein the HA conjugated molecule or derivative thereof also comprises a carrier molecule.

4. The method of claim 1, wherein the blocking agent is administered prior to administering the HA conjugated molecule or derivative thereof.

5. The method of claim 4, wherein the blocking agent is administered 10 minutes to 15 hours prior to administering the HA conjugated molecule or derivative thereof.

6. The method of claim 5, wherein the blocking agent is administered 60 minutes to 240 minutes prior to administering the HA conjugated molecule or derivative thereof.

7. The method of claim 6, wherein the blocking agent is administered 120 minutes prior to administering the HA conjugated molecule or derivative thereof.

8. The method of claim 2, wherein the anti-cancer agent comprises a cytotoxic agent, a chemotherapeutic agent, a cytokine, an antitubulin agent, a radioactive isotope, a combretastatin antagonists, a calcium ionophore, a calcium-flux inducing agent, or a combination thereof.

9. The method of claim 2, wherein the anti-cancer agent comprises 5-fluorouracil, 9-aminocamptothecin, amine-modified geldanomycin, Taxol®, vincristine, vinblastine, vinorelbine, and vindesine, calicheamicin, QFA, BCNU, streptozoicin, neomycin, podophyllotoxin, TNF-alpha, .alpha$_v$beta$_3$ colchicine, or a combination thereof.

10. The method of claim 2, wherein the anti-cancer agent is doxorubicin.

11. The method of claim 3, wherein the carrier molecule comprises a macromolecule of at least 5,000 daltons.

12. The method of claim 3, wherein the carrier molecule comprises a macromolecule having a molecular weight of from 10,000 daltons to 25,000 dalton.

13. The method of claim 3, wherein the carrier molecule comprises a polymer produced by the polymerization of an ethylenically unsaturated monomer.

14. The method of claim 13 wherein the monomer is an acrylate or methacrylate.

15. The method of claim 13 wherein the monomer is N-(2-hydroxypropyl)methacrylamide.

16. The method of claim 1, wherein the derivative of hyaluronic acid comprises hyaluronic acid modified with a dihydrazide compound.

17. The compound of claim 16 wherein the dihydrazide compound is adipic dihydrazide.

18. The compound of claim 3, wherein the anti-cancer agent is directly attached to the carrier molecule by a covalent bond.

19. The method of claim 3, wherein the anti-cancer agent is indirectly attached to the carrier molecule by a linker, wherein the anti-cancer agent and the carrier molecule are individually attached to the linker via a covalent bond.

20. The method of claim 19 wherein the linker comprises a peptide.

21. The method of claim 3, wherein the carrier molecule is directly attached to the hyaluronic acid or the derivative thereof by a covalent bond.

22. The method of claim 3, wherein the carrier molecule is attached to the hyaluronic acid or the derivative thereof by a covalent bond, and the hyaluronic acid or the derivative thereof is attached to the anti-cancer agent by a covalent bond.

23. The method of claim 3, wherein the carrier molecule is attached to the anti-cancer agent by a covalent bond, and the anticancer agent is attached to the hyaluronic acid or the derivative thereof by a covalent bond.

24. The method of claim 3, wherein the anti-cancer agent is doxorubicin, the carrier molecule is a polymer of N-(2-hydroxypropyl)methacrylamide, and the hyaluronic acid modified with adipic dihydrazide.

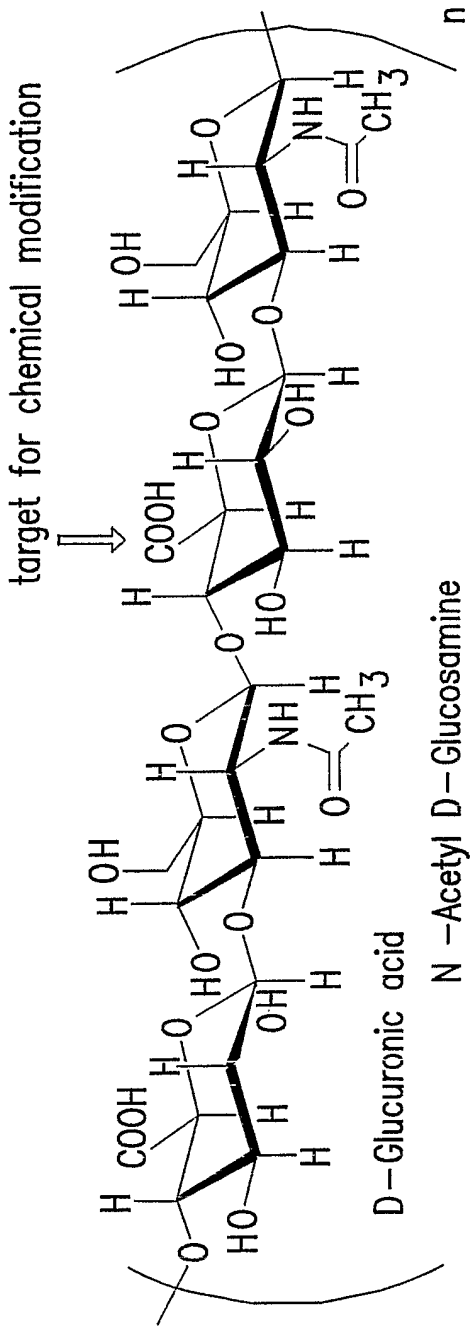
25. The method of claim 1, wherein the blocking agent is chondroitin 4-sulfate, chondroitin 6-sulfate, heparin, heparin sulfate, dextran sulfate, keratan, or keratan sulfate.

26. A method of inhibiting cancer cell proliferation comprising administering to the subject a blocking agent and an HA conjugated molecule or a derivative thereof.

27. A method of treating a patient with cancer comprising administering to the subject a blocking agent and an HA conjugated molecule or a derivative thereof.

28. A method of treating a patient comprising administering to the subject a blocking agent and an HA conjugated molecule or a derivative thereof.

29. The method of claim 1, wherein the blocking agent is administered concurrently with the HA conjugated molecule or a derivative thereof.


target for chemical modification
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D-Glucuronic acid
N -Acetyl D-Glucosamine

FIG. 1

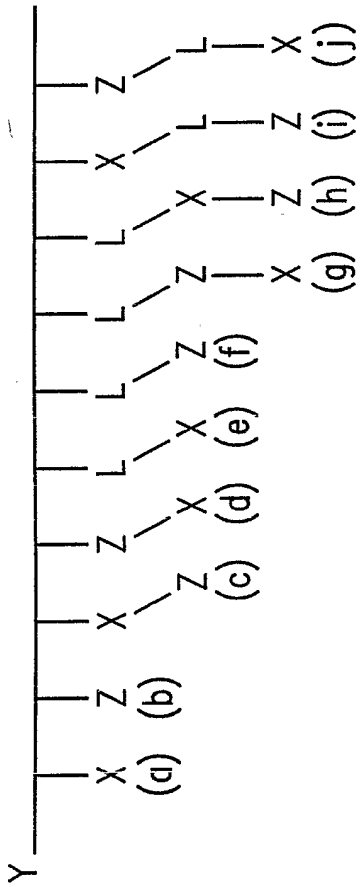


FIG.2

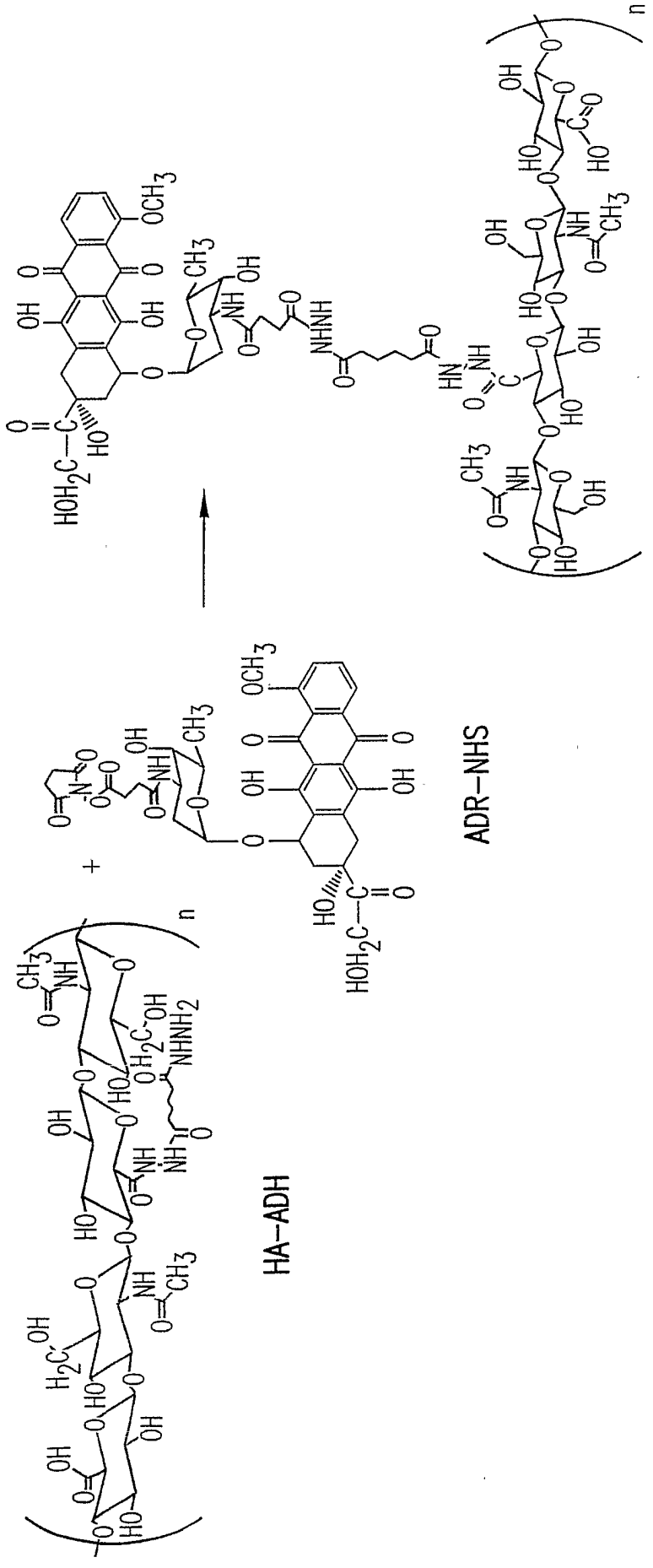


FIG.3

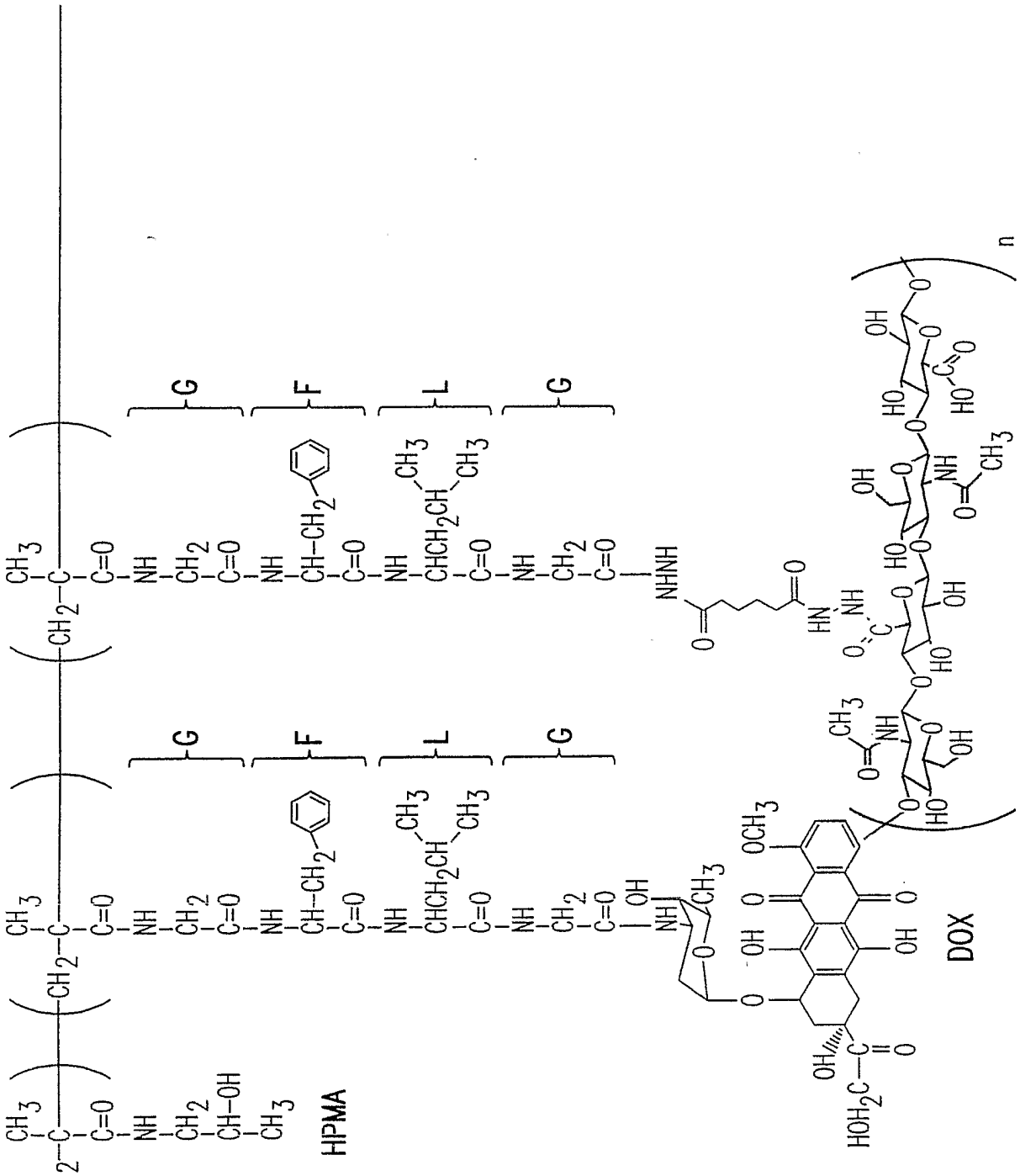


FIG. 4

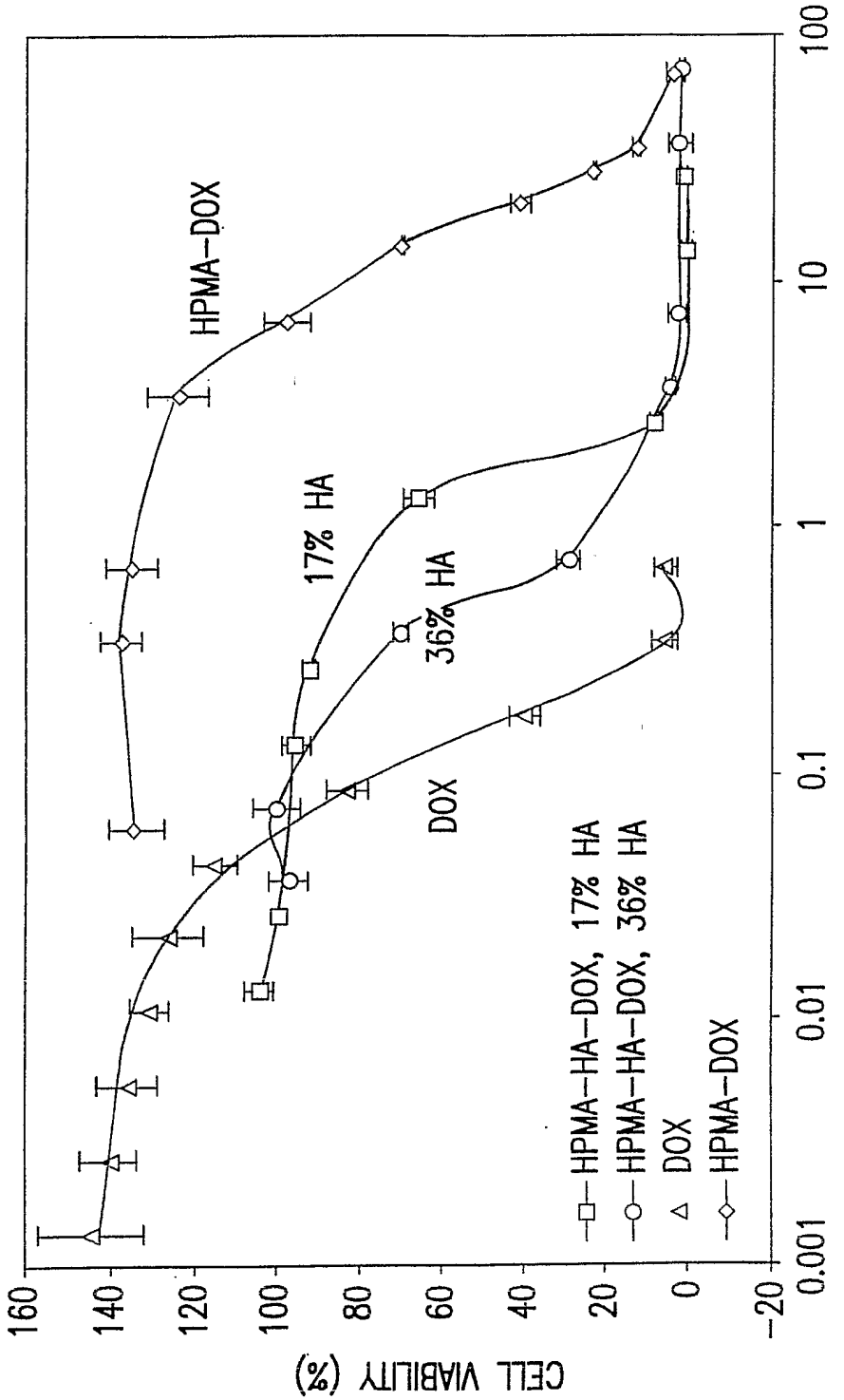

CELL VIABILITY (%)
160
140
120
100
80
60
40
20
0
−20
0.001
0.01
0.1
1
10
100
DOX equivalent (μM)
HPMA−DOX
17% HA
36% HA
DOX
HPMA−HA−DOX, 17% HA
HPMA−HA−DOX, 36% HA
DOX
HPMA−DOX

FIG. 5

binding of HPMA-HA-ADR conjugate on the cell surface HA-receptors

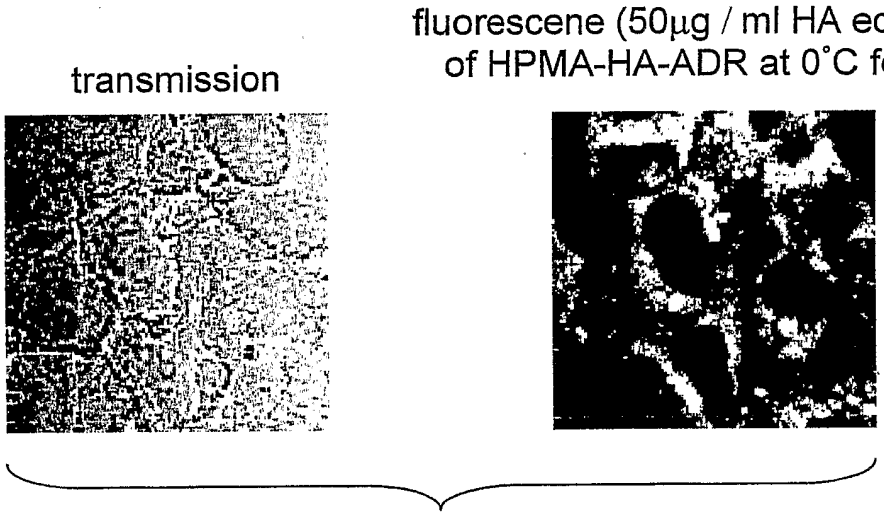


FIG.6

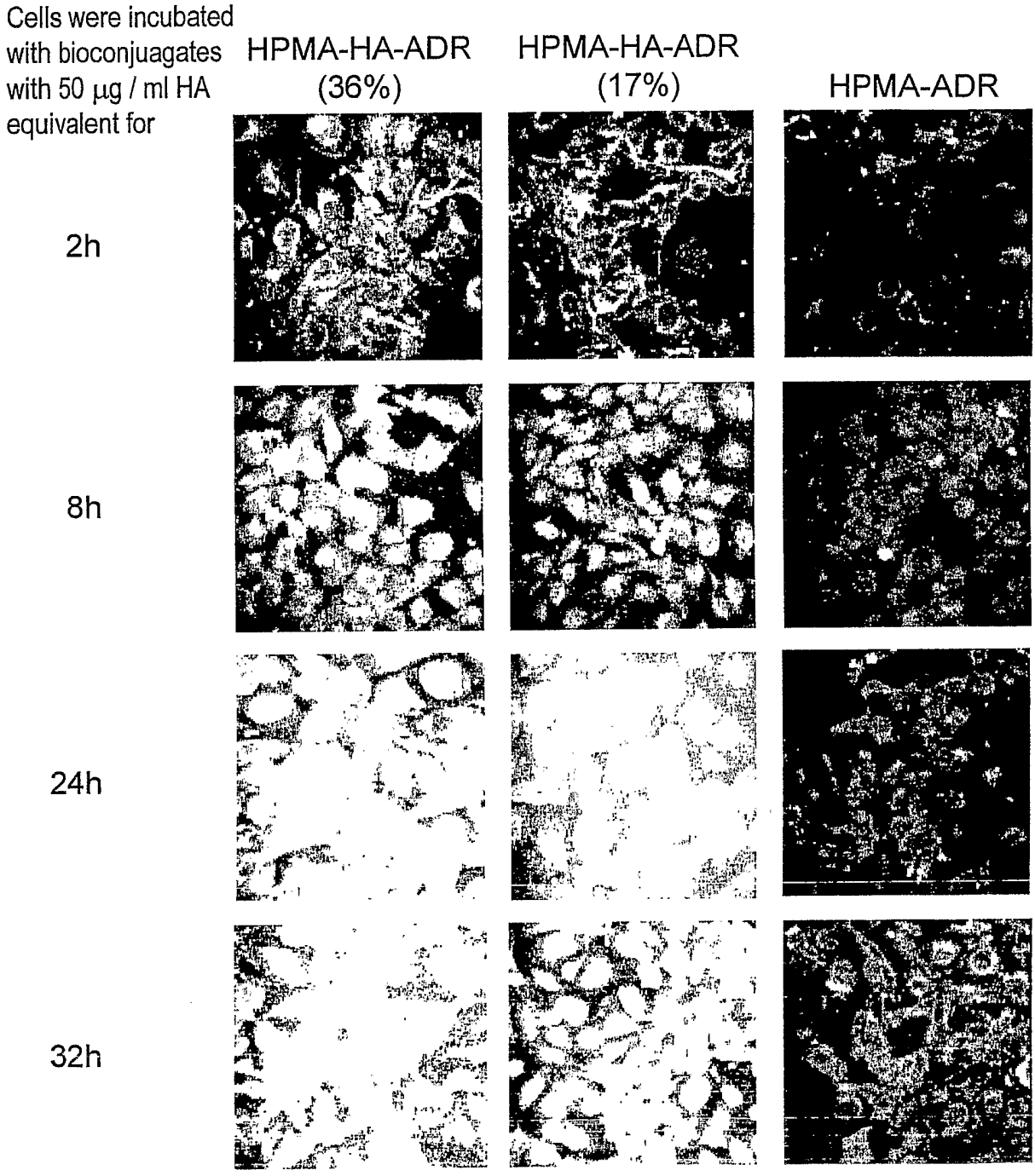

Cells were incubated with bioconjuagates with 50 μg / ml HA equivalent for
HPMA-HA-ADR (36%)
HPMA-HA-ADR (17%)
HPMA-ADR
2h
8h
24h
32h

FIG.7

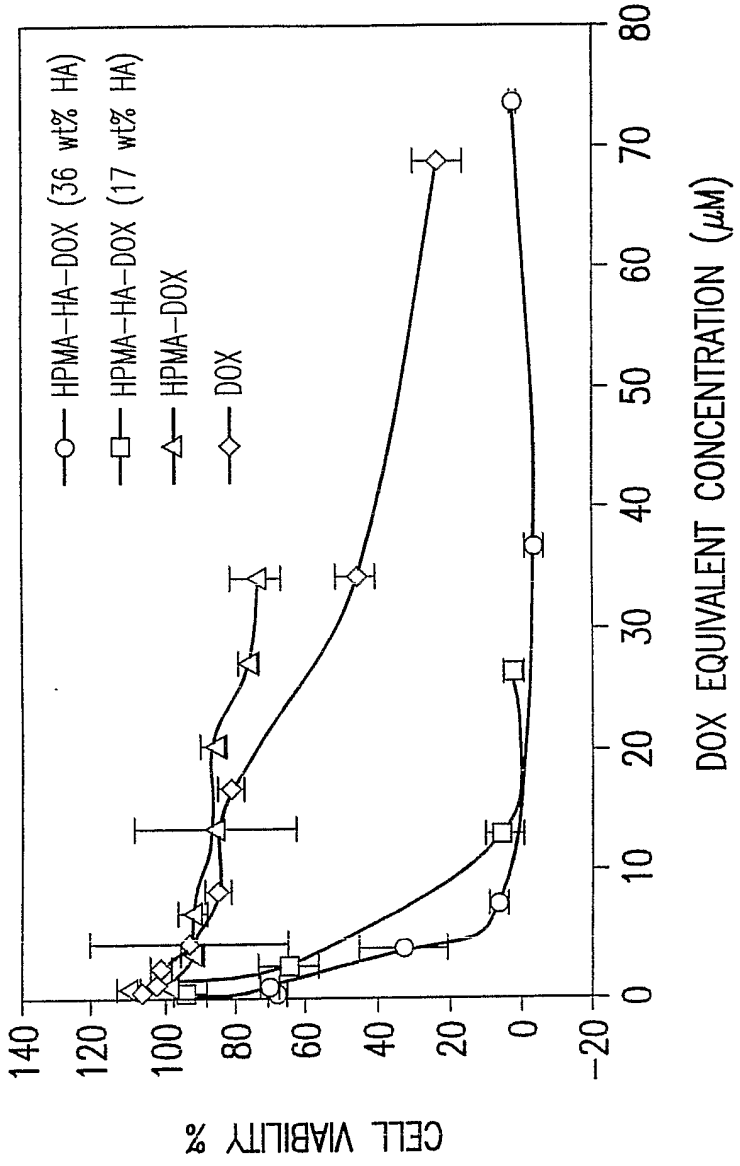

HPMA-HA-DOX (36 wt% HA)
HPMA-HA-DOX (17 wt% HA)
HPMA-DOX
DOX
CELL VIABILITY %
140
120
100
80
60
40
20
0
-20
0
10
20
30
40
50
60
70
80
DOX EQUIVALENT CONCENTRATION (μM)

FIG.8

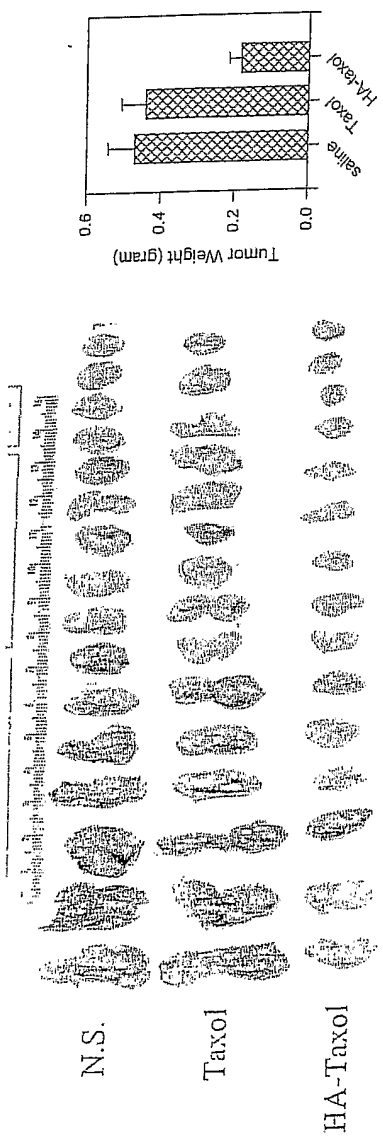

N.S.
Taxol
HA-Taxol

FIG. 9A

Tumor Weight (gram)
0.0
0.2
0.4
0.6
saline
Taxol
HA-taxol

FIG. 9B

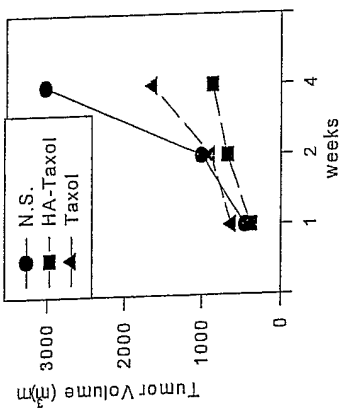
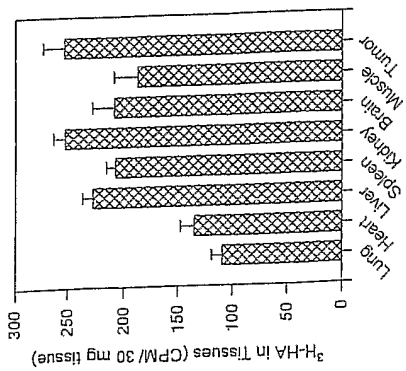
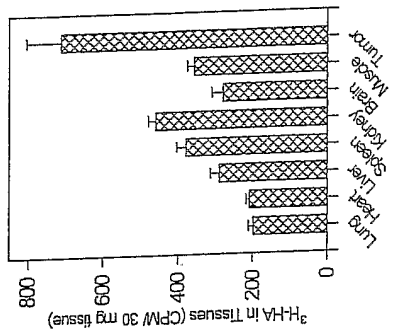


FIG. 10

FIG. 11A



11B



11C

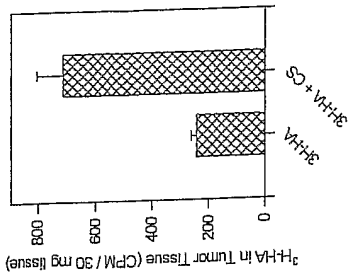
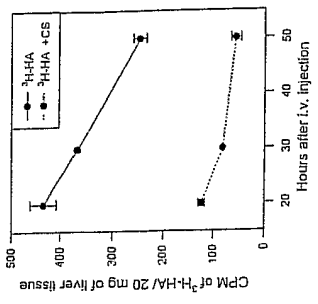
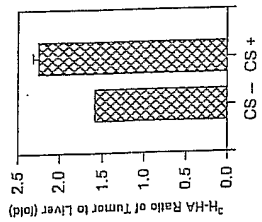


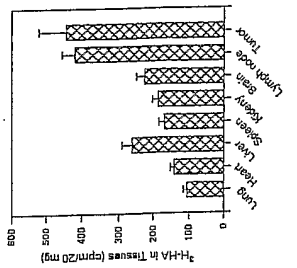
FIG. 11D



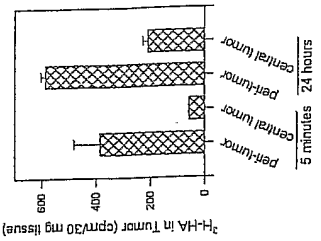
11E



11F



11G



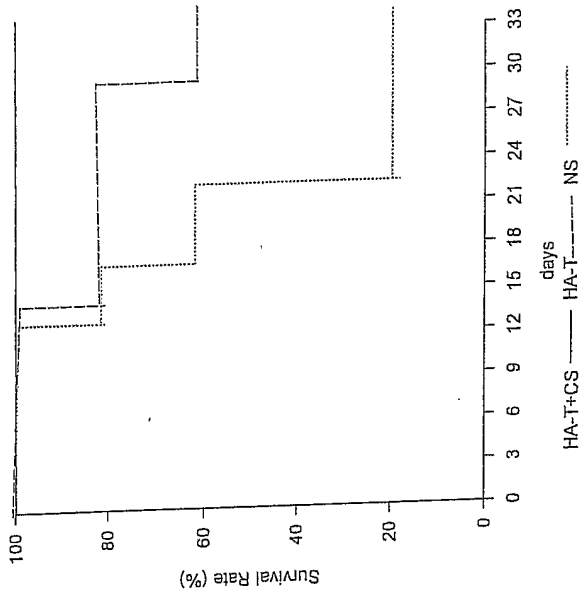
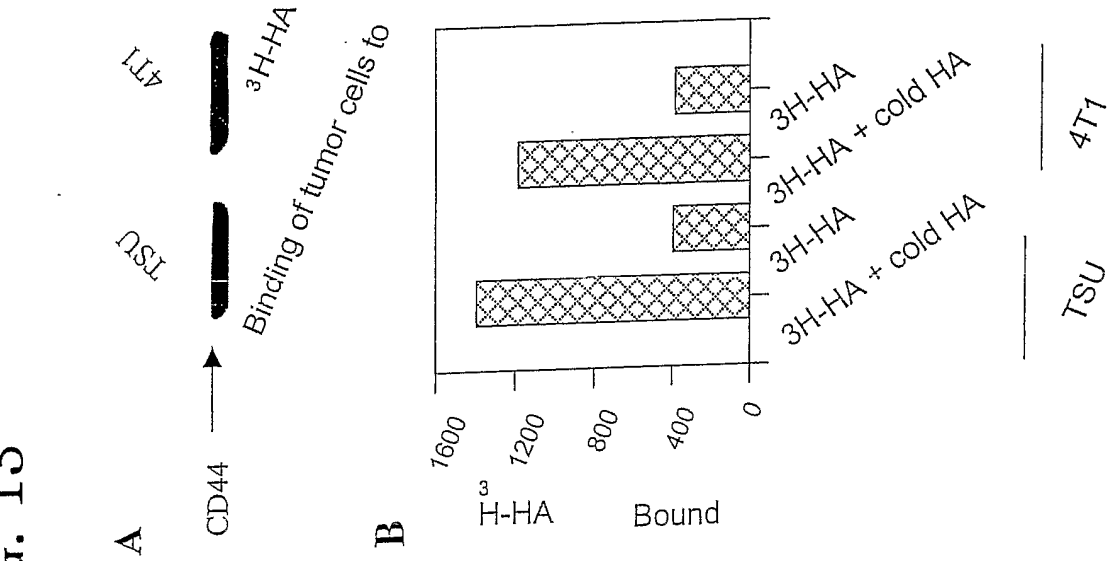

Survival Rate (%)
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24 27 30 33
days
HA-T+CS
HA-T
NS

FIG. 12

FIG. 13


A
CD44
TSU
4T1
^{3}H-HA
Binding of tumor cells to
B
^{3}H-HA Bound
1600
1200
800
400
0
3H-HA
3H-HA + cold HA
3H-HA
3H-HA + cold HA
TSU
4T1
C
TSU
Degraded ^{3}H-HA (cpm)
2000
1500
1000
500
0
3H-HA
3H-HA+HA
3H-HA+KM201
3H-HA+Chloroq
D
4T1
Degraded ^{3}H-HA (cpm)
1600
1200
800
400
0
3H-HA
3H-HA+HA
3H-HA+KM201
3H-HA+Chloroq

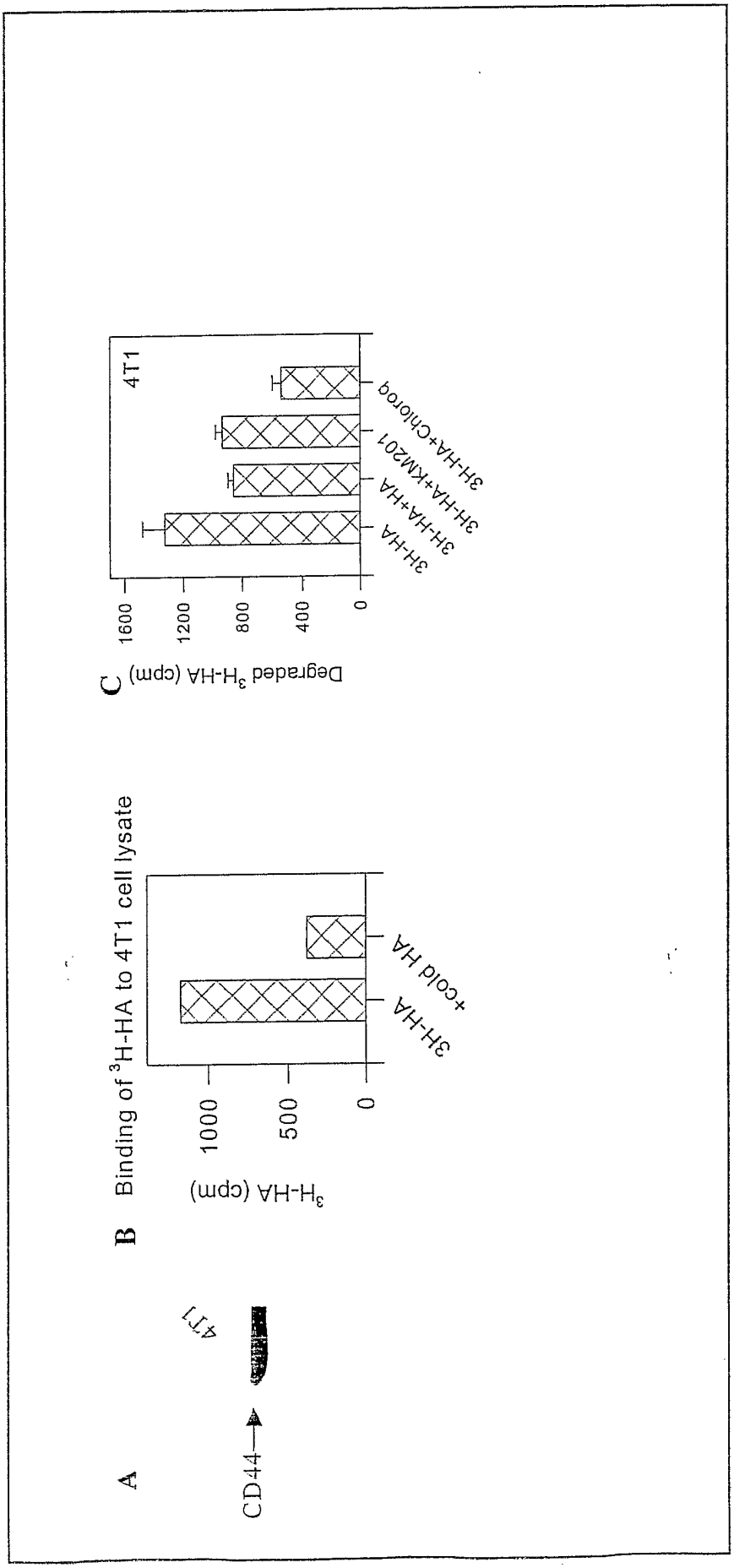
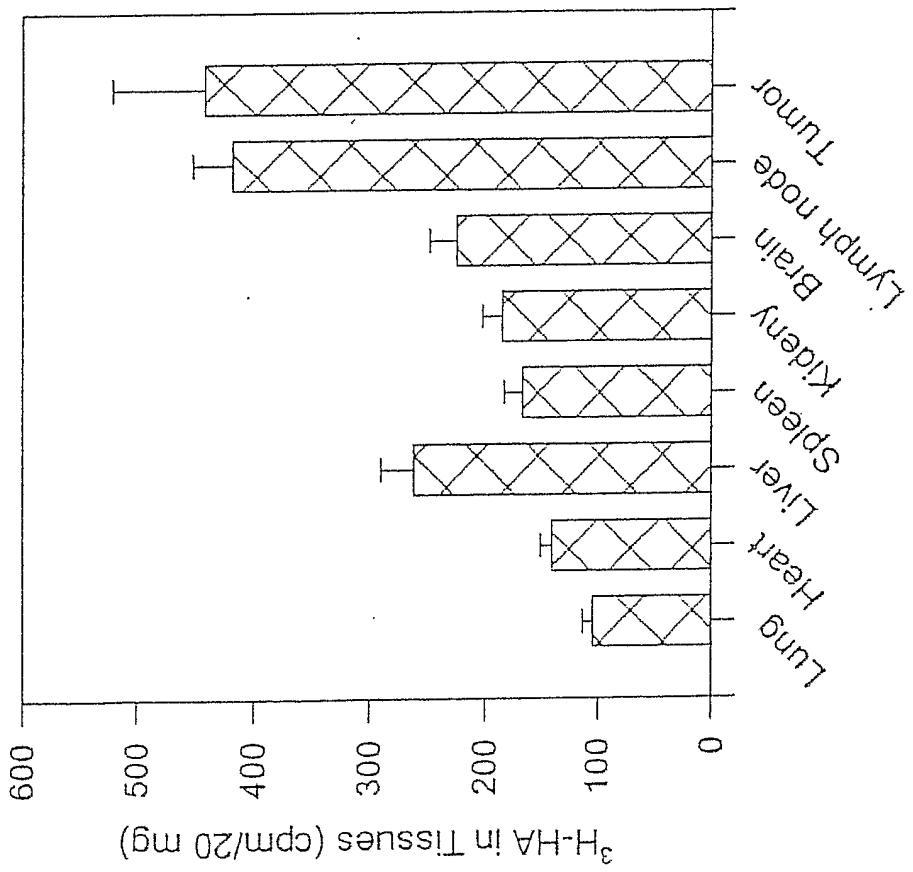

A
CD44
4T1
B
Binding of ^{3}H-HA to 4T1 cell lysate
^{3}H-HA (cpm)
1000
500
0
3H-HA
+cold HA
C
Degraded ^{3}H-HA (cpm)
4T1
1600
1200
800
400
0
3H-HA
3H-HA+HA
3H-HA+KM201
3H-HA+Chloroq

FIG. 14

FIG. 15


^{3}H-HA in Tissues (cpm/20 mg)
0
100
200
300
400
500
600
Lung
Heart
Liver
Spleen
Kideny
Brain
Lymph node
Tumor

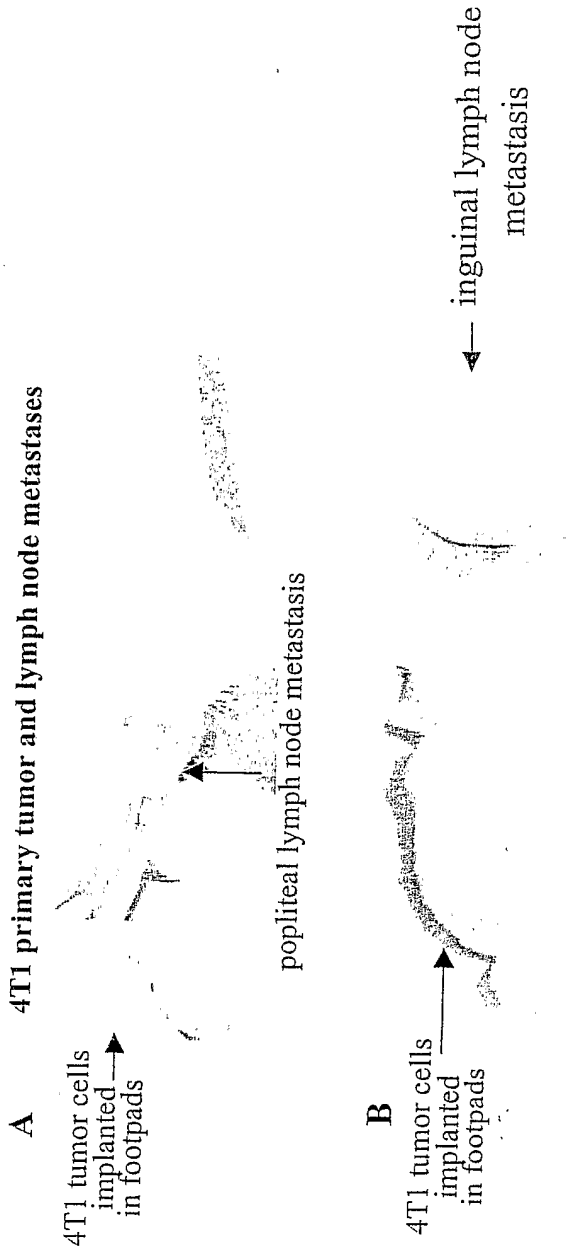

A
4T1 primary tumor and lymph node metastases
4T1 tumor cells implanted in footpads
popliteal lymph node metastasis
B
4T1 tumor cells implanted in footpads
inguinal lymph node metastasis

FIG. 16

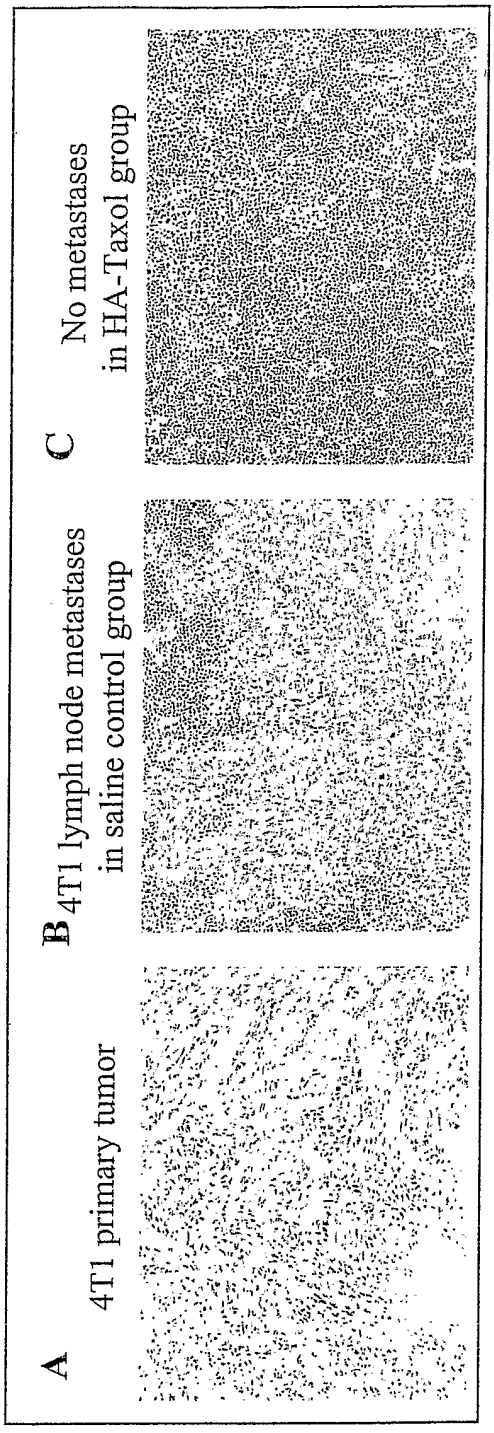

A
4T1 primary tumor
B
4T1 lymph node metastases
in saline control group
C
No metastases
in HA-Taxol group

FIG. 17