Paclitaxel–Hyaluronic NanoConjugates Prolong Overall Survival in a Preclinical Brain Metastases of Breast Cancer Model

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Abstract

Brain (central nervous system; CNS) metastases pose a life-threatening problem for women with advanced metastatic breast cancer. It has recently been shown that the vasculature within preclinical brain metastasis model markedly restricts paclitaxel delivery in approximately 90% of CNS lesions. Therefore to improve efficacy, we have developed an ultra-small hyaluronic acid (HA) paclitaxel nanoconjugate (~5 kDa) that can passively diffuse across the leaky blood–tumor barrier and then be taken up into cancer cells (MDA–MB–231Br) via CD44 receptor-mediated endocytosis. Using CD44 receptor-mediated endocytosis as an uptake mechanism, HA-paclitaxel was able to bypass p-glycoprotein–mediated efflux on the surface of the cancer cells. In vitro cytotoxicity of the conjugate and free paclitaxel were similar in that they (i) both caused cell-cycle arrest in the G2–M phase, (ii) showed similar degrees of apoptosis induction (cleaved caspase), and (iii) had similar IC50 values when compared with paclitaxel in MTT assay. A preclinical model of brain metastases of breast cancer using intracardiac injections of Luc-2 transfected MDA–MB–231Br cells was used to evaluate in vivo efficacy of the nanoconjugate. The animals administered with HA–paclitaxel nanoconjugate had significantly longer overall survival compared with the control and the paclitaxel-treated group (P < 0.05). This study suggests that the small molecular weight HA–paclitaxel nanoconjugates can improve standard chemotherapeutic drug efficacy in a preclinical model of brain metastases of breast cancer. Mol Cancer Ther; 12(11); 2389–99. ©2013 AACR.

Introduction

The incidence of brain metastases is increasing and represents an emerging cause of mortality in women with metastatic breast cancer (1, 2). Current therapeutic options involving chemotherapy, surgery, and/or radiation have generally had limited efficacy in eradicating metastatic lesions (3, 4). A major reason for the failure of chemotherapy is that the normal blood–brain barrier (BBB) and the resultant blood–tumor barrier (BTB) limit drug distribution to subtherapeutic levels (5, 6). To overcome poor drug distribution to metastatic lesions, we report here the formulation of a small hyaluronic acid (HA) paclitaxel nanoconjugate that uses a passive targeting mechanism to increase drug distribution into the tumor interstitial space and an active targeting mechanism to improve the intracellular uptake of paclitaxel into the metastatic cancer cells.

The intact BBB is the vascular interface between the blood and the brain, and protects the central nervous system (CNS) from harmful toxins, by acting as a physical, enzymatic, and transport barrier. This barrier functionally limits brain penetration of many small and large therapeutic molecules (7). It has been suggested that more than 98% of CNS drugs fail to go into clinical trials due to poor brain penetration (8). In contrast, the BTB (the vasculature within and potentially immediately adjacent to a brain metastasis) is generally more permeable than the BBB. In our previous work (5), it was observed that BTB in metastatic lesions is permeable enough to allow initial paracellular diffusion of a approximately 4 kDa dextran into brain metastases.

To take advantage of the increased paracellular permeability, we have conjugated paclitaxel to an approximately 3 to 5 kDa polymerized HA, which mimics the size of the dextrans that were able to penetrate the metastatic lesions (5). We hypothesize that this polymer conjugate of paclitaxel will increase drug concentrations...
in the metastatic lesions by (i) allowing paracellular diffusion of the paclitaxel conjugate from blood through the BTB openings to the tumor interstitial space and (ii) paclitaxel will no longer be subject to various BBB and BTB efflux transporters [e.g., p-glycoprotein (P-gp), breast cancer resistance protein (BCRP)] that normally limit paclitaxel brain and tumor distribution (9, 10).

We hypothesize that once the paclitaxel conjugate is in the tumor interstitial space, the ultra-small nanoconjugate (~2–3 nm) actively targets the metastatic cancer cell by CD44 receptor-mediated endocytosis (11, 12). CD44 (clusters of differentiation 44) is a cell surface receptor that binds to hyaluronic acid and undergoes receptor-mediated endocytosis to internalize hyaluronic acid (13). CD44 has been shown to be overexpressed in many tumors including breast and metastatic brain (14–18). The polymeric hyaluronic acid exhibits a number of properties as an ideal drug carrier system. Hyaluronic acid is water soluble, biocompatible, and as a polysaccharide, it has multiple different functional groups for potential chemical conjugation (11, 19–21) with numerous chemotherapeutic agents such as doxorubicin and paclitaxel (22–25). Consequently, we propose the use of hyaluronic acid to target CD44-mediated endocytosis as a novel BTB transit pathway (Fig. 1).

In this report, we show that the synthesized HA–paclitaxel nanoparticulate conjugate is (i) able to bypass P-gp efflux transporters at the BTB and the 231Br metastatic cancer cells, (ii) internalized into tumor cells via CD44 receptor-mediated endocytosis, (iii) causing G2–M cell-cycle arrest and cytotoxicity at levels similar to free paclitaxel, and (iv) significantly increasing the median survival time in a preclinical model of triple-negative brain metastasis of breast cancer.

Figure 1. Schematic diagram of HA–paclitaxel nanoconjugates hypothetical mechanism of action using CD44 receptor-mediated endocytosis.
Materials and Methods

Chemicals
Ultra-low molecular weight hyaluronic acid was purchased from R&D Systems (~4–5 kDa). 14C-paclitaxel (specific activity 75.6 mCi/mmol) was purchased from Moravek Biochemicals Inc. Paclitaxel and Texas Red (TX Red) hydrazide were purchased from Molecular Probes Invitrogen. Cyclosporin A was purchased from Toronto Research Chemicals Inc. 2-Chloro-1-methyl pyridinium iodide, triethylamine, 4-dimethyl aminopyridine (DMAP), dicyclohexyl carbodiimide (DCC), poly (ethylene glycol) dimethyl ether (dmPEG; MW = 2,000 Da), Cremophor EL, and anhydrous dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. All other chemicals used were of analytic grade and used as supplied.

Cell culture
Human breast cancer cells, MCF-7, and P-gp overexpressing, adriamycin-resistant, MCF-7/AdrR cells were kindly donated by Dr. U.S. Rao (Texas Tech University Health Sciences Center, Amarillo, TX; refs. 26, 27). The cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% (v/v) FBS, and antibiotics (penicillin, 100 U/mL; and streptomycin, 100 μg/mL). Luc-2–transfected human MDA-MB-231Br cells were kindly provided by Dr. Patricia Steeg (National Cancer Institute, Bethesda, MD; not authenticated by our laboratory). The cells were maintained in DMEM, supplemented with 10% (v/v) FBS. Cells were grown in a 37°C humidified incubator with 5% CO2.

Western blot analysis
The protein concentrations after lysis of cells were quantified using a BCA Pierce Assay Kit (Pierce Chemical Co.). Protein samples (20 μg/lane) were resolved by SDS-PAGE and transferred to polyvinylidyene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat dry milk for 1 hour, after which, membranes were incubated with anti-CD44 antibody at 4°C overnight. Membranes were then washed and incubated with respective HRP-conjugated secondary antibodies. Proteins were visualized using SuperSignal West Pico Chemiluminescence system (Pierce Chemical Co.).

Immunocytochemistry
Cells were grown to approximately 70% confluency on tissue culture–treated cover slips. Cells were fixed with ice-cold acetone methanol (1:1, v/v), washed with PBS, and then incubated with 5% nonfat dry milk for 1 hour. Then, the cells were incubated overnight with an anti-CD44 antibody (Cell Signaling Technology) at a dilution of 1:200. The primary antibody was removed by repetitive washings with PBS. Afterwards, cells were incubated with nuclear stain 4’,6-diamidino–2–phenylindole (DAPI) and Alexa Fluor–conjugated secondary antibodies (Molecular Probes, Invitrogen) for 1 hour. Fluorescent images were taken with an Olympus IX81 microscope (Olympus America Inc.).

Conjugation of hyaluronic acid with paclitaxel or TX Red
Paclitaxel was conjugated to hyaluronic acid using a previously published method (Fig. 2; 28). Initially, a nanocomplex between hyaluronic acid and dmPEG was

Figure 2. Chemical structure and synthetic scheme for HA–paclitaxel. Briefly, in the first step, the sodium hyaluronic acid was desalted using dialysis against water. In the second step, a nanocomplex was formed between hyaluronic acid and dmPEG. In the last step, the HA–dmPEG complex was conjugated to paclitaxel using a carbodiimide reaction.
formed (28). The hyaluronic acid polymer used was purified and desalted, in which 1 g of hyaluronic acid (~4–5 kDa) was dissolved in 100 mL of deionized water, and dialyzed for 24 hours Molecular Weight Cut Off (MWCO) = 1kDa, after which the sample was lyophilized. Then the desalted hyaluronic acid, and dmPEG (MW = 2,000 Da; at a molar ratio of 1:10) were dissolved in deionized water and stirred for 8 hours and then lyophilized to give a white cotton-like product. Then the HA/dmPEG was conjugated to paclitaxel using DMAP/DCC as a coupling agent. The hydroxyl groups of paclitaxel were conjugated to the carboxylic groups of hyaluronic acid to form an acid-cleavable ester linkage. HA/dmPEG, DCC, and DMAP were added to DMSO and the resulting solution was stirred at room temperature for 1 hour. Afterwards, paclitaxel in DMSO was added to the above mixture and stirred at room temperature for 1 hour. Afterwards, paclitaxel was conjugated to hyaluronic acid was activated with 2-chloro-1-methyl hydrazide (5 mg in 1 mL of DMSO) was added and the mixture was refluxed for 24 hours, followed by 48-hour dialysis against water, and the resultant compound was lyophilized. The yield of conjugates was approximately 60%. Particle size of HA–paclitaxel conjugate was measured by dynamic light scattering using a Malvern Zetasizer Nano ZS90 (Malvern Inc.). The size measurement was carried out at a concentration of 7.0 mg/mL of conjugate in PBS at room temperature. 1H–NMR spectra of HA–paclitaxel conjugate was obtained by Bruker nuclear magnetic resonance (NMR) spectrometer (Bruker) using deuterated DMSO-d6 (Supplementary Fig. S1). The amount of paclitaxel conjugated to hyaluronic acid was determined by high-performance liquid chromatography (HPLC) using a 50:50 mixture of acetonitrile and water as cosolvent. Eluted compounds were detected at 227 nm using a UV–Vis detector. Free paclitaxel was used as standard to generate a standard curve and the paclitaxel conjugated to hyaluronic acid was determined by counting the dpms (disintegrations per minute) using scintillation counter. Uptake of14C–paclitaxel was normalized with respect to total protein content determined using Bradford Assay.

Cellular uptake and competitive inhibition studies

For competitive inhibition studies, the MDA-MB-231Br cells were incubated in 2 mL of serum-free media containing hyaluronic acid (0.625 μmol/L) with hyaluronic acid conjugates for 90 minutes. For fluorescent microscopy studies with HA–TX Red, the cells were washed twice in PBS and fixed with ice-cold acetone:methanol (50:50). The intracellular accumulation of HA–TX Red was visualized using an Olympus IX81 stereo microscope. The hydrophilic properties of HA–paclitaxel conjugates, after incubation period, the cells were washed in PBS, followed by lysis in radioimmunoprecipitation assay (RIPA) lysis buffer. The radioactivity in cell lysates was determined using a Beckman liquid scintillation counter. To determine if the formulation bypassed P-gp efflux; P-gp overexpressing cells, grown in 12-well plates, were used to determine the cellular uptake of14C–paclitaxel and 14C–HA–paclitaxel. Cells were washed three times with PBS, then incubated with or without 10 μmol/L cyclosporin A (a classical p-gp inhibitor) for 30 minutes in serum-free media. Then the tracers were added and further incubated for 60 minutes. The cells were washed three times with ice-cold PBS, and cell lysis was accomplished using lysis buffer. The amount of tracer in cell lysates was determined using a Beckman liquid scintillation counter with appropriate correction for quench, background, and efficiency. An aliquot of cell lysate was used to determine protein content.

Receptor-mediated endocytosis of HA–paclitaxel nanoconjugates in 231Br cells

231Br cells were seeded in 12-well plates at a density of 5 × 10⁴ cells per well and incubated for 24 hours. After checking for confluency, media was aspirated from the wells and cells were washed with PBS three times. The cells were incubated with different endocytic inhibitors [methyl-β-cyclodextrin (MβCD; 5 mmol/L), chlorpromazine hydrochloride (10 μg/mL), and nystatin (25 μg/mL)], for 30 minutes. Then, 14C–HA–paclitaxel was added to each well and incubated for 60 minutes. After incubation, treatment solutions were aspirated and cells were washed three times with ice-cold PBS. Then the cells were lysed with RIPA lysis buffer and an aliquot of lysate was transferred to scintillation vial containing scintillation fluid. Amount of 14C–HA–paclitaxel present in the lysate was determined by counting the dpms (disintegrations per minute) using scintillation counter. Uptake of 14C–HA–paclitaxel was normalized with respect to total protein content determined using Bradford Assay.

Cell-cycle analysis using flow cytometry

231Br cells were grown to approximately 70% confluence and treated with equimolar concentrations of either paclitaxel or HA–paclitaxel for 24 hours. After treatment, cells were trypsinized, centrifuged, washed twice with PBS, and then fixed with 70% ice-cold ethanol for 24 hours. Fixed cells were washed twice in PBS to remove excess ethanol, after which, cells were incubated at 37°C with DNA staining solution containing propidium iodide (50 μg/mL). Cells were analyzed with a fluorescence-activated cell sorting (FACS) Scanner flow cytometer (BD Biosciences).

In vitro cytotoxicity

231Br cells were seeded at a density of 10⁴ cells per well in 48-well plates. Cytotoxicity was determined by treating the cells with equimolar concentrations of either paclitaxel or HA–paclitaxel for 48 hours. Cell viability was determined using MTT assay. Sixty microliter (2mg/mL) of...
dimethyl thiazolyl diphenyl tetrazolium salt (MTT) in 1× PBS was added to the cells, which were incubated for an additional 4 hours at 37°C. Afterward, the media was removed and 250 μL DMSO was added to each well to dissolve the formazan crystals. The absorption was measured at 570 nm using a BioTek Instrument ELx800 microplate reader. Each sample was prepared in triplicate and the data were reported as mean ± SEM. The percentage cell viability of each sample was determined relative to the control (untreated) cells.

**In vivo efficacy of HA–paclitaxel nanoconjugates**

The antitumor activity of the conjugate was determined in a preclinical model of brain metastasis of breast cancer. The 231Br breast cancer cell line was previously selected for brain tropism on the basis of multiple rounds of in vivo passage in immune-compromised mice (1, 30). To develop brain metastases, immune compromised NuNu female mice were anesthetized with isoflurane (0.5%–2%) and inoculated with 1.75 × 10⁷ human 231Br/100 μL into the left cardiac ventricle in serum-free media using a stereotaxic apparatus (Stoelting). Tumors seeded the brain and developed lesions over ~2 to 6 weeks, or until neurologic symptoms appeared. Three days after the tumor cell injection, animals were randomly divided into one of the three treatment groups: control, paclitaxel, or HA–paclitaxel. The animals were treated with vehicle alone, or paclitaxel, or HA–paclitaxel [6 mg/kg (paclitaxel equivalent), once a week, intravenously for five doses]. Paclitaxel was dissolved in a vehicle composed of a 1:1 blend of Cremophor EL (polyethoxylated castor oil) and ethanol was then diluted (seven parts of saline to three parts of blend) with normal saline for administration. HA–paclitaxel was dissolved in normal saline. All the formulations were prepared freshly on the day of dosing. Animals were monitored over a period of 90 days for neurologic symptoms (within 90-day window), animals were sacrificed and animals were imaged for bioluminescent signal to confirm presence of metastases in the brain.

**Statistical analysis**

Data in all experiments represent mean ± SEM. Experiments were analyzed using ANOVA followed by Bonferroni multiple comparison test. For the survival data, 24-hours after intracardiac injection, animals were verified to have bioluminescent signal from brain and were then randomized to the three groups. A Kaplan–Meier curve was obtained and a log-rank value was determined. A significance level of P < 0.05 was used for all experiments (GraphPad Prism 5 software).

**Results**

**CD44–HA as a novel BTB transit pathway**

CD44 is an extensively studied adhesion molecule that binds hyaluronic acid found in the extracellular matrix (11). Once hyaluronic acid is bound to the CD44 receptor, CD44 undergoes receptor-mediated endocytosis to internalize hyaluronic acid (13). In this study, the ability of 231Br cells to internalize the approximately 3 to ~5 kDa HA–paclitaxel conjugate through the CD44 receptor–mediated endocytosis pathway was investigated as a novel method to improve the uptake of paclitaxel. CD44 was observed to have a relatively high degree of expression in 231Br cells (Fig. 3A and B). Furthermore, this cellular phenotype was retained in vivo in brain metastatic lesions (Fig. 3C). Consequently, we hypothesized that conjugation of hyaluronic acid to normally impermeant molecules would permit their uptake into brain metastases.

After we observed the presence of CD44 in our model system, we conjugated hyaluronic acid to TX Red, a sensitive marker of BBB and BTB permeability (5), to quantify uptake of hyaluronic acid polymers within the 231Br cells. Fluorescent conjugates of hyaluronic acid were synthesized by condensation of TX Red hydrazide to hyaluronic acid. The HA-TX Red conjugate accumulated in the 231Br cells over a 90-minute incubation (Fig. 4A). Cellular accumulation of the HA-TX Red conjugate was inhibited by approximately 50% with the addition of 0.625 μmol/L of unlabeled large molecular weight (1,600kDa) hyaluronic acid [P < 0.05; (−)HA, 100.0% ± 34.4%; (+)HA, 48.4% ± 25.2%] due to competitive inhibition at the CD44 receptor (Fig. 4A; left bars).

To determine if the HA–CD44 interaction was capable of delivering therapeutically relevant concentrations of drugs into experimental brain metastases, similar studies were conducted with hyaluronic acid conjugated to paclitaxel. The HA/dmPEG was conjugated to paclitaxel using DMAP/DCC as a coupling agent (Fig. 2). The HA–¹³C–paclitaxel conjugate accumulated in the metastatic cells and was inhibited by the presence of free hyaluronic acid [Fig. 4A; right bars; P < 0.05; (−)HA, 100.0% ± 31.2%; (+)HA, 51.8% ± 4.2%] due to competitive
inhibition. These data suggest HA-conjugation facilitates cellular uptake via receptor-mediated endocytosis of both TX Red and paclitaxel.

**Mechanisms of cellular internalization**

To address the mechanisms of cellular internalization of the HA–paclitaxel conjugate, we observed the uptake of HA–14C–paclitaxel in 231Br cells for 60 minutes in the presence of three endocytosis inhibitors, MJFCD (5 mmol/L), chlorpromazine hydrochloride (10 μg/mL), and nystatin (25 μg/mL). In separate experiments, the inhibition of clathrin-mediated endocytosis (31) with chlorpromazine hydrochloride (10 μg/mL) and the inhibition of caveolae-mediated uptake with nystatin (25 μg/mL; ref. 32) did not alter the cellular uptake of 14C–HA–paclitaxel. However, inhibition of the CD44 lipid raft pathway (33) by MJFCD (5 mmol/L; ref. 34) reduced the cellular uptake of 14C–HA–paclitaxel by approximately 50% compared with control (Fig. 4B, P < 0.05). The data further confirm that an active mechanism is present that facilitates uptake of the HA–paclitaxel conjugate into the cell.

To test whether the hyaluronic acid conjugates would also bypass P-gp, the uptake of 14C-paclitaxel and HA–14C–paclitaxel in P-gp-overexpressing cells in the presence or absence of the P-gp inhibitor cyclosporine A was determined. Figure 5 shows that 14C–paclitaxel uptake in 231Br cells is significantly (~7–8 fold; P < 0.0001) increased in the presence of cyclosporine A (~ cyclosporin A (0.97 ± 0.45 μCi/g); (+)cyclosporin A (6.5 ± 1.7 μCi/g)). Furthermore, the uptake of HA–14C–paclitaxel is significantly (P < 0.05) higher than the 14C–paclitaxel control (~12-fold: 11.6 ± 2.1 μCi/g) and is not further increased by the addition of cyclosporine A (Fig. 5). This suggests that the hyaluronic acid conjugate bypasses P-gp and may enhance delivery of paclitaxel in metastatic brain lesions.

**HA-paclitaxel conjugates brain metastatic cells cytotoxicity**

Paclitaxel acts by binding and stabilizing the microtubules, which ultimately results in a G2-M arrest of the cell-cycle (35, 36). To determine whether HA–paclitaxel retained the same chemotherapeutic activity as paclitaxel, a cell-cycle analysis was performed for both compounds using flow cytometry. Briefly, 231Br cells were treated with equimolar paclitaxel (100 nmol/L) concentrations of either paclitaxel or HA–paclitaxel for 24 hours, after which, the cells were stained with propidium iodide and DNA content was analyzed. As shown in Fig. 6A, both paclitaxel and HA–paclitaxel caused significant (n = 3, P < 0.05) cell-cycle arrest at the G2–M phase (70.0% ± 12.2% and 56.0% ± 10.2% of cells respectively) as compared with control (17.0% ± 7.0%). Furthermore, after paclitaxel and HA–paclitaxel treatment, there was a similar degree of apoptosis as evidenced by cleaved caspase-3 staining (data not shown). Paclitaxel and HA–paclitaxel had similar (P > 0.05) in vitro cytotoxicity toward the 231Br breast cancer cell line with an IC50 of 3.3 nmol/L for HA–paclitaxel and 4.4 nmol/L for free paclitaxel (Fig. 6B).

**In vivo efficacy of HA–paclitaxel**

In the final set of experiments, the HA–paclitaxel conjugate was evaluated for in vivo efficacy in a preclinical model of brain metastases of breast cancer. One day after the intracardiac injection of the 231Br cell lines, animals were imaged using bioluminescence to verify the presence of metastatic cells in the brain region (Supplementary Fig. S2). After verification, animals were randomized to one of three groups: vehicle, intravenous paclitaxel (6 mg/kg), or intravenous HA–paclitaxel (6 mg/kg paclitaxel equivalent). Animals were treated weekly for five doses. In the animals receiving the HA–paclitaxel conjugate, overall survival was longer compared with control (P = 0.0345, by log-rank test) and paclitaxel-treated groups (P = 0.0283, by log-rank test). Similarly, lesion burden in brain was significantly decreased in the HA–paclitaxel group compared with the control and paclitaxel groups (P < 0.05) using bioluminescence imaging (Fig. 7A). The median survival time was 37, 42, and 49 days for control, paclitaxel, and HA–paclitaxel respectively (Fig. 7B). Terminal bioluminescence imaging was verified by histology in animals (representative image shown in supplementary Fig. S3).
Discussion

Brain metastases pose a significant problem for women with advanced breast cancer. Younger age HER-2 and triple-negative status may be the greatest risk factor for CNS metastasis development (37). Triple-negative patients who develop CNS metastases have approximately one half the time between initial diagnosis and cerebral relapse (22 vs. 51 months), and a significantly decreased survival time after initial diagnosis (4 vs. 8–15 months; ref. 37). Although there has been work completed on developing new drugs to treat brain metastases of breast cancer, clinical trials of standard, novel, and combinatorial chemotherapeutic regimens have been consistently disappointing. One aspect of this problem is that the brain remains, at least in part, a sanctuary site by the continued presence of some fraction of the BBB. New methods to traverse the BBB may hold widespread promise for the development of effective brain metastasis treatments and preventatives.

Herein, we present data showing that the widely expressed receptor-ligand pair CD44 and hyaluronic acid may be engineered small enough to pass through the leaky vasculature of the BTB and still retain the ability to be taken up into cancer cells via receptor-mediated endocytosis. Furthermore, the HA–paclitaxel nanoconjugate bypasses P-gp efflux mechanism in 231Br cells and accumulates intracellularly to a greater degree than free paclitaxel. Ultimately, the HA–paclitaxel inhibits the cell-cycle at G2–M phase, induces apoptosis and induces cytotoxicity at similar concentrations of free paclitaxel. In vivo efficacy studies showed that the median survival of mice improved significantly with HA–paclitaxel in breast metastasis to brain tumor model.

This novel formulation facilitates paclitaxel accumulation in 231Br cells through receptor-mediated endocytosis and bypasses P-gp efflux transporters present on the cell membrane. Similar to other tumor cell lines, the 231Br cell line overexpresses CD44 (38, 39) and is able to internalize a HA–drug conjugate. This type of active targeting has been used previously to increase cellular uptake of doxorubicin-loaded liposomes (28, 41–43). However, this work is different from most prior studies, in that the other studies used high molecular weight hyaluronic acid (75 kDa) as the drug carrier. We chose to use the smaller molecular weight hyaluronic acid as our previous work showed that in the preclinical model an approximately 4-kDa dextran was able to accumulate in numerous metastatic lesions, but the 70-kDa dextran had a more limited penetration (5). Importantly, the size of our formulation was observed to be in two distinct populations:

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**Figure 4.** HA–TX Red and 14C–HA–paclitaxel accumulate in 231Br cells via CD44 receptor-mediated endocytosis. A, the in vitro uptake of HA–TX Red and 14C–HA–paclitaxel was reduced approximately 50% by the presence of free 1,600 kDa hyaluronic acid (0.625 μmol/L), which effectively competes for CD44 receptor-mediated endocytosis. B, pharmacological inhibition of clathrin-mediated endocytosis by incubation with chlorpromazine hydrochloride (10 μg/mL) for 30 minutes, and caveolae-mediated endocytosis uptake by 25 μg/mL nystatin did not inhibit HA–paclitaxel uptake into 231Br cells (P > 0.05). However, inhibition of the lipid raft endocytosis pathway with 5 mmol/L MβCD significantly reduced the uptake of 14C–HA–paclitaxel into 231Br cells as compared with control (P < 0.01). These data suggest that the conjugate is actively taken into 231Br cells via CD44 mediated endocytosis. Data represent Mean ± SEM; n = 6. *, P < 0.05; **, P < 0.01; ***, P < 0.001. ns, not significant, P > 0.05.
one of 2 to 3 nm and the other at approximately 80 nm. We believe that the smaller conjugates can self-assemble into larger particles similar in structure to a lipid membrane. On the basis of previous work, it is unlikely that the larger particles enter into the brain metastases. It is likely that the single molecules, as well as single molecules released from the aggregates are the active component and responsible for the effect.

One concern in using the lower molecular weight hyaluronic acid is the binding affinity of hyaluronic acid to CD44, which depends on the number of disaccharides present on the hyaluronic acid molecule (44). However, our data showed that the ultra-low molecular weight hyaluronic acid polymer used in this study to conjugate to paclitaxel retained the ability to be internalized into the cell via receptor-mediated endocytosis. These data are consistent with previous studies showing oligomers of hyaluronic acid with as low as 4 to 14 sugar residues, were still able to undergo receptor-mediated endocytosis (44).

Paclitaxel has low water solubility (13) and has limited brain distribution primarily due to the BBB efflux transporter, P-gp (10, 45, 46). Several strategies have been used to circumvent P-gp at BBB, including chemical modification, (47) encapsulating in nanoparticles (48), and conjugating to peptide vectors (49–52). Our data suggest that HA–paclitaxel bypasses P-gp, and the cellular uptake of the conjugate is approximately 10-fold greater than that of free paclitaxel. These data are in agreement with prior reports, in which the conjugation of drugs such as doxorubicin and paclitaxel to peptides resulted in decreased affinity of the compound for P-gp at BBB (52, 53). For example, ANG1005, a conjugate of angiopep-2 with three molecules of paclitaxel showed approximately 5-fold increase in brain uptake than free paclitaxel in P-gp knockout mice (52, 54). Similarly, when doxorubicin was conjugated to a peptide (penetratin), the brain parenchymal accumulation was increased approximately four to five times greater than doxorubicin (55).

![Figure 5. HA–paclitaxel bypasses P-gp-mediated efflux of paclitaxel. In control experiments, an approximately 8-fold increase in 14C-paclitaxel cellular accumulation was observed in the presence of the P-gp inhibitor cyclosporine A (10 μmol/L) in 30 minutes in vitro uptake experiments (left bars). In contrast, cellular uptake of 14C-HA-paclitaxel was approximately 11-fold greater than control 14C-paclitaxel uptake and was not reduced by the presence of 10 μmol/L cyclosporine A (right bars). Data represent Mean ± SEM; n = 6. ***, P < 0.001 (ANOVA).](image)

![Figure 6. Paclitaxel and HA–paclitaxel exert similar cytotoxicity profiles. A, representative cell-cycle analysis graphs of control, paclitaxel- (100 nmol/L) treated and HA–paclitaxel- (100 nmol/L paclitaxel equivalent) treated 231Br cells. Both paclitaxel and HA–paclitaxel caused significant (n = 3, P < 0.05) cell-cycle arrest at the G2–M phase (70% ± 12% and 56% ± 10% of cells, respectively) as compared with control (17% ± 7%). B, in vitro cytotoxicity of HA–paclitaxel and paclitaxel in 231Br cells. 231Br cell cultures were incubated with paclitaxel or HA–paclitaxel for 48 hours at concentrations ranging from 0.1 nmol/L to 10 μmol/L. The number of viable cells at 48 hours was determined using MTT assay. Data represent mean ± SEM; n = 6 for all points. IC50 was calculated using log dose-response curve.](image)
Paclitaxel stabilizes microtubules and interferes with the G₂ and M phases of the cell-cycle (35, 36, 56). Our data show that HA–paclitaxel causes a similar arrest at the G₂–M phase of cell-cycle. Furthermore, the HA–paclitaxel conjugate had a slightly lower IC₅₀ in the 231Br cells than that of free paclitaxel. From these data, we speculate that HA–paclitaxel retains similar cytotoxicity mechanisms as that of paclitaxel. This may be possible as the HA–conjugate is designed to be cleaved by cytosolic hyaluronase enzymes (57) into hyaluronic acid and free paclitaxel, which has the ability to act on microtubules. It is entirely possible the HA–paclitaxel conjugate can act on the microtubules without being enzymatically cleaved.

The overarching goal of this work was to determine if our ultra-small nanoconjugate would have efficacy in a preclinical triple-negative brain metastases of breast cancer model. In agreement with our previous reports (5) paclitaxel did not improve the median survival time (control: 37 days; paclitaxel: 42 days; P = 0.4524). However, as shown in Fig. 7B HA–paclitaxel significantly improved the overall survival time from 37 days to 49 days (P = 0.035). To best of our knowledge, this is the first report to show the in vivo efficacy of HA–paclitaxel bioconjugate in a preclinical model of brain metastases of breast cancer. It is of interest, that 24 hours after intracardiac injection of the Luc-2 transplanted 231Br cells, most animals had luminescent signal only in the brain region, whereas a few animals had bioluminescent signal in the kidney, liver, and bladder region. We speculate that the 231Br cells when injected, rapidly seed the brain (within 24 hours) and most if not all remaining metastatic cells are cleared from the body.

The results presented in this article suggest that low molecular weight hyaluronic acid could be effective in delivering drug to brain metastases of breast cancer. Further work should be completed to determine if this platform is effective in the delivering other chemotherapeutics or potentially combinatorial therapy. These data could be used in the development of other preclinical studies evaluating pharmacokinetics and toxicity.

**Disclosure of Potential Conflicts of Interest**

Q.R Smith is a consultant/advisory board member of AngioChem. No potential conflicts of interest were disclosed by the other authors.

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