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CD44-Targeted Microparticles for Delivery of Cisplatin to Peritoneal Metastases

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Abstract: Intraperitoneal (ip) chemotherapy increases the survival of optimally debulked patients with ovarian cancer due to direct access of the drug to tumor nodules growing on the peritoneal surface. CD44 is overexpressed in many ovarian cancers. To further improve efficacy, we sought to develop a cisplatin-loaded microparticle that would target to CD44 on cancer cells when injected ip. Hyplat microparticles were produced by cross-linking hyaluronan via its carboxylate groups with cisplatin at a high temperature. Hyplat particles had an average diameter of 580 nm, and cisplatin was incorporated with an efficiency of ~50%. Drug release varied with chloride concentration but not pH. Flow cytometric analysis and confocal microscopy confirmed that CD44 positive cells (OV2008, A2780) internalized Hyplat more efficiently than CD44 negative cells (UCI101); uptake was compromised by knocking down CD44 expression. Clearance of Hyplat from the mouse peritoneum was reduced by 7-fold and tumor uptake was increased by 2- to 3-fold in CD44-positive but not CD44-negative tumor models compared to that attained with free cisplatin. Hyplat was more effective than cisplatin at slowing the growth of intraperitoneally inoculated A2780 ovarian cancer cells and improving survival thus demonstrating the potential of Hyplat to enhance the efficacy of ip chemotherapy.

Keywords: Intraperitoneal metastasis; hyaluronan-cisplatin microparticles; ovarian cancer; CD44

1. Introduction

Ovarian carcinomas characteristically spread primarily throughout the peritoneal cavity where they form large numbers of micro- and macroscopic tumor nodules on the surface of the peritoneum. Multiple randomized trials have demonstrated that intraperitoneal (ip) therapy with cisplatin and paclitaxel can improve survival in patients with optimally debulked advanced ovarian cancer.[1−8] The key pharmacologic principle of this approach is that, when cisplatin and paclitaxel are instilled via the ip route, the exposure for the peritoneal cavity is far greater than for the systemic circulation, and that, although these drugs are delivered to peritoneal tumor nodules via capillary flow, an additional increment of drug enters the nodules via free surface diffusion. The peritoneal/plasma AUC (area under the curve) ratio, a measure of this differential exposure, is ~15 for cisplatin and ~1000 for paclitaxel.[9−11] Approaches that increase local

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drug concentration even more and/or decrease systemic exposure are attractive for further improving the ip therapy.

Several particulate systems (liposomes, nanoparticles, microparticles) have been used to increase peritoneal exposure by retaining encapsulated drugs in the peritoneal cavity.[12−15] However, in addition to simply increasing the peritoneal exposure, it would be advantageous to have micro- or nanoparticle drug carriers that can actually attach themselves to tumor cells once they have made contact with tumor nodules growing on the peritoneal surface. Such particles would then be in position either to be ingested by the tumor cells or to release their drug load at the cell surface to create very high local drug concentrations. CD44 is one of the receptors that is overexpressed on the surface of ovarian cancer cells, and can thus be used as a molecular target of a delivery vehicle. The standard form of CD44 (CD44S) is responsible for the binding of ovarian carcinoma cells to mesothelium.[16] Inhibition of CD44S by preincubating human ovarian cancer cells with the antibodies to CD44S before ip inoculation has been reported to dramatically reduce the number of ip nodules that formed.[17] Kayastha et al.[18] and Rodriguez-Rodriguez et al.[19] found that CD44 expression was closely associated with survival of ovarian cancer patients; patients with CD44-positive tumors had a mean survival of 25 months compared with patients with CD44-negative tumors whose mean survival was 52 months.[18] CD44 expression was independently related to survival in the multivariate Cox regression analysis after adjusting for age, histology, stage and differentiation.[18] CD44S was reported to be expressed in 38−51% of the examined ovarian tumor samples.[18,19]

We have developed a simple and unique method to produce a microparticle (Hyplat) that encapsulates large amounts of cisplatin as well as targets itself to ovarian cancer cells. This particle is composed of cisplatin coupled to the naturally occurring polymer hyaluronan which is a natural ligand for CD44. The hyaluronan serves both as a polymer scaffold onto which the cisplatin is linked and as a targeting moiety for CD44. Hyaluronan was also selected because of its favorable properties as a drug carrier, such as the fact

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that it is biodegradable, biocompatible and nontoxic.[20] Hyaluronan is a high molecular weight extracellular matrix component with the polymeric repeat of D-glucuronic acid and *N*-acetylglucosamine and is synthesized normally in human body with a turnover rate ~5 g per day.[20]

We report here on the characterization of Hyplat, its *in vitro* tumor targeting efficiency, *in vivo* pharmacokinetics and tissue distribution, and its efficacy against a xenograft model of human ovarian cancer. Although previous reports have shown that hyaluronan can form stable conjugates with cisplatin,[21−23] we report on an improved and simplified method to prepare this type of drug conjugate with increased incorporation efficiency (27.3% drug content, w/w). Hyplat is the first among the hyaluronan-cisplatin particles[21−23] to show targeting ability to CD44 expressing cells, and superior pharmacokinetics and pharmacodynamics to free cisplatin in an animal model thus demonstrating its potential for improving ip chemotherapeutics.

2. Materials and Methods

2.1. Chemicals and Reagents. Cisplatin, hyaluronan (sodium salt, from *Streptococcus equi* sp., MW 0.6−1.2 × 10^6 g/mol) and fluorescein (FITC)-labeled hyaluronan were purchased from Sigma-Aldrich (St. Louis, MO). The mouse antibody against human CD44 and the secondary goat antibody against mouse IgG labeled with FITC were from Santa Cruz Biotechnologies (Santa Cruz, CA).

2.2. Cells and Cell Culture. A2780, OV2008, HEY and UCI101 cells were purchased from the American Type Culture Collection and were cultured in PRMI-1640 medium (HyClone Laboratories, Inc., South Logan, UT) supplemented with 10% fetal bovine serum and antibiotics. To engineer a DsRed expressing line of A2780, the cells were infected with lentivirus-DsRed and sorted by a flow cytomter (FACS Vantage DiVa I, BD Biosciences, San Diego, CA) at The Scripps Research Institute, La Jolla, CA.

2.3. Experimental Animals. Female BALB/c mice (~20 g) and female nu/nu mice age 5−6 weeks were purchased from Charles River Laboratories (Wilmington, MA). All work performed with animals was in accordance with and approved by the IACUC at the University of California, San Diego.

2.4. Preparation and Characterization of Hyplat. The details of the formulation development and optimization are described in the Supporting Information. Batches of the final formulation of Hyplat were produced by incubating 200 μL of a solution of cisplatin (15 mg/mL) and 800 μL of hyaluronan solution (5 mg/mL) in deionized water at 90−95 °C for 1 h followed by cooling on ice for 10−30 min and dialysis against sterile water to remove unbound platinum (Pt) using a dialysis cassette (Slide-A-Lyzer dialysis cassette, molecular weight cutoff 10,000, Promega, Rockford, IL). The final concentration of Pt incorporated into Hyplat was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Perkin-Elmer ICP-OES (model Optima 3000 XL) at the Analytical Facility at the Scripps Institute of Oceanography (La Jolla, CA). Indium was added to each sample at 1 ppb as a control for flow variation. The concentration ranged from 0.6 to 0.9 mg Pt/mL. Hyplat was analyzed by the Zetasizer ZS (Malvern Instruments, Malvern, Worcestershire, U.K.) for intensity averaged particle size at 25 ± 0.1 °C and zeta potential and the atomic composition of Hyplat was determined by a scanning electron microscope (XL 30 ESEM FEG SEM, FEI Company) after being dried on a TiO_2 plate. To prepare FITC-labeled Hyplat, hyaluronan was mixed with FITC-hyaluronan (1:1 weight ratio at a final concentration of 5 mg/mL) and then the same preparation procedure was employed.

2.5. *In Vitro* Pt Release from Hyplat. One hundred microliters of Hyplat was mixed with 900 μL of medium and incubated at 37 °C. At different time points, 100 μL aliquots were removed and diluted with 400 μL of deionized water, followed by centrifugation in a Microcon (molecular weight cutoff 3,000) at 10,000 rpm for 10 min. Fifty microliters of the flow-through was collected, and the Pt concentration was determined by ICP-OES.

2.6. Cellular Accumulation and Flow Cytometric Analysis. Cells were seeded in 6-well plates (0.5−1 × 10^6 cells/2 mL/well) one day before the uptake study. Cells were incubated with 125 μL of FITC-labeled Hyplat in 1 mL of culture medium (~90 μg Pt/mL) at 37 °C for 6 h. Cells were washed with PBS 3 times and trypsinized for flow cytometric analysis (BD FACSCalibur, BD Biosciences, San Diego, CA), and the signal from live cells was gated using propidium iodide staining. Alternatively, the cells were fixed on a coverslip, counterstained with DAPI and observed by confocal microscopy (Zeiss LSM, Carl Zeiss MicroImaging, Inc., Thornwood, NY). Cell surface expression of CD44 was accessed by flow cytometry as previously described.[24]

2.7. Cellular Uptake and Cytotoxicity Assay. A confluent monolayer of cells was used in the experiments to avoid nonspecific binding of Hyplat to the positively charged culture plates, and a 10 min exposure to drug was chosen

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based on the observation that accumulation in CD44-positive cells slowed after 10 min. Cells (OV2008, A2780, HEY and UCI101) were seeded in 24-well plates at a density of 2−4 $\times$ 10^5/0.5 mL/well and cultured overnight or until a confluent monolayer of cells had formed. For uptake studies, the cells were treated with cisplatin or Hyplat at 100 μM for 10 min followed by wash with 1 mL of PBS. The cells were dissolved in 70% nitric acid, and the cell-associated Pt levels were determined by ICP-OES. For cytotoxicity studies, cells were incubated with cisplatin or Hyplat at 600 μM for 10 min followed by wash with 1 mL of PBS. Cells were trypsinized and subcultured in a new well after being diluted 1:5. Forty-eight hours later, cells were trypsinized and counted using the trypan blue exclusion method. Cells that were stained with the dye were considered dead. A high concentration of Pt (600 μM) was used to compensate the short exposure time (10 min) to obtain significant cell killing.

2.8. Determination of the Maximum Tolerated Dose (MTD). Cisplatin formulations (free cisplatin and Hyplat) in 1 mL of PBS were injected ip into the nude mice, and body weight was monitored until it recovered to baseline. The MTD was defined as that which caused $\leq$20% body weight loss and no animal death.

2.9. Ip Pharmacokinetic Study. The experimental method was adopted from our previously reported articles with some modification.[25,26] BALB/c mice were anesthetized with ketamine and xylazine, and a small incision was made in the abdomen. Free cisplatin or Hyplat (0.1 mg Pt) was diluted in 1 mL of PBS and instilled into the peritoneal cavity. At different time points (<1 h), 100 μL aliquots were sampled from the peritoneal cavity and analyzed for Pt level by ICP-OES. For time points longer than 1 h, mice were injected ip with ~0.5 mL of PBS 5−10 min before sacrifice and 100 μL aliquots were sampled.

2.10. Blood Pharmacokinetics and Tissue Distribution Study. BALB/c mice were given either free cisplatin or Hyplat in 1 mL of PBS ip at a dose of 5 mg Pt/kg. At different time points, blood was collected from a tail artery and the mice were euthanized for tissue harvest (lung, liver, spleen, kidney and muscle). Blood (100 μL) and tissues were digested in 70% nitric acid, and the Pt levels were quantified and normalized to the sulfur (S) levels by the ICP-OES.

2.11. Tumor Uptake Study. Nu/nu mice were injected ip with 5 $\times$ 10^6 A2780, OV2008, HEY or UCI101 cells in 1 mL of RPMI-1640 medium. Thirty to forty days later, mice were given the free cisplatin (10 mg/kg) or Hyplat (5 mg Pt/kg) via the ip route. At different time points, the mice were sacrificed and the tumor nodules, liver and kidney were collected for measurement of Pt content normalized to S content by ICP-OES.

2.12. Efficacy Study. Nu/nu mice were injected ip with 5 $\times$ 10^6 A2780 that had been engineered to express a DsRed gene in 1 mL of RPMI-1640 medium thereby rendering them fluorescent. Fourteen days later, mice were treated with 1 mL of PBS (n = 10), cisplatin (10 mg/kg, n = 7) or Hyplat (5 mg Pt/kg, n = 7) in 1 mL of PBS via the ip route. The mouse body weight was monitored as a measure of toxicity and the tumor burden was quantified by external imaging using the Xenogen IVIS 200 (Caliper LifeSciences, Mountain View, CA) on day 45. Mice that gained or lost more than 20% of their initial body weight due to disease progression or the formation of ascites or became moribund were euthanized, and the survival rate was recorded throughout the experiment. The Kaplan−Meier plot was analyzed by GraphPad Prism5.

2.13. Statistical Analysis. Data are presented as mean $\pm$ SD. The statistical significance was determined by using the analysis of variance (ANOVA) among $\geq$3 groups or Student t test between 2 groups. P values of <0.05 were considered significant.

3. Results

3.1. Formulation Optimization. It was found that microparticles could be generated by incubating cisplatin and hyaluronan together at 90−95 °C for 1 h and then cooling on ice for 10−30 min. Figure 1A shows the sequence of steps in the preparation process. The formation of a microparticle is believed to be the result of cross-linking of the hyaluronan polymers when the free carboxyl groups on the hyaluronan displace the chloride groups of the cisplatin, known as the ligand replacement reaction. As shown in Figure 1 in the Supporting Information, when mixed at a hyaluronan/cisplatin weight ratio 4:3, particle size varied with both temperature and time. A temperature of 90 °C produced particles of smaller sizes compared to that at 45 °C, and increasing the incubation time significantly reduced the size of the final particles. When cisplatin and hyaluronan were incubated together at 45 °C, the final products displayed a wide size distribution (polydispersity index (PDI) > 0.8) and very high viscosity; therefore an incubation temperature of 90−95 °C was chosen for further studies. The characteristics of the particles prepared at the weight ratio of 4/3 (hyaluronan/cisplatin) at 90−95 °C for different time periods are summarized in Table 1. As the incubation time was increased from 10 min to 2 h, the size of the particles decreased from ~1,000 nm to ~220 nm, the cisplatin incorporation efficiency increased from ~30% to 40−50%, the zeta potential of the particles increased from −43 mV to −37.7 mV, and the estimated carboxylate/Pt ratio decreased from 3.2 to 2.2−2.6. A 1 h incubation time was selected for preparation of subsequent batches since this yielded the highest incorporation efficiency and a relatively desirable particle size. After removal of the unincorporated Pt by dialysis, the mean diameter of the final product increased to 580.6 $\pm$ 88.5 nm

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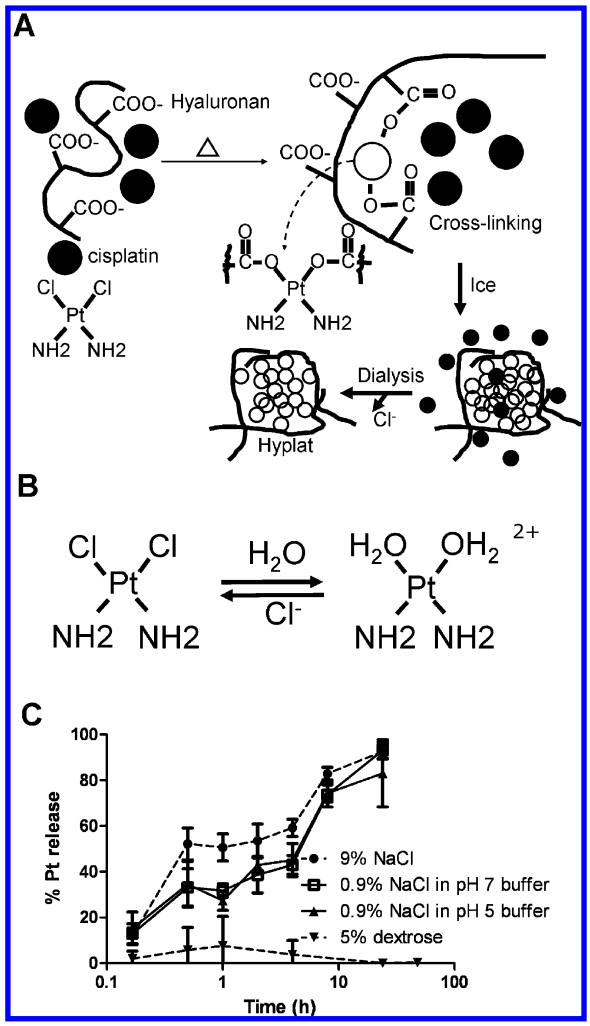

Figure 1. Preparation and characterization of Hyplat. (A) Illustration of the preparation method for Hyplat. (B) Formation of aquated platinum upon ligand replacement by water molecules. (C) Drug release profiles of Hyplat in different medium. Each data point represents mean ± SD, $n = 3$.

(PDI = 0.1−0.2, $n = 5$) and there was a slight and statistically insignificant change in zeta potential (40.3 ± 6.9 mV). The size distribution of Hyplat is shown in Figure 2 in the Supporting Information. Hyplat did not aggregate in the presence of serum. The characteristics of Hyplat as determined by electron diffraction spectroscopic analysis are presented in Table 1 in the Supporting Information. Of particular interest, Cl was barely detectable in Hyplat, suggesting that the ligand replacement reaction proceeded nearly to completion as illustrated in Figure 1A.

Cisplatin is very stable in 0.9% NaCl; however, in non-chloride-containing media it becomes aquated as water displaces the chlorides to form a Pt−O bond (illustrated in Figure 1B). If the chloride concentration is increased, the equilibrium shifts back and the oxygen is displaced. Since in the Hyplat particles cisplatin is held in place via a Pt−O bond, the particles were expected to be stable in non-chloride-containing medium but to release cisplatin in chloride-containing medium. As shown in Figure 1C, there was indeed no progressive release of Pt from the Hyplat microparticles for up to 2 days when the particles were incubated in 5% dextrose. However, when placed in 0.9% NaCl, 30% of the cisplatin was released in 30 min and this was followed by a slower but sustained release of Pt out to 24 h. Increasing the NaCl concentration to 9% increased the amount released over the first 30 min to 55% but did not alter the subsequent rate of release. Varying the pH of the 0.9% NaCl buffer between 5 and 7 had little effect on either initial or subsequent cisplatin release rate. Hyplat stored at 4 °C in sterile deionized water and protected from light was stable for at least 1 month without change in particle size, drug release or potency as determined by its ability to kill ovarian carcinoma cells *in vitro*. We determined that Hyplat could be prepared in 5% sucrose and lyophilized for storage and be reconstituted by water with no change in particle size and little drug release (<10%).

3.2. CD44 Targeting of Hyplat. As shown in Figure 2A, both A2780 and OV2008 cells expressed high levels of surface CD44 whereas the UCI101 cells had little expression. There was a strong association between the expression of CD44 and the cellular association of FITC-labeled Hyplat as quantified by flow cytometry as both A2780 and OV2008 cells exhibited much stronger association than UCI101 cells (Figure 2B). That a fraction of the cell-associated drug was intracellular was confirmed by the confocal microscopic images presented in Figure 2C. To further document that the uptake of Hyplat microparticles was mediated through interaction with CD44, the parental OV2008 and A2780 cells were infected with a lentivirus expressing a shRNA targeted to CD44 mRNA. The flow cytometric analysis of the amount of cell surface CD44 presented in Figure 3 in the Supporting Information demonstrated that CD44 expression was reduced in these sublines; the CD44-shRNA-lentivirus infected sublines displayed only 40−50% as much CD44 as the parental lines. This reduction was accompanied by a reduction in the cell-associated FITC-labeled Hyplat in the knockdown lines. Interestingly, the knockdown lines displayed a different morphology, a decreased proliferation rate and increased sensitivity to cisplatin (data not shown), confirming previous reports that CD44 is a biomarker of the aggressive phenotype of ovarian cancer cells.[27]

3.3. Cellular Pt Uptake and Cytotoxicity. Hyplat efficiently bound to the CD44-positive lines during an exposure of 10 min and increased the cell-associated Pt levels by 2- to 3-fold compared to cisplatin, but showed no targeting to the CD44-negative UCI101 cells (Figure 3A). The cytotoxicity results were in agreement with the uptake data; Hyplat showed enhanced killing of the CD44-positive A2780 cells compared to cisplatin, while it displayed differential no cytotoxicity against the receptor negative cells (Figure 3B).

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Table 1. Characteristics of Hyaluronan-Cisplatin Microparticles

incubation time	mean particle size (SD),[a] nm	PDI[b]	encapsulation efficiency[a]	zeta potential (1 mM KCl),[a] mV	calcd carboxylate/ Pt ratio
10 min	1160.2 (164.4)	0.65−0.80	33.6 ± 4.8%	−43.6 ± 4.8	3.2
20 min	750.0 (82.6)	0.55−0.60	35.6 ± 2.0%	−39.8 ± 7.0	3.0
1 h	349.5 (38.7)	0.19−0.26	49.0 ± 2.6%	−38.3 ± 4.8	2.2−2.6
2 h	221.5 (13.9)	0.15−0.28	40.7 ± 7.7%	−37.7 ± 4.0	

[a] $N \geq 3$. [b] Polydispersity index, given as a range based on multiple measurements.

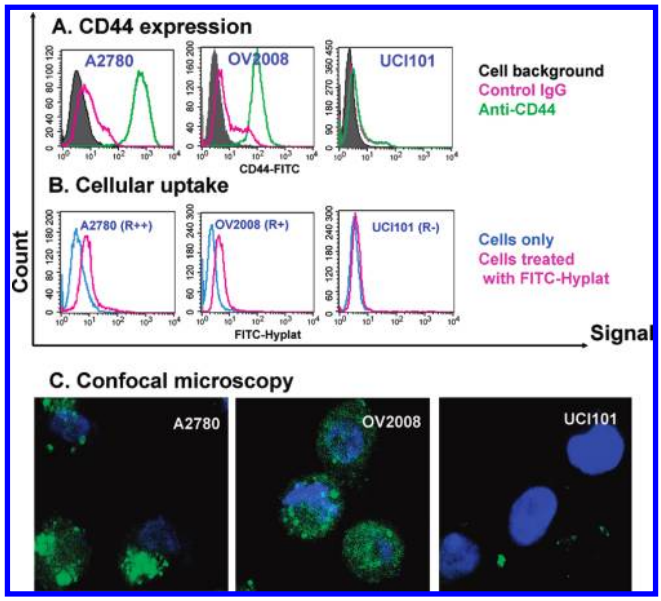

Figure 2. Cellular uptake of FITC-Hyplat in ovarian cancer cell lines expressing different levels of CD44 when exposed to ~90 μg Pt/mL for 6 h. (A) Cell surface expression of CD44 determined by flow cytometry. (B) Cellular uptake of FITC-Hyplat detected by flow cytometry. (C) Cellular uptake of FITC-Hyplat examined by confocal microscopy. Magnification, 1890×.

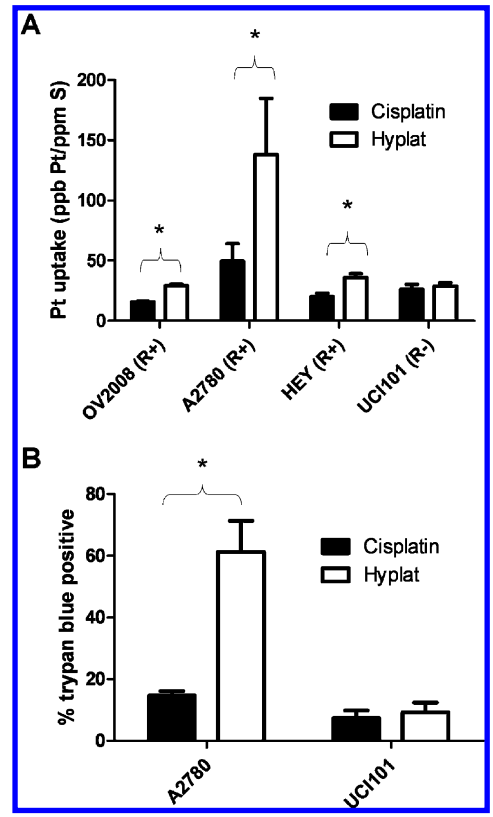

Figure 3. Comparison of the cellular uptake and cytotoxicity of Hyplat and cisplatin against CD44-positive and CD44-negative ovarian cancer cells. (A) Cellular Pt uptake after incubation with cisplatin or Hyplat at 100 μM for 10 min. (B) Cytotoxicity to cells treated with 600 μM cisplatin or Hyplat for 10 min. Data = mean ± SD; * indicates $P < 0.05$.

3.4. Pharmacokinetics in Peritoneal Cavity and Blood. As shown in Figure 4A, free cisplatin was rapidly cleared from the peritoneal cavity of BALB/c mice with a half-life of ~18 min (first order elimination, model fitting $r^2 = 0.98$). The concentration of Pt in whole blood reached its peak (~4 μg/mL) at 10 min and became undetectable by 16 h. Hyplat exhibited a substantially lower clearance from the peritoneal cavity with a half-life of ~124 min (first order elimination, model fitting $r^2 = 0.91$). The C_{max} in whole blood (~17 μg/mL) occurred at 4 h after ip injection of Hyplat, and blood levels were slower to fall as Pt was still detectable (>200 ng/mL) at 72 h. The AUCs for cisplatin and Hyplat in the blood and peritoneal cavity are presented in Table 2.

3.5. Tissue Distribution. The tissue distribution of Pt as a function of time in the major organs was determined in BALB/c mice that received equal (5 mg Pt/kg) ip doses of free cisplatin or Hyplat. As shown in Figure 4B, following ip injection of free cisplatin Pt levels rose rapidly in all the organs sampled with a particularly high level found in the kidneys. C_{max} was reached at 6 h, and levels in these tissues declined quickly and subsequently, with the exception of the kidneys, remained relatively steady out to 16 h. Most of the injected free cisplatin distributed to the liver and the kidneys, and very little accumulated in the muscle. Hyplat produced a very different distribution of Pt and favored accumulation in the liver and the spleen. In these two organs, the Pt level reached a peak by 16 h and then plateaued at a relatively high level for at least 3 days. The AUC in the liver and the spleen was ~2-fold higher compared to that after injection of free cisplatin (Table 2). The peak Pt levels were substantially higher in the lungs and lower in the kidneys following injection of Hyplat than administration of free cisplatin. Of particular interest was the fact that, once having reached its peak, the rate of elimination of Pt from the kidneys was much slower for Hyplat than for free cisplatin,

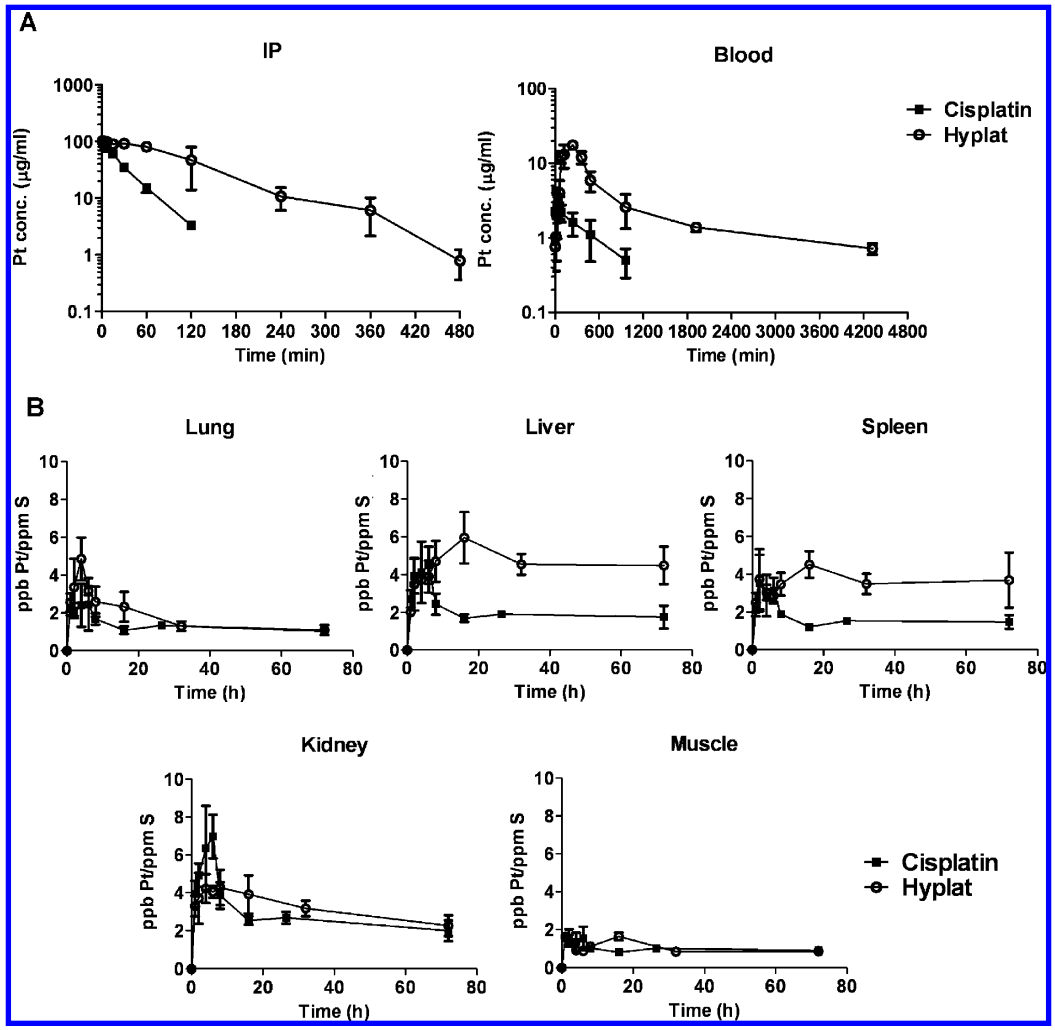

Figure 4. Pharmacokinetics and tissue distribution of free cisplatin and Hyplat. Mice were given an ip dose (5 mg Pt/kg) of free cisplatin or Hyplat. (A) Pharmacokinetic profiles in the mouse peritoneum and whole blood. (B) Tissue distribution profiles. Each data point represents the mean ± SD, $n = 3-5$.

Table 2. Total Drug Exposure Following Ip Injection of Cisplatin or Hyplat at a Dose of 5 mg Pt/kg in Tumor-Free Mice

	AUC (μg·min/mL)			AUC (ppb Pt·h/ppm S)		
drug	peritoneum	blood	peritoneal/blood AUC ratio	kidney	liver	spleen
cisplatin	3,071	1,263	2.4	201	147	117
Hyplat	13,990	11,944	1.2	227	334	263

indicating sustained drug release from Hyplat in the blood and delayed elimination via the kidney.

3.6. Tumor Uptake and *in Vivo* Efficacy Study. The MTD of free cisplatin in nu/nu mice is 10 mg/kg, and that of Hyplat was found to be 5 mg Pt/kg. Nu/nu mice were inoculated ip with OV2008, A2780, HEY or UCI101 human ovarian carcinoma cells, and the tumors were allowed to grow for 30−40 days prior to injection of either 10 mg/kg cisplatin or Hyplat at a dose of 5 mg Pt/kg. The mice were sacrificed 4 h after injection, and the Pt content of resected tumor nodules was measured. As shown in Figure 5A, the Pt levels in the CD44 positive tumor nodules (OV2008, A2780 and HEY) were 2- to 3-fold higher in the mice given Hyplat than in those given cisplatin, while no significant difference was found in the CD44 negative UCI101 tumor model. An additional time course study was performed in mice inoculated with A2780 cells and injected ip with the equitoxic doses of either 10 mg/kg free cisplatin or 5 mg Pt/kg Hyplat. As shown in Figure 5B, in mice injected ip with free cisplatin the AUCs for the A2780 tumor nodules, liver and kidney were similar to each other. However, in the mice that received Hyplat, the AUC for the A2780 tumor nodules was 2.8-fold higher than that for the liver and 2.3-fold higher than that for the kidney (Figure 5C and Table 3). The Pt levels in both the tumor and the kidney declined with time whereas the liver continued to accumulate Pt throughout the period of sampling. The AUC for the A2780 tumor nodules was ~3-fold higher in the mice receiving Hyplat than in those who received free cisplatin, while no difference was observed for that for the kidney

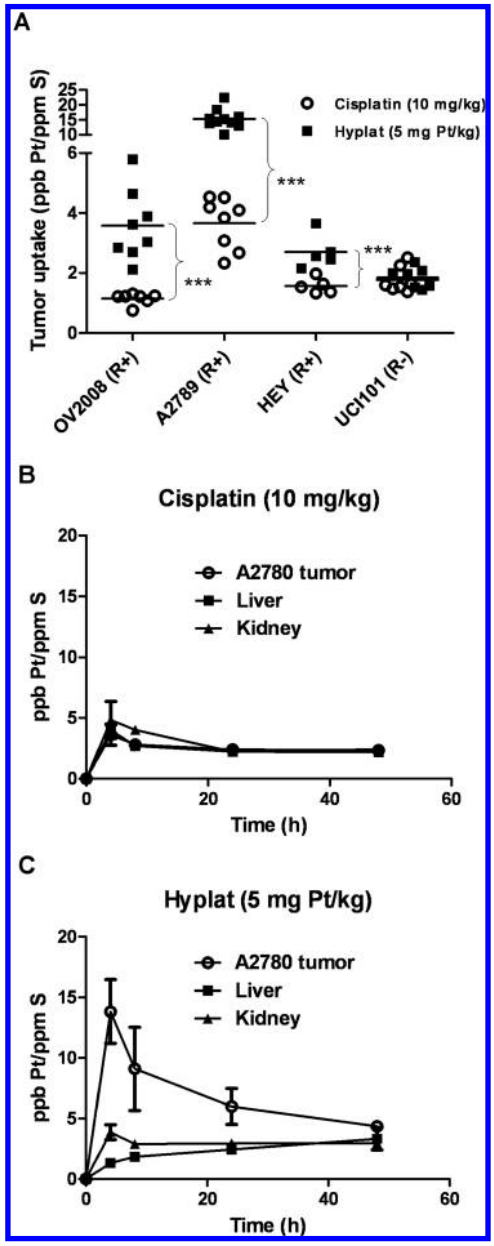

Figure 5. Tumor uptake in the human ovarian cancer xenograft models in nude mice. (A) Pt levels in the tumor nodules 4 h after an ip dose of free cisplatin (10 mg/kg) or Hyplat (5 mg Pt/kg). Each data point represents a nodule randomly collected from 3 mice in each group. Bar, mean. *** indicates $P < 0.005$. (B) Tissue distribution profiles of Pt in A2780 tumor-bearing nu/nu mice after receiving an ip dose of free cisplatin (10 mg/kg). (C) Tissue distribution profiles of Pt in A2780 tumor-bearing nu/nu mice after receiving an ip dose of Hyplat (5 mg Pt/kg). Each data point is the mean ± SD, $n = 4$.

and liver. Thus, for equivalent levels of accumulation of Pt in the kidneys, where the dose-limiting toxicity occurs for cisplatin, Hyplat delivered ~3-fold more Pt to the tumor.

Table 3. Summary of Pharmacokinetics in the A2780 Tumor-Bearing Mice after an Ip Dose of Either 10 mg/kg Cisplatin or 5 mg Pt/kg for Hyplat

	AUC (ppb Pt·h/ppm S)		
drug	A2780 tumor	kidney	liver
cisplatin	118	132	113
Hyplat	318	139	112

Nu/nu mice were inoculated ip with A2780 cells molecularly engineered to express the DsRed gene and treated with a single dose of 10 mg/kg free cisplatin or 5 mg Pt/kg Hylat starting on day 14. Tumor burden was subsequently quantified by live animal imaging on day 45. Figure 6 shows that a single injection of free cisplatin had a moderate effect on slowing the growth of the tumor whereas Hyplat significantly inhibited the progress of the disease, resulting in much prolonged survival in the treated mice. The overall survival on day 100 for untreated, cisplatin treated (10 mg/kg) and Hyplat treated (5 mg/kg) mice was 10%, 42.9% and 85.7%, respectively, and that on day 150 was 0%, 0% and 28.6%, respectively.

4. Discussion

Hyaluronan is a polysaccharide polymer that contains carboxylate groups and is the natural ligand for CD44. A strategy was sought that would permit cisplatin to be loaded onto microparticles of hyaluronan in a manner that would not destroy its ability to bind to CD44 and that would allow the gradual release of the cisplatin once bound to cells. The ability of Pt(II) to exchange chlorides for carboxylates in polymers has been utilized by other groups to prepare cisplatin-containing microparticles.[28−30] The reaction rate is slow and thus requires several days of incubating the polymer and drug under agitation. Here, we developed an improved and simplified approach to prepare Hyplat using hyaluronan, another carboxylate-containing polymer. Hyaluronan and cisplatin were mixed at a high concentration at 90−95 °C for 1 h, conditions that permitted the dissolution of cisplatin and facilitated the Cl replacement reaction. Agitation was only required at the beginning to homogeneously distribute the drug and polymer in the heating tube, and the cross-linking reaction proceeded after that without the need for further mixing. Treating cisplatin at such a high temperature did not decrease the cell killing activity of the drug (data not shown). Under these conditions, the chlorides of cisplatin were exchanged efficiently with the carboxyl groups of the D-glucuronic acid in the polymeric repeat of hyaluronan and in the process the polymer became cross-linked by the drug following which microparticles formed spontaneously. Incubation at 45 °C yielded products with significantly larger sizes and much wider size distribution (PDI > 0.8), indicating inefficient polymer cross-linking. Increasing the reaction time allowed more complete chemical reaction between cisplatin and hyaluronan as evidenced by the increase in incorporation efficiency and the decrease in particle size which suggested increased cross-linking. Interestingly, as the incubation time

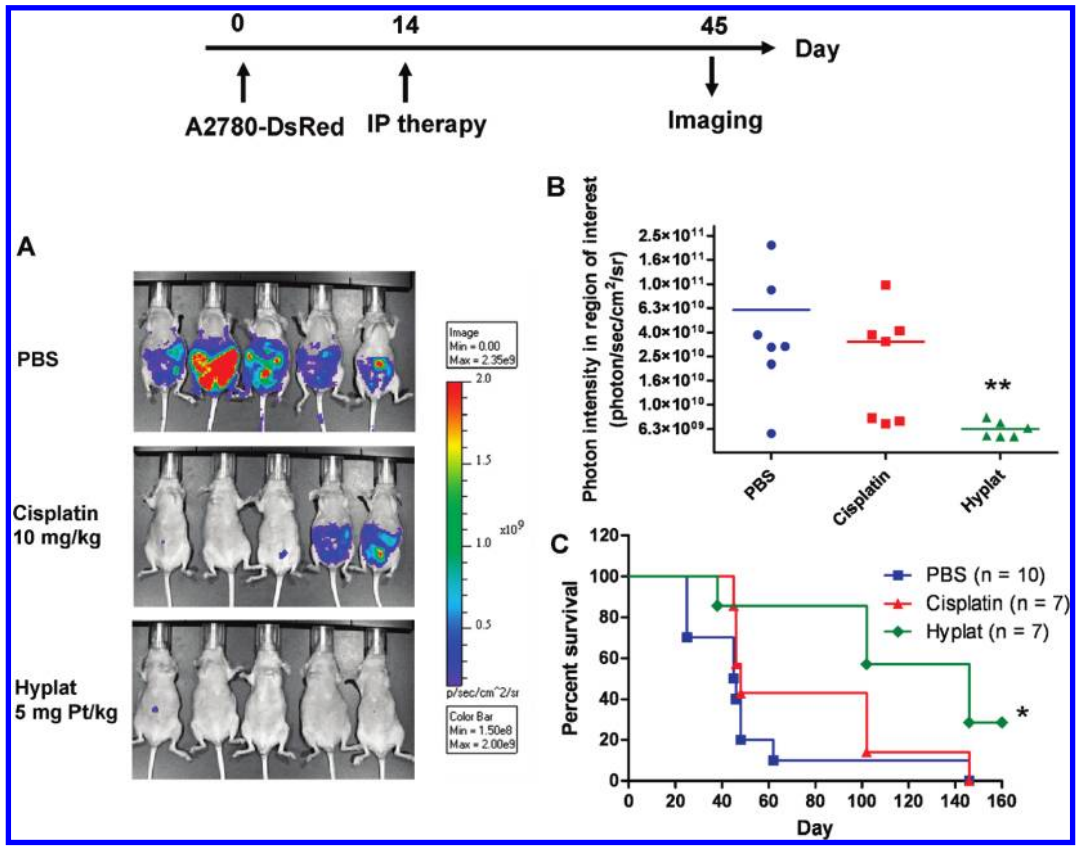

Figure 6. Antitumor efficacy of cisplatin and Hyplat in mice inoculated ip with A2780-DsRed. (A) Images of A2780-DsRed tumors in mouse peritoneum on day 45 obtained using the Xenogen IVIS imaging system. Images of 5 randomly picked mice from each group are shown. (B) Quantitative analysis of A2780-DsRed tumors in mouse peritoneum on day 45 by the Xenogen IVIS 200. Background level was between 5×10^9 and 1×10^{10} photons/s/cm^2/sΓ. Each data point represents 1 mouse. (C) Kaplan−Meier plot of the tumor-bearing mice after receiving different treatments. ** indicates $P < 0.01$, and * indicates $P < 0.05$. $N = 7-10$.

increased, the zeta potential of the particles increased from −43.6 mV to −37.7 mV indicating the consumption of free carboxylates, the only negatively charged group in Hyplat, which is consistent with the decreased carboxylate/Pt ratio. An incubation time of 1 h appeared optimal since it resulted in high incorporation efficiency and a particle size still large enough to exhibit delayed clearance from the peritoneal cavity. It has been shown that particle size is an important determinant of peritoneal clearance.[31] The increase in size from ∼350 nm to ∼600 nm that occurred after dialysis of the particles is likely due to the removal of the unincorporated and potentially partially aquated positively charged cisplatin that interacted with the hyaluronan via charge−charge interaction and whose removal resulted in expansion of the particle.

The electron diffraction spectroscopic analysis showed that Hyplat had no Cl, confirming that the ligand exchange reaction happened during the preparation as Cl was released from cisplatin and dialyzed out. This method provides clear advantages for the production of hyaluronan-cisplatin particles over the others reported recently.[21−23] First, unlike others that utilized a long period of agitation or $AgNO_3$ to improve drug incorporation, our method is simple and the formulation can be prepared in a single day. Second, the drug incorporation efficiency for Hyplat was higher than for the other methods (drug contents (%,w/w): 27.3% for Hyplat versus 11.8%[22] and 19.4%[21]). A high drug to carrier ratio is of importance for drug delivery. Third, Hyplat of different sizes can be prepared by manipulating the reaction conditions (i.e., temperature and incubation time) for different purposes (i.e., iv, ip, subcutaneous or intratumoral injection). The key variables controlling the particle size include incubation temperature and time. When prepared in a volume of 1.5 mL cooling speed appeared to be not critical for this small-scale preparation as cooling the formulation at room temperature yielded similarly sized particles. This is probably because the small volume of the reaction mixture allowed homogeneous heating and efficient cooling. However, when prepared on a large scale, it is likely that both heating and cooling speeds will need to be controlled.

The release of cisplatin from Hyplat was dependent on the chloride concentration but not the pH of the medium. Drug release in the medium containing 9% NaCl was significantly faster than that in 0.9% NaCl-containing buffer. Under physiologic conditions (0.9% NaCl) there

was an initial burst release of 30% of the cisplatin over the first 30 min followed by an almost constant and sustained release for the following 24 h. The burst release may be due to a rapid reverse ligand exchange on the particle surface in the presence of chloride. A similar effect was reported by other groups.[28−30] Accurate measurement of cisplatin release in serum is problematic due to the high protein binding property (>90%) of cisplatin (through hydrophobic interaction or formation of S−Pt bond later) and the difficulty of separating protein bound and polymer bound drug. It is important to note that additional mechanisms of release may be active *in vivo* where hyaluronic acid can be expected to degrade or erode at a faster rate.

In the Hyplat formulation, hyaluronan serves as both a polymer backbone for carrying cisplatin and a targeting moiety for CD44. Several lines of evidence suggest that Hyplat is targeting cisplatin via its interaction with CD44. FITC-labeled Hyplat displayed differential binding to CD44-expressing cells as documented by flow cytometric analysis, and differential uptake as shown by confocal microscopy. Downregulation of CD44 compromised the targeted delivery. The targeting of Hyplat to CD44 contributed to improved cellular uptake of Pt and enhanced cell killing for receptor positive cells compared to cisplatin, but not for receptor negative cells.

As anticipated based on their structure, the pharmacokinetics of Hyplat and cisplatin in the peritoneal cavity and blood were quite different. The key finding was that Hyplat was cleared more slowly from both compartments. The half-life and AUC of Pt in the mouse peritoneal cavity were ∼7-fold and ∼5-fold higher, respectively, following injection of Hyplat than the half-life and AUC for free cisplatin. Interestingly, the AUC of Pt in the blood following injection of Hyplat was ∼10-fold greater than that produced by free cisplatin. The liver, the spleen and the kidney were the three major organs that accumulated Pt following administration of Hyplat. The scavenger receptor HARE on the liver and spleen sinusoidal endothelial cells that is responsible for removing circulating hyaluronan by receptor-mediated endocytosis[32] likely accounts for the accumulation of Hyplat in these two tissues. However, it is the accumulation in the kidneys and spinal ganglia rather than the liver and spleen that limits the dose of cisplatin. The prolonged half-life of Hyplat in the systemic circulation relative to that of cisplatin provides an explanation for why Hyplat is more potent than cisplatin with respect to systemic toxicity and why it has a lower MTD. While Hyplat is more potent with respect to systemic toxicity, this is offset by its much higher level of selectivity for the tumor such that when the two drugs are administered at equitoxic doses Hyplat delivers substantially more drug to the tumor than cisplatin. Nevertheless, the toxicological profile of Hyplat will be carefully studied and monitored together with its pharmacokinetics in larger species of animals, such as rat and dog, for further clarification.

Hyplat produced a 2- to 3-fold increase in the delivery of Pt to peritoneal tumor nodules compared to free cisplatin in the CD44-positive tumor models (OV2008, A2780 and HEY), but offered no advantage for the CD44-negative ovarian tumor model (UCI101). The increased Pt uptake in the receptor positive nodules likely resulted from the improved peritoneal retention, sustained Pt release and tumor targeting via CD44. For future clinical application, CD44 might serve as a biomarker to predict the advantage of Hyplat over cisplatin for ip chemotherapy, and allow preselection of patients that might optimally benefit from Hyplat. Particle size is another parameter that can be varied during the synthesis of Hyplat and is likely to impact the peritoneal/plasma AUC ratio; additional studies are needed to define how size influences efficacy. The Pt uptake in the nodules was dependent on the anatomy of the tumors; the A2780 tumors which grow as more diffuse miliary nodules that are highly vascular took up significantly more Pt compared to the others. Free cisplatin showed little tumor selectivity, as the AUCs for the A2780 tumor, the liver and the kidney were all similar. However, Hyplat directed relatively more of the Pt to the A2780 tumor nodules in the peritoneal cavity, and the AUC for tumor was ∼3-fold higher than that for the liver and ∼2-fold higher than that for the kidney. Moreover, when compared to the liver and kidney in the tumor-free mice, the liver and kidneys in the tumor-bearing mice showed a decreased uptake, suggesting that Hyplat targeted A2780 nodules in the peritoneum and thus its systemic bioavailability and potential side effects were decreased. Although Hyplat did not improve the peritoneal/blood AUC ratio and the MTD was only half of that for cisplatin due to its dramatically prolonged systemic half-life, Hyplat still delivered 2- to 3-fold more Pt than cisplatin when given at their respective MTDs in multiple animal models. Finally, the enhanced delivery of Pt to the tumor produced by Hyplat resulted in improved therapy for the ip metastases in the A2780 ovarian cancer in the xenograft model; although the results were statistically significant, the reader is cautioned that this was a small study in which neither dose nor schedule was optimized. In addition it is important to note that the enhanced delivery of Pt to the tumor may be due to both

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the prolonged exposure of Hyplat in the peritoneal cavity and the ability of Hyplat to target to CD44, and the relative contribution cannot be distinguished from the data available.

5. Conclusion

We have developed a hylauronan-based microparticle formulation with high incorporation efficiency for cisplatin using a unique but simple and highly reproducible method. Hyplat targeting cisplatin to CD44-positive tumor cells had favorable pharmacokinetic characteristics and was more effective than free cisplatin in inhibiting the growth of ovarian cancer nodules growing on the peritoneal surface when administered by the ip route.

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Supporting Information Available: Details of materials and methods, tables of electron diffraction spectroscopic analytical data and formulation of hyaluronan-cisplatin microparticles, and figures depicting average diameter of hyaluronan-cisplatin particles as a function of incubation temperature and time, particle size distribution of Hyplat after removal of unincorporated cisplatin by dialysis, and cellular uptake of FITC-Hyplat in parent lines and the CD44 knockdown (KD) lines. This material is available free of charge via the Internet at http://pubs.acs.org.

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