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Hyaluronan-lysine cisplatin drug carrier for treatment of localized cancers: pharmacokinetics, tolerability, and efficacy in rodents and canines

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Abstract

The purpose of this study was to develop a safe and efficacious drug delivery platform for sustained release of cisplatin after locoregional administration. We successfully synthesized hyaluronan-cisplatin nanoconjugates (HA-Lys-Pt) using an N-Ac-lysine linker, which formed a thermodynamically stable 5-membered ring with the platinum. The conjugate was characterized for release kinetics, *in vitro* anti-proliferative activity, degradability, impurity content, formation of Pt-DNA adducts, pharmacokinetics, tolerability in rodents and canines, and for efficacy in rodents. The 75kD HA-Lys-Pt (75HALys-Pt) sustained release of platinum with a 69 hour half-life in phosphate buffered saline without substantial burst release. Compared to intravenous cisplatin, subcutaneously injected 75HA-Lys-Pt formed 3.2-fold more Pt-DNA adducts in rat peripheral blood mononuclear cells compared to intravenous cisplatin over 96 hours. Subcutaneous 75HA-Lys-Pt was tolerable in rats at 40 mg/kg (4× the LD50 of conventional cisplatin) and resulted in 62.5% partial response and 37.5% stable disease in murine xenografts of head and neck squamous cell cancer (20 mg/kg/wk × 3 wk). 75HA-Lys-Pt demonstrated extended t_{max} and improved area-under-the-curve compared to cisplatin in rats and canines. Canine safety was demonstrated by liver enzyme and electrolyte levels, complete blood count, and urinalysis.

Introduction

Cisplatin and its analogues have been among the most widely utilized anticancer drugs in the world for over three decades. Its success has lead to the development of over one thousand

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Conflict of Interest

No potential conflicts of interest were disclosed by the other authors.

analogues since the 1960s to improve tolerability, broaden activity, and to overcome drug resistance. Yet, less than 20 platinum drugs have made it into human clinical trials, and only 3 have won regulatory approvals in the US. They are the original cisplatin initially approved for ovarian and testicular cancers in 1978, carboplatin approved for palliative treatment of recurrent ovarian cancer in 1988, and oxaliplatin approved for second line treatment of metastatic colorectal cancer in 2002. Cisplatin remains the most potent in most indications, but these analogues generally have improved tolerability and equivalent overall survival and/or remission rates in the approved indications. [1–5]. There remains a need for platinum-based anti-cancer drugs with improved tolerability and efficacy.

Despite different chemical structures, cisplatin and its analogues undergo similar ligand hydrolysis reactions *in vivo* that form the active platinum species $\text{cis-}[\text{Pt}(\text{X})_2(\text{OH}_2)(\text{Y})]^+$, (Pt-monoaqua) and $\text{cis-}[\text{Pt}(\text{X})_2(\text{OH}_2)_2]^{2+}$, (Pt-diaqua). The aqua ligands on the Pt-monoaqua and Pt-diaqua are easily displaced by DNA bases, preferentially adenine and guanine, forming $[\text{Pt}(\text{X})_2(\text{Y})(\text{adenine-DNA})]^+$ and $[\text{Pt}(\text{X})_2(\text{Y})(\text{guanine-DNA})]^+$, subsequently $[\text{Pt}(\text{X})_2(\text{adenine-DNA})_2]^{2+}$ and $[\text{Pt}(\text{Y})_2(\text{guanine-DNA})_2]^{2+}$ via the displacement of the other ligands (Supporting Information Figure 4). The formation of intra- and inter-strand DNA crosslinks in cancer cells interfere with cell division and DNA repair, and in turn trigger cell death [6]. The ligands function primarily to alter the reactivity to the DNA bases and lipophilicity and hence alter tissue distribution and pharmacokinetics. The mechanisms of cisplatin activation and cell death are a complex process and may involve multiple possible pathways, which has been reviewed elsewhere [7].

Platinum chemotherapy is also highly damaging to normal cells that divide rapidly. Approximately 30% of patients who received 50–100 mg/m² cisplatin as an initial dose developed severe nephrotoxicity, due to the damaged pars recta of the proximal tubules of the kidney [8], and over half of patients develop hypomagnesaemia and hypocalcaemia [9]. In contrast, nephrotoxicity is greatly reduced in the carboplatin regimen, but thrombocytopenia, which is uncommon for cisplatin, is dose limiting in 25% of carboplatin patients [10].

The distinct toxicity profiles of cisplatin and carboplatin are likely attributed to the differences in the chemical reactivity, pharmacology, and toxicology of the two platinum compounds. The cyclobutane-dicarboxylate ligands of carboplatin are slowly hydrolyzed to form the same membrane impregnable aquated species as cisplatin, but the hydrolysis rate of carboplatin is approximately 10-fold slower than cisplatin [11], which causes significant changes in the pharmacokinetics, distribution and tolerability. In terms of excretion, 25% of cisplatin is excreted unchanged in the urine, while 90% of carboplatin is excreted unchanged in the urine. In regards to metabolism, cisplatin undergoes inactivation by sulfhydryl groups *in vivo*, while carboplatin is not significantly metabolized. These differences in pharmacokinetics may contribute to the longer elimination half-life and improved toxicity profile of carboplatin [12].

In our previous studies, we developed a first-generation cisplatin-based polymeric conjugate, HylaPlat (HA-Pt), using the biocompatible and non-immunogenic polysaccharide hyaluronan (HA) for locoregional delivery of platinum chemotherapy to treat spontaneous

canine cancers [13]. This conjugate overcame the lack of targeting inherent to most polymeric cisplatin delivery vehicles, such as HPMA, PEG, and polyamino acid conjugates [14], which relied on enhanced permeability and retention (EPR) effects [15] found in some tumors, but these conjugates lacked tumor-receptor specific targeting. The first generation hyaluronan carrier was based on a direct attachment of cisplatin to the polymer, and it released active aquated cisplatin over several hours ($t_{1/2}$ 10 hrs). It demonstrated superior pharmacokinetics in rodents and canines compared to cisplatin, and showed a 23% cure in heterogeneous oral squamous cell carcinomas in dogs (compared to 7% for cisplatin [16]). The conjugate did not demonstrate renal toxicity in either rodents or dogs, which is the dose-limiting side effect of cisplatin chemotherapy. However, it resulted in myelosuppression, hepatic, and cardiac toxicities in some patients in a Phase I/II canine clinical trial. Platinum chemotherapeutics rarely cause hepatic and cardiac toxicities in clinical practice [13]. We believe the unexpected toxicity was due to rapid release of the Pt-diaqua from the conjugate, altered deposition of the HA bound platinum, and prolonged retention in the liver during HA metabolism. To address potential hepatic and cardiac toxicities, we developed a second-generation stabilized ring-conjugate, HA-Lys-Pt, via a linker chemistry, with extended release half life and improved safety as detailed in this work. Our hypothesis was slower release would decrease burst release after injection, allow equilibrium to the non-aquated forms upon release from the polymer, prolong release into tissues, and alleviate toxicities.

Materials and Methods

Materials

Unless noted, all reagents were of highest grade available from Fisher Scientific (Pittsburgh, PA). The MDA-1986 human oral squamous carcinoma and the 4T1.2-neu murine breast cancer cell lines were kindly provided by Dr. Jeffery Myers (M.D. Anderson Cancer Center, Houston, TX), and Dr. Zhaoyang You (The University of Pittsburgh, Pittsburgh, PA), respectively. The human melanoma cell line was purchased from ATCC (Manassas, VA). Water for all aqueous solutions was ASTM Type 1. All reactions were conducted at ambient temperature, ca. 20°C, unless noted otherwise.

Synthesis of Hyaluronic Acid-Tetrabutylammonium (HA-TBA) Salt

Sodium hyaluronate (35- or 75-kDa, Lifecore Biomedical, Chaska, MN) was dissolved in water (1 g in ca. 300 mL) and then stirred overnight with 10 grams of cation exchange resin (Dowex 50W-X8, Bio-Rad, Hercules, CA). After filtration, the filtrate was neutralized with tetrabutylammonium hydroxide solution (TBA-OH, 55–60% w/v in water), and then freeze dried to obtain an orange to pink powder. The products were named 35HA-TBA and 75HA-TBA to reflect the molecular weights of the HA used.

Synthesis of HA-N^α-Acetyl-L-lys (HA-Lys)

Two hundred milligrams of HA-TBA was dissolved in 10 mL of dimethyl sulfoxide (DMSO) and after 10 minutes the solution turned clear and homogenous. The polymer was activated with 123.6 mg of N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC•HCl) and 140 mg of N-hydroxysulfosuccinimide (sulfo-NHS), and after 2 hours of stirring, 121.3 mg of N^α-Acetyl-L-lysine (Chem-Impex International Inc., Wood Dale, IL) in

4 mL of DMSO was added and stirred for another 22–24 hours. The product was dialyzed (10,000 MWCO, Fisher Scientific) against water for 4 hours, 100-mM sodium chloride for 2 hours, followed by two water changes over 18 hours. The first dialysis in water was to remove DMSO; the second dialysis in NaCl solution was to remove TBA, EDC/EDU, Sulfo-NHS and excess N-Ac-lysine; and the third and fourth dialyses in water were to remove any small molecule residues and NaCl. The product was filtered (0.22- μ m PES syringe filter) and concentrated using a centricon filter (10,000 MWCO, Fisher Scientific). The products were named 35HA-Lys and 75HA-Lys to reflect the molecular weights of the HA used. Synthesis of the 6.4HA-Lys is in the supplemental data.

Synthesis of HA-cis-[Pt(NH₃)₂(N^α-Acetyl-L-Lysine)] (HA-lys-Pt)

Cisplatin (60 mg, 200 μ mol, Qilu Pharmaceutical Inc., Shandong, China) was suspended in 3 mL of ca. 35°C water and then stirred with AgNO₃ (68 mg, 400 μ mol) for 1 day at ca. 20°C in the dark. The AgCl₂ precipitate was removed by centrifuging (4,000 rpm, 10 minutes), and the supernatant was filtered (0.22- μ m nylon syringe filter) to obtain *cis*-[Pt(NH₃)₂(OH)₂].

The HA-lys (35HA-Lys or 75HA-Lys, 125 mg) was dissolved in 15 mL of water, to which 815 μ L of *cis*-[Pt(NH₃)₂(OH)₂] (27 mg/mL on cisplatin basis) was added, and the pH of the mixture periodically adjusted to ~5.0 using 1-N NaOH. The reaction proceeded at 40 °C for 1 day and 50 °C for 2 days in the dark (Figure 1). The resulting solution was filtered (0.22- μ m nylon syringe filter) and then dialyzed (10,000 MWCO) against water for 4 hours, sodium phosphate solution (100-mM NaCl, 10-mM NaH₂PO₄, pH 7.4) for 2 hours, and two water changes over 18 hours. A detailed procedure for 6.4-kDa HA-Lys-Pt is in the Supporting Information S4.

Release study of Platinum from HA-Lys-Pt

A HA-lys-Pt solution (0.1 mg/mL on cisplatin basis) was dialyzed (10,000 MWCO) against phosphate buffered saline release medium (PBS, mM sodium phosphate, 137-mM NaCl, pH 7.4) at 37 °C. At pre-determined intervals, 100- μ L samples were collected and frozen at –20 °C until analysis. Samples were diluted 10,000 fold using 1% HNO₃ prior to ICP-MS analysis.

Degradation by Hyaluronidase

The biodegradability of HA-Lys-Pt was examined by treatment with bovine testicular hyaluronidase. HA-Lys-Pt (1.5 mg/mL on HA basis) was incubated with 5- μ g/mL hyaluronidase (HAase, from bovine testes, type I-S, 400–1000 units/mg solid, Fisher Scientific) in sodium phosphate buffer (140-mM NaCl, 16-mM NaH₂PO₄, and 7-mM Na₂HPO₄ at pH 6.4) at 37 °C for up to 24 hours. Timed samples were stored at –80 °C until analysis by size exclusion chromatography (SEC). Samples (N=3) were thawed, diluted 5-fold using mobile phase, and analyzed with a Shodex OHpak SB-804 HQ column using a refractive index (RI) detector. Conditions were 5-mM ammonium acetate (pH 5.0) mobile phase at 0.8 mL/min, column temperature of 40°C, and injections of 80 μ L. Known molecular weights of HA were used as calibration standards.

Analysis for aquated species by HPLC

Small molecular platinum species including cisplatin, Pt-monoaqua, and Pt-diaqua were quantified by HPLC. The mobile phase was sodium phosphate solution (10-mM, pH 3.0) at a flow rate of 0.25 mL/min on a Alltech® 250 mm × 4.6 mm M/M RP8/cation column thermostated at 25°C. The internal standard, a mixture of platinum species containing cisplatin, monoaquated and diaquated platinum, was prepared by dissolving 10 mg of cisplatin in 10 mL of water and stirring for 48 hours. A series of cisplatin solutions in saline were used as calibration standards. Prior to HPLC analysis, 1 mL of HA-lys-Pt solution (1.8 mg/mL on cisplatin basis) was incubated at 4 °C for 4 days, and then separated on a Centricon centrifugal filter unit (10,000 MWCO, 4000 rpm for 10 min at 4°C). The filtrate was immediately injected into the HPLC. The peaks of cisplatin, Pt-monoaqua, and Pt-diaqua were detected by UV detection at 283 nm.

Anti-proliferation

Human head and neck squamous cell carcinoma cells, MDA-1986, were maintained in Dulbecco's Modified Eagle Medium (DMEM, Corning, Manassas, VA) with 4.5 g/L glucose, 4.5 g/L L-glutamine, and 10% fetal bovine serum in a humidified incubator at 37°C and 5% CO₂. Cells were seeded into 96-well plates (3,000 cells/well in 90 µL of media) and allowed to attach overnight. Cisplatin, HA-Pt, or HA-lys-Pt was dissolved in water, added to cells (7 concentrations in duplicate; 10-µL addition), and incubated at 37 °C for 72 hours. All doses were on cisplatin basis. Resazurin Blue (Acros Organics, Geel, Belgium) in PBS was added to each well (5-µM final concentration; 10-µL addition) and incubated at 37 °C for 4 hours. Fluorescence (λ_{ex} 550 nm, λ_{em} 605 nm) was quantified with a SpectraMax Gemini XS plate-reader (Molecular Devices, Sunnyvale, CA). The relative growth of cells incubated with each compound concentration was normalized to vehicle-treated controls (100% proliferation). Data from at least three separate experiments performed in duplicate was analyzed using non-linear regression (GraphPad Prism 5.0) to generate IC₅₀ values.

Pt-DNA Adduct and Plasma Pt Quantitation

Female Sprague-Dawley rats (225–275g; Charles River, Wilmington, MA) were treated with cisplatin (10 mg/kg, i.v. tail vein) or 75HA-Lys-Pt (10 mg/kg cisplatin basis, s.c. cheek directly below ear) under 4% isoflurane (VetOne, Boise, ID) anesthesia. After 15 minutes to 96 hours, whole blood was collected via cardiac puncture into heparin collection tubes. Plasma and DNA from white blood cells were isolated based on a published procedure with modifications [17]. The detailed procedure is included in Supporting Information Section S5. Cisplatin is a known vesicant, thus, subcutaneous injection of cisplatin is excluded in the study design as it is not a clinically feasible option due to the side effect to the local skin tissue [18]. A previous generation of intravenously injected carried platinum resulted in high C_{max} immediately after injection, thus, i.v. HA-Lys-Pt was excluded from the study design due to potential ototoxicity and renal toxicity. In the current study, s.c. HA-Lys-Pt and i.v. cisplatin are compared [19].

Toxicity in Rodents

Groups of Balb/c mice were dose escalated to determine the maximum tolerated dose; animals were injected subcutaneously with 20 (N=2), 30 (N=3), and 40 mg/kg (N=2) of the 75HA-Lys-Pt. Another group were dosed with 8.86 (N=2), 13.3 (N=2), 17.7 (N=2), 20 (N=2), 30 (N=2), and 40 mg/kg (N=1) of the 33HA-Lys-Pt. Their body weights and symptoms of toxicities were monitored twice per week for up to 2 months.

Anticancer Efficacy in Mice

The MDA-1986 cells were prepared in PBS at a concentration of 2×10^7 cells/mL. Female NU/NU mice were anesthetized under 2% isoflurane in O₂, and 50 μ L of cell solution was injected into the oral sub-mucosa of the mice using a 27-ga needle. Tumor growth was monitored twice weekly by measurement with a digital caliper, and the tumor volume was calculated as $0.52 \times (\text{width})^2 \times (\text{length})$. The primary cheek tumors reached 50–100 mm³ typically after 2 weeks, and HA-Lys-Pt was administered peritumorally subcutaneously at 20 mg/kg on the basis of cisplatin. Cisplatin was administered intravenously via tail veins at a dose of 10 mg/kg, the maximum determined previously [13]. The drugs were given weekly for 3 consecutive weeks. All procedures in the animal study were approved by the Institutional Animal Care and Use Committee of the University of Kansas.

Pharmacokinetics and Toxicology in a Healthy Dog and Three Dogs with Oral Squamous Cell Carcinomas

A pilot dose-finding study of 75HA-Lys-Pt was conducted in a normal dog at 1.5 and 2.0 mg/kg. The safety of the 75HA-Lys-Pt was evaluated in 3 dogs with oral squamous cell carcinomas in an open-label Phase I clinical trial at the University of Missouri. All animal procedures have been approved by the Institutional Animal Care and Use Committee. Pharmacokinetic and toxicological data was collected. Due to the limited number of animals included in this pilot study, the study design and result are included in the Supporting Information S2 and S3.

Results

Synthesis of HA-Lys-Pt

We successfully synthesized a series of HA-Lys-Pt conjugates with multiple molecular weights of the hyaluronan carrier and a cisplatin loading degree of 6–8% wt.

NMR Characterization

Hyaluronic Acid-Tetrabutylammonium (HA-TBA) Salt: ¹H-NMR (400 MHz, D₂O) δ (ppm): 4.46 (d, J = 6.8 Hz, 1H), 4.36 (d, J = 6.8 Hz, 1H), 3.74–3.25 (m, 10H), 3.12–3.08 (t, J = 8.4 Hz, 8H), 1.92 (s, 3H), 1.60–1.52 (tt, J = 8 Hz, 8H), 1.27 (qt, J = 7.2 Hz, 8H), 0.85 (t, J = 7.2 Hz, 12H).

HA-N^α-Acetyl-L-lys (HA-Lys): ¹H-NMR (400 MHz, D₂O) δ (ppm): 4.57–4.29 (m, 2H), 4.06 (dd, J = 5.6 Hz, J = 7.6 Hz, 1H), 3.74–3.25 (m, 10H), 1.95 (s, 3H), 1.94 (s, 3H), 1.66–1.58 (m, 2H), 1.43 (tt, J = 6.4 Hz, 2H), 1.12 (m, 2H).

HA-cis-[Pt(NH₃)₂(N^α-Acetyl-L-lysine)] (HA-lys-Pt): ¹³C-NMR (500 MHz, D₂O) δ (ppm): 190.2, 178.0, 174.9, 174.0, 103.1, 100.5, 82.4, 79.8, 76.2, 75.3, 73.5, 72.4, 68.4, 65.2, 60.5, 54.3, 42.7, 36.0, 28.4, 24.1, 22.5, 21.7.

Release of Platinum Species from HA-Lys-Pt

The release profiles of platinum from HA-Lys-Pt were evaluated via dialysis against PBS at the physiological pH of 7.4, and against acetate buffer at pH 5.5 and 37 °C to simulate the acidic environment of tumor interstitium and cell endosomes. The platinum concentrations in tubing were measured and plotted as the percentage of cumulative drug released against time (Figure 2). The drug release from 75HA-lys-Pt followed pseudo-first-order kinetics; release rates in PBS were 0.036 h⁻¹ in the first 4 hours and 0.034 h⁻¹ over the first 24 hours, indicating little or no apparent burst release and an initial half life of ca. 21 hours. Over the entire 50 day period the release rate slowed to 0.010 h⁻¹, or an overall half life of 69 hours. Drug release was more rapid in acetate buffer, with a release rate of 0.070 h⁻¹ in the first 24 hours, initial half life of 9.9 hours, and overall rate of 0.022 h⁻¹ over 600 hours, or an overall half life of 32 hours. The more rapid release of Pt(II) ions in an acidic environment was anticipated. This is attributed to the acid labile nature of the N^α-acetylamido ligand that is protonated easily under acidic condition, followed by de-chelation from the Pt(II). Pt(II) was released from the 75HA-Lys carriers in the form of Pt-diaqua complex. This suggests that the enhanced release of the Pt aqua-active intermediate form at a lower pH may be advantageous to the formation of Pt-purine-DNA adducts in tumor cells.

Statistical analysis was conducted using t-test for the release study (GraphPad Software). The 75HA-Lys-Pt (pH 7.4) plot was statistically different with all other 4 plots with a p value of less than 0.05. In addition, the 33HA-lys-Pt (pH 7.4) and the 6.4HA-Lys-Pt (pH 7.4) were significantly different from each other. They both were significantly different from the cisplatin control.

Degradation by Hyaluronidase

Both HA-Lys and HA-Lys-Pt maintained similar rates of degradation in the presence of HAase (p > 0.6, Figure 3). Within the incubation time of 24 hours, only a small amount of drug was liberated, and it is possible that the carrier is only recognized and degraded by the enzyme once the platinum releases from the carrier. The result of the degradation study was analyzed for statistical significance. Result indicated that all three groups degraded in a similar pattern. The p values between either two groups were greater than 0.05.

Purity Analysis by HPLC

Aquated platinum species are potentially toxic impurities in cisplatin-based formulations. The diaquated cisplatin may induce hepatic injury and may interfere with a vital nervous system function at the neuromuscular junction [20], and monoaquated cisplatin is 3 fold more nephrotoxic than cisplatin [21]. The purity of the HA-Lys-Pt (33 and 75 kDa) solution after being stored at 4 °C for 4 days was tested by HPLC. The level of diaqua was below the limit of quantitation of the assay (18.4 µg/mL). Neither Pt-monoaqua nor Pt-diaqua was detected in the 75HA-Lys-Pt (Figure 4).

Anti-proliferative Activity of HA-Lys-Pt

When cisplatin is directly conjugated to the carboxylate groups of hyaluronan, it forms a HA-Pt ionic and covalent complex that has been evaluated in our previous studies [19, 22–29]. HA-Pt exhibited a similar anti-proliferative activity as cisplatin against the human head and neck squamous cell carcinoma (HNSCC) cell line, MDA-1986. The *in vitro* toxicity of the conjugates was due to the release of the active forms of Pt species upon hydrolysis. The cytotoxicity of HA-Pt and HA-Lys-Pt conjugates was likely due to either carrier-mediated endocytosis of the conjugates prior to the cellular release of the drug and/or free drug internalization via passive diffusion following the extracellular cleavage of the drugs.

The anti-proliferative effect of HA-Lys-Pt was evaluated using MDA-1986 human head and neck cancer, A2058 human melanoma, and 4T1.2-neu murine breast cancer cell lines (Figure 5, Supporting Information Table 1). In all three cell lines, the positive control, cisplatin, and HA-Pt show equivalent growth inhibition potencies ($IC_{50} \approx 8 \mu M$), as well as complete inhibition of growth at the highest doses (full efficacy). The 75- and 33-kDa HA-Lys-Pt conjugates also show full anti-proliferative efficacy in MDA-1986 cells, but they are significantly less potent ($IC_{50} \approx 40$ and $21 \mu M$, respectively) than cisplatin or HA-Pt. This is attributed to the extended release of the free drug from the conjugate ($t_{1/2} = 69$ hours in PBS) compared to HA-Pt ($t_{1/2} = 10$ hours in PBS). The active Pt species were almost completely cleaved from HA-Pt during the 3-day incubation in cells. However, within the same duration, HA-Lys-Pt partially released the hydrolyzed free drug, resulting in decreased *in vitro* toxicity. The 6.4-kDa conjugate inhibited cell growth <50% at the highest dose tested (300 μM), and therefore was not evaluated further.

Pt-DNA Adduct and Plasma Pt Quantitation

The formation of DNA-platinum adduct contributes to the cytotoxicity of cisplatin [17, 30, 31] and DNA-platinum adduct formation in white blood cells are often used as a proxy for adduct formation in tumors [17, 32, 33]. Therefore, we compared DNA-platinum adduct formation in circulating white blood cells of healthy rats treated with cisplatin or 75HA-Lys-Pt (Figure 6A). The PBMC of cisplatin treated animals had near constant levels of adduct formation (11–16 pg Pt/ μg DNA) over 96 hours with an area-under-the-curve (AUC) of 1466 pg Pt $\times$ h/ μg DNA). In contrast, HA-Lys-Pt shows significantly greater adduct formation, increasing over time and peaking at 48 hours (106 pg Pt/ μg DNA), followed by a reduction over the next 2 days. The resulting AUC for 75HA-Lys-Pt is 4730 pg Pt $\times$ h/ μg DNA. Simultaneously, we measured Pt content in plasma from the same animals (Figure 6B). Cisplatin shows an early spike in plasma Pt concentration at 15 minutes of 7 μg Pt/mL plasma with an AUC of 102.2 pg Pt $\times$ h/mL. In contrast to cisplatin, but similar to adduct formation, 75HA-Lys-Pt shows increasing plasma Pt levels over time plateauing between 24 and 72 hours with concentration between 4–5 μg Pt/mL plasma with an AUC of 398.5 pg Pt $\times$ h/mL.

Two-way ANOVA was performed for the Pt-DNA adduct formation study. DNA adduct formation differed significantly between treatment groups at 24 and 48 hours ($p < 0.05$). The plasma Pt also differed significantly between treatment groups at 48 and 72 hours.

Toxicity in Mice and Rats

To assess the tolerability of HA-Lys-Pt, healthy Balb/C mice were given six different doses of the drug, and their body weights and toxic reactions were monitored twice per week for 40 days (Figure 7). All mice maintained normal body conditions and had some degree of weight loss during the treatment, but returned to pre-dose weights at the completion of the study. Mice that received a dose of 20 or 30 mg/kg (on cisplatin basis) began to lose weight one-week post treatment but returned to pre-dose weight within another two weeks. It took one additional week for the mice that received the highest dose (40 mg/kg) to return to pre-dose weight. Acute toxic reactions including lethargy, paralysis, and respiratory depression were not observed at any dose levels during the entire study.

Statistical analysis was performed for mice that received 30 mg/m² of the 75HA-Lys-Pt treatment. The average body weights between day 0 and day 10 were significantly different; whereas the body weights between day 0 and day 18 were not significantly different. This result suggested that the mice experienced a transient weight loss after the treatment, but they returned to pre-treatment weight 18 days after the injection. Body weights from day 18 to 40 were not compared to day 0 as the mice had gained weight during this timeframe and their average body weight was greater than pre-treatment. Results from the other treatment groups were not analyzed for statistical power due to the limited number of animals. Future studies will include additional numbers of animals.

Anticancer Efficacy in Mice

An orthotopic xenograft model of human HNSCC was established to examine the anti-cancer efficacy of HA-Lys-Pt. Tumors in the control group reached a size of approximately 1000 mm³ within 6 weeks after tumor cell implantation. In comparison, the tumor growth rate slowed in the 75HA-Lys-Pt arm immediately after the first treatment on week 3. Tumor progression was delayed by four weeks after subcutaneous 75HA-Lys-Pt therapy (student t test, $p < 0.05$). The treated animals developed tumors of an average size less than 200 mm³ by the end of the sixth week (Figure 8A), and the survival rate was significantly extended relative to the control and cisplatin treated groups (Figure 8D; Log-rank (Mantel-Cox) test, $p < 0.05$). A single dose of 20 mg/kg HA-Lys-Pt resulted in less than 5% of weight loss, whereas three weekly doses of 20 mg/kg caused more than 20% weight loss after the last injection, although body weight returned to normal three weeks after the last injection (Figure 8C).

The treatment outcome of each individual animal is reported in Table 1 and Figure 8B. The rates of partial response (PR) and stable disease (SD) were 62.5% and 37.5% for 75HA-Lys-Pt treated animals and 75% and 25% for 33HA-Lys-Pt treated animals, respectively. Neither group contained any animals with progressive disease (PD). However, the rates of PR, SD, and PD were 37.5%, 12.5%, and 50% for cisplatin treated groups, which suggests significantly lower effectiveness of the standard-of-care IV therapy. As a control arm, 100% of the saline treated animals developed PD shortly after tumor implantation.

In the tumor efficacy study, statistical analysis was performed based on tumor size between HA-Lys-Pt and cisplatin treated mice, as well as between HA-Lys-Pt and saline treated mice.

The HA-Lys-Pt groups demonstrated smaller tumor size compared to the other two groups. For example, the p values between HA-Lys-Pt and cisplatin groups and between HA-Lys-Pt and saline groups on day 30 were 0.0222 and 0.0027, respectively. After day 30 (approximately 4 weeks), the number of animals in the saline and cisplatin groups began to decrease relatively quickly due to the large tumor size and low body weight.

In the toxicity study of tumor-bearing nude mice, we compared the weight losses of saline, cisplatin, or HA-Lys-Pt treated animals (Fig 8C). One-way ANOVA suggested that cisplatin and HA-Lys-Pt treated animals had a similar pattern of weight loss. Mice in both groups lost about 30% weight at week 3 but returned to pre-treatment weight at about 5 weeks. Notably, mice received 20 mg/m² of HA-Lys-Pt but 10 mg/m² of cisplatin, suggesting that the HA-Lys-Pt could be less toxic than the cisplatin if an equivalent dose was given to both groups. Statistical analysis also showed that both treatments had resulted in greater weight loss than the saline control group ($p < 0.05$).

In the survival study of the tumor-bearing nude mice (Fig 8D), we found that the HA-Lys-Pt significantly prolonged the lifespan of the mice compared to the survival status of the mice that received cisplatin or saline. Results of the saline and cisplatin treated groups were not statistically different from each other.

Discussion

Polymer-based chemotherapeutics create the opportunity to deliver potent anti-cancer drugs more efficiently to tumor cells via localized drug administration, whereas intravenous infusion often results in poor tumor and lymph node penetration. Direct injection of many chemotherapeutics is not possible due to their severe vesicant properties [34]. The idea of linking a platinum agent to a water-soluble, biocompatible polymer was evaluated by Gianasi *et al.* in 1999 [35].

The carboxylate of hyaluronan's glucuronic acid is not an ideal ligand for chelation of cisplatin platinum species due to rapid release of the active drug and the formation of the toxic diaquated cisplatin. Thus a lower maximum tolerated dose and higher *in vitro* cytotoxicity were observed for both HPMA-spacer-COO-Pt [35] and HA-COO-Pt [13] in accordance with the rapid hydrolytic release profiles at both pH 7.4 and 5.5. More tunable and sustained release of platinates such as observed for the HA-Lys-Pt would be more desirable. In this work, a thermodynamically stable N,O-amidomalonate cis-diamineplatinum (II) complex was found when a kinetically stable O,O-amidomalonate cis-diamineplatinum (II) underwent rearrangement [36].

Unlike common polymeric carriers such as PEG and HPMA, HA has an innate ability to target cancer cells through strong interaction with the CD44 receptor over expressed in many cancers [37–40]. While many normal cells express CD44, including T and B cells, the CD44 normally exists in a glycosolated low-affinity form. Increased expression of CD44 and CD44v6 (and others) is associated with tumor aggressiveness, and these variants forms have increased binding affinity for HA compared to normal CD44.

We designed hyaluronan platinum chelates using the carboxyl groups on N-Ac-L-lysine through the C6-amine moiety of L-lysine to take advantage of the desired release kinetics of the Pt-N,O-chelates and utilize our previously established HA-Pt delivery strategy for localization to tumor and lymph nodes. The use of amino acids as Pt ligands along with the five-member ring chelate formation was initially reported by Erickson *et al* in 1968 [41], followed by extensive investigation of anti-tumor activity *in vitro* and comprehensive characterization by Appleton *et al* in the 1990s [42–45]. A polyacrylic acid conjugate was reported by Lee in 2010, but it was not studied *in vivo* [46].

The HA-Lys-Pt displayed tunable release profiles in PBS at pH 7.4 (initial 24 hour $t_{1/2} \sim 1$ days) and in acetate buffer at pH 5.5 (initial 24 hour $t_{1/2} \sim 10$ hours), which can be attributed to the acid labiality of the N_{α} -acetylamido ligand. This indicates that HA-Lys-Pt may distribute in the body under normal physiological condition without an initial burst release of the active Pt-species, which was consistent with the extended T_{max} observed for the HA-Lys-Pt treated the rats and dog (Figure 6 and Supporting Information Table 2).

Although no burst release was observed, the release rate of Pt from conjugates slowed after the initial 24 hour period. This may be a result of reduced diffusivity of larger hyaluronans, which form highly hydrated gels, and increased clustering of drug in hydrophobic domains. Whereas, the 74, 35 and 6.4kD had similar release rates in the first 24 hours (0.034, 0.047 and 0.041 h^{-1} , respectively), the smaller 6.4 and 35kD HA both had faster overall release rates (0.026 and 0.019 h^{-1} , respectively) than the larger 75kD HA (0.010 h^{-1}). This is consistent with reduced diffusivity and clustering effects in the larger HA carriers. Transmission electron microscopy (TEM) of the 75HA-Lys-Pt (see Supporting Information section S6) revealed highly heterogeneous speckled areas within the 25–50 nm particles. Based on hydrated gel models of HA, the overall size of a single polymer 75-kD HA is expected to be ca. 30 nm [47]. Since no staining was used with the samples, the dark regions indicate high atomic number species and are consistent with densely platinated regions within the particle.

Unlike the carboxylate bound Pt that had a T_{max} of 0.5 or 1 h in dog, the HA-Lys-Pt had a T_{max} of 6 h in dog. In rats, HA-Lys-Pt had a T_{max} of 48 hours for Pt-DNA adduct and 72 hours for total Pt in plasma. Once the conjugates enter the tumor microenvironment, a rapid release of Pt species from the HA-Lys may be activated due to the low pH environment of the tumor. Our hypothesis for the enhanced platinum release rate at a lower pH is that the N_{α} -acetyl group may be protonated primarily in the highly acidic conditions and thus dechelated from the Pt center; subsequently, N,O-chelate may be first converted back to the intra-polymer or inter-polymer O,O-chelate. It has been previously determined that approximately 20% and 68% of the N,O-chelates were converted to the O,O-chelates at pH 5.0–5.5 and 4.0–4.5, respectively, within 20 hours [48]. It is beneficial for therapeutic efficacy that the triggered release of the active Pt molecules occurs at the lysosomal pH (4.0–4.5) for the enzymatic degradation of macromolecules under endocytosis.

The biocompatibility and biodegradability of HA have lead to its usage in dermatology, wound healing, and drug delivery. HA is degraded *in vivo* by radical attacks, hydrolysis, and enzymatic digestions [49]. The carboxyl group of the glucuronic acid is the recognition site

of HA receptors, such as CD44 [50], and hyaluronidase enzymes, such as HYAL1/2, which cleave HA at the β (1,4) link, thus significant modification may alter its biological interactions and degradability [51]. To avoid this, we limit the chemistry modification to the carrier to less than 15%.

Our previous study demonstrated that direct conjugation of platinum to HA lead to the formation of a less stable conjugate, which released Pt relatively quickly in the form of Pt-diaqua. The Pt-diaqua species induced severe hepatic and neurological toxicity in mice and dogs [13]. To eliminate the harmful side effects of aquated cisplatin release, the conjugation chemistry was modified to yield a much safer formulation that is still biocompatible and biodegradable, but is free of the toxic Pt-diaqua species. The improved safety of the 75HA-Lys-Pt conjugate was confirmed by mouse dose-escalation study, which revealed a 3-fold increase in MTD compared to the HA-Pt [13]. We believe the greater tolerability was partially due to the differences in pharmacokinetics and deposition of the conjugate. For example, the t_{max} of HA-Pt and 75HA-Lys-Pt (first dose) in dogs were 0.5 and 6 h, respectively (Supporting Information Table 2). The longer period of time for platinum from 75HA-Lys-Pt to reach C_{max} in blood may translate to a more efficient re-chlorination reaction of Pt-diaqua in the physiological condition, thus reducing the potential toxicity of the drug.

Of the three molecular weights we evaluated, the 75-kDa conjugate was superior to the other two lower molecular weight conjugates in terms of extended release half-life, reduced toxicity, and improved efficacy against xenografts of head and neck squamous cell carcinoma. Thus, we selected the 75kD conjugate as the lead compound for further canine pharmacokinetics and efficacy study. We believe the improved *in vivo* characteristics are likely attributed to the more sustained drug release, the slower clearance from the injection site, and enhanced lymphatic uptake due to increase in particle size. In our previous study, we investigated the molecular weight dependency on lymphatic uptake using a series of HA-dye conjugates with increasing molecular weights of HA (6.4, 35, 75, 132, 357, and 697 kDa). The HA-dye was injected into the footpad of mice and the lymphatic uptake and deposition of the conjugates in the draining axillary lymph nodes were determined. The result suggested that the 75-kD conjugate exhibited the greatest area-under-the-curve in axillary lymph nodes, indicating superior localization of the conjugate and advantageous pharmacokinetics [47]. It is also possible that higher molecular weight HAs may further increase retention at the injection site; however, viscosity rapidly increases with molecular weight of HA. The 75-kD was a balance that provided low viscosity suitable for injection through a <20 ga needle at typical concentrations, which would be beneficial if injecting against the high interstitial pressure of many tumors.

Systemic administration of cisplatin results in an initial spike in plasma Pt C_{max} , which may be associated with the drug's toxicity [52]. We have replicated this result in the present study, as well as previous studies [53]. In contrast, 75HA-Lys-Pt showed very different pharmacokinetics. The more gradual increase in Pt plasma levels and greater AUC is consistent with both the delayed diffusion of the compound from the injection site and the slow release of platinum from the carrier. This improved PK and increased platinum coordination to the polymer are likely responsible for the increase in tolerability in mice.

Notably, nanoparticles and conjugates have often much greater bioavailabilities and AUCs compared to intravenously administered parent drug because the clearance rates are different between the two. Also, clearance rate could vary significantly in patients with compromised renal function and other disease conditions.

The formation of DNA-platinum adduct contributes to the cytotoxicity of cisplatin [17, 30, 31]. While some studies have correlated DNA-platinum adduct formation in white blood cells with clinical results [32, 33], other studies have failed to show this correlation [54, 55]. Whether or not this correlation exists likely depends on the type of cancer and the specific drug being examined. Regardless, DNA adduct formation in PBMC demonstrates that HA-Lys-Pt is able to enter cells and that the active drug can bind DNA, thereby preserving the known mechanism of action of platinum drugs. Further, the AUC of adduct formation is consistent with the plasma Pt results, suggesting that HA-Lys-Pt results in greater exposure to the drug than cisplatin and that this exposure shows a more gradual onset and is sustained over time. In this study, the greater AUC for both DNA adduct formation and plasma Pt are consistent with greater efficacy seen in our mouse model.

A variety of polymer conjugation chemistries and polymer platforms for cisplatin delivery have been reported, including HPMA, dextrans, and PEG-polymer conjugates [14]. The hyaluronic acid platinum chelates that we reported in this manuscript have several distinct benefits over other polymers. First, most polymers such as HPMA act as a solubilization aid and to avoid rapid renal and hepatic clearance. These polymers have no specific targeting capacity for cancer cells, except via the proposed EPR effect in some solid tumors that exhibit leaky vasculature. We have shown in prior studies that hyaluronan can target drugs to the lymph nodes and increase endocytic uptake by tumor cells via an interaction with the CD44 receptor [28]. Second, non-targeted polymers generally will greatly reduce the *in vitro* efficacy of conjugated drugs because they reduce or eliminate passive non-specific drug uptake, and drug release may be substantially delayed so that only part of the drug is released during a typical 72 or 96 hour cytotoxicity study. In our prior studies of a faster releasing cisplatin-HA conjugate ($t_{1/2}$ 10 h), the efficacy was similar to free cisplatin in culture. However, efficacy was superior *in vivo* and in canine clinical trials [13]. The chelates we report here have less activity than cisplatin in culture, most likely due to the long release half-life ($t_{1/2}$ 69 h). However, they are superior to the HPMA chelate platform [56]. In the HPMA studies, much higher doses of the chelates were administered (e.g. 75 mg/kg) to obtain similar tumor inhibition [56], suggesting that though the HPMA chelate was well tolerated, it was significantly less effective than the hyaluronan chelates on a dose basis. We have shown in prior studies that hyaluronan conjugates increase cellular uptake through CD44 receptor uptake. Third, the hyaluronan chelate formed a significantly higher amount of Pt-DNA adducts *in vivo* compared to cisplatin. However, the Pt-DNA adduct concentrations achieved with the HPMA chelate were much lower than those achieved with cisplatin [57]. This suggests that the chelates were not effectively taken up by cells, which would explain the potential lack of efficacy on a dose equivalent basis. Fourth, we used a potentially more biodegradable chelation chemistry than HPMA conjugates. Reported HPMA conjugates used a malonate, whereas we developed chelates based on acetylated lysine, which is found naturally in tissues [58]. The degradation products of our carrier are

hyaluronan monomers and acetylated lysine, both natural compounds, unlike non-degradable HPMa and the referenced linkers.

Conclusion

We have demonstrated the therapeutic efficacy of 75HA-Lys-Pt polymeric conjugates through a synergy of the tunable release of active Pt species from locoregional treatment of HA-Lys-Pt and improved tumoral uptake. These results suggest that the HA-lys-Pt nanoplatform can provide a new therapeutic approach for the treatment of a wide spectrum of cancers. In addition to the attenuated systemic toxicity in mice, rats, and dog, the unique characteristics of HA polymer and the locoregional route of delivery can be explored for the targeted intralymphatic delivery of active Pt species. A Phase I/II trial in canines with spontaneous cancers is ongoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ML Forrest, D Aires, WC Forrest, S Cai, C Groer, T Zhang and E Mohr are employees and/or have ownership interest in HylaPharm.

Reference

1. Taylor AE, et al. Long-term follow-up of the first randomized study of cisplatin versus carboplatin for advanced epithelial ovarian cancer. *J Clin Oncol.* 1994; 12(10):2066–2070. [PubMed: 7931475]
2. Rosell R, et al. Phase III randomised trial comparing paclitaxel/carboplatin with paclitaxel/cisplatin in patients with advanced non-small-cell lung cancer: a cooperative multinational trial. *Ann Oncol.* 2002; 13(10):1539–1549. [PubMed: 12377641]
3. Montagnani F, et al. Effectiveness and safety of oxaliplatin compared to cisplatin for advanced, unresectable gastric cancer: a systematic review and meta-analysis. *Gastric Cancer.* 2011; 14(1):50–55. [PubMed: 21340667]
4. Misset JL, et al. Multicenter phase II–III study of oxaliplatin plus cyclophosphamide vs. cisplatin plus cyclophosphamide in chemonaïve advanced ovarian cancer patients. *Ann Oncol.* 2001; 12(10):1411–1415. [PubMed: 11762813]
5. Fakhrian K, et al. Cisplatin- vs. oxaliplatin-based radiosensitizing chemotherapy for squamous cell carcinoma of the esophagus: a comparison of two preoperative radiochemotherapy regimens. *Strahlenther Onkol.* 2014; 190(11):987–992. [PubMed: 24737541]
6. Siddik ZH. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene.* 2003; 22(47):7265–7279. [PubMed: 14576837]
7. Basu A, Krishnamurthy S. Cellular responses to Cisplatin-induced DNA damage. *J Nucleic Acids.* 2010; 2010
8. Barabas K, et al. Cisplatin: a review of toxicities and therapeutic applications. *Vet Comp Oncol.* 2008; 6(1):1–18. [PubMed: 19178659]
9. Schilsky RL, Anderson T. Hypomagnesemia and renal magnesium wasting in patients receiving cisplatin. *Ann Intern Med.* 1979; 90(6):929–931. [PubMed: 375794]

10. Go RS, Adjei AA. Review of the comparative pharmacology and clinical activity of cisplatin and carboplatin. *J Clin Oncol*. 1999; 17(1):409–422. [PubMed: 10458260]
11. Pavelka M, Lucas MF, Russo N. On the hydrolysis mechanism of the second-generation anticancer drug carboplatin. *Chemistry*. 2007; 13(36):10108–10116. [PubMed: 17896336]
12. Lokich J, Anderson N. Carboplatin versus cisplatin in solid tumors: an analysis of the literature. *Ann Oncol*. 1998; 9(1):13–21. [PubMed: 9541678]
13. Cai S, Zhang T, Forrest WC, Yang Q, Groer C, Mohr E, Aires DJ, Axiak-Bechtel SM, Flesner BK, Henry CJ, Selting KA, Tate D, Swarz JA, Bryan JN, Forrest ML. Phase I/II Clinical Trial of Hyaluronan-Cisplatin Nanoconjugate for the Treatment of Spontaneous Canine Cancers. *Am J Vet Res*. 2015 (Accepted).
14. Oberoi HS, et al. Nanocarriers for delivery of platinum anticancer drugs. *Adv Drug Deliv Rev*. 2013; 65(13–14):1667–1685. [PubMed: 24113520]
15. Maeda H, et al. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J Control Release*. 2000; 65(1–2):271–284. [PubMed: 10699287]
16. Knapp DW, et al. Cisplatin therapy in 41 dogs with malignant tumors. *J Vet Intern Med*. 1988; 2(1):41–46. [PubMed: 3221354]
17. Brouwers EE, et al. Inductively coupled plasma mass spectrometric analysis of the total amount of platinum in DNA extracts from peripheral blood mononuclear cells and tissue from patients treated with cisplatin. *Anal Bioanal Chem*. 2008; 391(2):577–585. [PubMed: 18385987]
18. Bairey O, et al. Severe tissue necrosis after cisplatin extravasation at low concentration: possible "immediate recall phenomenon". *J Natl Cancer Inst*. 1997; 89(16):1233–124. [PubMed: 9274921]
19. Cai S, et al. Pharmacokinetics and disposition of a localized lymphatic polymeric hyaluronan conjugate of cisplatin in rodents. *J Pharm Sci*. 2010; 99(6):2664–2671. [PubMed: 19960530]
20. Cleare MJ, Hoeschele JD. Studies on the Antitumor Activity of Group VIII Transition Metal Complexes. Part 1. Platinum (II) Complexes. *Bioinorg Chem*. 1973; 2:187–210.
21. Jones MM, et al. The relative nephrotoxicity of cisplatin, cis-[Pt(NH₃)₂(guanosine)₂]²⁺, and the hydrolysis product of cisplatin in the rat. *Cancer Chemother Pharmacol*. 1991; 29(1):29–32. [PubMed: 1742846]
22. Cai S, et al. Intralymphatic chemotherapy using a hyaluronan-cisplatin conjugate. *J Surg Res*. 2008; 147(2):247–252. [PubMed: 18498877]
23. Xie Y, et al. Pulmonary delivery of cisplatin-hyaluronan conjugates via endotracheal instillation for the treatment of lung cancer. *Int J Pharm*. 2010; 392(1–2):156–163. [PubMed: 20363303]
24. Cohen MS, et al. A novel intralymphatic nanocarrier delivery system for cisplatin therapy in breast cancer with improved tumor efficacy and lower systemic toxicity in vivo. *Am J Surg*. 2009; 198(6):781–786. [PubMed: 19969129]
25. Yang Q, A D, Cai S, Fraga GR, Zhang D, Li CZ, Forrest ML. In vivo efficacy of nano hyaluronan-conjugated cisplatin for treatment of murine melanoma. *J Drugs Dermatol*. 2014; 13(3):283–287. [PubMed: 24595572]
26. Venable R, et al. Hyaluronan cisplatin conjugate in five dogs with soft tissue sarcomas. *Am. J. Vet. Res*. 2012; 73(12):1969–1976. [PubMed: 23176425]
27. Cohen SM, et al. Efficacy and toxicity of peritumoral delivery of nanoconjugated cisplatin in an in vivo murine model of head and neck squamous cell carcinoma. *JAMA Otolaryngol Head Neck Surg*. 2013; 139(4):382–387. [PubMed: 23599074]
28. Cai S, A A, Yang Q, Forrest WC, Shnyder Y, Forrest ML. Cellular uptake and internalization of hyaluronan-based doxorubicin and cisplatin conjugates. *J Drug Target*. 2014; 22(7):648–657. [PubMed: 24892741]
29. Cai S, et al. Carrier-based intralymphatic cisplatin chemotherapy for the treatment of metastatic squamous cell carcinoma of the head & neck. *Ther Deliv*. 2010; 1(2):237–245. [PubMed: 21339844]
30. Jamieson ER, Lippard SJ. Structure, Recognition, and Processing of Cisplatin-DNA Adducts. *Chem Rev*. 1999; 99(9):2467–2498. [PubMed: 11749487]
31. Wong E, Giandomenico CM. Current status of platinum-based antitumor drugs. *Chem Rev*. 1999; 99(9):2451–2466. [PubMed: 11749486]

32. Schellens JH, et al. Relationship between the exposure to cisplatin, DNA-adduct formation in leucocytes and tumour response in patients with solid tumours. *Br J Cancer*. 1996; 73(12):1569–1575. [PubMed: 8664132]
33. Reed E, et al. Platinum-DNA adduct in leukocyte DNA of a cohort of 49 patients with 24 different types of malignancies. *Cancer Res*. 1993; 53(16):3694–3699. [PubMed: 8339278]
34. Balazsovits JA, et al. Analysis of the effect of liposome encapsulation on the vesicant properties, acute and cardiac toxicities, and antitumor efficacy of doxorubicin. *Cancer Chemother Pharmacol*. 1989; 23(2):81–86. [PubMed: 2491964]
35. Gianasi E, et al. HPMa copolymer platinates as novel antitumour agents: in vitro properties, pharmacokinetics and antitumour activity in vivo. *Eur J Cancer*. 1999; 35(6):994–1002. [PubMed: 10533484]
36. Stewart, Donald R.; Rice, John R.; St, JJV. N,O-amidomalonate platinum complexes. In: USPTO. , editor. Google Patent. US: Access Pharmaceuticals, Inc.; 2004.
37. Goodarzi N, et al. CD44-targeted docetaxel conjugate for cancer cells and cancer stem-like cells: a novel hyaluronic acid-based drug delivery system. *Chem Biol Drug Des*. 2014; 83(6):741–752. [PubMed: 24444042]
38. Thomas RG, et al. Paclitaxel loaded hyaluronic acid nanoparticles for targeted cancer therapy: in vitro and in vivo analysis. *Int J Biol Macromol*. 2015; 72:510–518. [PubMed: 25224289]
39. Negi LM, et al. Hyaluronic acid decorated lipid nanocarrier for MDR modulation and CD-44 targeting in colon adenocarcinoma. *Int J Biol Macromol*. 2015; 72:569–574. [PubMed: 25220787]
40. Bourguignon LY, Shiina M, Li JJ. Hyaluronan-CD44 interaction promotes oncogenic signaling, microRNA functions, chemoresistance, and radiation resistance in cancer stem cells leading to tumor progression. *Adv Cancer Res*. 2014; 123:255–275. [PubMed: 25081533]
41. Erickson LE, D A, Uhlenhopp JC. Proton magnetic resonance studies of amino acid complexes of platinum(II). II. Kinetics of N-H and C-H proton exchange. *J. Am. Chem. Soc*. 1968; 91(10): 2510–2513.
42. Appleton TG, H J, Ralph SF. Reactions of platinum(II) aqua complexes. 3. Multinuclear (nitrogen-15, platinum-195, carbon-13, and proton) NMR study of reactions of aqua and hydroxo complexes with glycine and (methylimino)diacetic acid. *Inorg. Chem*. 1985; 24(5):673–677.
43. Appleton, Trevor G.; JRH; Prenzler, Paul D. Reaction of the cis - Diamminediaquaplatinum(II) Cation with N-Acetylglycine. *Inorg. Chem*. 1989; 28:815–819.
44. Appleton TG, H J, Hambley TW, Prenzler PD. Reactions of the cis-diamminediaquaplatinum(II) cation with glycylglycine, N-glycylglycine, and N-(N-glycylglycyl) glycine. Crystal structure of a complex with two diammineplatinum(II) cations bound to glycylglycinate. *Inorg. Chem*. 1990; 29(18):3562–3569.
45. Appleton, Trevor G.; JRH; Neale, Donald W.; Thompson, Craig SM. Reactions of the cis-Diamminediaquaplatinum(II) Cation with 2-Aminomalonate and Its Homologues, Aspartate and Glutamate. Rearrangements of Metastable Complexes with Carboxylate-Bound Ligands to N,O-Chelates and Formation of Di- and Trinuclear Complexes. *Inorg. Chem*. 1990; 29:3985–3990.
46. Lee SM, O'Halloran TV, Nguyen ST. Polymer-caged nanobins for synergistic cisplatin-doxorubicin combination chemotherapy. *J Am Chem Soc*. 2010; 132(48):17130–17138. [PubMed: 21077673]
47. Bagby TR, et al. Impact of molecular weight on lymphatic drainage of a biopolymer-based imaging agent. *Pharmaceutics*. 2012; 4(2):276–295. [PubMed: 24300232]
48. Sood P, et al. Synthesis and characterization of AP5346, a novel polymer-linked diaminocyclohexyl platinum chemotherapeutic agent. *Bioconjug Chem*. 2006; 17(5):1270–1279. [PubMed: 16984138]
49. Vercruysse KP, Lauwers AR, Demeester JM. Kinetic investigation of the degradation of hyaluronan by hyaluronidase using gel permeation chromatography. *J Chromatogr B Biomed Appl*. 1994; 656(1):179–190. [PubMed: 7952027]
50. Oh EJ, et al. Control of the molecular degradation of hyaluronic acid hydrogels for tissue augmentation. *J Biomed Mater Res A*. 2008; 86(3):685–693. [PubMed: 18022803]
51. Schanté, Carole E., et al. Improvement of hyaluronic acid enzymatic stability by the grafting of amino-acids. *Carbohydrate Polymers*. 2012; 87:2211–2216.

52. Launay-Vacher V, et al. Prevention of cisplatin nephrotoxicity: state of the art and recommendations from the European Society of Clinical Pharmacy Special Interest Group on Cancer Care. *Cancer Chemother Pharmacol.* 2008; 61(6):903–909. [PubMed: 18317762]
53. Cai S, et al. Pharmacokinetics and disposition of a localized lymphatic polymeric hyaluronan conjugate of cisplatin in rodents. *J Pharm Sci.* 2010; 99(6):2664–2671. [PubMed: 19960530]
54. Hoebbers FJ, et al. Prediction of treatment outcome by cisplatin-DNA adduct formation in patients with stage III/IV head and neck squamous cell carcinoma, treated by concurrent cisplatin-radiation (RADPLAT). *Int J Cancer.* 2006; 119(4):750–756. [PubMed: 16550603]
55. Fichtinger-Schepman AM, et al. Kinetics of the formation and removal of cisplatin-DNA adducts in blood cells and tumor tissue of cancer patients receiving chemotherapy: comparison with in vitro adduct formation. *Cancer Res.* 1990; 50(24):7887–7894. [PubMed: 2253228]
56. Rice JR, et al. Preclinical efficacy and pharmacokinetics of AP5346, a novel diaminocyclohexane-platinum tumor-targeting drug delivery system. *Clin Cancer Res.* 2006; 12(7 Pt 1):2248–2254. [PubMed: 16609041]
57. Rademaker-Lakhai JM, et al. A Phase I and pharmacological study of the platinum polymer AP5280 given as an intravenous infusion once every 3 weeks in patients with solid tumors. *Clin Cancer Res.* 2004; 10(10):3386–3395. [PubMed: 15161693]
58. Lundby A, et al. Proteomic analysis of lysine acetylation sites in rat tissues reveals organ specificity and subcellular patterns. *Cell Rep.* 2012; 2(2):419–431. [PubMed: 22902405]

Figure 1.
Synthesis of HA-Lys-Pt using 35- or 75-kDa HA

Figure 2.
In vitro release of Pt(II) from HA-Lys-Pt in PBS at pH 7.4 or acetate at pH 5.5 and 37 °C.

Figure 3.
Degradation of HA, HA-Lys, and 75HA-Lys-Pt by HAdase in 24 hours.

Figure 4.
Chromatograms of Pt species mixture solution and the filtrate of HA-Lys-Pt solution generated by cation exchange HPLC.

Figure 5. Anti-proliferative activity of HA-Lys-Pt conjugates in MDA-1986 human head and neck squamous cell carcinoma cell line. Cells were treated with increasing concentrations of compound for 72 hours and cell proliferation was quantified using Resazurin Blue. Data from at least three separate experiments performed in duplicate was analyzed by non-linear regression. Representative curves are shown. Complete data set is in Supporting Information Table 1.

Figure 6.

Pt-DNA Adduct and Plasma Pt Quantitation. Rats were treated with cisplatin (i.v., 10mg/kg) or HA-Lys-Pt (s.c.; 10mg/kg cisplatin basis). Pt content was quantified by ICP-MS in white blood cell DNA and plasma. A) DNA adduct formation (mean $\pm$ S.E.M.; $n=3-6$). Cisplatin $C_{max} = 19.0 \pm 4.2$ pg Pt/ μ g DNA; $T_{max} = 48$ hr; AUC = 1466 pg Pt $\times$ h/ μ g DNA. HA-Lys-Pt $C_{max} = 106.4 \pm 26.9$ pg Pt/ μ g DNA; $T_{max} = 48$ hr; AUC = 4730 pg Pt $\times$ h/ μ g DNA. B) Plasma Pt (mean $\pm$ S.E.M.; $n=3-6$). Cisplatin $C_{max} = 7.0 \pm 2.1$ μ g Pt/mL plasma; $T_{max} = 0.25$ hr; AUC = 102.2 pg Pt $\times$ h/mL. HA-Lys-Pt $C_{max} = 5.7 \pm 1.1$ μ g Pt / mL plasma; $T_{max} = 72$ hr; AUC = 398.5 pg Pt $\times$ h/mL.

Figure 7.

A) Percent weight loss after a single injection of 75HA-Lys-Pt at 20 (N=2), 30 (N=3), and 40 mg/kg (N=2). B) Percent weight loss after a single injection of 33HA-Lys-Pt at 8.86 (N=2), 13.3 (N=2), and 17.7 (N=1) mg/kg.

Figure 8.

A) Growth of head and neck tumors after three weekly injections of saline (i.v., N=9), 75HA-Lys-Pt (20 mg/kg s.c., N=8), and cisplatin (10 mg/kg i.v., N=8); B) Tumor growth in individual mouse that received three weekly injections of 20 mg/kg 75HA-Lys-Pt subcutaneously; C) Percent weight loss of mice that received saline, 75HA-Lys-Pt, or cisplatin; D) Survival rate of the animals (HA-Lys-Pt: 40% survival at approximately 10 weeks; cisplatin: 40% survival at approximately 4 weeks).

Table 1

Tumor response based on Modified RECIST Criteria.

Treatment	#1	#2	#3	#4	#5	#6	#7	#8
75HA-Lys-Pt	PR	PR	PR	SD	SD	PR	SD	PR
33HA-Lys-Pt	PR	PR	SD	PR	PR	SD	PR	PR
Cisplatin	PR	PD	SD	PR	PD	PR	PD	PD
Saline	PD	PD	PD	PD	PD	PD	PD	PD

PR=Partial Response (>30% reduction); PD=Progressive Disease (>30% tumor growth); SD=Stable Disease (neither PR nor PD criteria met).