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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 five-membered ring with the platinum. The conjugate was characterized for release kinetics, *in vitro* antiproliferative activity, degradability, impurity content, formation of Pt-DNA adducts, pharmacokinetics, tolerability in rodents and canines, and for efficacy in rodents. The 75 kD HA-Lys-Pt (75HA-Lys-Pt) sustained release of platinum with a 69 h 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 h. Subcutaneous 75HA-Lys-Pt was tolerable in rats at 40 mg/kg (4 × 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 weeks). 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.

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Introduction

Cisplatin and its analogs have been among the most widely utilized anticancer drugs in the world for over 3 decades. Its success has led to the development of over 1000 analogs 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 USA. 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 analogs 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 anticancer drugs with improved tolerability and efficacy.

Despite different chemical structures, cisplatin and its analogs undergo similar ligand hydrolysis reactions *in vivo* that form the active platinum species *cis*-$[Pt(X)_2(OH_2)(Y)]^+$, (Pt-monoaqua) and *cis*-$[Pt(X)_2(OH_2)_2]^{2+}$, (Pt-diaqua). The aqua ligands on the Pt-monoaqua and Pt-diaqua are easily displaced by DNA bases, preferentially adenine and guanine, forming $[Pt(X)_2(Y)$ (adenine-DNA)$]^+$ and

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$[Pt(X)_2(Y)(guanine\text{-}DNA)]^+$, subsequently $[Pt(X)_2(adenine\text{-}DNA)_2]^{2+}$ and $[Pt(Y)_2(guanine\text{-}DNA)_2]^{2+}$ via the displacement of the other ligands (Supporting Information Fig. 4). The formation of intra- and interstrand 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 have 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/m^2 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 the patients develop hypomagnesemia and hypocalcemia.[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 2 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 nonimmunogenic 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 N-(2-Hydroxypropyl)methacrylamide (HPMA), polyethylene glycol (PEG), and polyamino acid conjugates,[14] which relied on enhanced permeability and retention effects[15] found in some tumors, but these conjugates lacked tumor-receptor-specific targeting. The first-generation HA 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 h). 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 that 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, HA-*cis*-$[Pt(NH_3)_2(N^{\alpha}$-Acetyl-L-Lysine)], via a linker chemistry, with extended release half-life and improved safety as detailed in this work. Our hypothesis was that slower release would decrease burst release after injection, allow equilibrium to the nonaquated 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 Salt

Sodium hyaluronate (33 or 75 kDa; Lifecore Biomedical, Chaska, MN) was dissolved in water (1 g in approximately 300 mL) and then stirred overnight with 10 g of cation exchange resin (Dowex 50W-X8; Bio-Rad, Hercules, CA). After filtration, the filtrate was neutralized with tetrabutylammonium hydroxide solution (55%-60% wt/vol in water), and then freeze dried to obtain an orange to pink powder. The products were named 33HA-TBA and 75HA-TBA (hyaluronic acid-tetrabutylammonium) to reflect the molecular weights of the HA used.

Synthesis of HA-N^{α}-Acetyl-L-Lys

Two hundred milligrams of HA-TBA was dissolved in 10 mL of dimethyl sulfoxide (DMSO) and after 10 min 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 h 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 h. The product was dialyzed (10,000 MWCO; Fisher Scientific) against water for 4 h, 100-mM sodium chloride for 2 h, followed by 2 water changes over 18 h. The first dialysis in water was to remove DMSO; the second dialysis in NaCl solution was to remove TBA, EDC, 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-μm polyethersulfone syringe filter) and concentrated using a centricon filter (10,000 MWCO; Fisher Scientific). The products were named 33HA-Lys and 75HA-Lys (HA-N^{α}-Acetyl-L-Lys) to reflect the molecular weights of the HA used. A smaller molecular weight HA-Lys was also made using 6.4 kD HA. Synthesis of the 6.4HA-Lys is in the Supporting Information.

Synthesis of HA-cis-[$Pt(NH_3)_2(N^{\alpha}$-Acetyl-L-Lysine)]

Cisplatin (60 mg, 200 μmol; Qilu Pharmaceutical, Inc., Shandong, China) was suspended in 3 mL of approximately 35°C water and then stirred with $AgNO_3$ (68 mg, 400 μmol) for 1 day at approximately 20°C in the dark. The AgCl precipitate was removed by centrifugation (4000 rpm, 10 min), and the supernatant was filtered (0.22-μm nylon syringe filter) to obtain *cis*-$[Pt(NH_3)_2(OH)_2]$.

The HA-lys (33HA-Lys or 75HA-Lys, 125 mg) was dissolved in 15 mL of water, to which 815 μL of *cis*-$[Pt(NH_3)_2(OH)_2]$ (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 (Fig. 1). The resulting solution was filtered (0.22-μm nylon syringe filter) and then dialyzed (10,000 MWCO) against water for 4 h, sodium phosphate solution (100-mM NaCl, 10-mM NaH_2PO_4, pH 7.4) for 2 h, and 2

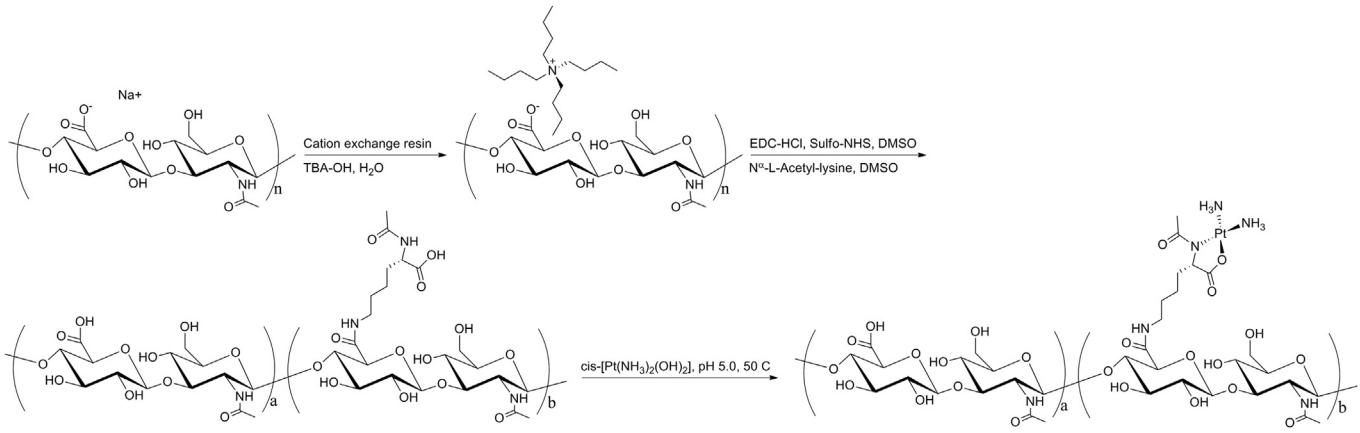

Figure 1. Synthesis of HA-Lys-Pt using 33- or 75-kDa HA.

water changes over 18 h. A detailed procedure for 6.4-kDa HA-Lys-Pt is in the Supporting Information S4.

Release Study of Platinum from HA-Lys-Pt

An HA-lys-Pt solution (0.1 mg/mL on cisplatin basis) was dialyzed (10,000 MWCO) against phosphate buffered saline release medium (PBS, 10mM sodium phosphate, 137 mM NaCl, pH 7.4) at 37°C. At predetermined intervals, 100-μL samples were collected and frozen at −20°C until analysis. Samples were diluted 10,000-fold using 1% HNO_3 prior to inductively coupled plasma mass spectrometry (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-μg/mL hyaluronidase (HAdase, from bovine testes, type I-S, 400-1000 U/mg solid; Fisher Scientific) in sodium phosphate buffer (140 mM NaCl, 16 mM NaH_2PO_4, and 7 mM Na_2HPO_4 at pH 6.4) at 37°C for up to 24 h. Timed samples were stored at −80°C until analysis by size exclusion chromatography. 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 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 10 μL. 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 an 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 h. 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 high performance liquid chromatography (HPLC). The peaks of cisplatin, Pt-monoaqua, and Pt-diaqua were detected by UV detection at 283 nm.

Antiproliferation

Human head and neck squamous cell carcinoma (HNSCC) cells, MDA-1986, were maintained in Dulbecco's modified eagle medium (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% CO_2. Cells were seeded into 96-well plates (3000 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 h. 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 h. 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 3 separate experiments performed in duplicate were analyzed using nonlinear regression (GraphPad Prism 5.0) to generate IC_{50} values.

Pt-DNA Adduct and Plasma Pt Quantitation

Female Sprague-Dawley rats (225-275 g; Charles River, Wilmington, MA) were treated with cisplatin (10 mg/kg, intravenous [i.v.] tail vein) or 75HA-Lys-Pt (10 mg/kg cisplatin basis, subcutaneous [s.c.] cheek directly below ear) under 4% isoflurane (VetOne, Boise, ID) anesthesia. After 15 min to 96 h, 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 (HA-Pt) 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 this 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 O_2, and 50 μL of cell solution was injected into the oral submucosa 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 mm^3 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 University of Missouri Institutional Animal Care and Use Committee. Pharmacokinetic and toxicological data were collected. Due to the limited number of animals included in this pilot study, the study design and result are included in 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 HA carrier and a cisplatin loading degree of 6-8%wt.

Nuclear Magnetic Resonance (NMR) Characterization

HA-TBA salt: ^{1}H-NMR (400 MHz, D_2O) 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-Lys: ^{1}H-NMR (400 MHz, D_2O) 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-lys-Pt: ^{13}C-NMR (500 MHz, D_2O) 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 (Fig. 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 h and 0.034 h^{-1} over the first 24 h, indicating little or no apparent burst release and an initial half-life of approximately 21 h. Over the entire 50-day period, the release rate slowed to 0.010 h^{-1}, or an overall half-life of 69 h. Drug release was more rapid in acetate buffer, with a release rate of 0.070 h^{-1} in the first 24 h, initial half-life of 9.9 h, and overall rate of 0.022 h^{-1} over 600 h, or an overall half-life of 32 h. 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.

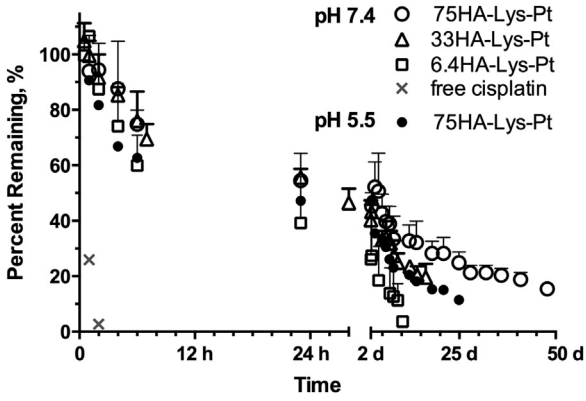

Figure 2. *In vitro* release of Pt(II) from HA-Lys-Pt in PBS at pH 7.4 or acetate at pH 5.5 and 37°C.

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 <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 HAdase ($p > 0.6$; Fig. 3). Within the incubation time of 24 h, only a small amount of drug was liberated,

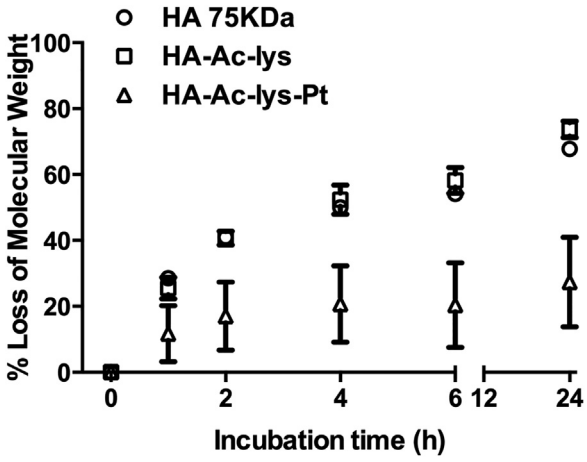

Figure 3. Degradation of HA, HA-Lys, and 75HA-Lys-Pt by HAdase in 24 h.

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. The result indicated that all 3 groups degraded in a similar pattern. The p values between either 2 groups were greater than 0.05.

Purity Analysis by HPLC

Aquated platinums 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 (Fig. 4).

Antiproliferative Activity of HA-Lys-Pt

When cisplatin is directly conjugated to the carboxylate groups of HA, it forms an HA-Pt ionic and covalent complex that has been evaluated in our previous studies.[19,22-29] HA-Pt exhibited a similar antiproliferative activity as cisplatin against the human 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 free drug internalization via passive diffusion following the extracellular cleavage of the drugs.

The antiproliferative 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 (Fig. 5, Supporting Information Table 1). In all 3 cell lines, the positive control, cisplatin, and HA-Pt show equivalent growth inhibition potencies ($IC_{50} \cong 8$ μM), 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 antiproliferative efficacy in MDA-1986 cells, but they are significantly less potent ($IC_{50} \cong 40$ and 21 μM, respectively) than cisplatin or HA-Pt. This is attributed to the extended release of the free drug from the conjugate ($t_{1/2} = 69$ h in PBS) compared to HA-Pt ($t_{1/2} = 10$ h 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.

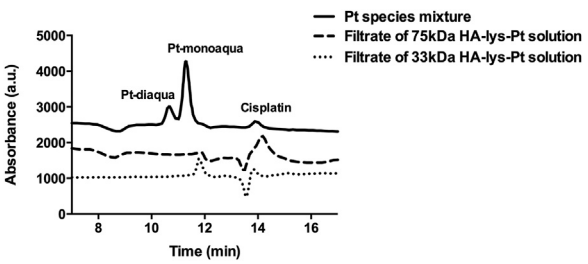

Figure 4. Chromatograms of Pt species mixture solution and the filtrate of HA-Lys-Pt solution generated by cation exchange HPLC.

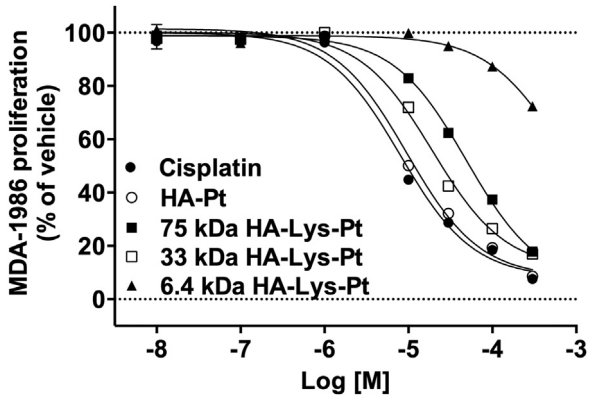

Figure 5. Antiproliferative activity of HA-Lys-Pt conjugates in MDA-1986 human HNSCC cell line. Cells were treated with increasing concentrations of compound for 72 h and cell proliferation was quantified using Resazurin Blue. Data from at least 3 separate experiments performed in duplicate were analyzed by nonlinear regression. Representative curves are shown. Complete data set is in Supporting Information Table 1.

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 (Fig. 6a). The peripheral blood mononuclear cell (PBMC) of cisplatin treated animals had near-constant levels of adduct formation (11-16 pg Pt/μg DNA) over 96 h with an area-under-the-curve (AUC) of (1466 pg Pt × h/μg DNA). In contrast, HA-Lys-Pt shows significantly greater adduct formation, increasing over time and peaking at 48 h (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 × h/μg DNA. Simultaneously, we measured Pt content in plasma from the same animals (Fig. 6b). Cisplatin shows an early spike in plasma Pt concentration at 15 min of 7 μg Pt/mL plasma with an AUC of 102.2 pg Pt × 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 h with concentration between 4 and 5 μg Pt/mL plasma with an AUC of 398.5 pg Pt × h/mL.

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

Toxicity in Mice and Rats

To assess the tolerability of HA-Lys-Pt, 6 groups of healthy BALB/c mice were given 6 different doses of the drug, and their body weights and toxic reactions were monitored twice per week for 40 days (Fig. 7). All mice maintained normal body conditions and had some degree of weight loss during the treatment, but returned to predose 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 1-week posttreatment but returned to predose weight within another 2 weeks. It took 1 additional week for the mice that received the highest dose (40 mg/kg) to return to predose 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/m^2 of the 75HA-Lys-Pt treatment. The average body weights

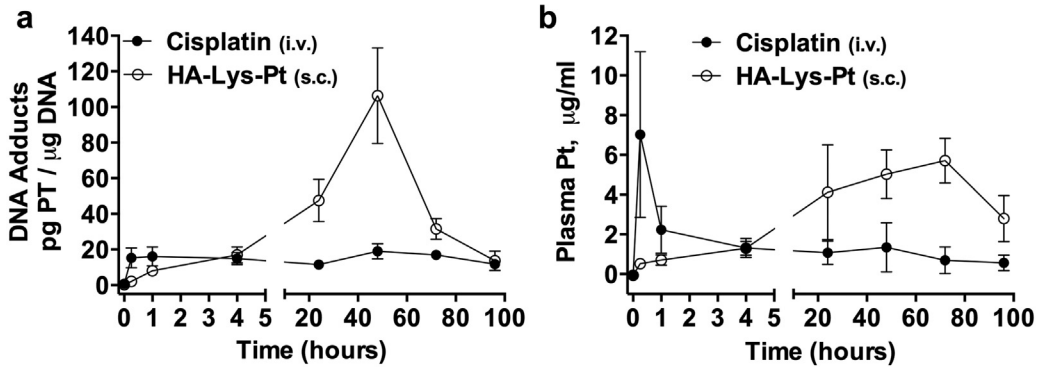

Figure 6. Pt-DNA adduct and plasma Pt quantitation. Rats were treated with cisplatin (i.v. 10 mg/kg) or HA-Lys-Pt (s.c. 10 mg/kg cisplatin basis). Pt content was quantified by ICP-MS in white blood cell DNA and plasma. (a) DNA adduct formation (mean ± standard error of the mean; $n = 3$-6). Cisplatin $C_{max} = 19.0 \pm 4.2$ pg Pt/μg DNA; $T_{max} = 48$ h; AUC = 1466 pg Pt × h/μg DNA. HA-Lys-Pt $C_{max} = 106.4 \pm 26.9$ pg Pt/μg DNA; $T_{max} = 48$ h; AUC = 4730 pg Pt × h/μg DNA. (b) Plasma Pt (mean ± standard error of the mean; $n = 3$-6). Cisplatin $C_{max} = 7.0 \pm 2.1$ μg Pt/mL plasma; $T_{max} = 0.25$ h; AUC = 102.2 pg Pt × h/mL. HA-Lys-Pt $C_{max} = 5.7 \pm 1.1$ μg Pt/mL plasma; $T_{max} = 72$ h; AUC = 398.5 pg Pt × h/mL.

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 pretreatment weight 18 days after the injection. Body weights from day 18 to 40 were not compared with day 0 as the mice had gained weight during this timeframe and their average body weight was greater than pretreatment. 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 orthotropic xenograft model of human HNSCC was established to examine the anticancer efficacy of HA-Lys-Pt. Tumors in the control group reached a size of approximately 1000 mm^3 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 4 weeks after subcutaneous 75HA-Lys-Pt therapy (Student t-test, $p < 0.05$). The treated animals developed tumors of an average size <200 mm^3 by the end of the sixth week (Fig. 8a), and the survival rate was significantly extended relative to the control and cisplatin treated groups (Fig. 8d; log-rank [Mantel–Cox] test, $p < 0.05$). A single dose of 20 mg/kg HA-Lys-Pt resulted in <5% of weight loss, whereas 3 weekly doses of 20 mg/kg caused more than 20% weight loss after the last injection, although body weight returned to normal 3 weeks after the last injection (Fig. 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 partial response, stable disease, and PD were 37.5%, 12.5%, and 50% for cisplatin treated groups, which suggest 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 2 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 (ca. 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

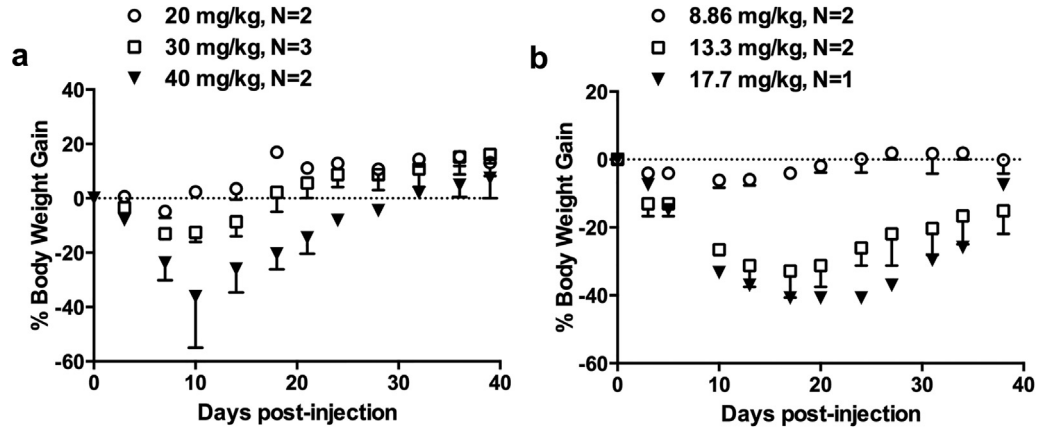

Figure 7. (a) Percent weight loss after a single injection of 75HA-Lys-Pt at 20 ($n = 2$), 30 ($n = 3$), and 40 mg/kg ($n = 2$). (b) Percent weight loss after a single injection of 33HA-Lys-Pt at 8.86 ($n = 2$), 13.3 ($n = 2$), and 17.7 ($n = 1$) mg/kg.

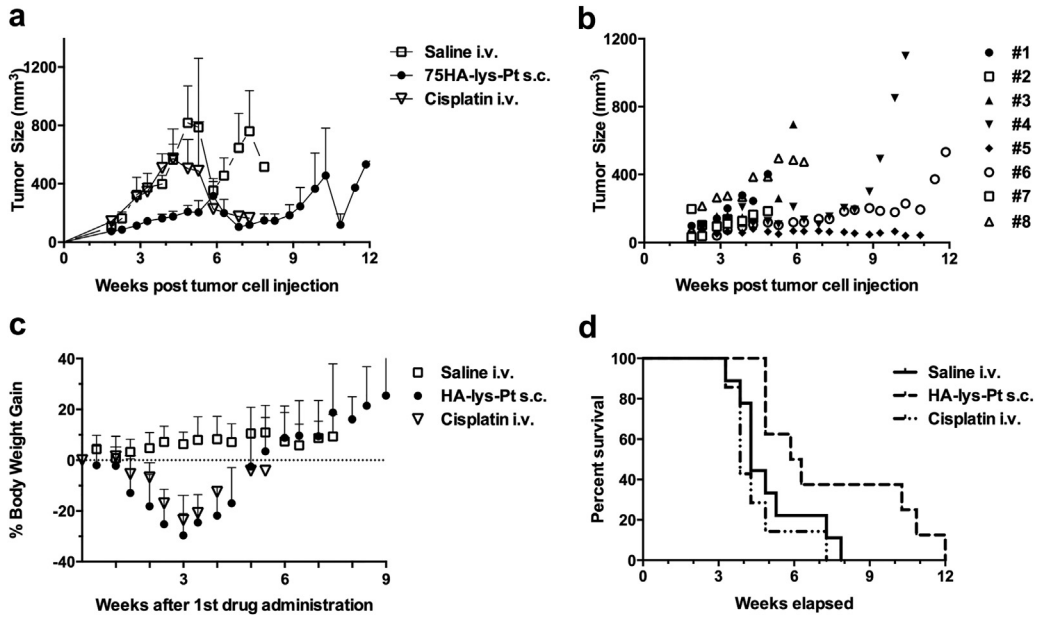

Figure 8. (a) Growth of head and neck tumors after 3 weekly injections of saline (i.v., $n = 9$), 75HA-Lys-Pt (20 mg/kg s.c., $n = 8$), and cisplatin (10 mg/kg i.v., $n = 8$). (b) Tumor growth in individual mouse that received 3 weekly injections of 20 mg/kg 75HA-Lys-Pt subcutaneously. (c) Percent weight 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).

pretreatment weight at about 5 weeks. Notably, mice received 20 mg/kg of HA-Lys-Pt but 10 mg/kg 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 anticancer 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.[35] in 1999.

The carboxylate of HA'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]

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).

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] Although 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 HA 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 5-member ring chelate formation was initially reported by Erickson et al.[41] in 1968, followed by extensive investigation of antitumor activity *in vitro* and comprehensive characterization by Appleton et al.[42-45] in the 1990s. A polyacrylic acid conjugate was reported by Lee et al.[46] in 2010, but it was not studied *in vivo*.

The HA-Lys-Pt displayed tunable release profiles in PBS at pH 7.4 (initial 24 h $t_{1/2}$ ~ 1 day) and in acetate buffer at pH 5.5 (initial 24 h $t_{1/2}$ ~ 10 h), 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 (Fig. 6 and Supporting Information Table 2).

Although no burst release was observed, the release rate of Pt from conjugates slowed after the initial 24-h period. This may be a result of reduced diffusivity of larger HAs, which form highly hydrated gels, and increased clustering of drug in hydrophobic

domains, whereas the 74, 33, and 6.4 kD had similar release rates in the first 24 h (0.034, 0.047, and 0.041 h^{-1}, respectively), the smaller 6.4 and 33 kD HA both had faster overall release rates (0.026 and 0.019 h^{-1}, respectively) than the larger 75 kD HA (0.010 h^{-1}). This is consistent with reduced diffusivity and clustering effects in the larger HA carriers. Transmission electron microscopy 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 approximately 30 nm.[47] Because 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 h for Pt-DNA adduct and 72 h 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 intrapolymer or interpolymer 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 h.[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 led 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 <15%.

Our previous study demonstrated that direct conjugation of platinum to HA led 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 cardiac toxicities 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 maximum tolerated dose compared to the HA-Pt.[13] We believe that 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 rechlorination reaction of Pt-diaqua in the physiological condition, thus reducing the potential toxicity of the drug.

Of the 3 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 HNSCC. Thus, we selected the 75 kD conjugate as the lead compound for further canine pharmacokinetics and efficacy study. We believe that 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 AUC 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.[19] 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] Although 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.[53,54] 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. Furthermore, 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 article 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 enhanced permeability and retention effect in some solid tumors that exhibit leaky vasculature. We have shown in prior studies that HA can target drugs to the lymph nodes and increase endocytic uptake by tumor cells via an interaction with the CD44 receptor.[28] Second, nontargeted polymers generally will greatly reduce the *in vitro* efficacy of conjugated drugs because they reduce or eliminate passive nonspecific drug uptake, and drug release may be substantially delayed so that only part of the drug is released during a typical 72- or 96-h 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.[55] In the HPMA studies, much higher doses of the chelates were administered (e.g., 75 mg/kg) to obtain similar tumor inhibition,[55] suggesting that although the HPMA chelate was well tolerated, it was significantly less effective than the HA chelates on a dose basis. We have shown in prior studies that HA conjugates increase cellular uptake through CD44 receptor uptake. Third, the HA 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.[56] 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.[57] The degradation products of our carrier are HA monomers and acetylated lysine, both natural compounds, unlike nondegradable 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.

Acknowledgment

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