Phase I-II clinical trial of hyaluronan-cisplatin nanoconjugate in dogs with naturally occurring malignant tumors

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OBJECTIVE
To conduct a phase I-II clinical trial of hyaluronan-cisplatin nanoconjugate (HA-Pt) in dogs with naturally occurring malignant tumors.

ANIMALS
18 healthy rats, 9 healthy mice, and 16 dogs with cancer.

PROCEDURES
HA-Pt was prepared and tested by inductively coupled plasma mass spectrometry; DNA-platinum adduct formation and antiproliferation effects of cisplatin and HA-Pt were compared in vitro. Effects of cisplatin (IV) and HA-Pt (SC) in rodents were tested by clinicopathologic assays. In the clinical trial, dogs with cancer received 1 to 4 injections of HA-Pt (10 to 30 mg/m², intratumoral or peritumoral, q 3 wk). Blood samples were collected for pharmacokinetic analysis; CBC, serum BUN and creatinine concentration measurement, and urinalysis were conducted before and 1 week after each treatment. Some dogs underwent hepatic enzyme testing. Tumors were measured before the first treatment and 3 weeks after each treatment to assess response.

RESULTS
No adverse drug effects were detected in pretrial assessments in rodents. Seven of 16 dogs completed the study; 3 had complete tumor responses, 3 had stable disease, and 1 had progressive disease. Three of 7 dogs with oral and nasal squamous cell carcinoma (SCC) that completed the study had complete responses. Myelosuppression and cardiotoxicosis were identified in 6 and 2 dogs, respectively; none had nephrotoxicosis. Four of 5 dogs with hepatic enzymes assessed had increased ALT activities, attributed to diaquated cisplatin products in the HA-Pt. Pharmacokinetic data fit a 3-compartment model.

CONCLUSIONS AND CLINICAL RELEVANCE
HA-Pt treatment resulted in positive tumor responses in some dogs, primarily those with SCC. The adverse effect rate was high.

IMPACT FOR HUMAN MEDICINE
Oral SCC in dogs has characteristics similar to human head and neck SCC; these results could be useful in developing human treatments. (Am J Vet Res 2016;77:1005–1016)

Hyaluronan-cisplatin nanoconjugate is a novel nanoparticle formulation that delivers cisplatin to tumors and draining lymphatics after intratumoral and peritumoral injection. In mice that received xenografts from human head and neck SCC, the formulation was shown to provide better tumor control, compared with conventionally administered (IV) cisplatin. Complementing this enhanced local efficacy, the pharmacokinetic profile of the formulation was found to have a lower Cmax but greater AUC than IV-administered cisplatin, resulting in lower incidence of nephrotoxicosis, enhanced local efficacy, and extended systemic tumor cell exposure, compared with results for cisplatin-treated rodents in other studies. In a preliminary study of HA-Pt in 5 dogs with spontaneously occurring soft tissue sarcomas, use of the formulation resulted in similarly favorable tumor and plasma distributions of platinum. The toxic effects and efficacy of HA-Pt in rodents have also been reported.
Hyaluronan-cisplatin nanoconjugate is highly water soluble (the solubility of cisplatin is 1 mg/mL, whereas solubility of HA-Pt is > 10 mg/mL) and is injectable via a 27-gauge needle. The HA-Pt nanoparticles are approximately 20 to 25 nm in size and have an in vitro release half-life of 10 hours in PBS. The antiproliferative activity of HA-Pt is similar to that of cisplatin in multiple cancer cell lines, including human head and neck SCC (eg, MDA-1986 and JMAR), breast cancer (eg, MCF-7 and MDA-MB-231), lung cancer (eg, A549), and melanoma (eg, A2058) cells, and mouse mammary gland tumor (4T1), lung carcinoma (Lewis), and melanoma (B16) cells. It enters human head and neck SCC cells via CD44-mediated endocytosis.

In dogs, as in humans, IV administration of cisplatin requires a prolonged fluid diuresis to avoid nephrotoxicity. One representative protocol includes 3 hours of diuresis before cisplatin administration and another hour of diuresis afterward, while other protocols recommend longer durations of diuresis (eg, 4 to 6 hours). In addition to saline (0.9% NaCl) solution diuresis, concurrent administration of mannitol or furosemide is often used in veterinary practices to ameliorate cisplatin-induced renal injury by increasing urinary excretion. The characteristics of the HA-Pt formulation could allow investigation of its efficacy in treatment of dogs with malignant tumors, and the results could offer information relevant to human medicine, with a potential for more convenient localized injections and improved antitumor efficacy without the need for prolonged diuresis.

Cisplatin chemotherapy has been investigated in dogs with osteosarcoma, peritoneal and pleural mesotheliomas, transitional cell carcinomas of the urinary bladder, mast cell tumors, and other malignancies. The objectives of the phase I-II study reported here were to determine the pharmacokinetics of HA-Pt in dogs with naturally occurring malignant tumors and to assess its safety and efficacy in these patients.

Materials and Methods

Drug preparation and in vitro experiments

Preparation of HA-Pt—Synthesis of HA-Pt was performed under sterile conditions. Water for injection (7.5 L) was combined with cisplatin (15 g). Subsequently, 30 g of sodium hyaluronate was added, and the pH was adjusted to 6.5 with 6N NaOH. After 4 days, the unreacted cisplatin and unwanted reaction products were removed by tangential flow filtration with a 10-kDa molecular-weight cutoff polyethersulfone membrane, followed by concentration to a final value of 6 to 8 mg of cisplatin/mL via the tangential flow filtrate system. The concentrated HA-Pt was terminally sterilized by passage through 2 sterile 0.22-micron filters, drawn up into 5-mL ready-to-use sterile plastic syringes, and stored ≤3 months until use in patients.

The platinum concentration of HA-Pt was determined by ICP-MS. A calibration curve was generated from 1 to 50 ppb platinum (acceptable criterion, R² > 0.995) and 50-ppb bismuth was used as an internal standard. Unknown samples were interweaved with a 20-ppb platinum quality control; the acceptance criterion was an internal standard recovery of 80% to 120%.

DNA-platinum adduct formation—Adduct formation was evaluated as a means of assessing the potential efficacy of the platinum-containing compound in vitro (where it is accepted that cisplatin exerts its therapeutic efficacy through binding to DNA bases, preferentially the highly nucleophilic N-7 positions of adenine and guanine bases). A commercially prepared DNA (type I calf thymus sodium salt) was dissolved in tris-EDTA buffer (10mM tris, 1mM EDTA, pH 8.0 in H₂O) to a concentration of 1 µg/µL. Cisplatin and HA-Pt were each dissolved in water to a 1 mg/mL concentration. Sterile water (used as a vehicle control), cisplatin (2.5 µg; positive control), or HA-Pt (2.5 µg on cisplatin basis) were added to PBS containing 20 µg of DNA in a 400-µL total reaction volume and incubated in a 37°C water bath for 72 hours. The DNA was precipitated and rehydrated in water as described by Brouwers et al, and concentration and purity of the DNA were determined by measurement of absorbance at 260 and 280 nm. The DNA content ratios (calculated as the measurement at 260 nm divided by the measurement at 280 nm) were routinely between 1.8 and 1.9. Samples were digested in 1% nitric acid at 70°C, and platinum concentrations were determined by ICP-MS analysis. Each test was performed in duplicate, and the mean ± SEM platinum concentration (ng/µg of DNA) of 4 independent experiments was used in analyses.

Antiproliferation assay—An in vitro anti-proliferation assay was conducted to evaluate the potency of cisplatin or HA-Pt against cancer cells prior to the in vivo experiments. Human head and neck SCC (MDA-1986) cells were maintained in Dulbecco modified Eagle medium containing glucose (4.5 g/L) and l-glutamine (4.5 g/L) with fetal bovine serum added to a final concentration of 10% in a humidified incubator at 37°C with 5% CO₂. Cells were seeded into 96-well plates (3,000 cells/well) and allowed to attach overnight in the incubator. Cisplatin and HA-Pt were dissolved in water, added to cells (7 concentrations from 10nM to 0.3mM in duplicate), and incubated at 37°C for 72 hours. After a 72-hour incubation with the assigned drug, resazurin blue in PBS was added to each well (5µM final concentration) followed by incubation at 37°C for 4 hours. Fluorescence (λex [excitation] 550 nm; λem [emission] 605 nm) was quantified with a plate reader. Metabolically viable cells reduce nonfluorescent resazurin to fluorescent resorufin, allowing quantification of viable cells. The relative growth of cells incubated with each compound concentration was normalized to vehicle (water)-treated controls (100%...
proliferation) according to the following calculation: experimental value/mean vehicle value \( \times 100 \). Each test was performed in duplicate, and the mean ± SEM of \( \geq 6 \) independent experiments was used in analyses. Growth inhibition potencies (IC\(_{50}\)) were calculated by plotting the logarithm of the molar concentration of the compound (log\(_{10}\) [M]) versus the normalized growth percentage and analyzing with a commercially available\(^1\) software package by nonlinear regression, by use of the following equation: \( Y = \text{bottom} + (\text{top-bottom})/(1 + 10^{X-\log IC50}) \), where bottom and top represent the lowest and highest points of the inhibitory dose-response curve.

**Assessment of toxic effects of cisplatin and HA-Pt in rodents**

**Animals**—Twenty-four apparently healthy adult rodents (15 female Sprague-Dawley rats\(^2\) and 9 female BALB/c mice\(^3\)) were used to assess toxic effects of HA-Pt. Throughout the study period, rodents were housed individually in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility at the University of Kansas. All experimental procedures were approved by the University of Kansas Institutional Animal Care and Use Committee.

The 15 Sprague-Dawley rats weighed 0.20 to 0.25 kg and were arbitrarily assigned to 3 groups of 5 animals each by means of blindly selecting rats for each study group. Five rats each received 1 dose of cisplatin (6 mg/kg, IV), HA-Pt (6 mg/kg on a cisplatin basis, SC), or saline (0.9% NaCl, IV; volume equivalent to that for cisplatin) solution. The same SC injection site was used in all rats. The animals were monitored daily for signs of toxicosis. Twenty-four-hour urine samples were collected 5 and 30 days after injection of the assigned treatment (day 1). Urine samples were centrifuged at 2,500 \( X g \) and 4°C for 10 minutes, and the supernatants were frozen at –80°C until analysis.

The 9 BALB/c mice weighed 0.02 to 0.03 kg and were arbitrarily assigned to 3 groups of 3 animals by the same method described for rats. Three mice each received 1 dose of cisplatin (10 mg/kg, IV), HA-Pt (10 mg/kg on a cisplatin basis, SC), or saline solution (IV; volume equivalent to that for cisplatin). Animals were monitored daily for signs of toxicosis and euthanized by overdose of isoflurane 24 hours after injection of the assigned treatment. Heart blood samples (0.5 mL) were collected immediately after death was confirmed and sent to the Kansas State Veterinary Diagnostic Laboratory for a CBC and analysis of serum ALT, AST, and ALP activities.

**Phase I-II clinical trial in dogs**

**Clinical trial design and enrollment criteria**—An open-label, multiple dosage phase I-II clinical trial of HA-Pt was conducted at the University of Missouri Veterinary Medical Teaching Hospital. The clinical trial protocol was approved by the University of Missouri Institutional Animal Care and Use Committee. Informed client consent was required for each dog to be enrolled in the study.

Dogs of any age, sex, or breed that weighed \( \geq 10 \) kg, were evaluated by the facility’s oncology service, and had a histologically confirmed diagnosis of anal gland adenocarcinoma, sarcoma, oral SCC, oral melanoma, nasal SCC, or digital SCC were considered for enrollment. Dogs with metastatic cancers were excluded. The weight criterion was established to allow for safe collection of samples for pharmacokinetic analysis.

To be included in the study, dogs were required to have a performance score of 0 or 1 on a 5-point scale modified from a scale developed for use in humans\(^19\) (where 0 = normal activity; 1 = restricted activity; 2 = compromised activity; 3 = disabled; and 4 = deceased) as assessed by physical examination by the attending clinician (SMAB, BKF, CJH, KAS, or JNB), and could not have any identified comorbid conditions expected to limit life expectancy to \( < 9 \) weeks. Additionally, eligible dogs had to meet the following criteria: a tumor \( \geq 2 \) cm in its longest dimension and disease fully staged with a CBC, serum biochemical analysis, thoracic radiographs (for patients with SCC of the mouth or a forepaw, oral melanoma or nasal SCC), abdominal ultrasound images (for patients with SCC of a hind paw or anal gland adenocarcinoma), and cytologic evaluation of a fine needle aspirate of the draining lymph node. Finally, dogs were excluded if they had received NSAIDs \( \leq 7 \) days before starting the trial or at any time during the trial; if they had received immunotherapy, other chemotherapy, or radiation therapy \( \leq 3 \) weeks before starting the trial; or if they had renal dysfunction serum as graded according to the following scale (because conditions such as renalolithiasis, tumors, bladder paralisis, dehydration, and congestive heart disease can affect circulating BUN concentration, renal dysfunction was assessed on the basis of serum creatinine concentration): 0 = normal (creatinine < 1.6 mg/dL); 1 = mild dysfunction (creatinine, 1.7 to 2.5 mg/dL); 2 = moderate dysfunction (creatinine, 2.6 to 5.0 mg/dL); 3 = severe dysfunction (creatinine, 5.1 to 10.0 mg/dL); and 4 = renal failure (creatinine \( \geq 10.0 \) mg/dL), persistent neutropenia or thrombocytopenia, or a clinically important infection of the tumor that could not be readily managed according to the attending clinician’s opinion.

**Treatment and monitoring protocols**—Tumor diameter in the largest dimension was measured by use of calipers and recorded for all targeted lesions prior to the first treatment. All dogs were examined before and after each treatment administration by the attending clinician (SMAB, BKF, CJH, KAS, or JNB). An indwelling jugular catheter was placed to facilitate collection of blood samples at the start of each treatment. Dogs were sedated or anesthetized for each treatment, and
1 to 3 spinal needles (22-gauge) were preplaced to allow drug delivery. Dogs received injections of HA-Pt (intratumoral or into peritumoral submucosa) at doses of 10 to 30 mg/m² (on a cisplatin basis) once every 3 weeks for up to 4 planned doses unless toxicosis or progressive disease was observed. The starting dose was 10 mg/m², and doses were adjusted at the discretion of the attending clinician. A 2-mL blood sample was collected from the jugular catheter at 0.5, 1, 2, 4, and 24 hours after the first drug administration (time 0) for subsequent pharmacokinetic analysis. Blood samples were centrifuged at 2,500 Xg, and plasma was collected and frozen ≤ 2 hours after the sample was collected. Immediately prior to and 1 week after each treatment, an additional blood sample was collected for a CBC and measurement of serum BUN and creatinine concentrations, and a urine sample was collected via a catheter for urinalysis. Posttreatment follow-up examinations were performed every 1 to 3 weeks according to the clinician’s discretion and client’s willingness.

Posttreatment CBC and serum BUN and creatinine concentration measurements were performed for samples collected between days 4 and 148 and days 2 and 120, respectively. For platelet and neutrophil assessments, the median of all pretreatment samples (before the first treatment) and median of the minimum value among all post-treatment samples (all samples after the first treatment until the end of the study) were used in the analyses. In the platelet nadir analysis, the median of the minimum (nadir) value of all post-treatment samples (all samples after the first treatment until the end of the study) was used in the analysis. Dogs were grouped on the basis of the number of injections received. For serum creatinine and BUN analyses, the median of all pretreatment samples (before the first treatment) and median of the maximum value of all posttreatment samples (all samples after the first treatment until the end of the study) were used. Not all dogs completed the posttreatment follow-up. Some dogs that were suspected to have adverse effects of drug administration had complete serum biochemical analysis performed, including assessment of serum ALT and bilirubin concentrations. Tumor measurements were repeated 3 weeks after each drug injection to assess response. A complete response was defined as the tumor completely resolved with no recurrence in 6 months, a partial response was defined as ≥ 50% decrease in tumor size, and stable disease was defined as any change in tumor size between a 30% decrease and a 20% increase. Progressive disease was defined as ≥ a 20% increase in tumor size.

Dogs were withdrawn from the study if nephrotoxicosis of grade ≥ 3 (scale of 1 [least severe] to 5 [most severe]), a local reaction of grade ≥ 3 (scale of 1 [least severe] to 5 [most severe]), evidence of systemic toxicosis (eg, cardiotoxicosis) or intolerance of the treatment, or progressive disease was identified. Dogs were also removed from the study if requested by the client. Necropsy was requested for any dog that died during the study period so that patients could be assessed for tumor response to treatment and evidence of systemic toxicosis.

Pharmacokinetic modeling and analysis of HA-Pt—Plasma samples collected from dogs for pharmacokinetic analysis were diluted 20-fold in 1% HNO₃ and vortexed prior to ICP-MS analysis. The ICP-MS was performed as previously described for preparation of the HA-Pt. Pharmacokinetics of the HA-Pt formulation was modeled with a commercially available analysis software package.

Statistical analysis
A 1-way ANOVA was used to assess differences in the mean ± SEM DNA-bound platinum content among DNA samples treated by addition of cisplatin, HA-Pt, or saline in vitro, followed by a Tukey multiple comparisons test. Proliferation rates of MDA-1986 cells exposed to cisplatin or HA-Pt were analyzed by nonlinear regression, and mean ± SEM IC₅₀/s were compared between treatments by use of a Student t test. A 2-way ANOVA followed by a Sidak test for multiple comparisons was used to compare creatinine concentrations in 24-hour urine samples on 2 study days from rats that received cisplatin, HA-Pt, or saline solution injections. Data were assumed to have a normal distribution; available tools for determining distributions such as Kolmogorov-Smirnov, Shapiro-Wilk, and D’Agostino-Pearson are not sufficiently robust to detect non-Gaussian distributions in small sample sizes.

For the clinical trial data, Hct, BUN concentration, and creatinine concentration over time were assessed by linear regression with calculation of 95% confidence intervals. Hematologic and biochemical variables of interest before and after each treatment were compared by Wilcoxon matched-pairs signed rank tests, and association between platelet nadir (lowest platelet count for each patient during the study) and the number of treatments (1, 2, or ≥ 3) was explored with a Kruskal-Wallis test.

Statistical analyses were performed with commercially available software. Values of P < 0.05 were accepted as significant.

Results
Drug preparation and in vitro experiments
HA-Pt production—The HA-Pt was successfully synthesized. A typical batch produced 12 to 15 g of the HA-Pt conjugate (2 g on a cisplatin basis) with a cisplatin loading degree of 12% to 18% wt/wt and a cisplatin concentration of 6 to 8 mg/mL. The chemical structure of HA-Pt is shown (Figure 1).

DNA-platinum adduct formation—The mean ± SEM DNA-bound platinum content following exposure in vitro to cisplatin (13.90 ± 2.69 ng/µg DNA) or HA-Pt (17.97 ± 2.72 ng/µg DNA) was significantly (P < 0.01
and $P < 0.001$, respectively) greater than that following the vehicle treatment ($0.22 \pm 1.34$ ng/µg DNA). The difference in DNA-bound platinum content between cisplatin- and HA-Pt-treated samples was non-
significant ($P > 0.05$; Figure 1).

**Antiproliferation effects of cisplatin and HA-Pt**—Cisplatin and HA-Pt treatment of MDA-1986 cells resulted in $> 80\%$ inhibition of growth relative to vehicle (water) control (Figure 1). The mean ± SEM IC$_{50}$ of cisplatin ($8.4 \pm 1.1$µM) and HA-Pt ($8.4 \pm 0.6$µM) did not differ significantly ($n = 6$ and 8 independent experiments, respectively; $P > 0.05$).

**Evaluation for toxic effects of cisplatin and HA-Pt in rodents**

The creatinine concentrations in 24-hour urine samples collected on days 5 and 30 from rats did not differ significantly between those that received cisplatin or HA-Pt treatment and those that received the control (saline solution) treatment at each time point (Figure 2). Also, results between the 2 drug treatment groups did not differ significantly ($P > 0.05$). No rats died or required euthanasia because of clinical signs during the 30-day period.

The CBC results were similar ($P > 0.05$) among groups of mice that received a 10 mg/kg dose of cisplatin or HA-Pt (dosed on a cisplatin basis) or a control (saline solution) treatment (Table 1). There were also no significant ($P > 0.05$) differences in hepatic enzyme (ALT, AST, and ALP) activities between any 2 of the 3 treatment groups (cisplatin vs control, cisplatin vs HA-Pt, or HA-Pt vs control).

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**Figure 1**—Diagram depicting the chemical structure of HA-Pt (A) and results of in vitro analysis of the HA-Pt compound used in a phase I-II clinical trial of dogs with naturally occurring malignant tumors (B and C). A—The depicted compound was successfully synthesized and platinum content was assessed by ICP-MS; a typical batch produced 12 to 15 g of the conjugate (2 g of cisplatin), and was concentrated to 6 to 8 mg of cisplatin/mL prior to final sterilization. B—Graphic representation of DNA-bound platinum content following in vitro exposure of commercially prepared calf thymus DNA (20 µg) to sterile water (vehicle), cisplatin (2.5 µg), or HA-Pt (2.5 µg on cisplatin basis) for 72 hours at 37°C. The DNA was precipitated and rehydrated, and platinum analysis was performed by ICP-MS. Data are the mean and SEM of 4 independent experiments (2 samples/experiment; $n = 8$). Each drug treatment resulted in significantly ($P < 0.05$; 1-way ANOVA followed by Tukey test for multiple comparisons) greater DNA-platinum adduct formation than did the vehicle. The difference between cisplatin and HA-Pt was nonsignificant. C—Representative curves depicting antiproliferative activity of cisplatin (black circles) and HA-Pt (white circles) in MDA-1986 human head and neck SCC cells in vitro. Cells were treated with increasing concentrations of the assigned drug or a vehicle control (water) for 72 hours, followed by incubation with resazurin blue and quantification by fluorophotometry. Data from ≥ 6 separate experiments performed in duplicate were analyzed by nonlinear regression. Both treatments inhibited cell growth $> 80\%$. Potencies (IC$_{50}$) did not differ significantly ($P > 0.05$; Student t test) between treatments. *$P = 0.01$. †$P < 0.001$. M = Molar concentration. PT = Platinum.
values, indicating myelosuppression induced by the following treatment (n = 12) were also significantly compromised. Median platelet counts at nadir (treatment considered day 1) values (14), suggesting the pretreatment (day 0 [with the day of first HA-Pt n = 13) were significantly (P = 0.002) decreased from the pretreatment group and the saline solution (control) group were nonsignificant (P > 0.05; 2-way ANOVA followed by Sidak multiple comparisons test).

Phase I-II clinical trial

Dogs—Sixteen dogs (9 males [8 neutered and 1 sexually intact] and 7 females [all spayed]) were enrolled in the study. The median age was 9.25 years (range, 4.75 to 15.75 years) and median weight was 26.8 kg (range, 11.5 to 44.0 kg); breeds included Labrador Retriever (n = 4), mixed (4), Golden Retriever (2), and American Foxhound, Australian Shepherd, Basset Hound, Boxer, Miniature Schnauzer, and Pembroke Welsh Corgi (1 each). Cancer types included oral SCC (n = 9 dogs), nasal SCC (4), anal gland adenocarcinoma (2), and soft tissue sarcoma (on the roof of the mouth; 1). Initial tumor size ranged from 2 cm to 11 cm in the longest dimension. Seven dogs completed the study, and 9 dogs were withdrawn from the study between days 1 and 62. Reasons for withdrawal were disease progression (n = 2), tumor ulceration (1), and drug-related toxicity (n = 6; 2 with drug-related cardiotoxicosis and 4 with drug-related hepatic toxicosis). Among dogs that completed the study protocol, the follow-up examination period ranged from 8 to 910 days.

Clinicopathologic and histopathologic effects—Linear regression analysis of Hct revealed no significant change over time (P = 0.964), suggesting HA-Pt did not affect RBC production during the study (Figure 3). However, median minimum posttreatment neutrophil counts (obtained between days 4 and 148; n = 13) were significantly (P = 0.002) decreased from the pretreatment (day 0 [with the day of first HA-Pt treatment considered day 1]) values (14), suggesting that ability of bone marrow to produce these cells was compromised. Median platelet counts at nadir following treatment (n = 12) were also significantly (P = 0.001) decreased from the pretreatment (12) values, indicating myelosuppression induced by the treatment; however, the decrease was not related to the number of doses (P = 0.431).

Linear regression of serum creatinine and BUN concentrations revealed that, although both variables were briefly increased shortly after treatment in some dogs, creatinine concentrations decreased to approximate the pretreatment concentrations, with no significant (P > 0.05) change over time (Figure 4). The BUN concentrations decreased significantly (P = 0.025) over time after treatment. This suggested that HA-Pt did not cause nephrotoxicosis over time and that renal function was not greatly affected by the drug. This was supported by similar median specific gravity values of urine measured before (1.031 ± 0.011; n = 14) and after (1.031 ± 0.014; 11) treatment.

Serum activities of hepatic enzymes were compared in samples collected before and after treatment for 5 patients suspected to have adverse reactions to HA-Pt. One patient had ALT activities before and after the study that were similar (120 and 113 U/L, respectively; laboratory reference range, 5 to 107 U/L), and the remaining 4 had increased posttreatment ALT activities at the completion of the study, compared with their ALT activities prior to any treatment. One had a slightly increased value (from 49 to 59 U/L), 2 had moderately increased values (from 30 to 53 U/L in one and from 24 to 99 U/L in the other), and 1 had a markedly increased value (from 34 to 416 U/L). The maximum ALT activity was detected on study days 115, 61, 100, 29 and 22 for these dogs. Two of these dogs (with maximum ALT activity detected on days 115 and 29) were included in long-term follow-up through study days 489 and 92, respectively. In both of these patients, the ALT activities decreased to approximate the pretreatment values by the end of monitoring. In addition, mean ± SD serum bilirubin concentrations before the first treatment for these 5 dogs was 0.16 ± 0.09 mg/dL, and mean ± SD maximum serum bilirubin concentration for the same dogs was 0.80 ± 0.60 mg/dL (reference range, 0.1 to 0.4 mg/dL). Although the mean maximum bilirubin concentration exceeded the upper limit of the reference range, the data were not significantly different from the pretreatment values for the same dogs (Wilcoxon matched-pairs signed rank test, P > 0.05).

During the course of the study, 1 dog (with maximum ALT activity of 434 U/L on study day 115) underwent liver biopsy, and 3 dogs underwent necropsy. Histologic evaluation of the biopsy sample from 1 dog and liver samples from 2 of 3 necropsied dogs revealed disruption of the hepatocellular cords, loss of hepatocytes, and dissecting fibrous connective tissue in the centrolobular region typical of a toxic injury. Similar pathologic changes were not found on histologic evaluation of the liver for the remaining deceased dog.

Because of the finding that some treated dogs had hepatic injury, to investigate whether toxic liver changes might be attributable to the presence of a hydrolysis product (diaquated cisplatin), additional experiments approved by the University of Kansas
Institutional Animal Care and Use Committee were performed in rats. We injected 2 apparently healthy (0.21- and 0.22-kg) female Sprague Dawley rats with diaquated cisplatin (cis-[Pt(NH$_3$)$_2$(OH)$_2$]$_2^{2+}$) at doses of 0.1 (n = 1) and 0.25 mg/kg (1), IV. The diaquated cisplatin was prepared by incubating 1.0 g of cisplatin with a stoichiometric amount of silver nitrate in 1.0 mL of water for 24 hours at room temperature (approx 22°C) in the dark. The solution was then centrifuged at 2,500 X g for 20 minutes. The supernatant was filtered through a sterile 0.22-µm polyethersulfone membrane to remove the silver chloride precipitate. The obtained cis-[Pt(NH$_3$)$_2$(OH)$_2$]$_2^{2+}$ solution was used for injection without further purification. Both animals were euthanized at the end of the experiment as described for mice, and 8 mL of blood was collected from each rat immediately after confirmation of death. At the lower dose, no signs of toxicosis were observed until day 6 (time of euthanasia; the day of injection was considered day 1), but high serum ALT (48 U/L [reference range, 17.5 to 30.2 U/L]) and AST (93 U/L [reference range, 45.7 to 80.8 U/L]) activities were detected. At the higher dose, the rat became lethargic on the day after the injection and was euthanized on day 3 because signs did not improve; high serum ALT activity (63 U/L) was identified. One additional rat, a 0.222-kg female, was injected with 4 doses of diaquated cisplatin.

Figure 3—Hematologic data for 16 client-owned dogs with cancer immediately before (day 0) and after treatment with HA-Pt. The first dose of HA-Pt (10 to 30 mg/m$^2$, intratumoral or peritumoral, every 3 weeks for up to 4 treatments) was given on day 1; the starting dose was 10 mg/m$^2$ and was subsequently adjusted at the clinician’s discretion for remaining treatments. Samples were collected before, and 1 week after, each treatment until the end of the study. Dogs were removed from the study if signs of toxicosis (including nephrotoxicosis or local reaction of grade ≥ 3 [each on a scale of 1 to 5] or other signs of severe toxicosis) or progression of the tumor were detected, if the patient died, or if the client requested the patient be withdrawn; 7 dogs completed the study. A—Scatterplot showing Hct over time. The solid line represents the line of best fit in the linear regression model; dashed lines represent upper and lower limits of the 95% confidence interval for the best-fit line. The values did not differ significantly (P = 0.964) over time. B—Box-and-whisker plots of neutrophil counts before HA-Pt treatment (day 0; n = 13) and minimum neutrophil counts after HA-Pt treatment (lowest value of 1 to 14 samples/dog, collected between days 4 to 148; 13). Results differed significantly (P = 0.002; Wilcoxon matched-pairs signed rank test) between time points. C—Box-and-whisker plots of platelet counts before (day 0; n = 12; 1 sample/dog) and at the nadir after HA-Pt treatment (lowest value of 2 to 15 samples/dog collected between days 4 to 148; 12). Results differed significantly (P = 0.001; Wilcoxon matched-pairs signed rank test) between time points. D—Median and range platelet counts at the nadir for dogs grouped according to the number of doses of HA-Pt received (1 [n = 7; circles], 2 [2; squares], or ≥ 3 [3; triangles]). Platelet count at nadir was not significantly (P = 0.431; Kruskal-Wallis test) associated with the number of doses. In panels B and C, upper and lower limits of each box represent the 75th and 25th percentiles, respectively; horizontal lines within each box indicate the median. In panel D, upper and lower limits of each vertical line indicate the maximum and minimum values of platelet counts except for 2 outliers in the 1-treatment group that were excluded from analysis; horizontal lines at midpoint indicate median.
(0.1 mg/kg, IV) at 4-day intervals but had no clinical signs observed. The rat underwent serum biochemical evaluation for liver function on day 17 (with the day of first injection considered day 1); high ALT (50 U/L) and AST (89 U/L) activities were detected, and the rat was euthanized on day 19.

**Treatment responses to HA-Pt and outcomes**—Of the 7 dogs that completed the study, 3 had complete remission of disease, 3 had stable disease, and 1 had progressive disease at the end of the study. The complete response rate to HA-Pt treatment in dogs with naturally occurring, heterogeneous, locally advanced cancers was 3 of 16. The complete response rate in dogs with SCC of the head and neck, including oral and nasal SCC, was 3 of 13. One of the 3 dogs with oral SCC had a complete response according to study criteria (complete resolution with no recurrence in 6 months) but had tumor recurrence after the 6-month period had ended.

Of the 9 dogs that were withdrawn from the study, 2 had progressive disease, with no signs of drug-related toxicosis observed. One dog with oral SCC was euthanized; necropsy findings for this dog indicated tumor ulceration, and no signs of toxicosis were observed. Two dogs that had anal gland adenocarcinoma had acute cardiac death after 1 treatment; both had underlying cardiomyopathy identified on necropsy, with no other signs of toxicosis observed. The remaining 4 dogs (including 1 with oral sarcoma affecting the palate) had signs of drug-related hepatic toxicosis (high liver enzyme activities and histopathologic liver changes). Of the 16 dogs, 2 received 4 injections, 2 received 3 injections, 3 received 2 injections, and 9 received 1 injection (dogs that had progressive disease or signs of toxic effects after a treatment were not given the next treatment).

One of the dogs with oral SCC that had a complete response to treatment had received 4 intratumoral injections of HA-Pt at 3-week intervals (2 doses of 30 mg/m², followed by 1 dose each at 20 mg/m² and 10 mg/m²). No signs of toxicosis were seen, and swelling and inflammation associated with the tumor had decreased substantially by 7 days after the first treatment. Results of a CT scan and biopsy 910 days...

**Figure 4**—Plots depicting serum creatinine (A and B) and BUN (C and D) concentrations for the same dogs as in Figure 3. A—Scatterplot of creatinine concentrations over time. There was no significant ($P > 0.05$) difference for this variable over time after treatment. B—Box-and-whisker plots of creatinine concentrations before HA-Pt treatment ($n = 11; 1$ sample/dog) and maximum creatinine concentration after HA-Pt treatment (highest value of 1 to 12 samples/dog collected between days 2 and 120; 11). Results did not differ significantly ($P > 0.05$; Wilcoxon matched-pairs signed rank test) between time points. C—Scatterplot of BUN concentrations over time. Values decreased significantly ($P = 0.025$) over time after the start of treatment. D—Box-and-whisker plots of BUN concentrations before HA-Pt treatment ($n = 12; 1$ sample/dog) and maximum BUN concentration after HA-Pt treatment (highest value of 1 to 12 samples/dog collected between days 2 and 120; 11). Results did not differ significantly ($P > 0.05$; Wilcoxon matched-pairs signed rank test) between time points. See Figure 3 for remainder of key.
after the first treatment confirmed a complete tumor response with no signs of recurrence; no further follow-up was available.

The dog with oral SCC that had a complete response to treatment but developed recurrence after the 6-month criterion was met had received 4 intratumoral injections of HA-Pt at 3-week intervals (10 mg/m² for 3 doses, followed by 1 dose at 12.5 mg/m²). Twenty-one days after the first treatment, swelling and inflammation was substantially reduced, and CT examination revealed sclerosis of the affected bone with resolution of the contrast-enhancing mass. At subsequent examinations up to 6 months, the dog had no erythema or gingival bleeding. At the follow-up examination 196 days after the first treatment, progression was identified; radiation treatment resulted in complete regression of the tumor, with no recurrence identified at last follow-up (329 days after the injection [study day 330]).

Circulating platinum concentrations in dogs treated with HA-Pt—Plasma platinum concentrations were measured in samples from 12 dogs by ICP-MS. The remaining 4 dogs were excluded because blood samples were not collected. The plasma drug concentration-versus-time curves are shown (Figure 5). For clarity, dogs with different types of cancer (oral SCC vs nasal SCC and sarcoma) were plotted separately. Most dogs (11/12) had a Cmax at 0.5 hours. The Cmax varied from 0.20 to 3.60 µg/mL.

Pharmacokinetic modeling—Models that employed 1 or 2 compartments failed to reflect the complexity of the pharmacokinetics of an intratumorally administered polymer-drug conjugate, resulting in unacceptable data fitting. A 3-compartment model (injection site, plasma, and tissue) was used to model the plasma concentration-versus-time data, as this properly represented the physiologic distribution of HA-Pt in 12 of the 16 dogs in Figures 3 and 4. A and B—Plasma platinum concentration-versus-time curves for dogs with oral SCC (A; n = 8) and dogs with nasal SCC or oral sarcoma (B; 4). Each symbol represents an individual dog; data for 4 dogs were not included because blood samples were not collected. Time 0 was the time of injection for the first drug treatment. C—Diagrammatic representation of a 3-compartment model of HA-Pt pharmacokinetics showing the injection site (q3), plasma compartment (q1), and a tissue compartment (q2). The solid circle (s1) reflects a sampling event from the plasma compartment. Three transfer constants, k(1,3), k(1,2), and k(2,1), and 1 loss constant, k(0,1), were assigned to the model. The k(1,3) constant defined the drug absorption process allowing the transfer of HA-Pt from the injection site compartment to the plasma compartment. A reverse k(3,1) constant was not assigned because a sink condition was assumed. Owing to the concentration gradient between the injection site and the plasma compartment, drug redistribution back to the injection site was not expected. The loss constant k(0,1) represented the renal clearance of either unchanged cisplatin or its metabolites. Drug transfer and equilibrium between the plasma and tissue compartments were represented by k(1,2) and k(2,1).
platinum after local injection of a polymer-based chemotherapeutic agent (Figure 5).

The pharmacokinetic data from 11 dogs in the clinical trial were successfully fitted to the 3-compartmental model. One dog was excluded from this analysis because insufficient serum samples were available to model the terminal phase. One dog had a Tmax of 1 hour, and all other dogs had a Cmax at 0.5 hours after drug administration, indicating a rapid distribution of the HA-Pt from the injection site into the systemic circulation. The Cmax varied up to 18-fold among individual dogs (range, 0.20 to 3.60 µg/mL), although the dose of HA-Pt received varied by only 3-fold (range, 10 to 30 mg/m²).

The drug elimination process varied greatly among patients, whereas Tmax was fairly consistent. Specifically, 6 patients had an elimination half-life in the range of 0.5 to 8.5 hours, and 3 patients had calculated drug elimination half-lives that ranged from 51 to > 1,000 hours. This may have been partially attributable to incomplete drug elimination at 24 hours and variations in the renal health among individual dogs. The remaining 2 patients had incomplete sample collection (only 5/6 samples were successfully collected) and were excluded from PK modeling. Owing to travel distances and owner compliance considerations, collection of samples > 24 hours after drug administration was not performed in this trial.

Data from the 5 patients with incomplete drug elimination or missing data at the 24-hour time point were excluded from the clearance and elimination half-life (ie, t1/2 [β]) calculations because of the incomplete drug elimination at 24-hour post dose, but were not excluded from the Vdss and AUC calculations as they did not differ significantly (P > 0.05) from the results for the remaining dogs. The mean ± SD Vdss (normalized for patient weight) was 2.119 ± 1.502 L/kg. The clearance (mean ± SD, 0.0435 ± 0.0203 L/kg/h) was also reported on a per-weight basis because the range of patient weights was substantial (11.5 to 40.7 kg). The mean ± SD AUC and elimination half-life were calculated as 6.330 ± 3.578 µg•h/mL and 3.846 ± 2.907 hours, respectively.

**Discussion**

The phase I-II clinical trial of the present study included calculation of the pharmacokinetics of HA-Pt in dogs with spontaneously occurring malignant tumors. The doses ranged from 10 to 30 mg/m², or approximately 0.3 to 1.0 mg/kg. The Vdss was determined to be 2.119 L/kg, with clearance and elimination half-life of 0.0435 L/h/kg and 3.846 hours, respectively, excluding patients that did not have a complete elimination phase by 24 hours after injection. Vaden et al²³ conducted a pharmacokinetic study of cisplatin in dogs following IV administration in the 1990s. Those dogs received 1 mg of cisplatin/kg as a bolus injection, and the Vdss, clearance, and elimination half-life were determined to be 0.45 L/kg, 8.6 mL/kg/min, and 38 minutes, respectively. The greater Vdss of 2.119 ± 1.302 L/kg following intratumoral HA-Pt injection in the present study, compared with that reported by Vaden et al,²³ was likely attributable to the differences in the route of administration, slower clearance of the drug, and greater lymphatic and tissue penetration of the compound.²³,²⁴ Cisplatin administered IV would be cleared from the body at a much higher rate than the polymer drug conjugate because small molecules like cisplatin undergo rapid renal excretion when administered as an IV bolus. In comparison, large molecules may undergo a more complex process of clearance. Lymphatically targeted HA-Pt may preferentially distribute to the lymphatics before it enters the systemic circulation. Interactions between hyaluronan and its receptors or other cell-surface proteins in the body may also slow down the systemic clearance of the drug. The relatively short Tmax in the present study indicated a rapid distribution of the HA-Pt from the injection site to the systemic circulation. This is likely attributable to the rich blood supply in the oral and nasal mucosa (tumor sites for 13 of the 16 dogs in our study), which would expedite the systemic entrance of the drug. These findings were consistent with pharmacokinetics of a large-molecule protein therapeutic, where the SC absorption rate is highly dependent on injection site and can be influenced by a combination of increased lymphatic flow resulting from high hydrostatic pressure in confined tissues and the richness of blood perfusion in the tissue space.²³ Because these nanoparticle carriers have a distribution of sizes, it is expected that uptake could occur by both lymphatic and capillary routes. A variation in Cmax such as that found in the present study may be attributable to the heterogeneity of the tumors and their surrounding environments.²³ In other words, it suggests that variable leakiness of local blood capillaries and lymphatic vessels affected the plasma kinetics of the drug molecules. Patient-to-patient variability in morphological features of tumors and even heterogeneity within the same tumor can strongly affect the distribution of drug from the injection site and absorption by blood vessels.²⁶,²⁷ Incorporation of the vasoconstrictor epinephrine was required in a cisplatin-bovine collagen gel formulation for intratumoral administration to prevent rapid variable absorption of small cisplatin species, which could occur in highly perfused tissues.²⁸ Yet a high degree in variability in cisplatin pharmacokinetics among patients was cited by the FDA in its rejection of the new drug application for that product.²⁹ In the formulation used in the present study, release of cisplatin species is delayed by chemical conjugation to the carrier. Although the rate of release is dependent on pH and on the presence of chloride ions,³ which could vary with the perfusion and morphological characteristics of the injection site, the release rate is expected to be less variable than that in a system where release is controlled by diffusion only.

Cisplatin is known to induce nephrotoxicosis under various experimental conditions in animals including mice, rats, and dogs. At a cisplatin dose
of 50 to 100 mg/m², 127 of 401 (32%) human patients were reported to develop acute renal failure during or after treatment. Results of a study to investigate toxicological effects of cisplatin in dogs revealed that the nephrotoxicosis was initiated by proximal tubular impairment as the proximal re-absorption rates decreased significantly after IV administration of cisplatin in dogs. The prolonged localization of cisplatin, platinum metabolites, or cisplatin hydrolytic products in the kidney is attributed to the continuous excretion and active secretion by the kidney. The most striking morphological changes in the kidney occur in the pars recta of the proximal tubules of the lumen, which is the most active site of secretion. The underlying mechanism of cisplatin-induced renal toxicosis has not been clearly understood. However, evidence suggests cytotoxic renal effects are likely attributable to the inhibition of DNA synthesis and compromised self-recovery of the pars recta of the proximal tubules in the kidney. Our results regarding the potential nephrotoxic effects of HA-Pt suggested that, at the doses used, HA-Pt did not adversely affect renal function as assessed by urinary creatinine concentrations in rats or by serum BUN and creatinine concentrations in clinical canine patients. We consider it likely that this was attributable to the fact that cisplatin was bound to a natural carrier and slowly released from the carrier in vivo, modifying the kinetics and the renal deposition of the drug and thus preventing irreversible injury to the kidneys.

Cisplatin rarely causes toxic hepatic injury. Results of our experiments with laboratory mice prior to use of HA-Pt in dogs indicated that neither cisplatin nor HA-Pt significantly increased serum activities of liver enzymes such as ALT, AST, and ALP. Surprisingly, the use of HA-Pt in the phase I-II clinical trial of dogs with cancer resulted in toxic liver changes in several dogs. After the first HA-Pt administration, 5 dogs had hepatic enzyme activities monitored during follow-up. Two of the 5 dogs had moderately increased serum ALT activities, and 1 of 5 had a markedly increased serum ALT activity. In addition, 2 dogs with anal sac adenocarcinoma developed signs of acute toxicosis and died of acute cardiac arrest within a few hours after local injection of HA-Pt.

We believe the hepatic injury in study dogs may have been attributable to the altered pharmacokinetics and deposition of HA-Pt, compared with those of cisplatin as well as a rapid release of diaquated platinum in the liver. In contrast to cisplatin, which is rapidly excreted by the kidney, the polymer-based HA-Pt is too large to be cleared through the kidney. The HA-Pt may be broken down by the liver, because the carrier hyaluronan is naturally metabolized by the liver. As the liver metabolizes the polymer, diaquated free platinum molecules are likely liberated from the polymer backbone in the forms of cis-[Pt(NH3)2(OH2)Cl]2+ and cis-[Pt(NH3)2(OH2)2]2+. Subsequent entry of the diaquated platinum into hepatocytes would result in DNA damage to the cells and release of liver enzymes from necrotic cells into the circulation.

Results of experiments to assess the effects of the diaquated product in rodents led to the conclusion that, during the scale-up of drug production, changes in the synthesis and purification procedures resulted in the introduction of diaquated impurities in some batches, which were not detected before patient treatment. These impurities resulted in reduced tolerability and moderate or severe toxic effects in some dogs. Diaquated product not only causes safety concerns, but substantial amounts of diaquated product in batches may also reduce the efficacy of the drug, considering that highly charged diaqua molecules may not enter tumor cells as efficiently as cisplatin, resulting in insufficient local exposure to the drug. Cleare and Hoeschele reported similar findings in regard to toxic effects of diaquated platinum products in rodents, and suggested that these molecules may interfere with a vital nervous system function at the neuromuscular junction. In subsequent research (unpublished data), we eliminated diaqua contamination by including an additional purification step by dialysis. Alternate formulations of HA-Pt should ensure a product free of diaquated cisplatin while preserving the effectiveness of the drug in canine patients in future studies.

In the present study, complete response to intratumoral injection of HA-Pt in dogs with SCC of the head and neck (which remained 2/13 at the time of last follow-up) was higher than that reported for dogs that received cisplatin IV (0/16 [0%]) for treatment of such tumors in another study. The high adverse effect rate (9/16) in the present study was primarily attributed to the unexpected presence of a diaquated cisplatin impurity in the prepared product, and a sensitive analytical method is needed to verify the strength, purity, and quality of future drug lots. Similar to human clinical trial designs, a pilot study is needed to develop dose ranges in a small number of healthy dogs before future trials to evaluate the toxicological effects of the formulation in this species. Because our previous study suggested that the carrier was internalized into cells via CD44-mediated endocytosis, screening of the level of CD44 expression in tumors could potentially be incorporated in future patient eligibility evaluation to increase the response rate in the most appropriate patient subpopulations, sparing the potential nonresponders with low expression of tumor CD44 from the adverse effects of the chemotherapy.

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Footnotes

a. VWR, Radnor, Penn.
b. Qila Chemical Co, Zibo, China.
c. LifeCORE Biomedical, Chaska, Minn.
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h. DMEM, Corning, Manassas, Va.
i. Acros Organics, Geel, Belgium.
j. SpectraMax Gemini XS, Molecular Devices, Sunnyvale, Calif.
k. GraphPad Prism, version 5.0, GraphPad Software Co, LaJolla, Calif.
l. Charles River Laboratories, Raleigh, NC.
m. Sigma-Aldrich Corp, St Louis, Mo.

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