Hyaluronan cisplatin conjugate in five dogs with soft tissue sarcomas

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Abbreviations

HA hyaluronan

H&E haematoxylin-eosin

Objective-This study investigates intratumoral delivery of a novel hyaluronan-cisplatin nanocarrier with goals of reduced systemic toxicity and enhanced tumor and lymphatic chemotherapeutic penetration.

Animals-5 dogs with spontaneously-occurring soft tissue sarcomas (STSs) were enrolled.

Procedure-Approximately 1.5 mls of nanocarrier with 20 mg cisplatin were injected into one external STS per animal. Blood for pharmacokinetics was collected at ½hr, 1hr, 2hrs, 3hrs, 4hrs, 24hrs, and 96hrs. Urinalysis was performed at initiation and 96hrs. Each STS and its draining sentinel lymph node(s) were removed at 96hrs. Platinum levels were measured in blood, tumor, and lymph nodes via ICP-MS analysis.

Results-There were no observed tissue reactions 96 hours following injection into the tumor. Average AUC for unbound platinum was 661.5 +/- (228.4) ng/ml, and total platinum was 2355.5 +/- (897.7) ng/ml. C_{max} of unbound platinum was 56.47 +/- (20.9) ng/ml, and total platinum was 81.6 +/- (40.4) ng/ml. The $t_{1/2}$ were 2.49 and 42.9 hours respectively for unbound and total platinum. Average platinum concentrations ranged from 3324.5 ng/g to 8228.8 ng/g in STSs, and from 129.5 ng/g to 6066.0 ng/g in lymph nodes.

Conclusions-Hyaluronan-cisplatin nanocarrier was well tolerated following intratumoral injection. Systemic platinum exposure appeared to be reduced versus traditional intravenous delivery. Hyaluronan-cisplatin nanocarrier demonstrated up to 1000-fold higher levels in tumors versus systemic circulation, and was concentrated by local lymphatics at levels up to 100 fold greater than hemovascular circulation. These characteristics make it a promising new chemotherapy modality.

Introduction

Hyaluronan is a natural polysaccharide with alternating D-glucuronic acid and N-acetyl-D-glucosamine units. Hyaluronan and its breakdown products are cleared by the lymphatics via receptor-mediated endocytosis and lysosomal degradation. [1-4] Hyaluronan is part of the extracellular matrix, and found in synovial fluid, cartilage, and vitreous humor of the eye, and dermis. [3,5] It is involved in multiple important processes such as cell adhesion, organization of the extracellular matrix, growth, migration, tumor formation, and metastasis.[3,5,6] Hyaluronan is nonimmunogenic which makes it an ideal nanocarrier for many different drugs such as cisplatin, paclitaxel, doxorubicin, and mitomycin.[1,5,7]

Cisplatin (cis-diamminedichloroplatinum or CDDP) is a DNA damaging agent that inhibits protein and rRNA synthesis.[8,9] *In-vitro* causes of cisplatin cytoxicity include platinum binding to DNA, creation of interstrand cross-links, and formation of intrastrand bidentate N-7 adducts at d(GpG) and d(ApG).[10] It is cell cycle phase nonspecific and is renally cleared.[9] It is used to treat many human solid tumors including head and neck squamous sarcomas, lymphomas, small cell and nonsmall cell lung, testicular, ovarian, gastric, esophageal, and pancreatic cancers. It is also used in treatment of companion animal solid tumors including osteosarcomas, carcinomas, and sarcomas.[9,11] Use of cisplatin has been limited due to significant side effects and laborious administration. Common side effects include significant nephrotoxicity involving renal tubular inflammation and necrosis, leucopenia, nausea, anemia, and chronic neurotoxicity with ototoxicity.[2,11] The cisplantin toxicities seen in people are also seen in animals which limits its veterinary use.

Cisplatin toxicity increases with peak plasma levels, but its effectiveness does not.[11] Different treatment strategies have been proposed such as metronomic chemotherapy, and local injection

of cisplatin to affected tissues while isolating systemic circulation to decrease the peak plasma levels and therefore decrease the rate of toxicity especially nephrotoxicity.[11] These treatment options can be lengthy and fiscally unattractive as well as requiring specialized skills and equipment not widely available.[11]

Nanocarriers with HA combined with cisplatin represent a new treatment modality that may decrease peak plasma levels while maintaining cisplatin efficacy.[11] In addition to being nonimmunogenic, HA is a ligand for CD44 receptors which are located on lymphocytes and some cancer cells.[11,12] Once bound to CD44, HA is catabolized, brought into the cell via receptor-mediated endocytosis, degraded in lysosomes, and then sent into the lymphatic microcirculation.[11] When cisplatin is combined with HA, the nanoparticles are activated as the nanoconjugate comes in contact with hyaluronidase. In addition to lymph nodes, hyaluronidase is also expressed on many tumors and can in fact be a marker of tumorigenesis. After tumor hyaluronidase or receptor -mediated endocytosis activates the HA-cisplatin, then the cisplatin is released into the peri-tumoral microlymphatics.[3]

Soft tissue sarcomas (STS) in the dog are mesenchymal and arise from connective tissues. They represent approximately 8-17% of skin and subcutaneous tumors.[13,14] Theses tumors are locally invasive with wide local excision being the treatment of choice. Recurrence following surgery is reported to be 7 to 32%.[13,15,16] Cisplatin,doxorubicin, Mitoxantrone, and paclitaxel have been tried in a wide variety of sarcomas, adjuvant chemotherapy has not been fully evaluated in STS and its role is relatively unknown.[16] Doxorubicin has not been found to have an increased effectiveness as adjunctive therapy in high grade STS.[17] A protocol of doxorubicin in combination with cyclophosphamide found an overall response rate of 23%.[18]

Dogs did not tolerate locally-delivered Cisplatin suspension well, resulting in premature termination of the study.[19] Dogs with spontaneous osteosarcoma treated with a cisplatin-containing implant after limb sparing surgery showed a non-significant decrease in local recurrence.[20] The implants require DMSO, and the DMSO can deactivate cisplatin which could impair efficacy.

The aims of this study are to gain experience with HA as a nanocarrier for local tissue delivery of cisplatin in spontaneously-occurring soft tissue sarcomas in dogs; characterize pharmacokinetics of the HA-cisplatin conjugate; and assess whether HA-cisplatin conjugate has preferential local lymphatic penetration. Our hypothesis is that canine STS will respond to local injection of HA-cisplatin, and that HA-delivered cisplatin will be concentrated within tumor-draining lymph nodes as compared to intravascular concentrations. A second hypothesis is that the the plasma concentration of cisplatin will be lower than tumor concentration, and there will be no evidence of systemic toxicity.

Material and Methods

Animals-Five client owned dogs weighing greater than 10 kg with spontaneously occurring soft tissue sarcomas presenting to the Animal Cancer Center at Colorado State University for surgical treatment were enrolled. The STS pretreatment diagnosis was done by needle core biopsy or incisional biopsy. Routine blood work including a complete blood count, chemistry panel and urinalysis were performed. All clients signed a consent form prior to study enrollment and all of the procedures were approved by the Institutional Animal Care and Use Committee and had hospital clinical board approval.

Synthesis of Hyaluronan-Cisplatin conjugates-Cisplatin was conjugated to HA using a previously reported procedure.[11] An ionic complex was formed between cisplatin and HA

containing ca. 20% by weight bound platinum, which releases cisplatin over several days into the local lymphatics. The platinum content was validated by atomic absorption spectroscopy (AAS) (Varian SpectrAA GTA-110 with graphite furnace).

HA-Cisplatin administration-The tumor was clipped of fur and measured with calipers in minimally two or three dimensions when possible. All dogs were sedated for intratumoral injection pending individual dog disposition and 20 mg of HA-cisplatin conjugate in a volume less than 2 milliliters was injected into the center of the tumor in all dogs. After 96 hours following injection the tumor was again measured.

Regional lymphoscintigraphy –After 96 hours following HA-cisplatin conjugate administration, the dogs returned for sentinel lymph node mapping and surgical tumor removal. All dogs were sedated for regional lymphoscintigraphy imaging and were injected with 125 microCuries of filtered technetium sulfur colloid peritumorally in four quadrants. Single photon emission computer tomography images were taken using a GE Millenium VG gamma camera every five minutes until the first draining lymph node basin was visualized.

Surgical procedure-All dogs' tumor sites were clipped including the area of the draining lymph node basin. Intracavitary sentinel lymph nodes were not removed within the chest or abdomen unless it was part of the planned tumor resection. Methylene blue (0.4 mL, 5 mg/mL) was injected around the tumor in four quadrants. Tumors were excised via wide local excision per routine clinical practice. A handheld gamma probe was used intraoperatively to identify individual draining lymph nodes for intraoperative lymphoscintigraphy. Separate instruments were used for lymph node extirpation. Once removed, the tumor and lymph nodes were remeasured. The tumors were dissected into quadrants. Core samples were collected from the

center of the tumor and at approximately every 2 cm radiating outward for larger tumors, placed in cryotubes and frozen at -80˚C for later platinum analysis. Remaining ex vivo tissue was placed in10% neutral buffered formalin for H & E staining and image analysis to determine percent tumor necrosis and histology. The lymph nodes were sectioned in half, and half of the tissue was placed in cryotubes and frozen at -80˚C for later platinum analysis and the other half was placed in 10% neutral buffered formalin for H & E staining for histology.

Pharmacokinetic Evaluation- - Approximately two mLs of whole blood in serum tubes and heparinized blood collected in EDTA tubes were obtained to measure unbound (plasma) and total (serum) platinum concentrations at 0 minutes, 30 minutes, 1 hr, 2 hrs, 3 hrs, and 4 hrs from an indwelling catheter following drug administration. The dogs then returned in 24 hrs and 96 hrs for additional blood samples. Also blood for a complete blood count and chemistry panel as well as urine for a urinalysis was done at 0 and 96 hrs. The whole blood was centrifuged and the serum portion was collected and placed in a cryotube prior to freezing as well as the EDTA tubes were centrifuged and the plasma collected. The plasma and serum were collected into cryotubes and immediately frozen at -80˚C. Plasma and serum were thawed at the time of assessment. The serum was placed in a centrifugal filter unit with 10,000 MWCO. Centrifugation was performed at 7,500 x *g* for 20 minutes. The filter was then removed and volume of filtrate recorded, and then sufficient 6% nitric acid was added to obtain a 1:10 dilution to obtain the ultrafiltration portion. Plasma was also collected and sufficient 6% nitric acid was added to obtain a 1:10 dilution.

Platinum determination by ICP-MS- Plasma (unbound) and serum (total) were prepared as described above and frozen tumor and lymph node sections were thawed at room temperature and then analyzed. Preparation method was similar for all sample types. Tissue (0.5 g wet

weight) or plasma samples were placed in 15-mL centrifuge tubes, and then 0.75mL of concentrated nitric acid was added and samples were heated to 95˚C for 6 hrs. After cooling the samples, 0.5 ml of 30% hydrogen peroxide was added and heated to 80˚C for 30 minutes. After the second cooling, 0.25 mL of concentrated hydrochloric acid was added and heated to 80˚C for 30 minutes. After the third cooling, the samples were brought to approximately 5 mL final volume with purified water, and the exact volumes were determined gravimetrically. Samples were grouped into sets of approximately 20, and each set was prepared with a blank, a spiked blank, a standard reference material (NRC Canada's DOLT-4), a duplicate sample, and a spiked sample. Digestates were diluted 10x in purified water for platinum analysis by ICP-MS (PerkinElmer Sciex Elan DRC II) and were analyzed undiluted for other elements using ICP-OES (PerkinElmer Optima 7300).

Pharmacokinetics modeling- C_{max} was determined from plasma and serum concentration-time profiles. Area under the curve (AUC) was calculated by linear trapezoidal summation from time zero to infinity. The $t_{1/2}$ was calculated using the elimination rate constant with $K_{el}=0.693/t_{1/2}$ from 4 to 96 hours.

Histology and pharmocodynamics - The tumor was longitudinally sectioned. Sections representing the complete longitudinal plane of the tumor were fixed in formalin for 24 to 48 hours. The sections were processed with an extended protocol, paraffin-embedded, sectioned at 4 microns, and H&E stained. All sections from a sample were completely scanned using an AxioCam HRc Carl Zeiss camera coupled to a Carl Zeiss Axioplan 2 microscope with a mechanical stage and utilizing Carl Zeiss Axiovision analysis software. The scanned images were viewed simultaneously with the H&E stained sections. Total region of tumor and regions of necrosis were outlined on the images. Regions outlined on the images were then analyzed with

the Axiovision image analysis software to determine tumor area and the area of tumor necrosis. Total tumor and necrosis areas for a tumor were determined by adding results of all sections for that tumor. Percent necrosis for a tumor was determined as total area of necrosis/total tumor area. The lymph nodes were longitudinally sectioned. Sections representing the complete longitudinal central plane of the lymph node were fixed in formalin for 24-48 hrs. The sections were processed with an extended protocol, paraffin-embedded, sectioned at 4 microns, and haematoxylin-eosin (H&E) stained. Histology was evaluated by a pathologist.

Results

Five client-owned dogs were enrolled in the study. Two were mixed breed, one Labrador retriever, one Golden retriever, and one Chesapeake Bay retriever. All dogs were of similar age with a median age of 9 years old (range 7.5 to 10 years). There were three male castrated and two female spayed. They all had similar weight with a median weight of 38 kg (range 27.1 kg-43.1 kg). Each dog had only one STS examined for the study, and the tumor locations were antebrachium, stifle, ventral thorax, lateral flank, and medial hock. Four of five tumors were greater than 5 cm in diameter and the average largest tumor diameter was 8.9 cm (4.4 cm to 13.7 cm). There was no evidence of metastasis at the time of presentation.

Tumor response-Following injection all tumors remained stable in size as determined by serial measurements at the 96 hour time point. All tumors were completely removed via histopathologic assessment of surgical tumor margins. The STS histologic grading system evaluates cell differentiation, mitosis, and percent necrosis on a scale of I to III.[15] The dogs in this study had a median tumor grade of II (range I-III).

Toxicity- One dog had a grade one dermal reaction (VCOG consensus statement 2004) [21] 24 hours post HA-cisplatin injection in the tumor which consisted of two areas of mild erythema one at the injection site and the other at the distal aspect of the mass. No other dogs had any reactions at the injection site or noted in the tumor at the 96 hour time point.

Blood values prior to injection and 96 hrs post injection were not significantly different between the complete blood cell counts or serum chemistry. One dog had a grade one thrombocytopenia at the 96 hour complete blood count.[21] One dog was hyposthenuric prior to treatment and at 96 hrs. All renal values remained within normal reference intervals.

Two dogs with amputations performed to remove the STSs developed mild seromas ten days post amputation. Two dogs developed dehiscence at the surgical site located on the stifle and ventral thorax. The stifle resection developed partial dehiscence nine days postoperatively from the distal aspect of the incision to the point of maximal tension over the joint and was approximately 8.8 cm in length. The original tumor on the stifle was 9 cm in the largest diameter. This was managed as an open wound and then surgically closed five days later. The ventral thorax resection was large with the largest diameter being 7.4 cm and developed partial dehiscence at the cranial aspect of the incision at the level of the drain approximately 2.2 cm in diameter 22 days postoperatively. This was managed as an open wound and healed. One dog developed a seroma fourteen days post operatively at the lateral flank that developed into an abscess eight days later. This was lanced and treated with antibiotics.

Sentinel lymph nodes- Regional lymphoscintigraphy was performed in three out of five dogs. The draining lymph nodes were identified with lymphoscintigraphy and gamma camera. Sentinel lymph nodes were obtained surgically in four of the five dogs. One dog had a draining

lymph node identified by regional lymphoscintigraphy, but the lymph node could not be found at the time of surgery with intraoperative lymphoscintigraphy and blue dye mapping. The two other dogs not receiving regional lymphoscintigraphy had the local draining lymph nodes identified by intraoperative methylene blue injection and hand held gamma probe guidance and were removed. Histology of all lymph nodes was reactive hyperplasia and/or chronic histiocytosis. No evidence of neoplasia was found in any of the lymph nodes. The tumor locations and associated draining lymph nodes are described in Table 1.

Platinum levels in the lymph nodes, tumors and blood-The platinum concentration in the STSs was greater than in the lymph nodes and blood. The average platinum concentrations within excised STSs ranged from 3324.5 ng/g to 8228.8 ng/g. The platinum concentrations within the draining lymph nodes were 129.5 ng/g to 6066 ng/g. The average AUC, $t_{1/2}$, and C_{max} for the unbound and total platinum are listed in Table 2 through Table 4.

Percent tumor necrosis- The averaged percent tumor necrosis ranged from 0.25% to 6.61%.

Discussion

Platinum levels were significantly higher in the tumor and its lymph nodes than in the systemic circulation. This has also been demonstrated in rodent studies evaluating this nanoconjugate.[4,11,12] Platinum levels remained much higher at 96 hours in lymph node and tumor, with average platinum concentration in tumors approximately one-thousand-fold greater than plasma and one-hundred-fold greater than serum. The lymph node platinum concentration ranges at the 96 hour time point were ten-fold to one-hundred-fold greater than in the plasma and serum. Hyaluronan-cisplatin achieved preferential uptake into the local regional draining lymph

nodes indicating it is a good treatment delivery system, especially for neoadjuvant or adjuvant chemotherapy when there is a high likelihood of lymphatic spread.

Nanoconjugated HA cisplatin showed lower toxicity than cisplatin alone. Studies examining the pharmacokinetics of HA-cisplatin conjugates administered subcutaneously and intravenously in rodents found increased plasma AUC with decreased peak plasma levels compared to conventional intravenous cisplatin.[11] Higher concentrations in the tumor and lower concentrations in circulation help decrease renal and other toxicity. Acute renal toxicity due to cisplatin usually develops within the first 24 hours.[10] In this study no dogs developed any systemic toxicity up to 96 hours post injection. Mild thrombocytopenia in one dog was likely not related to the drug exposure because the half-life of platelets is much greater than 96 hours.

In this study all five dogs had varying types of complications at the surgical site. Not surprisingly the two dogs with amputations developed mild seromas, which are anecdotally not unusual following limb amputation. As in the case of the dog that developed a seroma on the lateral flank that then progressed into an abscess, seroma formation two weeks postoperative is not uncommon following wide surgical excision in this location. It is also not uncommon for seromas to become secondarily infected. The tumors in both of these dogs were a considerable distance away from the amputation site making less likely that the seromas were related to the tumor or HA-cisplatin injection site. The three other dogs developed complications at their incision sites, which might have been due to the wide 2-3 cm tumor excision margins in locations with little or no redundant skin. This study selected for STS greater than 2 cm which can make complete wide-margin surgical removal and primary wound closure challenging. All tumors were completely removed for histologic evaluation. Dehiscence in a taut, high motion area overlying the stifle is a normal risk factor of tumor removal in routine clinical practice.

Similarly, the ventral thorax tissue is notoriously taut without redundant tissue; dehiscence at this location is a common risk and can transpire if no undue tension is present with primary closure. There is the possibility that the HA-cisplatin conjugate interferes with wound healing. This has not been evaluated in rodent models.[4,11,12] Other studies evaluating cisplatin administered locally have also found high complication rates. Earlier studies evaluating OPLA-cisplatin implants in dogs found a wound complication rate of 47.5% to 60%.[20,22] Another recent study in dogs with surgically removed STS placed a cisplatin biodegradable implant delivery system and found a wound complication rate of 84.2%.[23] This study had a wound complication rate of 3/5 dogs (60%). Further studies evaluating wound healing with this nanoconjugate are needed to further elucidate if the drug inhibits healing, or if complications are simply those associated with the wide margins needed to obtain complete removal of these tumors.

This study determined the regional draining lymph nodes for each tumor by sentinel lymph node mapping by regional lymphoscintigraphy using a gamma camera and also intraoperatively by using methylene blue dye and a hand held gamma probe. No lymph nodes were found to have evidence of STS metastasis in this group of dogs. Soft tissue sarcoma metastasis more commonly occurs in the lungs as it is spread hematogenously, and it is less likely to occur in the lymph nodes.[15] Greater risk of metastasis can be associated with tumor grade.[13-15] The average STS grade in this group of dogs was grade II, which has an uncertain metastatic rate but has been reported to be less than 15%.[15] Soft tissue sarcoma is a good model for this nanocarrier in a pilot study where it is of interest to instill HA-cisplatin into a tumor that can be completely excised and whose draining lymph nodes can easily be determined. This tumor model assessed product tolerability in the dog model and there was minimal morbidity associated with lymph node excision, and no complications occurred related to lymph node extirpation.

The data demonstrate that the study drug increased uptake of platinum in the regional draining lymph nodes; this has been previously noted in rodent HA-cisplatin studies that found increased platinum accumulation in local regional lymph nodes and low-level sustained systemic release that allowed for decreased peak plasma concentrations.[4,11] Hyaluronan enters into the microlymphatics from the interstitial space and is activated within the lymphatic system making the HA-cisplatin conjugate a new modality to treat lymphatically spread and metastatic cancer. Hyaluronan-cisplatin given intratumorally is also taken up intracellularly by tumor cells because many tumor cells express the HA ligand CD44, as do lymph nodes.[11,12] Lymph is partially comprised of tissue interstitial fluid that enters into the lymphatic circulation via active processes of opening lymph endothelial valves, after which the fluid travels unidirectionally through a series of collecting lymphatic vessels and nodes.[24] Hyaluronan-cisplatin enters preferentially into the lymphatic system, and the carrier is cleaved from the conjugate by lysosomal degradation, which activates the drug.[11,12] This new treatment delivery system has been used in murine models for breast cancer, intrapleural injection for malignant pleural mesothelioma, and endotracheal instillation for lung cancer.[2,5,12] Doxorubicin conjugated to HA for breast cancer has also been examined in rodents.[1]

The tumors remained stable in size 96 hours post injection. Chemotherapy has not been evaluated extensively in dogs with measurable STS. A response rate of 15% to 74% has been reported and this has been with varying systemic therapies and agents.[16] Another study evaluating two doses of doxorubicin found the response rate for STS to be 22%.[18] There was not a large amount of tumor necrosis in the histologic and percent tumor necrosis evaluations. However, significant necrosis would not be expected at this early time point.

This is the first study evaluating the pharmacokinetics of HA-Cisplatin nanoconjugate in dogs. The AUC and C_{max} in our study were less than previously described with this nanoconjugate in a rodent model.[11] This may be due to the fact that we administered HA-cisplatin intratumorally and not subcutaneously as was done in the rodent model. Also, we gave 20 mg to each dog which averaged to 0.56 mg/kg, while the rodents received 3.3 mg/kg. The $t_{1/2}$ was greater in our study, but this is not surprising as dogs have a slower heart rate and metabolism than rodents. Our $t_{½}$ is within the range of multiple reported studies for $t_{½\beta}$ in people in the second phase of cisplatin clearance (total 30.5 hr to 130 hr) with the first phase representing the terminal half-life of unbound platinum and the second phase being total platinum.[25]

There is limited data available for AUC for cisplatin in dogs. The AUC can be extrapolated from a previous study that evaluated the effect of hyperthermia on the pharmacokinetics of cisplatin in normal dogs, and our AUC for total and unbound is very similar with our unbound platinum AUC within 400(ng hr)/ml difference, and our total platinum AUC was 1,629 (ng hr)/ml greater.[26] An early study evaluating intravenous cisplatin administration at 1 mg/kg in normal dogs found much higher plasma concentrations in the first four hours, approximately one-thousand-fold higher than seen in this study.[27] (Table 5) Although a part of this much higher systemic cisplatin exposure is no doubt due to the higher dose in that study, most of the difference is likely due to the nature of the study drug in this study. Hyaluronan-cisplatin nanoconjugate helps maintain lower systemic levels while achieving higher concentrations locally in the tumor and regional lymphatic tissue. The lower systemic levels help to prevent systemic toxicities.

Theon and coworkers [28] conducted a study of intratumorally-injected cisplatin in canines with oral malignant melanoma using a study dose (0.25 mg/kg or ca. 5 mg/m^2) much closer to the

dose we administered (0.56 mg/kg or ca. 11 mg/m^2). Compared to Theon's study, the intratumoral HA-cisplatin demonstrated a 1.1-fold reduction in the C_{max}, 50.2- and 14.9-fold increases in the $t_{1/2}$ and AUC, respectively, even though the animals in our study were treated with a 1.2-fold higher dose of the drug. The findings suggested that our HA-cisplatin formulation has significantly enhanced the retention and accumulation of the drug in vivo at lower doses, which could be utilized as maintenance chemotherapy, reducing the cycle number of the treatment. In addition, Theon et al reported the incidence of grade 1, 2, and 3 toxicities following the intratumoral cisplatin chemotherapy. In comparison, dogs treated with HA-cisplatin were free of drug-induced severe toxicities (grade 2 & 3) for the duration of the study.

Cisplatin has been evaluated in the dog in different delivery modalities. Cisplatin has been given intra-arterially to dogs with osteosarcoma treated with limb spare surgery and radiation therapy.[29] Cisplatin has also been given subcutaneously and intracavitary. High toxicity was observed in subcutaneous administration, but not in intracavitary administration.[19,30] Intracavitary administration however is best for a small subset of cancer treatments: palliation of pleural and/or abdominal effusions. Another modality studied evaluated cisplatin release from a D, L-Polylactic acid (OPLA) implant in dogs with soft tissue sarcomas, nasal tumors, and osteosarcoma treated with limb sparing surgery.[20,22,31-33] These studies did not find any statistically significant clinical improvement with the cisplatin implant. A study evaluating cisplatin release from D, L-polylactic acid implant in normal beagle dogs adjacent to a cortical allograft found a much higher AUC for both dogs with the implants and beagles given cisplatin intravenously compared to our study. The dose used in these dogs was much higher than ours. The beagles with implants received 54.4 mg/m^2 and 81.6 mg/m^2 (AUC 27,050 ug·min/ml) and the dogs given cisplatin intravenously received 70 mg/m^2 (AUC 940.3 ug·min/ml).[33] In our

study the dogs' dosages ranged from 16 mg/m^2 to 22 mg/m^2. It is also difficult to compare the variable administration routes. In a study evaluating cisplatin impregnated polymethylmethacrylate in healthy dogs dosed at 25.68 mg/m^2 or 20 mg/dog, plasma concentrations were approximately one hundred fold greater than in our study.[34] The AUC was less than our study (AUC 1.093 ug·min/ml) which can be attributed to the different delivery routes and dosages.[34] Although this study dosage was similar to ours (20 mg/dog), our HA-cisplatin conjugate showed less systemic absorption and more sustained release from carrier. Soft tissue sarcomas are a good tumor model because these are tumors that can be easily measured and amenable to a drug injection directly into the tumor. Soft tissue sarcomas have a low metastatic rate to the local lymph node and lung, yet it is straightforward determining if there is presence of cisplatin is in the sentinel lymph node(s) which tend to be externally located and readily accessible. Cisplatin levels were higher in the tumor and lymph nodes compared to systemic circulation. Tumors remained stable in size and did not progress over the 96 hour time frame. Further investigation into possible inhibition of healing at the surgical site is needed, although in clinical practice excision soon after intratumoral chemotherapy would likely not be standard procedure.

In conclusion this study demonstrated that HA-cisplatin nanoconjugate can be given intratumorally to dogs resulting in higher cisplatin concentration in tumors and draining lymph nodes with preferential regional lymphatic uptake, lower cisplatin concentration in plasma, and no systemic toxicities noted within the first 96 hours.

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Table 1- Tumor location and associated draining lymph nodes found via regional lymphoscintigraphy and intraoperative sentinel lymph node mapping using blue dye and hand held gamma probe.

Patient	Tumor location	Regional lymphoscintigraphy	Sentinel lymph nodes surgically excised
Dog 1	Medial hock	Not performed	Popliteal, inguinal
Dog 2	Lateral flank	Axillary	Not obtained
Dog 3	Cranial ventral thorax	Axillary, superficial cervical	Axillary
Dog 4	Caudal antebrachium	Not performed	Prescapular and axillary
Dog 5	Cranial stifle	Popliteal, inguinal, and medial iliac	Popliteal and inguinal

Table 2- Pharmacokinetics averages of unbound (plasma) and total (serum) platinum levels in the blood

PK parameter	Unbound	Total
C_{max} (ng/ml)	56.47 (+/-20.9)	81.6 (+/-40.4)
$AUC_{0\text{-}inf}$ (ng h/ml)	774.6 (+/-222.1)	3562.1 (+/-2031.1)
$t_{1/2}$ (4-96h)	33.6 (+/- 16.1)	51.2 (+/- 29.1)

Table 3-Pharmacokinetics of unbound (plasma) platinum levels in the blood of the individual dogs

PK parameter	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5
C_{max} (ng/ml)	22.88	69.41	48.91	70.97	70.2
$AUC_{0\text{-}inf}$ (ng h/ml)	556.87	1147.1	745.32	746.69	677.1
$t_{1/2}$ (4-96h)	30.47	23.64	24.2	62.1	27.9

Table 4-Pharmacokinetics of total (serum) platinum levels in the blood of the individual dogs

PK parameter	Dog 1	Dog 2	Dog 3	Dog 4	Dog 5
C_{max} (ng/ml)	37.98	95.91	53.94	142.02	78.15
$AUC_{0\text{-}inf}$ (ng h/ml)	1457.48	4719.82	6502.43	2788.74	2341.86
$t_{1/2}$ (4-96h)	37.28	58.19	99.1	27.54	34.06

Table 5- Comparative pharmacokinetics of HA-cisplatin with other routes

	Intratumoral HA-cisplatin	Intravenous cisplatin[35]	Intratumoral cisplatin[19]	Intratumoral cisplatin [28]
Dose, mg/m^2	11≈	10	70	5≈
C_{max}, ng/mL (serum)	81.6 ± 40.4	294 ± 60	770*	170*ǂ
$t_{1/2}$, hrs	51.2 ± 29.1	29.1 ± 15.7	96*ǂ	1*ǂ
$AUC_{0\text{-}inf}$, ng·h/mL (serum)	3562.1 ± 2031.1	663 ± 52	58800*	224*ǂ
Tumor concentration, ng/g	5650.0 (3324.5 - 8228.8)×	-	-	-
LN concentration, ng/g	2485.0 (129.5 - 6066.0)×	-	-	-
Bioavailability	488%	100%	1270%	116%

* Standard deviation not reported by authors

ǂ Determined from concentration-time curve provided by authors

× Tissue concentrations measured at 96 h

≈ Converted from mg/kg dose based on Freireich et al [36]

Notes: