GEMCITABINE IN COMBINATION WITH CARBOPLATIN EXHIBITS BIOLOGIC ACTIVITY AGAINST CANINE OSTEOSARCOMA

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ABSTRACT

Gemcitabine, an analog of cytosine arabinoside, inhibits the growth of human osteosarcoma (OSA) cell lines \textit{in vitro} as well as in mouse xenograft models. The purpose of this study was to evaluate the activity of gemcitabine against canine OSA cell lines alone or in combination with compounds known to have activity against OSA \textit{in vitro}, including the bisphosphonate pamidronate and the chemotherapeutic carboplatin. Subsequent to the \textit{in vitro} study, we evaluated whether gemcitabine administered in combination with carboplatin would improve outcome in dogs with OSA. We hypothesized that gemcitabine in combination with carboplatin would significantly improve 1-year survival rates in dogs with OSA following amputation compared to carboplatin alone. Additionally, we predicted that the toxicity of the gemcitabine and carboplatin combination would be comparable to carboplatin alone in dogs with OSA post-amputation.

OSA cell lines OSA8, OSA16, OSA32, and OSA36 were treated with gemcitabine alone or in combination with pamidronate or carboplatin. Loss of cell viability was assessed using the water soluble tetrazolium-1 (WST-1) assay, cell cycle analysis was evaluated using propidium iodide staining, and apoptosis was assessed by measuring caspase-3/7 activation. Synergy was quantified by combination index (CI)
analysis using CalcuSyn software. Following the preclinical study, forty-five dogs with stage II appendicular OSA were enrolled into a multicenter, prospective clinical trial. Each dog received carboplatin $300\text{mg/m}^2$ and gemcitabine $2\text{mg/kg}$ following amputation for four treatment cycles. Dogs were monitored for metastatic disease during the treatment course and every three months after the completion of chemotherapy.

In the preclinical study, treatment of canine OSA cell lines with gemcitabine induced growth inhibition, cell cycle arrest, and apoptosis. No synergistic or additive activity was noted when canine OSA cell lines were treated with gemcitabine and pamidronate. However, when OSA cell lines were treated with gemcitabine and carboplatin in combination, a significant decrease in cell viability was observed compared to gemcitabine alone, and the drug combination was synergistic. This activity was greater when cells were treated with carboplatin prior to gemcitabine, versus gemcitabine prior to carboplatin in 3 of 4 OSA cell lines. In the clinical study, the median follow-up time from diagnosis was 171 days, and 38 of the 45 dogs were still alive at the time of writing; therefore, disease free interval and median survival time could not be calculated. No grade 3 or 4 hematologic or gastrointestinal toxicities were noted in the patients for which data was available; however, one death was attributed to chemotherapy toxicity.

Gemcitabine exhibits biologic activity against canine OSA cell lines, and carboplatin combined with gemcitabine exhibits synergistic activity at biologically
relevant concentrations. The efficacy of gemcitabine administered in combination with carboplatin in dogs with OSA can not be established at this time. Gemcitabine in combination with carboplatin is well tolerated with minimal and acceptable toxicity.
To Althea; my faithful friend for the last 13 years.
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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Osteosarcoma (OSA) is the most common bone tumor in dogs, reportedly occurring in over 8,000 dogs each year in the United States and representing up to 85% of all bone malignancies\(^1\). OSA occurs most often in the long bones and is locally aggressive causing lysis of bone leading to lameness. Treatment most commonly involves amputation of the affected limb followed by chemotherapy. Despite the fact that less than 15% of dogs have evidence of gross pulmonary or osseous metastases at the time of diagnosis\(^2\), approximately 90% of dogs with OSA will die of metastatic disease, usually to the lungs, within 2 years of amputation and chemotherapy\(^1\). No significant changes in survival times have been achieved for dogs with appendicular OSA in the past 10 years despite modification of existing chemotherapy protocols. When amputation or limb sparing is the only treatment, the median survival time of dogs with OSA is 3-4 months\(^3\). Median survival times are extended to 8-12 months if adjuvant chemotherapy with doxorubicin, cisplatin, carboplatin, or a combination of doxorubicin and a platinum compound is used\(^4-8\). Carboplatin is currently used as a standard of care in the treatment of canine OSA, but only 35% of dogs will survive 1 year post amputation\(^5,6\). If
significant improvements in survival time are to occur, new therapeutic approaches need
to be explored.

Gemcitabine is a synthetic analog of cytosine arabinoside that has anti-tumor
activity in a variety of human cancers. Recent evidence suggests it can inhibit the
viability and growth of human OSA cell lines in vitro as well as in mouse xenograft
models\textsuperscript{9,10}. In this study we evaluated the anti-tumor activity of gemcitabine against
several canine OSA cell lines to assess whether this chemotherapeutic agent has potential
clinical utility. Additionally, the ability of gemcitabine to work synergistically with the
bisphosphonate pamidronate and the chemotherapeutic carboplatin, both of which have
some activity against OSA, was evaluated. We hypothesized that gemcitabine would
have biologic activity in canine OSA cell lines, and that gemcitabine would interact
synergistically with carboplatin at biologically relevant concentrations. Additionally, if
we found our first hypothesis to be true, we proposed that gemcitabine administered in
combination with carboplatin would improve outcome in dogs with OSA. Therefore, our
second hypothesis is that carboplatin plus gemcitabine would significantly improve 1-
year survival rates compared to carboplatin alone in dogs with OSA following amputation
without an increase in clinically significant toxicity.

1.2 Gemcitabine

Gemcitabine (2’, 2’-difluorodeoxycytidine) is a synthetic analog of cytosine
arabinoside. Anti-tumor activity is achieved through inhibition of DNA replication and
cell growth by incorporation of gemcitabine into replicating DNA and through inhibition
of repair mechanisms by masked DNA chain termination\textsuperscript{11}. Gemcitabine has biologic
activity in a variety of human cancers and has been approved for use as a single agent in
the treatment of human pancreatic adenocarcinoma and in combination protocols for patients with non-small cell lung cancer, metastatic breast cancer, and ovarian cancer.\textsuperscript{12-17} In addition, clinical trials of gemcitabine in people, either as a single agent or as part of a multi-agent protocol, have demonstrated stabilization of disease and response rates in several solid tumors, including transitional cell carcinoma, advanced biliary carcinoma, and soft tissue sarcomas.\textsuperscript{18-21}

Gemcitabine has not been used extensively in veterinary medical oncology. A phase I study determined that gemcitabine can be administered to dogs biweekly at 675mg/m\textsuperscript{2} IV with minimal toxicity.\textsuperscript{22} The combination of 2mg/kg gemcitabine IV over 20 to 30 minutes and 10mg/kg carboplatin as an IV bolus has been evaluated clinically in dogs with cancer resulting in acceptable toxicity, but low response rates.\textsuperscript{23}

1.3 Gemcitabine in OSA

The potential activity of gemcitabine against OSA has been investigated \textit{in vitro} and \textit{in vivo}. In one study, gemcitabine inhibited the viability and growth of human OSA cell lines \textit{in vitro} and human OSA lung metastases in a mouse model when administered by intraperitoneal injection.\textsuperscript{9} Additionally, aerosolized gemcitabine inhibited the growth of OSA xenografts as well as lung metastases in mice.\textsuperscript{10} While gemcitabine has not been evaluated extensively in human patients with OSA, one study demonstrated stabilization of metastatic disease in five of seven patients with progressive localized or metastatic chemo-resistant OSA for 13 to 96 weeks.\textsuperscript{21} These data suggest that gemcitabine may have clinical utility for the treatment of OSA.

1.4 Gemcitabine and bisphosphonates

Another potential therapeutic approach for the treatment of OSA has been the use
of bisphosphonates. These are analogs of endogenous pyrophosphate, an inhibitor of bone metabolism. Bisphosphonates have been used to treat a variety of diseases and conditions including osteoporosis, hypercalcemia, primary bone tumors, and bone metastasis. Pamidronate disodium is a bisphosphonate that is administered intravenously and binds to bone at sites of active remodeling, thereby inhibiting bone resorption without affecting bone growth and mineralization\textsuperscript{24}. Pamidronate has been shown to decrease the viability of canine OSA cell lines \textit{in vitro}\textsuperscript{24}. Furthermore, a recent study demonstrated that bisphosphonates enhance the cytotoxic effects of gemcitabine \textit{in vitro}\textsuperscript{25}. Given the apparent synergistic activity of gemcitabine and pamidronate, one of the aims of this study is to determine if the bisphosphonate pamidronate will enhance the cytotoxicity of gemcitabine against canine OSA cell lines.

1.5 Gemcitabine and platinum compounds

The ability of gemcitabine to work synergistically with platinum compounds has been investigated both \textit{in vitro} and in human clinical trials. For example, gemcitabine and carboplatin were found to induce synergistic killing of several human non-small cell lung cancer cell lines\textsuperscript{26,27}. Gemcitabine plus cisplatin demonstrated cytotoxicity and synergy in human squamous cell carcinoma and platinum resistant ovarian carcinoma cell lines when co-administered \textit{in vitro}\textsuperscript{28}. Finally, cytotoxicity was significantly improved when cisplatin or carboplatin was added to gemcitabine in the treatment of human endometrial carcinoma cell lines compared with any single agent alone\textsuperscript{29}. With respect to clinical patients, gemcitabine plus carboplatin has been approved for use in women with platinum-sensitive recurrent ovarian cancer\textsuperscript{17}. In advanced non-small cell lung cancer, the combination of gemcitabine with carboplatin or cisplatin has significantly improved
response rates and survival times in affected patients\textsuperscript{14}. Based on this evidence from the human literature and the need to identify new therapeutic approaches that will significantly improve survival times in dogs with OSA, this study seeks to evaluate the biologic activity of gemcitabine and carboplatin in combination in canine OSA cell lines, as well as in clinical patients with OSA following amputation.
CHAPTER 2

MATERIALS AND METHODS

Preclinical Study

2.1 Cell lines and reagents. All OSA cell lines (OSA8, OSA16, OSA32, and OSA36) were provided by Dr. Jaime Modiano (University of Minnesota, College of Veterinary Medicine and Cancer Center, Minneapolis, MN) and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco/Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Gemini, Sacramento, CA), nonessential amino acids, sodium pyruvate, HEPES, penicillin, streptomycin, and L-glutamine. Cells were treated with gemcitabine (Eli Lilly, Indianapolis, IN), carboplatin (Hospira, Inc., Lake Forest, IL), pamidronate (Teva Pharmaceuticals, North Wales, PA), or drugs in various combinations. Doxorubicin (Bedford Laboratories, Bedford, OH) was used as the positive control in all assays³⁰.

2.2 Cell viability assays. The WST-1 assay (Roche, Indianapolis, IN) was used to assess cell viability. OSA cells (2,000 per well) were seeded in 96-well plates in 150µl RPMI-1640 medium with 1% fetal bovine serum and incubated overnight at 37°C and 5% CO₂. The cells were then treated with gemcitabine, carboplatin, pamidronate, or drugs in various combinations. Doxorubicin at a concentration of 0.5µM was used as the
positive control and untreated cells served as the negative control. Cell viability was assessed using the WST-1 assay after 72 hours of culture according to manufacturer’s specifications. With the exception of the sequence of administration experiments, OSA cells were exposed to drug for 72 hours. For the sequence of administration experiments, OSA cells were exposed sequentially to gemcitabine and carboplatin, each for 2-hour incubations. OSA cells were washed (medium was removed by suction and cells were rinsed three times with phosphate buffer saline (PBS) solution) after each 2-hour drug exposure, followed by incubation in 150μl RPMI-1640 medium with 1% fetal bovine serum until assessment with the WST-1 assay 72 hours later. For all cell viability assays, absorbance was quantified using an ELISA plate reader (Molecular Devices, Sunnyvale, CA) at 440nm. Cell viability was calculated as a percentage of the control wells: absorbance of sample/absorbance of untreated cells. Samples were analyzed in triplicate and each experiment was repeated three times.

2.3 Evaluation of synergy. To evaluate for synergistic interactions between carboplatin and gemcitabine, cell viability was established after 72 hours of culture using the WST-1 assay for each drug alone, and the drugs in combination. The drug combination was evaluated at a fixed ratio (1 to 100 of gemcitabine to carboplatin) of equipotent concentrations. Serial dilutions of the highest concentration combination of gemcitabine to carboplatin were used to generate a dose response curve. Synergy was quantified by the median-effect equation and CI analysis using Calcusyn software (Biosoft, Version 1.2, Cambridge, UK). The CI values were determined at different effect levels and different dose levels. The dose-reduction index (DRI) was determined by comparing the ratio of the concentrations required to reach a given degree of growth.
inhibition for gemcitabine and carboplatin individually and in combination.

2.4 **Cell cycle analysis.** To determine cell cycle status, OSA cells were seeded at 5 x 10^5 cells per well in 6-well plates in 3ml of RPMI-1640 medium with 1% fetal bovine serum and incubated overnight at 37°C and 5% CO₂. Gemcitabine at concentrations of 1, 10, and 100μM were evaluated alone and in combination with carboplatin at a concentration of 250μM. Doxorubicin at a concentration of 0.5μM was used as the positive control. Untreated cells served as the negative control. After 48 hours of drug exposure, cells were collected, fixed in 70% ethanol, incubated with 0.5ml of propidium iodide staining solution (50μg/ml PI and 10μg/ml RNAse in 0.1% glucose-PBS solution), and analyzed by flow cytometry. Data analysis was performed using the Cell Quest Pro software (Cell Quest Pro software, Becton-Dickinson Biosciences, San Jose, CA). Samples were analyzed in duplicate and each experiment was repeated three times with each cell line.

2.5 **Measurement of caspase activation.** To evaluate activation of the caspase-3/7, OSA cells (5,000 per well) were seeded in 96 well plates in 150μl RPMI-1640 medium with 1% fetal bovine serum and incubated overnight at 37°C and 5% CO₂. Gemcitabine at concentrations of 1, 10, and 100μM were evaluated alone and in combination with carboplatin at a concentration of 250μM. Doxorubicin at a concentration of 0.5μM was used as the positive control. Untreated cells served as the negative control. After 24 of drug exposure, caspase-3/7 activity was assessed using the SensoLyte Homogenous AMC Caspase-3/7 Assay Kit according to manufacturer’s specifications (AnaSpec, San Jose, CA). Fluorescence was quantified using an ELISA plate reader at an excitation of 354nm and emission of 442nm. Samples were analyzed in triplicate and each experiment was
repeated three times with each cell line.

2.6 Statistical Analysis. *In vitro* experiments were performed at least three times and data are summarized as mean ± standard deviation. All data were transformed to the natural log-scale prior to statistical analysis in order to stabilize variances across treatment groups and to achieve approximate normality. For the cell viability and caspase-3/7 assays, Dunnett’s multiple comparisons method was used to compare treatment groups to positive or negative control. Dunnett’s method was implemented at an overall \( \alpha = 0.05 \), except for the cell viability experiments involving pamidronate and gemcitabine where a significance level of 0.025 was used to adjust for tests performed at two different doses of gemcitabine (10 and 100\( \mu \)M). For the sequence of administration analysis, the two different treatment schedules (gemcitabine/carboplatin vs. carboplatin/gemcitabine) were compared by use of a two way ANOVA and an interaction between order and dose of gemcitabine was tested. If the interaction was significant (\( p < 0.05 \)), a pairwise comparison of the different treatment schedules was performed at each dose of gemcitabine using a Bonferroni-corrected \( \alpha = 0.017 \). If the interaction was not significant, we tested the average effect of order across dose of gemcitabine. Statistical analyses were performed using commercially available software (SAS Institute, Version 9.1, Cary, NC).

Clinical Study

2.7 Number of subjects. Reported 1-year survival rates in dogs with OSA treated with amputation and carboplatin chemotherapy are 35\%\textsuperscript{5,6}. Assuming 80\% power and 95\% confidence, 52 dogs would be required to demonstrate a clinically significant increase in 1-year survival rate of 35\% to 60\% (Table 3.1). An early withdrawal rate of
5% is assumed based on the expected tolerability of the protocol and low required client commitment. Because single-agent carboplatin has been used extensively in the treatment of canine OSA a historical control was used for comparison. As the addition of gemcitabine was not expected to negatively influence survival outcomes, a one sided sample size was chosen.

2.8 Inclusion criteria. Each dog enrolled met all of the following criteria: (1) weigh > 15 kg; (2) have a histopathologically confirmed OSA of the appendicular skeleton with disease clinically confined to the leg; (3) have no evidence of distant metastasis to the lungs based on evaluation of 3-view thoracic radiographs at the time of presentation; (4) undergo amputation or limb-sparing procedure to remove the tumor; (5) have adequate organ function as indicated by complete blood count, serum biochemistry profile, and urinalysis; (6) have received no prior chemotherapy or radiation therapy prior to amputation; (7) discontinue NSAIDs or corticosteroids 48 hours prior to initiation of chemotherapy and for 48 hours before and after each chemotherapy treatment; (8) owner must be willing to give written informed consent; (9) owner must be willing to return to the hospital for follow-up after the completion of chemotherapy.

2.9 Drug administration. Treatment was initiated 7 to 14 days after amputation. Carboplatin was administered at a dose of 300 mg/m² as an intravenous bolus prior to gemcitabine. The timing of this administration was determined based on preliminary data indicating better inhibition of OSA cell viability when cells are exposed to carboplatin first (Fig. 3.7), as well as a trend for improved survival times in human lung cancer patients when carboplatin is administered 4 hours prior to gemcitabine. Gemcitabine
was diluted in 20ml 0.9% NaCl and administered at a dose of 2 mg/kg as a 20 minute infusion 4 hours post-carboplatin administration. This dose was chosen based on previous use of gemcitabine with carboplatin as well as pharmacokinetic data indicating that 2mg/kg achieves a peak blood concentration of at least 10uM, the concentration demonstrated in the preclinical evaluation to produce a significant decrease in OSA cell viability. To decrease the likelihood of gastrointestinal toxicity, maropitant 1mg/kg was administered subcutaneously on the day of chemotherapy administration and prescribed at an oral dose of 2mg/kg once a day to be administered as needed for nausea and vomiting. Carboplatin plus gemcitabine chemotherapy was administered every 21 days for a total of four cycles.

2.10 Patient assessment. Initial evaluation included a complete blood count (CBC), biochemical profile, and urinalysis. Thoracic radiographs were completed to rule out gross pulmonary metastasis. Histopathology was completed following amputation or limb-spare procedure. Each patient was assessed once per week during the first cycle, then on the first day of every cycle thereafter. Patient weight, physical examination, and CBC were performed during these visits. Three-view thoracic radiographs were obtained at initial evaluation, at the time of the fourth cycle of therapy, and every three months thereafter.

Treatment delays were permitted by the attending clinician if the patient was systemically ill at the time of the chemotherapy appointment or if hematologic toxicity was observed at the time of the chemotherapy appointment defined as an absolute neutrophil count less than 2,000/uL or a platelet count was less than 100,000/uL. If treatment is delayed, the patient will be re-evaluated for treatment with a physical
examination and the appropriate routine laboratory tests within five days. Treatment delays of more than seven days was avoided.

Serious hematologic and gastrointestinal toxicities were not anticipated in this clinical study. However, if grade 3 or 4 hematologic or non-hematologic toxicities occurred in three dogs during the study (defined by standard Veterinary Cooperative Oncology Group – Common Terminology for Adverse Events (VCOG-CTCAE) criteria in Appendix I), the dose of carboplatin was reduced by 25% increments until no grade 3 toxicities occurred.

2.11 Statistical analysis to be performed following completion of the clinical trial

Disease-free interval is defined as the period from definitive diagnosis (the date of amputation or limb spare) to the date that the patient develops detectable metastasis. Survival is defined as the period from definitive diagnosis to the date that the patient dies or is euthanized. Every attempt was made to obtain a complete or partial necropsy at the time of death or euthanasia. Disease-free interval, median survival time, and 1-year survival rate will be calculated using the Kaplan-Meier product limit method. Survival data will be compared with historical data on dogs with OSA treated with amputation and single-agent carboplatin.

Standard descriptive statistics will be used to report changes in hematologic and biochemical parameters following the administration of carboplatin and gemcitabine. All toxicities will be reported and appropriate toxicity grades will be assigned according to VCOG-CTCAE criteria.
CHAPTER 3

RESULTS

Preclinical Study

3.1 Gemcitabine induces loss of cell viability and apoptosis of canine OSA cell lines. To evaluate the effects of gemcitabine on canine OSA cell lines, cells were treated with varying concentrations of gemcitabine and cell viability was assessed using the WST-1 assay after 72 hours of culture. All canine OSA cell lines evaluated exhibited growth inhibition in the presence of gemcitabine in a dose-dependent manner (Figure 3.1). The calculated concentrations required for 50% inhibition of cell viability (IC\textsubscript{50}) for the cell lines were 5.7, 10.3, and 15.3\(\mu\)M for OSA8, OSA16, and OSA36, respectively. The IC\textsubscript{50} value could not be calculated for the relatively resistant OSA32 line as a 50% decrease in cell viability was not observed at any of the gemcitabine concentrations evaluated.

To determine whether growth inhibition was due to induction of cell death, we cultured OSA cell lines with gemcitabine and assessed cells for cell cycle arrest and apoptosis using propidium iodide staining and flow cytometry. As shown in Figure 3.2, an increase in the proportion of dead cells (sub-G\textsubscript{0}/G\textsubscript{1} phase) was detected in all OSA cell lines after incubation with gemcitabine. This increase in the percent of cells in the sub-
\[ G_0/G_1 \] phase of the cell cycle was observed at concentrations of \( 1\mu M \) or greater for OSA8, OSA16, and OSA36. In the more resistant OSA32 line, \( 10\mu M \) gemcitabine was required to produce evidence of cell death. To further evaluate the mechanism of cell death, the ability of gemcitabine to induce activation of caspases 3 and 7 was assessed. A significant increase in caspase-3/7 activity was detected following treatment of OSA8 and OSA36 with gemcitabine at \( 1\mu M \) or greater. An increase in caspase-mediated apoptosis was not observed at any of the gemcitabine concentrations evaluated for OSA16 and OSA32.

3.2 Loss of cell viability in OSA cell lines following gemcitabine treatment is not enhanced in the presence of pamidronate. To investigate whether the effect of gemcitabine and the bisphosphonate pamidronate would produce a greater decrease in cell viability when compared to gemcitabine alone, OSA cell lines were treated with gemcitabine at two concentrations (10 and \( 100\mu M \)) with or without pamidronate, or with pamidronate alone and evaluated for loss of cell viability (Figure 3.3). When OSA lines were treated with pamidronate alone, concentrations equal to or greater than \( 100\mu M \) were required to significantly decrease cell viability in all but the OSA32 line, for which there was no decrease in cell viability. For OSA16 and OSA32, a decrease in cell viability was observed when \( 10\mu M \) gemcitabine was combined with \( 100\mu M \) pamidronate when compared to gemcitabine alone. However, a difference was not observed at any additional concentrations of gemcitabine or pamidronate tested in combination. Further, in OSA8 and OSA36 there was no difference in cell viability observed when gemcitabine was combined with pamidronate when compared to gemcitabine alone.

3.3 Inhibition of cell viability in OSA cell lines by gemcitabine is enhanced in the
presence of carboplatin, resulting in synergistic activity. OSA cell lines were treated with gemcitabine and carboplatin to determine if the combination would produce a greater decrease in cell viability when compared to carboplatin alone (Figure 3.4). To mimic in vivo drug exposure, a carboplatin concentration of 250μM was chosen as this correlates with the peak plasma level in dogs after a standard dose of 300mg/m² of carboplatin is administered as a bolus. Carboplatin was combined with 1, 10, and 100μM gemcitabine. In all four canine OSA cell lines, a significant decrease in cell viability was demonstrated when carboplatin and gemcitabine were used in combination in comparison to carboplatin alone. For the relatively gemcitabine resistant OSA32 line, a significant decrease in cell viability of approximately 60% was achieved when either 10 or 100μM gemcitabine was combined with carboplatin, compared to a decrease in viability of 10% and 20% for carboplatin alone and gemcitabine alone, respectively.

To evaluate for a synergistic effect of gemcitabine combined with carboplatin on canine OSA cell lines, cells were treated with gemcitabine alone, carboplatin alone, and equipotent concentrations of carboplatin and gemcitabine in combination. Synergy was quantified by CI analysis. As shown in Figure 3.5, gemcitabine and carboplatin interacted synergistically over a range of concentrations in all cell lines evaluated. The CI indicated strong synergism (CI < 0.3) at concentrations equal to or less than 1μM/100μM of gemcitabine/carboplatin in all cell lines except at the lowest dose level (0.0625μM/6.25μM). Moderate to slight synergism was observed in all cell lines at concentrations greater than 1μM/100μM of gemcitabine/carboplatin. The DRI showed a considerable dose reduction for gemcitabine and carboplatin as a result of their synergism. When using gemcitabine and carboplatin in combination at the corresponding
dose levels, the DRI indicated that the concentration of gemcitabine necessary to inhibit growth of 50% of OSA cells could be reduced by 15.5-fold (OSA8), 18.8-fold (OSA16), 46.6-fold (OSA32), and 21.4-fold (OSA36).

To determine whether growth inhibition was due to induction of caspase activity, OSA cell lines were cultured with gemcitabine and/or carboplatin for 24 hours. As shown in Figure 3.6, a significant increase in caspase-3/7 activity was detected in the OSA8, OSA16, and OSA36 lines following gemcitabine treatment at all gemcitabine concentrations evaluated in combination with carboplatin in comparison to carboplatin alone. In the more resistant line OSA32, there was a significant increase in caspase-3/7 activity at 1 and 100uM gemcitabine, but not at 10uM gemcitabine in combination with carboplatin.

3.4 Inhibition of cell viability is enhanced when OSA cells are treated with carboplatin prior to gemcitabine. Data generated from both in vitro and in vivo studies suggests that for certain cancers, synergistic activity of gemcitabine and platinum drugs may depend on the sequence of drug administration. To investigate the effects of sequence of drug exposure on OSA cell viability, we used two different treatment schedules. OSA cells were exposed to 1, 10, and 100µM gemcitabine for 2 hours, washed out of drug, and then exposed to 250µM carboplatin for 2 hours. The sequence of drug exposure in reverse (carboplatin before gemcitabine) was evaluated in parallel (Figure 3.7). The mean optical density was significantly and consistently lower when carboplatin was administered first in OSA8 and OSA36. For OSA8, the test of the order-by-gemcitabine interaction was insignificant suggesting that the magnitude of the difference was consistent with dose of gemcitabine. For OSA36, although the magnitude
of the difference changes with dose of gemcitabine, mean optical density was consistently lower when carboplatin was administered first. For OSA16, order effects differ with dose of gemcitabine and schedule dependency was only observed at the 1 and 100\(\mu\)M gemcitabine concentrations (p-value < Bonferroni-corrected \(\alpha = 0.017\)). Conversely, for OSA32, the sequence of drug administration did not alter the resultant effects on inhibition of cell viability.

**Clinical study**

3.5 **Patient characteristics.** Starting in February 2008, patients with histologically confirmed stage II appendicular OSA were enrolled in a prospective, multi-institutional clinical trial. Institutions and number of cases per institution included The Ohio State University (20 cases), New England Veterinary Oncology Group (NEVOG, 14), and the University of Wisconsin (11).

Patient characteristic data were available for 31 dogs at the time of writing. The median age was 7 years (range; 2 – 15 years) and the median weight was 33.5kg (range; 22.8-51.8kg). Two dogs were intact females, 15 were spayed female, 1 was an intact male, and 13 were castrated males. There were 8 mix breed dogs, 3 Labrador Retrievers, 3 Greyhounds, 3 Great Danes, 2 Golden Retrievers, 2 Great Pyrenese, 2 Irish Setters, 2 German Shepherds, and 1 of each of the following breeds: Boxer, Doberman pincher, German Shorthair Pointer, Rottweiler, Saint Bernard, and Samoyed.

OSA was diagnosed in the forelimb in 17 dogs, and the hindlimb in 14 dogs. The most common site was the proximal humerus (n=10), followed by distal femur (n=8), distal radius (n=7), proximal tibia (n=3), and distal tibia (n=3).

3.6 **Hematologic and gastrointestinal toxicity.** Toxicity data was available for 16
dogs that had completed the chemotherapy protocol at OSU. There were 19 episodes of
grade 1 neutropenia and one episode of grade 2 neutropenia at days 8, 15, or 21 of the
chemotherapy cycle. The median neutrophil count at the start of chemotherapy was 6.7 ×
10³/µL (range; 2.0–22.7 × 10³/µL). The median neutrophil count at days 8 and 15 was 3.2
× 10³/µL (range; 1.7-6.5 × 10³/µL) and 4.0 × 10³/µL (range; 2.0-12.7 × 10³/µL),
respectfully. No episodes of grade 3 or 4 neutropenia were observed in the 16 dogs for
which data was available.

There were four episodes of grade 1 thrombocytopenia and two episodes of grade
2 thrombocytopenia at day 8 and 15 of the chemotherapy protocol. The median platelet
count at the start of chemotherapy was 461 × 10³/µL (range; 81–1,212 × 10³/µL). The
median platelet count at days 8 and 15 was 291 × 10³/µL (range; 61–792 × 10³/µL) and
145 × 10³/µL (range; 50–507 × 10³/µL), respectfully. No episodes of grade 3 or 4
thrombocytopenia were observed.

Six of sixteen dogs had signs of gastrointestinal toxicosis for a total of 14
episodes. The most commonly recorded adverse event was grade 1 (5 episodes) and
grade 2 (3 episodes) anorexia. Grade 1 vomiting was noted in one dog and grade 2
vomiting was noted in two dogs. Grade 1 diarrhea was recorded in two dogs and neither
required medical intervention. No dogs experienced grade 3 or 4 gastrointestinal
toxicosis. One dog, an 8 year-old female spayed mixed breed dog, experienced grade 5
gastrointestinal toxicosis (death) after the first treatment with carboplatin and
gemcitabine. This dog developed severe vomiting and diarrhea two days after treatment.
Clinical signs progressed and the dog died at home five days after treatment. The
oncology service was not notified about the adverse event until the dog had died.
3.7 **Outcomes.** At the time of writing, 38 of 45 dogs enrolled were still alive. Nine dogs were diagnosed with metastatic disease to the following sites: lung (4), bone (4), skin (1), and visceral organs (1). Eight dogs had metastasis to one site and one dog had metastatic disease to two sites. The **median time to development of metastasis in these dogs was 88 days.** Seven dogs were euthanized due to clinical signs associated with metastatic disease. The median time follow-up time from diagnosis was 171 days (range; 27–418 days) for 44 dogs enrolled. **Patient enrollment and data accrual is ongoing and the statistical analyses will be performed at 6 and 12 months after enrollment of the last patient into the study to allow for data maturation.**
Table 3.1 Calculation of Study Population. Assuming 80% power and 95% confidence, 52 dogs will be required to demonstrate a clinically significant increase in 1-year survival rate of 35% to 60%. An early withdrawal rate of 5% is assumed based on the expected tolerability of the protocol and low required client commitment. Because single-agent carboplatin has been used extensively in the treatment of canine OSA a historical control will be used for comparison. As the addition of gemcitabine is not expected to negatively influence survival outcomes, a one sided sample size was chosen.
Figure 3.1  Gemcitabine inhibits the viability of canine OSA cell lines. Mean +/- SD cell viability for canine OSA cell lines (OSA8 [open triangle], OSA16 [closed circle], OSA32 [open circle], and OSA36 [closed square]) treated with gemcitabine at increasing concentrations. Cell viability was determined using the WST-1 reagent at 72 hours of treatment and is expressed as a percentage of the mean absorbance of treated cells divided by the mean absorbance of untreated cells.
Figure 3.2  Gemcitabine induces cell cycle arrest in OSA cell lines in a dose dependent manner. Mean +/- SD percentage of cells in the sub-G₀/G₁ phase in canine OSA cell lines treated with various concentrations of gemcitabine for 48 hours. The percent of sub-G₀/G₁ cells was assessed by flow cytometry following propidium iodide staining.
Figure 3.3  Inhibition of cell viability in OSA cell lines by gemcitabine in the presence of pamidronate. OSA cell lines OSA8 (A), OSA16 (B), OSA32 (C), and OSA36 (D) were treated with 0μM (open triangle), 10μM (closed circle), and 100μM (open square) gemcitabine with or without pamidronate at various concentrations. Cell viability was determined using the WST-1 reagent after 72 hours of treatment and is expressed as a percentage of the mean absorbance of treated cells divided by the mean absorbance of untreated cells. Pairwise comparisons were performed using the raw optical densities rather than the standardized values. * p < 0.05
Inhibition of cell viability in OSA cell lines by carboplatin is enhanced in the presence of gemcitabine. OSA cell lines OSA8 (A), OSA16 (B), OSA32 (C), and OSA36 (D) were treated with 1, 10, and 100µM gemcitabine alone (closed square) or in combination with 250µM carboplatin (open square). Cell viability was determined using the WST-1 reagent after 72 hours of treatment and is expressed as a percentage of the mean absorbance of treated cells divided by the mean absorbance of untreated cells. Pairwise comparisons were performed using the raw optical densities rather than the standardized values. *p < 0.05
Figure 3.5  Gemcitabine and carboplatin interact synergistically in canine OSA cell lines. OSA cells were exposed to gemcitabine and carboplatin and after 72 hours of culture, the WST-1 assay was performed to assess cell viability. Synergy was quantified by CI analysis and expressed as CI vs. fraction affected (Fa). By this method, CI < 1 is synergistic, CI = 1 is additive, and CI > 1 is antagonistic. The CI is plotted as a function of the fractional affect for cell lines OSA8 (A), OSA16 (B), OSA32 (C), and OSA 36 (D).
Figure 3.6  Caspase 3/7 activity in OSA cell lines treated with gemcitabine with or without carboplatin. OSA cell lines OSA8 (A), OSA16 (B), OSA32 (C), and OSA36 (D) were treated with 1, 10, and 100 μM gemcitabine alone (closed square) or in combination with 250 μM carboplatin (open square). After 24 hours of drug exposure, caspase-3/7 activity was assessed using the SensoLyte Homogenous AMC Caspase-3/7 Assay Kit. Caspase-3/7 activity is expressed in relative fluorescence units (RFU). *p < 0.05
Figure 3.7 Effect of sequence of administration of carboplatin and gemcitabine. OSA cell lines were treated with carboplatin and gemcitabine in combination. Two administration schemes were evaluated: (i) a 2-hour incubation with gemcitabine directly followed by a 2-hour incubation with carboplatin (dashed line with closed circle) and (ii) the sequence in reverse order (carboplatin before gemcitabine, solid line with open circle). Cells were washed in between drug exposure. OSA cells were treated with gemcitabine at varying concentrations (0 to 100 μM) and carboplatin at a concentration of 250 μM. Cell viability was assessed using the WST-1 assay after 72 hours of culture. Mean optical density is plotted as a function of gemcitabine dose in cell lines OSA8 (A), OSA16 (B), OSA32 (C), and OSA36 (D). **p < 0.0001, *p < 0.01
CHAPTER 4

DISCUSSION

Within the last several years, gemcitabine has been used extensively in human oncology to treat a variety of cancers. In general, gemcitabine’s activity has been best in the setting of drug combinations, particularly with platinum based chemotherapeutics. It is now approved for the treatment of several human tumors including pancreatic cancer, non-small cell lung cancer, metastatic breast cancer, and ovarian cancer.\textsuperscript{12-17} Gemcitabine has only recently been evaluated in veterinary oncology and a phase I study established a maximum tolerated dose of this agent.\textsuperscript{22} However, few studies have studied the biologic activity of gemcitabine against specific tumors in veterinary cancer patients. Evidence generated from \textit{in vitro} work as well as mouse models of OSA indicated that gemcitabine has activity against human OSA cell lines.\textsuperscript{9} While objective responses against metastatic OSA were not observed in human patients after treatment with gemcitabine as a single agent, disease stabilization and improvement in quality of life were noted.\textsuperscript{21} Therefore, we hypothesized that gemcitabine may have activity against canine OSA.

In the current study, we evaluated the activity of gemcitabine in canine OSA cell lines alone or in combination with compounds known to have activity against OSA. A
dose-dependent inhibition of cell viability was observed in canine OSA cell lines treated with gemcitabine with IC$_{50}$ concentrations ranging from 5.7 to 15.3$\mu$M. These results are similar to those reported for human OSA cells treated with gemcitabine in vitro. To establish if these in vitro concentrations were achievable in vivo, previously established gemcitabine pharmacokinetics in the dog were reviewed. A dose of 22mg/kg (675mg/m2, the established maximum tolerated dose of gemcitabine), would achieve plasma levels of approximately 20 to 30$\mu$g/ml, equivalent to 67 to 100$\mu$M. Therefore, biologically active concentrations of gemcitabine can readily be achieved in canine patients with OSA.

Our experiments revealed a dose-dependent increase in the sub-G$_0$/G$_1$ phase population in all OSA cell lines evaluated following gemcitabine treatment. In cell lines OSA8 and OSA36, a corresponding increase in caspase-3/7 activity was demonstrated supporting apoptosis as the mechanism of cell death. For the cell lines OSA16 and OSA32, the increase in the sub-G$_0$/G$_1$ population of gemcitabine-treated OSA cells was modest. Further, there was no significant increase in caspase-3/7 activity indicating that OSA16 and OSA32 were more resistant to the effects of gemcitabine. These data suggest that gemcitabine may in part be acting primarily as an inhibitor of cell viability in resistant OSA cell populations rather than directly inducing cell death. Moreover, it likely explains the fact that in human clinical trials of metastatic OSA, gemcitabine primarily caused disease stabilization.

Given the potential resistance of OSA cells to single-agent chemotherapy, we evaluated gemcitabine in combination with compounds known to have activity against OSA to determine if additive or synergistic effects could be identified. Pamidronate has
been identified as a potentially active compound against canine OSA based on previous
in vitro work\textsuperscript{24}. However, recent clinical trials have not found a significant survival
benefit when pamidronate is added to chemotherapy treatment post-amputation (Kozicki
et al., personal communication, Veterinary Cancer Society Conference, Seattle, WA,
October 2008) or during palliative radiation therapy in combination with chemotherapy\textsuperscript{34}.
Our data indicate that at biologically relevant drug concentrations, pamidronate does not
exhibit significant inhibition of OSA cell viability as a single agent or in combination
with gemcitabine. As such, it is unlikely that combinations of pamidronate and
gemcitabine would exhibit biologic activity \textit{in vivo}.

Carboplatin and gemcitabine are ideal candidates for use in combination as they
have different but complementary mechanisms of action and acceptable toxicity profiles.
Indeed, these chemotherapeutics have demonstrated synergistic activity in a variety of
human malignancies. In the current study, a significant decrease in OSA cell viability
was demonstrated when carboplatin and gemcitabine were used in combination in
comparison to carboplatin alone. Interestingly, for the more resistant lines OSA16 and
OSA32, the gemcitabine/carboplatin combination decreased cell viability by greater than
50\% at gemcitabine concentrations equal to or greater than 10\textmu M evaluated in
combination. For all cell lines tested, the magnitude of loss of cell viability in OSA cell
lines achieved by either drug alone was not as great as that found when the drugs were
combined. Importantly, the combination of gemcitabine and carboplatin was synergistic
in all OSA cell lines evaluated. These data are consistent with findings in a variety of
human cancer cell lines.

Based on a previous clinical study of carboplatin and gemcitabine administered
on the same day to dogs, the recommended doses of these drugs are 10mg/kg carboplatin and 2mg/kg gemcitabine, given 4 hours apart\textsuperscript{23}. These clinical doses correspond to concentrations of approximately 10\(\mu\)M gemcitabine and 250\(\mu\)M carboplatin, and are therefore considered clinically relevant and biologically achievable cytotoxic doses \textit{in vivo}. Moreover, these drug concentrations correspond to those at which synergistic drug activity was observed.

When carboplatin and gemcitabine are given together it is hypothesized that following platinum induced DNA adduct formation, gemcitabine is incorporated into DNA during the repair process, thereby contributing to inhibition of further DNA replication and repair. Based on this, it has been suggested that administration of gemcitabine should occur before platinum compounds to ensure that sufficient intracellular concentrations of gemcitabine are present prior to initiation of DNA damage by the platinum compound. In the present study, a significant and consistent decrease in cell viability was achieved when OSA cell lines OSA8 and OSA36 were treated with carboplatin prior to gemcitabine in comparison to the treatment schedule in reverse. This was also true for OSA16 for the majority of gemcitabine concentrations tested in combination with carboplatin. Conversely, for OSA32, the sequence of administration did not affect the magnitude of loss of cell viability. The effects of drug sequence have been evaluated extensively in solid tumors in people leading to inconsistent results. In one study, carboplatin plus gemcitabine resulted in synergistic activity against a lung carcinoma cell line only when carboplatin was administered prior to gemcitabine\textsuperscript{27}. It was further demonstrated that the administration of carboplatin 4 hours prior to gemcitabine was associated with higher response rates and longer survival times in
clinical patients with non-small-cell lung cancer when compared to patients who were treated with drug in the reverse order. In contrast, another study demonstrated that the sequence of administration of carboplatin and gemcitabine did not affect toxicity, pharmacodynamics, the maximum tolerated dose, or response rates in patients with non-small cell lung cancer. These conflicting data suggest that the actual sequence of gemcitabine/platinum administration may not be the determining factor regarding response to therapy. Based on the data presented here, administering carboplatin first may be beneficial and would be unlikely to negatively impact outcome when compared to administering carboplatin after gemcitabine.

Given the exciting preclinical data, we initiated a multi-center, prospective clinical trial to evaluate the whether gemcitabine administered in combination with carboplatin will improve outcome in dogs with OSA. Our hypothesis was that carboplatin plus gemcitabine would significantly improve 1-year survival rates compared to carboplatin alone in dogs with OSA following amputation. At the time of writing, the median follow-up time from diagnosis was 171 days; therefore, the efficacy as determined by disease free interval and survival time of the protocol can not be assessed.

Additionally, we hypothesized that gemcitabine in combination with carboplatin would not result in increased toxicity compared to carboplatin alone in dogs with OSA post-amputation. Hematologic toxicity was mild and clinically insignificant in the patients for which that data was available for analysis. In addition, no to minimal gastrointestinal toxicity was reported in the majority of dogs assessed to date. Surprisingly, one dog died five days after chemotherapy administration after having vomiting and diarrhea for the three days prior. However, a necropsy performed on this
patient could not determine the cause of death. In the small number of patients evaluated here, toxicity was similar to that reported in a previous study evaluating carboplatin alone in dogs with OSA following amputation\textsuperscript{6}. One death secondary to chemotherapy toxicity was reported in that study with otherwise acceptable hematologic and gastrointestinal toxicity. Regarding the chemotherapy associated death in our study, it is possible that the death could have been prevented had the clients called to report the adverse events.

In summary, our data demonstrate that similar to the case with human OSA, gemcitabine has biologic activity against canine OSA cell lines, and that this activity is synergistic when combined with carboplatin at biologically relevant drug concentrations. In addition, gemcitabine in combination with carboplatin has acceptable toxicity compared to carboplatin alone when administered to dogs with OSA following amputation. The efficacy of gemcitabine administered in combination with carboplatin in dogs with OSA will be determined following completion of patient enrollment.
BIBLIOGRAPHY


