Antitumor effects of combined carboplatin and gemcitabine
in canine transitional cell carcinoma

A Thesis

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Abstract

Gemcitabine has demonstrated synergistic antitumor activity in a variety of human cancer cells when combined with carboplatin. The purpose of this study was to evaluate the antitumor activity of gemcitabine in canine TCC cell lines alone and in combination with carboplatin. We hypothesized that combined gemcitabine and carboplatin would have synergistic effects in vitro in canine TCC. Demonstration of synergistic antitumor activity in canine TCC cell lines would provide a rationale for effective treatment of canine TCC with this combination of drugs.

Five TCC cell lines were treated with gemcitabine, carboplatin, and the combination. Cell proliferation was assessed using CyQUANT® cell proliferation assay, cell cycle was evaluated using propidium iodide staining, and apoptosis was assessed by measuring caspase-3/7 enzymatic activity. Synergy was quantified by combination index analysis using CompuSyn software.

Treatment of canine TCC cell lines with carboplatin or gemcitabine decreased cell proliferation, increased apoptosis, and induced cell cycle arrest. When TCC cell lines were treated with gemcitabine and carboplatin in combination at therapeutically relevant concentrations, a significant decrease in cell proliferation was observed compared to gemcitabine or carboplatin alone and the drug combination was synergistic in 3 of 5 cell
lines and additive in the other two. Drug sequence did not reliably affect cell proliferation amongst cell lines during combination treatment.

We conclude, gemcitabine exhibits biologic activity against canine TCC cells and when combined with carboplatin the two exhibit synergistic activity. Our results support further evaluation of this drug combination in dogs with TCC to determine its clinical efficacy.
Dedication

To my parents, Eustaquito and Cassia for their unconditional love and support; to my dear sister Juliana for her lasting friendship; to my wife Cristiane for her ∞ love, understanding and partnership; and to my dear cats Layla and Lila that taught me what companion animals represent to their guardians.
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Chapter 1: Introduction and Literature Review

1.1 Introduction

Transitional cell carcinoma of the urinary bladder in dogs (TCC) is a challenging disease to diagnose and treat. Unfortunately, only approximately 12 to 25% of affected dogs receiving chemotherapy have an objective response to treatment.\(^1\) Clearly, new therapeutic strategies are needed.

The prevalence of TCC at university-based veterinary hospitals is approximately 1.5 – 2.0% and appears to be increasing.\(^2\) TCC is the most common form of canine urinary bladder cancer, accounting for approximately 60% to 90% of all reported cases.\(^3-6\) The etiology of canine TCC is not clear, but is likely multifactorial. Risk factors that have been identified include exposure to topical insecticides for flea and tick control, exposure to marshes that have been sprayed for mosquito control, obesity, possibly cyclophosphamide administration, female gender, and breed (e.g., Scottish terrier).\(^1-3,7,8\) Other more commonly affected breeds include Shetland sheepdog, beagle, wirehaired fox terrier, and West Highland white terrier. Ingestion of vegetables has been associated with a lower risk of TCC in Scottish terriers that ate vegetables at least three times a week, along with their normal diet TCC.\(^9\)

TCC occurs most commonly in the trigone region of the bladder, often presenting as a grossly thickened bladder wall with papillary projections extending into the bladder.
lumen. These lesions can lead to partial or complete urinary tract obstruction. In one reported series of dogs affected with TCC, the tumor involved the urethra as well as the bladder in 56% (57/102) of cases and involved the prostate in 29% (11/38) of cases. In this same study, metastasis was present in 14% of dogs at the time of diagnosis and in 49% of dogs at the time of death. The majority of dogs die of complications associated with local disease.

Many different treatment strategies have been investigated for treating TCC in dogs, including surgery, radiation therapy, chemotherapy, and non-steroidal anti-inflammatories (NSAIDs). In contrast to humans, where TCCs are commonly diagnosed early as superficial cancers of the urinary bladder’s transitional cell epithelium, the majority of TCCs in dogs are infiltrative tumors of intermediate or high-grade malignancy. For this reason, common therapies used to treat superficial tumors in people are not successful for treating the large, exophytic, and invasive tumors that are seen in dogs. Surgical excision followed by urinary tract reconstruction generally is ineffective and is associated with high morbidity in canine patients. Because of the limited surgical options and potential for metastases, cytotoxic chemotherapy has been advocated for the treatment of TCC in dogs similar to human patients with advanced invasive bladder cancer.

Historically, surgical treatment was recommended to dogs with TCC; however, it was found that because of the high prevalence of metastasis and diffuse microscopic spread of TCC, surgery was not a viable option. Thereafter, cytotoxic drugs became the mainstay of treatment. Cisplatin was the first cytotoxic drug evaluated in a series of dogs with TCC. Although median survival time was not reported in this study, overall
response (percentage of patients that had either complete or partial remission) was 13%; 4 of 8 dogs had at least stable disease for a minimum 30 weeks.\textsuperscript{27} In another study, fifteen dogs with TCC were then treated with cisplatin; overall response was 20% and 12 of these dogs showed improvement with a median survival time of 180 days.\textsuperscript{23} However, in a subsequent study where 18 dogs were treated at a higher dose (60 mg/m\textsuperscript{2} IV) of cisplatin every three weeks, the median survival time was only 130 days and overall response was 17%.\textsuperscript{25} The use of doxorubicin, mitoxantrone, and actinomycin D as single agents has been reported in small numbers of dogs with TCC. A single partial response was reported for mitoxantrone and actinomycin D\textsuperscript{29,30}; however, the numbers of dogs treated in these studies were too small to adequately address activity. The toxicity profile of cisplatin (e.g. myelosuppression, nephrotoxicity, gastrointestinal toxicity) prompted clinicians to search for a less toxic alternative. Another platinum compound, carboplatin, was evaluated in 14 dogs. No objective tumor responses were seen and the median survival time was 132 days.\textsuperscript{26}

Piroxicam, a nonsteroidal anti-inflammatory drug (NSAID), is currently the most commonly used drug for the treatment of TCC in dogs. The majority of dogs treated with piroxicam show an improvement in clinical signs (e.g., stranguria, hematuria, pollakiuria); however, significant objective tumor regression is relatively uncommon.\textsuperscript{40} The use of piroxicam in dogs with cancer was first reported in a phase I clinical trial of dogs with various tumors. Three of 10 dogs with TCC showed partial remission after treatment with piroxicam.\textsuperscript{39} This study prompted further evaluation of piroxicam as a treatment for canine TCC. In a subsequent study of 34 dogs with TCC treated with piroxicam, the resulting median survival time was 181 days and the overall response rate
In an attempt to improve survival time in dogs with TCC, combinations of cytotoxic drugs and NSAIDs were attempted. In an initial study of this approach 8 dogs were treated with cisplatin followed by piroxicam while 14 dogs were treated with the two drugs combined. The median survival time was 309 and 246 days for cisplatin followed by piroxicam (overall response 71%) and cisplatin + piroxicam combination groups (overall response 50%), respectively.\(^{21}\) The longest reported median survival time in dogs with TCC was 329 days, with an overall response rate of 50% in dogs treated with piroxicam (0.3 mg/kg daily) for 4 weeks followed by cisplatin (60 mg/m\(^2\) every 21 days).\(^{42}\) This protocol was evaluated again with lower cisplatin dose (initially 50 mg/m\(^2\), then reduced to 40 mg/m\(^2\) every 3 weeks). Even though these dogs had a median survival time of 307 days, overall response was only 11% (compared to 50% previously noted), renal toxicity was frequent and dose limiting, even with cisplatin dose reduction.\(^{32}\) The combination of mitoxantrone (5 mg/m\(^2\) every 21 days) given for 4 cycles and piroxicam (0.3 mg/kg daily for duration of treatment), resulted in a median survival time of 291 days (overall response 35%).\(^{20}\) This was slightly shorter than the combination of cisplatin and piroxicam; however, it was much better tolerated with minimal associated toxicities. In general, the platinum-based cytotoxic drugs cisplatin and carboplatin have shown disappointing results in dogs with TCC due to toxicity and lack of efficacy.\(^{25-27}\) As mentioned above, other cytotoxic chemotherapy drugs have not been rigorously evaluated for treatment of canine TCC in a significant number of patients and none have been reported to have significant antitumor activity in dogs with TCC.\(^{28-30}\)
1.2 Gemcitabine

Gemcitabine (2’, 2’-difluorodeoxycytidine) is a synthetic analog of cytosine arabinoside that has anti-tumor activity in a variety of human cancers. This antimetabolite cytotoxic prodrug undergoes complex cellular uptake and metabolism. Gemcitabine was the first compound licensed based on antitumor efficacy and on improved quality-of-life score in the pivotal study for people with pancreatic carcinoma. This drug, like other nucleoside analogs, is hydrophilic and cannot transverse cell membranes by passive diffusion. The prodrug gemcitabine enters cells through the activity of specialized transporter systems. After entering the cell, gemcitabine is phosphorylated to its monophosphate by deoxycytidine kinase and subsequently to its triphosphate (major metabolite). Phosphorylation of gemcitabine to monophosphate is the rate-limiting step of drug activation and also essential for its biologic activity. The cellular elimination of the gemcitabine triphosphate possibly determines its therapeutic efficacy. However, combination studies with cisplatin, where the two drugs acted synergistically, have not shown accumulation of this metabolite as a reason for their synergism. Gemcitabine also increases its own intracellular concentration by a positive feedback loop of activation. Gemcitabine exhibits cell phase specificity, primarily killing cells undergoing DNA synthesis (S-phase) and also blocking the progression of cell through the G1/S phase boundary. Multiple “self-potentiation” mechanisms potentiate the activity of gemcitabine diphosphates and triphosphates and decreased elimination of gemcitabine. Gemcitabine can be inactivated by the action of deoxycytidine deaminase. The active metabolite of gemcitabine can be incorporated into both DNA and RNA. Resistance appears to be caused by reduced nucleoside
transport into the cells and also to decreased activity of the enzyme deoxycytidine kinase.⁴⁸

In summary, gemcitabine’s 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.⁵¹ 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, and metastatic breast cancer.⁵²-⁵⁷ 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.⁵⁸-⁶¹

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/m² IV with minimal toxicity.⁶² A recent study described 38 dogs with TCC treated with gemcitabine weekly at 800 mg/m² IV in conjunction with piroxicam (0.3 mg/kg PO q 24 hours).⁶³

**1.3 Carboplatin**

Platinum compounds react with a multitude of cellular components that have nucleophilic sites such as DNA, RNA, proteins, membrane phospholipids, cytoskeletal microfilaments, and thiol-containing molecules. The primary cellular target for platinum complexes is generally thought to be DNA. Approximately 1% of the total cellular
platinum binds to DNA, resulting in inter- and intra-strand cross-linking.\textsuperscript{64} It has been suggested that platinum-damaged DNA causes G2 phase arrest to repair damage. In the absence of adequate repair, cells undergo an abortive attempt at mitosis that results in cell death via an apoptotic mechanism.\textsuperscript{64} Platinum compounds may also inhibit DNA synthesis by other mechanisms. Carboplatin is a second-generation platinum compound characterized by significantly reduced toxicity (i.e. nephrotoxicity) compared to cisplatin. Carboplatin has been commonly used as adjuvant treatment in canine osteosarcoma.\textsuperscript{65} It is known to increase disease-free interval and survival time in these dogs. Even though well-tolerated, neutropenia is considered dose-limiting toxicity. Despite not being nephrotoxic, myelosuppression is thought to be exacerbated in patients with decreased kidney function.

1.5 Gemcitabine and canine transitional cell carcinoma

The combination of gemcitabine and piroxicam was evaluated in dogs with TCC in a phase I clinical trial. Thirty-three dogs with confirmed TCC were treated with 800 mg/m\textsuperscript{2} IV of gemcitabine once weekly and daily oral piroxicam at 0.3 mg/kg. In dogs developing toxicities, gemcitabine treatment intervals were increased to 10-14 days, then, if necessary, doses were decreased by 25%. The median number of gemcitabine doses administered was 8, with a range of 1 to 38 doses. Nausea or vomiting occurred in 66% of the patients, but was generally mild (grade 1 or 2). Neutropenia was the most common hematological toxicity (nadir 3 to 7 days) and no febrile episodes or septic events were observed. Thrombocytopenia and anemia were rare. Clinical improvement of stranguria, pollakiuria, and hematuria was reported in all dogs. These dogs had an overall response
of 27% and a median survival time of 230 days. The combination of 2 mg/kg gemcitabine IV over 20 to 30 minutes and 10mg/kg carboplatin as an IV bolus has been evaluated clinically in dogs with various carcinomas resulting in acceptable toxicity, but low response rates.

1.6 Carboplatin and canine transitional cell carcinoma

There are two studies evaluating the efficacy of carboplatin in canine TCC. One study looked at carboplatin alone, where the median survival time was 132 days (only 1 in 14 dogs had stable disease), however these dogs were treated with various rescue drugs. The other study specifically evaluated the combination of carboplatin and piroxicam, where the median survival time was 161 days (overall response 38%). The combination of carboplatin and piroxicam caused frequent toxicity, limiting survival.

1.7 Synergistic activity of combination gemcitabine and platinum compounds

The use of these two drugs in combination is attractive from a clinical point of view, because these drugs have relatively non-overlapping toxicity profiles and different mechanisms of action. A recent study in a human TCC cell line showed that synergism occurred when gemcitabine was given prior to (4 hours) or at the same time as carboplatin. When carboplatin was given prior to gemcitabine, only additive effects were noted. Similarly, pre-incubation (4 hours) with cisplatin was synergistic in 2 of 3 ovarian cancer cells lines, while pre-incubation with gemcitabine was synergistic in 1 of these cells lines. In the latter study, the lack of an effect of cisplatin on gemcitabine
accumulation was interpreted to indicate that the mechanism for synergism is unlikely to be related to enhanced gemcitabine accumulation. In fact, when used in combination, cisplatin has been shown to decrease the cellular uptake of decitabine (cytosine analog) while promoting an increase in DNA platination. This suggests that the mechanism of interaction between cisplatin and gemcitabine is unrelated to gemcitabine uptake. Thus, it seems more likely that gemcitabine affects DNA repair of damage caused by cisplatin. It has been demonstrated that cytosine arabinoside markedly delayed the recovery of DNA synthesis inhibited by cisplatin. The in vitro synergism of gemcitabine and platinum compounds is possibly related to reduced repair of platinum-induced DNA damage, a recognized mechanism of platinum resistance.

Synergy analysis results depend on the timing of drug treatments. For a given cell line, synergism may occur when combining gemcitabine and a platinum drug after a certain drug exposure and but not when exposed for a different time period or drug sequence. For example, human ovarian cancer cell line (A2780) showed antagonism after 1 and 4 hours of drug exposure. However, this same cell line showed synergism at 24 and 72 hours of drug exposure according to the combination index. Additionally, confounding results exist when comparing pre-treatment with either drug in different cell lines.

1.8 The use of combined gemcitabine and platinum compounds in human transitional cell carcinoma

Initially, before the development of effective combination chemotherapy protocols, the median survival time for unresectable and metastatic human bladder cancer
did not exceed 3 to 6 months. With combination chemotherapy, median survival times of 1 year are now commonly achieved. In people, most combination chemotherapy protocols used for the treatment of bladder cancer are cisplatin-based. Initially, the most commonly used combination was methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC), which was developed over 2 decades ago. MVAC became the standard of care when it was shown to provide a survival advantage over treatment with cisplatin alone. However, treatment with MVAC is associated with substantial toxicity, which prompted studies with other chemotherapeutic compounds. One of these compounds was gemcitabine, which had been used as a single agent for the treatment of metastatic bladder cancer. Based on its mechanisms of action, gemcitabine was thought to have the potential to be synergistic with cisplatin. Further in vitro and in vivo studies confirmed this synergism, which prompted clinical use of this combination.

Gemcitabine, or one of its metabolites, also may interact with the mechanism of action of cisplatin, modulating the extent or nature of DNA platination or the process of DNA repair. These findings led to investigation of the use of combined gemcitabine and cisplatin (GCis) in patients with TCC. When known survival and toxicity profiles from previous MVAC clinical trials were compared to four GCis phase II clinical trials, GCis seemed to have comparable activity with a seemingly better toxicity profile. These results led to a larger randomized, open label, multicentric comparison between MVAC and GCis for advanced or metastatic TCC. Similar results were achieved in this study resulting in the shift of the standard of care from MVAC to GCis for patients with advanced TCC. Given the success achieved with cisplatin, several human studies evaluated combined carboplatin and gemcitabine as carboplatin has a much more
favorable toxicity profile.\textsuperscript{77-83} For instance, in a randomized study that evaluated 110 humans with advanced TCC, nephrotoxicity was present in 26\% of patients in gemcitabine/cisplatin group while only 16\% in gemcitabine/carboplatin group.\textsuperscript{82} Median time to progression of disease and median survival times were similar amongst the two groups.

Advanced stage bladder cancer in people closely mimics bladder cancer as it is seen in dogs. Consequently, combined gemcitabine and carboplatin treatment was recently studied in dogs with various carcinomas, including TCC. An empirically chosen dosage schedule of gemcitabine (2 mg/kg, 20-30 minute infusion IV) on days 1 and 8 and 4 hours later, carboplatin (10 mg/kg IV) on day 1 was used. The cycle was repeated every 3 weeks. An overall response rate of 13\% was observed.\textsuperscript{35} Thirty-seven dogs were evaluated in this study; however, only two were TCCs. Although the results obtained were modest, much still needs to be learned regarding dosages, treatment schedule, toxicity, and efficacy of this drug combination in canine cancers.

The positive results obtained with the use of combination gemcitabine and carboplatin in human bladder cancer prompted us to look further into this combination in canine TCC. The purpose of this study was to evaluate the cytotoxic effects of gemcitabine and carboplatin in various TCC cell lines, determine whether synergistic activity occurs and whether it is drug sequence dependent, and determine the effects of these drugs on cell cycle and apoptosis. The results of this study may offer support for further evaluation of this combination in dogs with TCC.
Chapter 2: Materials and Methods

2.1 Cell lines and reagents

Five canine transitional cell carcinoma cell lines (TCC-Kiss [KISS], K9TCC-PU [JS], K9TCC-PU-AxA [AXA], K9TCC-PU-AxC [AXC], and K9TCC-PU-Sh [SH] maintained in Dulbecco’s Modified Eagle Medium (D-MEM) (Gibco/Invitrogen, Grand Island, NY) with L-glutamine and glucose, and supplemented with 1% fetal bovine serum (Gibco), 1% newborn calf serum (Gibco), penicillin and streptomycin (Gibco) were used in the study. K9TCC-PU [JS], K9TCC-PU-AxA [AXA], K9TCC-PU-AxC [AXC] and K9TCC-PU-Sh [SH] were kindly provided by Dr. Deborah W. Knapp (Purdue University, West Lafayette, IN). The cell lines provided by Dr. Knapp have been described elsewhere. TCC-Kiss [KISS] was provided by Dr. William C. Kisseberth (The Ohio State University, Columbus, OH).

2.2 Effect of single-agent gemcitabine and carboplatin on cell proliferation

CyQUANT® cell proliferation assay (Molecular Probes Inc, Eugene, OR) was used to assess the effect of gemcitabine, carboplatin and the combination on cell proliferation. In brief, TCC cells (2,500 cells/well) were seeded in 96-well plates in 200 µL of D-MEM medium without phenol red and incubated overnight at 37°C and 5% CO₂. Gemcitabine (Eli Lilly, Indianapolis, IN) at different concentrations (0.001, 0.01, 0.1, 0.5,
1, 2.5, 5, 10, 100 µM) was then added to the wells and plates were incubated for an additional 72 hours. TCC cells were similarly treated with carboplatin (Hospira Inc, Lake Forest, IL) at different concentrations (0, 10, 50, 75, 100, 250, 375, 500, 750, 1000 µM). Cell proliferation was then assessed using the CyQUANT® assay, performed in accordance with the manufacturer’s instructions. Untreated cells were used as the negative control as described previously.\(^ {66,85}\) Fluorescence (indirect quantitative measurement of nucleic acids) was quantified with an ELISA plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 485 nm (excitation) and 538 nm (emission), and cell proliferation was calculated as a percentage of proliferation for the negative control wells (i.e., 100 x fluorescence of the treated wells/fluorescence of untreated wells). All samples were done in triplicates, and each experiment was repeated 3 times with each of the cell lines. For each of the TCC cell lines, linear regression after the logarithm transformation of cell proliferation was used to calculate the IC\(_{50}\) (50% inhibition of cell proliferation) for gemcitabine and carboplatin.

2.3 Effect of gemcitabine in combination with carboplatin on cell proliferation

To determine the effect of combined gemcitabine and carboplatin, cell proliferation was assessed using the CyQUANT® assay after incubation of TCC cells for 72 hours with gemcitabine alone (0.01, 0.1, 1, 10, 100 µM), carboplatin alone (50 µM and 150 µM), and gemcitabine + carboplatin combination (same concentrations) treated at the same time. Gemcitabine concentrations chosen were based on single-agent cell proliferation results. The concentrations of carboplatin (50 and 150 µM) were chosen because 250 µM (reported peak plasma concentration achieved in dogs following IV
administration of standard dose of 300 mg/m²) caused significant cytotoxicity making evaluation of combined drug treatments at the 250 μM peak carboplatin concentration impossible.  

To determine whether sequence of administration of gemcitabine and carboplatin had an effect on cell proliferation, TCC cells were exposed sequentially to gemcitabine alone (0.01, 0.1, 1, 10, 100 μM), carboplatin alone (50 μM and 150 μM) and gemcitabine + carboplatin combination (same concentrations) treated 4 hours apart. More specifically, cells were treated with gemcitabine 4 hours prior to carboplatin, at the same time as carboplatin, and with gemcitabine 4 hours after carboplatin.

2.4 Evaluation of synergy

To identify a synergistic interaction between gemcitabine and carboplatin, cell proliferation was assessed via CyQUANT® assay after incubation of TCC cells for 72 hours with each drug alone and with a combination of gemcitabine and carboplatin. For the drug combination assays, cells were incubated with medium containing both drugs at a fixed ratio. This ratio was based on the IC₅₀ of each individual drug for each cell line. The highest drug concentration was 8 times the IC₅₀ while lowest was 1/16 of the respective IC₅₀. Synergy was analyzed by the use of the CI (combination index) method, as described, with standard software (CompuSyn, version 3.0.1, ComboSyn Inc, Paramus, NJ, [www.combosyn.com]). The amount of each drug, either alone or in combination, needed to achieve a given effect level (i.e. reduction in cell proliferation) was used to calculate CI values, which were then used to define the nature of the drug interaction, with a CI < 1 indicating synergism, a CI > 1 indicating antagonism, and a CI
= 1 indicating additivity. The dose-reduction index was determined by comparing the ratio of the concentrations required to obtain a given degree of growth inhibition for gemcitabine and carboplatin individually and in combination. The magnitude of decrease was expressed in fold-change beyond the expected when the combination treatment is used.

2.5 Effect of treatment on cell cycle distribution

Propidium iodide staining was used to assess the effect of gemcitabine and carboplatin alone, as well as in combination, on cell cycle distribution. In brief, TCC cells (30 x 10^5 cells/well) were seeded in 6-well plates in 3 mL of D-MEM medium with 1% fetal bovine serum and incubated overnight at 37°C and 5% CO₂. For this experiment, drug concentrations were chosen based on the average 25% and 75% cell proliferation results for both drugs amongst all cell lines. The cells were treated with gemcitabine (0.1 and 1.0 µM) and carboplatin (20 and 200 µM) alone and in combination (0.1 µM gemcitabine + 20 µM carboplatin, 0.1 µM gemcitabine + 200 µM carboplatin, 1 µM gemcitabine + 20 µM carboplatin, 1 µM gemcitabine + 200 µM carboplatin), and plates were incubated for an additional 24 hours. Cells were then collected, fixed in 70% ethanol, incubated with 0.5 mL of propidium iodide staining solution consisting of propidium iodide (25 µg/mL) and RNAse (10 µg/mL) in PBS solution containing 0.1% glucose, and analyzed by means of flow cytometry (Beckon-Dickinson dual laser [448 nm and 635 nm] FACSCalibur system, BD Biosciences, San Jose, CA). Data were analyzed by means of standard software (Modfit LT software, version 3.2, Verify Software House, Topsham, ME). Untreated cells served as negative control. The
percentage of sub-G₀/G₁ phase was calculated based on the total number of gated counts. All samples were done in duplicate, and each experiment was repeated 3 times with each cell line.

2.6 Measurement of treatment on apoptosis

Apoptosis was assessed by measuring caspase-3/7 activity. In brief, TCC cells (7,500 cells/well) were seeded in 96-well plates in 150 µL of D-MEM with 1% fetal bovine serum and incubated overnight at 37°C and 5% CO₂. For this experiment, drug concentrations were chosen based on the average 25% and 75% cell proliferation results for both drugs amongst all cell lines. The cells were treated with gemcitabine (0.1 and 1.0 µM) and carboplatin (20 and 200 µM) alone and in combination (0.1 µM gemcitabine + 20 µM carboplatin, 0.1 µM gemcitabine + 200 µM carboplatin, 1 µM gemcitabine + 20 µM carboplatin, 1 µM gemcitabine + 200 µM carboplatin), and plates were incubated for an additional 24 hours. Caspase-3/7 enzymatic activity was then measured with a commercial assay (SensoLyte Homogenous AMC Caspase-3/7 Assay Kit, AnaSpec, San Jose, CA) performed according to the manufacturer’s instructions. Fluorescence was quantified with an ELISA plate reader at an excitation wavelength of 354 nm and emission of 442 nm. Untreated cells were used as negative control. Relative fluorescence units (RFU) were calculated by subtracting mean fluorescence measurement of media alone from all samples. Samples were analyzed in triplicate, and each experiment was repeated 3 times with each of the cell lines.
2.7 Statistical analysis

Data were summarized as mean ± SD of data from the 3 independent experiments. When evaluating cell proliferation, 2-way ANOVA was used to analyze the effect of treatment combinations groups (gemcitabine alone, gemcitabine + carboplatin 50 µM, and gemcitabine + carboplatin 150 µM) and levels of gemcitabine concentrations. If interaction was significant (P < 0.001), a post-hoc test with Bonferroni adjustment was used for pairwise comparisons of treatment groups at each concentration of gemcitabine.

To compare sequence of treatment in cell proliferation, 2-way ANOVA was used to analyze the effect of treatment orders (gemcitabine 4 hours before carboplatin, carboplatin 4 hours before gemcitabine, and both drugs given at the same time) and levels of gemcitabine concentration. For this experiment, a P < 0.05 was considered significant in an attempt to identify small differences in cell proliferation due to treatment order. If interaction was significant (P < 0.05), a post-hoc test with Bonferroni adjustment was used for pairwise comparisons of the two administration sequences at each concentration of gemcitabine. Significant differences in treatment order were further evaluated for best treatment sequence (sequence that yielded lowest cell proliferation) in all gemcitabine concentrations for each treatment (gemcitabine alone, gemcitabine + carboplatin 20 µM, gemcitabine + carboplatin 200 µM) independently in each cell line. The best treatment order was based on the best treatment sequence performance among different treatment groups and gemcitabine concentrations for each cell line. Sequence of treatment was considered relevant if the majority of cell lines behaved similarly.

For apoptosis assay and cell cycle analysis, 2-way ANOVA was used to analyze the effect of treatment combination groups (carboplatin 0 µM, carboplatin 20 µM +
gemcitabine, and carboplatin 200 µM + gemcitabine) and different concentrations of gemcitabine (0, 0.1, and 1 µM). If interaction was significant (P < 0.05), post-hoc test with Bonferroni adjustment was used for pairwise comparisons of treatment groups at each concentration of gemcitabine. Other P values were also described for these experiments. Statistical analyses were performed using commercially available software (Prism, version 5.0, GraphPad Software Inc, La Jolla, CA).
Chapter 3: Results

3.1 Effect of individual drugs on proliferation

3.1.1 Effect of gemcitabine on cell proliferation

For all 5 canine TCC cell lines, percentage of cell proliferation after incubation with gemcitabine for 72 hours decreased as the concentration of the drug increased (Figure 3.1A). For AXA, AXC, KISS, JS, and SH cell lines, IC\textsubscript{50} concentrations were 0.63, 0.27, 0.32, 0.59 and 2.4 µM, respectively.

3.1.1 Effect of carboplatin on cell proliferation

For all canine TCC cell lines, percentage of cell proliferation after incubation with carboplatin for 72 hours decreased as the concentration of the drug increased (Figure 3.1B). For AXA, AXC, KISS, JS, and SH cell lines, IC\textsubscript{50} concentrations were 52.4, 130.3, 111.2, 131.1, and 140.5 µM, respectively.

3.2 Effects of gemcitabine in combination with carboplatin on cell proliferation

For all 5 canine TCC cell lines, cell proliferation was significantly decreased when cells were incubated with a combination of gemcitabine and carboplatin, compared with proliferation when cells were treated with gemcitabine alone at most concentrations.
(Figure 3.2). This difference was greatest when comparing gemcitabine alone to gemcitabine + carboplatin 150 µM (†). When comparing differences in cell proliferation of gemcitabine + carboplatin 50 µM and gemcitabine + carboplatin 150 µM (‡), AXA and SH were the only cell lines with significant differences (P < 0.001) at more than one gemcitabine concentration. Cell proliferation among groups was least affected at high gemcitabine concentrations regardless of treatment group. This was due to the overall low cell proliferation at these concentrations. Decrease in cell proliferation in TCC cell lines by gemcitabine is enhanced in the presence of carboplatin, resulting in synergistic activity.

Treatment order did not consistently affect cell proliferation in all cell lines (Figure 3.3). When evaluating significant differences in treatment order among treatment groups and gemcitabine concentrations, gemcitabine given first yielded best results (i.e. lower cell proliferation) in AXA and SH cell lines, while giving both drugs at the same time offered best results in AXC and KISS cell lines. For JS cell line, a significant interaction between order of drug administration and concentration of gemcitabine was not detected, indicating that the magnitude of the difference did not differ with concentration of gemcitabine. Overall, no specific pattern was identified with respect to treatment order.

Evaluation of CI values (Figure 3.4) indicated that for 3 of 5 TCC cell lines, the gemcitabine and carboplatin combination was synergistic, in that most CI values were < 1 over a range of drug effect levels. For the synergistic cell lines, CI values < 0.3 (indication of strong synergism) occurred in at least 1/3 of concentrations evaluated at drug concentrations < 1.5 µM for gemcitabine and < 150 µM for carboplatin in all cell
lines. These concentrations (<1.5 \mu M for gemcitabine and <150 \mu M for carboplatin) are considered biologically relevant (achievable in vivo). For the remaining 2 TCC cell lines, effect was considered additive based on the range of CI values. Calculation of dose-reduction index indicated that the concentration of carboplatin necessary to inhibit 50% of TCC cells could be reduced by 14.2-fold (AXA), 5.9-fold (AXC), 5.4-fold (KISS), 1.5-fold (JS), and 1.2-fold (SH) with the addition of gemcitabine (Figure 3.5A).

Furthermore, the concentration of gemcitabine necessary to inhibit 50% of TCC cell could be reduced by 1.1-fold (AXA), 3.0-fold (AXC), 4.3-fold (KISS), 3.8-fold (JS), and 19.3-fold (SH) with the addition of carboplatin (Figure 3.5B).

### 3.3 Effect of treatment on cell cycle distribution

For 4 of 5 TCC cell lines, combination of carboplatin (200 \mu M) and gemcitabine (0.1 or 1.0 \mu M) was associated with a substantial increase in the percentage of dead cells (sub-G0/G1 phase; Figure 3.6) when compared to either treatment alone. For 4 of 5 cell lines, P < 0.001 when comparing carboplatin (200 \mu M) + gemcitabine (0.1 \mu M) to carboplatin (20 \mu M) + gemcitabine (0.1 \mu M) or gemcitabine alone (0.1 \mu M). The same was true for concentrations of gemcitabine 1.0 \mu M. In 4 of 5 TCC cell lines, the percentage of dead cells for carboplatin treatment was not different than control at 24 hours. SH was the only cell line that did not show substantial increase in the percentage of dead cells when treated with gemcitabine in addition to carboplatin.

### 3.4 Effect of treatment on apoptosis

For all 5 TCC cell lines, treatment combination of carboplatin (200 \mu M) and gemcitabine
(0.1 or 1.0 µM) was associated with significant increases in caspase-3/7 activity (Figure 3.7) when compared to either treatment alone at 24 hours post-treatment. For all cell lines, P < 0.001 when comparing carboplatin (200 µM) + gemcitabine (1 µM) to carboplatin (20 µM) + gemcitabine (1 µM) or gemcitabine alone (1 µM). The same was true for concentrations of gemcitabine 0.1 µM in 4 of 5 TCC cell lines. In 3 of 5 TCC cell lines, apoptosis in carboplatin treatment group (200 µM) was significantly higher (P < 0.05) than control at 24 hours.
Figure 3.1. Effect of gemcitabine and carboplatin alone on cell proliferation. Mean +/- SD cell proliferation, determined by use of CyQUANT® assay, for 5 canine TCC cell lines incubated with: (A) gemcitabine (0, 0.001, 0.01, 0.1, 0.5, 1, 2.5, 5, 10, 100 µM); (B) carboplatin (0, 10, 50, 75, 100, 250, 375, 500, 750, 1000 µM) for 72 hours. Cell proliferation was calculated as mean fluorescence of treated cell divided by mean fluorescence of untreated cells. Each experiment was performed three times in triplicates.
Figure 3.2. Effects of gemcitabine in combination with carboplatin on cell proliferation. Mean +/- SD cell proliferation determined by the use of CyQUANT® assay, for TCC cell lines AXA (A), AXC (B), KISS (C), JS (D), SH (E) following incubation with gemcitabine (0, 0.01, 0.1, 1, 10, 100 µM) alone (open circles), or in combination with carboplatin 50 µM (open squares) or carboplatin 150 µM (open triangles) for 72 hours. *Significantly (P<0.001) different when comparing gemcitabine alone to carboplatin (50 µM); †significantly (P<0.001) different when comparing gemcitabine alone to carboplatin (150 µM); ‡significantly (P<0.001) different when comparing carboplatin (50 µM) to carboplatin (150 µM). Cell proliferation was calculated as mean fluorescence of treated cell divided by mean fluorescence of untreated cells. Each experiment was performed three times in triplicates.
Figure 3.3. Effect of sequence of administration of carboplatin and gemcitabine on cell proliferation. Mean +/- SD cell proliferation, determined by use of CyQUANT® assay, for canine TCC cell lines AXA (A), AXC (B), KISS (C), JS (D), SH (E) following
incubation with gemcitabine (0, 0.01, 0.1, 1, 10, 100 μM) alone (1), or in combination with carboplatin 50 μM (2) or carboplatin 150 μM (3) for 72 hours. Cells were treated with gemcitabine 4 hours prior to carboplatin (open circles), at the same time as carboplatin (open squares), or with gemcitabine four hours after carboplatin (open triangles). *Significantly (P<0.001) different when comparing gemcitabine before carboplatin and drugs given at the same time; †significantly (P<0.001) different when comparing gemcitabine given first and carboplatin given first; ‡significantly (P<0.001) different when comparing both drugs given at the same time and carboplatin given first.

Cell proliferation was calculated as mean fluorescence of treated cell divided by mean fluorescence of untreated cells (% of control). Each experiment was performed three times in triplicates.
Figure 3.4. Synergistic effect of combination of carboplatin and gemcitabine on cell proliferation. Combination index values for canine TCC cell lines AXA (A), AXC (B), KISS (C), JS (D), SH (E) following incubation with gemcitabine and carboplatin alone and in combination at a fixed gemcitabine-to-carboplatin concentration ratio (8, 4, 2, 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$ times IC$_{50}$) based on the IC$_{50}$ of each drug in each cell line for 72 hours. Fraction affected equates to percentage reduction in cell proliferation. A CI value < 1 indicates synergy. Experiments were performed three times in triplicates.
Figure 3.5. Dose reduction index when combining gemcitabine and carboplatin.

Dose reduction index (DRI) expressed as % reduction in IC\textsubscript{50} for carboplatin in combination with gemcitabine, compared to their individual IC\textsubscript{50}s. (A) Carboplatin (solid bars), combination with gemcitabine (hollow bars). (B) Gemcitabine (solid bars), combination with carboplatin (hollow bars). \textit{In vitro} drug combination studies in five canine TCC cell lines: AXA, AXC, KISS, JS, SH according to the constant ratio experimental design. Mean value is shown. The experiment was performed three times in triplicates.
Figure 3.6. Cell cycle changes when combining gemcitabine and carboplatin. Mean +/- SD percentage of cells in sub-G_0/G_1 phase (i.e. dead cells), determined by use of the propidium iodide staining method, following incubation of 5 canine TCC cell lines AXA (A), AXC (B), KISS (C), JS (D), SH (E) following incubation with gemcitabine (0, 0.1, 1 µM) alone, or in conjunction with carboplatin (20 µM or 200 µM) for 24 hours. ***,*** Significantly (P<0.05, P<0.01, P<0.001 respectively) different from value obtained in respective treatment combinations (in brackets). Experiments were performed three times in duplicates.
Figure 3.7. Caspase-3/7 activity after treatment with gemcitabine and carboplatin.

Mean +/− SD caspase 3/7 activity, measured in relative fluorescence units (RFU) with a commercial assay, in canine TCC cell lines AXA (A), AXC (B), KISS (C), JS (D), SH (E) following incubation with gemcitabine (0, 0.1, 1 µM) alone, or in conjunction with carboplatin (20 µM or 200 µM) for 24 hours. ***Significantly (P<0.05, P<0.01, P<0.001 respectively) different from value obtained in respective treatment combinations (in brackets). Experiments were performed three times in triplicates.
Chapter 4: Discussion

In the present study, we evaluated the in vitro activity of gemcitabine and carboplatin alone and in combination in five canine TCC cell lines. All cell lines showed similar dose-dependent decreases in cell proliferation. For 4 of 5 cell lines, IC$_{50}$ concentrations ranged from 0.27 to 0.59 µM (for the remaining cell line [SH], the IC$_{50}$ was 2.4 µM) when treated with gemcitabine. The results in the sensitive cell lines are similar to those reported for human TCC cells treated with gemcitabine in vitro. The effect of gemcitabine on canine cancer cell lines has only been reported in one other study. In that study, the IC$_{50}$ of gemcitabine ranged from 5.7 to 15.3 µM in 3 of 4 canine osteosarcoma cell lines (due to resistance, the IC$_{50}$ could not be calculated in the remaining cell line), all of which were higher than in our report. Although it is impossible to directly compare our IC$_{50}$ results to other studies, especially when different methodologies are used, these findings may suggest that TCC cells are comparatively more sensitive to single agent gemcitabine than other tumor types.

Single agent carboplatin had relatively low IC$_{50}$ values in this study. For all cell lines, the IC$_{50}$ was below the peak plasma concentration achieved in the dog at recommended dosing (300 mg/m$^2$ IV bolus), which is approximately 250 µM. In fact, for the combination study, where the carboplatin dose was fixed, the initial concentration had to be decreased to 50 µM and 150 µM because 250 µM (dose initially used, data not
shown) caused a dramatic decrease in cell viability. The higher dose impaired our ability to see changes in cell viability when both drugs were combined. The IC$_{50}$ reported for carboplatin in a human TCC cell line was 289.3 µM, which was higher than any of the cell lines in our study.$^{66}$

Given the potential resistance of TCC cells to single-agent chemotherapy, we evaluated gemcitabine in combination with carboplatin. 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 the treatment of a variety of malignancies in human patients, including TCC.$^{66}$ Some even consider this combination as the standard of care therapy for human metastatic TCC.$^{77}$ Despite the poor response of canine TCC to single agent carboplatin$^{26}$, we speculated that response to therapy might be significantly improved if the two drugs had synergistic effects. In the present study, 3 of 5 cell lines showed synergism by combination index, while the remaining 2 showed additive effects. These results are consistent with previously published results in other human TCC cell lines$^{66,89}$ and canine osteosarcoma cell lines.$^{85}$

Although the combination of gemcitabine and carboplatin has been given to dogs with various types of cancer$^{35}$ with modest results, the dosage and treatment cycle were extrapolated from human medicine. Unfortunately, in that study only two of the dogs had TCC. The ideal dosage and treatment sequence remains unknown for canine patients. It is possible that better responses could be achieved if we knew the ideal treatment protocol for this combination in the dog. Several pharmacokinetic studies have been performed in dogs.$^{49,90-93}$ Based on the pharmacokinetics of gemcitabine in the dog, a 3 mg/kg dose
should achieve a peak blood concentration of approximately 9 µM (demonstrated to be clinically relevant).\textsuperscript{92,93} In these studies the $C_{\text{max}}$ ranged from 1.36 to 4.05 µg/ml (4.53 – 13.51 µM) when gemcitabine was given at 3 mg/kg IV and the elimination half-life was very similar in the two studies (1.75 vs. 1.38 hours).\textsuperscript{92,93} Extrapolating from rat studies, if the distribution ratio is maintained (4.5\% of $C_{\text{max}}$ at 24 hours and 0.8\% of $C_{\text{max}}$ 5 days later in the urinary bladder tissue); a dog treated with 3 mg/kg IV ($C_{\text{max}}$ of 13.51 µM)\textsuperscript{93} would have a urinary bladder tissue concentration of gemcitabine equivalent to 0.6 µM and 0.1 µM at 24 hours and 5 days post-treatment respectively.\textsuperscript{91,93} If one considered the $C_{\text{max}}$ of 4.53 µM\textsuperscript{92} obtained in a different study, the concentration in the bladder would be 0.2 µM and 0.04 µM for 24 hours and 5 days respectively.\textsuperscript{92} These findings suggest that it may be possible to achieve better results with gemcitabine if it were used more frequently than on days 1 and 8 (in a 3-week cycle), as it was previously done in dogs.\textsuperscript{35} According to the IC$_{50}$ results achieved in the majority of the cell lines tested here, lower doses of gemcitabine are likely to still be effective in TCC. Future \textit{in vivo} studies are recommended to evaluate whether lower, more frequent doses of gemcitabine is a viable option for canine TCC treatment. The significant decrease in cell proliferation with relatively low concentrations of gemcitabine along with the limited concentration of this drug in tissues support the use of lower dosages at more frequent intervals \textit{in vivo}.

Even though our results did not show a consistent increase in efficacy with any particular sequence of treatment, others have found the sequence of treatment to be important. In this study, 2 cell lines showed more significant decrease in cell proliferation when the two drugs were given simultaneously (AXC and KISS) while 2 other cell lines had more significant decrease in cell proliferation when gemcitabine was given first.
(AXA and SH). This was consistent with results obtained from another in vitro study in a human TCC cell line where a greater decrease in cell viability was seen with either simultaneous treatment or when gemcitabine was given first. In a different study, 3 of 4 canine osteosarcoma cells behaved in the opposite way. However, it has been hypothesized that when carboplatin and gemcitabine are given together, gemcitabine inhibits DNA repair of platinum-induced damage through its inhibition of ribonucleotide reductase. Gemcitabine is also incorporated into DNA during the repair process, thereby contributing to inhibition of further DNA replication and repair. Thus, it has been suggested that when gemcitabine is administered in conjunction with a platinum compound, the gemcitabine be administered before the platinum compound to ensure that a sufficient intracellular concentration of gemcitabine is present prior to initiation of DNA damage by the platinum compound. Our findings are consistent with the underlying cell-killing mechanisms of these two drugs. Carboplatin kills cells mainly through induction of DNA adducts. Gemcitabine is a nucleoside analog in which the hydrogen on the 2’ carbon of deoxycytidine is replaced by a fluorine atom. During DNA replication, gemcitabine triphosphate is incorporated into the DNA strands and terminates DNA replication. It is hypothesized that if drug sequence plays a role in drug efficacy, treatment with carboplatin prior to gemcitabine induces DNA damaged and cell cycle arrest, decreasing the incorporation of gemcitabine triphosphate into DNA, and mitigating the cytotoxic effects of gemcitabine. It is possible that DNA structural changes by incorporation of gemcitabine favor the binding of platinum drugs, and gemcitabine inhibits DNA repair of platinum-DNA adducts. Furthermore, platinum inhibits ribonucleotide reductase, and further enhances the incorporation of gemcitabine
triphosphate into DNA. Nucleotide excision repair is the major pathway responsible for the removal of platinum-DNA adducts. Gemcitabine might reduce the effectiveness of nucleotide excision repair through its inhibition of ribonucleotide reductase.\textsuperscript{66} However, it is also possible that gemcitabine administration may not be the determining factor regarding response to therapy.

Further support of the efficacy of this combination is based on dose-dependent increase in the proportion of cells in the sub-$G_0/G_1$ phase in all cell lines and increased caspase-3/7 activity, especially when carboplatin and gemcitabine were used in combination. For the most part, this increase was several-fold higher than when either drug was used alone. These results may be another indication of synergism. The mild increase in the proportion of cells in the sub-$G_0/G_1$ phase at 24 hours when cells were treated with either drug alone is consistent with previous data available in a human TCC cell line.\textsuperscript{66,88} When evaluating apoptosis, for the most part, no significant change was noted in caspase activity at 24 hours post-treatment when comparing control to carboplatin treatment alone. This may be because platinum compounds have been shown to diminish cellular respiration after many hours of drug treatment. However, when evaluating caspase levels at 22 hours, cisplatin treated cells had a much higher increase in caspase activity compared to carboplatin. Caspase activation leads to impaired cellular respiration and decreased cellular ATP. Thus, the mitochondria are rapidly targeted by caspases.\textsuperscript{97} Carboplatin is known to take more than 12 hours post-treatment to cause mitochondrial dysfunction.\textsuperscript{97} This may explain the lack of difference seen at 24 hours when comparing the control group to the carboplatin treated cells. The modest increase in caspase activation noted when cells were treated with single agent gemcitabine is also
consistent with previous data available in a human TCC cell line. The magnitude of the activation was much higher at 48 and 72 hours. The mechanism for the significant difference in caspase activation when both gemcitabine and carboplatin were used in combination is unclear.

In summary, our data demonstrate that, as is the case with human TCC cell lines, gemcitabine has biological activity against canine TCC cell lines and that this activity is synergistic when gemcitabine is combined with carboplatin at biologically relevant concentrations. Furthermore, treatment sequence does not reliably affect cell proliferation amongst cell lines. These results lay the groundwork for future evaluations of gemcitabine administered alone or in combination with carboplatin for dogs with TCC.
Chapter 5: Bibliography


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