Effects of gemcitabine and gemcitabine in combination with carboplatin on five canine transitional cell carcinoma cell lines

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Objective—To evaluate in vitro effects of gemcitabine alone and in combination with carboplatin on canine transitional cell carcinoma (TCC) cell lines.

Sample—In vitro cultures of 5 canine TCC cell lines.

Procedures—Cells were treated with gemcitabine, carboplatin, or a combination of both at various concentrations. Cell proliferation was assessed via a fluorescence-based microplate cell proliferation assay. Cell cycle was evaluated via propidium iodide staining, and apoptosis was assessed by measurement of caspase 3 and 7 enzymatic activity. Synergy between gemcitabine and carboplatin was quantified via combination index analyses.

Results—Treatment of 5 canine TCC cell lines with gemcitabine or carboplatin decreased cell proliferation, increased apoptosis, and induced cell cycle arrest. Cell cycle arrest and apoptosis were markedly increased when cell lines were treated with both gemcitabine and carboplatin simultaneously or sequentially. Order of administration during sequential treatment did not consistently affect cell proliferation results in TCC cell lines. When TCC cell lines were treated with gemcitabine and carboplatin in combination at therapeutically relevant concentrations (gemcitabine concentration, < 10μM; carboplatin concentration, < 250μM), a significant decrease in cell proliferation was observed, compared with cell proliferation following treatment with gemcitabine or carboplatin alone. In combination, the effects of gemcitabine and carboplatin were synergistic in 3 of 5 cell lines and additive in the other 2.

Conclusions and Clinical Relevance—Gemcitabine had antitumor effects on canine TCC cells in vitro, and the combination of gemcitabine and carboplatin had synergistic activity at biologically achievable concentrations. (*Am J Vet Res* 2012;73:1262–1272)

Transitional cell carcinoma of the urinary bladder in dogs is a challenging disease to diagnose and treat. Only 12% to 25% of affected dogs that receive conventional antineoplastic drugs have an objective response to treatment[1]; thus, new treatment strategies are needed. Many strategies have been investigated for treating TCC in dogs, including surgery,[2–5] radiation therapy,[6–10] chemotherapy,[9,11–27] and NSAIDs.[9,11–13,24,26,28–36] The longest reported median survival time for dogs with TCC was 329 days, with an overall response rate of 50% among 12 dogs treated with piroxicam alone for 4 weeks followed by piroxicam combined with cisplatin.[34] Even though that protocol was efficacious, renal toxicosis was frequent and dose limiting. In general, the platinum-based cytotoxic drugs cisplatin and carboplatin have had disappointing results in dogs with TCC of the urinary bladder because of their toxic effects and lack of efficacy.[17–19] The combination of mitoxantrone and piroxicam has also been used to treat 48 dogs with TCC of the urinary bladder with an overall response rate of 35%.[12] Currently, piroxicam is the most commonly used drug to treat TCC of the urinary bladder in dogs, and its use results in an improvement in clinical signs (eg, stranguria, hematuria, and pollakiuria) for most dogs. However, substantial objective tumor regression in piroxicam-treated dogs is uncommon.[32]

Abbreviations

CI	Combination index
Cmax	Peak plasma concentration
DMEM	Dulbecco modified Eagle medium
IC_{50}	Concentration required for 50% inhibition of cell proliferation
TCC	Transitional cell carcinoma

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Gemcitabine (2′, 2′-difluorodeoxycytidine) is a synthetic analog of cytosine arabinoside that has activity against a variety of human cancers.[37] This antimetabolite cytotoxic prodrug undergoes complex cellular uptake and metabolism.[38] Gemcitabine was the first compound licensed on the basis of antitumor efficacy and improved quality-of-life score in a study[39] involving humans with pancreatic carcinoma. Gemcitabine has cell phase specificity, primarily killing cells undergoing DNA synthesis (S phase) and also blocking the progression of cells from the growth (G1) phase to the S phase.[39,40] Multiple self-potentiation mechanisms augment the activity of gemcitabine diphosphates and triphosphates and decrease elimination of gemcitabine.[37] The active metabolite of gemcitabine can be incorporated into both DNA and RNA.[37,41] Gemcitabine has been used on a limited basis in species of veterinary interest. Results of a phase I study[42] indicated that gemcitabine (675 mg/m^2, IV, q 2 weeks) can be administered with minimal toxic effects to dogs. In another study[43] that involved 38 dogs with TCC of the urinary bladder, administration of gemcitabine (800 mg/m^2, IV, q 1 week) in conjunction with piroxicam (0.3 mg/kg, PO, q 24 h) resulted in clinical improvement of stranguria, pollakiuria, and hematuria for all treated dogs; 10 of 38 (26%) dogs responded at least partially to treatment and the median survival time for all study dogs was 230 days.

Platinum compounds react with cellular components that have nucleophilic sites such as DNA, RNA, proteins, membrane phospholipids, cytoskeletal microfilaments, and thiol-containing molecules. Following administration of a platinum compound, approximately 1% of the total platinum that is absorbed intracellularly binds to DNA, resulting in inter- and intrastrand cross-linking.[44] It has been suggested that platinum-damaged DNA causes cells to arrest at the premitotic (G2) phase to repair the damage.[44] In the absence of adequate repair, cells undergo an abortive attempt at mitosis that results in cell death via an apoptotic mechanism.[44] Platinum compounds may also inhibit DNA synthesis by other mechanisms. Carboplatin is a second-generation platinum compound that has reduced toxic effects (ie, nephrotoxicosis), compared with that of cisplatin. Carboplatin has been commonly used as an adjuvant treatment for osteosarcoma in dogs,[45] in which it typically increased the disease-free interval and survival time. Carboplatin is generally well tolerated by dogs, although some may develop neutropenia that limits the dose of carboplatin that can be administered. Even though carboplatin is not considered nephrotoxic, myelosuppression may be exacerbated in patients with impaired kidney function. Two studies[11,18] have been conducted to evaluate the efficacy of carboplatin administration in dogs with TCC of the urinary bladder. One study[18] was conducted to evaluate the administration of carboplatin alone, and median survival time of treated dogs was 132 days (1/14 dogs had stable disease); however, these dogs also received various rescue drugs. The other study[11] was conducted to specifically evaluate the administration of a combination of carboplatin and piroxicam, and median survival time of treated dogs was 161 days (11/29 [38%] dogs had partial remission).

The administration of a combination of gemcitabine and cisplatin has been investigated in humans with metastatic urinary bladder cancer and is currently considered the standard of care.[46] Given the success achieved with cisplatin, investigators in several studies[46–52] that involved humans have evaluated combinations of gemcitabine and carboplatin because carboplatin has fewer toxic effects than cisplatin. The use of carboplatin and gemcitabine in combination is beneficial from a clinical standpoint because these drugs have different mechanisms of action, toxicity profiles that do not overlap, and possible synergism. Carboplatin might interact with gemcitabine metabolism at its activation site or at the DNA level. Carboplatin can also inhibit ribonucleotide reductase. Gemcitabine or one of its metabolites may interact with the mechanism of action for platinum compounds, such as the extent or nature of platinum incorporation into DNA or the process of DNA repair.[37] Results of a study[53] that involved the use of a TCC cell line of human origin indicated that there was synergism between gemcitabine and carboplatin when gemcitabine was given 4 hours prior to or at the same time as carboplatin. When carboplatin was given prior to gemcitabine, only additive effects were detected. Similarly, incubation with cisplatin for 4 hours prior to gemcitabine treatment resulted in synergistic antitumor effects in 2 of 3 ovarian cancer cell lines, whereas incubation with gemcitabine prior to cisplatin treatment resulted in synergistic antitumor effects in only 1 of those cell lines.[41] In that study,[41] cisplatin had no apparent effect on gemcitabine accumulation; therefore, the investigators hypothesized that the mechanism of synergism between gemcitabine and cisplatin was not associated with enhanced gemcitabine accumulation.

Advanced stage cancer of the urinary bladder in humans closely resembles cancer of the urinary bladder in dogs. Consequently, a study[27] was conducted to evaluate the combined administration of gemcitabine and carboplatin for treatment of various carcinomas in 37 dogs; however, only 2 dogs had TCC of the urinary bladder. An empirically chosen dosing schedule was used to treat dogs in that study[27]: gemcitabine (2 mg/kg, IV, as a 20- to 30-minute infusion) on days 1 and 8 with carboplatin (10 mg/kg, IV) administered 4 hours after the end of the gemcitabine infusion on day 1. That treatment protocol was repeated every 3 weeks. Although the overall tumor response rate among the 37 dogs in that study[27] was modest (13%), further research regarding dosages, treatment schedule, toxic effects, and efficacy of the administration of gemcitabine in combination with carboplatin for the treatment of carcinomas in dogs is warranted. The purpose of the study reported here was to evaluate the cytotoxic effects of gemcitabine and carboplatin on various TCC cell lines of canine origin and determine whether there was synergism between the 2 drugs and whether such synergism was dependent on the sequence of drug administration.

Materials and Methods

TCC cell lines—Five TCC cell lines (K9TCC-PU-AxA[a] [AXA], K9TCC-PU-AxC[a] [AXC], K9TCC-PU[a]

[JS], K9TCC-PU-Sh[a] [SH], and TCC-Kiss[b] [KISS]) that originated from dogs with TCC were used in the study reported here. All cell lines were maintained in DMEM[c] with L-glutamine and glucose and supplemented with 1% fetal bovine serum,[c] 1% newborn calf serum,[c] penicillin,[c] and streptomycin.[c] Four of the cell lines (AXA, AXC, JS, and SH) have been described elsewhere.[54]

Treatments—Treatments consisted of gemcitabine[d] alone, carboplatin[e] alone, and combinations of gemcitabine and carboplatin. Gemcitabine was evaluated at concentrations of 0.001, 0.01, 0.1, 0.5, 1, 2.5, 5, 10, and 100µM, and carboplatin was evaluated at concentrations of 0, 10, 50, 75, 100, 250, 375, 500, 750, and 1,000µM. Combination treatments consisted of various concentrations of gemcitabine (0.01, 0.1, 1, 10, and 100µM) each with carboplatin at concentrations of 50 or 150µM. For the combination treatments, the 2 drugs were administered at the same time or sequentially with gemcitabine administered 4 hours before carboplatin and vice versa.

Effect of treatment on cell proliferation—Cell proliferation following treatment (gemcitabine alone, carboplatin alone, and combinations of gemcitabine and carboplatin) was assessed by use of a fluorescence-based microplate cell proliferation assay[f] in accordance with the manufacturer's specifications. Briefly, 96-well plates were prepared by seeding approximately 2.5×10^3 TCC cells suspended in 200 µL of DMEM without phenol red in each well, and then plates were incubated overnight (approx 24 hours) at 37°C in 5% CO_2. The respective treatment was added to each of the wells, and the plates were incubated for an additional 72 hours at 37°C in 5% CO_2. Untreated cells were used as the negative control sample, in accordance with methods described previously.[53,55] Fluorescence (indirect quantitative measurement of nucleic acid concentration) was quantified by use of an ELISA plate reader[g] at an excitation wavelength of 485 nm and emission wavelength of 538 nm. Cell proliferation was calculated as a percentage of proliferation for the negative control wells (ie, [fluorescence of the treated wells/fluorescence of untreated wells] × 100). Cell proliferation was determined for each treatment and concentration in triplicate for each TCC cell line.

Evaluation of synergy between gemcitabine and carboplatin—To evaluate whether there was synergy between gemcitabine and carboplatin, TCC cells were incubated with medium containing each drug alone and in combination at fixed gemcitabine-to-carboplatin concentrations (8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 times the IC_{50} of each drug, respectively, for each TCC cell line) for 72 hours at 37°C in 5% CO_2. For each TCC cell line, cell proliferation was then assessed via a cell proliferation assay,[f] and the concentrations for each drug alone and in combination that achieved a reduction in cell proliferation were determined. Those concentrations were then used to calculate the CI value via standard software[h] as described.[56] The interaction between gemcitabine and carboplatin was considered synergistic when the CI value was < 1, antagonistic when the CI value was > 1, and additive when the CI value was 1. The dose-reduction index was a measure of the reduction in TCC cell proliferation achieved with the combination treatment of gemcitabine and carboplatin in excess of that achieved with treatment of gemcitabine or carboplatin alone. It was calculated as the percentage reduction in IC_{50} for the combination of gemcitabine and carboplatin, compared with the IC_{50} for gemcitabine and carboplatin, respectively, and was expressed as a fold decrease relative to the IC_{50} for gemcitabine or carboplatin. For each TCC cell line, all calculations were performed in triplicate for each treatment and concentration.

Effect of treatment on cell cycle—The effect of treatment with gemcitabine alone, carboplatin alone, and combinations of gemcitabine and carboplatin on the cell cycle of TCC cells was evaluated via propidium iodide staining. The drug concentrations used for this part of the study were chosen on the basis of the mean 25% and 75% cell proliferation results for both drugs among all cell lines. Briefly, 6-well plates were prepared by seeding 3×10^6 TCC cells suspended in 3 mL of DMEM with 1% fetal bovine serum in each well, and then plates were incubated overnight at 37°C and 5% CO_2. Subsequently, the cells were treated with gemcitabine (0.1 and 1.0µM) alone, carboplatin (20 and 200µM) alone, or 1 of 4 combinations of gemcitabine and carboplatin (0.1µM gemcitabine and 20µM carboplatin, 0.1µM gemcitabine and 200µM carboplatin, 1µM gemcitabine and 20µM carboplatin, or 1µM gemcitabine and 200µM carboplatin). Untreated cells were used as negative controls. The plates were incubated for an additional 24 hours at 37°C and 5% CO_2. Cells were then collected, fixed in 70% ethanol, and incubated for 30 minutes at 20°C with 0.5 mL of propidium iodide staining solution that consisted of propidium iodide[i] (25 µg/mL) and RNAse[i] (10 µg/mL) in PBS solution containing 0.1% glucose. The stained cells were analyzed by means of flow cytometry,[j] and data were analyzed via standard software.[k] The percentage of cells in the sub–resting (G0)–G1 phase (ie, dead cells) was calculated from the total number of gated counts. For each TCC cell line, each treatment and concentration was replicated 3 times, and all samples were analyzed in duplicate.

Effect of treatment on apoptosis—The effect of treatment with gemcitabine alone, carboplatin alone, and combinations of gemcitabine and carboplatin on the apoptosis of TCC cells was evaluated via measurement of caspase 3 and caspase 7 (caspase 3/7) activity. The drug concentrations used for this part of the study were chosen on the basis of the mean 25% and 75% cell proliferation results for both drugs among all cell lines. Briefly, 96-well plates were prepared by seeding 2.5×10^3 TCC cells suspended in 150 µL of DMEM with 1% fetal bovine serum in each well, and then plates were incubated overnight at 37°C and 5% CO_2. Subsequently, the cells were treated with gemcitabine (0.1 and 1.0µM) alone, carboplatin (20 and 200µM) alone, or 1 of 4 combinations of gemcitabine and carboplatin (0.1µM gemcitabine and 20µM carboplatin, 0.1µM gemcitabine and 200µM carboplatin, 1µM gemcitabine and 20µM carboplatin, or 1µM gemcitabine

and 200µM carboplatin). Untreated cells were used as negative control samples. The plates were incubated for an additional 24 hours at 37°C and 5% CO_2. Caspase 3/7 enzymatic activity was then measured via a commercial assay[l] in accordance with the manufacturer's specifications. Fluorescence was quantified by means of an ELISA plate reader at an excitation wavelength of 354 nm and emission wavelength of 442 nm. For each well, relative fluorescence units were calculated by the subtraction of the mean fluorescence measurement of medium alone from the fluorescence measurement of the well. For each TCC cell line, each treatment and concentration was replicated 3 times, and all samples were analyzed in triplicate.

Statistical analysis—Statistical analyses were performed with commercially available software.[m] Data were summarized as mean ± SD. Prior to analyses, the distribution of the data was tested for normality by use of the D'Agostino and Pearson omnibus test.

For all cell lines, a logarithmic transformation was used to normalize the distribution of the cell proliferation data. To evaluate the effect of treatment on TCC cell proliferation, linear regression was used to determine the IC_{50} for each concentration of gemcitabine and carboplatin, respectively. A 2-way ANOVA was used to compare the effects of the various treatment combinations (gemcitabine alone, and each concentration of gemcitabine [0.01, 0.1, 1, 10, and 100µM] with carboplatin at a concentration of 50 or 150µM, respectively) on cell proliferation. When interaction between gemcitabine and carboplatin was significant ($P < 0.001$), pairwise comparisons of treatment at each concentration of gemcitabine were performed with the use of a Bonferroni adjustment for multiple comparisons.

A 2-way ANOVA was used to compare the effect of treatment order (gemcitabine administered 4 hours before carboplatin, carboplatin administered 4 hours before gemcitabine, and both drugs administered at the same time) and concentration of gemcitabine (0.01, 0.1, 1.0, 10, and 100µM) on TCC cell proliferation. When interaction between order of drug administration and gemcitabine concentration was significant ($P < 0.05$), pairwise comparisons for each drug administration sequence at each concentration of gemcitabine were performed with the use of a Bonferroni adjustment for multiple comparisons. For each TCC cell line, the order of drug administration that resulted in the lowest cell proliferation for each combination of gemcitabine and carboplatin (gemcitabine [0, 0.01, 0.1, 1.0, 10, 100µM] alone, gemcitabine [0, 0.01, 0.1, 1.0, 10, 100µM] and carboplatin [50µM], and gemcitabine [0, 0.01, 0.1, 1.0, 10, 100µM] and carboplatin [150µM]) was determined. The effect of treatment (gemcitabine [0.1 or 1.0µM] alone, carboplatin [20 or 200µM] alone, gemcitabine [0.1µM] and carboplatin [20µM], gemcitabine [0.1µM] and carboplatin [200µM], gemcitabine [1.0µM] and carboplatin [20µM], and gemcitabine [1.0µM] and carboplatin [200µM]) on cell cycle and apoptosis was evaluated by use of a 2-way ANOVA. When interaction between gemcitabine and carboplatin was significant ($P < 0.05$), pairwise comparisons of treatment at each concentration of gemcitabine were performed with the use of a Bonferroni adjustment for multiple comparisons.

Results

Effect of treatment on cell proliferation—For all 5 TCC cell lines, the percentage of cell proliferation after incubation with gemcitabine for 72 hours decreased as the concentration of the drug increased (**Figure 1**). The IC_{50}s of gemcitabine were 0.63, 0.27, 0.32, 0.59, and 2.4µM for the AXA, AXC, KISS, JS, and SH cell lines, respectively. Similarly, for all TCC cell lines, the percentage of cell proliferation after incubation with carboplatin for 72 hours decreased as the concentration of the drug increased. The IC_{50}s of carboplatin were 52.4, 130.3, 111.2, 131.1, and 140.5µM for AXA, AXC, KISS, JS, and SH cell lines, respectively.

A decrease in TCC cell proliferation was enhanced by the administration of carboplatin in addition to gemcitabine. For all TCC cell lines, the percentage of cell proliferation was significantly decreased when cells were incubated with a combination of gemcitabine

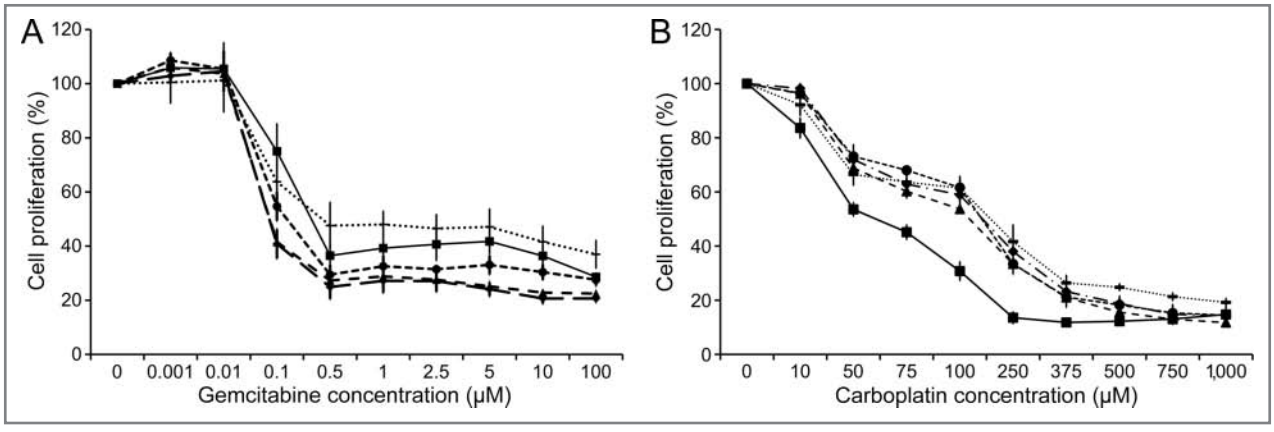

Figure 1—Mean ± SD percentage of cell proliferation determined by use of a fluorescence-based microplate cell proliferation assay 72 hours after administration of gemcitabine (0, 0.001, 0.01, 0.1, 0.5, 1, 2.5, 5, 10, and 100µM; A) or carboplatin (0, 10, 50, 75, 100, 250, 375, 500, 750, and 1,000µM; B), compared with that for untreated (control) cells in 5 TCC cell lines (K9TCC-PU-AxA [AXA; squares], K9TCC-PU-AXC [AXC; diamonds], TCC-Kiss [KISS; triangles], K9TCC-PU-JS [JS; circles], and K9TCC-PU-SH [SH; dashes]) of canine origin. Percentage of cell proliferation was calculated as the mean fluorescence of treated cells divided by the mean fluorescence of untreated cells. For each TCC cell line, cell proliferation was determined for each treatment (gemcitabine and carboplatin) and concentration in triplicate.

and carboplatin, compared with the percentage of cell proliferation when cells were treated with gemcitabine alone (**Figure 2**). At all concentrations of gemcitabine, TCC cell proliferation was decreased to a greater extent when 150µM of carboplatin was administered than when 50µM of carboplatin was administered. When comparing differences in cell proliferation of gemcitabine and carboplatin (50µM) and gemcitabine and carboplatin (150µM), AXA and SH were the only cell lines with significant ($P < 0.001$) differences at > 1 gemcitabine concentration.

The sequence in which gemcitabine and carboplatin was administered did not consistently affect cell proliferation in all cell lines. For the AXA and SH cell lines, the lowest TCC cell proliferation was achieved when gemcitabine was administered 4 hours before carboplatin. However, for the AXC and KISS cell lines, the lowest TCC cell proliferation was achieved when gemcitabine and carboplatin were administered at the same time. For the JS cell line, no interaction between order of drug administration and gemcitabine concentration was detected, which suggested that the magnitude of cell proliferation was not dependent on gemcitabine concentration.

Evaluation of synergy between gemcitabine and carboplatin—Evaluation of CI values (**Figure 3**) indicated that for 3 of 5 TCC cell lines (AXA, AXC, and KISS), the combination treatment of gemcitabine and carboplatin was synergistic in that most CI values were significantly < 1 over the range of fixed gemcitabine-to-carboplatin concentration ratios considered. For these TCC cell lines, at least one-third of the CI values were < 0.3 (ie, an indication of strong synergism) when the combination treatment consisted of a gemcitabine concentration < 1.5µM and carboplatin concentration < 150µM, which are achievable in vivo peak serum concentrations when the drugs are administered IV. For the remaining 2 TCC cell lines (JS and SH), the combination treatment of gemcitabine and carboplatin was classified as additive on the basis of the range of CI values. The dose-reduction index indicated that the addition of gemcitabine to carboplatin resulted in a 14.2-, 5.9-, 5.4-, 1.5-, and 1.2-fold reduction in the IC_{50} of carboplatin for the AXA, AXC, KISS, JS, and SH cell lines, respectively (**Figure 4**). Furthermore, the addition of carboplatin to gemcitabine resulted in a 1.1-, 3.0-, 4.3-, 3.8-, and 19.3-fold reduction in the IC_{50} of gemcitabine for the AXA, AXC, KISS, JS, and SH cell lines, respectively.

Effect of treatment on cell cycle—For the AXA, KISS, JS, and SH cell lines, the combinations of gemcitabine (0.1 or 1.0µM) and carboplatin (200µM) were associated with a substantial increase in the percentage of cells in the sub-G0–G1 phase (ie, dead cells; **Figure 5**), compared with that achieved by gemcitabine treatment alone. For the AXA, AXC, KISS, and JS cell lines, the combinations of gemcitabine (0.1 or 1.0µM) and carboplatin (200µM) were associated with a substantial increase in the percentage of dead cells, compared with that achieved by carboplatin treatment alone. For 4 of 5 (AXA, KISS, JS, and SH) cell lines, significant ($P < 0.001$) differences were found when the combination treatment of gemcitabine (0.1µM) and carboplatin (200µM) was compared with gemcitabine (0.1µM) and carboplatin (20µM) or gemcitabine alone (0.1µM). For all 5 cell lines, significant ($P < 0.05$) differences in the percentage of dead cells were found when the combina-

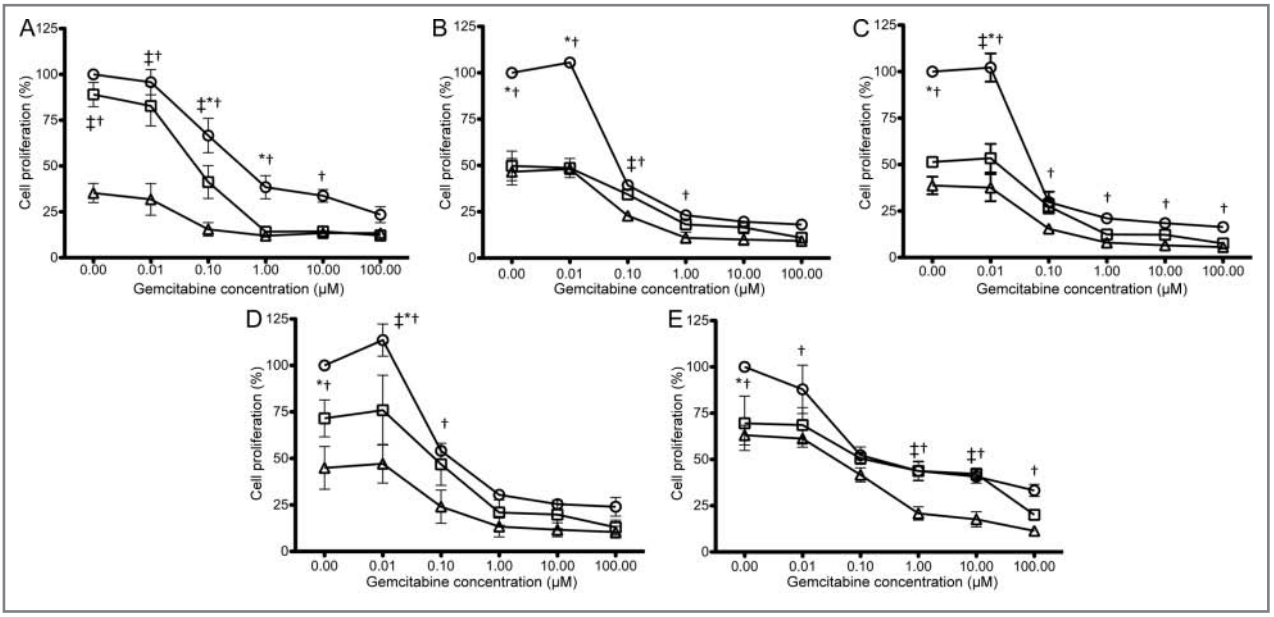

Figure 2—Mean ± SD percentage of cell proliferation 72 hours after administration of various combination treatments of gemcitabine (0, 0.01, 0.10, 1.0, 10.0, and 100.0µM) and carboplatin (0µM [circles], 50µM [squares], and 150µM [triangles]) in 5 TCC cell lines (AXA [A], AXC [B], KISS [C], JS [D], and SH [E]) of canine origin. For each TCC cell line, cell proliferation was determined for each combination treatment in triplicate. *At this concentration of gemcitabine, value for treatment with gemcitabine alone is significantly ($P < 0.001$) different from the value for gemcitabine with carboplatin (50µM). †At this concentration of gemcitabine, value for treatment with gemcitabine alone is significantly ($P < 0.001$) different from the value for gemcitabine with carboplatin (150µM). ‡At this concentration of gemcitabine, value for treatment with gemcitabine and carboplatin (50µM) is significantly ($P < 0.001$) different from the value for treatment with gemcitabine and carboplatin (150µM). *See* Figure 1 for remainder of key.

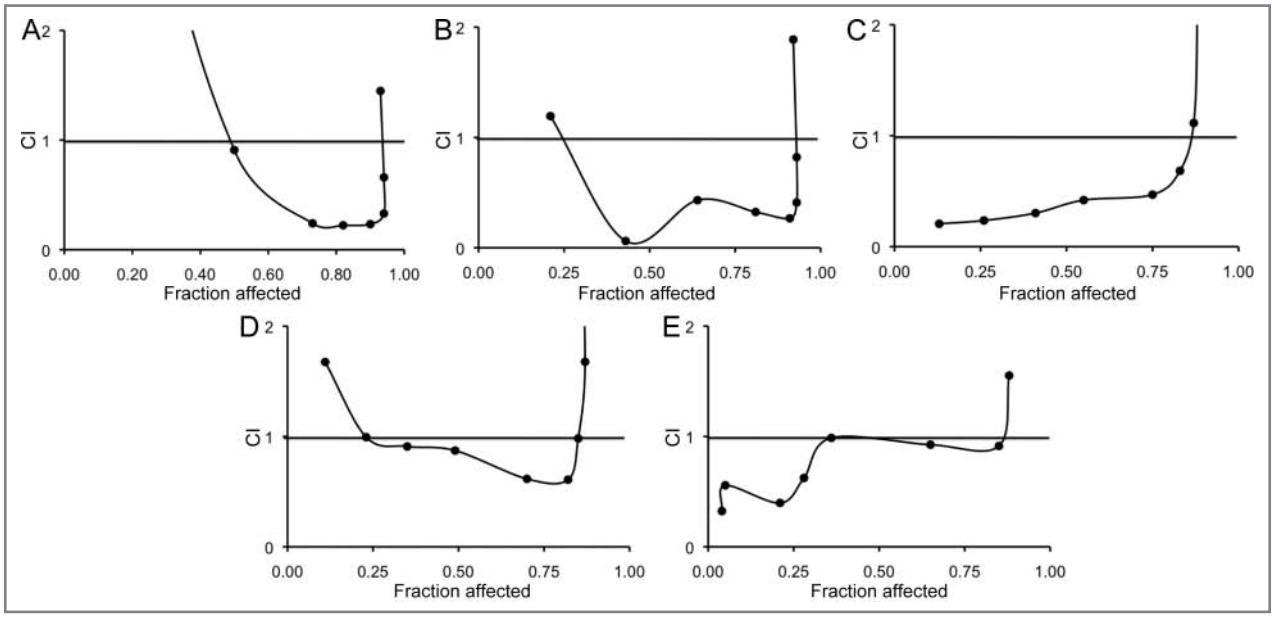

Figure 3—Mean CI values for the combination treatment of gemcitabine and carboplatin determined for 5 TCC cell lines (AXA [A], AXC [B], KISS [C], JS [D], and SH [E]) of canine origin 72 hours after administration of the combination treatment at fixed gemcitabine-to-carboplatin concentrations (8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 times the IC_{50} of each respective drug). Those concentrations were then used to calculate the CI value as described.[57] A CI value equal to 1 indicates an additive effect, < 1 indicates a synergistic effect, and > 1 indicates an antagonistic effect. Combination index values > 2 (AXA = 3.62 and KISS = 2.10) are not graphically depicted. Fraction affected denotes the proportion of cells affected (ie, percentage reduction in cell proliferation). For each TCC cell line, CI values were determined for each combination treatment in triplicate. *See* Figure 1 for remainder of key.

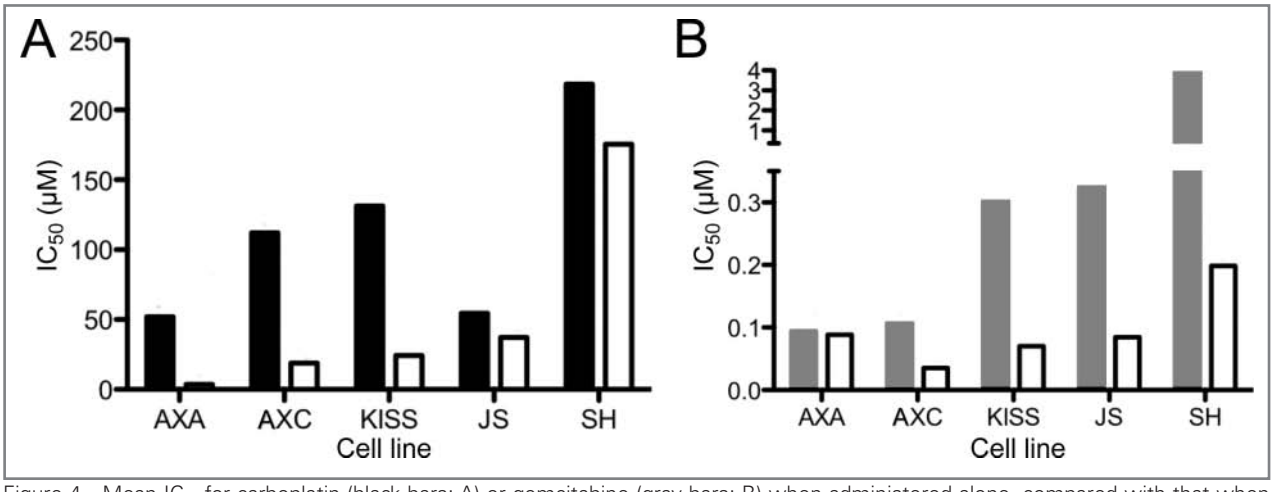

Figure 4—Mean IC_{50} for carboplatin (black bars; A) or gemcitabine (gray bars; B) when administered alone, compared with that when administered as a combination treatment (white bars) of gemcitabine and carboplatin in 5 TCC cell lines (AXA, AXC, KISS, JS, and SH) of canine origin. For each cell line, the dose reduction index was calculated as the IC_{50} for each drug alone divided by the IC_{50} for the combination treatment. The addition of gemcitabine to carboplatin resulted in a 14.2-, 5.9-, 5.4-, 1.5-, and 1.2-fold reduction in the IC_{50} of carboplatin for the AXA, AXC, KISS, JS, and SH cell lines, respectively. The addition of carboplatin to gemcitabine resulted in a 1.1-, 3.0-, 4.3-, 3.8-, and 19.3-fold reduction in the IC_{50} of gemcitabine for the AXA, AXC, KISS, JS, and SH cell lines, respectively. *See* Figure 1 for remainder of key.

tion treatment of gemcitabine (1.0μM) and carboplatin (200μM) was compared with gemcitabine (1.0μM) and carboplatin (20μM) or gemcitabine alone (1.0μM). In 4 of 5 (AXA, AXC, KISS, and JS) TCC cell lines, the percentage of dead cells for the carboplatin treatment was not different from that for the control sample (no treatment) at 24 hours. The only cell line that did not have a substantial increase in the percentage of dead cells when treated with gemcitabine in addition to carboplatin was SH.

Effect of treatment on apoptosis—For all 5 TCC cell lines, treatment combinations of gemcitabine (0.1 or 1.0μM) and carboplatin (200μM) were associated with significant increases in caspase 3/7 activity **(Figure 6)**, compared with that for carboplatin alone. For the AXA, AXC, KISS, and JS cell lines, treatment combinations of gemcitabine (0.1 or 1.0μM) and carboplatin (200μM) were associated with significant increases in caspase 3/7 activity, compared with that for gemcitabine alone. For the SH cell line, the caspase 3/7

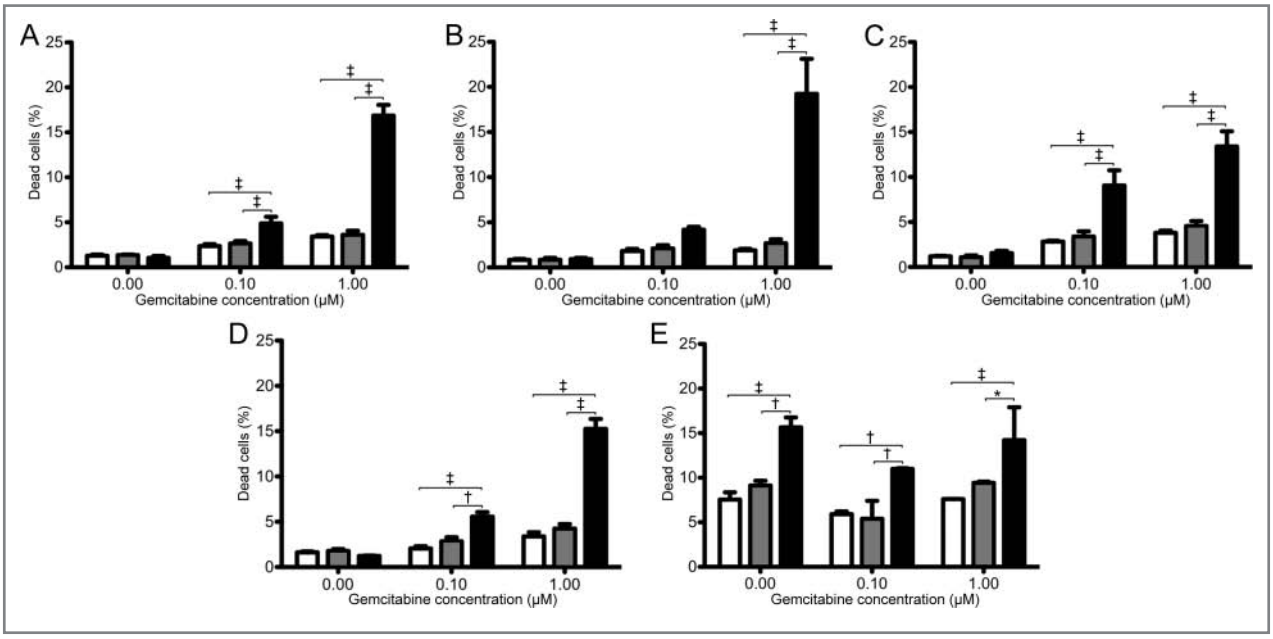

Figure 5—Mean ± SD percentage of dead cells (ie, cells in the sub-G0–G1 phase) determined via a propidium iodide staining method following incubation of 5 TCC cell lines (AXA [A], AXC [B], KISS [C], JS [D], and SH [E]) of canine origin for 24 hours after treatment with gemcitabine (0, 0.1, and 1μM) in combination with carboplatin (0 [white bars], 20 [gray bars], or 200μM [black bars]). For each cell line, the percentage of dead cells was determined for each treatment (gemcitabine and carboplatin) and concentration in triplicate. *†‡Bracketed values are significantly (*$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$) different. *See* Figure 1 for remainder of key.

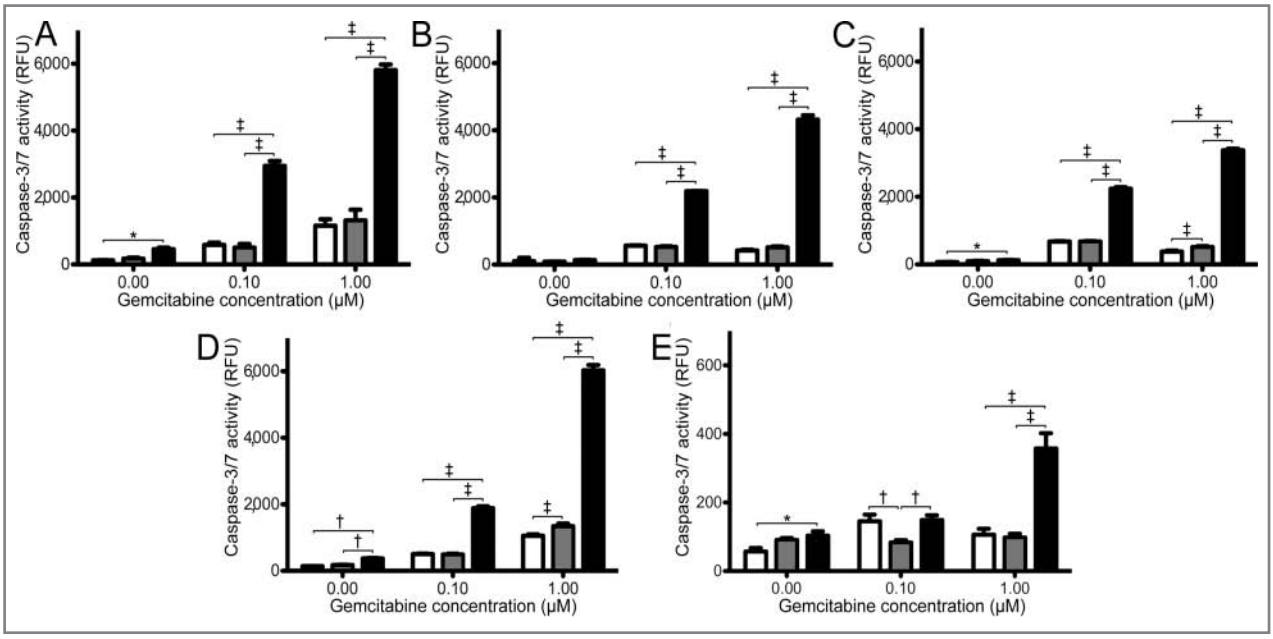

Figure 6—Mean ± SD caspase 3/7 activity determined via a commercial assay following incubation of 5 TCC cell lines (AXA [A], AXC [B], KISS [C], JS [D], and SH [E]) of canine origin for 24 hours after treatment with gemcitabine (0, 0.1, and 1μM) in combination with carboplatin (0 [white bars], 20 [gray bars], or 200μM [black bars]). RFU = Relative fluorescence units. *See* Figure 5 for remainder of key.

activity of the combination treatment of gemcitabine (0.1μM) and carboplatin (200μM) was not significantly different from that of gemcitabine (0.1μM) alone. For all cell lines, significant ($P < 0.001$) differences in caspase 3/7 activity were detected when the combination of gemcitabine (1μM) and carboplatin (200μM) was compared with the combination of gemcitabine (1μM) and carboplatin (20μM) or gemcitabine alone (1μM). The same was true for 4 of 5 (AXA, AXC, KISS, and JS) TCC cell lines when the concentration of gemcitabine used was 0.1μM. In 4 of 5 (AXA, KISS, JS, and SH) TCC cell lines, apoptosis in the carboplatin treatment group (200μM) was significantly higher than that for the control treatment group at 24 hours.

Discussion

In the present study, the in vitro antitumor effects of gemcitabine alone, carboplatin alone, and various

combinations of gemcitabine and carboplatin concentrations on 5 TCC cell lines of canine origin were determined. All TCC cell lines had similar dose-dependent decreases in cell proliferation in response to treatment. The IC_{50} for the gemcitabine-only treatment ranged from 0.27 to 0.59μM for the AXA, AXC, KISS, and JS cell lines and was similar to the IC_{50} for in vitro gemcitabine treatment of TCC cells of human origin.[53,57] The effect of gemcitabine on cancer cell lines of canine origin has been evaluated in only 1 other study,[55] in which the IC_{50} of gemcitabine ranged from 5.7 to 15.3μM in 3 of 4 osteosarcoma cell lines (the IC_{50} could not be calculated in the remaining cell line because of resistance), values much higher than those detected in the present study. Although it is impossible to directly compare the IC_{50} results for gemcitabine in the present study to those of other studies because various methods were used, these findings may indicate that TCC cells are more susceptible to treatment with gemcitabine administered alone than are other tumor types.

Carboplatin administered alone had low IC_{50} values in the present study. For all TCC cell lines evaluated, the IC_{50} was lower than the Cmax (approx 250μM) achieved in dogs at the recommended dose (300 mg/m^2, as an IV bolus).[58] In fact, the carboplatin concentration we planned to use in combination with gemcitabine in the present study was 250μM but had to be decreased to 50 and 150μM because the 250μM concentration had such a dramatic effect that the changes in cell viability could not be determined when both drugs were used in combination. The IC_{50} for carboplatin in a TCC cell line of human origin was 289.3μM,[53] which was higher than that for any of the cell lines of canine origin evaluated in the present study.

Because of the potential for resistance of TCC cells to single-agent chemotherapy, a treatment combination of gemcitabine and carboplatin was evaluated in the present study. Gemcitabine and carboplatin are ideal drugs for use in combination treatment because they have different but complementary mechanisms of action and acceptable toxicity profiles. These 2 chemotherapeutics have synergistic activity for the treatment of a variety of malignancies in human patients, including TCC.[53] Some oncologists consider administration of gemcitabine in combination with carboplatin as the standard-of-care treatment for metastatic TCC in humans.[46] Despite the poor response of TCC of the urinary bladder in dogs to carboplatin treatment alone,[18] it was speculated that response to treatment might be substantially improved if 2 drugs were used that had synergism with each other. In the present study, gemcitabine and carboplatin had a synergistic effect on decreasing cell proliferation in 3 of 5 TCC cell lines and an additive effect on decreasing cell proliferation in the other 2 TCC cell lines evaluated. These results are similar to those of other studies involving TCC cell lines of human origin[53,59] and osteosarcoma cell lines of canine origin.[55]

The combination of gemcitabine and carboplatin has been given to dogs with various types of cancer[27] with modest results; however, the dosage and treatment protocol were extrapolated from human medicine. In that study,[27] only 2 dogs had TCC of the urinary bladder; therefore, the ideal dosage and treatment sequence for gemcitabine and carboplatin could not be determined for dogs with TCC of the urinary bladder. It is possible that better patient responses could be achieved if the ideal treatment protocol for gemcitabine and carboplatin were known for dogs with TCC of the urinary bladder. Several studies[40,60–63] have been conducted to evaluate the pharmacokinetics of gemcitabine in dogs. Results of 2 of those studies[62,63] indicated that 3 mg of gemcitabine/kg will generally achieve a clinically relevant Cmax of approximately 9μM.[62,63] In those studies,[62,63] when 3 mg of gemcitabine/kg was administered IV to dogs, the Cmax ranged from 1.36 to 4.05 μg/mL (4.53 to 13.51μM), and the elimination half-life ranged from 1.38 to 1.75 hours. With the extrapolation of data obtained from studies involving rats[61,63] and the assumption that the distribution ratio for gemcitabine (ie, gemcitabine concentration in the urinary bladder tissue was 4.5% of the drug's Cmax at 24 hours after administration and 0.8% of the drug's Cmax 5 days after administration) would be similar for dogs, a dog treated with 3 mg of gemcitabine/kg, IV (Cmax, 13.51μM), would have a gemcitabine concentration in the urinary bladder tissue of 0.6μM 24 hours after administration and 0.1μM 5 days after administration. If the Cmax of gemcitabine (4.53μM) obtained in another study[62] were used with the same assumptions, the gemcitabine concentration in the urinary bladder tissue would be 0.2 and 0.04μM at 24 hours and 5 days, respectively, after administration. These findings suggest that better response to treatment may be achieved if gemcitabine were administered more frequently than at 7-day intervals (ie, days 1 and 8 in a 3-week cycle), a protocol that was used in another study[27] involving dogs. In the present study, the IC_{50} of gemcitabine for most of the TCC cell lines evaluated suggested that a gemcitabine dose < 3 mg/kg IV might be effective for the treatment of TCC of the urinary bladder in dogs. However, a limitation of the present study was that it was conducted in TCC cells in vitro, and the drug exposure times may not mimic the pharmacokinetics of these drugs in vivo. Studies conducted in vivo are recommended to determine whether lower, more frequently administered doses of gemcitabine are a viable treatment option for TCC of the urinary bladder in dogs.

Results of the study reported here did not indicate a consistent effect for sequence of gemcitabine and carboplatin administration on TCC cell proliferation; however, results of other studies[53,55] indicated there was a significant association between sequence of drug administration and tumor cell proliferation. In the present study, 2 cell lines (AXC and KISS) had more substantial decreases in cell proliferation when the gemcitabine and carboplatin were administered simultaneously, whereas 2 other cell lines (AXA and SH) had more substantial decreases in cell proliferation when gemcitabine was administered 4 hours before carboplatin. This decrease in cell proliferation observed following the simultaneous administration of gemcitabine and carboplatin or administration of gemcitabine before carboplatin was similar to results obtained from another study[53] conducted in vitro with a TCC cell line of human origin. In another study,[55] cell proliferation was decreased in

3 of 4 osteosarcoma cell lines of canine origin when carboplatin was administered before gemcitabine. It has been hypothesized that gemcitabine and platinum compounds have synergistic effects because platinum compounds induce DNA damage in dividing tumor cells and because gemcitabine inhibits ribonucleotide reductase, which is required for DNA repair.[64–66] Also, gemcitabine becomes incorporated into DNA during the repair process, which further inhibits DNA replication and repair. Thus, it has been suggested that when a combination treatment of gemcitabine and a platinum compound is used, the gemcitabine should be administered before the platinum compound to ensure a sufficient amount of gemcitabine is present intracellularly prior to the induction of DNA damage caused by the platinum compound.[53]

The findings of the present study are consistent with the cell-killing mechanisms of gemcitabine and carboplatin. The primary mechanism by which carboplatin kills cells is the 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 DNA strands and terminates DNA replication.[67,68] It is hypothesized that if carboplatin is administered prior to gemcitabine, the carboplatin will induce DNA damage, which will then arrest the cell cycle and decrease the opportunity for gemcitabine triphosphate to become incorporated into the DNA, thereby mitigating the cytotoxic effects of gemcitabine.[53] The incorporation of gemcitabine triphosphate in DNA may result in structural changes that favor the binding of platinum drugs, and repair of the platinum-DNA adducts is inhibited by gemcitabine.[66] Furthermore, platinum compounds inhibit ribonucleotide reductase and further enhance the incorporation of gemcitabine triphosphate into DNA. Finally, the primary method by which platinum-DNA adducts are removed is via nucleotide excision. Gemcitabine might reduce the effectiveness of nucleotide excision repair by the inhibition of ribonucleotide reductase.[53]

In the present study, there was a dose-dependent increase in the proportion of dead cells (cells in the sub-G0–G1 phase) and increased caspase 3/7 activity for all TCC cell lines evaluated, especially when gemcitabine and carboplatin were administered in combination. Generally, the combination treatment of gemcitabine and carboplatin resulted in a proportion of dead cells that was several times as high as that achieved when either drug was administered alone, which provided another indication of synergism between the 2 drugs. The small increase in the proportion of dead cells 24 hours after administration of either gemcitabine alone or carboplatin alone, compared with the proportion of dead cells at the time of administration, was similar to results obtained from studies[53,57] that involved TCC cell lines of human origin. Results 24 hours after treatment provide the initial impression that TCC cells are resistant to these drugs. However, in the present study, the proportion of dead cells was substantially increased 72 hours after administration of carboplatin, compared with that at the time of administration. Similarly, there was no significant change in caspase 3/7 activity in TCC cells at 24 hours after carboplatin administration, compared with caspase 3/7 activity in cells that received no treatment (control). The reason for this may be that platinum compounds do not cause a decrease in cellular respiration until many hours after administration.[69] However, cisplatin-treated cells had a much higher increase in caspase 3/7 activity from the time of administration to 22 hours after administration, compared with that for carboplatin-treated cells.[70] Caspase activation leads to impaired cellular respiration and decreased production of ATP; thus, mitochondria are rapidly affected by activated caspases.[70] Mitochondrial dysfunction does not develop until > 12 hours after carboplatin administration,[70] which may be the reason there was no significant difference in caspase 3/7 activity in TCC cells at 24 hours after administration of carboplatin, compared with that for TCC cells that received no treatment. The modest increase in caspase 3/7 activity of cells that were treated with gemcitabine alone is also consistent with results of a study[58] that involved use of a TCC cell line of human origin. The magnitude of the caspase 3/7 activity was much higher at 48 and 72 hours after administration of gemcitabine, compared with that at 24 hours after gemcitabine administration.[57] In the present study, the mechanism for the significant increase in caspase 3/7 activity in TCC cells after administration of both gemcitabine and carboplatin, compared with caspase 3/7 activity following administration of either drug alone, is unclear.

Results of the present study indicated that gemcitabine has antitumor effects when administered to TCC cell lines of canine origin in vitro and that these effects were enhanced when gemcitabine was administered in combination with carboplatin at concentrations that were biologically relevant. These results provide baseline information for future studies to evaluate the effectiveness of in vivo treatment with gemcitabine alone or in combination with carboplatin for the treatment of TCC in dogs.

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d. Eli Lilly, Indianapolis, Ind.
e. Hospira Inc, Lake Forest, Ill.
f. Molecular Probes Inc, Eugene, Ore.
g. Elisa plate reader, Molecular Devices, Sunnyvale, Calif.
h. CompuSyn, version 3.0.1, ComboSyn Inc, Paramus, NJ.
i. Sigma-Aldrich, St Louis, Mo.
j. Beckton-Dickinson dual laser (448nm and 635 nm) FACSCalibur system, BD Biosciences, San Jose, Calif.
k. Modfit LT software, version 3.2, Verify Software House, Topsham, Me.
l. SensoLyte Homogenous AMC Caspase-3/7 Assay Kit, AnaSpec, San Jose, Calif.
m. Prism, version 5.0, GraphPad Software Inc, La Jolla, Calif.

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