Introduction

While bladder cancer is relatively uncommon in dogs (2% of all malignancies), it is a difficult disease to diagnose, stage, and treat. Transitional cell carcinoma (TCC) is the most common histological type of bladder cancer described in dogs, and canine TCC has been proposed as a good model of human invasive bladder cancer. Treatment options for TCC include surgery, radiation therapy, chemotherapy, and cyclooxygenase inhibitors. Chemotherapy is considered the main treatment option in dogs with nonresectable or metastatic TCC. Chemotherapeutic options include cisplatin, edecloreoplatinum, carboplatin, mitoxantrone, and doxorubicin. Only 12% to 24% of treated dogs have a response to treatment, and median survival typically does not extend past one year. Further investigation into novel treatment options is warranted if improved survival is to be realized.

Gemcitabine (2’-deoxy-2’,3’-difluorodeoxycytidine, dFdC) is an analog of deoxycytidine, one of the four bases of DNA. This fraudulent nucleoside has been shown to have broad spectrum of activity against human epithelial malignancies. The primary mechanism of action is incorporation into the genome of tumor cells undergoing DNA synthesis during 5 phase or repair, which causes growth phase (G1) and leads to apoptosis.

Gemcitabine use in veterinary medicine has not been well studied. An in vitro study showed gemcitabine alone had antitumor effects on canine TCC cell lines, and the combination of gemcitabine with carboplatin had synergistic effects. An in vivo study of 38 dogs with TCC showed administration of gemcitabine and piroxicam improved clinical signs for all treated dogs. Twenty-six percent of dogs partially responded to treatment and the median survival time was 230 days. While this study did not improve long-term survival, gemcitabine dosing had fewer adverse effects when compared to other treatment options. Despite these encouraging pre-clinical and clinical responses, the optimal dosing of gemcitabine in canine TCC has not been determined. Because gemcitabine is a small, uncharged molecule and is rapidly undergoing biotransformation at the cellular level, it will improve our understanding of this potentially clinically useful therapeutic molecule and may help to guide its clinical application.

Objectives

1. Determine and quantify the effects of dFdC on cellular survival and proliferation in both a time- and concentration-dependent manner.
2. Quantitate the intracellular accumulation and elimination of dFdCTP, the active metabolite.
3. Quantificate the formation of dFdCMP-DNA in a time- and concentration-dependent manner.

Methods

Two canine TCC cell lines (TCC and AKC) maintained at 37°C with 5% CO2 in MEM X medium supplemented with 15% fetal bovine serum, nonessential amino acids, penicillin, streptomycin, and sodium pyruvate (sMEM) were used in this study.

Effect of gemcitabine on cell survival and proliferation – A clonogenic assay was used to assess the long term effect of varying concentrations of gemcitabine on cell survival and proliferation. TCC cells (2,500 cells/well) were seeded in 6-well plates with 2 mL sMEM and incubated for 24 hours at 37°C and 5% CO2 to allow adhesion to the plastic. Gemcitabine (0, 1, 3, 10, 30 mM) was added to the wells, and plates were incubated for an additional 4 hours. The drug-containing media was then replaced with warm drug-free media and plates incubated for an additional 10 days to allow colonies to form. At the conclusion of the incubation, the media was removed, cells were washed with DPBS 1X, fixed with methanol, and stained with crystal violet. Semi-quantitative analysis of colony numbers was performed with AlphaView software.

An MTS assay was done to quantify the short term effect of gemcitabine on cell survival and proliferation. TCC cells (500 cells/well) were seeded in 96-well plates with 100 μl sMEM and incubated for 24 hours as described above. Gemcitabine (0, 1, 3, 10, 30 mM) was added to the wells, and plates were incubated for an additional 4 or 24 hours. The drug-containing media was then replaced with warm drug-free media and plates incubated for an additional 5 days. MTS reagent was added to samples after the incubation period, and was used as an indirect measurement of cellular viability. The amount of MTS reagent conversion was quantified by use of a spectrophotometer.

Intracellular accumulation and elimination of dFdCTP – Cells were incubated at various concentrations of the drug for 4 hours, 1, 3, 10, 30 mM and one concentration of drug (10 μM) for 24 hours. At 24 hours, aliquots were taken and the cells pelleted. Nucleotides were extracted from the cell pellet using the perchloric acid method and analyzed by high performance liquid chromatography (HPLC). The intracellular concentrations of nucleotides and dFdCTP in the extracts was calculated from a given number of cells of a known volume. This calculation assumed the nucleotides were uniformly distributed in the total cell volume. The lower limit of sensitivity of this assay was 10 pmol in an extract of 5 x 105 cells, corresponding to a cellular concentration of 1 μM.

To determine the elimination of dFdCTP, cells were incubated with the dFdC for 24 hr. At the end of incubation, cells were washed, and re-supplied with drug-free media. At various times, aliquots were taken and nucleotides were analyzed using HPLC as described above.

Figure 1: Clonogenic Survival of TCC after 4 hour exposure to gemcitabine.

Figure 2: MTS of TCC and AKC after 4 and 24 hour exposure to gemcitabine.

Future Directions

• Intracellular accumulation and elimination of the activated metabolite (dFdCTP) will be determined using HPLC.
• DNA incorporation of the active metabolite will be confirmed and quantitated using LC/MS/MS.
• Optimal in vivo dosing of gemcitabine will be predicted.
• An in vivo clinical trial will be conducted guided by data from this project.

Discussion

Colony formation was inhibited following four hours of gemcitabine treatment and ten days of incubation for TCC cell line. Colony density decreased as gemcitabine concentration increased. IC50 was between 10 μM and 30 μM. Similar results were obtained for AKC cell line (data not shown). Experiments were completed twice in triplicate.

In the present study, the in vitro effects of gemcitabine were determined at various concentrations. Both cell lines were shown to be sensitive to gemcitabine at clinically relevant and achievable concentrations (10 μM – 30 μM) and exposure times (4 hours).

The findings in this study are consistent with previously reported studies in vitro human cell lines. As gemcitabine works as a nucleoside analog, it must first be incorporated into the DNA where it can halt DNA replication. Therefore, gemcitabine can affect cells only as they replicate (S-phase). The doubling times of these cell lines have been reported (TCC 24 ± 0.7 hours, AKC 36 ± 2.9 hours).

The clonogenic assay was useful in observing long-term (10 day) cellular proliferation following a one-time, short-duration (4 hour) exposure to gemcitabine. By studying colony formation 10 days after treatment, gemcitabine was given enough time to affect cells attempting to replicate.

The MTS assay was used to quantify these effects. Because of the relatively slow replication rate of these two cell lines, inhibition of proliferation was not evident between 24 and 96 hours after 4- or 24-hour exposure with the MTS (data not shown). Five days following treatment, gemcitabine affected cellular proliferation and this was significant. Longer post-incubation periods were not able to be used as there was overgrowth of cells in the control wells.

Results from this ongoing study will help to better understand the relationship between drug exposure, incorporation into DNA, efficacy, and toxicity at the cellular level with a hope to translate these bench-top findings to clinical care-side application.

References

6. Creation of a 36” x 48” clinical poster. You can use it to create your research presentation poster. This PowerPoint 2007 template produces a 36”x48”...