THESIS

GLUCOSE TRANSPORTER-1 EXPRESSION AND THE ANTIPROLIFERATIVE EFFECTS OF 2-DEOXY-D-GLUCOSE IN OSTEOSARCOMA MODELS

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY JANET C. LORI ENTITLED GLUCOSE TRANSPORTER-1 EXPRESSION AND THE ANTIPROLIFERATIVE EFFECTS OF 2-DEOXY-D-GLUCOSE IN OSTEOSARCOMA MODELS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

GLUCOSE TRANSPORTER-1 EXPRESSION AND THE ANTIPROLIFERATIVE EFFECTS OF 2-DEOXY-D-GLUCOSE IN OSTEOSARCOMA MODELS

Osteosarcoma (OSA) is the most common bone tumor in the dog, more common in large to giant breed dogs. 90% of dogs diagnosed with OSA will die of metastatic disease within one year of diagnosis. There have been no great advances in therapy for canine OSA over the last 20 years. Hypoxia in tumors has been associated with an increased resistance to radiation and chemotherapy, and increased metastatic potential. Hypoxia-inducible factor 1-α (HIF-1α) is a transcription factor stabilized by hypoxia. Glucose transporter 1 (GLUT-1), a downstream product of HIF-1α pathway activation, is over-expressed in a variety of human tumors. We sought to determine if GLUT-1 is expressed in canine OSA and if expression is related to tumor necrosis and outcome. Immunohistochemistry was performed on 44 histologically confirmed OSA tissue samples to assess expression of GLUT-1. Of 44 cases, 27 (61%) expressed GLUT-1. There was no statistical correlation between GLUT-1 and disease-free interval, survival time, or percent necrosis. As hypothesized, GLUT-1 is present in most canine

appendicular OSA. A more objective evaluation of GLUT-1 and other proteins in the HIF-1α pathway may be warranted.

Some cells within a tumor may be poorly perfused, and therefore less susceptible to traditional chemotherapy. Cancer cells, especially those hypoxic cells that are distant from the stromal blood vessels, require more glucose than normal cells as they utilize anaerobic glycolysis, rather than oxidative phosphorylation, to survive. 2-deoxy-D-glucose (2-DG) is a glucose analog that is preferentially captured by cancer cells, blocking the first step of glycolysis. We evaluated the sensitivity of various OSA (canine and murine) cell lines to 2-DG, and attempted correlation to the protein GLUT-1 with western analysis. There was no statistical correlation between 2-DG and GLUT-1 or Akt expression, although it did correlate with total ERK expression. In a murine OSA model, 2-DG was shown to inhibit metastasis, possibly through the inhibition of invasion and migration, as assessed by Boyden chamber assays *in vitro* using the same OSA murine cell line.

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Chapter 1: Literature Review

Canine Osteosarcoma-Biology and Therapy

Osteosarcoma (OSA) is the most common bone tumor in the dog, encompassing 85% of all skeletal malignancies.[1-3] There are approximately 8,000 cases recognized annually. It is more common in middle-aged to older dogs, and mostly of large and giant breeds. Approximately 75% of cases occur in the appendicular skeleton in the metaphyseal regions of the long bones.[1,3] The front limbs are most commonly affected, with the distal radius and proximal humerus being the most frequently reported sites.[4] The most common presenting clinical sign is lameness of the affected limb, and possibly swelling of the site.[3] Radiographs are the most useful diagnostic tool, with changes ranging from mostly bony lysis to almost purely osteoblastic.[5] A diagnosis may be suspected using a combination of patient signalment, clinical signs, and radiographic changes, although tissue biopsy is the gold standard for diagnosis.[3]

Osteosarcoma is a mesenchymal tumor, which produces an extracellular matrix of osteoid. This matrix is the basis for histopathological diagnosis.[3] The etiology of the disease in generally unknown. There have been associations with metallic implants and previous fracture sites.[6-8] Osteosarcoma is characterized by very aggressive local invasion as well as distant metastasis, with 90% of cases having micrometastatic disease at the time of diagnosis.[3] The standard of care therapy is amputation or limb salvage

followed by adjuvant chemotherapy. Reported survival times of dogs with OSA with standard of care therapy range from 262 to 540 days, with approximately 30 to 50% of dogs alive at one year. Most dogs die of metastatic disease.[2,3,9-11] There have been no significant advances in therapy of this disease in the last 20 years.

Hypoxia and Hypoxia Inducible Factor-1α (HIF-1α)

Hypoxia is a condition that exists in tissue when there is a reduced O_2 availability or O_2 partial pressures are decreased below critical thresholds. This condition limits or even eliminates the functions of different organs, tissues or cells.[12] The metabolism that is present within a solid tumor is vastly different from that of normal tissues. The most recognized reasons for this alteration are low levels of O_2, acidosis and increased interstitial fluid pressure, which are largely the result of poorly formed blood vessels within the tumor.[13] Thus, in tumors, hypoxia generally is a consequence of disruptions of circulation and the decline of diffusion conditions.[12] There are two types of hypoxia seen in tumors: acute and chronic. Chronic hypoxia results from the limited diffusion distance of oxygen through the tissues, while acute hypoxia results from the temporary occlusion of a blood vessel, most notably from malformed vessels. Both forms drive malignant progression.[14]

The presence of hypoxia in human tumors has been shown to correlate with a worse prognosis. Hypoxia has been associated with an increased resistance to radiation and chemotherapy, as well as an increased metastatic rate.[12,15] In the absence of oxygen, damage produced by the indirect actions of ionizing radiation may be repaired. Hypoxia can also confer chemoresistance from fluctuations in blood flow, drug diffusion distance,

and decreased proliferation. In addition, some antineoplastic agents which damage DNA, such as doxorubicin, become less efficient in hypoxic environments due to a decrease in oxygen-derived free-radical generation.[14] The association of hypoxia with a poor prognosis may be due in part to expression of proteins that are modulated via the Hypoxia Inducible Factor-1α (Hif-1α) pathway.

Hif-1 is a transcription factor that is up-regulated in hypoxic conditions, and whose downstream protein products contribute to tumor survival in the harsh micro-environment. Hif-1 is a heterodimer composed of the Hif-1α and Hif-1β subunits. Hif-1α is constitutively expressed and destabilized in the presence of O_2 by proline hydroxylation. It is then targeted for proteosomal degradation by the von Hippel-Lindau (VHL) ubiquitin ligase. Under conditions of low oxygen, proline hydroxylation is inhibited.[16-21] Hif-1 is stabilized in low oxygen conditions, and the Hif-1complex moves to the nucleus where it binds to hypoxia response elements (HREs) in the promoter region of target genes, thus promoting their activation.[17] The Hif-1α subunit and its ability to activate transcription are regulated by cellular oxygen concentrations, as well as oncogene activation and loss of tumor suppressors.[13] There are approximately 30 target genes that have been identified to be activated by Hif-1, including vascular endothelial growth factor (VEGF), glucose transporters-1 and 3 (GLUT-1; GLUT-3), and transferrin.[22] There are a wide variety of markers of hypoxia in tissues, including 2-nitroimidazole, carbonic anhydrase 9, GLUT-1, GLUT-3 and HIF-1α.

Glucose Transporter 1 (GLUT-1)

Glucose transporter 1 (GLUT-1) is a facilitative glucose transporter whose expression is regulated through activation of the HIF-1a pathway.[12] Hypoxia increases transcription of GLUT-1, to mediate glucose transport across the cell membrane. The expression level of GLUT-1 typically correlates with the rate of cellular glucose metabolism.[23] There is a family of glucose transporters, ranging from GLUT-1 to GLUT-12. These are all responsible for passive transport of glucose and the first 4 are most widely characterized. GLUT-1 and GLUT-3 have been more heavily studied in tumors.

GLUT-1 is expressed in normal human tissues, including red blood cells, placental trophoblasts, endothelial cells of the brain, Kupffer cells of the liver, and perineurium.[24] In humans, GLUT-1 has been shown to be present in neoplastic tissues, including carcinomas of the colon, lung, liver, stomach, esophagus, breast and skin.[24-30] In tumors, GLUT-1 can be up-regulated in response to hypoxia, activated oncogenes, or loss of tumor suppressor genes. This allows hypoxic tumor cells that are distant from stromal blood vessels to generate ATP via anaerobic glycolysis, which requires increased amounts of glucose when compared with oxidative phosphorylation. Increased expression has been shown to correlate with a worse prognosis in several tumor types. In colorectal carcinoma, GLUT-1 expression correlates with a higher incidence of lymph node metastasis and is a negative prognostic factor, while in liver vascular tumors (hemangioma and angiosarcoma) in children, expression has been reported to correlate with higher cell proliferation and mortality rates.[25,27,29] More recently, GLUT-1 has been employed as a molecular marker to indicate the degree of hypoxia experienced by

tumors. This was performed by measuring GLUT-1 mRNA in the blood of colorectal carcinoma patients and correlating this to tumor hypoxia. An increase was noted in advanced stage versus early stage patients.[31] GLUT-1 expression has not been evaluated previously in canine tumors, nor has it been evaluated in human OSA.

The "Warburg Effect" and 2-deoxy-D-glucose

Warburg proposed in the 1920's that tumor cells are dependent on glycolysis to support their metabolic requirements, as they have lost their ability for oxidative phosphorylation. This was noted to occur even under conditions of normal oxygen tension.[32] This phenomenon became known as the Warburg effect. The Warburg effect was highly controversial during the 1950's, although more widely accepted today.[33-36] Glycolysis is a much less efficient process than oxidative phosphorylation, which implies that tumor cells are dependent on large amounts of glucose to generate energy and support metabolic functions.[37]

The Warburg theory proposed that this shift was caused by defects in "respiration" in the mitochondria. Evidence for defective respiration is scant, but Thompson's model links the Warburg effect to mutations in the pathways that govern glucose uptake, mainly activation of the Akt kinase pathway.[33] Akt is a serine/threonine kinase which can promote cell survival, but also has additional functions which can affect the biology of transformed cells. Akt often has constitutive activity in cancer cells.[38] The Akt oncogene is associated with enhanced glucose uptake and aerobic glycolysis with no associaton with the actions of Hif-1. Akt brings glucose transporters to the cell surface where hexokinase 2 is activated to trap the glucose intracellularly. In this way,

Akt enhances the rate of glycolysis with no affect on oxidative phosphorylation by the mitochondria. Akt's effect on glucose metabolism reiterates Warburg's 1924 theory.[38,39] Thompson's theory views the Akt kinase pathway as the main driver of the Warburg effect.

Dang et.al., another proponent of metabolism as a strong driver of carcinogenesis, showed that another oncogene, *myc* can activate glycolysis. He proposes that a shift to glycolysis is necessary for tumor development.[33,39] The challenge in proving that these changes are causative rather than secondary effects of cancer, is demonstrating that these changes are not simply an adaption to hypoxia.[33] From a treatment perspective, whether enhanced glycolysis is a cause or by-product of cancer is of less relevance, as it is vital for survival of the cancer cell. Previous work has also shown a relationship between HIF-1 activity and p42/44 mitogen activated protein kinases (p42/44 MAPK; ERK1/2), showing that cell signaling is critical for HIF-1 activation in response to hypoxia.[40] MAPK's, like Akt, are involved in cell signaling, and have important functions in cancer.

Aggressive tumors often have an increased dependency on the glycolytic pathway. This results in a more acidic environment due to the conversion of lactate to pyruvic acid. This local acidosis leads to invasion and metastasis through the inhibition of gap junction conductance, and activation of metalloproteinases promoting degradation of the extracellular matrix and basement membranes.[23,41-45] Ptitsyn showed through evaluation of expression profiles of colorectal and breast cancer tumor panels, that one of the most strongly and consistently altered pathways in metastatic cancer involves glucose utilization, specifically up-regulation of genes in the glycolytic pathway and down-regulation of major components of oxidative phosphorylation.[46] In a second publication

using an alternative method of analysis, oxidative phosphorylation and glycolysis were again found in biological pathways separating metastatic and non-metastatic expression.[47]

Tumor cells' enhanced glucose utilization may be exploited from a treatment standpoint by several strategies. Depriving cells of glucose can be achieved using glucose analogs, which have been found to inhibit glucose metabolism.[48] 2-deoxy-D-glucose (2-DG) has been shown to be effective in inhibiting glucose metabolism and ATP production. It is a structural analog of glucose that is different at the second carbon atom by substitution of hydrogen for a hydroxyl group. It appears to selectively accumulate in cancer cells by metabolic trapping.[49] Hexokinase, which catalyzes the first step of glycolysis, is another target to block glycolysis. A drug which blocks hexokinase, 3-bromopyruvate, has been shown in mice to completely eradicate advanced glycolytic tumors.[50] Analogs of this drug have also been developed for clinical trials.[51]

It has been shown that 2-DG inhibits cell proliferation and stimulates cell death in murine and human tumor cell lines. Also, several studies have shown in murine and rodent tumor models, as well as xenografted human tumors that 2-DG can inhibit tumor growth.[15,52-56] When initially used to treat cancer patients, 2-DG was not successful and was abandoned due to the toxicity seen at the doses required to reach therapeutic levels when given chronically.[57] Later results have been more encouraging, especially when combined with other treatment modalities, and the drug is currently in phase III clinical trials in combination with radiation therapy in people.[58,59] It has also been investigated as an adjuvant to chemotherapy with some success.[60,61] Most chemotherapy drugs target rapidly dividing cells and leave the slower growing hypoxic cells behind. The goal of

using 2-DG along with cytotoxic chemotherapeutic drugs is to more selectively target these hypoxic cells, with less effect on the normally oxygenated cells.

Hypoxia Increases Tumor Cell Sensitivity to Glycolytic Inhibitors and Up-Regulates Glucose Transporters

A major obstacle in the treatment of solid tumors is the presence of slow growing, hypoxic cells, as these are not targeted by traditional therapies. This population of cells is generally found in the core of tumors, as they have less availability to oxygen and rely more on glycolysis for ATP synthesis and survival. Previous studies show that this population of cells, which is considered hypoxic due to decreased oxygen tension, is more sensitive to glycolytic inhibitors such as 2-DG.[15] Hypoxia stabilizes HIF-1, which increases transcription of glucose transporters.[22] Maher et al. recently showed that sensitivity to 2-DG correlates with GLUT-1 expression by evaluating two different lines of pancreatic tumor cells.[62]

Therefore, in the tumor environment which is composed of both well-oxygenated and hypoxic tissues, a combination of a traditional cytotoxic chemotherapeutic drug such as doxorubicin (DOX) with a glycolytic inhibitor such as 2-DG could result in superior anti-tumor efficacy. This has been demonstrated in a human OSA xenograft model.[60] Because canine OSA is a common cancer where novel therapies are desperately needed, evaluation of newer treatment options is imperative.

Project Rationale

The treatment of canine OSA has remained static over the course of many years. The aim of this project was to find markers of increased glucose uptake in canine OSA, by evaluating the presence of GLUT-1, for prognostic value, and as a potential predictor of treatment response to drugs that inhibit glycolysis. Once the presence of the protein was established in OSA, we evaluated the sensitivity of different canine OSA cell lines to 2-DG, and attempted to correlate this sensitivity to the expression of GLUT-1, as well as other markers of cell signaling. Then a mouse model of OSA was created to evaluate the anti-metastatic effect 2-DG as an adjuvant treatment to amputation, in an attempt to find an alternative therapy for canine OSA.

We found that 61% of canine OSA samples expressed GLUT-1 when evaluated by immunohistochemistry. GLUT-1 was not found to correlate with outcome following treatment. The distribution and location of the staining was very similar to that reported in human cancer. The precise role of GLUT-1 in canine tumors is unknown at this time, but further investigation is warranted.

We subsequently showed that canine and mouse OSA cell lines have variable sensitivities to 2-DG. This sensitivity did not correlate with the presence of GLUT-1, or phosphorylated or total Akt, but was correlated with total Erk. The canine OSA cell lines were more sensitive to 2-DG under hypoxic conditions, although this sensitivity did not correlate with the change in GLUT-1 expression in hypoxic conditions. A mouse model for metastatic OSA was developed in which 2-DG was used as an adjuvant therapy after amputation of the primary tumor. This model showed that 2-DG significantly prolonged the time to metastasis, and also inhibited *in vitro* invasion and migration. This drug

should be further evaluated in the dog model, in our quest to find better treatments for canine OSA.

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Chapter 2: Glucose Transporter 1 Expression in Canine Osteosarcoma

Introduction

The presence of hypoxia has been shown to correlate with a poorer prognosis in some human tumors. Hypoxia has been associated with increased resistance to radiation and chemotherapy, as well as increased metastatic potential.[1-3] This may be due to increased expression of proteins mediated by the hypoxia inducible factor-1α (HIF-1α) pathway.[4,5] Hypoxia inducible factor-1α is a transcription factor that is stabilized in hypoxic conditions, and whose downstream protein products contribute to tumor survival in the harsh microenvironment.[5] Glucose transporter 1 (GLUT-1) is a facilitative glucose transporter whose expression is regulated through HIF-1α. Hypoxia increases transcription of GLUT-1, to mediate glucose transport across the cell membrane, allowing hypoxic tumor cells that are distant from patent blood vessels to generate ATP via anaerobic glycolysis.

GLUT-1 is located in some normal human tissues, including red blood cells, placental trophoblasts, endothelial cells of the brain, Kupffer cells of the liver, and perineurium.[6] In humans, GLUT-1 is present in neoplastic tissues, including carcinomas of the colon, lung, liver, stomach, esophagus, breast and skin.[6-12] Increased GLUT-1 expression in several tumor types has been shown to correlate with a worse outcome. In colorectal carcinoma, GLUT-1 expression correlates with a higher incidence of lymph

node metastasis and is a negative prognostic factor,[7,8] while in liver vascular tumors (hemangioma and angiosarcoma) in children, expression has been reported to correlate with higher cell proliferation and mortality rates.[11]

Osteosarcoma (OSA) is the most common bone tumor in the dog, encompassing 85% of all skeletal malignancies. There are approximately 8,000 cases recognized annually. OSA is characterized by very aggressive local tissue destruction and distant metastasis, with 90% of cases having micrometastatic disease at the time of diagnosis.[13] The standard of care is amputation or limb salvage followed by adjuvant chemotherapy. Reported survival times range from 262 to 540 days, with approximately 30 to 50 % of dogs alive at one year, however most dogs die of metastatic disease within two years.[13-17]

To date, GLUT-1 expression has not been evaluated in animal tumors. GLUT-1 has mainly been evaluated in carcinomas in humans. However, there have been no great advances in canine OSA outcome in recent history. Novel therapeutic targets and prognostic factors for canine and human OSA are necessary.

The purpose of this study was to evaluate the expression of GLUT-1 in canine OSA and determine if expression was correlated to outcome in dogs treated with surgery and chemotherapy. Since necrosis indicates a loss of vital cellular functions and thus may be an indirect measure of tumor hypoxia,[3] we also attempted to correlate GLUT-1 expression with tumor necrosis. We hypothesized that GLUT-1 would be expressed in canine OSA and expression would be positively correlated with tumor necrosis and inversely related to disease-free interval and survival time.

Materials and Methods

Patient selection

Forty-four cases of histopathologically confirmed canine appendicular OSA from the Colorado State University Veterinary Medical Center that were seen between 1997 and 1999 were selected. All patients were staged prior to presentation with a complete blood count, serum biochemistry profile, urinalysis, 3-view thoracic radiographs, and ^{99}M-Technicium scintigraphy. All patients were determined to be free of visible metastasis prior to amputation. Treatment consisted of amputation followed by adjuvant doxorubicin starting 14 days post amputation and every 2 weeks thereafter, for a total of 5 treatments, as long as metastasis was not detected. Patients were restaged every 3 months with a complete blood count, serum biochemistry profile, urinalysis and 3-view thoracic radiographs up to 24 months or until relapse. The first re-staging period was at the time of the last chemotherapy, or during chemotherapy if clinically indicated. Thirty of the patients were concurrently enrolled in a clinical trial where they received either a placebo or investigational drug post-chemotherapy.

Immunohistochemistry

At the time of amputation, tumor samples were placed in neutral-buffered 10 % formalin and processed by routine histologic methods. Bone samples were decalcified in an HCl-ethylenediaminetetraacetic acid (EDTA) decalcifying solution (Richard Allen Scientific, Kalamazoo, MI) for variable times (1-5 days) based on the content of the bone. Immunohistochemical staining was performed on an automated stainer (Discovery,

Ventana Medical Systems, Tucson, AZ) using standard technique. Briefly, 4-μm sections were cut and mounted on positively charged slides. The sections were deparaffinized and then rehydrated with descending alcohol concentrations to buffer. Heat induced epitope retrieval with citrate buffer (pH 6.0) for 30 minutes was followed by endogenous peroxidase blocking with 3 % hydrogen peroxide and incubation with the primary antibody at room temperature for 10 hours.

The antibody used was a polyclonal rabbit anti-rat-GLUT-1 antibody raised against a synthetic peptide corresponding to the C-terminus of rat GLUT-1 (Chemicon International, Temecula, CA) at a dilution of 1:500. A prediluted, universal biotinylated secondary antibody and a DAB MAP detection kit (Ventanna Medical Systems, Tucson, AZ) were utilized to detect the immunoreactive complexes. The slides were then counterstained with Meyer's hematoxylin. The specificity of the immunostaining was determined using two control slides with each set of slides stained. The positive control slide consisted of primary antibody applied to canine brain, lymph node, nerve and kidney. Negative controls were the same tissues used for positive controls with the primary antibody replaced by antibody diluent (**Figure 2.1**).

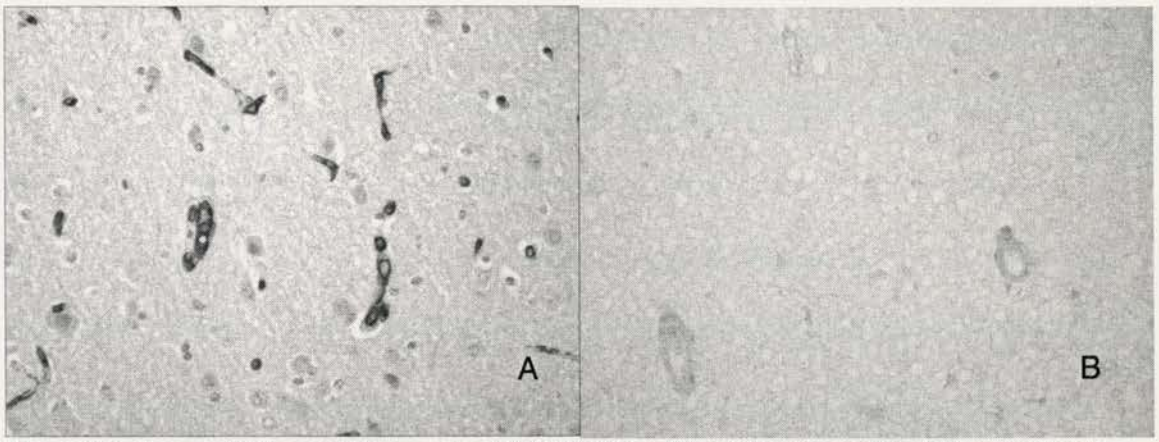

Figure 2.1. Image of Glucose Transporter-1 (GLUT-1) positive (A) and negative (B) canine brain control tissues. In 1A, the endothelial cells of the brain are staining positive (dark brown) for GLUT-1 seen at 20x. In 1B, there is no dark brown staining associated with the endothelial cells of the canine brain.

Western Blot

A western blot was performed to support the specificity and cross-reactivity of the primary antibody with canine tissues. Fresh flash frozen samples of canine lymph node, kidney, whole blood, and brain, were homogenized with RIPA lysis buffer (Santa Cruz Biotech, Santa Cruz, CA) following manufacturer directions. Whole tissue samples were mechanically homogenized in ice cold lysis buffer containing protease inhibitors. The Abrams canine OSA cell line was passaged on plastic in complete minimal essential medium supplemented with 5% fetal bovine serum and 5% newborn calf serum, and seeded in T150 tissue culture flasks. When approximately 75% confluent, one flask was treated with 100 uM desferrioxamine (DFO, Sigma, St. Louis, MO), an iron chelator used to simulate conditions of hypoxia, for 24 hours. Whole cell lysates from each flask were extracted using Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL) to which protease inhibitors (Complete Mini, Roche Molecular Biochemicals, Indianapolis, IN), 1 mM sodium orthovanadate and 1 mM PMSF are added. Sodium

dodecyl sulfate (SDS) was added to make a 1% solution. Samples were centrifuged at 4°C for 5 minutes at 10,000 RPM. Supernatants were loaded to a maximum volume of 40 µL, adding equal amounts of protein to each lane, including 4x sample buffer containing 0.5 M Tris-HCl (pH 8.5), 20% sucrose, 8% SDS (wt/vol), and 0.005% bromophenol blue and reducing agent. Samples were electrophoresed under reducing conditions on a 4-12% Bis-Tris SDS polyacrylamide electrophoresis (PAGE) gel at 200 V for 50 minutes. Separated proteins were transferred to a polyvinylidine fluoride (PVDF) membrane by using Bis-Tris transfer buffer at 30 V for one hour. The membrane was blocked in 5% non-fat dry milk for one hour before incubation in the affinity purified rabbit polyclonal antibody against GLUT-1 diluted in a blocking solution to 1:1000 for one hour. The membrane was washed in 0.1 % Tween 20 Tris buffered saline (TTBS) and then incubated in secondary biotin-conjugated goat anti-rabbit antibody (Rockland Immunochemicals, Gilbertsville, PA) at 1:25,000 for 3 hours followed by washing in TTBS. The membrane was soaked in 10 mL of Super Signal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) for 5 minutes, placed in a plastic sheet protector, and positioned in an autoradiography cassette electrophoresis system where it was exposed to Kodak BioMax light film (Carestream Health France, Paris, France) for 1 minute. It was processed using an automatic film processor.

Scoring Method

Immunoreactivity scoring was performed by one author (JCP). The original scoring system used was similar to those used in human studies.[7] The samples were

evaluated for percentage of tumor cells staining over an average of 5 high-powered fields. The samples were scored 0 through 2, assigned as follows: score of 0=0%; score 1= 1-50%; score 2= >50% positive staining cells. The samples were also evaluated for average intensity of cellular staining. An intensity score of 0 had no staining, 1 had weak staining, and 2 was strong staining as compared to positive control tissues. A product of the 2 scores, a final immunoreactivity score, was assigned. Negative expression (score 0) was a product of 0. Poor to moderate expression (score 1) was a product of 1-2, and strong expression (score 2) was a product of 4.

Necrosis scoring

Thirty-nine of the original hematoxylin and eosin (H&E) stained slides that were evaluated by a pathologist to confirm OSA were also evaluated for percentage of necrosis. Areas of necrosis were characterized by cells with nuclear changes consisting of pyknosis, karyorrhexis, and karyolysis, cytoplasmic changes of eosinophilia and decreased cytoplasm, as well as loss of cell outline or entire cells.[18] Necrosis was scored as a 1=< 10 %; score 2= 11-50 %, and score 3= > 50%.

Statistical analysis

Expression of GLUT-1 in relation to clinicopathologic data was compared. The variables that were evaluated included age, sex, weight, serum alkaline phosphatase, disease-free interval (DFI), survival time (ST), as well as study drug usage. Serum alkaline phosphatase and the study drug were compared to GLUT-1 final scores using multivariate Cox regression. The effect of GLUT-1 on outcome was analyzed by

Kaplan-Meier survival analysis for DFI and ST. Log-Rank *p* values were calculated to compare groups for DFI and ST. Disease free interval was defined as the time between amputation and the detection of metastasis. Survival time was defined as the time between amputation and death. Patients were censored from DFI and ST analysis if they were disease-free/alive at the time of the last recheck, died from another unrelated cause, or were lost to follow-up. A *P* value of less than 0.05 was considered statistically significant.

Results

Clinical Findings

The median age of the dogs was 9 years, with a range of 5 to 15 years. There was 1 intact female, 18 spayed females, and 25 castrated males. The median weight was 32.3 kg, with a range of 19 to 68.4 kg. Twelve of 44 dogs had elevated serum ALP before amputation (normal range 20-142 IU/L). Serum alkaline phosphatase elevation had no significant association with outcome in this study. The median DFI was 190 days with a range of 28 to 1978 days. The median ST was 220 days, with a range of 28 to 1978 days. Thirty-three (75%) of the dogs that were included in the study died of metastatic disease. The remainder of the dogs died of unrelated causes and were censored from survival analysis.

Expression of GLUT-1

Positive control tissues, canine brain, lymph node, nerve and kidney, were immunoreactive for GLUT-1 as has been described in human literature. The brain had

distinct strong staining of vascular endothelial cells (**Figure 2.1**). Of the 44 OSA samples that were examined for GLUT-1 expression, 27 samples (61.4 %) expressed GLUT-1. The staining pattern of tumor cells was mostly membranous, with some staining or light dusting in the cytoplasm, as is characteristic of GLUT-1 immunohistochemistry in human tumor studies (**Figure 2.2**).[6,7] Staining was heterogeneous throughout the tumors. In tumors with a high percentage of necrosis, staining was often greater and more intense at the junction between viable and non-viable tissues (**Figure 2.3**), but staining was not always limited to regions of necrosis. Normal bone tissue was negative for GLUT-1 immunoreactivity (not shown). With respect to final immunoreactivity score, 17 (38.6%) of the 44 samples had a GLUT-1 immunoreactivity score of 0, 24 (54.5%) had a score of 1, and 3 (6.8%) had a score of 2.

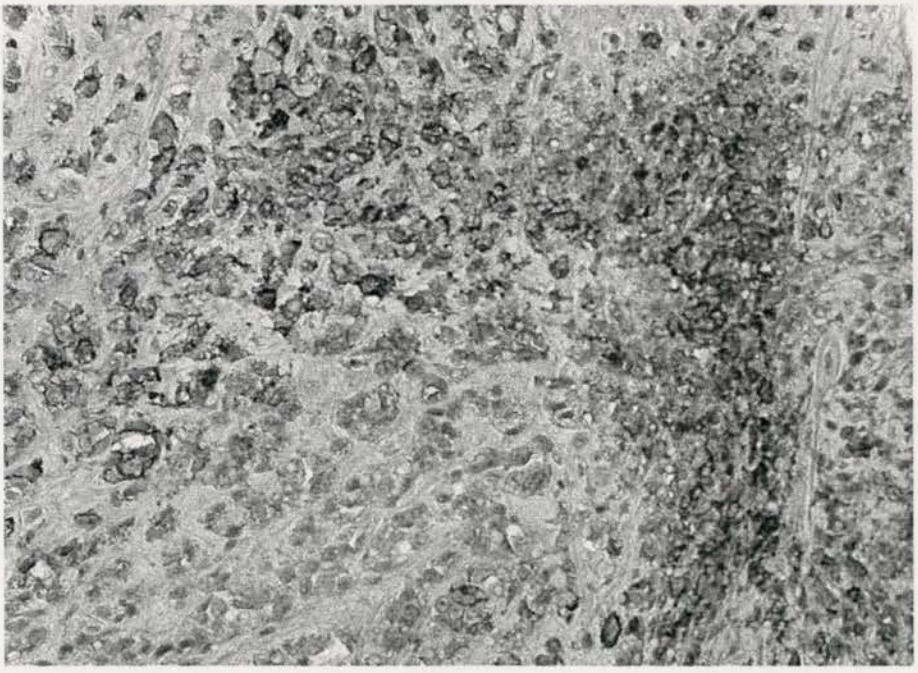

Figure 2.2. Image of Glucose Transporter-1 (GLUT-1) staining with immunohistochemistry and hematoxylin counterstaining at 20x. The dark brown staining that appears cell membrane associated

is consistent with positive GLUT-1 expression. The more intense staining was membranous, although there is staining or light dusting seen in the cytoplasm.

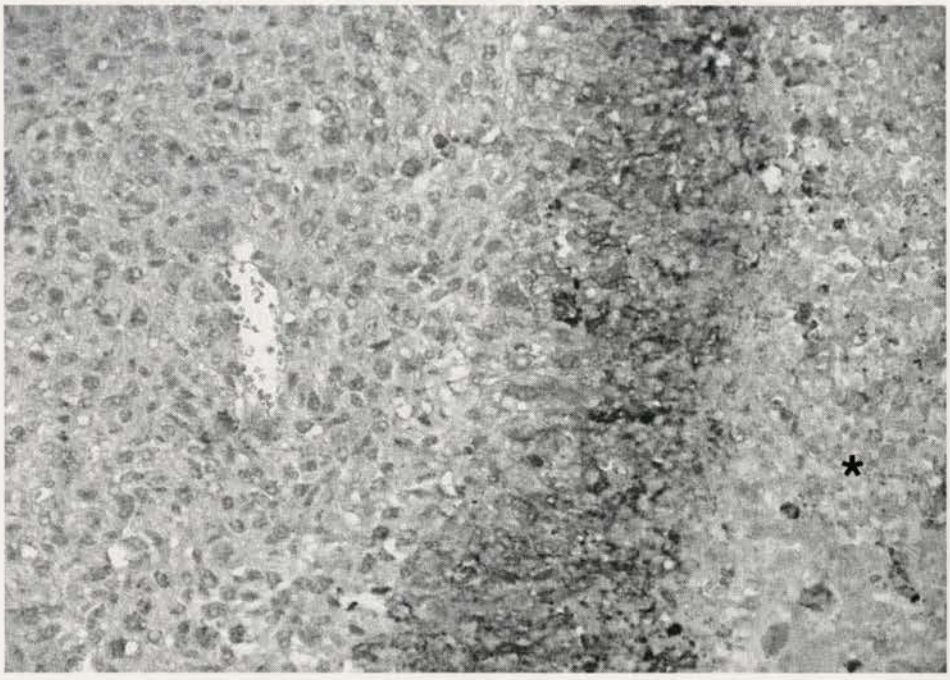

Figure 2.3. Image of Glucose Transporter-1 (GLUT-1) immunohistochemistry at 20x with more intense staining that localizes to the edge of viable and non-viable tissues visualized. (*) labels area of necrosis.

Western Blot

One band of expected size for GLUT-1 was identified in rat brain, canine brain, kidney, whole blood, lymph node, and a canine OSA sample that was incubated with or without DFO to mimic hypoxia (**Figure 2.4**). The DFO-stimulated cells show an up-regulation of GLUT-1 as compared to un-stimulated cells where no GLUT-1 band is seen. The presence of a single band at the expected molecular weight range, and the ability to upregulate expression of GLUT-1 with simulated hypoxia strongly supports the

cross-species specificity of the primary antibody. The bands ranged in size from 43 to 50 kDa, correlating with previous reports of GLUT-1 expression showing a band from 42 to 50 kDa.[19,20] The variation in band size could be explained by species differences, as well as post-translational modifications, such as glycosylation in tissues.

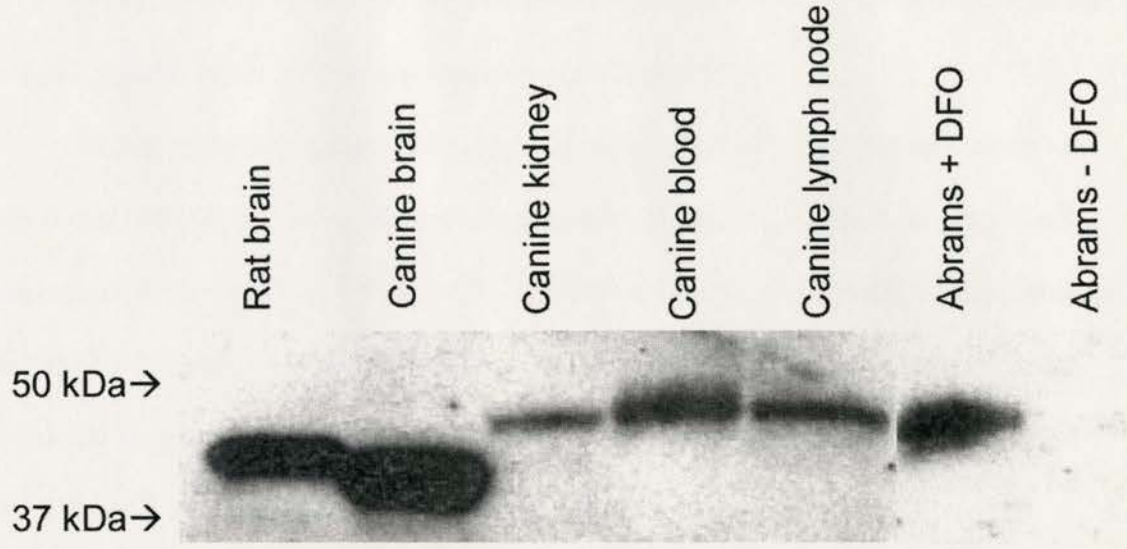

Figure 2.4. Western immunoblot for Glucose Transporter-1 (GLUT-1) with rat brain serving as the positive control. Canine brain, kidney, blood, lymph node and osteosarcoma cell line all show a band in the range of 40 to 48 kDa. The canine osteosarcoma line Abrams is shown with and without desferrioxamine stimulation. The stimulated cell line shows an up-regulation of GLUT-1 as compared to un-stimulated where no band is seen.

GLUT-1 and Percent Necrosis

Thirty-nine of the 44 samples had H&E slides available for evaluation of percent necrosis. There were 10 samples (25.6%) with a score of 1, 17 (43.6%) with a score of 2, and 12 (30.7%) with a score of 3. There was no significant association between percent tumor necrosis and the GLUT-1 percent staining score, the intensity score, or the final

immunoreactivity score. Subjectively, areas of necrosis often demonstrated increased GLUT-1 expression and intensity of staining.

Outcome Assessment

There were no significant correlations between GLUT-1 expression and age, sex, weight, placebo or study drug administration, DFI, or ST.

When using the Kaplan-Meier and log-rank analysis, no significant correlation was found between GLUT-1 percent of cells staining, intensity of cells staining, final immunoreactivity score, and DFI or ST. The DFI and survival curves for the patients in the GLUT-1 immunoreactivity score 1 group were affected by 2 censored values with unusually long DFI and ST (**Figures 2.5 and 2.6**). The *p* value for GLUT-1 and DFI and ST was 0.461 and 0.493, respectively.

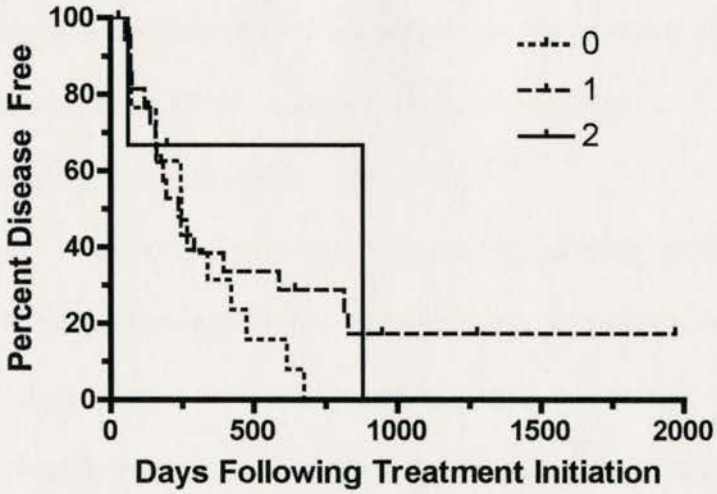

Figure 2.5. Kaplan-Meier curves depicting disease-free interval for canine OSA patients with Glucose Transporter (GLUT-1) final immunoreactivity scores 0, 1, and 2 (P = 0.4606).

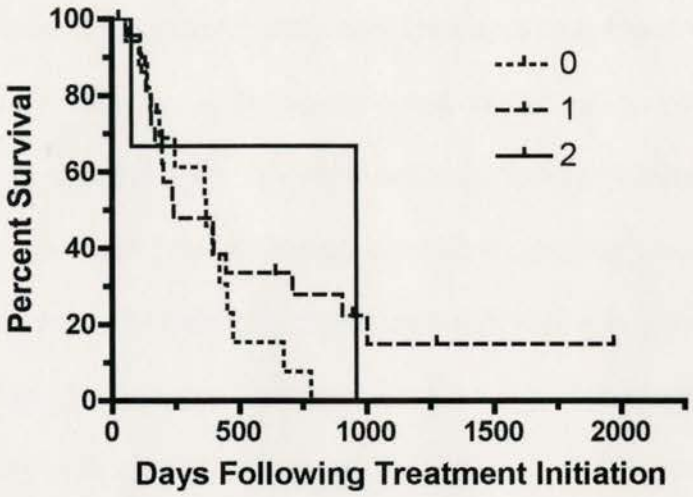

Figure 2.6. Kaplan-Meier curves depicting survival time for canine OSA patients with Glucose Transporter (GLUT-1) final immunoreactivity scores 0, 1, and 2 expression groups (P = 0.493).

Discussion

The results of this study suggest that GLUT-1 is present in a meaningful percentage of canine OSA. This is an important finding, as GLUT-1 expression, to the authors' knowledge, has never been evaluated in canine tumors. There are also no known assessments of GLUT-1 expression in human OSA. In humans, GLUT-1 expression has primarily been reported in carcinomas.[7,9,10]

The primarily membranous staining pattern identified in this study is very similar to what has been reported in human tumors. In humans, red blood cells serve as internal positive controls for identification of GLUT-1. The lack of staining of red blood cells in canines is noteworthy. However, the primary glucose transporter in canine red blood cells, to date, has not been identified in the dog. Furthermore, identification of a protein band with western analysis of the appropriate molecular weight in the blood suggests that

GLUT-1 is a glucose transporter for canine red blood cells, but also may suggest a species and sensitivity difference between western blot and immunohistochemistry.

Also similar to human reports are the high proportion of cases with low to no expression, and the more abundant and intense expression in perinecrotic areas. The high proportion of cases with weak to moderate staining and the low number (6.8%) of the samples with the highest immunoreactivity score could reduce enthusiasm for GLUT-1 as a therapeutic target. However, weak immunohistochemical staining may not correlate with weak expression as assessed by other means. Furthermore, the amount of GLUT-1 necessary to functionally transport glucose is not clear. The more abundant and intense staining in perinecrotic areas may suggest these areas have a greater need for glucose due to hypoxia. However in this study, there was no correlation between GLUT-1 and percentage of overall necrosis within the tumor. This may be due to the fact that necrosis was evaluated on only one small section of tumor, which may not be representative of global tumor necrosis. Evaluation of a larger section of tumor volume or the use of more objective markers of necrosis may have been more accurate and correlated better with GLUT-1 expression. Though necrosis and hypoxia can be correlated, hypoxia may exist independent of necrosis, providing another possible explanation for the lack of correlation between GLUT-1 and percent necrosis in the tumor.

A limitation of this study was the subjective method of measuring GLUT-1. Finding a more objective method, such as quantitative image analysis, could potentially change the results of this study. Additionally, the sample size was small, which may contribute to the lack of significance of the data as well due to small numbers in each group. Hypoxia is a common finding in canine OSA. This is a plausible explanation for

a lack of significance of hypoxia and/or GLUT-1 expression with outcome data, as well as the probability that other factors help to drive the course of the disease.

Hypoxia in human tumors has been associated with a worse outcome, as well as resistance to radiation and chemotherapy.[1-3] The HIF-1α transcription factor, a key regulator of GLUT-1 expression, is one of the most widely studied markers of tumor hypoxia, as are its downstream products; however, HIF-1α expression has not been evaluated previously in canine tumors. Our demonstration of GLUT-1 expression also indirectly suggests that HIF-1α may be an active transcription factor in canine OSA.[5] However, there are other mechanisms of GLUT-1 up-regulation including signaling through cytokines such as TGF-β and IL-1, and others which may contribute to expression of GLUT-1 independent of HIF-1α. In humans, GLUT-1 expression has been positively correlated with increased uptake of ^{18}F 2-fluoro-2-deoxy-D-glucose (FDG) using positron emission tomography (PET).[12,21] A future direction of this study is to evaluate the expression of GLUT-1 in veterinary tumors, and to correlate this data to ^{18}FDG uptake as assessed by PET.

Molecules designed to interfere with glucose transport pathways may show therapeutic promise in hypoxic tumor cells, which may be the most resistant to conventional therapies. Recent studies in mice using human xenograft models of OSA and non-small cell lung tumors have combined glycolytic inhibitors with traditional chemotherapy to target slow-growing, hypoxic tumor centers that are not targeted with traditional radiation or chemotherapy.[22] The expression of GLUT-1 in a majority of canine OSA suggests that therapeutic approaches targeting glycolysis may be useful in the future.

In conclusion, this study demonstrates the presence of GLUT-1 in the majority of canine OSA tumors. GLUT-1 was not found to correlate with outcome following treatment. The distribution and location of the staining is similar to that reported in human cancer. Although GLUT-1 expression co-localized with regions of necrosis in some OSA, its association could not be confirmed statistically with the small sample size evaluated. The precise role of GLUT-1 in canine tumors is unknown at this time, but further investigation is warranted.

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Chapter 3: The Anti-Tumor Effects of 2-Deoxy-D-Glucose in Osteosarcoma Models

Introduction:

Osteosarcoma is the most common bone tumor in the dog, encompassing 85% of all skeletal malignancies.[1-3] Standard of care therapy is amputation or limb salvage followed by adjuvant chemotherapy. Reported survival times of dogs with OSA treated with standard of care therapy range from 262 to 540 days, with approximately 30 to 50% of dogs alive at one year. Most dogs die of metastatic disease.[3-10] There have been no great advances in therapy of this disease in the last 20 years. Because canine OSA is a common cancer where novel therapies are desperately needed, evaluation of newer treatment options is imperative.

GLUT-1, a facilitative glucose transporter whose expression is regulated by the Hif-1α pathway, has been previously shown to be present in canine osteosarcoma.[11] In this study, expression did not correlate with outcome, as in some human tumors.[12-14] The expression level of GLUT-1 generally correlates with the rate of cellular glucose metabolism.[15] Thus, the fact that it is increased in many tumors may indicate a higher glucose requirement in these tissues. Several studies have indicated that glucose transport via GLUT-1 is the rate limiting step in anaerobic glycolysis in various cancers.[16-18]

The Warburg effect proposed that tumor cells are dependent on glycolysis to support their metabolic requirements, as they have lost their ability for oxidative

phosphorylation through mitochondrial defects, even under conditions of normal oxygen tension.[15] Glycolysis is a much less efficient process than oxidative phosphorylation, making tumor cells dependent on large amounts of glucose to generate energy and support metabolic functions.[19] Thompson's model links the Warburg effect to mutations in the pathways that govern glucose uptake, mainly activation of the Akt kinase pathway. The Akt family has a major role in signal transduction, and has been shown to increase transcription of the GLUT-1 transporter, as well as increase the overall rate of glycolysis.[20-22] Aggressive tumors frequently have an increased dependency on the glycolytic pathway. This results in a more acidic environment due to the conversion of lactate to pyruvic acid. Local acidosis leads to invasion and metastasis through the inhibition of gap junction conductance, and activation of metalloproteinases promoting degradation of the extracellular matrix and basement membranes.[15,23-27] Through evaluation of expression profiling of colorectal and breast cancer tumor panels, Ptitisyn showed that one of the most strongly and consistently altered pathways in metastatic cancer is involving glucose utilization, specifically up-regulation of genes in the glycolytic pathway and down-regulation of major components of oxidative phosphorylation.[28]

Tumor cells' increase in glucose utilization may be exploited from a treatment standpoint by several strategies. 2-deoxy-D-glucose (2-DG) has been shown to be effective in inhibiting glucose metabolism and ATP production. It is a structural analog of glucose that differs at the second carbon atom by substitution of hydrogen for a hydroxyl group. It appears to selectively accumulate in cancer cells by metabolic trapping.[29] It has been shown that 2-DG inhibits cell proliferation and stimulates cell

death in murine and human tumor cell lines, as well as inhibits tumor growth in murine and rodent tumor models, as well as xenografted human tumors.[30-35] Most chemotherapy drugs target rapidly dividing cells and leave the slower growing, hypoxic cells behind. The goal of using 2-DG in combination is to more selectively target these hypoxic cells.

The population of slow growing, hypoxic cells are generally found in the core of tumors, as they have less availability to oxygen and rely more on glycolysis for ATP synthesis and survival. Hypoxia stabilizes HIF-1, which increases transcription of glucose transporters.[36] One group, recently showed that sensitivity to 2-DG correlates with GLUT-1 expression in two different pancreatic tumor cell lines.[37] Previous work has also shown a relationship between HIF-1 activity and p42/44 mitogen activated protein kinases (p42/44 MAPK; ERK1/2), showing that cell signaling is critical for HIF-1 activation in response to hypoxia.[38] MAPK's, like Akt, are involved in cell signaling, and have important functions in cancer.

Therefore, in a tumor environment which is composed of both well-oxygenated and hypoxic tissues, a combination of a traditional cytotoxic chemotherapeutic drug such as doxorubicin (DOX) with a glycolytic inhibitor such as 2-DG should result in superior anti-tumor efficacy. This has been demonstrated in a human OSA xenograft model.[39] The purpose of this study was to evaluate 2-DG, and the combination of 2-DG and DOX, in canine OSA, correlate sensitivity to 2-DG with the expression and activation of GLUT-1, Akt, and ERK, and to evaluate 2-DG in an orthotopic OSA murine model of metastasis. We hypothesized that 2-DG will dose-dependently induce cell growth inhibition and apoptosis in OSA, that sensitivity to 2-DG will correlate with glucose transporter, Akt or ERK expression, that 2-DG will enhance cell killing when used in

combination with DOX, and that 2-DG will delay time to metastasis in an orthotopic murine model.

Materials and Methods

Osteosarcoma Cell Culture

In this study, we used 5 established canine OSA cell lines and 1 established murine OSA cell line. The D17 cell line was from American Type Culture Collection (ATCC, Manassass, VA), while the Moresco cell line was developed from a clinical case from the University of Wisconsin-Madison School of Veterinary Medicine. Abrams, Gracie and MacKinley cell lines were previously isolated and cultured from clinical cases treated at The Animal Cancer Center at Colorado State University. The DLM8 cell line was generously provided by Dr. E. Kleinerman (M.D. Anderson Cancer Center) and luciferse-engineered as described.[40] Cells were cultured in complete minimal essential media (MEM) supplemented with 5% fetal bovine serum and 5% newborn calf serum (C5/5 MEM) in adherent T75 plates and incubated in a humidified atmosphere at 37°C in 5% CO_2 until they were at least 75% confluent and serially passaged by trypsinization.

Osteosarcoma 2-DG Growth Inhibition Curves

Cells were seeded in 96 well plates at a density of 2×10^3 $well^{-1}$ in C10 MEM media and incubated at 37°C in 5% CO_2. After 24 h, the media was aspirated, and the wells were treated with C10 MEM with varying concentrations of 2-DG (Sigma-Aldrich, St. Louis, MO), ranging from 100 to 10,000 ug/mL. The cells were incubated at 37°C in 5% CO_2 with the drug for 72 h. With the canine OSA cell lines, half of the samples

were incubated in normoxic conditions, and half in hypoxic conditions. For the hypoxic samples, cells were incubated in a hypoxic chamber controlling the level of oxygen to 2% for the 72 hour period. After 72 h, the bioreductive compound Alamar Blue (Invitrogen Corporation, Carlsbad, CA) was added to the wells at 1/10 of the volume of media, and the plates were read using a fluorescence plate reader according to manufacturer directions. Relative viable cell number was expressed as a percentage of untreated cells and plotted using Prism 5 software (Graph Pad Inc, San Diego, CA). Inhibitory concentration values at 50% (IC_{50}) were calculated for the five cell lines using non-linear regression of the growth inhibition curves created from a mean curve of 4 independent experiments. The growth inhibition percentage at 370 ug/mL was also recorded from the mean curve.

To evaluate 2-DG/DOX combinations, cells were seeded in 96 well plates at a density of 2×10^3 $well^{-1}$ in C10 MEM media and incubated at 37°C in 5% CO_2. After 24h, the cells were treated with 3 concentrations of 2-DG (Sigma-Aldrich, St. Louis, MO), ranging from 0 to 100 ug/mL. Cells were concurrently treated with DOX at concentrations ranging from 0 to 200 ng/mL. Similar to the 2-DG protocol, the cells were incubated at 37°C in 5% CO_2 with the drugs for 72 h. After 72 h, relative viable cell number was determined as above.

GLUT-1 Immunocytochemistry

For immunocytochemistry, the 5 canine OSA cell lines were passaged on plastic in complete C10 MEM, and seeded in chamber slides at a density of 1×10^4 cells per well. The chamber slides were made in duplicate, so that one could be treated in

normoxia (21% O_2) and one in hypoxia (2% O_2). The cells were left in 21% oxygen at 37°C in 5% CO_2 overnight to become adherent. One slide was then placed in each of the above described conditions for 24 hours. After incubation, the slides were rinsed 3 times in 0.1% phosphate buffered saline with Tween 20 (PBST). They were fixed in methanol for 5 minutes at -20°C. There were then rinsed again 3 times in 0.1% PBST. They were permeabilized in 0.2% PBST at room temperature for 10 minutes. They were blocked in 0.1% PBST with 1% BSA for 45 minutes at room temperature. The primary antibody (affinity purified rabbit polyclonal antibody against GLUT-1) was diluted in Tris-Buffered Saline Tween-20 (TBST) at a dilution of 1:500 and incubated at room temperature for 45 minutes. The slides were then rinsed three times in 0.1% PBST. They were incubated with a FITC-conjugated anti-rabbit secondary antibody at a dilution of 1:250 for 1.5 hours at room temperature. The slides were then rinsed three times in 0.1% PBST. One drop of 4',6-diamidino-2-phenylindole (DAPI) was added to each well and a coverslip was placed. The slides were evaluated using a fluorescent microscope.

Western Analysis

Western analysis was performed for GLUT-1, phospho-Akt, phospho-p44/42 MAPK (ERK1/2), total Akt, and total ERK 1/2 expression on the 5 OSA canine cell lines. The 5 cell lines (Abrams, D17, Gracie, MacKinley, and Moresco) were passaged on plastic in C10 MEM, and seeded in T150 tissue culture flasks. The cells were incubated at 37°C in 5% CO_2, with one flask for each cell line at 21% O_2 and one at 2% O_2. When approximately 75% confluent, whole cell lysates from each flask were extracted using Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockford,

IL) to which protease inhibitors (Complete Mini, Roche Molecular Biochemicals, Indianapolis, IN), 1 mM sodium orthovanadate and 1 mM PMSF are added. Sodium dodecyl sulfate (SDS) was added to make a 1% solution. Samples were centrifuged at 4°C for 5 minutes at 10,000 RPM and protein quantified using the BCA Protein Assay Kit. Supernatants were loaded to a maximum volume of 18 µL of liquid per well, adding equal amounts of protein to each lane, including 6x sample buffer containing 0.5 M Tris-HCl (pH 8.5), 20% sucrose, 8% SDS (wt/vol), and 0.005% bromophenol blue and reducing agent. Samples were electrophoresed under reducing conditions on a 4-12% Bis-Tris SDS polyacrylamide electrophoresis (PAGE) gel at 200 V for 45 minutes. Separated proteins were transferred to a polyvinylidine fluoride (PVDF) membrane by using Bis-Tris transfer buffer at 30 V for one hour. The membranes was blocked in various blocking agents according to the antibody used for one hour before incubation in the primary antibody diluted in a blocking solution for one hour (see **Table 3.1** for antibody, manufacturer, concentration, and blocking agent used). The membrane was washed in 0.1 % Tween 20 Tris buffered saline (TTBS) and then incubated in secondary biotin-conjugated goat anti-rabbit antibody (Rockland Immunochemicals, Gilbertsville, PA) at 1:20,000 in 5% non-fat dry milk for 2 hours followed by washing in TTBS. The membrane was soaked in 1 mL of Super Signal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL) for 5 minutes, placed in a plastic sheet protector, and positioned in an autoradiography cassette electrophoresis system where it was exposed to Kodak BioMax light film (Carestream Health France, Paris, France) for 1 minute. It was processed using an automatic film processor. The membranes were stripped using a solution of 2% SDS, 100 mM beta-mercaptoethanol and 6.25 mM Tris

with a pH of 6.8. The membranes were soaked in 25 mL of solution for 40 minutes at 60°C on a shaker. They were then rinsed 3 times with TBST for 5 minutes. Once stripped and rinsed, the membranes were able to be re-probed with β-actin as a housekeeping protein control for equal loading (**Table 3.1**) using the same technique described above.

The western blots for GLUT-1, phospho-Akt, total Akt, phosphor-ERK, total ERK, and the corresponding β-actin blots were analyzed for densitometry using Image J software (National Institutes of Health, Bethesda, MD). Each individual blot lane was analyzed and standardized to the densitometry for the corresponding actin blot, or in the case of the phospho-antibodies, were standardized to the corresponding total blot of the same protein. These numbers were analyzed using linear regression to compare blot intensity to the corresponding IC_{50} and growth inhibition percentages for each cell line.

Table 3.1. Primary Antibodies used in Western Blots and characteristics

Primary Antibody	Manufacturer	Concentration	Blocking Agent
rabbit polyclonal antibody against GLUT-1	Abcam (Cambridge, MA)	1:1,000	5% non-fat dry milk in TBST
rabbit polyclonal against phospho-Akt (Ser473)	Cell Signaling (Danvers, MA)	1:1,000	SuperBlock T20 (PBS) Blocking Buffer (Thermo Scientific, Rockford, IL)
rabbit polyclonal against phospho-p44/42 MAPK (ERK1/2)	Cell Signaling (Danvers, MA)	1:1,000	SuperBlock T20 (PBS) Blocking Buffer (Thermo Scientific, Rockford, IL)
rabbit polyclonal against Akt	BD Pharmingen Biosciences (San	1:1,000	5% non-fat dry milk in TBST

	Jose, CA)		
rabbit polyclonal against p44/42 MAPK (ERK1/2)	Cell Signaling (Danvers, MA)	1:1,000	5% non-fat dry milk in TBST
rabbit polyclonal against β-actin	Abcam (Cambridge, MA)	1:1,000	5% non-fat dry milk in TBST

Caspase 3/7 Apoptosis Assay

The Alamar Blue Assay gives a relative number of viable cells, but cannot distinguish between growth inhibition and cell death. Thus, an assay to quantify activated caspase-3,7 was used to determine the amount of apoptotic cells after treatment with selected concentrations of 2-DG. Five canine OSA cell lines were plated at a density of 5.0×10^4 cells/well in C10 MEM in 6 well plates with each cell line occupying 3 wells. The plates were incubated overnight in 21% oxygen at 37°C in 5% CO_2 to become adherent. Each cell line was treated with 0, 1,000, or 10,000 ug/mL of 2-DG and incubated with the drug for 24 hours. The media was then removed from the treated cells and saved. The adherent cells were trypsinized and added to the tubes of media already pipetted off of each well. The tubes were centrifuged at 1400 rpm for 5 minutes and the pellet was resuspended in 300 uL 1X lysis buffer. This solution was then transferred to 1.5 mL Eppendorf tubes and placed on a rotating apparatus in 4°C for 30 minutes. The cell suspension was the centrifuged at 2500 g for 10 minutes at 4°C. The supernatant was collected into new Eppendorf tubes. Sixty uL of supernatant were added in duplicate to the wells of a 384 well plate for each sample. Then, 20 uL of Caspase-3/7 working solution was added to each well using the Sensolyte Homogenous AMC Caspase 3/7 Assay Kit (AnaSpec, San Jose, CA). The plate was covered with an aluminum foil wrapped lid to protect from the light and placed on a plate shaker at 200 rpm for 60

minutes. The fluorescence was read at 354 nm/442 nm. The fluorescence intensity correlates with the amount of Caspase 3/7 activity.

Orthotopic DLM8 murine model treated with 2-DG

All animal studies were performed in an AALAC-approved facility, with approval of the Colorado State University Institutional Animal Care and Use Committee. Six to eight week old female C3H mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). DLM8-luc-M1 was injected into mice using $2x10^6$ cells per tumor challenge. This cell line was created from the DLM8 cell line.[40] For orthotopic injection, mice were first anesthetized with isoflurane. The surgical site was prepared by first shaving and then cleaning with ethanol. A 23G needle was then used to drill a hole into the proximal tibia. Tumor cells ($2x10^6$) were re-suspended in PBS and injected into the proximal tibia in 50 μL to enhance focal tumor formation. Mice received 0.05 mg/kg of buprenorphine every 12 hours for 72 hours post-tumor challenge to manage pain associated with tumor challenge. The mice were then amputated 16 days post-tumor challenge as determined from previous experiments to ensure that all mice developed metastases. Treatment was then initiated 24 hours post-amputation. Mice (n=5-8 per group) were left untreated or treated with 2-DG (500 mg/kg three times weekly IP) for one month postoperatively. Mice were imaged twice a week after amputation to observe ible Factor 1, alpha Subunit/metabolismtastases. Mice were euthanized immediately upon showing signs of distress due to metastatic disease. Time to metastasis and survival data was analyzed using Kaplan-Meier log-rank analysis. For all analyses, a p-value of less than 0.05 was considered statistically significant.

DLM 8 and Abrams Invasion and Migration Assays

Invasion and migration was assessed using 24-well Boyden chamber assays (BD Matrigel, San Jose, CA). DLM8 cells were prepared in a cell suspension of Complete Minimal Essential Media with 0.1% fetal bovine serum (C0.1 MEM) containing 2.5×10^5 cells/mL for the invasion assay and 2.0×10^5 cells/mL for the migration assay. The positive controls were cells in C0.1 media in the inserts and C10 EMEM in the wells. The negative controls consisted of the cells suspended in C0.1 in the inserts and C0.1 in the wells. Three concentrations of 2-DG (10, 100, and 1,000 ug/mL) were added to the cell line suspended in C0.1 MEM at the previously described cell densities for the invasion and migration assays. Conditions were made in duplicate.

Wells were incubated for 22 hours in a tissue incubator at 37°C and 5% CO_2. The non-invading cells were removed by inserting a cotton-tipped swab into the insert with firm, but gentle pressure while moving the tip over the membrane surface. This was repeated a second time with a cotton-tip moistened with media. The cells on the lower surface were stained with Diff-Quik and the bottoms of the inserts were cut out using a scalpel blade and applied to a microscope slide. Inserts were evaluated with a microscope at 40x magnification. Each insert was counted in 10 random fields. Numbers for each insert were calculated by averaging fields. Statistical analyses were performed by parametric one-way ANOVA with Bonferroni.

Results

2-DG Inhibits Growth in Canine and Murine Osteosarcoma

The 5 canine OSA cell lines demonstrated variable sensitivities to 2-DG. Growth inhibition curves from the 5 OSA cell lines are shown below (**Figs. 3.1-3.6**). Figures 1a and 1b group the 5 cell lines according to those treated in normoxic **(Fig. 3.1a)** and hypoxic **(Fig. 3.1b)** conditions to show the varying sensitivities among the cell lines. **Figures 3.2-3.6** are for the individual 5 canine OSA cells lines and show that at 2% O_2 the cells are more sensitive to 2-DG as evidenced by the curve having a steeper slope. Non-linear regression was used to generate IC_{50}'s. These are shown in **Table 3.2**. There was extreme variation between the cell lines with regard to drug sensitivity, with Gracie being the most sensitive with an IC_{50} of less than 100 ug/mL and Abrams being the least sensitive with a value of 7993 ug/mL. All of the canine OSA cell lines, with the exception of MacKinley, showed an increased sensitivity to 2-DG, as evidenced by the decreased IC_{50} and increased % growth inhibition at 370 ug/mL in hypoxic conditions. **Table 3.3** outlines the percent growth inhibition at 370 ug/mL for the 5 cell lines. **Figure 3.7** is the growth inhibition curve for the murine OSA cell line DLM8 in normoxic conditions treated with 2-DG.

Figures 3.8-3.12 show the results of the growth inhibition curves for the five canine OSA cell lines for concurrent treatment with 2-DG and DOX. The two drugs appear to have additive effects for each cell line.

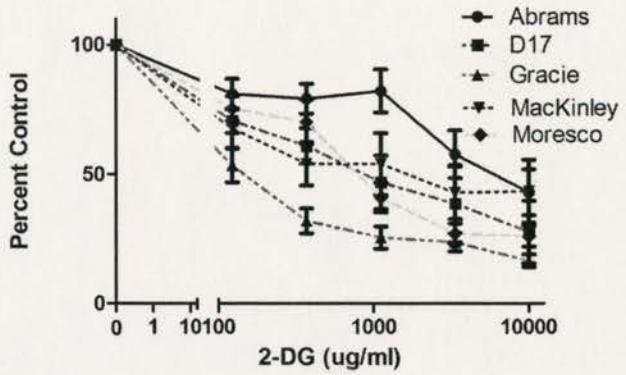

Figure 3.1a. 5 OSA cells lines in normoxic (21% O_2) conditions treated with various concentrations of 2-DG

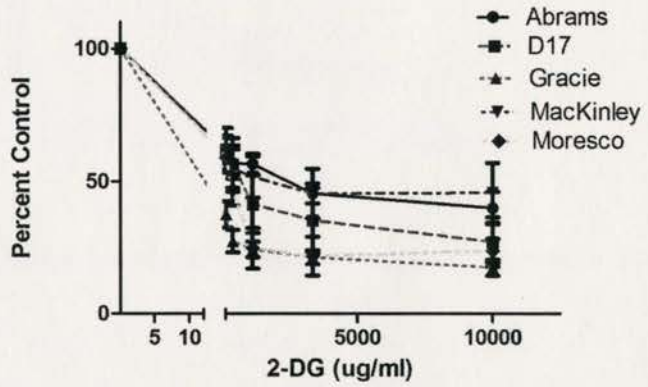

Figure 3.1b. 5 OSA cells lines in hypoxic (2% O_2) conditions treated with various concentrations of 2-DG

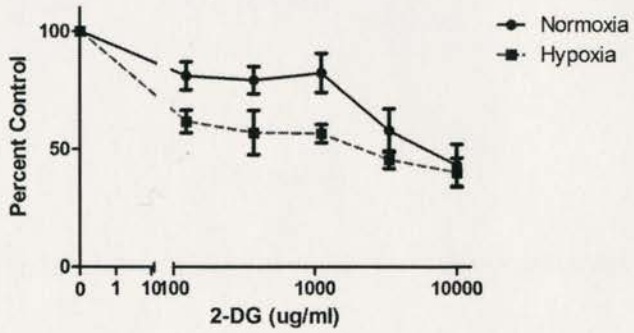

Figure 3.2. Abrams cell line comparing normoxic to hypoxic conditions

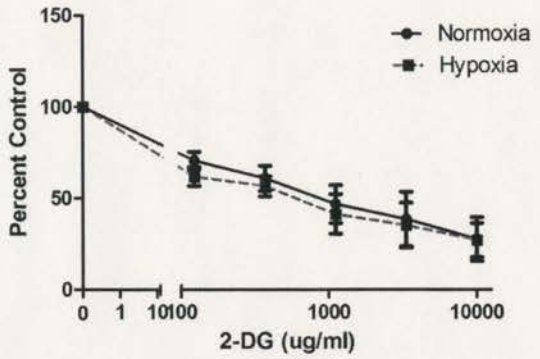

Figure 3.3. D17 cell line comparing normoxic to hypoxic conditions

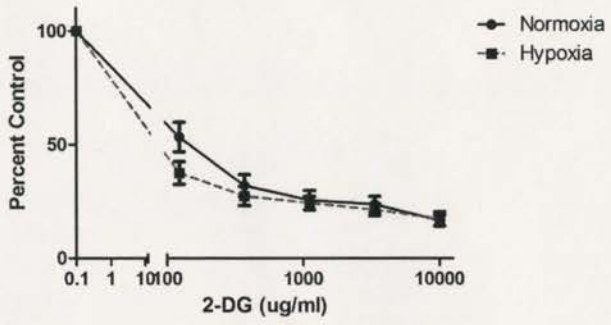

Figure 3.4. Gracie cell line comparing normoxic to hypoxic conditions

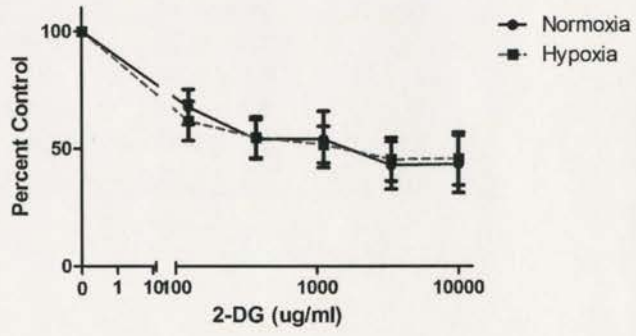

Figure 3.5. MacKinley cell line comparing normoxic to hypoxic conditions

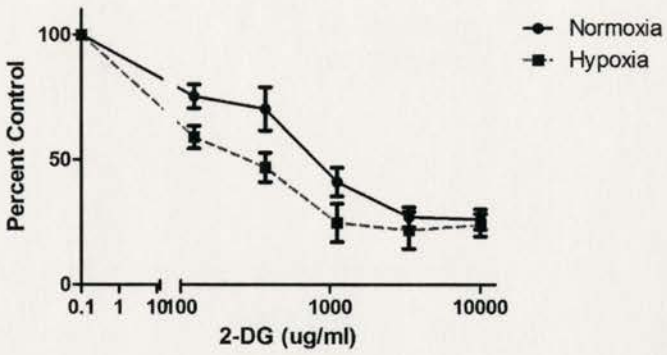

Figure 3.6. Moresco cell line comparing normoxic to hypoxic conditions

Table 3.2. IC_{50}s of 5 OSA cell lines to 2-DG

IC 50 (ug/mL)		
Cell Line	Normoxia	Hypoxia
Abrams	7993	3100
D17	902.6	864.9
Gracie	<100	<100
MacKinley	222.9	361.1
Moresco	651.8	221.7

Table 3.3 Percent growth inhibition at 370 ug/mL in 5 canine OSA cell lines

Growth Inhibition at 370 ug/mL (%)		
Cell Line	Normoxia	Hypoxia
Abrams	20.8 + 5.76	43.2 ± 9.39
D17	39.1 ± 6.9	43.5 ± 5.5
Gracie	68.1 ± 4.8	72.7 ± 4.2
MacKinley	46.0 ± 8.4	45.3 ± 8.7
Moresco	29.8 ± 8.7	53.2 ± 5.9

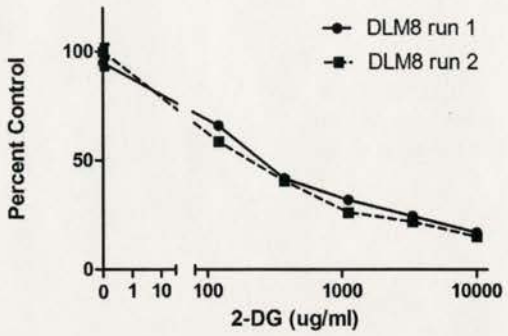

Figure 3.7 Growth inhibition curve for DLM8 cell line treated with varying concentrations of 2-DG

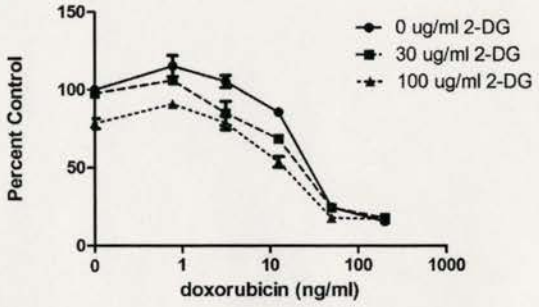

Figure 3.8 Growth inhibition curve for Abrams cell line with concurrent treatment with varying concentrations of 2-DG and DOX.

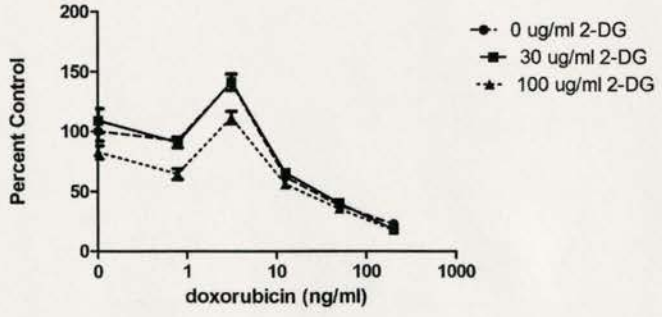

Figure 3.9 Growth inhibition curve for D17 cell line with concurrent treatment with varying concentrations of 2-DG and DOX.

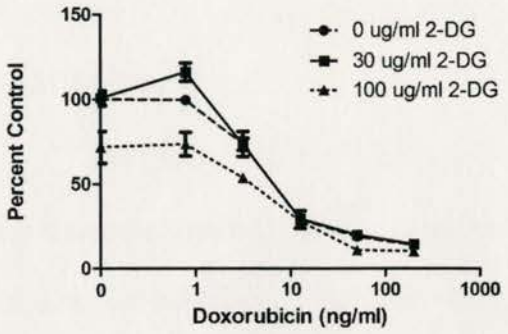

Figure 3.10 Growth inhibition curve for Gracie cell line with concurrent treatment with varying concentrations of 2-DG and DOX.

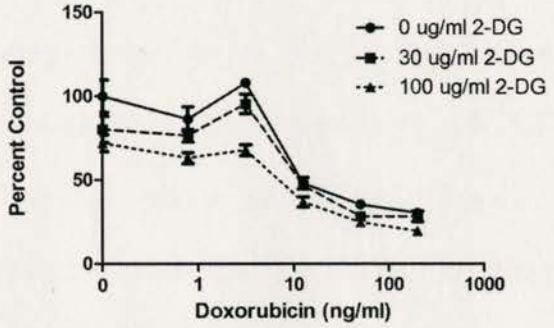

Figure 3.11 Growth inhibition curve for MacKinley cell line with concurrent treatment with varying concentrations of 2-DG and DOX.

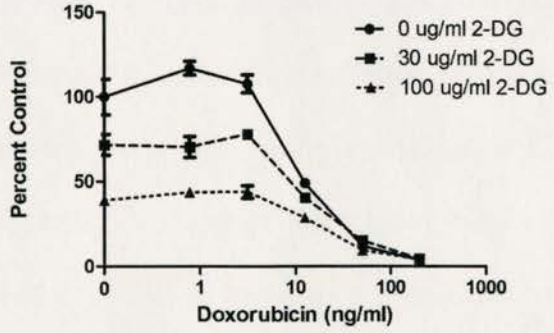

Figure 3.12 Growth inhibition curve for Moresco cell line with concurrent treatment with varying concentrations of 2-DG and DOX.

GLUT-1 Expression

The up-regulation of GLUT-1 was demonstrated in canine OSA cells following incubation in normoxic (21% O_2) and hypoxic (2% O_2) conditions using fluorescence immunocytochemistry **(Fig. 3.13).** The DAPI is used to bind to DNA in the nuclei serving as a control between the two conditions, while the FITC binds to the GLUT-1 antibody, showing the difference between the two conditions. When western analysis was performed to compare the two conditions, there was a large amount of variation between the cell lines with respect to GLUT-1 expression, although there was not an abundant overexpression of GLUT-1 seen in hypoxic conditions, when compared to normoxic conditions **(Fig. 3.14)**. This difference was much more appreciable when evaluating the immunocytochemistry, showing the up-regulation of GLUT-1.

This was shown in **Table 3.4** when image analysis was performed to compare the bands for each cell line and condition. Higher densitometry correlates with lower expression as it is measuring white rather than dark. The Abrams, MacKinley and Moresco cell lines all showed increased GLUT-1 expression in hypoxic conditions compared to normoxic conditions, as evidenced by the higher numbers in the last column. Moresco expressed the highest amount of GLUT-1. When linear regression was performed to compare the expression level of GLUT-1 to the respective IC_{50} and percent growth inhibition for each cell line, there were no significant correlations between sensitivity to 2-DG and expression of GLUT-1. The fold change of GLUT-1 induction between normoxic and hypoxic conditions for each cell line was also compared to the

fold change of the IC50's and percent growth inhibition using linear regression; these did not show a significant correlation with *p*-values of 0.069 and 0.210, respectively.

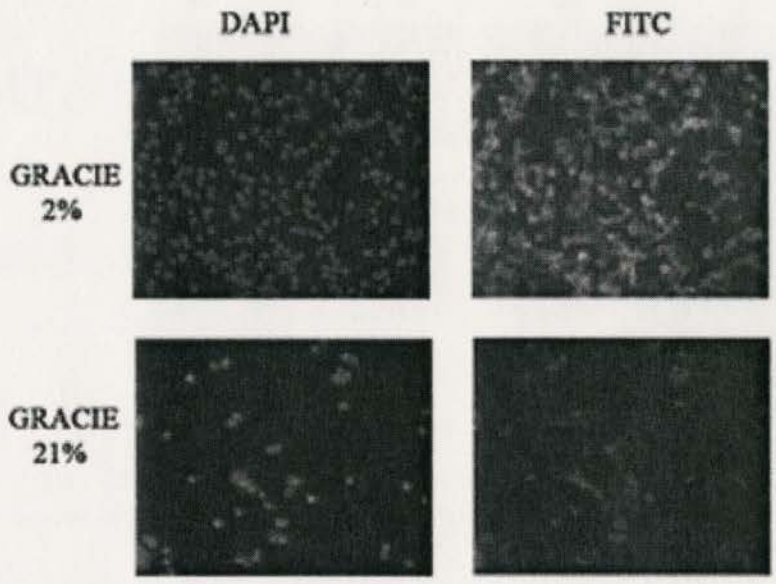

Figure 3.13. GLUT-1 expression in canine OSA cells following exposure to conditions of normoxia and hypoxia using immunocytochemistry

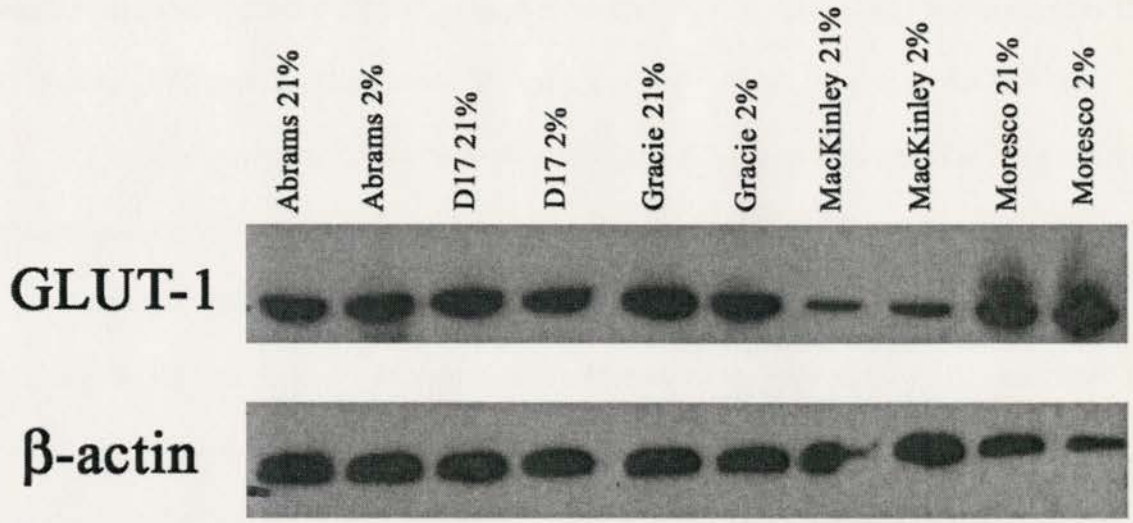

Figure 3.14. GLUT-1 expression in various canine OSA cells following exposure to conditions of normoxia and hypoxia using western blot

Table 3.4. Image Analysis data for GLUT-1 Western Blot compared to β-actin control

Cell line	Mean GLUT densitometry	mean actin densitometry	GLUT/actin	1/(GLUT/actin)
Abrams 21%	62.56	53.74	1.16	0.86
Abrams 2%	55.68	57.57	0.97	**1.03**
D17 21%	51.98	56.28	0.92	1.08
D17 2%	62.62	59.66	1.05	0.95
Gracie 21%	55.32	52.51	1.05	0.95
Gracie 2%	68.12	59.02	1.15	0.87
MacKinley 21%	90.28	75.29	1.20	0.83
MacKinley 2%	73.06	62.66	1.17	**0.86**
Moresco 21%	59.69	60.73	0.98	1.01
Moresco 2%	54.34	93.93	0.58	**1.73**

Akt Expression

Western analysis was performed to measure basal phosphorylated and total Akt expression. There was some variation between the cell lines with respect to p-Akt

expression, with Moresco exhibiting high amounts of p-Akt relative to the other cell lines **(Fig. 3.15)**. When evaluating total Akt, there was little variability among the cell lines.

This was shown in **Table 3.5** where image analysis was performed to compare the bands for each cell line. Again, higher densitometry correlates with lower expression as it is measuring white rather than dark. Moresco expressed the highest amount of pAkt and second highest of total Akt; interestingly Moresco expressed the highest amount of GLUT-1. When linear regression was performed to compare the expression level of GLUT-1 to the respective IC_{50} and percent growth inhibition for each cell line, there were no significant correlations shown between the sensitivity to 2-DG and the expression of pAkt and tAkt.

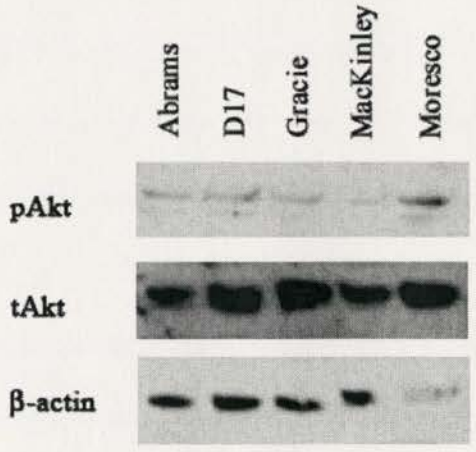

Figure 3.15 Basal phospho and total Akt expression in various canine OSA cells using western blot

Table 3.5 Image analysis data comparing pAkt blot to total Akt blot and total Akt blot to β-actin

pAkt blot	mean pAkt	mean tAkt	pAkt/tAkt	1/(pAkt/tAkt)
Abrams	201.26	40.71	4.94	0.202
D17	198.26	28.91	6.86	0.146
Gracie	207.32	28.67	7.23	0.138

MacKinley	215.67	32.968	6.54	0.153
Moresco	152.36	49.55	3.07	**0.325**

tAkt blot	mean tAkt	mean β-actin	tAkt/actin	1/(tAkt/actin)
Abrams	40.71	118.19	0.344	2.903
D17	28.91	67.86	0.426	2.347
Gracie	28.67	121.05	0.237	**4.222**
MacKinley	32.968	93.83	0.351	2.846
Moresco	49.55	202.83	0.244	4.093

ERK1/2 Expression

Western analysis was also performed to compare the five canine OSA cell lines for basal p-ERK and total ERK expression. There was variation between the cell lines with respect to pERK and tERK expression, more so among the total ERK **(Fig. 3.16)**.

This was shown in **Table 3.6** where image analysis was performed to compare the bands for each cell line. Moresco again expressed the highest amount of phosphorylated ERK, like Akt and GLUT-1. Similarly, Gracie also expressed the highest relative amount of tERK. When linear regression was performed to compare the expression level of GLUT-1 to the respective IC_{50} and percent growth inhibition for each cell line, there was a significant correlation between the amount of tERK expression and IC_{50} with a p-value of 0.03 and an r^2 value of 0.8034. The association found increased basal tERK expression correlated with decreased sensitivity to the drug. **Figure 3.17** is the linear regression plot of tERK and IC_{50}.

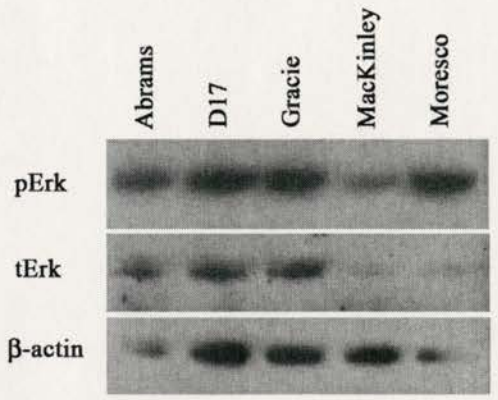

Figure 3.16 Basal phospho and total ERK expression in various canine OSA cells using western blot

Table 3.6 Image analysis data comparing phospho-ERK blot to total ERK blot and total ERK blot to β-actin

pERK blot	mean pERK	Mean tERK	pERK/TERK	1/(pERK/tERK)
Abrams	87.24	103.17	0.846	1.18
D17	70.85	100.25	0.707	1.41
Gracie	69.7	91.38	0.763	1.31
MacKinley	113.75	158.58	0.717	1.39
Moresco	76.98	162.82	0.473	**2.12**

tERK blot	mean tERK	mean β-actin	tERK/actin	1/(tERK/actin)
Abrams	103.17	118.63	0.870	1.15
D17	100.25	72.2	1.389	0.72
Gracie	91.38	70.25	1.301	**0.77**
MacKinley	158.58	75.39	2.103	0.48
Moresco	162.82	111.55	1.460	0.69

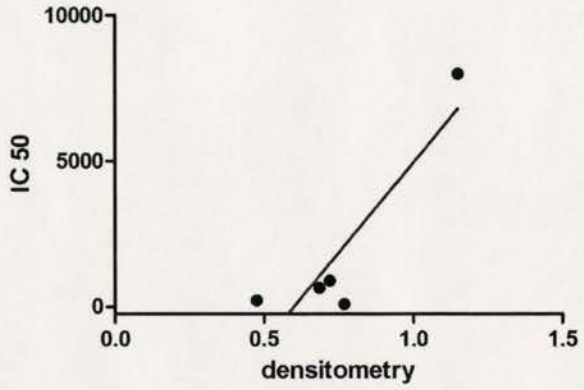

Figure 3.17 Linear regression plot comparing tERK expression to IC_{50}

2-DG Increases Caspase 3/7 Activity in Canine OSA

When the Caspase 3/7 assay was used to determine caspase activity, all five of the canine OSA cell lines demonstrate a dose-dependent increase in activity. This correlates with an increase in cells dying by apoptosis at increasing doses of 2-DG (**Figure 3.18).**

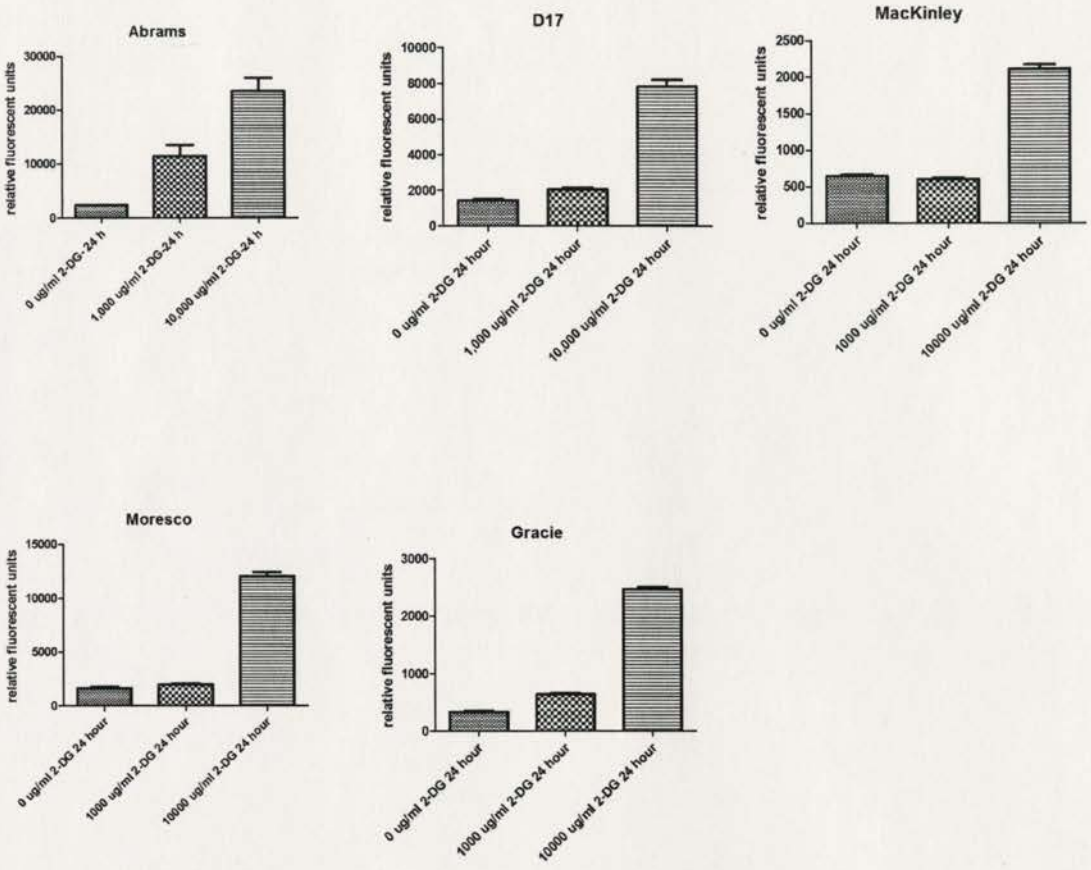

Figure 3.18 Caspase 3/7 activity in the five canine OSA cell lines at varying concentrations of 2-DG indicating cell death from apoptosis.

2-DG delays time to metastasis in DLM8 orthotopic murine model

In the murine model of orthotopically injected mouse OSA cells, it was found that mice treated three times weekly with 500 mg/kg of 2-DG had a clinically detectable delay in developing metastatic disease (**Figure 3.19**), with untreated mice having a median disease free interval (DFI) of 24 days and 2-DG treated mice having a median DFI of 49.5 days (p=0.0493). Survival on the other hand, was not different between the two groups (**Figure 3.20**), with median survival times of 42 days for both groups (p= 0.4952).

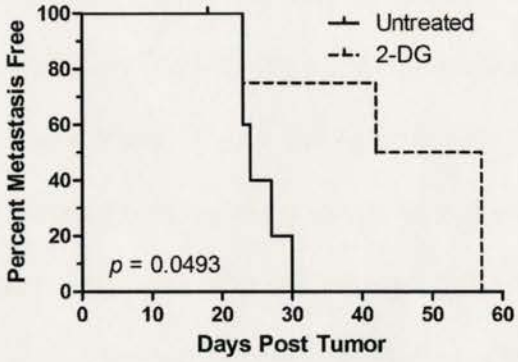

Figure 3.19 Kaplan Meier curve showing delay of metastasis in mice that received 2-DG versus untreated mice.

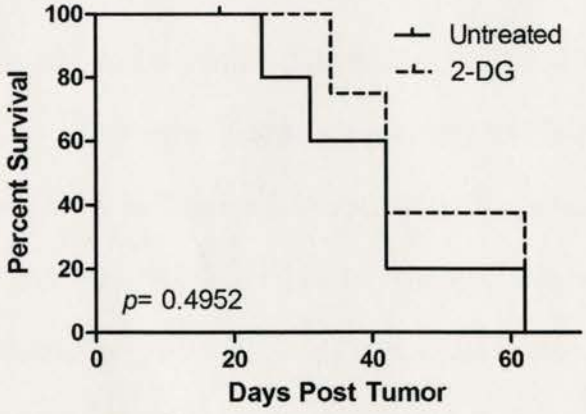

Figure 3.20 Kaplan Meier curve showing survival times in mice that received 2-DG versus untreated mice.

2-DG Inhibits Invasion and Migration of DLM8 cells *in vitro*

The invasion assays test the ability of the cells to invade through a basement membrane matrix preparation, one of the hallmarks of the metastatic cancer phenotype.[41]

In the DLM8 cell line, this test was performed to determine why 2-DG caused a delay in time to metastasis, combined with the results of Ptitsyn study's showing that the glycolytic pathway is one of the most consistently altered pathways in metastasis. This invasion assay revealed that treatment with 2-DG inhibits invasion through a basement membrane. With increasing concentrations of 2-DG, there were fewer cells that invaded the membrane. This is shown in **Figure 3.21**. There was a statistically significant difference between the positive control and 100 ug/mL 2-DG ($p = 0.0015$) and between the positive control and 1,000 ug/mL 2-DG ($p < 0.0001$).

Figure 3.22 shows the results of the migration assay for DLM8. The cells migrate through the insert. The migration insert is the control insert for the invasion inserts, without the matrigel basement membrane. The migration assay results were almost identical to the invasion assay, with 2-DG treatment inhibiting migration through the porous insert. All treatment values for 2-DG were significantly different from the positive control, with p-values less than 0.0001.

The same tests were performed for the canine OSA cell line Abrams. The results are shown in **Figures 3.23 and 3.24**. These results were similar to what was seen in the DLM8 murine OSA cell line. Treatment values were significantly different from the positive control, with p-values less than 0.0001, with the exception of the positive control and 10ug/ml 2-DG in the invasion assay.

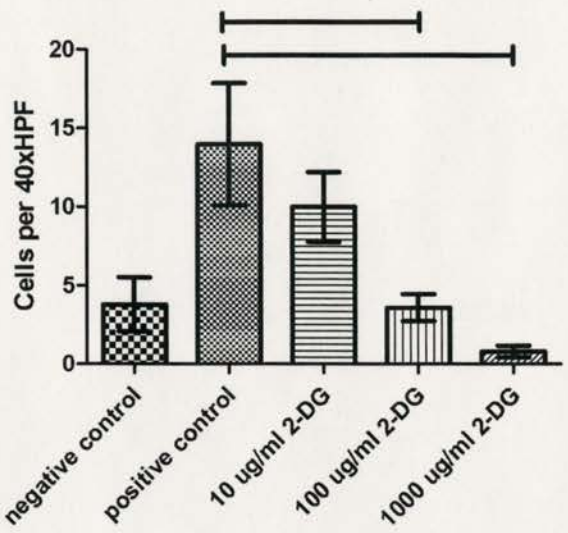

Figure 3.21 Invasion Assay of DLM8 cells comparing values to negative and positive controls at a range of concentrations of 2-DG.

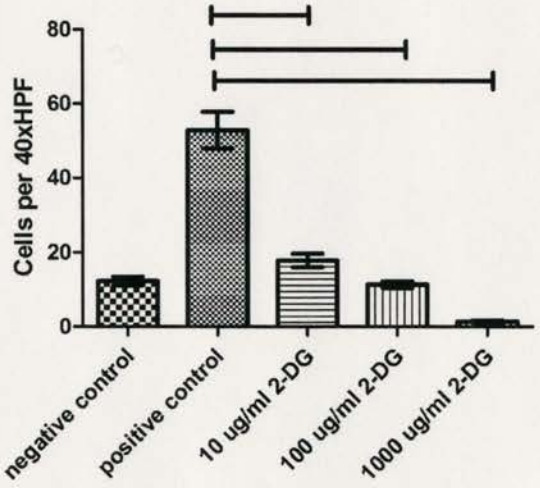

Figure 3.22 Migration assay of DLM8 cells comparing values to negative and positive controls at a range of concentrations of 2-DG.

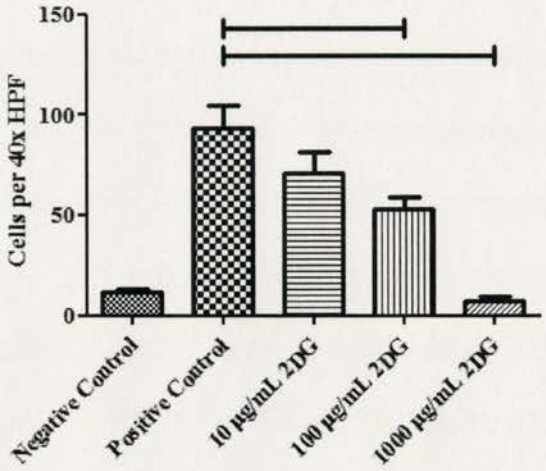

Figure 3.23 Invasion assay of Abrams cells comparing values to negative and positive controls at a range of concentrations of 2-DG.

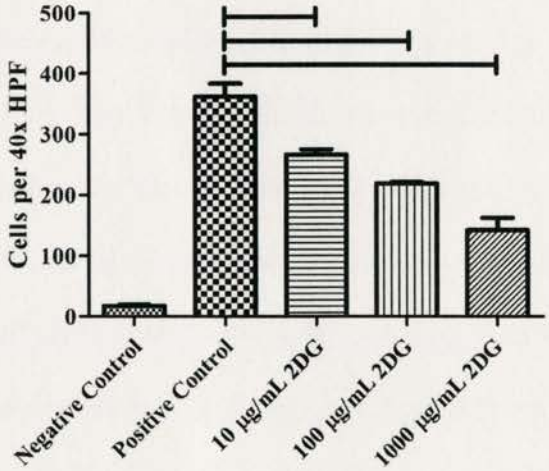

Figure 3.24 Migration assay of Abrams OSA cells comparing values to negative and positive controls at a range of concentrations of 2-DG.

Discussion

OSA is the most common bone tumor in both humans and dogs.[42,43] Novel therapies are desperately needed, thus evaluation of newer treatment options is imperative. Therefore, 2-DG was explored as a potential therapy for OSA in an *in vitro* setting as well as *in vivo* in an OSA murine model.

The purpose of this study was to evaluate 2-DG, and the combination of 2-DG and DOX, in OSA, correlate OSA sensitivity to 2-DG with the expression of GLUT-1, Akt, and ERK, as well as evaluate 2-DG in an orthotopic OSA murine model of metastasis. The results suggest that different OSA cell lines show varying sensitivity to 2-DG, and that overall sensitivity to 2-DG does not correlate with the expression of GLUT-1 or Akt, but did correlate with tERK. 2-DG has an impact on metastasis in an orthotopic, postsurgical murine OSA model by increasing disease free interval. 2-DG also inhibits invasion and migration in the same murine OSA line, as well as one of the canine OSA cell lines in an *in vitro* setting.

The growth inhibition curves for the 5 canine OSA and one murine OSA cell lines showed a variable dose-dependent sensitivity to 2-DG. There is one published study evaluating the pharmacologic effects of 2-DG in cancer patients, that measured blood 2-DG and blood glucose levels in patients following IV dosing ranging from 50-200 mg/kg and oral dosing at 60 mg/kg. None of these doses led to serious adverse events in people. This study showed peak blood levels post injection ranging from 200-450 ug/mL.[44] A dog study showed that blood levels of about 900 ug/mL were achievable in the nephrectomized, eviscerated dog after an infusion of 250 mg/kg of 2-DG. These were peak levels, which remained above 250 ug/mL for 3 hours, although with a 30% renal

clearance found in the human study, makes the blood levels difficult to extract from a nephrectomized patient.[45]

In our experiment evaluating IC_{50}'s in canine OSA cell lines, values ranged from <100 to 7993 ug/mL, with two of five OSA cell lines having IC_{50}'s below that of peak drug levels reached with one IV infusion of 200 mg/kg in a person. The DLM8 murine OSA line had a mean IC_{50} of 206 ug/mL, showing a greater sensitivity than most of the canine lines. However, our models evaluated 72 hours of drug exposure, thus it is hard to correlate peak drug levels to these values. Threshold Pharmaceuticals conducted a phase I study in 2004, and found that daily unpublished doses did not appear to cause serious adverse events in people.[46] The goal of this study was to establish a maximally tolerated dose and the pharmacokinetics of the drug, but this data remains unpublished. It is reasonable to assume with such varying sensitivity in the canine cell lines, that extremely variable responses might be expected among clinical patients based on the doses that are able to be given in dogs. Landau, et al. found that doses of 500 mg/kg caused death in a patient.[45]

Hypoxic cells have a higher sensitivity to 2-DG.[30,47] This was also seen in our model evaluating hypoxia at 2% in canine OSA cell lines, although to a lesser extent than in previous publications. We suspect that the reason for this difference is related to the difference in the percentage of oxygen we used versus the two studies using human cell lines evaluating the same concept. One model evaluated the conditions at 0.1% oxygen and the other model a range of 0-21%, with 4 out of 6 hypoxic conditions being less than 2%. All studies utilized a single human OSA cell line. In reality, hypoxia varies greatly among regions of tumors, and perhaps a lower percentage of oxygen would have been

more representative. There may have been an enhanced effect of 2-DG at lower oxygen levels in our canine OSA model.

We have previously shown the presence of GLUT-1 in canine OSA by IHC and western blot.[37] In this experiment, we attempted to correlate GLUT-1 expression with sensitivity to 2-DG at normoxia, as has been previously shown in human pancreatic carcinoma cell lines. We were unable to find any correlation between sensitivity and expression. In humans, the hope is to correlate GLUT-1 expression in tumors to those that would be sensitive to 2-DG. This does not seem to be the case for canine OSA in this experiment. We also evaluated GLUT-1 expression between normoxic and hypoxic conditions. Three of the canine OSA cell lines, Abrams, MacKinley and Moresco, expressed increased amounts of GLUT-1 when grown in 2% oxygen versus in normoxia at 21% oxygen. These cell lines also had the largest subjective change in IC50 between normoxic and hypoxic conditions, although these differences weren't statistically significant. It is important to note that there are many other glucose transporters that were not evaluated in this study. Thus, lack of correlation with GLUT-1, does not mean that is the only mechanism available for glucose transport.

Thompson's work links the Warburg effect to mutations in the pathways that govern glucose uptake, mainly activation of the Akt kinase pathway. The Akt family has been shown to increase transcription of the GLUT-1 transporter, as well as increase the overall rate of glycolysis.[20-22] We sought to evaluate if there was a relationship between basal expression of Akt and ERK, total and phosphorylated, and the sensitivity of different canine OSA cell lines to 2-DG. We found that tERK expression had a significant correlation with sensitivity to 2-DG, such that cell lines that were less

sensitive to 2-DG had a higher level of basal tERK expression. pERK is the active form and the expectation would be a correlation of greater pERK with greater sensitivity. There is no obvious explanation for why a higher tERK expression would correlate with a decreased sensitivity to 2-DG.

2-DG significantly delayed postoperative metastasis in the DLM8 orthotopic murine OSA model, although no survival advantage was found. 2-DG also inhibited *in vitro* invasion and migration. This makes sense to inhibition of metastasis, or at least slowing the process down. There is no good explanation for why metastasis would be significantly delayed, but not survival, unless the metastatic process was sped up once therapy was discontinued. The mice were treated for 30 days, and per the Kaplan-Meier, very few mice had evidence of metastasis at this time. After discontinuation of therapy, they shortly developed metastasis which made the mice die quickly. It seemed as though the untreated mice lived longer with disease.

In conclusion, the purpose of this study was to evaluate 2-DG, and the combination of 2-DG and DOX, in OSA, correlate OSA sensitivity to 2-DG with the expression of GLUT-1, Akt, and ERK, as well as evaluate 2-DG in an orthotopic OSA murine model. These results suggest that different OSA cell lines show varying sensitivity to 2-DG, and that overall sensitivity to 2-DG does not correlate with the expression of GLUT-1 or Akt, but does correlate with tERK using western analysis. 2-DG has an impact on an orthotopic murine OSA model by increasing metastasis free interval, but not survival, and also inhibits invasion and migration in the same murine OSA line in an *in vitro* setting.

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Chapter 4: Concluding Remarks

Canine OSA is the most common bone tumor in the dog.[1-3] With amputation and chemotherapy with platinum based drugs being the standard of care, the treatment and clinical outcome of canine OSA has not improved significantly over the course of many years.[4-6] This disease is an excellent model for OSA in the pediatric human.[7] Thus, newer treatments are needed for this devastating disease to improve outcomes in both species.

In the first portion of this study, we sought to find markers of increased glucose uptake in canine osteosarcoma, by evaluating the presence of GLUT-1, for prognostic value, as well as a potential predictor of how the tumor may be treatable by drugs which inhibit glycolysis.[8] We found that 61% of canine OSA samples expressed GLUT-1 when evaluated by immunohistochemistry, and expression was subjectively enhanced in perinecrotic, and presumably hypoxic, tumor regions. GLUT-1 did not correlate with outcome following treatment. The distribution and location of the staining was very similar to that reported in human cancer. The precise role of GLUT-1 in canine tumors is unknown at this time, but further investigation is warranted.

Once the presence of the protein was established in OSA, we evaluated the sensitivity of canine and murine OSA cell lines to 2-DG, and attempted to correlate this sensitivity to the expression of GLUT-1, as well as other markers of cell signaling. Canine and mouse OSA have variable sensitivities to 2-DG. The sensitivity to 2-DG was

not statistically correlated with expression of GLUT-1, or phosphorylated or total Akt, although it was correlated with total Erk expression. The canine OSA cell lines were more sensitive to 2-DG under hypoxic conditions, although this did not correlate with the change in GLUT-1 expression in hypoxic conditions.

Later, a mouse model was developed using 2-DG as an adjuvant therapy for OSA after amputation of the primary tumor.[9] We determined that 2-DG in the adjuvant setting in a murine orthotopic model of OSA delayed metastasis. 2-DG was shown to inhibit invasion and migration in the same mouse OSA cell line *in vitro*. Seeing as the canine OSA (Abrams) cell line that was evaluated seemed to have the same inhibition of invasion and migration as was seen in the murine cell line, it is possible that therapy with 2-DG may also delay metastasis in the dog model. This drug should be further evaluated in the dog model, in our quest to find better treatments for canine and human OSA.

Future Directions

2-deoxyglucose should next be evaluated in a phase I study of dogs with cancer to establish a maximally tolerated dose. Once this is established, 2-DG could be evaluated for efficacy in canine OSA in the adjuvant setting post amputation. Since the efficacy of 2-DG in canine OSA is unknown, this would be more ethically evaluated in a model combining 2-DG therapy with the standard of care chemotherapy for the dog, which is either carboplatin, cisplatin or the combination of carboplatin and doxorubicin. This would be best evaluated later in a randomized, placebo controlled trial with dogs receiving either the standard of care platinum based drugs, followed by 2-DG or placebo. The combination of these drugs should target both the hypoxic cells and tumor cells that

are more susceptible to traditional chemotherapy, likely making this a more successful treatment for canine OSA. As was seen in the mouse model, we would expect a delay in time to metastasis in the patients treated with the addition of 2-DG, and hopefully survival time as well.

Although GLUT-1 did not appear to correlate with 2-DG sensitivity in the five OSA cell lines evaluated in this model, and had no prognostic value in the 44 cases evaluated in chapter 2, it would be potentially beneficial to evaluate GLUT-1 expression, and potentially the expression of other glucose transporters, in a prospective setting at the time of amputation, and then to follow these cases through adjuvant therapy to attempt a correlation with response to 2-DG, as well as to potentially make further prognostic associations. This would be done by evaluating for at least GLUT-1 and GLUT-3 in the samples at the time of evaluation by IHC. These samples would be scored as the were in Chapter 2. IHC scores would be correlated with outcome data like DFI and ST.

It would also be potentially beneficial to go back and evaluate the presence of GLUT-3 in canine OSA. This, unrelated to the previously discussed clinical trial should initially be evaluated by IHC in a prospective population of dogs at the time of amputation. By performing prospectively, one could insure that all samples were processed the same, and were available for necrosis evaluation. Dogs should also have PET-CT scans prior to amputation to evaluate glucose uptake in the tumors in an effort to correlate PET uptake to the presence of glucose transporters. GLUT-1 should be evaluated concurrently to establish presence in a prospective setting, and multiple tumor samples of the same tumor should be evaluated to attempt to correlate a higher percentage of the tumor, and these values averaged. Then, several of the evaluated

canine lines should be established in cell culture. These samples could be treated in vitro, with 2-DG at varying concentrations to establish an IC_{50} and again attempt correlation to glucose transporters prior to the evaluation of 2-DG further in a clinical trial setting. Other glucose transporters could be evaluated, but GLUT-1 and GLUT-3 have been the most established in human tumors so this seems like the best starting point.

It would also be beneficial to evaluate GLUT-1 and GLUT-3 in panels of other tumor types. Since this is more commonly evaluated in carcinomas in humans, likely because of the difference in the number of carcinomas versus sarcomas seen in human medicine, it would also be interesting to evaluate the presence in other commonly seen canine tumors and attempt a correlation with outcome.

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