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Pharmacokinetics and Toxicity of the Novel Oral Demethylating Agent Zebularine in Laboratory and Tumor Bearing Dogs

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PHARMACOKINETICS AND TOXICITY OF THE NOVEL ORAL DEMETHYLATING AGENT ZEBULARINE IN LABORATORY AND TUMOR BEARING DOGS

A Thesis

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Christopher M. Fulkerson, DVM

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For my loving wife, Caroline Van Tassel Fulkerson, who supports me unconditionally.

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5-Aza-CR – 5-azacytidine, azacytidine (Vidaza®, Celgene Corp., Summit, NJ, USA)

5-Aza-CDR – 5-aza-2'-deoxycytidine, decitabine (Dacogen®, SuperGen, Inc., Dublin, CA, USA)

ALP – alkaline phosphatase

ALT – alanine aminotransferase

AO – aldehyde oxidase

CDA – cytosine deaminase

CK – creatine kinase

CBC – complete blood count

CpG – cytosine guanine dinucleotide

DNA – deoxyribonucleic acid

DNMT – DNA methyltransferase

DLT – dose-limiting toxicity

InvTCC – invasive transitional cell carcinoma, invasive urothelial carcinoma

LDH – lactate dehydrogenase

MTD – maximum tolerated dose

NSAID – non-steroidal anti-inflammatory drug

PUVTH – Purdue Veterinary Teaching Hospital

VCOG-CTCAE v1.1 – Veterinary Cooperative Oncology Group common terminology criteria for adverse events v1.1

Zeb – zebularine, 1-β-D-Ribofuranosyl-2(1*H*)-pyrimidinone, NSC 309132

ABSTRACT

Fulkerson, Christopher M. D.V.M, M.S., Purdue University, August 2014. Pharmacokinetics and Toxicity of the Novel Oral Demethylating Agent Zebularine in Tumor Bearing Dogs. Major Professor: Deborah Knapp.

The aim of this thesis is to investigate the pharmacokinetics and toxicity of the novel oral demethylating agent zebularine (zeb) in laboratory dogs and tumor bearing dogs. This thesis focuses on the application of this therapeutic strategy in dogs with naturally occurring invasive transitional cell carcinoma (InvTCC), which serve as a relevant model of the human disease.

DNA hypermethylation in the promoter region is a common epigenetic change in cancer that silences tumor suppressor genes. Zeb is an oral cytidine analog that acts as a demethylating agent. Zeb has been investigated extensively *in vitro* as well as in mice, rodents and rhesus monkeys. Prior to the investigation described in this thesis, zeb had not been investigated in the dog. The authors set out to investigate zeb in dogs with naturally occurring InvTCC with an eventual goal of applying this treatment strategy to humans with this devastating cancer.

The first part of this thesis reviews the importance of DNA methylation in cancer development, the development and application of zeb as a novel oral demethylating agent

and the application of demethylating agents in InvTCC in the dog. The second part of this thesis presents initial pharmacokinetics and toxicity data following high dose oral zeb in laboratory dogs (n=3) and tumor bearing dogs (n=3). Daily high dose oral zeb at 4 mg kg^{-1} resulted in remarkable, but reversible, hematologic toxicity in the form of neutropenia in laboratory dogs. Dose adjustment revealed that a dose intensity of 4 mg kg^{-1} once every 21 days was well tolerated in tumor bearing dogs. The third part of this thesis presents the results to date of a phase I dose escalation trial of oral zeb in 26 dogs with InvTCC. At daily doses up to 0.5 mg kg^{-1}, zeb was well tolerated. At higher doses, dose-limiting toxicity (DLT) was detected. In a population in which 73% of dogs had failed previous treatment, overall disease control rate of 73.3% was detected, and median progression free survival time was 86 days (95% CI, 47.5-124.5). Of particular interest is that one dog has experienced disease stabilization for more than 511 days.

Remarkable, but reversible, neutropenia was detected in laboratory dogs treated with high dose daily zeb. Low dose daily zeb at doses up to 0.5 mg kg^{-1} was well tolerated in dogs with InvTCC. Initial results indicate promising disease control rates and progression free survival in a population of dogs that was heavily pretreated. These results warrant further investigation as a treatment strategy in dogs with InvTCC with potential applications to the human disease. Further investigation is required to determine if the optimal effects of zeb may be in combination with cytotoxic chemotherapy drugs and to document that tumor responses are due to DNA demethylation and reactivation of silenced genes.

CHAPTER 1. INTRODUCTION

1.1 Introduction

Epigenetic changes are heritable changes that alter gene expression without changing the DNA sequence.[1,2] DNA methylation is one of the most studied epigenetic changes in mammals. DNA methylation may become dysregulated due to altered DNA methyltransferase (DNMT) activity and other changes associated with chronic inflammation and exposure to carcinogens.[1] DNA methylation in mammals occurs on cytosine residues in the 5' position to guanine residues in a cytosine guanine dinucleotide (CpG).[2,3]

CpGs are unevenly distributed throughout the genome and are concentrated within short repeats called CpG islands.[3] These CpG islands are concentrated within the promoter regions of half or more of all mammalian genes.[2,3] In normal cells, promoter region CpG islands are usually unmethylated, whereas in cancer cells, these regions are often hypermethylated. Hypermethylation of promoter region CpG islands results in transcriptional silencing.[3] Hypermethylation of DNA within a gene promoter region prevents binding of the transcriptional apparatus by physically blocking its interaction with DNA.[1] Methylation attracts methyl-CpG binding proteins which attract histone and chromatin remodeling complexes that alter the conformation of chromatin from the transcriptionally active euchromatin to transcriptionally silent heterochromatin.[1,2]

DNA methylation is mediated by DNMTs.[3,4] DNMTs are responsible for establishing and maintaining the patterns of methylation throughout DNA synthesis.[4] DNMTs transfer methyl groups from *S*-adenosyl-l-methionine to CpGs as DNA is replicated during S-phase.[1,4] When the CpG on one daughter strand pairs with a CpG on the complementary strand of DNA, the resultant pair can be unmethylated, partially methylated (hemi-methylated) or completely methylated. DNMT1 preferentially binds to partially methylated CpG pairs, restoring a completely methylated state.[4] DNMT1 is largely responsible for maintenance methylation, while DNMT3a and DNMT3b are responsible for *de novo* methylation.[2,3,5] *De novo* methylation is associated with aging and inflammation, and likely occurs early in tumor development.[3] Expression of DNMT3a and 3b is high during embryogenesis, and low in adult cells.[2] Expression of DNMT is essential to development as indicated by the fact that mice deficient in either DNMT1 or DNMT3b are embryonic lethal, and mice deficient in DNMT3a die before they are 4 weeks old.[2]

Tumor suppressor gene function can be lost due to hypermethylation of gene promoter regions, mutations and DNA loss through deletions.[4] DNA hypermethylation is frequently the "second hit" in familial cancers, leading to gene inactivation.[3] Transcriptional silencing by promoter hypermethylation has been demonstrated in important genes involved in apoptosis, DNA repair, cell cycle regulation, metastasis/invasion and hormone receptors.[2,3] The list of human genes that have been documented as silenced through DNA methylation include *ATM* (ataxia telangiectasia mutated), *APC* (adenomatosis polyposis coli), *BRCA2* (breast cancer 2, early onset), *CDH1* (E-cadherin), *CDKN2A* (cyclin-dependent kinase

inhibitor 2A), *GSTP1* (glutathione S-transferase pi), *P14^{ARF}, P15, P16^{INK4A} PTEN* (phosphatase and tensin homologue), *RASSF1A* (Ras association domain containing protein 1a) *RB1* (retinoblastoma 1) and *TIMP3* (tissue inhibitor of metalloproteinase 3).[3]

DNA hypermethylation can also contribute to genomic instability and tumor progression by promoting genetic mutation.[2,3] Silencing of *MLH1* (mutL homologue 1), a mismatch repair gene, is often seen in colon and endometrial cancers with microsatellite instability. Hypermethylation of *MLH1* has also been demonstrated within adjacent normal colonic epithelium of people with colon cancer, and hyperplastic tissue in people with endometrial cancer.[3] Hypermethylation of *MGMT* (O^6-methylguanine-DNA methyltransferase), a gene that repairs carcinogen induced DNA adducts, has been detected in a variety of malignancies, and may facilitate mutations in *p53* and *K-RAS*.[1,3]

Unlike genetic mutations, DNA methylation is a potentially reversible heritable change.[2,3,6] There are currently two Food and Drug Administration approved injectable demethylating agents in use for the treatment of human myelodysplastic syndrome, 5-azacytidine (Vidaza®, Celgene Corp., Summit, NJ, USA; 5-Aza-CR) and decitabine (Dacogen®, SuperGen, Inc., Dublin, CA, USA; 5-Aza-CDR).[1] 5-Aza-CR has been investigated in dogs with invasive transitional cell carcinoma (InvTCC), resulting in 22% partial remission and 50% stable disease for more than 8 weeks. Since demethylating agents function by interfering with the activity of DNMTs during DNA replication, continuous exposure in the form of daily oral administration may be the most effective delivery strategy.[1,7]

Zebularine (1-β-D-Ribofuranosyl-2(1*H*)-pyrimidinone; zeb) is another demethylating agent that is very similar in structure to 5-Aza-CR and 5-Aza-CDR, but it is stable under a wide range of conditions and can be administered orally.[6,8] The purpose of this work is to describe initial results of investigation of zeb in the dog including initial pharmacokinetics and toxicity in laboratory and tumor bearing dogs, and a phase I dose escalation trial in dogs with InvTCC.

References

1. Howell PM, Liu Z and Khong KT. Demethylating agents in the treatment of cancer. *Pharmaceuticals.* 2010 3(7):2022-2044.

2. Kanwal R and Gupta S. Epigenetic modifications in cancer. *Clinical Genetics.* 2012 81(4):303-311.

3. Jones PA and Baylin SB. The fundamental role of epigenetic events in cancer. *Nature Reviews Genetics*. 2002 3(6):415-428.

4. Gowher H and Jeltsch A. Mechanism of inhibition of DNA methyltransferases by cytidine analogs in cancer therapy. *Cancer Biology & Therapy*. 2004 3(11):1062-1068.

5. Marquez VE, Barchi JJ, Kelley JA, Rao KV, Aqbaria R, Ben-Kasus T, et al. Zebularine: a unique molecule for an epigenetically based strategy in cancer chemotherapy. The magic of its chemistry and biology. *Nucleosides, Nucleotides, and Nucleic Acids.* 2005 24(5-7):305-318.

6. Yoo CB, Cheng JC, Jones PA. Zebularine: a new drug for epigenetic therapy. *Biochemical Society Transactions*. 2004 32(6):910-912.

7. Hahn NM, Bonney PL, Dhawan D, Jones DR, Balch C, Guo Z, et al. Subcutaneous 5-azacytidine treatment of naturally occurring canine urothelial carcinoma: a novel epigenetic approach to human urothelial carcinoma drug development. *The Journal of Oncology*. 2012 187(1):302-309.

8. Marquez VE. Recent developments of two NCI epigenetic drugs: zebularine and 3-deazneplanocin-A. In: *American Association for Cancer Research 101st Annual Meeting Education Book*; 2010 Apr 17-21; Washington, DC. Philadelphia: AACR; 2008. pp 17-22.

CHAPTER 2. LITERATURE REVIEW

2.1 Introduction

This chapter will begin with a review of the available literature on zebularine's (zeb) mechanisms of action as a cytidine deaminase (CDA) inhibitor, DNA methyltransferase (DNMT) inhibitor and radiosensitizer. Then, this chapter reviews zeb's activity *in vivo* and *in vitro* and its pharmacokinetics in rodents and monkeys. Finally, this chapter provides an introduction to human and canine invasive transitional cell carcinoma (InvTCC), and the basis for the application of demethylating therapy in this disease.

2.2 Zebularine

Zeb is the 4-desamino analog of cytidine.[1-4] Zeb was first synthesized in 1961.[3] Zeb was originally identified as a bacteriostatic drug, then determined to be a potent CDA inhibitor and more recently determined to be a DNMT inhibitor.[3-5] In addition, zeb acts as a radiosensitizer.[6,7] Zeb's physical characteristics make it ideal for oral administration. Zeb has a half-life of 44 hours at 37°C in phosphate buffered saline at pH 1.0, and 508 hours at pH 7.0.[2] The triazine ring of 5-azacytidine (Vidaza®, Celgene Corp., Summit, NJ, USA; 5-Aza-CR) and decitabine (Dacogen®, SuperGen, Inc., Dublin, CA, USA; 5-Aza-CDR) rapidly decompose even at neutral pH.[3] Unlike 5-Aza-CR and 5-Aza-CDR, which rapidly decompose even at neutral pH, zeb is stable over a broad pH

range and is not subject to rapid inactivation by CDA.[3,4] The stability of zeb over a broad pH range allows for oral administration, unlike 5-Aza-CR and 5-Aza-CDR.[2] While zeb has not been demonstrated to be as potent as 5-Aza-CR or 5-AZa-CDR *in vitro* or *in vivo*, its stability and reported lack of toxicity allows for chronic or continuous administration.[3] Once incorporated into DNA, zeb is as potent as 5-Aza-CR.[5]

2.3 Zebularine inhibits the activity of cytidine deaminase

After zeb's initial synthesis, it was determined to be an inhibitor of CDA.[3,4,8,9] CDA is normally involved in the pyrimidine salvage pathway and converts cytidine and deoxyctidine to uridine and deoxyuridine. CDA is important in the metabolic disposition of nucleoside analogs including cytosine arabinoside and 5-Aza-CDR. CDA rapidly deaminates cytosine arabinoside and 5-Aza-CDR, resulting in their inactivation.[4,9]

Unlike other nucleoside analogs, zeb is not subject to deamination by CDA, but rather inhibits the enzyme's activity. Zeb can enhance the activity of 5-Aza-CDR and cytosine arabinoside by inhibiting their CDA mediated degradation.[4,8,] The combination of zeb and either cytosine arabinoside or 5-Aza-CDR resulted in improved survival in tumor bearing mice with L1210 or P388 leukemia.[3] Upregulation of CDA occurs in relapsed leukemias, and can be an important mechanism of resistance to 5-Aza-CDR and cytosine arabinoside.[8]

2.4 Zebularine is incorporated into DNA and inhibits DNMTs

Cytidine analogs are incorporated into DNA in place of cytosine residues.[1] Once incorporated into DNA, zeb forms a complex with DNMTs, thus inhibiting methylation. The mechanism of action of zeb's demethylating activity is essentially identical to 5-Aza-CR and 5-Aza-CDR.[1,4,10] The target for DNMTs is normally the second cytosine base in a GCGC sequence. Normally, a cytosine base undergoes methylation at the 5' position when it rotates out of the DNA helix through a process called base flipping.[1,3] When zeb rotates out of the DNA helix, a covalent bond forms between the zeb incorporated DNA and the DNMT, resulting in a stable nucleoprotein complex.[1]

As these zeb-DNA-DNMT complexes form throughout the genome, DNMTs are sequestered and depleted, resulting in decreased DNMT activity.[11] Following zeb treatment, DNMT1's activity is decreased first. *In vitro*, the activity of DNMT1 is frequently completely inhibited. The activity of DNMT3b and then DNMT3a are also decreased, but not as robustly. A lack of change in DNMT RNA transcripts following zeb treatment supports that zeb's mechanism of action is sequestration of DNMT in stable nucleoprotein complexes.[3]

Activation of zeb is complicated and inefficient. Zeb must undergo phosphorylation and conversion into its deoxynucelotide before it can be incorporated into DNA.[3,4] Zeb is converted to 2'-deoxyzebularine-5'monophosphate (dZMP), 2'-deoxyzebularine-5'diphosphate (dZDP), 2'-deoxyzebularine-5'triphosphate (dZTP), 2'-deoxyzebularine-5'diphosphocholine (ZDP-chol) and 2'-deoxyzebularine-5'diphosphoethanolamine. The metabolites dZTP and ZDP-chol are the most

common, but only the triphosphate dZTP can be incorporated into DNA. The rate limiting step in the zeb's activation and incorporation into DNA is the conversion of zebularine-5'diphosphate to dZDP via ribonucleotidediphosphate reductase. Up to sevenfold more zeb is incorporated into RNA than DNA.[3]

2.5 Zebularine is preferentially incorporated into tumor cells compared to normal fibroblasts

One concern with the use of demethylating agents is the induction of global genome hypomethylation, which has been associated with cancer and genomic instability.[1,10] While demethylation of the hypermethylated promoter region of a tumor suppressor gene would be beneficial in cancer therapy, removing repressive methylation from the promoter region of a proto-oncogene within a tumor could have deleterious results.[1] Interestingly, zeb appears to be preferentially incorporated into the DNA of human tumor cells compared to normal human fibroblasts *in vitro*.[10]

Zeb's activity was investigated in seven human cancer cell lines (T24, HCT15, CFPAC-1, SW-48, HT-29, PC3 & CALU-1) and four normal human fibroblast cell lines (LD98, T-1, LD419 & CCDD-1070Sk). Following continuous treatment with 1,000 μM zeb, the doubling time increased in all seven cancer cell lines. The cancer cell lines appeared to be more susceptible to the affects of zeb than normal fibroblasts. Doubling times increased from 33 to 68% in the tumor cell lines and by 12 to 21% in fibroblast cell lines. A two- to sevenfold increase in *p21*, which encodes a protein that binds to proliferating cell nuclear antigen and halts DNA replication, was

detected in the cancer cell lines following zeb treatment. No significant changes in *p21* were detected in fibroblasts.[10]

Promoter methylation resulting in transcriptional silencing of *p16* was known to be present in all seven cancer cell lines, while normal *p16* was present in the fibroblast cell lines. Following 1,000 μM zeb treatment, *p16* mRNA levels remained unchanged in human fibroblasts, but *p16* was induced in five cancer cell lines (T24, HCT15, CFPAC-1, SW48 and HT-29). With continued zeb treatment, *p16* expression was induced in a sixth cancer cell line (CALU-1). The growth inhibitory affects of zeb appear to be due to a combination of upregulation of *p21* in all seven cell lines and induction of *p16* in five of the cell lines.[10]

Levels of DNMT1 were evaluated and determined in all seven cancer cell lines and human fibroblasts. DNMT1 activity was decreased in the normal fibroblasts and two cancer cell lines (PC3 and CALU-1), but residual activity was still present. Levels of DNMT1 were almost completely depleted in T24, HCT-15, CFPAC-1, SW48 and HT-29 cell lines. DNMT1 mRNA levels were unchanged, supporting that depletion occurred due to sequestration of DNMT within zeb-DNA-DNMT complexes. Zeb's growth inhibitory effects were not as great in the cancer cell lines that did not have complete depletion of DNMT1 (PC3 and CALU-1) and normal human fibroblasts.[10] Overall, cancer cells were more susceptible to zeb induced growth inhibition, and DNMT1 was almost completely inhibited in five of the cell lines.

Differences in zeb's activity in cancer cells compared to normal cells may be due to differences in metabolic pathways. Uridine/cytidine kinase is responsible for

zeb phosphorylation, which is necessary for zeb activation and incorporation into DNA. The activity of uridine/cytidine kinase was three to 40 times greater in cancer cell lines compared to human fibroblasts. The fact that CALU-1, a cancer cell line that was resistant to zeb, had low uridine/cytidine kinase activity supports that this pathway is important in responsiveness to zeb. The uridine/cytidine kinase pathway likely regulates how much zeb is eventually incorporated into DNA and this enzyme is more expressed in many cancer cell lines compared to normal fibroblasts.[10] The potential for sparing normal tissues is a significant advantage, and may allow for accumulation of zeb within tumor tissues while avoiding toxicity to normal cells.

2.6 Zebularine is a radiosensitizer

Dote et al. investigated zeb's influence on DNA methylation and radiosensitivity in human cell lines from histologies that are normally treated with radiation therapy including MiaPaCa (pancreatic carcinoma), U251 (glioblastoma) and DU145 (prostate carcinoma). Methylation-specific polymerase chain reaction was used to detect methylation of *RASSF1A*, *HIC-1* and *14-3-3σ*, three genes that are frequently methylated in human cancers. All three genes were methylated in MiaPaCa and U251 cell lines. *RASSF1A* was methylated, *HIC-1* was partially methylated and *14-3-3σ* was unmethylated in the DU145 cell line.[6]

Following treatment with zeb for 48 hours at 200 μM (MiaPaCa) and 300 μM (U251 and DU145), increased unmethylated *RASSF1A* and *HIC-1* were detected in all three cell lines, and unmethylated *14-3-3σ* was detected in MiaPaCa and U251 tumor cells. Reverse transcription-PCR analysis revealed increased expression of *RASSF1A*

and *HIC-1* in all three cell lines, and increased expression of *14-3-3σ* in MiaPaCa and U251 cell lines. Unmethylated *RASSF1A* was detectable within 24 hours of zeb treatment. Treatment of all three cell lines with zeb for 24 hours prior to treatment with up to 8 gray (Gy) increased radiosensitivity. The maximum increase in radiosensitivity was observed following zeb treatment for 48 hours prior to irradiation.[6]

In vivo effects of the combination of zeb and radiation were investigated in male NCr *nu/nu* mice implanted with U251 xenografts. To determine the demethylating activity of zeb in U251 xenografts, zeb was administered at 350 mg kg^{-1} by intraperitoneal injection for 5 days and tumor samples were evaluated by PCR for *RASSF1A*, *HIC-1* and *14-3-3σ*. Expression of all three genes was maximized following three days of zeb treatment compared to controls, with no increase detected with additional days of treatment. Mice were randomized to no treatment, zeb alone, radiation alone (4 Gy) or zeb and radiation, and then tumor growth was evaluated. The time for tumors to reach a size of 1,400 mm^3 in mice treated with the combination of zeb and radiation was significantly increased compared to all other treatment groups.[6]

Kim et al. investigated six DNMT inhibitors including 5-Aza-CR, 5-Aza-CDR, zeb, hydralazine, epigallocatechin gallate (EGCG) and psammaplin A on radiosensitivity *in vitro* in A549 (human lung cancer with a wild type *p53*) and U373MG (human glioblastoma with an inactive *p53* mutant) cell lines. A zeb concentration of 800 μM was used because this was the *in vitro* IC_{50} for these cell lines. Treatment with 5-Aza-CDR, zeb and psammaplin A before irradiation with up

to 8 Gy resulted in significantly enhanced radiation cell killing in both cell lines. Western blot revealed depletion of DNMT1 and DNMT3a in both cell lines following treatment with 5-Aza-CDR, zeb and psammaplin A, but no change in DNMT3b.[7]

γH2AX expression, which is associated with radiation induced double strand DNA breaks, is increased following irradiation with zeb pretreatment.[6,7] In MiaPaCa, U251 and DU145 cell lines pretreated with zeb followed by irradiation, *γH2AX* expression was not increased one hour following irradiation, but was significantly greater at 24 hours when compared to controls treated with irradiation alone.[6] Similarly, *γH2AX* expression in A549 and U373MG cell lines was no different 1 hour after irradiation with 5-Aza-CDR, zeb and psammaplin A pretreatment, but increased significantly over time compared to irradiation alone.[7] Increased *γH2AX* expression suggests persistent, unrepaired double strand breaks and that zeb enhanced radiosensitivity may be due to inhibition of DNA repair.[6,7]

Meador et al. determined that the DNA-dependent protein kinase (DNA-PK) deficient human glioblastoma cell line MO59J was more sensitive to zeb treatment up to 300 μM compared to MO59K, a DNA-PK proficient human glioblastoma cell line. DNA-PK is a member of the phosphatidylinositol-3 kinase super family and normally functions to remove DNA damage such as double strand breaks or adducts. DNA-PK deficient cells are inherently sensitive to radiation due to an inability to repair DNA damage. Prolonged G_2/M arrest is usually detected in DNA-PK deficient cells, but this arrest was not detected following zeb treatment in the MO59J cell line. Following treatment with zeb, the MO59J cell line demonstrated increased genomic instability indicated by increased numbers of centrosomes per cell, polyploid cells and

micronuclei formation indicating chromosome breakage and mis-segregation. DNMT1 is normally involved in the repair of UV-laser induced double strand breaks, and its depletion by zeb may result in impaired DNA repair. Defects in DNA repair and cell cycle checkpoint mechanisms resulted in enhanced zeb cytotoxicity as a single agent in the DNA-PK deficient MO59J cell line.[12]

Demethylation and reactivation of specific genes and increased radiosensitivity has been demonstrated following zeb pretreatment *in vitro* and *in vivo*.[6,7] Results of these studies suggest that the combination of radiation therapy and zeb pretreatment may be clinically useful. Evidence suggests that radiosensitivity may be enhanced following zeb pretreatment due to inhibition of DNA repair, potentially due to depletion of DNMT1 or enhancement of inherently dysregulated cellular pathways within a tumor such as DNA-PK.[6,7,12] Zeb's increased uptake in tumor cells compared to normal cells[10] would support its utility as a radiosensitizer, as it would be expected to accumulate in tumor tissue and not nearby normal tissues. While the use of high concentrations of zeb have been reported prior to irradiation, the minimum zeb concentration required to achieve enhanced radiosensitivity remains unknown. Future efforts should be aimed not only at determining the optimal delivery schedule, but the minimum zeb dose required to achieve the desired radiosensitization.

2.7 Zebularine inhibits tumor growth and reactivates silenced genes

Zeb administered by intravenous or intraperitoneal injection, oral gavage or in drinking water is reported to be well tolerated *in vivo* in genetically tumor-prone

mice[13], transgenic mice[14], mice harboring tumor xenografts[15-17] and mice with radiation-induced tumors.[18] Demethylating activity that correlates with tumor responses including stable disease, delayed or decreased tumor development and decreases in tumor volume compared to untreated controls have been reported.[13-18]

The reported dose of zeb administered to mice has varied between studies. Neuriter et al. reported the use of a relatively high dose of 1,000 mg kg^{-1} by intraperitoneal injection in mice with pancreatic tumor xenografts. Male NMRI mice with human Panc-89 cells implanted into the flank were allowed to develop seven millimeter tumors. Mice were then treated daily for seven days with either zeb or saline by intraperitoneal injection. Tumors in zeb treated mice remained stable in size over the seven day period, while there was a statistically significant increase in size of tumors in saline treated controls. Untreated tumors increased in size by 33% over the same period of time.[15] While shrinking tumors is often considered the goal of chemotherapy, arresting tumor growth may be sufficient to improve patient outcomes in many tumor types.

Herranz et al. used a lower zeb dose in a long-term injectable treatment protocol in C57BL/6J mice treated with gamma irradiation to induce T-cell thymic lymphoma. Mice were treated with 400 mg kg^{-1} by intraperitoneal injection for 78 days or phosphate buffered saline. Control mice treated with phosphate buffered saline died, as expected, of thymic lymphoma within six months of irradiation. Mice treated with zeb had significantly improved survival compared to controls. Of 30 mice treated with zeb, 40% (n=12) were still alive 12 months after irradiation. Decreased levels of DNMT1 and a 30% decrease in 5-methylcytosine were detected

in the thymus of mice treated with zeb that survived for 12 months when compared to thymic lymphomas that developed in controls. Demethylation of hypermethylated promoters of *p16*^{Ink4a}, *MGMT*, *MLT*-1, and *E-cadherin* was detected along with expression of the protein products. No remarkable toxicity was detected and thorough necropsies were performed.[18] This is one of the longest reported zeb treatment protocols in mice, and demonstrated that continuous zeb administration was feasible and safe in mice.

Zeb was the first demethylating agent to demonstrate effective demethylation and antitumor effects following oral administration. Cheng et al. treated mice with bladder cancer xenografts with up to 1,000 mg kg^{-1} zeb by oral gavage or intraperitoneal injection. Male BALB/c nu/nu mice had EJ6 bladder cancer cells implanted into the flank, and were then treated with zeb for 18 days. Minimal weight loss and no deaths were reported in treated mice. Tumor growth was significantly inhibited following treatment with 1,000 mg kg^{-1} orally or by intraperitoneal injection. The *p16* promoter, which is normally methylated in EJ6 cells, was significantly hypomethylated in tumors from mice treated with 500 or 1000 mg kg^{-1} orally or intraperitoneally compared to untreated controls. The detection of tumor growth inhibition and demethylating effects *in vivo* following oral administration confirmed zeb's stability and demonstrated its clinical potential.[17]

Zeb also appeared to be effective following oral administration at even lower doses in mice. Female BALB/c nu/nu mice had BGC823 gastric cancer cells implanted into the flank, and were then treated with up to 100 mg kg^{-1} by oral gavage once every four days for 20 days. No morbidity or mortality was reported, and a

significantly decreased tumor weight was detected at day 20 for doses of 10 mg kg^{-1}, 50 mg kg^{-1} or 100 mg kg^{-1} compared to controls. Expression of p16 and BAX increased and expression of BCL-2 decreased as zeb concentration increased.[16]

Alternatives to oral gavage including spiking drinking water with zeb have also been reported. Treatment with zeb in drinking water was well tolerated in a majority of *Min* mice and resulted in tumor reduction in female mice. These mice harbor a defect in the *APC* gene that leads to development of benign intestinal adenomas. Most mice treated with 0.2 mg mL^{-1} zeb in drinking water from birth for 120 days appeared to be normal and healthy, but 14% died between weeks five and seven of treatment. Importantly, this mortality may be a result of an exaggerated sensitivity to zeb in *Min* mice as a dose of 1 mg mL^{-1} resulted in 100% death and a dose of 0.1 mL^{-1} resulted in 100% survival. In female *Min* mice, average polyp number decreased from 58 to one. In male *Min* mice, there was no change in the average polyp number. There were no reported differences in toxicity based on sex.[13] This was the first report of a sex difference in mice treated with zebularine. This difference may be explained by differential activity of aldehyde oxidase in male mice compared to females.[19] The role of aldehyde oxidase in zeb metabolism is further discussed later in this chapter.

While *Min* mice died following a dose of 1 mg mL^{-1} zeb in drinking water, transgenic female FVB/N-Tg(MMTV-PyMT)^{634Mul} mice tolerated treatment with 5 mg mL^{-1} zeb in drinking water. These mice spontaneously develop mammary tumors by 60 days of age. Zeb treatment began at 46 days of age and resulted in a statistically significant 10 day delay in the onset of tumor development compared to

untreated controls. Mice were treated for 48 days and then sacrificed to determine total tumor burden, which was significantly decreased in treated mice compared to controls. Treatment morbidity and mortality were not reported.[14]

Overall, zeb is reportedly well tolerated over a wide range of doses and routes of administration in mice. Significant tumor responses and changes in DNA methylation have been detected *in vivo*, providing strong evidence that zeb may be clinically useful.

2.8 Zebularine pharmacokinetics in mice, rats and rhesus monkeys

Initial investigation into zeb's pharmacokinetics was performed in mice, rats and rhesus monkeys. Animal subjects consisted of five to six week old male CD_2F_1 mice, seven to eight week old male Fischer 344 rats and six to nine year old male and female rhesus monkeys (*Macaca mulatta*). Mice received a zeb dose of either 100 mg kg^{-1} intravenously or 1,000 mg kg^{-1} orally. Rats received a zeb dose of either 50 mg kg^{-1} intravenously, 250 mg kg^{-1} orally or 500 mg kg^{-1} orally. One male and one female rhesus monkey each received a zeb dose of either 500 or 1,000 mg kg^{-1} intravenously on day one and then the same dose orally 16 days later. Serial blood samples were collected over the next 24 to 48 hours and processed to plasma.[20]

Peak zeb plasma concentrations following intravenous injection in mice were between 354 and 702-μg mL^{-1} (1,550 to 3,070 μM), half-life was 40 minutes with an AUC of 7,323 μg mL^{-1}. Peak zeb plasma concentrations following oral administration in mice were between 28 and 35 μg mL^{-1} (120 to 150 μM) at 90

minutes. Plasma zeb remained above the lower limit of quantification (0.03 μg mL^{-1}) for 960 minutes. AUC was 4,935 μg mL^{-1} and oral bioavailability was 6.7%.[20]

Peak zeb plasma concentrations following intravenous injection in rats were between 165 and 238-μg mL^{-1} (723 to 1,040 μM), half-life was 363 minutes with an AUC of 12,526 μg mL^{-1}. Peak zeb plasma concentrations following oral administration of 250 mg kg^{-1} in rats were between 10 and 11 μg mL^{-1} (44 to 48 μM) at 120 minutes. AUC was 1,969 μg mL^{-1} and oral bioavailability was 3.1%. Peak zeb plasma concentrations following oral administration of 500 mg kg^{-1} in rats were between 31 and 32 μg mL^{-1} (135 to 140 μM) at 120 minutes. The AUC was 7,612 μg mL^{-1} and oral bioavailability was 6.1%.[20]

Intravenous administration of 500 mg kg^{-1} resulted in a peak zeb plasma concentration of 1,094 μg mL^{-1} (4,794 μM) in a male rhesus monkey and 537 μg mL^{-1} (2,350 μM) in a female rhesus monkey at five minutes. Plasma zeb concentration decreased to below the level of quantification after 480 minutes in the male monkey and 240 minutes in the female monkey. Estimated half-life was 70 and 76 minutes, AUC were 88,020 and 46,080 μg mL^{-1}. Oral administration of 500 mg kg^{-1} resulted in a peak zeb plasma concentration of 0.37 and 0.18 μg mL^{-1} (1.6 and 0.79 μM) at 60 and 30 minutes. AUC was 84 and 12 μg mL^{-1}. Oral bioavailibilities following an oral dose of 500 mg kg^{-1} were 0.1% and 0.026%.[20]

Intravenous administration of 1000 mg kg^{-1} resulted in a peak zeb plasma concentration of 1,656 μg mL^{-1} in a male rhesus monkey and 2,853 μg mL^{-1} in a female rhesus monkey at five minutes. Plasma zeb concentration was above the level of quantification for 24 hours following administration. Estimated half-life was 150

and 147 minutes, and AUC was 104,520 and 281,220 μg mL^{-1}. Oral administration of 1,000 mg kg^{-1} resulted in peak zeb plasma concentrations of 0.5 and 0.92 μg mL^{-1} at 30 minutes and 8 hours. AUC was 42 and 1,128 μg mL^{-1}. Oral bioavailabilities following an oral dose of 1,000 mg kg^{-1} were 0.04% and 0.4%.[20]

Based on the pharmacokinetic parameters determined in mice, rats and rhesus monkeys, frequent administration of zeb was recommended in order to achieve desired plasma levels. The relatively low bioavailability, particularly in rhesus monkeys, was surprising and led to the suggestion that continuous intravenous administration or frequent oral dosing would be required. The reason for the low bioavailability, particularly in rhesus monkeys, was unknown, but suggested causes included first pass metabolism by aldehyde oxidase or saturation of an absorption pathway in the intestines.[20]

Interestingly, when male cynomolgus monkeys (*Macaca fascicularis*) were treated with continuous zeb infusions for five days out of seven designed to achieve plasma concentrations of 10 or 15 μM, toxicity was detected. Toxicity included elevated white blood cell counts, reticulocyte counts, alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK). Treatments designed to yield plasma concentrations of 25 to 50 μM or bolus intravenous injections of 500, 750 or 1000 mg kg^{-1} proved lethal. Rapid rises in zeb plasma concentrations prior to death were suggestive of impaired zeb clearance, possibly due to liver or kidney injury. Bolus doses $\leq$ 250 mg kg^{-1} resulted in elevated renal and liver values but no deaths.[21]

2.9 Aldehyde oxidase and zebularine metabolism

Zeb is ultimately metabolized to uridine, uracil and dihydrouracil, all of which are normally present in the body. In a study of zeb metabolism in male CD_2F_1 mice, approximately 38% of zeb was oxidized to uridine by aldehyde oxidase (AO).[22] AO activity was evaluated in hepatic cytosol from mice, rats, humans, a male cynomolgus monkey and a male Beagle dog. Among the species in which activity was detectable, the activity was highest in male mice and lowest in female mice. Activity was not detectable in hepatic cytosol from a male Beagle dog. A 50-fold difference was detected between male and female mice, but a similar magnitude of difference was not detected between sexes in rats or humans.[19] there is often greater AO homology between mice and humans than monkeys and humans.[4]

Supraphysiologic concentrations of uridine have been reported to cause hyperthermia and hypothermia in mice. In humans, prolonged infusions of uridine have been reported to cause hyperthermia and shivering has been reported during shorter high dose infusions. The shivering reported in humans does not appear to be due to changes in body temperature, and may be due to interference in the metabolism of neurotransmitters like dopamine and neuropeptide. Based on body surface area, a dose of 100 mg kg^{-1} in the mouse would be equivalent to a dose of 8 mg kg^{-1} in humans. This dose would result in a uridine exposure of 3 mg kg^{-1}, which is much lower than pharmacologic doses ranging from 150 to 1,000 mg kg^{-1} d^{-1} currently used to treat human diseases. The production of uridine can also decrease the efficacy of fluoropyrimidines like 5-fluorouracil.[22] While uridine levels were not

assessed, zeb did decrease the activity of 5-fluorouracil against human oral squamous cell carcinoma cell lines *in vitro* when the two drugs were combined.[23]

While exposure to uridine is unlikely to pose a significant risk, the production of hydrogen peroxide when AO converts zeb to uridine may pose a risk. In all species that express AO, its activity is highest in the liver.[22] Elevations in ALT and LDH were detected in male cynomolgus monkeys treated with zeb to achieve plasma concentrations of 10 to 15 μM. Zeb was lethal in cynomolgus monkeys treated to target plasma concentrations of 25 to 50 μM or with bolus intravenous injections greater than 500 mg kg^{-1}. In these monkeys, zeb plasma concentrations rapidly increased prior to death, suggesting that liver or renal injury resulted may have decreased zebularine clearance. Bolus intravenous dosing of ≤ 250 mg kg^{-1} did not result in any deaths, but elevations in hepatic and renal parameters were detected.[21] Production of large amounts of hydrogen peroxide resulting in massive hepatocellular injury could account for the apparent liver injury observed in monkeys.

If differences in AO activity were responsible for differences in toxicity, then a difference in toxicity would be expected in mice since there is a 50-fold difference in AO activity between the sexes. While no difference in toxicity has been detected between male and female mice, differences were detected in tumor prevention in *Min* mice. Following treatment with zeb, the number of tumors that develop in female mice is significantly decreased, but not in male mice.[13] The implication of the lack of AO activity in the dog is unclear, as is the true cause of the hepatic toxicity in monkeys. It is unknown whether or not similar toxicity would be observed in dogs or humans treated with zeb.

2.10 Invasive transitional cell carcinoma in man and the dog

Around the world, human bladder cancer accounts for 3.3% of newly diagnosed cases and 2.1% of all cancer deaths.[24] Bladder cancer is the 5th most common cancer diagnosed in the United States. Treatment is often very involved and costly due to frequent tumor recurrence. While most humans develop non-invasive, superficial bladder tumors that can be successfully treated locally by instillation of therapeutic agents directly into the bladder or transurethral tumor resection, some will go on to develop muscle InvTCC. Others will have invasive cancer already present at diagnosis.[25] In 2008, an estimated 386,300 new cases of bladder cancer were diagnosed, and 150,200 people died due to bladder cancer around the world.[26] More than 14,000 people in the United States died due to InvTCC in 2011.[27]

In contrast to humans in which lower grade superficial bladder tumors predominate, InvTCC is the most common cancer affecting the urinary tract in the dog.[28] While the true prevalence is unknown, InvTCC is estimated to account for up to 2% of all canine cancers.[27,28] Risk factors for the development of InvTCC in the dog include use of older generation topical insecticides and dips, obesity, female sex and breed.[28] Scottish Terriers are at a 20-fold increased risk.[28] Exposure of Scottish Terriers to phenoxy herbicides like 2,4-D further increases the risk of InvTCC.[29] Other breeds including Beagles, Shetland Sheepdogs, Wire Hair Fox Terriers and West Highland White Terriers are also at an increased risk. Half or more of dogs diagnosed with InvTCC will develop distant metastasis by the time of death. The most common sites of metastasis are the lungs and regional lymph nodes. Death often occurs prior to the development of metastasis due to urinary tract obstruction.[28]

Dogs with naturally occurring InvTCC offer a highly relevant model of aggressive bladder cancer in humans due to similar clinical, cellular and biological features, and responses to chemotherapy.[27,30] Most InvTCC-related deaths in dogs and humans are due to non-resectable chemotherapy-resistant cancer. Developing novel strategies to improve treatment efficacy is essential for both dogs and humans. Overexpression of genes including *bFGF*, *COX1* (*PTGS1*) and *COX2* (*PTGS2*) and mutations in *TP53* are present in both human and canine InvTCC.[30]

While the methylation patterns in canine InvTCC have not been investigated[27], methylation patterns in human superficial bladder cancer and InvTCC have been investigated. The development of bladder cancer in humans is known to involve multiple oncogenes and tumor suppressor genes, including *FGFR3*, *HRAS*, *CDH1*, *CDKN2A* (*p16INK4a* and *p14ARF*), *HOXB2*, *RASSF1A*, *RB1*, *SFRP2* and *WAF1*.[25,30,31] DNA hypermethylation is known to cause transcriptional silencing of many of these genes in human bladder cancer.[30] Hypermethylation of specific genes is frequently reported in human bladder cancer, but with inconsistent results.[32] For example, Zhu et al. reported hypermethylation of *MLH1*, *HOXA9*, *SLT2* and *HIC-1* in a variety or bladder carcinomas compared to adjacent normal tissues, but were unable to demonstrated hypermethylation of *RASSF1A*, *MGMT* and *p16,* which had previously been reported. Other changes important in urothelial carcinogenesis include deletions of chromosome 9 and defects in p53.[31] Global hypomethylation is more consistently reported in human bladder cancers, which may result in, or reflect, genomic instability.[32]

Specific methylation patterns are not consistent across all human bladder tumors, but methylation status is associated with advanced tumors and tumor progression. Yates et al. investigated promoter methylation within 17 genes from 96 malignant urothelial cancers and 30 normal samples. Statistically significant higher rates of methylation were detected in eight of 17 genes (*WIF-1*, *TNFRSF25*, *EDNRB*, *APC*, *MGMT*, *BCL2*, *hTERT*, *IGFBP3*) for malignant tumors versus normal tissue. No statistically significant differences were detected for *RASSF1A*, *DAPK*, *RAR*β, *CYCLIND2*, *TIMP3* and *HIC-1* or *CDH1*, *CDH4* and *P16* after applying Bonferonni's correction. Methylation at these 17 loci was significantly increased in poorly differentiated urothelial cancers compared to well-differentiated tumors, and invasive tumors compared to superficial tumors. Tumors that had methylated *TNFRSF25*, *EDNRB*, *RASSF1A*, *APC* and *CDH1* were significantly more likely to progress to a more aggressive stage of disease and result in patient death. No tumors that lacked methylation or had methylation of one gene progressed. Patients could be stratified based on their risk of developing disease progression using subsets of these genes, suggesting that assessment of methylation status may be useful in determining what patients should undergo more vigilant monitoring.[33] The ability to predict which patients are unlikely to develop tumor recurrence could result in significant cost savings, and direct resources toward patients more likely to develop tumor recurrence.

2.11 Investigation of DNMT1 in canine InvTCC cell lines

Expression of DNMT1 has been demonstrated in human InvTCC and other cancers. Dhawan et al. demonstrated that DNMT1 is also upregulated in canine

InvTCC.[27] Microarray gene expression profiling demonstrated a 2.28-fold increase in DNMT1 expression in biopsy samples from four dogs with InvTCC compared to samples of bladder mucosa from four normal dogs. Positive DNMT1 immunoreactivity (>10% of cells with positive nuclear staining) was detected in 45% (n=10) of canine biopsy samples and no immunoreactivity was detected in bladder samples from normal dogs (n=6).[27] DNMT1 would be expected in a greater proportion in rapidly proliferating tissues as it is highly expressed during S-phase.

Increased DNMT1 expression in canine InvTCC appeared to be a rational target for treatment with DNMT1 inhibitors, and further investigation was performed in three canine InvTCC cell lines (K9TCC, K9TCC-PU-Sh, K9TCC-PU-Nk). Cell lines were treated with the DNMT inhibitor 5-Aza-CR, the histone deactylase inhibitor trichostatin A or the combination. Treatment with trichostatin A alone resulted in morphologic changes in all three cells lines. Treatment with either 5-Aza-CR or trichostatin A resulted in decreased cell counts, but the combination resulted in a larger decrease. Decreased DNMT1 protein was detected by Western blot in two of three cell lines (K9TCC and K9TCC-PU-Sh) following treatment with 5-Aza-CR. Decreased DNMT1 protein was detected in all cell lines following treatment with trichostatin A.[27]

Following treatment with 5-Aza-CR, p16 was slightly increased in all three cell lines, and p21 was decreased in one cell line (K9TCC-PU-Sh). Following treatment with trichostatin A or combination 5-Aza-CR and trichostatin A, there was increased expression of p16 and decreased expression of cyclin D1, p21, pRb,

survivin & PARP in all three cell lines. Expression of p16 was slightly greater following combination treatment in K9TCC than with trichostatin A alone.[27]

The *p16* gene is often silenced by hypermethylation in human cancers, and therefore, the increase in p16 protein following treatment of canine InvTCC cell lines with 5-Aza-CR, TSA or the combination is of considerable interest. These results suggest that while the DNMT inhibitor 5-Aza-CR has some activity alone, the addition of an HDAC inhibitor like TSA may yield better results. As the safety, toxicity and efficacy of most DNMT inhibitors and HDAC inhibitors have not been assessed in dogs with cancer, further investigation of these agents alone and then in combination is indicated.[27]

2.12 Investigation of 5-Aza-CR in dogs with InvTCC

A phase I study of subcutaneous 5-Aza-CR was performed in dogs with naturally occurring InvTCC to determine if further studies should be performed in humans with InvTCC. This dog study was performed to assess toxicity, antitumor activity and a dose range for subsequent work. 5-Aza-CR was administered subcutaneously once daily for five days once every 14 days or 28 days. Dogs were initially treated with a dose of 0.2 mg kg^{-1} per day, which is equivalent to 10% of the FDA approved dose of 5-Aza-CR for the treatment of myelodysplastic syndrome in humans. Dose-limiting toxicity (DLT) was defined as any grade 3 or 4 Veterinary Cooperative Oncology Group common terminology criteria for adverse events v1.1 (VCOG-CTCAE v1.1) adverse event, and the maximum tolerated dose (MTD) was defined as the dose at which no more than one out of six dogs within a cohort

experienced a DLT. A total of 19 dogs were enrolled. One dog was excluded from evaluation of tumor response due to concurrent non-steroidal anti-inflammatory drug (NSAID) administration. Eleven dogs were treated for five consecutive days once every 28 days, and eight dogs were treated for five consecutive days once every 14 days. Of the dogs enrolled, 73% had failed prior therapies.[30]

The most common adverse events following treatment with 5-Aza-CR were myelosuppression, including grade 3 or 4 neutropenia, and gastrointestinal upset, including grade 3 or 4 nausea and anorexia. The MTD was determined to be 0.1 and 0.2 mg kg^{-1} per day when 5-Aza-CR was given for five consecutive days once every 14 days or 28 days, respectively. Decreased tumor volume was detected in 55.6% (n=10) of dogs, and 22.2% (n=4) achieved partial remission (PR; ≥50% or greater decrease in tumor volume). While no complete remissions (CR; complete resolution of all lesions) were detected, the disease control rate, defined as the combination of CR, PR and stable disease (SD; <50% increase or decrease in tumor volume for at least 8 weeks), was 72.2% (n=9).[30]

Samples of peripheral blood mononuclear cells (PBMCs) and bladder tumor tissue were collected for gene-specific (*CDKN2A/p14ARF*) and global methylation assays. No significant differences in global DNA methylation were detected in paired pre- and post-treatment PBMCs from 12 dogs. Non-statistically significant decreases in global DNA methylation ranging from 0.74% to 8.43% were detected in six dogs. No significant differences in global DNA methylation were detected in paired pre- and post-treatment tumor biopsy samples from 10 dogs. Non-statistically significant decreases in global DNA methylation ranging from 1.55% to 17% were detected in

seven dogs. A 6.8% decrease in *CDKN2A* (*p14ARF*) methylation was detected in one dog that achieved PR and had paired tumor samples for analysis.[30]

Treatment with 5-Aza-CR was generally well tolerated in dogs with InvTCC, with reversible myelosuppression and gastrointestinal toxicity as the most clinically relevant toxicity. While no complete remissions were detected, the overall response rate of 72.2% and observation of a decrease in tumor volume in 56% of dogs was promising, particularly when taking into account that 73% of treated dogs had failed prior therapy. The lack of statistically significant DNA hypomethylation and myelosuppression observed in this study may indicate that the tumor responses observed were due to cytotoxic effects as opposed to demethylating activity. Cytotoxicity is known to occur at some doses of 5-Aza-CR, and these doses have not been well defined in the dog. Similarly, tumor responses without detectable changes in DNA methylation have been observed in human cancers treated with drugs thought to work through DNA demethylation. The optimal timing of post-treatment sample collection is unknown, and the lack of significant changes could reflect that samples were not collected at the optimal time interval after initiating demethylating therapy.[30] Another possible explanation for lack of detection of gene-specific hypomethylation is inappropriate target selection. *CDKN2A* (*p14ARF*) is frequently lost through deletions in human bladder cancer[32], thus it may not even be present to undergo demethylation. Similarly, genes other than *CDKN2A* (*p14ARF*) may have undergone gene-specific hypomethylation, but were not investigated.

2.13 Conclusion

Investigation of demethylating agents is just beginning in pet dogs. Treatment of dogs with InvTCC 5-Aza-CR was generally well tolerated and had a promising disease control rate of 72.2%.[30] Canine InvTCC cell lines appear to have increased activity of DNMT1 that can be impaired by treatment with 5-Aza-CR, TSA or the combination.[27] These studies provide a basis for the further exploration of demethylating therapy in pet dogs with InvTCC. While 5-Aza-CR has shown promise, treatment involved frequent subcutaneous injections.[30] Zeb's stability offers a significant advantage over 5-Aza-CR[2,3,4,17], particularly in the treatment of pet dogs, because it can be easily administered orally on a continuous schedule. Despite its apparent low bioavailability in the initial pharmacokinetic study[20], zeb was the first agent to demonstrate *in vivo* demethylating activity following oral administration.[17] Zeb was well tolerated in rodents, but marked hepatic toxicity and death were reported following high dose treatment in male cynomolgus monkeys.[21] The cause of toxicity in cynomolgus monkeys is unknown, but the use of such high doses of zeb in the future would be unlikely.

To the author's knowledge, prior to the work described in the following chapters, no *in vivo* evaluation of zeb has been performed in the dog. The purpose of the work described in the following chapters was to evaluate the pharmacokinetics and toxicity of zeb in laboratory dogs and tumor bearing dogs, and provide an initial description of zeb's activity in dogs with InvTCC.

References

1. Gowher H, Jeltsch A. Mechanism of inhibition of DNA methyltransferases by cytidine analogs in cancer therapy. *Cancer Biology & Therapy*. 2004 3(11):1062-1068.

2. Yoo CB, Cheng JC, Jones PA. Zebularine: a new drug for epigenetic therapy. *Biochemical Society Transactions*. 2004 32(6):910-912.

3. Marquez VE, Barchi JJ, Kelley JA, Rao KV, Aqbaria R, Ben-Kasus T, et al. Zebularine: a unique molecule for an epigenetically based strategy in cancer chemotherapy. The magic of its chemistry and biology. *Nucleosides, Nucleotides, and Nucleic Acids.* 2005 24(5-7):305-318.

4. Marquez VE. Recent developments of two NCI epigenetic drugs: zebularine and 3-deazneplanocin-A. In: *American Association for Cancer Research 101st Annual Meeting Education Book*; 2010 Apr 17-21; Washington, DC. Philadelphia: AACR; 2008. pp. 17-22.

5. van Bemmel DM, Brank AS, Eritja R, Marquez VE, Christman JK. DNA (cytosine-C5) methyltransferase inhibition by oligodeoxyribonucleotides containing 2-(1H)-pyrimidinone (zebularine aglycon) at the enzymatic target site. *Biochemical Pharmacology*. 2009 78(6):633-641.

6. Dote H, Cerna D, Burgan WE, Carter DJ, Cerra MA, Hollingshead MG, et al. *Clinical Cancer Research*. 2005 11(12):4571-4579.

7. Kim HJ, Kim JH, Chie EK, Young PD, Kim IA, Kim IH. DNMT (DNA methyltransferase) inhibitors radiosensitize human cancer cells by suppressing DNA repair activity. *Radiation Oncology*. 2012 7:39. Epub 2012 March 20.

8. Laliberte J, Marquez VE, Momparler RL. Potent inhibitors for the deamination of cytosine arabinoside and 5-aza-2'-deoxycytidine by human cytidine deaminase. *Cancer Chemotherapy and Pharmacology*. 1992 30(1):7-11.

9. Lemaire M, Momparler LF, Raynal NJ, Bernstein ML, Momparler RL. Inhibition of cytidine deaminase by zebularine enhances the antineoplastic action of 5-aza-2'-deoxycytidine. *Cancer Chemotherapy and Pharmacology*. 2009 63(3)411-416.

10. Cheng JC, Yoo CB, Weisenberger DJ, Chuang J, Wozniak C, Liang G, et al. Preferential response of cancer cells to zebularine. *Cancer Cell*. 2004 6(2):151-158.

11. Zhou L, Cheng X, Connolly BA, Dickman MJ, Hurd PJ, Hornby DP. Zebularine: a novel DNA methylation inhibitor that forms a covalent complex with DNA methyltransferases. *Journal of Molecular Biology*. 2002 321(4):591-599.

12. Meador JA, Yanrong S, Ravanat J, Balajee AS. DNA-dependent protein kinase (DNA-PK)-deficient human glioblastoma cells are preferentially sensitized by zebularine. *Carcinogenesis*. 2010 31(2):184-191.

13. Yoo CB, Chuang JC, Byun H, Egger G, Yang AS, Dubeau L, et al. Long-term epigenetic therapy with oral zebularine has minimal side effects and prevents intestinal tumors in mice. *Cancer Prevention Research*. 2008 1(4):233-240.

14. Chen M, Shabashvili D, Nawab A, Yang SX, Dyer LM, Brown KD, et al. DNA methyltransferase inhibitor, zebularine, delays tumor growth and induces apoptosis in a genetically engineered mouse model of breast cancer. *Molecular Cancer Therapeutics*. 2012 11(2):370-382.

15. Neureiter D, Zopf S, Leu T, Dietze O, Hauser-Kronberger C, Hahn EG, et al. Apoptosis, proliferation and differentiation patterns are influence by zebularine and SAHA in pancreatic cancer models. *Scandinavian Journal of Gastroenterology*. 2007 42(1):103-116.

16. Tan W, Zhou W, Yu H, Luo H, Shen L. The DNA methyltransferase inhibitor zebularine induces mitochondria-mediated apoptosis in gastric cancer cells in vitro and in vivo. *Biochemical and Biophysical Research Communications*. 2013 430(1):250-255.

17. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, et al. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *Journal of the National Cancer Institute*. 2003 95(5):399-409.

18. Herranz M, Martin-Caballero J, Fraga MF, Ruiz-Cabello J, Flores JM, Desco M, et al. The novel DNA methylation inhibitor zebularine is effective against the development of murine T-cell lymphoma. *Blood*. 2006 107(3):1174-1177.

19. Klecker RW, Cysyk RL, Collins JM. Zebularine metabolism by aldehyde oxidase in hepatic cytosol from humans, monkeys, dogs, rats and mice: influence of sex and inhibitors. *Bioorganic & Medicinal Chemistry*. 2006 14(1)62-66.

20. Holleran JL, Parise RA, Joseph E, Eiseman JL, Covey JM, Glaze ER, et al. Plasma pharmacokinetics, oral bioavailability, and interspecies scaling of the DNA methyltransferase inhibitor, zebularine. *Clinical Cancer Research*. 2005 11(10):3862-3868.

21. Johnson WD, Harder JB, Naylor J, McCormick DL, Detrisac CJ, Glaze E, et al. 2006. A pharmacokinetic/pharmacodynamic approach to evaluating the safety of zebularine in non-human primates. In: Proceedings of the 97th Annual Meeting of the American Association for Cancer Research; 2006 Apr 1-5; Washington, DC. Philadelphia: AACR; 2006. p 309. Abstract nr 1311.

22. Beumer JH, Joseph E, Egorin MJ, Parker RS, D'argenio DZ, Covey JM, et al. A mass balance and disposition study of the DNA methyltransferase inhibitor zebularine (NSC 309132) and three of its metabolites in mice. *Clinical Cancer Research*. 2006 12(19):5826-5833.

23. Suzuki M, Shinohara F, Endo M, Sugazaki M, Echigo S, Rikiishi H. Zebularine suppresses the apoptotic potential of 5-fluorouracil via cAMP/PKA/CREB pathway against human oral squamous cell carcinoma cells. *Cancer Chemotherapy and Pharmacology*. 2009 64(2):223-232.

24. Bilgrami SM, Qureshi SA, Pervez S and Abbas F. Promoter hypermethylation of tumor suppressor genes correlates with tumor grade and invasiveness with urothelial bladder cancer. *Springer Plus*. 2014 3:178. Epub 2014 April 5.

25. Wolff EM, Chihara Y, Pan F, Weisenberger DJ, Siegmund KD, Sugano K, et al. Unique DNA methylation patterns distinguish non-invasive and invasive urothelial cancers and establish an epigenetic field defect in premalignant tissue. *Cancer Research*. 2010 70(20):8169-8178.

26. Zhu J, Zhimao J, Gao F, Hu X, Zhou L, Chen J, et al. A systematic analysis on DNA methylation and the expression of both mRNA and microRNA in bladder cancer. *PLoS One*. 2011 6:11. Epub 2011 November 30.

27. Dhawan D, Ramos-Vara JA, Hahn NM, Waddell J, Olbricht GR, Zheng R, et al. DNMT1: an emerging target in the treatment of invasive urinary bladder cancer. *Urologic Oncology: Seminars and Original Investigations*. 2013 31(8):1761–1769.

28. Knapp DW, José A. Ramos-Vara JA, Moore GE, Dhawan D, Bonney PL, Young KE. Urinary Bladder Cancer in Dogs, a Naturally Occurring Model for Cancer Biology and Drug Development. *ILAR Journal* 2014 55(1):100-118.

29. Glickman LT, Raghavan M, Knapp DW, Bonney PL, Dawson MH. Herbicide exposure and the risk of transitional cell carcinoma of the urinary bladder in Scottish Terriers. *Journal of the American Veterinary Medical Association*. 2004 224(8):1290-1297.

30. Hahn NM, Bonney PL, Dhawan D, Jones DR, Balch C, Guo Z, et al. Subcutaneous 5-azacytidine treatment of naturally occurring canine urothelial carcinoma: a novel epigenetic approach to human urothelial carcinoma drug development. *The Journal of Oncology*. 2012 187(1):302-309.

31. Wu X. Urothelial tumorigenesis: a tale of divergent pathways. *Nature Reviews Cancer*. 2005 5(9):713-725.

32. Neuhausen A, Florl A, Grim M, Schulz WA. DNA methylation alterations in urothelial carcinoma. *Cancer Biology & Therapy*. 2006 5(8):993-1001.

33. Yates DR, Rehman I, Abbod MF, Meuth M, Cross SS, Linkens DA, et al. Promoter hypermethylation identifies progression risk in bladder cancer. *Clinical Cancer Research*. 2007 13(7):2046-2053.

CHAPTER 3. PHARMACOKINETICS AND TOXICITY OF THE NOVEL ORAL DEMETHYLATING AGENT ZEBULARINE IN LABORATORY AND TUMOR BEARING DOGS

3.1 Authors

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Abbreviated Running Headline:
PK and toxicity of zeb in dogs

Key Words:
Zebularine; demethylating agent; dog pharmacokinetics and toxicity

3.2 Abstract

The purpose of this study was to determine the plasma pharmacokinetics and toxicity of zebularine (zeb), an oral cytidine analog with demethylating activity, in dogs. Plasma

zeb concentrations were determined by HPLC-MS/MS following an oral zeb dose of 8 or 4 mg kg^{-1}. Plasma zeb clearance was constant. Mean C_{max} was 23 ± 4.8 and 8.6 ± 1.4 μM following 8 and 4 mg kg^{-1}, respectively. Mean half-life was 5.7 ± 0.84 and 7.1 ± 2.1 following 8 and 4 mg kg^{-1}, respectively. A single 8 mg kg^{-1} dose was well tolerated. Daily 4 mg kg^{-1} treatment in 3 laboratory dogs resulted in grade 4 neutropenia (n=3), grade 1 anorexia (n=2) and grade 1 or 2 dermatologic changes (n=2). All adverse events resolved with supportive care. A 4 mg kg^{-1} dose every 21 days was well tolerated. A follow up dose escalation study is in progress with a lower starting dose.

3.3 Introduction

DNA methylation is an important epigenetic modification that often occurs on cytosines within cytosine guanine dinucleotides (CpGs).[1,2] CpGs are concentrated within gene promoter regions in half of all human genes.[1] Hypermethylation of DNA within the promoter region of a tumor suppressor gene can result in functional silencing of the gene and an increased risk of cancer development or progression. DNA methylation patterns are maintained by DNA methyltransferases (DNMTs) during DNA replication. Unlike genetic mutations, epigenetic changes are potentially reversible.[1,2]

Zeb (1-β-D-Ribofuranosyl-2(1*H*)-pyrimidinone) is a cytidine analog that was originally identified as a bacteriostatic drug, then determined to be a cytidine deaminase (CDA) inhibitor and more recently determined to be a DNMT inhibitor.[2,3] Zeb is incorporated into DNA in place of cytidine and forms a complex with DNMTs, thus inhibiting methylation. The mechanism of action of zeb's demethylating activity is

similar to that of 5-azacytidine (Vidaza®, Celgene Corp., Summit, NJ, USA; 5-Aza-CR) and decitabine (Dacogen®, SuperGen, Inc., Dublin, CA, USA; 5-Aza-CDR), which are currently FDA approved for the treatment of human cancers.[3] Unlike 5-Aza-CR and 5-Aza-CDR, zeb is stable at a broad pH range and is not subject to rapid inactivation by CDA.[2,3] In addition, zeb acts as a radiosensitizer *in vitro*, which may be due to inhibition of DNA repair.[4,5]

Dogs with naturally occurring cancer offer an excellent opportunity to evaluate and optimize new cancer treatment strategies including those involving demethylating agents.[6,7] The injectable DNMT inhibitor 5-Aza-CR has been investigated in dogs with invasive transitional cell carcinoma (InvTCC) of the urinary bladder with promising results. Subcutaneous injections were administered daily for five consecutive days at two or four week intervals in 18 dogs, resulting in 22% partial remission and 50% stable disease for more than eight weeks.[7] Daily oral administration is likely to be the most convenient dosing strategy and potentially the most efficacious since demethylating agents requires actively dividing cells, making zeb an attractive drug to investigate in subsequent studies.[8,9]

Previous pharmacokinetic and toxicity assessment of zeb has been performed in mice, rats and rhesus monkeys.[10] Zeb administered by intravenous or intraperitoneal injection, oral gavage or in drinking water was well tolerated *in vivo* in genetically tumor-prone mice, transgenic mice and mice harboring tumor xenografts. Demethylating activity correlated with tumor responses including stable disease, delayed or decreased tumor development and decreases in tumor volume compared to untreated control mice.[11-16] Following very high dose zeb treatment in cynomolgus monkeys,

clinicopathologic evidence of liver and kidney injury were detected. Further dose elevations were lethal.[17]

In vivo evaluation of zeb has not been performed in the dog. Previous study in the dog is limited to an *in vitro* zeb plasma protein binding assay performed on samples from Beagle dogs.[10] The purpose of this study was to evaluate the pharmacokinetics and toxicity associated with oral zeb administration in laboratory dogs and dogs with naturally occurring cancer.

3.4 Materials and Methods

Study subjects

All work was approved by the Purdue Animal Care and Use Committee. Laboratory dogs were obtained from Marshall Farms (North Rose, New York, USA) and then housed and evaluated in the Clinical Discovery Laboratory, College of Veterinary Medicine, Purdue University. Privately owned, tumor bearing dogs undergoing treatment at the Purdue University Veterinary Teaching Hospital (PUVTH) were enrolled following informed written pet owner consent. Entry criteria included a definitive diagnosis of cancer via histopathology, measurable cancer and expected survival time of at least 6 weeks. Dogs were allowed to enroll if they had failed other cancer therapy or if other therapy had been declined for any reason. Dogs were allowed to continue non-steroidal anti-inflammatory drugs (NSAIDs) if needed for pain control, and if cancer progression had been documented during NSAID treatment prior to zeb administration. Tumor bearing dogs lived at home with their owners, and were evaluated at the PUVTH.

Zebularine formulation

Pharmaceutical grade zeb was provided through the National Cancer Institute's Experimental Therapeutics (NExT) Program as a bulk powder. Zeb was compounded into gelatin capsules in sizes ranging from 5 to 20 mg by Diamondback Drugs, LLC (Scottsdale, AZ, USA).

Zebularine plasma pharmacokinetics

Plasma pharmacokinetic analysis was performed in samples from dogs following a single oral dose of 8 mg kg^{-1} or 4 mg kg^{-1} with food. Laboratory dogs (n=3) received 8 mg kg^{-1}, and pet dogs received 4 mg kg^{-1}. Doses were rounded to the nearest 5 mg due to capsule size. Serial venous blood samples were collected prior to the first treatment and at 0.5, 1, 1.5, 2, 4, 6, 8, 12 and 24 hours following zeb administration to obtain the plasma concentration versus time profile. Following collection into ethylenediamine-tetraacetic acid (EDTA) blood collection tubes, blood samples were immediately centrifuged (1,800 g, 15 min, 4°C), and plasma was collected. Samples were stored at -80 °C until analysis.

Analyses were performed by the Clinical Pharmacology Analytical Core Facility at Indiana University School of Medicine, Indianapolis, IN, USA. Zeb was quantified in plasma using 5-Aza-CR as the internal standard, liquid-liquid extraction, and HPLC-MS/MS (Thermo Accela pump and Thermo TSQ Quantum Ultra MS/MS; Thermo Fisher). Zeb and 5-Aza-CR were separated by gradient mobile phase (acetonitrile:5mM ammonium formate) and HPLC using a C8 column (Zorbax, 5 µm 250 X 4.6 mm). The Q1/Q3 settings (in positive mode) for zeb and 5-Aza-CR were 229/97 and 245/113,

respectively. The lower limit of quantification (LOQ) is 1 ng mL^{-1} using 200 µL of plasma.

Pharmacokinetic parameters for zeb including area under the curve (AUC), area under the moment curve (AUMC) and the elimination rate constant, k_{el}, were estimated using noncompartmental methods with add-ins on Excel®. The half-life ($t_{1/2}$) was estimated by 0.693 k_{el}^{-1}. Zeb AUC was estimated from time zero to infinity. The AUC from the last concentration, C_{last}, to infinity was estimated by C_{last} k_{el}^{-1}. The systemic clearance (Cl F^{-1}, where F is the bioavailability) was calculated from the dose and $AUC_{0-\infty}$ and the volume of distribution at steady state (Vd_{ss} F^{-1}) was calculated from the Cl and the mean residence time (MRT; MRT =(Dose/AUC)x(AUMC/AUC). The maximum plasma concentration (C_{max}) and the time of C_{max} (t_{max}) were obtained from the data.

Zebularine toxicity

Zeb toxicity was assessed following daily oral dose of 4 mg kg^{-1} in laboratory dogs and an oral zeb dose of 4 mg kg^{-1} once every 21 days in tumor bearing dogs. Toxicity was assessed by physical examination, serial complete blood counts and serial serum biochemistry panels. Serial complete blood counts and serum biochemistry panels were initially planned weekly and then performed as needed based on observed toxicity at the discretion of the attending clinician. Toxicity was graded using the Veterinary Cooperative Oncology Group common terminology criteria for adverse events v1.1 (VCOG-CTCAE v1.1).[18]

3.5 Results

Study subjects

Three laboratory dogs of approximately one year of age (one male intact Beagle, one female intact Beagle, and one female intact mixed breed dog) were enrolled. The dogs weighed 11.4, 7.7 and 16.9 kgs respectively. Initial pharmacokinetics assessment was performed following a single oral dose of approximately 8 mg kg^{-1} (range 7.8 to 8.0 mg kg^{-1}), and toxicity assessment was performed following daily dosing of approximately 4 mg kg^{-1} (range 3.8 to 3.9 mg kg^{-1}).

Three tumor bearing dogs (one 8.7 year old male neutered Beagle, one 9.6 year old male neutered mixed breed dog, and one 11.3 year old female spayed Labrador retriever) were enrolled. The dogs weighed, 16.9, 27.9 and 30.6 kgs, respectively. Two tumor bearing dogs had a histopathological diagnosis of InvTCC, and one had a diagnosis of a recurrent gastrointestinal stromal tumor (GIST). One dog with InvTCC had been previously treated with an NSAID (carprofen) for pain and continued to receive an NSAID (piroxicam) at the time of enrollment. One dog with InvTCC had previously failed one injectable chemotherapy drug (vinblastine) and one NSAID (firocoxib). The dog with GIST had undergone surgery to remove the tumor, and the tumor recurred seven months later at the time zeb was initiated. Pharmacokinetics assessment in these dogs was performed following the initial oral dose of approximately 4 mg kg^{-1} (range 3.8 to 3.9 mg kg^{-1}). The dogs continued to receive this dose at 21 days intervals for toxicity assessment.

Zebularine pharmacokinetics

Pharmacokinetics results are summarized in Table 3.1 and zeb concentration versus time curves are shown in Figure 3.1. Following an oral dose of 8 mg kg^{-1}, the mean t_{max} was 1.2 ± 0.62 hours, mean $t_{1/2}$ was 5.7 ± 0.84 hours, mean C_{max} was 5280 ± 0.84 ng ml^{-1}, and mean $AUC_{0-\infty}$ was 35,358 ± 6132.6 ng ml^{-1}. Following an oral dose of 4 mg kg^{-1}, the mean t_{max} was 2.3 ± 1.2 hours, mean $t_{1/2}$ was 7.1 ± 2.1 hours, mean C_{max} was 1962 ± 320.3 ng ml^{-1}, and mean $AUC_{0-\infty}$ was 17,206 ± 3221.4 ng ml^{-1}.

Zebularine toxicity – laboratory dogs treated daily with 4 mg kg^{-1} zebularine

All three laboratory dogs treated with a daily oral zeb dose of 4 mg kg^{-1} developed grade 2 (n=1) or grade 3 (n=2) neutropenia that progressed to grade 4 neutropenia. Zeb was discontinued when grade 2 or greater neutropenia was detected on day 14 (n=1), day 23 (n=1) and day 24 (n=1). Neutropenia progressed to grade 4 neutropenia by day 21 (n=1), day 29 (n=1) and day 30 (n=1). Grade 1 thrombocytopenia was detected in one dog. Grade 1 anorexia was detected immediately prior to grade 4 neutropenia in two dogs. No abnormalities were detected in serial serum biochemistry panels. After marked neutropenia developed, supportive care was initiated including antibiotics (n=3), antiemetics (n=2) and intravenous crystalloid fluids (n=2). All three dogs recovered, and neutrophil counts returned to normal within 16, 17 or 19 days, respectively. No abnormalities were detected in serial serum biochemistry panels.

Intermittent dermatologic changes were detected in two laboratory dogs including grade 2 rash/desquamation in the axilla (n=1) and grade 1 erythema of ventral abdomen

(n=1). Dermatologic changes resolved without specific treatment after zeb was discontinued.

The laboratory dogs underwent orchiectomy (n=1) or laparoscopic ovariohysterectomy (n=2), and had laparoscopic liver and kidney biopsies (n=3) performed prior to placement in permanent private homes. Specific pathology was not detected in any of the samples that were collected. Liver biopsies revealed non-specific, mild to moderate, reversible, hepatocellular degeneration in one dog and mild hydropic change in one dog. Renal pathology was not observed.

Zebularine toxicity – tumor bearing dogs treated once every 21 days with 4 mg kg^{-1} zebularine

Three tumor bearing dogs received six, four, and one dose(s) of zeb, respectively. Grade 1 thrombocytopenia was detected one week after the first zeb treatment in one dog. No dose adjustment was made, and thrombocytopenia did not recur. No other hematologic abnormalities, serum biochemical abnormalities or dermatologic changes were detected.

3.6 Discussion

The purpose of this study was to determine the plasma pharmacokinetics and assess toxicity of the novel demethylating agent, zeb, in laboratory and tumor bearing dogs. The work builds upon that previously reported in mice, rats, and monkeys, and provides pivotal information required for subsequent investigation of zeb's potential as a demethylating and antineoplastic agent in dogs.[10-16] Knowledge of the peak plasma

concentrations in dogs can be taken into account when interpreting *in vitro* and *in vivo* mechanisms studies. The constant plasma clearance and half-life of approximately four to nine hours, can be used in designing subsequent treatment studies in dogs. Epigenetic based treatment strategies can be applied across several cancer types, but work in naturally occurring canine InvTCC is particularly intriguing as it serves as a relevant model of the cancer in humans. Studies in dogs are expected to lead to the development of new treatment strategies that can be applied in both species.[6]

The dog study contributed to the understanding of the potential toxicity of zeb. Following daily treatment with 4 mg kg^{-1} zeb, grade 4 neutropenia developed in all three laboratory dogs. Although the myelosuppression resolved after zeb was discontinued, and all dogs recovered with supportive care, clearly lower zeb doses are indicated for follow-up studies. The myelosuppression in dogs was not completely unexpected as this has occurred with other demethylating agents in dogs and humans, especially when the drugs are given at higher, cytotoxic doses.[7,19] The degree of hematologic toxicity at the 4 mg kg^{-1} dose in dogs, however, was not expected when considering the available published *in vivo* study results of zeb in other species. Zeb was reported to be well tolerated in mice with no significant weight loss or mortality when administered for weeks to months.[11-15] Although clinicopathologic assessments were not reported in the mouse studies, it would be expected that should severe myelosuppression have occurred, that infection and sepsis would have been noted in the mice. It is also possible that higher doses of zeb in mice could result in myelosuppression. In male cynomolgus monkeys treated with five out of seven day intravenous infusions of zeb at <250 mg kg^{-1} per day for two consecutive weeks, an elevation in white blood cell count was observed, although

it is not known if the blood counts were performed when the neutropenia would have occurred if it were going to occur at that dosage. Regardless of whether the findings in dogs were expected or not, the documentation of the reversible, yet severe, myelosuppression in dogs is being used in the planning of additional studies of zeb in dogs. A dose of 4 mg kg^{-1} every 21 days resulted in no appreciable toxicity in tumor bearing dogs. A follow-up dog study is in progress in which the dose intensity of the starting dose cohort (daily 0.2 mg kg-1 zeb) is equivalent to the dose intensity of 4 mg kg^{-1} once every 21 days. This daily dose has been well tolerated to date in dogs.

The dog study results were encouraging in that major internal organ toxicity was not observed in dogs at any of the doses given. There has been concern for liver and kidney toxicity of zeb in other species. In male cynomolgus monkeys treated with five out of seven day intravenous infusions at 250 mg kg^{-1} per day of zeb for two consecutive weeks, clinicopathologic changes included elevated alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and creatine kinase (CK). Higher zeb doses of 500, 750 or 1,000 mg kg^{-1} given by continuous infusion or bolus resulted in lethal toxicity.[17] Zeb plasma concentrations rapidly increased prior to death in the cynomolgus monkeys treated with very high dose zeb, raising the possibility that liver or renal injury could have resulted in decreased zeb clearance.[17] While LDH and CK were not assessed in the current dog study, serum biochemistry panels did not reveal any evidence of liver or kidney injury following zeb treatment. Evaluation of post-treatment liver and kidney biopsies did not reveal any specific pathologic changes.

Differences in aldehyde oxidase (AO) activity or other not-yet-characterized enzymes involved in the metabolism of zeb could account for variation in drug

metabolism, efficacy and toxicity across species and individuals.[3,20,21] Among hepatic cytosol samples from mice, rats, humans and a male cynomolgus monkey, AO activity was highest in male mice and lowest in female mice. AO activity was not detected in hepatic cytosol from a male Beagle dog. A 50-fold difference was detected between male and female mice, but a similar magnitude of difference was not detected between sexes in rats or humans.[22] There is often greater aldehyde oxidase homology between mice and humans than monkeys and humans.[3] The effect of differential AO activity may account for differences in the effects of zeb on tumor development between female *Min* mice and male *Min* mice. These mice harbor a defect in the adenomatous polyposis coli (*APC*) gene that leads to development of benign intestinal adenomas. The number of tumors that developed in female, but not male, *Min* mice significantly decreased following treatment with zeb. In *Min* mice, there were no reported sex differences in zeb toxicity.[16] The lack of AO activity in the dog could result in improved bioavailability due to a decrease in first pass metabolism.

While tumor response was not a primary end point of this study, and a very small tumor bearing cohort of dogs was evaluated, it was noted that the two dogs with InvTCC treated with zeb had stable disease (<50% change in tumor size and no new lesions) when treated with zeb once every 21 days for 12 to 18 weeks. In dogs that have failed therapy with progressive disease, InvTCC often progresses rapidly, and lack of progression over 3-4 months suggests beneficial drug activity. In addition, when considering that the optimal dosing strategy for demethylating agents is likely daily administration[7,9], the dosing strategy employed in tumor bearing dogs was unlikely to reflect the optimum benefit that could be gained from zeb. It is also important to note that the demethylating

effects of zeb could occur at doses considerably lower than those which were toxic in dogs.[3] With a half-life in the dog ranging from approximately four to nine hours, near continuous exposure to zeb would be expected even with dosing once every 24 hours. Furthermore, the production of stable zeb metabolites may act as a depot for the drug, allowing for drug accumulation and recycling.[23]

In summary, this study provided important information concerning the pharmacokinetics and toxicity of zeb in dogs. A daily dose of zeb of 4 mg kg^{-1} resulted in unacceptable, but reversible, hematologic toxicity in laboratory dogs while a dose of 4 mg kg^{-1} every 21 days resulted in no appreciable toxicity in tumor bearing dogs. A daily dose of zeb (0.2 mg kg^{-1}), which is equivalent to the dose intensity of 4 mg kg^{-1} once every 21 days, has been well tolerated in dogs to date. Continued investigation of zeb is warranted in tumor bearing dogs to determine the optimal dosing strategy, activity as both a single agent and in combination with other drugs and characterization of demethylating activity *in vivo*.

3.7 Acknowledgements

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3.8 Source of Funding

This study was supported by a grant from the Purdue University Canine Disease Research Fund and private donations to the Purdue Comparative Oncology Program.

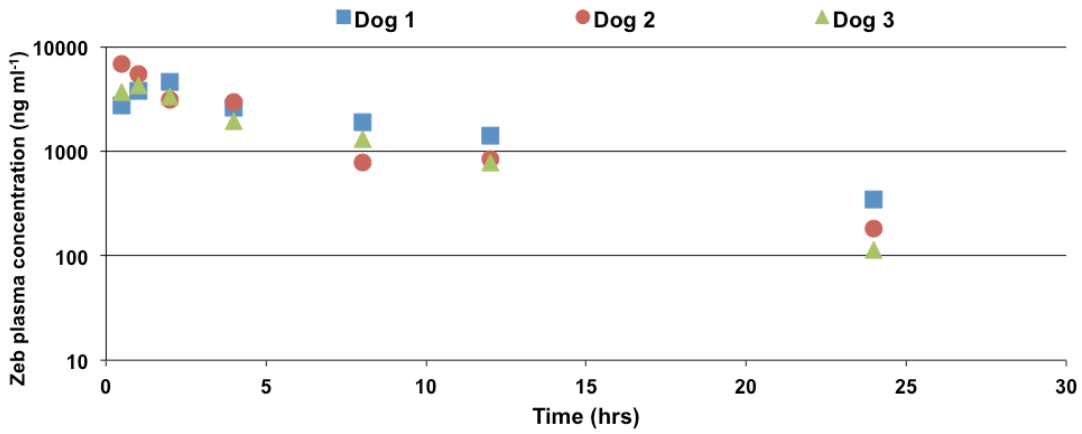
3.9 Conflict of Interest

The authors have no conflicts of interest to declare.

Table 3.1. Pharmacokinetic parameters from laboratory dogs treated with a single oral zebularine dose of 8 mg kg^{-1} (n=3) and tumor bearing dogs treated with a single oral zebularine dose of 4 mg kg^{-1}.

Dog	Weight (kg)	Dose (mg)	C_{max} (ng ml^{-1})	Mean C_{max} (µM)	t_{max} (hrs)	$AUC_{0-\infty}$ (ng ml^{-1} hr)	$t_{1/2}$ (hrs)	Cl/F (L/hr)	Vd_{ss}/F (L)
Laboratory dogs – 8 mg kg^{-1}									
1	16.9	135	4695	--	2.0	43584	6.8	3.1	29.2
2	7.7	60	6824	23 ± 4.8	0.5	33626	5.4	1.8	12.5
3	11.4	90	4322	--	1.0	28865	4.8	3.2	21.3
Tumor bearing dogs – 4 mg kg^{-1}									
1	16.9	65	1850	--	2.0	12665	8.3	5.1	52.7
2	27.9	110	2399	8.6 ± 1.4	1.0	19167	4.1	5.7	38.6
3	30.6	120	1639	--	4.0	19788	8.9	6.1	84.1

C_{max}: maximum concentration
t_{max}: time of maximum concentration
AUC_{0-inf}: area under the plasma concentration-time curve from 0-infinity
$t_{1/2}$: half-life
Cl/F: clearance/availability; if dosage is IV, then F=1
Vd/F: apparent volume of distribution



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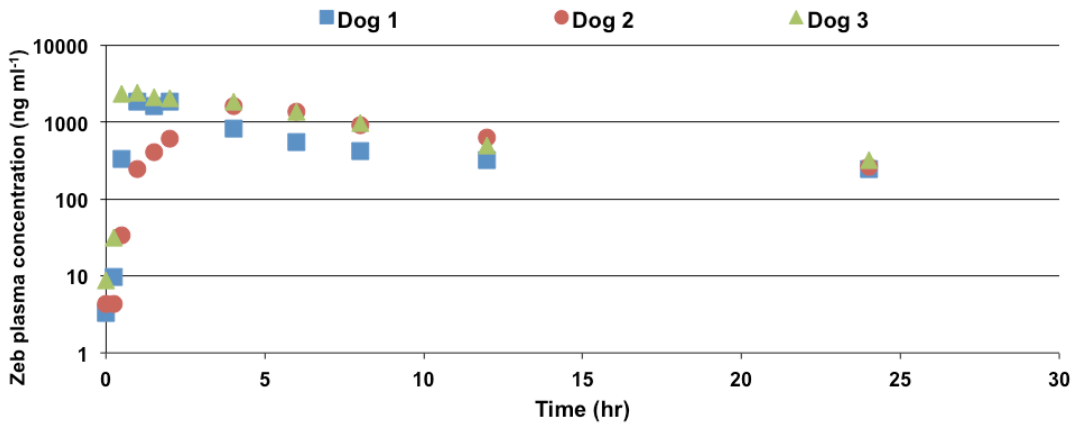

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Figure 3.1. Zeb clearance in three laboratory dogs following a single oral dose of 8 mg kg^{-1} (A) and three tumor bearing laboratory dogs following a single oral dose of 4 mg kg^{-1} (B).

References

1. Jones PA and Baylin SB. The fundamental role of epigenetic events in cancer. *Nature Reviews Genetics*. 2002 3(6):415-428.

2. Yoo CB, Cheng JC, Jones PA. Zebularine: a new drug for epigenetic therapy. *Biochemical Society Transactions*. 2004 32(6):910-912.

3. Marquez VE. Recent developments of two NCI epigenetic drugs: zebularine and 3-deazneplanocin-A. In: *American Association for Cancer Research 101st Annual Meeting Education Book*; 2010 Apr 17-21; Washington, DC. Philadelphia: AACR; 2008. pp 17-22.

4. Dote H, Cerna D, Burgan WE, Carter DJ, Cerra MA, Hollingshead MG, et al. *Clinical Cancer Research*. 2005 11(12):4571-4579.

5. Kim HJ, Kim JH, Chie EK, Young PD, Kim IA, Kim IH. DNMT (DNA methyltransferase) inhibitors radiosensitize human cancer cells by suppressing DNA repair activity. *Radiation Oncology*. 2012 7:39. Epub 2012 March 20.

6. Knapp DW, Ramos-Vara JA, Moore GE, Dhawan D, Bonney PL, Young KE. Urinary bladder cancer in dogs, a naturally occurring model for cancer biology and drug development. *Institute of Laboratory Animal Medicine Journal*. 2014 55(1):100-118.

7. Hahn NM, Bonney PL, Dhawan D, Jones DR, Balch C, Guo Z, et al. Subcutaneous 5-azacytidine treatment of naturally occurring canine urothelial carcinoma: a novel epigenetic approach to human urothelial carcinoma drug development. *The Journal of Oncology*. 2012 187(1):302-309.

8. Howell PM, Liu Z and Khong KT. Demethylating agents in the treatment of cancer. *Pharmaceuticals*. 2010 3(7):2022-2044.

9. Cowan LA, Talwar S and Yang AS. Will DNA methylation inhibitors work in solid tumors? A review of the clinical experience with azacitidine and decitabine in solid tumors. *Epigenomics*. 2010 2(1):71-86.

10. Holleran JL, Parise RA, Joseph E, Eiseman JL, Covey JM, Glaze ER, et al. Plasma pharmacokinetics, oral bioavailability, and interspecies scaling of the DNA methyltransferase inhibitor, zebularine. *Clinical Cancer Research*. 2005 11(10):3862-3868.

11. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, et al. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *Journal of the National Cancer Institute*. 2003 95(5):399-409.

12. Tan W, Zhou W, Yu H, Luo H, Shen L. The DNA methyltransferase inhibitor zebularine induces mitochondria-mediated apoptosis in gastric cancer cells in vitro and in vivo. *Biochemical and Biophysical Research Communications*. 2013 430(1):250-255.

13. Chen M, Shabashvili D, Nawab A, Yang SX, Dyer LM, Brown KD, et al. DNA methyltransferase inhibitor, zebularine, delays tumor growth and induces apoptosis in a genetically engineered mouse model of breast cancer. *Molecular Cancer Therapeutics*. 2012 11(2):370-382.

14. Neureiter D, Zopf S, Leu T, Dietze O, Hauser-Kronberger C, Hahn EG, et al. Apoptosis, proliferation and differentiation patterns are influence by zebularine and SAHA in pancreatic cancer models. *Scandinavian Journal of Gastroenterology*. 2007 42(1):103-116.

15. Herranz M, Martin-Caballero J, Fraga MF, Ruiz-Cabello J, Flores JM, Desco M, et al. The novel DNA methylation inhibitor zebularine is effective against the development of murine T-cell lymphoma. *Blood*. 2006 107(3):1174-1177.

16. Yoo CB, Chuang JC, Byun H, Egger G, Yang AS, Dubeau L, et al. Long-term epigenetic therapy with oral zebularine has minimal side effects and prevents intestinal tumors in mice. *Cancer Prevention Research*. 2008 1(4):233-240.

17. Johnson WD, Harder JB, Naylor J, McCormick DL, Detrisac CJ, Glaze E, et al. 2006. A pharmacokinetic/pharmacodynamic approach to evaluating the safety of zebularine in non-human primates. In: Proceedings of the 97th Annual Meeting of the American Association for Cancer Research; 2006 Apr 1-5; Washington, DC. Philadelphia: AACR; 2006. p 309. Abstract nr 1311.

18. Veterinary Cooperative Oncology Group. Veterinary cooperative oncology group – common criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. *Veterinary and Comparative Oncology*. Epub 2011 July 20.

19. Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H. Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. *Cancer*. 2008 112(11):2341-2351.

20. Sahi J, Khan KK, Black CB. Aldehyde oxidase activity and inhibition in hepatocytes and cytosolic fractions from mouse, rat, monkey and human. *Drug Metabolism Letters*. 2(3):176-183.

21. Sugihara K, Katsuma Y, Kitamura S, Ohta S, Fujitani M, Shintani H. Cynomolgus monkey liver aldehyde oxidase: extremely high oxidase activity and an attempt at purification. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 2000 126(1):53-60.

22. Klecker RW, Cysyk RL, Collins JM. Zebularine metabolism by aldehyde oxidase in hepatic cytosol from humans, monkeys, dogs, rats and mice: influence of sex and inhibitors. *Bioorganic & Medicinal Chemistry*. 2006 14(1)62-66.

23. Ben-Kasus T, Ben-Zvi Z, Marquez VE, Kelley JA, Agbaria R. Metabolic activation of zebularine, a novel DNA methylation inhibitor, in human bladder carcinoma cells. *Biochemical Pharmacology*. 2005 70(1):121–33.

CHAPTER 4. PHASE I DOSE ESCALATION TRIAL OF THE ORAL DEMETHYLATING AGENT ZEBULARINE IN DOGS WITH INVASIVE TRANSITIONAL CELL CARCINOMA

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Phase 1 of zeb in dogs with InvTCC

Key Words:
Zebularine; demethylating agent; dog pharmacokinetics and toxicity; transitional cell carcinoma

4.2 Abstract

A phase I clinical trial of zebularine (zeb) was performed in dogs with naturally occurring invasive transitional cell carcinoma (InvTCC). A total of 26 dogs were enrolled in six treatment cohorts ranging from 0.2 to 0.7 mg kg^{-1} per day. Dose-limiting toxicity (DLT)

included neutropenia following a daily zeb dose of 0.6 mg kg^{-1}. Based on results to date, the maximum tolerated dose (MTD) is likely to be 0.5 mg kg^{-1} per day. Of the 26 dogs treated, 73% had failed treatments including NSAIDs, oral and injectable chemotherapy. Median progression free survival was 86 days (95% CI, 47.5-124.5). Of 19 dogs evaluated for tumor response, 14 had stable disease for at least eight weeks, and five had progressive disease. Zeb was well tolerated up to 0.5 mg kg^{-1} per day and further investigation of zeb alone or in combination in the dog should be targeted at this dose.

4.3 Introduction

Zeb is an oral demethylating agent with the potential to reactive silenced genes in cancer.[1-5] Our group performed an initial investigation of the pharmacokinetics and toxicity of zeb in laboratory and tumor bearing dogs. A zeb dose of 8 mg kg^{-1} per day in laboratory dogs resulted in remarkable but reversible neutropenia. A zeb dose of 4 mg kg^{-1} once every 21 days was well tolerated in tumor bearing dogs (see Chapter 3). A daily dosing schedule has been proposed as the optimum dosing strategy for demethylating agents.[6,7]

Naturally occurring InvTCC in the dog is an excellent model of the disease in humans and has previously been used in the study of the injectable demethylating agent 5-Aza-CR with promising results.[8,9] The purpose of this study was to perform a phase I trial of zeb in dogs with naturally occurring InvTCC.

4.4 Materials and Methods

Study subjects

All work in pet dogs was approved by the Purdue Animal Care and Use Committee. Privately owned dogs with a histopathological diagnosis of invasive transitional cell carcinoma undergoing treatment at the Purdue University Veterinary Teaching Hospital (PUVTH) was enrolled following informed written pet owner consent. Entry criteria included a definitive diagnosis of InvTCC via histopathology, measurable disease and an expected survival time of at least eight weeks. Dogs were allowed to enroll if they had failed other cancer therapy or if other cancer therapy had been declined for any reason. Dogs that had previously been treated with non-steroidal anti-inflammatory drugs (NSAIDs) and had documented evidence of cancer progression were allowed to continue on NSAIDs after starting zeb. Dogs lived at home with their owners and were regularly evaluated at the PUVTH.

Dog monitoring and evaluation

Physical examination was scheduled at least monthly at the PUVTH and weekly at the dogs' local veterinary hospitals. Serial complete blood counts (CBC) were scheduled weekly and then biweekly, or more often at the discretion of the attending veterinarian based on observed toxicity. Serial serum biochemistry panels were scheduled monthly, or more often at the discretion of the attending veterinarian based on observed toxicity. All toxicity was graded using the Veterinary Cooperative Oncology Group common terminology criteria for adverse events v1.1 (VCOG-CTCAE v1.1).[10]

Staging tests including three view thoracic radiographs and complete abdominal ultrasound were scheduled at eight-week intervals. Ultrasound of the urinary tract using a previously published protocol was performed once every four weeks.[11] Urinary bladder ultrasound was performed by a single operator (DWK) when the urinary bladder was distended to a similar volume as the previous exam. Dogs were placed in right lateral recumbency, and measurements were recorded. Bladder tumor volume was estimated by multiplying the lesion area in the sagittal plane by the dorsal-ventral dimension in the transverse plane measurement and sagittal plane measurement.[11] Tumor response was defined as complete remission (CR) if there was complete resolution of all lesions, partial remission (PR) if there was ≥50% decrease in tumor volume but not complete resolution of all lesions, stable disease (SD) if there was <50% increase or decrease in tumor volume for at least eight weeks and progressive disease (PD) if a ≥50% increase in tumor volume was detected or new lesions developed.

Zebularine peak and trough concentration

After at least four weeks of zeb treatment, peak (two hours after an oral zeb dose with food) and trough (approximately 24 hours after the last zeb dose) blood samples were collected into ethylenediaminetetraacetic acid (EDTA) blood collection tubes and immediately centrifuged (1,800 g, 15 min, 4°C). Plasma was collected and stored at -80°C until analysis. Analysis to determine the plasma zeb concentration was performed by the Clinical Pharmacology Analytical Core Facility at Indiana University School of Medicine, Indianapolis, IN, USA as described in Chapter 3.

Zebularine formulation

Zeb was provided by the National Cancer Institute's Experimental Therapeutics (NExT) Program as a bulk powder and compounded into gelatin capsules from 0.5 to 20 mg by Diamondback Drugs, LLC (Scottsdale, AZ, USA).

Zebularine dose escalation

Pet dogs received an initial zeb dose of 0.2 mg kg^{-1} per day in food based on the data presented in Chapter 3. Enrollment of a minimum of three dogs per cohort was planned with dose escalation of 0.1 mg kg^{-1} per cohort. DLT was defined as any grade 3 or greater adverse event as described by VCOG-CTCAE v1.11.[10] Dose cohorts were expanded to include at least six dogs if a DLT was observed. MTD was defined as the dose at which a DLT occurred in no more than one of six dogs within a cohort. Treatment with zeb was continued as long as there was evidence of disease control, defined as CR, PR or SD, was detected. Dose escalation was allowed if there was evidence of PD or SD.

4.5 Results

Study subjects

A total of 26 dogs with InvTCC were enrolled in six treatment cohorts. There were 17 female spayed dogs and nine male neutered dogs. Median age was 11.6 years (range 6.8 to 17.3), and median weight was 17.5 kgs (range 3.8 to 36.7). The most common breeds were mixed breed (n=5), Scottish Terrier (n=4), West Highland White Terrier (n=4), Shetland Sheepdog (n=2) and Standard Poodle (n=2). Other breeds

represented by one dog each included Australian Shepherd, Basset Hound, Beagle, Collie, Dachshund, German Shepherd Dog, Golden Retriever, Labrador Retriever and Yorkshire Terrier. Of the 26 dogs, 73% (n=19) had failed previous treatments including NSAIDs, oral chemotherapy and injectable chemotherapy.

Two dose escalations were performed within one dog from 0.2 to 0.3 and then to 0.4 mg kg^{-1} without evidence of PD. Dose escalation was performed within one dog from 0.3 to 0.4 without evidence of PD. Dose escalation was performed within one dog from 0.5 to 0.6 mg kg^{-1} due to PD. Dose de-escalation was performed in one dog from 0.3 to 0.2 mg kg^{-1} due to grade 1 neutropenia. Patient characteristics for each treatment cohort are summarized in Table 4.1.

Zebularine toxicity

Hematologic toxicity is summarized in Table 4.2. Grade 1 neutropenia (n=1) and grade 1 thrombocytopenia (n=1) were detected in the 0.2 mg kg^{-1} cohort. Grade 1 neutropenia (n=1) was detected in the 0.3 mg kg^{-1} cohort. The dose of zeb in one dog was de-escalated to the 0.2 mg kg^{-1} cohort from the 0.3 mg kg^{-1} cohort, but grade 1 neutropenia persisted. Grade 1 neutropenia (n=1) and grade 2 thrombocytopenia (n=1) were detected in the 0.4 mg kg^{-1} cohort. Grade 1 neutropenia (n=1) and grade 1 thrombocytopenia (n=1) were detected in the 0.5 mg kg^{-1} cohort. Grade 3 neutropenia (n=1), grade 1 neutropenia (n=1) and grade 2 thrombocytopenia (n=1) were detected in the 0.6 mg kg^{-1} cohort.

Grade 2 anorexia was reported in one dog in the 0.4 mg kg^{-1} cohort. No other remarkable gastrointestinal adverse events were reported that were not present prior to starting zeb. No remarkable changes were noted in serial serum biochemistry panels.

One dog in the 0.3 mg kg^{-1} cohort with a history of seizures that were reportedly well controlled with potassium bromide had one seizure approximately 24 hours after the first dose of zeb. Zeb was discontinued, but the dog had additional seizures. An MRI was performed and revealed a T1 and T2 hypointense intra-axial mass within the left olfactory bulb with heterogeneous central contrast enhancement and strong peripheral contrast enhancement. The appearance of this lesion was consistent with a meningioma.

Several dogs enrolled in the 0.6 mg kg^{-1} cohort and the dog in the 0.7 mg kg^{-1} cohort have been enrolled for less than eight weeks at the time of this analysis. While no adverse events have been reported at the time of this analysis, these dog may not have been enrolled for long enough to detect adverse events.

Tumor response

Of 26 individual dogs, 19 dogs have been evaluable for tumor response after at least eight weeks of therapy. No complete or partial remissions were detected. SD was detected in 14 dogs (73.7%), and PD was detected in 5 dogs (26.3%). Overall disease control rate, defined as CR, PR or SD was 73.7%. Dogs were excluded from evaluation of tumor response if there was no evidence of tumor progression but dogs had been on treatment for less than eight weeks (n=3) or if they had stopped zeb for any reason other than tumor progression (n=4). One dog stopped zeb after one dose due to seizures. Subsequently, a suspected meningioma was diagnosed by MRI. One dog was euthanized

after 40 days of zeb treatment without evidence of tumor progression due to acute hind limb paresis due to suspected intervertebral disc disease (IVDD). IVDD was confirmed at necropsy. One dog with a history of syncopal episodes and pancreatitis died acutely at home after 10 days of treatment. A necropsy was declined in this case. One dog with a history of marked anorexia prior to starting zeb treatment stopped treatment after 22 days.

Progression free survival analysis was performed using IBM's SPSS Statistics 21. Dogs were censored from survival analysis if they were still alive and on zeb at the time of analysis (n=7), if zeb was stopped for any reason other than disease progression (n=4) or if they were lost to follow up (n=1). Median progression free survival calculated using the Kaplan Meier product limit was 86 days (95% CI, 47.5-124.5; see Figure 1).

At the time of analysis, two dogs have experienced ongoing long-term disease stabilization. One dog experiencing SD for more than 511 days has had the dose escalated without progressive disease from 0.2 to 0.3 and then 0.4 mg kg^{-1}. One dog experiencing SD for more than 182 days was treated at 0.4 mg kg^{-1}.

Zebularine peak and trough plasma concentrations

Peak and trough plasma samples following at least four weeks of daily zeb treatment were available from dogs treated daily with 0.2 mg kg^{-1} (n=6), 0.3 mg kg^{-1} (n=7) and 0.4 mg kg^{-1} (n=1). Following daily oral zeb treatment with 0.2, 0.3 and 0.4 mg kg^{-1}, mean zeb peak plasma concentrations were 179 ± 50 ng ml^{-1} (0.78 ± 0.22 μM), 233 ± 131 ng ml^{-1} (1.1 ± 0.57 μM) and 292 ng ml^{-1} (1.28 μM), respectively. Following daily oral zeb treatment with 0.2, 0.3 and 0.4 mg kg^{-1}, mean zeb trough plasma concentrations were

64 ± 17 ng ml^{-1} (0.28 ± 0.075 μM), 101 ± 40 ng ml^{-1} (0.44 ± 0.17 μM) and 67 ng ml^{-1} (0.29 μM), respectively.

4.6 Discussion

The purpose of this phase I trial was to determine the DLT and MTD of daily oral zeb in dogs with InvTCC. Naturally occurring InvTCC in the dog is a relevant model of human cancer, and discoveries in the dog can be translated into more effective treatments in humans. Globally, 2.1% of all human cancer deaths are attributed to bladder cancer.[12] Demethylating agents like 5-Aza-CR and 5-Aza-CDR are FDA approved for myelodysplastic syndrome,[2,5] and these agents are being investigated in the treatment of solid tumors.[6] Zeb was the first agent that resulted in successful demethylating activity following oral administration.[1]

Overall, zeb was well tolerated in dogs with InvTCC up to 0.5 mg kg^{-1}. Based on previous work by our group, hematologic toxicity was anticipated as dose increased (see Chapter 3). A DLT of grade 3 neutropenia was detected in one dog within the 0.6 mg kg^{-1} cohort. MTD was defined as the cohort in which one of six dogs experienced a DLT. By definition, MTD has not yet been reached at the time of this analysis. Based on toxicity that has been observed thus far, it is likely that 0.5 mg kg^{-1} will be the MTD for daily administration. Further information will be provided as dogs within the 0.5, 0.6 and 0.7 mg kg^{-1} cohorts continue to be evaluated.

While tumor response was not a primary outcome, the overall disease control rate of 73.3% and median progression free survival of 86 days were promising. Given the small sample size, the effect of censoring may impact these results, and further analysis

when the data is more mature is indicated. Investigation of the DNA methylation patterns in responders and non-responders may reveal a subset of patients that are more likely to respond to demethylating therapy. Of particular interest are 2 dogs with ongoing responses of 511 and 182 days.

Measurement of zeb plasma concentrations following chronic daily oral administration revealed that zeb was present in the plasma at detectable levels throughout a 24-hour period. The peak plasma concentrations of zeb were 179 ± 50 ng ml^{-1} (0.78 ± 0.22 μM), 233 ± 131 ng ml^{-1} (1.1 ± 0.57 μM) and 292 ng ml^{-1} (1.28 μM) following daily oral dosing of 0.2, 0.3 and 0.4 mg kg^{-1}, respectively. The plasma zeb concentration achieved following treatment with daily 0.5 mg kg^{-1} is likely greater than 1 μM. While these concentrations are far lower than concentrations previously investigated *in vitro* and *in vivo*, growing evidence suggests that such high concentrations may not be necessary for efficacy. Treatment of human hematopoietic and epithelial cancer cell lines and derivative mouse xenografts with nanomolar doses of either 5-Aza-CR or 5-Aza-CDR resulted in altered gene expression and DNA demethylation. At ultra low doses, 5-Aza-CR and 5-Aza-CDR appear to target stem-like populations within tumors. A stem-like population of cells would have to be depleted before a clinical response would be observed, which could account for the observed delay between treatment initiation and tumor response in humans with myelodysplastic syndrome.[13] Zeb plasma concentrations in the micromolar or nanomolar range are safely achievable in the dog and these concentrations may be sufficient to achieve the desired anti-neoplastic activity without undesirable toxicity.

In summary, daily treatment with zeb up to 0.5 mg kg^{-1} has been well tolerated in dogs with InvTCC and resulted in a promising disease control of 73.3%. Based on initial data analysis, the DLT of daily oral zeb is likely neutropenia at doses higher than 0.5 mg kg^{-1}, and MTD is likely to be 0.5 mg kg^{-1}. A daily dose of zeb up to 0.5 mg kg^{-1} is a rational starting point for investigating the potential of zeb in combination with other cytotoxic chemotherapy agents.

4.7 Source of Funding

This study was supported by a grant from the Purdue University Canine Disease Research Fund and private donations to the Purdue Comparative Oncology Program.

4.8 Conflict of Interest

The authors have no conflicts of interest to declare.

Table 4.1. Summary of characteristics of dogs with InvTCC enrolled in zeb dose escalation by dose cohort.

	0.2 mg kg^{-1} <0.25	**0.3 mg kg^{-1}** ≥0.25 but <0.35	**0.4 mg kg^{-1}** ≥0.35 but <0.45	**0.5 mg kg^{-1}** ≥0.45 but <0.55	**0.6 mg kg^{-1}** ≥0.55 but <0.65	**0.7 mg kg^{-1}** ≥0.65 but <0.75
Number	6[a]	9[b]	4[b,c]	4	7[d]	1
Age	12.2 yrs (range 6.9 to 14.4)	11.2 yrs (range 6.8 to 15.2)	12.0 yrs (range 8.5 to 13.7)	12.0 yrs (range 8.6 to 17.3)	11.6 yrs (range 9.8 to 17.3)	7.7 yrs
Sex	2 FS, 3 MN	6 FS, 3 MN	3 FS, 1 MN	3 FS, 1 MN	4 FS, 3 MN	1 FS
Weight	16.6 kgs (range 7.7 to 31.2)	17.0 kgs (range 7 to 32.2)	9.0 kgs (range 7.0 to 32.2)	13.6 kgs (range 7.7 to 36.7)	16.1 kgs (range 7.8 to 31.5)	29.6 kgs
# Prior Tx[e]	1 (n=2), 2 (n=3), 4 (n=1)	0 (n=3), 1 (n=2), 2 (n=2), 3 (n=1), 4 (n=1)	0 (n=1), 2 (n=1), 4 (n=1), 6 (n=1)	1 (n=1), 2 (n=3)	0 (n=3), 1 (n=1), 2 (n=2)	3 (n=1)
TNM	$T_2N_0M_0$ (n=4) $T_3N_0M_0$ (n=2[f])	$T_2N_0M_0$ (n=7) $T_3N_0M_0$ (n=1[f]) $T_3N_1M_0$ (n=1[f])	$T_2N_0M_0$ (n=2) $T_3N_0M_0$ (n=1[f]) $T_3N_1M_0$ (n=1[f])	$T_2N_0M_0$ (n=2) $T_2N_1M_0$ (n=1) $T_2N_0M_1$ (n=1) $T_3N_1M_0$ (n=1[f])	$T_2N_0M_0$ (n=5) $T_2N_0M_1$ (n=1) $T_3N_0M_0$ (n=1[f])	$T_2N_1M_0$ (n=1)
Breed	**Pure bred n=6** (1 each of Dachshund, German Shepherd Dog, Labrador Retriever, Scottish terrier, Shetland Sheepdog, Standard Poodle) **Mixed breed n=0**	**Pure bred n=8** (1 each of Australian Shepherd, Beagle, Dachshund, German Shepherd Dog, Golden Retriever, Scottish, West Highland White & Yorkshire Terrier) **Mixed breed n=1**	**Pure bred n=4** (1 each of Dachshund, Golden Retriever, Scottish Terrier, West Highland White Terrier) **Mixed breed n=0**	**Pure bred n=4** (1 each of Basset Hound, Scottish Terrier, Standard Poodle, West Highland White Terrier) **Mixed breed n=0**	**Pure bred n=3** (Collie n=1, West Highland White Terrier n=2, Shetland Sheepdog n=1) **Mixed breed n=3**	**Pure bred n=0** **Mixed breed n=1**

26 individual dogs were enrolled (17 FS, 9 MN; 21 pure bred dogs, 5 mixed breed dogs)

[a] *1 dog was dose de-escalated from 0.3 to 0.2 mg kg^{-1} due to grade 1 neutropenia*

[b] *1 dog was dose escalated from 0.2 to 0.3 to 0.4 mg kg^{-1} without evidence of progressive disease*

[c] *1 dog was dose escalated from 0.3 to 0.4 mg kg^{-1} without evidence of progressive disease*

[d] *1 dog was dose escalated from 0.5 to 0.6 mg kg^{-1} after developing progressive disease*

[e] *19 dogs had failed prior treatments including NSAIDs (carprofen, deracoxib, firocoxib & piroxicam,), oral chemotherapy drugs (chlorambucil) and injectable chemotherapy (carboplatin, mitoxantrone, vinblastine)*

[f] *Prostate involvement*

Table 4.2. Selected hematologic toxicity by dose cohort in dogs with invasive transitional cell carcinoma treated with zebularine.

	Grade of Neutropenia				Grade of Thrombocytopenia			
	1 1500 μL^{-1} to < lower limit of normal	**2** 1,000 to 1,499 μL^{-1}	**3** 500 to 999 μL^{-1}	**4** < 500 μL^{-1}	**1** 100,000 μL^{-1} to < lower limit of normal	**2** 50,000 to 99,000 μL^{-1}	**3** 25,000 to 49,000 μL^{-1}	**4** < 25,000 μL^{-1}
0.2 mg kg^{-1} n=6	1	0	0	0	1	0	0	0
0.3 mg kg^{-1} n=9	2	0	0	0	0	0	0	0
0.4 mg kg^{-1} n=4	1	0	0	0	0	1	0	0
0.5 mg kg^{-1} n=4	1	0	0	0	1	0	0	0
0.6 mg kg^{-1} n=7	1	0	1	0	0	1	0	0
[a]0.7 mg kg^{-1} n=1	0	0	0	0	0	0	0	0

[a]*The dogs enrolled in the 0.7 mg kg^{-1} cohort have not been enrolled long enough to evaluate toxicity at this time.*

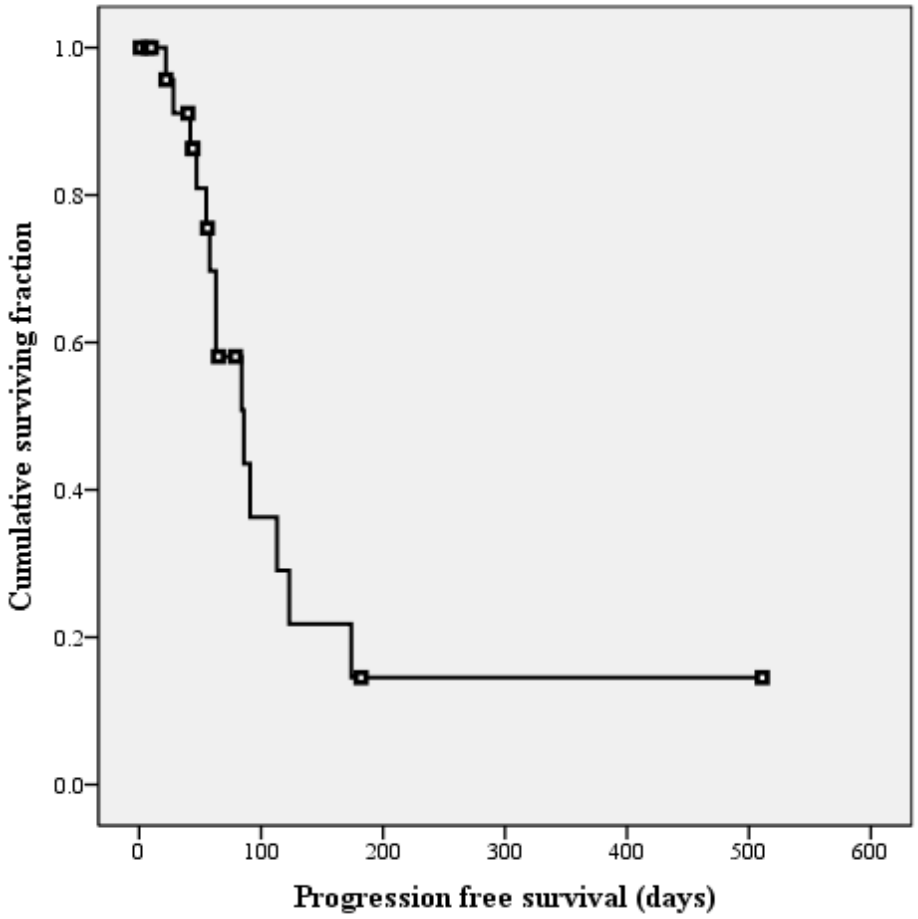

Figure 4.1. Progression free survival for 26 dogs treated with invasive transitional cell carcinoma treated with zebularine. Median progression free survival calculated using the Kaplan Meier product limit was 86 days (95% CI, 47.5-124.5). Dogs were censored (open squares) if they were still alive and on zeb at the time of analysis (n=7), if zeb was stopped for any reason other than disease progression (n=4) or if dogs were lost to follow up (n=1).

References

1. Cheng JC, Matsen CB, Gonzales FA, Ye W, Greer S, Marquez VE, et al. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *Journal of the National Cancer Institute*. 2003 95(5):399-409.

2. Yoo CB, Cheng JC, Jones PA. Zebularine: a new drug for epigenetic therapy. *Biochemical Society Transactions*. 2004 32(6):910-912.

3. Holleran JL, Parise RA, Joseph E, Eiseman JL, Covey JM, Glaze ER, et al. Plasma pharmacokinetics, oral bioavailability, and interspecies scaling of the DNA methyltransferase inhibitor, zebularine. *Clinical Cancer Research*. 2005 11(10):3862-3868.

4. Yoo CB, Chuang JC, Byun H, Egger G, Yang AS, Dubeau L, et al. Long-term epigenetic therapy with oral zebularine has minimal side effects and prevents intestinal tumors in mice. *Cancer Prevention Research*. 2008 1(4):233-240.

5. Marquez VE. Recent developments of two NCI epigenetic drugs: zebularine and 3-deazneplanocin-A. In: *American Association for Cancer Research 101st Annual Meeting Education Book*; 2010 Apr 17-21; Washington, DC. Philadelphia: AACR; 2008. pp 17-22.

6. Cowan LA, Talwar S and Yang AS. Will DNA methylation inhibitors work in solid tumors? A review of the clinical experience with azacitidine and decitabine in solid tumors. *Epigenomics*. 2010 2(1):71-86.

7. Howell PM, Liu Z and Khong KT. Demethylating agents in the treatment of cancer. *Pharmaceuticals.* 2010 3(7):2022-2044.

8. Hahn NM, Bonney PL, Dhawan D, Jones DR, Balch C, Guo Z, et al. Subcutaneous 5-azacytidine treatment of naturally occurring canine urothelial carcinoma: a novel epigenetic approach to human urothelial carcinoma drug development. *The Journal of Oncology*. 2012 187(1):302-309.

9. Mutsaers AJ, Wimdmer WR and Knapp DW. Canine transitional cell carcinoma. *Journal of Veterinary Internal Medicine*. 2003 17(2):136-144.

10. Veterinary Cooperative Oncology Group. Veterinary cooperative oncology group – common criteria for adverse events (VCOG-CTCAE) following chemotherapy or biological antineoplastic therapy in dogs and cats v1.1. *Veterinary and Comparative Oncology*. Epub 2011 July 20.

11. Chun R, Knapp DW, Widmer WR, Denicola DB, Glickman NW, Kuczek T, et al. Phase II clinical trial of carboplatin in canine transitional cell carcinoma of the urinary bladder. *Journal of Veterinary Internal Medicine*. 1997 11(5):279-83.

12. Bilgrami SM, Qureshi SA, Pervez S and Abbas F. Promoter hypermethylation of tumor suppressor genes correlates with tumor grade and invasiveness with urothelial bladder cancer. *Springer Plus*. 2014 3:178. Epub 2014 April 5.

13. Tsai H, Li H, Van Neste L, Cai Yi, Robert C, Rassool FV, et al. Transient Low Doses of DNA-Demethylating Agents Exert Durable Antitumor Effects on Hematological and Epithelial Tumor Cells. *Cancer Cell* 2012 21 (3):430-446.

CHAPTER 5. CONCLUSION

5.1 Conclusion

Work published to date in rodents suggested that chronic zebularine (zeb) administration was well tolerated. Limited information was available on the toxicity of zeb in animals larger than rats and suggested that zeb may be hepatotoxic. This thesis presented the findings of the first investigation of zeb in dogs. Initial pharmacokinetics in laboratory dogs (n=3) and tumor bearing dogs (n=3) revealed that zeb undergoes constant clearance in the dog. High dose daily zeb (4 mg kg^{-1}) resulted in remarkable but reversible neutropenia in laboratory dogs (n=3). High dose intermittent zeb (4 mg kg^{-1} once every 21 days) was well tolerated in tumor bearing dogs with no appreciable toxicity. Based on the results of the initial pharmacokinetics and toxicity study, a phase I dose escalation trial of zeb was initiated in dogs with InvTCC at a daily starting dose of 0.2 mg kg^{-1}. Low dose zeb was well tolerated at daily doses of up to 0.5 mg kg^{-1}, but dose-limiting toxicity (DLT) in the form of neutropenia was detected at daily doses of 0.6 mg kg^{-1}. Although evaluation of tumor response was not a primary goal of this study, the disease control rate and median progression free survival was detected in dogs with invasive transitional cell carcinoma (InvTCC).

High dose daily zeb resulted in unexpected and remarkable neutropenia in laboratory dogs. While suspected hepatotoxicity had previously been reported in

cynomolgus monkeys, remarkable neutropenia was not a feature of zeb toxicity that had been described. The mechanism for neutropenia is unknown, but may reflect a cytotoxic effect that can be seen when demethylating agents are used at high doses. The toxicity mirrored that which is seen following treatment with traditional cytotoxic chemotherapy as the most rapidly dividing granulocyte precursors appeared to be most affected. Importantly, this toxicity was reversible with supportive care. The unexpected myelosuppression at high zeb doses necessitated significant dose reductions before enrolling dogs with InvTCC in a phase I trial of low dose daily zeb.

Low dose daily zeb was well tolerated up to doses of 0.5 mg kg^{-1}. At 0.6 mg kg^{-1}, DLT in the form of grade 3 neutropenia was detected. At the time of analysis, only one dog had demonstrated a grade 3 neutropenia, so maximum tolerated dose (MTD) was not reached as defined in the study. As more dogs in the higher dose cohorts are evaluated, further toxicity may be identified. A total of 26 dogs with InvTCC were treated with low dose daily zeb. Of these 26 dogs, 19 were evaluable for tumor response. A disease control rate of 73.7% in a cohort of dogs that had been heavily pretreated is impressive. Furthermore, one dog has experienced remarkable stable disease beyond 511 days at the time of data analysis. These results support that low dose oral zeb is a promising therapy for the treatment of InvTCC in the dog. As the dog is a relevant model of human InvTCC, this work supports further investigation of zeb's potential in humans.

While a clear benefit following daily low dose zeb treatment was demonstrated in dogs with InvTCC, future research should be directed at confirming that this benefit is a result of DNA demethylation. Further work is needed to characterize DNA methylation

in canine InvTCC. A variety of canine InvTCC cell lines are readily available and may serve as an important tool to screen for gene specific methylation patterns that could then be used to target the investigation of methylation patterns in tumor samples from dogs and humans with InvTCC. Necropsy is a useful tool for banking large amounts of normal tissue from dogs unaffected by cancer and dogs that have naturally occurring cancer for use in this type of study. Exciting work has been performed in human clinical trials combining demethylating agents with cytotoxic chemotherapy and this is a next logical step that is currently undergoing pilot investigation at Purdue.

In conclusion, high dose daily zeb results in an unacceptable level of neutropenia that was reversible. A less frequent high dose of zeb was well tolerated. As the optimal dosing strategy for demethylating agents is likely frequent administration, a phase I dose escalation trial of low dose oral zeb was performed. Daily low dose zeb at doses up to 0.5 mg kg^{-1} was well tolerated and resulted in promising disease control rates in dogs with InvTCC. The work presented in this thesis supports further investigation of zeb in dogs and humans with InvTCC.

VITA

VITA

Education

Purdue University College of Veterinary Medicine
Master of Science in Veterinary Clinical Sciences – August 2014
Purdue University College of Veterinary Medicine
Doctor of Veterinary Medicine – May 2010
- Small Animal Practice Track

Purdue University College of Liberal Arts
Bachelor of Arts – May 2006
- Major: Behavioral Neuroscience
- Minor: Biology

Veterinary Residency

Purdue University College of Veterinary Medicine
Medical Oncology Resident – July 2011 to July 2014
- Included rotations in medical oncology, radiation oncology, small animal internal medicine, diagnostic imaging, anatomic and clinical pathology

Veterinary Internship

Texas A&M College of Veterinary Medicine and Biomedical Sciences
Small Animal Intern Instructor – June 2010 to July 2011
- Rotating small animal internship including anesthesiology, emergency and critical care, feline and canine internal medicine, general surgery, neurology and neurosurgery, oncology, orthopedic surgery, soft tissue surgery and primary care
- Internship included ~10 weeks of elective oncology rotations including exposure to both medical oncology and cobalt-60 radiotherapy

Veterinary Licensure

Indiana – License to Practice Veterinary Medicine
Indiana State Board of Veterinary Medical Examiners – June 2010 to present
Texas – Special License to Practice Veterinary Medicine
Texas State Board of Veterinary Medical Examiners – June 2010 to July 2011

Professional Organizations

American Veterinary Medical Association
Member – Spring 2010 to present

Veterinary Cancer Society
Member – Summer 2010 to present
American Association for Cancer Research
Member – Spring 2012 to present

Work Experience

Purdue University Department of Veterinary Clinical Sciences
Clinical Assistant Professor of Veterinary Oncology – July 2014 to present
Purdue University Veterinary Teaching Hospital
Small Animal Surgery Treatment Crew – December 2007 to January 2008 and December 2008 to January 2009
Small Animal Intensive Care Unit Treatment Crew – December 2006 to January 2007
Oncology Department Veterinary Assistant – June 2005 to August 2006
Pet Smart
Lead Pet Care Associate – April 2004 to August 2006
Pet Care Associate – May 2002 to April 2004

- Advised customers on proper care of pocket pets, amphibians, reptiles, birds and fish
- Performed initial health examinations on every pocket pet, amphibian, reptile and bird that entered the store, communicated with local veterinarians and executed treatment plans for in-store animals that were sick or injured
- Hand-reared psittacines including macaws, cockatoos, Amazons, African Greys, hawkhead parrots, parakeets, lorikeets and lories

Awards and Honors

Dr. Ann L. Johnson and Walter E. Hoffmann Residency Teaching Award for outstanding teaching by a resident in the Department of Veterinary Clinical Sciences at Purdue University
Recipient – April 2014
Veterinary Cancer Society E. Gregory MacEwen Memorial Award for outstanding oral abstract presentation of research in basic science
Recipient – April 2013
Veterinary Cancer Society Senior Student Award for interest/excellence in oncology
Recipient – Spring 2010
Central Indiana Veterinary Medical Association Award for outstanding communication skills
Recipient – Spring 2010
Veterinary Pet Insurance/VBMA Case Study Competition
PVM Chapter Winner – Spring 2010
J.E. Salsbury Scholarship for leadership, academic success, initiative and perseverance
Recipient – Fall 2009
Phi Zeta 1st Place Research Award for Third-Year Veterinary Students
Recipient – Spring 2009
Holly Watts Memorial Award for interest/proficiency in treatment/care of exotic pets
Recipient – Spring 2009
Omega Tau Sigma John C. Gordon Award for commitment/dedication to local chapter

Recipient – Fall 2008
Veterinary Software Associates Veterinary Learning Award for academics/leadership
Recipient – Spring 2008
Indiana Top Resident Scholarship for outstanding academic performance
Recipient – August 2001 to August 2005
Purdue University President's Leadership Class selected for leadership potential
Member – Fall 2001 to Spring 2002

Research Experience

Purdue University College of Veterinary Medicine
Thesis Master's and Resident Projects – Fall 2011 to July 2014
- Pharmacokinetics and toxicity of zebularine, an oral demethylating agent, in normal and tumor bearing dogs
- Evaluation of the combination of zebularine and chemotherapy drugs *in vivo* in dogs with transitional cell carcinoma
- Administration of carboplatin as a rescue agent for high grade canine lymphoma

Texas A&M College of Veterinary Medicine and Biomedical Sciences
Intern Project – Fall 2010 to Spring 2011
- The role of vascular endothelial growth factor expression in canine InvTCC

Purdue University Veterinary Teaching Hospital
Anesthesia Research Assistant – January 2009 to February 2009
- Assisted with a study on the efficacy of transmucosal buprenorphine administration for analgesia in dogs during the first 24 hours following spay or castration
- Performed regular physical exams, pain assessment and blood collection on research subjects

Surgical Assistant for DNR Rattlesnake Project – Summer 2009
- Assisted PUVTH faculty with radiotransmitter implantation in timber rattlesnakes
- Radiotransmitters were used to collect data on rattlesnake snake movements and habitats in southern Indiana and study the impact of human development on the rattlesnake population
- Anesthetized snakes using isoflurane, administered antibiotics, administered analgesics and monitored snakes during induction, surgery and recovery

Merck-Merial Summer Research Program
Research Fellow – Summer 2007 and Summer 2008
- Investigated the role of the protein LYAR in oncogenesis by developing a knock-out by gene-trap insertion mouse model that under-expressed the protein and a transgenic mouse model that over-expressed the protein
- Practiced a variety of laboratory techniques including PCR, electrophoresis, Western blot, immunoprecipitation, flow cytometry, radioimmunoassay, cell culture and mouse husbandry
- Wrote an abstract and created a poster to present research findings at local events and during the Merck-Merial Summer Research Symposium held at the National Institutes of Health in 2007 and Michigan State University in 2008
- Presented Grand Rounds discussion on research opportunities for veterinary students

Purdue University College of Liberal Arts Honors Colloquium
Presenter – Spring 2002
- Presented the findings of an original research paper, the culminating project of an honors research seminar on politics, social groups and the media, that explored the role of race in newspaper coverage of political scandals

Purdue University College of Liberal Arts Freshman Dean's Scholar Program
Participant – Fall 2001 to Spring 2002
- Assisted with a project studying the role of cholecystokinin (CCK) and the vagus nerve in hunger satiety using mouse and rat models
- A partial or complete vagal transection was performed on experimental subjects while controls received no surgery or a sham surgery; CCK was administered to all subjects via an intraperitoneal injection and post-injection glucose consumption was recorded
- Practiced restraint of mice and rats, injection techniques, collected data and maintained mechanized feeding and watering equipment

Grants

Veterinary Clinical Sciences Graduate Student Competitive Research Fund
Purdue University College of Veterinary Medicine – awarded December 2012
- Pilot study assessing the combination of a novel demethylating agent and carboplatin for more effective treatment of invasive bladder cancer

Peer-reviewed Publications

1. Fulkerson CM, Dhawan D, Jones DR, Fourez LM, Bonney PL and Knapp DW. Pharmacokinetics and toxicity of the novel oral demethylating agent zebularine in laboratory and tumor bearing dogs. 2014; *Manuscript in progress.*
2. Wang G, Fulkerson CM, Malek R, Ghassemifar S, Snyder PW, & Mendrysa SM. Mutations in Lyar and p53 are synergistically lethal in female mice. *Birth Defects Res A Clin Mol Teratol*. 2012; 94:729-737.
3. Cichocki B, Robarge M, Fulkerson CM, Scott-Moncrieff JC, & Ramos-Vara J. Multiple endocrine neoplasia-like syndrome in an 11-year-old dog with concurrent malignant oral melanoma and malignant pulmonary neoplasm. *Manuscript in progress.*

Teaching Experience

VM 83000 Applications & Integrations II
January to February 2013
- Served as a tutor for 1st year veterinary students in a problem-based learning course

VCS 22600 Principles of Veterinary Anesthesia Lab
February 2012
- Laboratory instructor for veterinary technology students learning how to perform general anesthesia and dentistry procedures

VCS 84500 Small Animal Medicine Lab
Fall 2011 and Fall 2012
- Laboratory instructor for 3rd year veterinary students learning how to perform bone marrow aspirates and needle-core biopsies

VCS 87102 Oncology (Clinical Rotation)
Summer 2011 to present
- Responsibilities include leading student topic rounds, working one-on-one with 4th year veterinary students to develop diagnostic and treatment plans for oncology referral cases and teaching biopsy and other diagnostic techniques to 4th year students and house officers

VCS 86100/87100 Small Animal Medicine I & II (Clinical Rotation)
January 2012
- Worked one-on-one with 4th year veterinary students to develop diagnostic and treatment plans for internal medicine referral cases

Presentations

American College of Veterinary Medicine Forum 2014
Pharmacokinetics and Toxicity of the Novel Oral Demethylating Agent Zebularine in Laboratory Dogs and Dogs With Transitional Cell Carcinoma – June 2014
- Selected as one of 2 presenters to present a 12 minute oral abstract originally presented at Veterinary Cancer Society Annual Meeting; reporting on pharmacokinetics and toxicity data in laboratory and tumor bearing dogs treated with the oral demethylating agent zebularine and preliminary results of a phase 1 dose-escalating trial in dogs with transitional cell carcinoma

Veterinary Cancer Society Annual Meeting
Pharmacokinetics and Toxicity of the Novel Oral Demethylating Agent Zebularine in Laboratory Dogs and Dogs With Transitional Cell Carcinoma – October 2013
- Presented 12 minute oral abstract reporting on pharmacokinetics and toxicity data in laboratory and tumor bearing dogs treated with the oral demethylating agent zebularine and preliminary results of a phase 1 dose-escalating trial in dogs with transitional cell carcinoma

Purdue Veterinary Medicine Phi Zeta Day 2013
Pharmacokinetics and Toxicity of the Novel Oral Demethylating Agent Zebularine in Laboratory and Tumor bearing Dogs – April 2013
- Presented poster with initial pharmacokinetics and toxicity data in laboratory and tumor bearing dogs treated with the oral demethylating agent zebularine

Purdue University Comparative Oncology Program Research Meeting
Epigenetics: A Primer – January 2013
- Presented a 50-minute review for the PCOP faculty and staff on genetics and epigenetic changes in cancer (with an emphasis on the current literature on DNA methylation, histone modifications and microRNAs)

Purdue University Veterinary Clinical Sciences Departmental Seminar
Pharmacokinetics and Toxicity of the Novel Oral Demethylating Agent Zebularine in Laboratory Dogs and Dogs With Transitional Cell Carcinoma – September 2013
- Presented 12 minute oral abstract reporting on pharmacokinetics and toxicity data in laboratory and tumor bearing dogs treated with the oral demethylating agent zebularine and preliminary results of a phase 1 dose-escalating trial in dogs with transitional cell carcinoma

Diagnosing and Treating Canine Lymphoma – October 2012

- Presented a 50-minute seminar reviewing lymphoma in the dog with an emphasis on the importance and prognostic significance of a specific histopathologic diagnosis in this disease; introduced an on-going clinical trial using carboplatin as a rescue agent for dogs with relapsed diffuse large B-cell lymphoma

Epigenetics and Cancer – April 2012

- Presented a 50-minute seminar introducing the topic of epigenetics with an emphasis on the use of demethylating agents in dogs with transitional cell carcinoma; introduced zebularine, a demethylating agent zebularine that is under clinical investigation in the dog

Purdue University Veterinary Teaching Hospital Histopathology Rounds

A Dachshund With a Multitude of Tumors – October 2012

- Presented a 20 minute clinical case presentation of a dachshund treated at the PUVTH for oral malignant melanoma and multiple cancers in endocrine organs in association with a presentation of pertinent histopathologic findings by an anatomic pathology resident

Telangiectactic Osteosarcoma (?) in the Rib of a Dog – October 2013

- Presented a 20 minute clinical case presentation of a mastiff-mix treated at the PUVTH for a rib tumor that was initially diagnosed as a telangiectatic osteosarcoma in association with a presentation of pertinent histopathologic findings by an anatomic pathology resident

Purdue University Veterinary Teaching Hospital Intern Rounds

Mast Cell Tumors – February 2013

- Presented a 50-minute update on canine mast cell tumors that incorporated a critical review of recent peer-reviewed literature using ancillary diagnostic tests to guide treatment recommendations

Principles of Chemotherapy – February 2012

- Presented a 50-minute introduction to the principles for using cytotoxic chemotherapy drugs, common drugs used in the clinic and managing relevant toxicity in the context of the emergency room

Texas A&M College of Veterinary Medicine Resident and Intern Seminar Series

VEGFR Expression in Canine Transitional Cell Carcinoma – May 2011

- Presented a 25-minute seminar introducing the topic of canine transitional cell carcinoma including diagnosis, treatment and prognosis, and reported on the results of a retrospective evaluation of immunohistochemical staining for vascular endothelial growth factor receptor in tissues obtained from dogs with transitional cell carcinoma at the time of necropsy

Continuing Education

American College of Veterinary Medicine Forum 2014

Attendee and oral abstract presenter – Nashville, TN; June 2014

Veterinary Cancer Society Annual Meeting

Attendee and oral abstract presenter – Minneapolis, MN; October 2013

Clinician Scientist Training Workshop

Attendee – University of Wisconsin-Madison, Madison, WI; October 2013

Purdue University Center for Cancer Research/Indiana University Simon Cancer Center Bladder Cancer Minisymposium
Attendee – Indianapolis, IN; March 2013
Veterinary Cancer Society and ACVR Annual Meeting
Attendee – Las Vegas, NV; October 2012
American Association for Cancer Researchers Annual Meeting
Attendee – Chicago, IL; March 2012
3rd Annual Dentistry for Small Animal Practitioners & Technicians Conference
Laboratory Instructor – College Station, TX; July 2010
- Instructor for a four hour hands-on wet lab covering regional anesthesia techniques for use in dental procedures in the dog and cat

Attendee – College Station, TX; July 2010
- Attended eight hours of discussion and lectures on dental conditions and procedures applicable to primary care veterinary practice

Purdue University Annual Fall Conference for Veterinarians and Veterinary Technicians
Attendee – September 2007, 2008, 2009, 2011 and 2012
Student American Veterinary Medical Association Symposium
Attendee – The Ohio State University; March 2009
Attendee – Auburn and Tuskegee Universities; March 2008
Attendee – North Carolina State University; March 2007
Merck-Merial Summer Research Symposium
Attendee – Michigan State University; August 2008
Attendee – National Institutes of Health; August 2007
- Presented original research as part of the Merck-Merial Summer Research Program and attended lecture series on cutting edge veterinary, comparative and translational research

6th Annual Veterinary Student Exotic Animal Symposium
Attendee – University of Tennessee; January 2008

Veterinary Study Abroad Experience

Introduction to Conservation Medicine in South Africa
Participant – May 2008
- Studied immobilization techniques, anesthetic drugs, field necropsy techniques and management strategies for communicable wildlife diseases in South Africa
- Practiced capture and immobilization techniques, intravenous catheter placement and sedation monitoring in giraffe, white rhinoceros and African hoofstock including cape buffalo, impala, kudu and wildebeest
- Performed screening tests on captive hoofstock for infectious diseases including theileriosis, tuberculosis and foot and mouth disease

Organizations and Leadership Activities

Phi Zeta, Omicron Chapter
Inducted April 2014

Alternate Graduate Student Representative to the PVM Grade Appeals Committee
November 2013 to July 2014
Purdue Veterinary Teach Hospital Continual Improvement Committee
October 2013 to present
Resident Representative to Purdue University Veterinary Clinical Sciences Faculty
September 2012 to July 2014
Veterinary Centers of America (VCA)
Senior Student Representative – July 2009 to May 2010
Student Representative – July 2008 to July 2009
Purdue University CVM Student-Faculty Liaison Committee
Student Member – Spring 2009 to Spring 2010
Purdue University CVM 50th Anniversary Executive Committee
Student Events Committee Co-Chair – Spring 2008 to Fall 2009
Omega Tau Sigma, Xi Chapter
President – Spring 2007 to Spring 2009
Grand Council Delegate – Fall 2006, Fall 2007 and Fall 2008
Member – Fall 2006 to Spring 2010

- As President, created a Philanthropy chair position to coordinate philanthropic activities and encourage community involvement by veterinary students; teamed with other student organizations for large scale projects for the local community and a national project for Heifer International
- Worked to promote unity within the PVM by planning social events that brought together the entire PVM community including veterinary students, veterinary technology students, faculty and staff

Student Chapter of the American Veterinary Medical Association (SCAVMA)
Budget and Documents Committee Member – Spring 2008 to Spring 2009
Executive Board Member – Spring 2007 to Spring 2009
Member – Fall 2006 to Spring 2010
Veterinary Business Management Association (VBMA)
Marketing Director – Spring 2007 to Fall 2007
Member – Fall 2006 to Spring 2010
Exotics Club
Member – Fall 2006 to Spring 2010
International Veterinary Student Association (IVSA)
Member – Fall 2006 to Spring 2010
Student Veterinary Emergency and Critical Care Society (SVECCS)
Member – Fall 2006 to Spring 2010
Purdue University College of Liberal Arts Dean's Ambassadors
Ambassador – Fall 2002 to Spring 2006
Alpha Lambda Delta and Phi Eta Sigma Undergraduate Honor Society
Senior Advisor – Spring 2003 to Spring 2004
President – Spring 2002 to Spring 2003

PUBLICATION

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1. Fulkerson CM, Dhawan D, Jones DR, Marquez VE, Jones PA, Fourez LM, Bonney PL and Knapp DW. Pharmacokinetics and toxicity of the novel oral demethylating agent zebularine in laboratory and tumor bearing dogs. 2014; *Manuscript in progress.*