DISSERTATION

CHEMOSENSITIZATION OF OSTEOSARCOMA BY THE HISTONE DEACETYLASE INHIBITOR VALPROIC ACID

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LUKE A. WITTENBURG ENTITLED CHEMOSENSITIZATION OF OSTEOSARCOMA BY THE HISTONE DEACETYLASE INHIBITOR VALPROIC ACID BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CHEMOSENSITIZATION OF OSTEOSARCOMA BY THE HISTONE DEACETYLASE INHIBITOR VALPROIC ACID

Osteosarcoma is the most common primary bone tumor in both canines and humans. Since the introduction of adjuvant chemotherapy to treatment protocols in the 1980's there has been very little improvement in long-term survival. The majority of canine patients will succumb to metastatic disease and likewise, this is the primary cause of death in humans with osteosarcoma. This underscores the urgent need for novel therapeutics in the treatment of osteosarcoma. Histone deacetylase inhibitors (HDACi) have recently emerged as a promising class of anti-cancer agents that are rapidly undergoing clinical evaluation in human cancer patients, yet very little information exists on the use of these agents in osteosarcoma. Studies on histone deacetylase inhibitors in canine cancer are even scarcer, and a better understanding of the mechanisms of action is required to generate rational treatment protocols utilizing these drugs in combination with traditional chemotherapy agents.

Utilizing a combination of *in vitro* assays we have been able to demonstrate that the HDACi valproic acid can sensitize both human and canine osteosarcoma cells to the anti-proliferative and pro-apoptotic effects of the DNA-targeting chemotherapeutic agent doxorubicin. We confirmed this finding in a xenograft model of canine osteosarcoma

which showed a significant delay in tumor growth associated with increased apoptosis and decreased tumor cell proliferation. These observations confirmed that, as in previously reported studies on human cancer cells, synergistic anti-tumor activity can be attained with the combination of valproic acid and doxorubicin.

We additionally sought to determine a maximum tolerated dose of valproic acid that can be administered to tumor-bearing dogs for 48 hours prior to a standard dose of doxorubicin. We discovered that the combination is well tolerated and that a biologically effective dose can be achieved without significant toxicity. In addition we showed that there were no effects on doxorubicin pharmacokinetics or potentiation of side effects when valproic acid was given prior to doxorubicin.

Lastly, through gene expression microarray and pathway analysis we identify the molecular pathways that are most affected by valproic acid treatment in OS cells and were able to confirm these results with a combination of quantitative real time RT-PCR and functional/biochemical assays, providing additional information on potential therapeutic modalities to combine with HDACi. The combination of studies presented herein provide evidence that canine and human OS cells have similar molecular responses to HDAC inhibition and provide further rationale for the use of spontaneously occurring OS in dogs as a model system for the human disease.

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Dedication

This dissertation is dedicated to the patients of the Animal Cancer Center at the James L. Voss Veterinary Teaching Hospital

Table of Contents

List of Tables and Figures

Chapter Two

Chapter 3

Chapter 4

Chapter One

Literature Review and Project Rationale

Osteosarcoma

Comparative biology and molecular pathogenesis

Osteosarcoma (OS) is a high-grade primary bone neoplasm of mesenchymal origin, with a reported incidence in humans ranging from 1-2 cases per million adults and 6 cases per million children annually (1, 2). Osteosarcoma is the most common malignant tumor of the bone, accounting for 5% of childhood tumors in the United States with approximately 900 new cases diagnosed each year (3). Similarly, OS accounts for 85% of primary bone tumors in canines and has an incidence approximately ten times that seen in humans, with nearly 8,000 new cases diagnosed each year (4). These neoplasms are characterized by tumor cell production of defective osteoid, or immature bone, which forms the basis for differentiating OS from other tumors of the bone. Histologic subclassifications of OS include osteoblastic, chondroblastic, fibroblastic, poorly differentatiated, and telangiectatic (4). In human patients, greater than 80% of OS tumors display a lack of normal bone differentiation (3). For both canines and humans there is a bimodal peak incidence. Dogs demonstrate a small peak incidence at 18 to 24 months and a larger peak incidence in middle-aged to older dogs, with a median age of 7

years (4). In humans, up to 75% of cases are reported in patients between the ages of 10-25, and a second, smaller peak incidence is seen in patients over the age of 50 (3). There is a slight male predilection in both species with a male:female ratio of 1.5:1 (1, 3). In both species these tumors arise primarily in the metaphases of long bones, with a predilection for the femur in humans (50% of cases) while the front limbs are affected twice as often as rear limbs in dogs (3, 4). These tumors show a much higher incidence in large and giant breed dogs which, along with the fact that they occur primarily in adolescent humans during their longitudinal growth spurt, may suggest a correlation between rapid bone turnover and osteosarcomagenesis. These tumors demonstrate an aggressive biological behavior and metastasize via the hematogenous route primarily to the lungs. In fact, the cause of death for the vast majority of OS patients is spread to the lungs, as improvements in local tumor therapy including limb-sparing surgical techniques have provided long-term primary tumor control. With the introduction of adjuvant multimodal therapy in the 1980's the overall five-year survival rates for humans with OS improved from approximately 30% to 75% (2). This has not held true for patients who present with metastatic disease at the time of diagnosis, and little improvement in survival has been appreciated in this group. In contrast, canines demonstrate a significantly shorter survival time and a more rapid disease progression following surgery and chemotherapy with one-year survival rates ranging from 30-50% and median survival times ranging from 260 to 540 days (4) While only 15% of canine patients may have radiographically detectable pulmonary metastasis at the time of diagnosis, the relatively rapid development of metastases early in the course of disease suggests the

existence of occult metastases and, in fact, the reported incidence of occult metastasis is ranges from 80-90% in dogs and humans (4, 5).

Relatively little is known about the exact etiology of osteosarcoma in humans and dogs. The lack of described benign pre-neoplastic lesions that progress to osteosarcoma has hampered studies into the causes of this disease. However, numerous studies have been implemented to identify possible genetic causes of osteosarcoma genesis in humans and dogs. These studies have also provided important information in basic tumor biology, exemplified by the discovery of the retinoblastoma gene and protein and cell cycle control. In humans there are a myriad of familial inherited syndromes that are associated with increased incidence of osteosarcoma including Li-Fraumeni (altered DNA damage response due to P53 mutation), Retinoblastoma (impaired cell-cycle control due to loss-of-heterozygosity of RB1 locus), Pagets disease (impaired IL-1/TNF and RANK signaling), Rothmund Thomson syndrome, Werner Syndrome, and Bloom Syndromme (all resulting in impaired DNA repair through defective helicase activity) (3). As is the case with most inherited syndromes resulting in predisposition to tumor formation, the genes involved function as recessive tumor suppressors. It is interesting to note that the incidence of OS in humans is relatively low considering the number of familial syndromes that lead to an increased incidence. Familial syndromes and their contribution to OS have not been intensively studied in canine cancer. However, the vast majority of OS in humans do not occur as part of familial syndromes, and sporadic OS in both species have been shown to have inactivating mutations in p53, retinoblastoma (Rb) protein, and their regulators. Interestingly, both canine and human OS tumors have p53 mutations at similar frequencies, although canine tumors do not demonstrate the large

deletions (primarily within DNA binding domains) that occur with relatively high frequency in human tumors, suggesting a possible difference in the initial mutagenic effector (6).

In contrast to what is seen in human OS, canine tumors do not appear to have direct mutations in the Rb gene but contain mutations that indirectly inactivate all three members of the Rb family; Rb, p107, and p130 simultaneously (7). The inactivation of Rb family proteins has important implications beyond deregulation of cell-cycle, as a bone-specific regulator of differentiation, *Runx2*, which activates the coordinated expression of genes characteristic of the osteoblast phenotype, is bound and transactivated by pRb; loss of pRb function would therefore result in deregulation or attenuation of terminal differentiation of osteoblasts, a common finding in osteosarcoma (8, 9) In addition to mutations in p53 and Rb family members, a myriad of other genes have been identified that potentially play a role in the development of sporadic OS and a few have been confirmed in canines and humans. The TWIST gene encodes a transcription factor that is implicated in bone and tissue differentiation and, in one study of high-grade OS, was found to have deletions in 41% of patients (10). Interestingly, this transcription factor contains a basic helix-loop-helix (bHLH) domain that contains a binding site for histone acetyltransferase (HAT) proteins, and deletions could potentially lead to an altered epigenetic profile of these tumors. The importance of epigenetics in skeletal development and OS are discussed in later sections. Other studies have discovered mutations in the putative tumor suppressor cell cycle inhibitors p16INK4a (CDKN2A) and p14ARF (CDKNA2) in 5-15% of evaluated cases. In addition to deletions and nonsense mutations seen in these genes, promoter methylation and

subsequent gene silencing was identified as a cause of decreased expression in OS (11, 12). In addition, another study reported that 60% of OS cases exhibit mutations or loss of heterozygosity in a region that encodes the fibroblast growth factor receptor-2 (FGFR2) which has important implications in tumor development as the basic fibroblast growth factor (bFGF) autocrine loop has been documented to control differentiation and phenotype of human OS cells *in vitro* (13, 14).

In addition to the deletions and LOH of some putative tumor suppressor genes, a number of genes that encode proteins essential in cell growth and proliferation are amplified in cases of sporadic OS. Studies by independent investigators have found amplifications in the chromosomal region containing the MDM2 gene in approximately 1/3 of cases, encoding a product that binds to and inhibits the tumor-suppressive effect of p53 (11, 15, 16). The *erb*B2 proto-oncogene encodes epidermal growth factor receptor 2 (HER2) and, while the specific role of this gene in human OS remains controversial with several studies showing overexpression and a correlation with poor patient survival (17-19) while others report that HER2 amplification is uncommon and/or does not play a significant role in OS (20, 21), overexpression of HER2 was shown in 86% of canine OS cell lines and 40% of OS tissue specimens suggesting a potential role for this growth factor-receptor pathway in the development of canine OS (22). In addition to HER2, EGFR has also been shown to be expressed in a number of OS tumors, although the EGFR mutations within the kinase domain that correlate with enhanced sensitivity to tyrosine kinase inhibitors in lung and breast cancer do not appear to be present in OS (23).

Other oncogenes that have been studied in human and canine OS include *c-sis*, *c-myc and MET*. The *c-sis* oncogene encodes another growth factor, platelet derived growth factor-β (PDGF-β), and one study evaluating levels of *c-myc*, *c-sis,* and the sis gene product PDGF-β found that not only did canine tissue samples have significant amplifications of both proto-oncogenes, the levels of PDGF-β by immunostaining were comparable to human OS tissue (24). The additional finding that canine OS cell lines express functional receptors for PDGF-β (25) provides evidence for an autocrine growth circuit in OS. In addition, another autocrine growth circuit has been shown to be active in both human and canine OS; the hepatocyte growth factor-scatter factor (HGF-SF)/c-Met tyrosine kinase receptor circuit. Binding of HGF to Met triggers receptor phosphorylation and results in a signaling cascade including activation of phosphotydilinositol-3 kinase (PI3K), extracellular signal-related kinase 1 and 2 (ERK1/2), focal adhesion kinase (FAK), and phospholipase C gamma (PLC-γ) leading to cellular responses such as scattering, motility, and increased proliferation (26). This circuit was found to be autocrine in canine OS, with expression of both the c-Met receptor and HGF ligand with constitutive activation of the receptor, while human OS cells appear to express Met protein and show receptor activation upon stimulation with exogenous HGF (27). The importance of the HGF/Met pathway is illustrated by studies aimed at inhibiting the pathway showing promising antitumor activity, as well as the discovery that human primary osteoblasts can be transformed to OS by MET overexpression (28). The use of small-molecule inhibitors of Met or Met-specific RNA interference in canine OS cell lines resulted in inhibition of Met receptor activation and reductions in phosphorylation of downstream targets such as Erk and Akt. In addition,

these studies also reported reduced migration, invasion, branching morphogenesis and colony formation resulting from Met inhibition (26, 29). An additional downstream target of HGF-Met signaling is the signal transducer and activator of transcription-3 (STAT3), and canine and human OS have been shown to express constitutively active STAT3; inhibition of STAT3 activation and/or DNA binding ability have also been shown to decrease OS cell proliferation and viability as well as induce apoptosis (30). Importantly, aside from determining the potential therapeutic relevance of targeting Met, these studies provided further validation of canine OS as a model system. In addition, the insulin-like growth factor-1 (IGF-1), IGF-1 receptor, and IGF binding proteins have an essential role in osteoblast function and physiology and have been shown to be expressed at high levels in some canine and human OS cell lines and tissue specimens, with increased levels of the receptor contributing to a more aggressive phenotype *in vitro* (31, 32). One study performed on clinical samples of human primary OS and metastatic lesions was unable to detect a difference in the level of expression of IGF-R or the ligands IGF-1/IGF-2; however, all three were expressed in primary and metastatic lesions and there was a correlation between IGF-R and IGF-1 expression which underscores the potential importance of this autocrine growth circuit in the development and possibly the metastatic spread of OS (31). Interestingly, a study performed in canines with spontaneously occurring OS aimed at evaluating the clinical potential of IGF growth factor suppression utilizing an analog of somatostatin (Octreotide pamoate) in combination with a traditional platinum-based chemotherapy found no improvement in outcome when compared to dogs receiving chemotherapy alone (33).

Further evidence of the importance of deregulated lineage determination and differentiation in OS development comes from studies that identify abnormalities in the Wnt/β-catenin pathway. The canonical Wnt pathway is essential during embryonic development for the differentiation of osteoblasts from a precursor common to both chondrocytes and osteoblasts (34). This pathway is activated by binding of Wnt ligands to Frizzled receptors and low-density lipoprotein receptor-related protein 5/6 (LRP5/6) which consequently blocks the phosphorylation and subsequent degredation of β-catenin, allowing nuclear translocation and formation of a transcriptional complex with the Tcf/Lef family of transcription factors resulting in gene transcription. In addition to early developmental osteoblast lineage determination, canonical Wnt signaling has various roles in bone homeostasis including osteoblast proliferation and maturation and attenuating osteoclastogenesis through transcriptional regulation of osteoprotegerin (34). This has led to efforts to identify the importance of Wnt signaling is OS and, as might be expected in a tumor of deregulated osteoblast differentiation and increased bone resorption by osteoclasts, inactivity of the pathway is found in human OS cell lines and up to 90% of OS biopsies, determined by a lack of nuclear β-catenin staining (2). In addition to identification of inactive Wnt signaling in OS samples, this same study also reported strong β-catenin signaling in benign osteoblastoma samples. Although a direct progression from benign osteoblastoma to malignant osteosarcoma has not been reported, these results suggest that the loss or deregulation of Wnt signaling is an important step in malignant transformation.

Osteosarcoma metastasis and metastasis-related genes

As mentioned previously, one of the similarities between canine and human OS is the propensity to metastasize early in the course of disease progression, primarily via hematogenous spread to the lungs. Elucidation of genes involved in metastasis of osteosarcoma has been a target of a number of researchers and the growing list of candidate genes includes the membrane-cytoskeletal linker Ezrin, Annexin-2, and chemokine receptor-4 (CXCR4) (35-38). It has been shown that the majority of canine primary OS tumors express CXCR4, and CXCR4 expressing canine OS cells demonstrate directional migration toward the ligand CXCL12 (39). This has important implications for the propensity to spread to the lung as high levels of CXCL12 are found within lung tissues and inhibition of this interaction has been shown to reduce metastasis to the lung in murine models (40). Studies have correlated the pattern of positive Ezrin staining of primary tumors with prognosis in both canine and human patients (36, 41). Furthermore, strategies that reduce Ezrin expression, such as antisense RNA or inhibition of the mTOR pathway by Rapamycin, were shown to reduce propensity of OS cell to metastasize to the lungs in a murine model of OS, providing additional in vivo evidence of the importance of Ezrin in OS metastasis (42).

Treatment of Osteosarcoma

The current standard of care for OS includes a combination of chemotherapy and surgery, either limb-spare techniques or amputation. In human patients, an intensive multi-modal chemotherapy regimen typically involving the use of ifosfamide, cisplatin, doxorubicin, and methotrexate (43, 44) is administered prior to surgical intervention, and there is a direct correlation between primary tumor response (percent necrosis) to

chemotherapy and prognosis (45). In canine patients, standard of care typically involves surgical intervention followed by chemotherapy using either platinum-based or doxorubicin-based protocols. The association between pre-surgical chemotherapy response and patient prognosis in dogs is not as clear, with one report showing only a correlation with local tumor control and another showing a direct correlation between percent necrosis and survival time following doxorubicin administration (46-48). Radiation therapy has limited utility in the treatment of OS as it is a relatively radioresistant tumor; however, it has been used in cases of unresectable tumors or where margins of resection are positive for tumor cells, as well as for palliation of pain from bony metastases (44).

Prognostic factors

Aside from the previously mentioned responsiveness to chemotherapy in human patients, the second most important prognostic factor is the development of metastasis, and even for patients developing an isolated pulmonary metastatic nodule after treatment the overall survival remains poor (43). Additional prognostic factors include serum alkaline phosphatase (ALP) levels at the time of diagnosis, with elevated ALP correlating with an approximately 50% reduction in survival regardless of chemotherapy protocol (49), histologic grade (50), and pre-treatment microvascular density which correlates to levels of vascular endothelial growth factor (VEGF), a negative prognostic indicator for metastasis and overall survival (51-54). In addition, over-expression of the *MDR1* gene product P-glycoprotein, a membrane drug efflux pump, is an important predictor of adverse clinical outcome for patients treated with chemotherapy (55). This is not due to an increased metastatic potential inherent within MDR over-expressing cells, but a

reduced ability of these cells to respond to chemotherapy, underpinning the need for novel therapeutics particularly in these tumors (56). Additional drug-related genes may also have predictive significance in OS, as one study found that inosine-monophosphate dehydrogenase II (IMPDH2), ATPase family, AAA domain containing 2 ($ATAD_2$), and ferritin light chain (FTL) upregulation in tumor cells were all associated with a decreased overall event-free survival (57). The expression of the telomere-length maintaining enzyme telomerase has also been shown to predict an unfavorable outcome in OS as high-expressing primary tumors may be associated with shorter progression free and overall survival (58).

Investigational therapies for OS

Because of the poor prognosis for patients presenting with metastatic disease and the reduced overall survival of patients who develop metastases after chemotherapy, novel therapeutic approaches are desperately needed for OS. However, due to the large number of varying genetic mutations and constitutively activated autocrine growth circuits in OS, determining the optimal target becomes difficult. In one study of human OS cells, an attempt to block the IFG-1/IGFR autocrine circuit which, as previously discussed, contributes to the malignant phenotype of OS had only limited effects. Upon further investigation of these cells it was determined that they also expressed autocrine circuits mediated by epidermal growth factor receptor (EGFR) interacting with both EGF as well as TGFα, nerve growth factor (NGF), low-affinity nerve growth factor receptor (NGF-R) and tyrosine receptor kinase A (trkA) (59). This same study found that treatment of OS with a therapy capable of blocking multiple growth circuits by non-

selectively interfering with ligand-receptor binding (suramin) was a promising alternative to direct targeting of a specific pathway.

Additionally, an immune-based therapy has been evaluated in both canine and human osteosarcoma. Liposomal muramyl tripeptide phosphatidylethanolammine (LMTP-PE) is a synthesized component of mycobacterium that is capable of stimulating the immune system through potent activation of monocytes and macrophages, which selectively take up the compound because of the liposome encapsulation. This compound was shown to significantly prolong overall survival time in dogs and in humans as well as provide a trend toward a better event-free survival in human patients (60-62).

Epigenetics

History and relevance to cancer

The vast majority of research into changes in gene expression that relate to the initiation and progression of cancer have historically focused on genetic alterations such as deletions, amplifications, or mutations. However, it has recently become apparent that altered epigenetic programs play an equally important role in tumor development by providing an additional means of heritable transmission of altered gene expression. The term *epigenetics* refers to heritable changes in somatic cells that result in altered gene expression that are not due to changes in the primary base sequence of DNA, and it is these epigenetically mediated gene expression profiles that allow for changes in cell phenotype, or differentiation, that occur against the uniform background of DNA sequence within an individual (63). Since the DNA sequence itself cannot determine which portions of the genome are expressed, this occurs by changing the accessibility of

specific regions of DNA to transcriptional machinery which in turn determines the gene expression profile of any given cell (63). The three main types of epigenetic information, all of which are interrelated include covalent methylation of DNA cytosines, genomic imprinting or parent-of-origin-specific allele silencing, and post-translational histone modifications (64-66). DNA within eukaryotic cells is compacted into higher order structures, the basic unit of which is the nucleosome containing approximately 147 base pairs of DNA wrapped around an octamer of histone proteins (67). The protein octamer is made up of the histone proteins H1, H2A, H2B, H3, and H4 each containing a flexible amino terminus (11-37 residues) with numerous positively charged basic amino acids which interact with the negatively charged phosphate backbone of DNA (67). These amino terminal tails contain residues, particularly lysine, that are subject to reversible modifications such as acetylation that determine the level of interaction between histones and DNA as well as between nucleosome and linker histones (67). It is this interaction between the amino terminal tails of histone proteins and DNA that is, in part, responsible for the condensation of DNA into higher order structures which in turn plays an essential role in accessibility of the DNA to transcriptional machinery and thus determines gene expression.

In addition to the effect of post-translational histone modifications, chromatin structure is influenced by the covalent methylation of cytosine residues that are located 5' to guanine residues (CpG dinucleotides) utilizing S-adenosyl-methionine as a methyl donor group and DNA methyltransferase enzymes (DNMT) to place the methyl groups upon DNA (66, 68). This modification marks regions of chromatin that are transcriptionally silent, and provides a means of stabilizing the heritable nature of

transcriptional silencing as a mechanism to prevent unwanted transcription of potentially deleterious elements such as viral insertions and repeat elements (63, 68, 69). Approximately half of the genes in the genome contain these CpG regions within the promoter, or CpG islands, and these regions are the target of the key epigenetic abnormalities found within cancer cells (65, 70, 71). The first epigenetic abnormality to be discovered in tumor versus normal tissues was a global hypomethylation of CpG islands resulting in gene activation (65, 66, 72). In fact, HRAS and KRAS were the first cellular oncogenes found to be activated in a panel of human carcinomas by means of hypomethylation of their promoter regions (73). Subsequently, hypomethylation induced overexpression was discovered in a number of human cancers including gastric carcinoma (cyclin D2), renal-cell carcinoma (MN/CA9), colon cancer (S100A4 metastasis-associated gene), and cervical cancer (HPV16) (74-77). Global hypomethylation potentially carries an additional mechanism for the development of tumors beyond simply allowing expression of proto-oncogenes. The pericentromeric regions of DNA are typically hypermethylated and transcriptionally silent, and mutations in DNA methyltransferase enzymes (DNMTs) have been linked to hypomethylation in these pericentromeric regions which in turn predisposes them to recombination events and increased chromosomal instability; recurrent chromosomal translocations within pericentromeric regions have been described in breast, ovarian, and Wilm's tumors (65, 78-80). In addition, studies have also linked global decreases in methylation with greater degrees of aneuploidy (65). Methylation of DNA sequences also serves an important function in preventing the unwanted expression of viral elements that have become trapped within the genome, and an additional role of hypomethylation serving as a

potential initiating event is the re-expression of latent *HPV16* resulting in cervical dysplasia and subsequently cervical cancer, and latent Epstein-Barr virus (*EBV*) hypomethylation in the generation of EBV-associated lymphomas (81, 82)

Conversely, hypermethylation is also linked to the development of cancer and it has been suggested that aberrant methylation patterns are found more frequently than mutations, in cancer, as a mechanism of loss of function of tumor-suppressor genes (83). The first correlation between gene hypermethylation and cancer was made with the *RB* gene, the first discovered tumor-suppressor (65). A number of cases of sporadic as well as hereditary retinoblastoma have promoter hypermethylation leading to reduced *RB* expression (84, 85). Additional tumor-suppressor genes discovered to be silenced in cancer by CpG promoter hypermethylation include *CDKN2A* (encoding p16/INK4A; previously mentioned as a gene commonly mutated in OS), the DNA mismatch repair gene *MLH1*, von-Hippel-Lindau (*VHL*), and E-cadherin (65, 72). In fact, evidence exists that aberrant methylation patterns may be the first detectable, or gatekeeper, mutation in some tumors. This evidence stems from research on Wilm's tumor and the biallelic expression of *IGF2* seen in these tumors resulting from a loss of imprinting (the second form of epigenetic information). In normal cells, the paternal copy of *IGF2* is silenced by promoter methylation which is maintained by a nearby differentially methylated region containing the *H19* gene, which is reciprocally imprinted (unmethylated). Interestingly, the earliest detectable genetic change in Wilm's tumors (and even nearby normal kidney) is a gain of methylation of the *H19* promoter which results in hypomethylation and expression of *IGF2* from the paternal allele (65, 66, 86-88). In addition to hypermethylation of specific target genes, another comprehensive method of

altering gene expression is the silencing of key transcription factors which in turn silences its downstream targets. This phenomenon has been documented OCT3/4 in testicular cancer, RUNX3 in esophageal cancer and NFATC1 in lymphoma (89-91). The argument could be made that this methylation phenotype is the result of malignant transformation instead of the causative factor; however, the finding of abnormal hypermethylation of the tumor-suppressor *CDKN2A* in atypical bronchial epithelia of smokers, classified histologically as pre-neoplastic lesions, lends support to the argument that epigenetic changes are early events that predispose to tumor formation, not resulting from tumor formation (92). Another example of early epigenetic alterations having a pivotal role in clonal expansion of progenitor cells which leads to increased predisposition to cancer involves the Wnt pathway in the progression of colon cancer. This pathway is canonically overactive in colon cancer via mutations in downstream genes such as *APC* and β-*catenin*. However, recent studies have found that epigenetic silencing of a family of secreted antagonists of the pathway, SFRPs, occur early during colon cancer development and suggest that breaching of this "epigenetic gatekeeper" is what then allows for the accumulation of mutations downstream in *APC* and *β-catenin*, or the "genetic gatekeeper" mutations and oncogenic pathway addiction (70).

The third type of epigenetic information is the post-translational modification of histone proteins, or specifically, the amino terminal tails of these proteins assists in both maintaining the structure of chromatin and controlling gene expression. Currently, there are more than 50 known sites of modification including lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, glutamic acid ADP-ribosylation, and lysine ubiquitination and suymoylation (93). Of these, lysine

acetylation and methylation are the best-characterized with regard to their role in the development of cancer (65). The post-translational modifications of histones have been proposed as a regulatory "code" for transcriptional activation and silencing that is read by non-histone proteins and protein complexes that make up the transcriptional machinery (94).

Histone methylation

Histone methylation, occurring primarily on lysine residues of histones H3 and H4, can result in either activation or repression of transcription, and is regarded as a mark of long-standing cellular memory. For example, sites of active gene transcription are marked by methylation of histone H3 on lysine 4 (H3-K4), 36 (H3-K36), or 79 (H3-K79) while transcriptionally inactive sites are marked by methylation of H3-K9, H3-K27, or H4-K20 (95). Multiple methyl groups can be added to lysine residues by histone methyltransferase (HMT) enzymes, a family of 17 proteins, which also interact with DNMTs and thereby can cooperatively result in silencing of tumor-suppressor genes (65). In addition to marking regions of active or inactive chromatin, these methylation patterns, with multiple methyl groups on specific residues, can also be markers of specific domains of heterochromatin; for example enrichment of trimethylated H3-K9 in pericentromeric regions, and trimethylated H3-K27 at the inactive-X chromosome (96). The functional consequences of methylated histones is, for the most part, determined by the proteins which recognize a specific modification, such as the repressive proteins heterochromatin protein 1 (HP-1) and Polycomb proteins which contain chromodomains that recognize methylated H3-K9 and H3-K27, while the activating H3-K4 mark is recognized by the chromodomain of an activating helicase DNA-binding protein 1 (65). It has recently

been discovered that histone methylation is a dynamic process and not necessarily irreversible, as the enzyme lysine-specific demethylase 1 (LSD-1) was found to remove methyl groups from H3-K4 (97). It is likely that because of the dynamic nature of gene expression more enzymes catalyzing the removal of methyl groups will be discovered. The link between aberrant expression of HMTs and cancer is clear as a number of these proteins have been found to be deleted, mutated, or overexpressed in a wide variety of cancers including acute leukemias, neuroblastoma, hepatocellular carcinoma, Wilm's tumor and small cell lung carcinoma (98-103).

Histone Acetylation

The acetylation of histone proteins is the result of balanced activities between histone acetyltransferases (HAT) and histone deacetylases (HDAC). In contrast to histone methylation, only a single acetyl group is added to any lysine residue. The net effect of histone acetylation is the neutralization of positive charges and a weakening of the interaction between histone proteins and DNA, resulting in formation of euchromatin, a relaxed form of chromatin that is more accessible to transcriptional machinery (65, 104-106). The acetylation of histones is a key process associated with gene transcription and is not a random process, with specific HATs preferentially acetylating specific histone tails (106, 107). HAT enzymes are categorized into one of three families based upon highly conserved structural motifs; the GNAT family (Gcn5-related N-acetyltransferase), the MYST family (containing MOZ, YBF2/SAS3, SAS2, and TIP60), and the p300/CBP family which differs from the first two families because they do not bind directly to DNA but are recruited to promoters by other DNA-bound transcription factors (107). The HAT enzymes also target other, non-histone, proteins such as the transcription factors p53,

E2F1, and GATA1 and disruption of HAT enzymes has been associated with carcinogenesis such as mutations in p300 in gastric and colorectal cancer (108). In addition, the developmental disorder Rubinstein-Taybi syndrome, which is characterized by >300 fold increased cancer risk, is associated with germline mutations in CBP, and somatic mutations of the other allele result in loss of HAT activity (65, 109). Gain-of-function mutations, such as chromosomal translocations that incorporate HATs into fusion proteins that lead to mistargeting and aberrant gene activation, have also been reported in leukemias (110).

Histone deacetylation

The removal of acetyl groups from lysine residues is catalyzed by HDAC enzymes. There are currently 18 known human HDACs which are divided into class I, class II, class III, or class IV based upon their homology to yeast orthologues Rpd3, HdaI, and Sir2 (111). These classes differ in their catalytic mechanism; class I, II, and IV are referred to as "classical" HDACs which are Zn^{2+} -dependent enzymes; removal of acetyl groups occurs via a charge-relay system that consists of two histidine residues, two aspartic acid residues, and one tyrosine residue residing within the catalytic pocket that contains the essential zinc ion (111). Class III HDACs, referred to as sirtuins, do not have a requirement for Zn^{2+} but require NAD^{+} as an essential cofactor (112). This difference in catalytic activity also translates into differences in their ability to be blocked; classical HDACs are inhibited by zinc chelating compounds and these have no effect on the sirtuins (113). Similar to the HATs, HDACs also target non-histone proteins such as the transcription factors p53, E2F1, GATA1, TFIIE, TFIIF, and glucocorticoid receptors (114-116).

Aside from catalytic activity, HDAC classes also differ in their expression patterns and cellular sub-localization. The class I HDACs, which include HDAC 1, 2, 3, and 8, are the most ubiquitously expressed and, with the exception of HDAC 8, are strictly nuclear in localization (113). Class II HDACs are divided into subclass *a* or *b* which indicates the cellular compartment in which they are found; class IIa includes HDAC 4, 5, 7, and 9 which are both nuclear and cytoplasmic, while class IIb includes HDAC 6 and 10 which are predominantly cytoplasmic as they lack a nuclear localization sequence (113). Class IV HDACs currently only contain one member, HDAC 11, which is both nuclear and cytoplasmic but little is know about its expression or function (113). This trafficking of HDACs, which is phosphorylation dependent and regulated by binding sites for 14-3-3 proteins, becomes essential in regulating the activity of some transcription factors and numerous signaling pathways regulate the phosphorylation of these 14-3-3 binding sites (113).

Many of the HDACs exist in multiprotein complexes where they function as transcriptional corepressors, and some have been shown to be recruited to hypermethylated CpG islands of tumor-suppressor genes by methyl-binding proteins (MBP) (117). In addition, HDACs can be recruited to gene promoters independently of methylation by oncogenic fusion proteins, such as the AML-ETO and PML-RARα proteins actively suppressing transcription by the aberrant recruitment of HDAC-containing corepressors (118, 119). HDAC 1 and 2 have both been found in the Co-REST repressive complex which is essential in inactivating expression of neuronal genes in non-neuronal tissues, as well as the NURD and SIN3 repressor complexes (120), and HDAC3 is associated with the N-COR and SMRT corepressor complexes (121).

Germline and somatic mutations of the HDAC enzymes are a fairly infrequent finding in cancers. However, a germline promoter polymorphism resulting in increased *in vitro* activity for HDAC10 has been associated with the development of hepatocellular carcinoma among patients with chronic Hepatitis B-virus infection (122), a somatic truncating mutation in HDAC2 has been found in a variety of tumor types that results in activation of cell-transforming pathways and confers resistance to HDAC inhibitor therapy (123, 124), and mutations in HDAC4 have been discovered in breast and colorectal cancers (125). A much more frequent finding is the overexpression and/or function of classical HDACs in cancer. The class I HDACs are frequently found to be overexpressed in tumors versus paired normal tissues and the significance of each relates to the function of the particular enzyme, which has been found to be cell-type specific (126).

The ubiquitously expressed, nuclear HDAC1 is a binding partner in repressive complexes including Sin3, Mi-2/NuRD, and CoREST, and its substrates include p53, MyoD, E2F1, and Stat3; knockout of HDAC1 in mice is embryonic lethal (113). HDAC1 appears to have an essential role in the control of cell proliferation as it represses the expression of p21 and p27 cyclin-dependent kinase inhibitors in osteosarcoma, and knockdown of HDAC1 results in cell cycle arrest at either the G(1) phase or the G(2)/M transition. In addition, knockdown of HDAC1 in breast and osteosarcoma cells leads to an increase in apoptosis and decreases in proliferation and the number of mitotic cells (127). Furthermore, prostate cancer cells overexpressing HDAC1 display a more undifferentiated phenotype in addition to increased proliferation (128). Consistent with an essential role in cellular differentiation and proliferation processes, overexpression of

HDAC1 has been found in gastric, pancreatic, colorectal, hepatocellular, and breast carcinomas (129-133). In addition, relatively high levels of HDAC1 have been found in advanced stage lung cancer compared to low stage cancers (134). This overexpression of HDAC1 in gastric cancers has been related to a significantly increased risk of nodal spread and was an independent prognostic marker for survival (130). The increased levels of HDAC1 expression in pancreatic cancer (along with HIF1α) have also been linked to poor prognosis as well as reduced differentiation and increased proliferation (131). In prostate cancers, elevated levels of HDAC1 have been associated with hormone refractory higher grade tumors when compared to low grade tumors or benign prostatic hyperplasia (128). In hepatocellular carcinoma, more invasive and poorly differentiated tumors show higher HDAC1 expression, and these tumors are more likely to have a higher TNM stage along with poor patient survival (133). Conversely, the increased expression of HDAC1 in breast carcinomas has been associated with negative lymph node status and a better overall survival, particularly in patients with small tumors (135). HDAC1 has additionally been shown to be a regulator of colon cancer cell sensitivity to oxidative stress through negative regulation of thioredoxin binding protein 2 (TBP-2); low levels of TBP-2 are associated with poor clinical outcome in colon cancer patients and correlates with a decreased sensitivity to chemotherapeutic agents such as cisplatin (136).

HDAC2 is also a ubiquitously expressed nuclear enzyme that, similar to HDAC1, is part of Sin3, Mi-2/NuRD, and CoREST repressive complexes. Substrates for HDAC2 mediated deacetylation include Bcl-6, Stat3, glucocorticoid receptor, and YY-1 protein (113). Knockout of HDAC2 in mice results in severe cardiac defects including excessive

hyperplasia of cardiac muscle and arrhythmia (113). Knockdown or selective depletion of HDAC2 has been associated with reductions in the expression of progesterone and estrogen (PR/ER) receptors and potentiation of tamoxifen-induced apoptosis in ER/PR positive breast tumor cells (137). In addition, knockdown of HDAC2 in chronic lymphocytic leukemia and prostate cancer cells was shown to be sufficient to sensitize these cells to TRAIL-induced apoptosis (138, 139). Along with HDAC1 and 3, HDAC2 overexpression has been shown in gastric, colorectal and prostate cancer and is associated with advanced stage disease (130, 132, 140).

HDAC3 also belongs to the ubiquitously expressed, nuclear localized class I HDAC family and functions as part of the N-CoR/SMRT corepressor complex. Substrates for HDAC3 include GATA-1, RelA, Stat3, MEF2D, YY-1, and short-heterodimer partner (SHP). Knockout of HDAC3 in mice is embryonic lethal, while conditional liver knockouts demonstrate hepatocyte hypertrophy and induction of metabolic genes (113). HDAC3 functions as a key component of osteoblast differentiation by associating with Runx2, an essential transcription factor for bone development, and appears to inhibit differentiation in this setting (141). In addition, HDAC3 is a component of the aberrant transcriptional regulation in PML-RARα expressing pro-myelocytic leukemia cells and knockdown of HDAC3 in these cells restores expression of retinoic acid dependent genes (142). In addition to HDAC1 and2 HDAC3 expression has been significantly associated with poor prognosis in gastric, prostate, and colorectal cancer samples (130, 132, 140). Interestingly, specific knockdown of HDAC3 in colorectal cancer cell lines does not appear to have the same growth inhibitory effects that knockdown of HDAC1 and 2 have, nor does it appear to

sensitize CLL cells to TRAIL-induced apoptosis as is seen with suppression HDAC1 and 2 (138, 140).

HDAC8 is the last member of the ubiquitously expressed class I HDACs and, contrary to the other three members, is localized to both the nucleus and cytoplasm. HDAC8 interacts with the Ever-Shortening Telomeres 1B protein (EST1B) and HDAC8 knockout mice are viable but exhibit craniofacial defects (113, 143). Of all the HDACs, only increased HDAC8 expression appears to be correlated with advanced stage disease, poor prognosis and shorter overall survival in childhood neuroblastoma, and knockdown of HDAC8 in neuroblastoma cells results in increased differentiation and cell cycle arrest along with decreased proliferation (144). The role of HDAC8 in tumorigenesis may lie in its association with ESTB1 protein and the subsequent control of telomerase activity, shown to be activated in a number of cancers including OS where expression predicts an unfavorable outcome (58, 143, 145). Specific inhibition of HDAC8 induces apoptosis in T-cell lymphomas and reduces proliferation of colon, lung, and cervical cancer cell lines (146, 147).

Class II HDACs, with more restricted tissue expression and an ability to be shuttled between the nucleus and cytoplasm, have also been linked to cancer. HDAC4 is normally expressed in heart, smooth muscle, and brain (113). This HDAC binds to the transcription factors ANKRA and RFXANK which play essential roles in the expression of MHC class II molecules on lymphocytes and HDAC4 activity results in decreased expression of MHCII (148). This action may implicate HDAC4 in the ability of tumor cells to evade detection by the immune system. HDAC4 is also able to interact with N-CoR/SMRT and the co-repressors Bcl-6-interacting co-repressor (BCoR) and c-terminal

binding protein (CtBP) (111). In addition, HDAC4 is able to interact with the fusion protein PLZF-RARα in acute promyelocytic leukemic cells and repress genes associated with differentiation (149). Substrates of HDAC4 deacetylase activity include GCMa, GATA-1, and HP-1. HDAC4 knockout mice are viable but exhibit premature and ectopic ossification and chondrocyte hypertrophy (113). Elevated HDAC4 expression has been found in renal, bladder, colorectal, and breast cancer samples with the highest expression belonging to breast cancer samples (150). Inhibition of HDAC4 may provide a method of tumor anti-angiogenic therapy as HDAC4 is able to bind to and augment the transcriptional activity of HIF-1α, and this activity is independent of VHL which is the canonical controller of HIF-1α activity (151). Along with the other class IIa members HDAC5 and 7, HDAC4 is able to bind to and inactivate myocyte-enhancer factor 2 (MEF2) which effectively blocks muscle cell differentiation. This inhibition of muscle cell differentiation can be relieved by a phosphorylation induced dissociation of HDAC4/5/7 and MEF2 by Ca^{2+}/calmodulin dependent kinase (CaMK) (111, 152).

HDAC5 acts in concert with HDAC4 and 7 in transcriptional control of muscle cell differentiation through interactions with MEF2 (152). HDAC5 knockout mice exhibit myocardial hypertrophy and abnormal cardiac stress response resulting from constitutive activation of stress signals (113, 153). In addition, HDAC5 plays a role in the inhibition of angiogenesis that is independent of binding to MEF2 or histone deacetylation activity. Of all the HDACs, only silencing of HDAC5 results in a pro-angiogenic state that is associated with upregulation of FGF2 and the angiogenic guidance factor Slit2 (154).

In addition to the previously mentioned role in controlling muscle cell differentiation through interaction with MEF2, HDAC7 has a critical role in the normal development of endothelial cells, and is expressed in heart, placenta, pancreas and smooth muscle tissues (113). An additional consequence of MEF2 binding by HDAC7 is the repression of matrix metalloproteinase 10 (MMP10), a function that is essential for maintaining vascular integrity of the endothelium during embryogenesis (155). Knockouts of HDAC7 result in embryonic lethality due to a lack of endothelial cell-cell adhesion and subsequent dilation of rupture of blood vessels (155). *In vitro* silencing of HDAC7 has been shown to inhibit endothelial cell migration which is a key process in tumor angiogenesis (156). HDAC7 is also transiently expressed in humans within CD4/CD8 double-positive thymocytes where it represses the expression of the pro-apoptotic gene *nurr77* which is involved in negative selection (157). A role for HDAC7 in the apoptosis of tumor cells has not been clearly defined. In addition to its role in regulation of angiogenesis, HDAC7 plays a role in the maturation of osteoblasts by associating with and repressing the activity Runx2, although the deacetylase activity is not required which suggests a role as a scaffold or recruiting protein (158). HDAC7 is highly expressed in colorectal cancers when compared to bladder, renal and breast cancer (150).

HDAC9 is the fourth member of the class IIa HDAC family, and there are three known splice variants for this protein; HDAC9a, HDAC9b, and HDRP/HDAC9c (159). As with the class I HDACs, and in contrast to the other class IIa members, HDAC9a and b have their catalytic domains within the N-terminus, while HDRP lacks a catalytic domain; however, HDRP is able to recruit HDAC3 similar to HDAC4/5/7 which

circumvents the lack of catalytic activity of this splice variant (111). HDAC9 is also able to interact with MEF2, and the presence of splice variants may provide an additional level of control in muscle cell differentiation (159). Currently there is no information on the specific expression and function of HDAC9 in cancer (113).

The class IIb HDAC family includes HDAC6 and 10. HDAC10 is the most recently discovered of the class II HDACs, and there appear to be two splice variants of this protein as well. In contrast to the class I HDACs this one has two putative catalytic domains, one N-terminal and one C-terminal, and can associate with HDACs 1, 2, 3, 4, 5, and 7. In addition, there are two Rb binding sites found on HDAC10 which suggests it might have a role in cell cycle regulation (160-163). A specific role in cancer has not yet been identified.

HDAC6 is another unique enzyme within the classical HDAC family with two catalytic domains arranged in tandem. In addition, HDAC6 is unique because it contains a domain on the C-terminus which acts a signal for ubiquitination which indicates that this particular HDAC is prone to degradation by the proteosome (164). HDAC6 demonstrates mostly cytoplasmic localization, but has been identified in the nucleus in association with HDAC11 (164). The expression patter in normal tissues includes kidney, liver, heart, and pancrease. HDAC6 knockout mice are viable and have no significant phenotypic defects but exhibit a global tubulin hyperacetylation and mouse endothelial fibroblasts fail to recover from oxidative stress (113). Substrates that have been identified for HDAC6 include α-Tubulin, HSP90, Smad7, and the small—heterodimer-partner (SHP) nuclear receptor transcription factor (113). Consistent with its association and action on HSP90, specific inhibition of HDAC6 leads to hyperacetylation

of HSP90 and disruption of its chaperone function, leading to a decrease in pro-growth survival client proteins, such as the Bcr-Abl fusion protein in leukemic cells (165). Moreover, HDAC6 plays a role in the HSP90/proteosome mediated regulation of vascular endothelial growth factor receptors 1 and 2 (VEGF1/2) and inhibition of HDAC6 results in reduced association of VEGFR1/2 with HSP90 and increased association with HSP70, leading reduced levels of both receptors in a proteosome-dependent manner (166).

HDAC6 has another potential role in the regulation of angiogenesis as it, along with HDAC4, has been found to associate with and control the activity of HIF-1α in a VHL-independent manner (151). In colon carcinoma cells, activation of epidermal growth factor (EGF) has been shown to result in nuclear translocation of β-catenin and activation of *c-myc*, and this activity is dependent upon HDAC6 deacetylation of β-catenin, preventing its phosphorylation and subsequent degredation (167). Consistent with this activity, inhibition of HDAC6 has been shown to inhibit EGF induced nuclear localization of β-catenin and reduce activation of *c-myc* in these cells, resulting in reduced proliferation (167). HDAC6 is overexpressed in a number of tumor types as well; however, the relevance of this overexpression appears to be tumor-type dependent as HDAC6 is correlated with higher grade, advanced stage oral squamous cell carcinomas while overexpression in breast cancers appears to correlate with a better prognosis particularly in ER positive tumors treated with tamoxifen where HDAC6 expression was shown to be an independent prognostic indicator (168-170).

The last class of HDAC inhibitors, a family of seven Sirtuins, differs from classical HDACs in that they require NAD^+ as a catalytic cofactor and are not inhibited

by conventional HDAC inhibitors. The role of sirtuins in cancer is controversial; although the expression of sirtuins is altered in cancer it is unclear whether they function as tumor suppressors or oncogenes (171). Initial studies on Sirt1 suggested that it functions as an oncogene as p53 was the first target identified, and deacetylation of p53 by Sirt1 resulted in reduced transactivation and inhibition of p53 mediated apoptosis (172, 173). Recently, Sirt1 was suggested to have a critical role in cancer through modulation of angiogenesis through deacetylation of the transcription factor FOXO1 and subsequent blockade of endothelial cell senescence and promotion of endothelial cell growth, sprouting, branching morphogenesis and blood vessel formation in mice (174). Other studies have identified a critical role in DNA repair and found that Sirt1 transgenic mice lived longer in models of colon cancer (175). Furthermore, Sirt1 mutant mice exhibit impaired DNA damage response, genome instability, and increased tumorigenesis (176). Some expression studies on human cancer samples have shown that Sirt1 is overexpressed, when compared to normal tissues, in colon, skin, breast and prostate cancers and leukemia while others have shown a reduced level of expression in prostate and bladder carcinoma, glioblastoma and ovarian cancer (171).

As eluded to earlier within this section, histones are not the only proteins affected by acetylation/deacetylation, and a wide variety of proteins that are relevant to the oncogenic process are also subjected to post-translational, reversible, acetylation. These include, but are not limited to, HMG proteins which have a proposed role in DNA repair, transcription factors such as p53, c-Jun, EKLF, GATA1, MyoD, TCF, TFIIE, TFIIF, NF-E2, ACTR, Runx2, and nuclear import factors (177). Differential acetylation of these proteins affects activities such as DNA binding affinity and protein-protein interactions.

In the case of transcription factors, the effect of acetylation depends on the location of the acetylated lysine residues; p53, E2F1, EKLF, and GATA-1 contain acetylation sites that are adjacent to the DNA binding domain and acetylation increases DNA binding and transactivation (177). In contrast, HMGI contains a lysine that is acetylated within the DNA binding domain and acetylation reduces binding affinity and transactivation providing an explanation for why acetylation is not always associated with increased transcription (177).

Based upon the information presented within this section, it is clear that HDACs play critical roles in normal development and differentiation processes and have significant roles in tumor development and progression, and inhibition has pleiotropic effects on phenotype depending upon which HDAC is inhibited and in what cellular context.

Epigenetic regulation of bone development

Skeletal development and maintenance are dynamic processes that occur throughout life, and the coupled and balanced activity of bone-resorbing osteoclasts and bone-forming osteoblasts is critical for maintaining skeletal health. Bone morphogenic proteins (BMP) are the key regulators of bone formation and BMP2 signals that induce the osteogenic phenotype of mesenchymal cells are directed to the nucleus through Smad heterodimers that interact with Runx2, an essential transcription factor for osteoblast development (178). Formation of the long-bones occurs through a process known as endochondral bone formation which involves a cartilaginous template that is invaded by osteoclasts which resorb bone and form the bone marrow cavity, followed by invasion by mesenchymal-derived osteoblasts which then lay down a collagenous matrix and the

proteins necessary for mineralization (179). Epigenetic activities have a crucial role in the process of endochondral bone formation and osteoblast differentiation, as Runx2 has been shown to positively or negatively regulate osteoblast gene expression through interactions with either co-activators (HATs p300, CBP, MOZ, and MORF) or co-repressors (HDACs 3, 4, 6 and 7) (141). In addition to interaction with HATs and HDACs, the induction of osteogenic genes, such as osteocalcin, by Runx2 has been linked to alterations in chromatin structure and requires the DNA-dependent ATPase chromatin remodeling complex SWI/SNF (180). Chromatin remodeling by the SWI/SNF complex also supports transcriptional control of myogenic, adipocytic, and myeloid differentiation and has been shown to be essential for the activation of p53-mediated transcriptional activation in cell-cycle control (181-183). The importance of the timed interactions between Runx2 and transcriptional co-repressors is illustrated by a study showing that HDAC4-null mice prematurely express the Runx2 target gene Indian hedgehog (Ihh), in addition to showing significant chondrocyte hypertrophy and premature and ectopic ossification (179, 184). In addition, the interaction between HDAC4 and MEF2, described previously, is important for proper endochondral bone formation as the ectopic bone formation that occurs in HDAC4 depleted mice can be partially rescued by reducing expression of MEF2 (185). HDAC3 also interacts with Runx2 to regulate the transcription of key osteoblast differentiation genes such as osteocalcin, osteopontin and bone sialoprotein and HDAC3 suppression causes premature mineralization (141). In addition, suppression of HDAC4 or 5 relieves the repression of the osteocalcin promoter and results in increased acetylation of Runx2 (186, 187).

The interaction between HDACs and Runx2 or other osteogenic transcription factors is temporally regulated in the developing skeleton. HDAC 3 and 7 are found in all osseous cells, HDAC1 appears to be limited to progenitor cells, and HDAC4 and 6 are found in mature osteoblasts (141, 188). Thus, as osteoblast differentiation progresses the cells may express multiple HDACs simultaneously that interact with Runx2, Smads, Twist (which contains intrinsic HAT activity), and pRb to control the coordinated gene expression required for proper osteoblast differentiation (141, 186, 188-191). Osteoclast differentiation is also dependent upon HDAC enzymes and HDAC activity results in suppression of osteoclast maturation as HDAC1 has been shown to be recruited by STAT3 to the promoter regions and inhibit the expression of key osteoclast maturation genes such as NFATc and OSCAR (179, 192). Interestingly, the osteoclast maturation gene NFATc has also been shown to be a co-repressor of the osteocalcin promoter during osteoblast differentiation by recruitment of HDAC3 to the osteocalcin promoter (193).

In addition to BMP2 mediated Runx2 activation, the Wnt pathway also plays a crucial role in the maturation of differentiation of osteoblasts. As previously mentioned, the Wnt pathway involves the binding of Wnt proteins to Frizzled/LRP5/6 receptors. The downstream effect is to stabilize β-catenin and allow nuclear translocation and gene transcription. This has been shown to be important to osteoblast maturation as overexpression of Wnt ligands such as Wnt3 increase osteoblast differentiation while receptor disruption of LRP5 results in decreased osteoblast proliferation (194, 195). Additional links between the Wnt pathway and Runx2-induced expression have been found through interactions between the DNA binding domains of Runx2 and LEF1, a transcription factor activated by β-catenin, as well as the finding that BMP2-induced

differentiation leads to the activation of β-catenin signaling (196, 197). Secreted frizzled-related proteins (sFRPs) are decoy receptors for Wnt signaling as they are truncated such that they do not contain either the transmembrane or cytosolic domains and their expression generally results in a decrease in Wnt signaling through sequestration of Wnt ligands (198-200). Interestingly, one particular decoy receptor, sFRP3, appears to have an effect on osteoblast proliferation and differentiation that may be independent of its action as an inhibitor of Wnt; this particular protein suppresses osteoblast proliferation and increases osteoblast differentation as determined by increased differentiation markers such as alkaline phosphatase and osteocalcin (198). This appears to be independent of Wnt/ β-catenin signaling because treatment of cells with endostatin, which degrades β-catenin, does not affect the sFRP3-induced increases in ALP (198). This important activity of sFRP3 is further supported by the finding that osteosarcoma cell lines and tissue samples exhibit a marked suppression of sFRP3 when compared to normal bone (201). The data on suppression of sFRP3 in OS, together with information from various other tumor types showing a loss of heterozygosity in the chromosomal region containing the *sFRP3* gene (202-204), suggests that sFRP3 may function as a tumor-suppressor gene in OS.

Histone deacetylase inhibitors

Cellular anaplasia, or a lack of differentiation, is a hallmark of malignant tumors and undifferentiated tumors have been associated with a poor prognosis and thus, the induction of differentiation resulting in inhibition of limitless replicative potential in tumors has become a promising therapeutic strategy (205). The initial impetus for the use of inhibitors of histone deacetylase enzymes came from studies on the aberrant

expression and function of HDACs in hematologic malignancies, particularly leukemias in which oncogenic fusion proteins have been shown to recruit HDAC-containing co-repressive complexes leading to a deregulation of normal differentiation processes (83, 118, 165, 206-214). Moreover, implications for the use of HDAC inhibitors in leukemia include the previously mentioned role of HDAC6 in the chaperone function of HSP90 for the oncogenic fusion protein BCR-ABL which is responsible for development of chronic myelogenous leukemia (CML); deacetylation by HDAC6 results in stabilization of BCR-ABL (165). In addition to the HDAC effects on myeloid malignancies, some types of lymphoma also demonstrate aberrant HDAC activity. Diffuse large B cell lymphoma (DLBC) and Follicular lymphoma have a mechanism of oncogenesis resulting from the overexpression of BCL6 which acts as a transcriptional corepressor in complexes with both Class I and Class II HDACs (215-217). Furthermore, the activity of BCL6 is controlled by its acetylation status by the HAT p300; acetylation of BCL6 results in an inability to recruit HDAC and transform cells (218). Expression levels of HDAC1, 2, and 6 have been shown to be elevated in patients with cutaneous T-cell lymphoma (CTCL) and DLBC and the expression level of HDAC2 in particular has been correlated with aggressiveness in CTCL (219, 220).

Currently, there are six classes of agents that are known to inhibit HDAC activity: short-chain fatty acids such as sodium butyrate and valproic acid; epoxides such as depudecin and trapoxin; cyclic tetrapeptides such as apicidin and Depsipeptide (Romidepsin/Istodax); hydroxamic acids such as trichostatin A (TSA), suberoylanilide hydroxamic acid (Vorinostat/Zolinza), oxamflatin, scriptaid, pyroxamide, LAQ824, Panobinostat, and Belinostat; benzamides such as Entinostat and CL-994; and hybrid

compounds such as CHAP (221). Importantly, although all of the listed compounds have been selected for their ability to inhibit histone deacetylation, they have widely varying specificity for individual HDAC isoenzymes and non-histone substrates as well as widely varying potency. Compounds in the hydroxamic acid class generally have *in vitro* potency in the high-nanomolar to low-micromolar range and are effective against Class I, IIa/b, and IV HDAC; the short-chain fatty acids are generally less potent with activity in the low-micromolar range and show efficacy against Class I and IIa HDAC; the cyclic tetrapeptide depsipeptide (romidepsin) has activity in the nanomolar range and has a more specific activity against Class I HDACs, particularly HDAC1 and 2; benzamide compounds show activity in the high-nanomolar to low-micromolar range and are particularly active against Class I HDAC1 and 3; the epoxides also have activity in the nanomolar range of concentrations and they demonstrate (irreversible) activity against Class I and IIa HDAC (221). All of the compounds that are pan-inhibitor appear to work equally well against Class I versus Class II HDAC, and there is currently only one HDACi that can efficiently discriminate between HDACs of the same class; tubacin for HDAC6 which does not affect histone acetylation levels, gene expression levels, or cell-cycle distribution (222, 223).

Given that HDACs contribute to a myriad of essential cellular processes it might seem that the therapeutic window of inhibitors would be very low; however, the existing data seems to show that transformed cells are more sensitive to HDAC-inhibitor induced apoptosis than normal cells which may be explained by addiction of tumor cells to certain epigenetically deregulated pathways, concomitant genetic defects, and a reduced ability of transformed cells to upregulate rescue pathways after a toxic insult (224-227).

Considering the variety of HDAC activities it is then not surprising that the inhibition of HDAC has pluripotent anti-tumor activities ranging from induction of differentiation in some cell types (including osteoblasts) to growth arrest, initiation of senescence at low doses and apoptosis at high doses, decreased angiogenesis and immuno-modulatory activities (228-232).

Induction of apoptosis by HDACi has been shown to proceed by both the extrinsic or death receptor, and intrinsic or mitochondrial pathways. Induction of death receptor genes such as death receptor 5 (DR5) as well as their cognate ligands and repression of inhibitory components of the death receptor pathway has been shown for HDACi treated cells leading to increased susceptibility of these cells to apoptosis via the extrinsic pathway (233-239). The importance of the regulation of death receptors and the extrinsic apoptotic pathway are emphasized by the fact that neutralizing antibodies or siRNA directed toward death receptors and their ligands can protect some tumor cells from the apoptotic effects of HDACi (237, 238). The pro-apoptotic effects of HDACi acting through the intrinsic pathway seem to result from an overall shift in the balance of apoptotic proteins toward those that promote apoptosis (240-243). In addition to shifting the balance toward pro-apoptotic proteins, HDACi can lead to post-translational changes in proteins that favor apoptosis such as cleavage and activation of Bid and Bim (244-246). The activation of apoptosis may also be dependent upon the generation of reactive oxygen species (ROS) an effect of HDACi that appears to be tumor-type specific. The generation of ROS is an early event that precedes loss of mitochondrial membrane potential which suggests that formation of ROS is not a consequence of apoptosis induction (244, 247). The generation of ROS has been described as a means of

promoting apoptosis in hematologic malignancies for a number of targeted agents (248). In addition, treatment of cells with scavengers of ROS such as n-acetylcysteine appears to abrogate the HDACi mediated apoptosis (229). The ability to mediate response to ROS provides another potential rationale for the tumor-cell selectivity seen with HDACi. In normal cells, HDACi treatment has been shown to induce the endogenous ROS scavenger thioredoxin (TRX) whereas transformed cells show an upregulation of thioredoxin-binding protein (TBP-2) a negative regulator of TRX activity leading to a decreased ability to cope with the HDACi generation of ROS (225, 249, 250). In contrast to the beneficial effects on TRX and TBP-2 activity, HDACi that target HDAC6 may actually increase tumor cell ability to cope with ROS, as the activity of peroxiredoxins I and II is mediated by HDAC6 induced deacetylation; inhibition of HDAC6 results in hyperacetylation of these proteins and increases their ability to reduce hydrogen peroxide which may contribute to HDACi resistance (251).

The anti-angiogenic effects of HDACi also constitute an attractive aspect for tumor therapy. Tumor growth and metastasis are dependent upon the development of neovasculature, a process that is dependent upon the balance between angiogenic stimulating and inhibitory factors factors (252-254). HIF-1α is a major regulator of angiogenesis that functions to induce neovascularization in response to hypoxia through upregulation of pro-angiogenic gene expression, while normal oxygen levels lead to hydroxylation of a domain within the protein that becomes a target of the E3 ubiquitin ligase VHL, ultimately resulting in proteosomal degradation of HIF-1α (255). HIF-1α activity and expression have been linked to regulation of metastasis and has been found to be overexpressed in a number of human tumor types (256-258). As mentioned

previously HDAC4 is capable of binding to HIF-1α and augmenting its activity, thereby providing a mechanism for HDACi based anti-angiogenic activity. Other reports have linked HDAC1 overexpression in tumors to angiogenesis through repression of tumor suppressor genes *p53* and *VHL* (259). In addition to directly inhibiting an interaction between HDAC and HIF-1α, HDACi are capable of modulating downstream signaling pathways such as VEGF. A study of TSA and SAHA showed that treatment of endothelial cells with HDACi reduced invasion, downregulated VEGF-induced expression of VEGF receptors and inhibited VEGF-induced angiogenesis while upregulating the expression of the VEGF competitor semiphaphorin III (260). Other studies have shown that HDACi are capable to reducing VEGF secretion by tumor cells (261). Furthermore, HDACi are capable of repressing the expression of endothelial nitric oxide synthase (eNOS), which is a molecule essential for angiogenic signaling in endothelial cells, an additional mechanism of anti-angiogenic activity (262, 263). The inhibition of angiogenesis does not appear to be HDACi class-specific as numerous studies encompassing all classes of HDACi have reached the same conclusion (264-270).

On of the most commonly reported cell-cycle modulating effects of HDACi is the induction of the cell-cycle checkpoint protein p21 (247, 271-278). The nuclear factor-κB (NF-κB) is a transcription factor that is partly responsible for the control of cell cycle regulating proteins, and shows altered activity dependent upon acetylation status (279-281). The mechanism of p21 induction appears to through the inhibition of NF-κB activity at specific sites within the p21 promoter; Sp1 and/or Sp3 (277, 282). In addition to upregulation of p21, the effect of NF-κB inhibition has also been shown to result in a decrease in cyclin D1, another mechanism for the observed cell cycle arrest seen in

HDACi treated cells (283, 284). Interestingly, the cyclin-dependent kinase inhibitor p19/INK4d, a member of the INK4 gene family which has been associated with osteosarcoma when downregulated, is also induced through an Sp1 site within its promoter following HDACi treatment (285, 286).

An additional mechanism proposed for the specific anti-tumor activity of HDACi is through altered energy metabolism. It is commonly accepted that tumor progression and metastasis are associated with a switch from oxidative-phosphorylation to aerobic glycolysis, a phenomenon referred to as the Warburg effect (287, 288). The net effect of this switch in metabolism is that tumor cells are dependent upon glycolysis as an energy source and as a source of TCA cycle intermediates for production of molecules necessary for rapid growth and proliferation such as nucleic acids, fatty acids, and amino acids. HDACi have been shown to target the glycolytic pathway and result in decreased glucose utilization through downregulation of the glucose transporter GLUT1 and enzymatic inhibition of hexokinase, the first enzyme in the glycolytic pathway (289).

Combination therapy with histone deacetylase inhibitors

With a clearly demonstrated ability to lower the apoptotic threshold in many tumor cells through tipping the balance of apoptotic proteins, combination therapies with HDACi and numerous chemotherapeutic agents as well as ionizing radiation have been evaluated. Part of the rationale for combining HDACi with ionizing radiation comes from the discovery that HDACi can regulate DNA repair through downregulation of genes involved in the DNA damage response such as Ku70, Ku80, Rad50, and DNA ligase IV (290). In fact, HDACi can independently induce the same pathways that are

activated following radiation such as activation of ATM which then leads to phosphorylation and activation of downstream effectors such as BRCA1, CHK2, and γH2AX (290). In addition to altering expression of DNA repair genes, inhibition of HDAC has been shown to increase the acetylation levels of Ku70 which reduces the ability to bind DNA double-strand breaks during non-homologous end joining (NHEJ) leading to prolonged γH2AX foci, a marker of DNA damage (291, 292). Other DNA damage response proteins that are regulated through acetylation include ATM, NBS1 and PARP1 (293-295). HDACi can also alter tumor cell ability to repair DNA damage through the nucleotide excision repair pathway, demonstrated in a study that found a lack of normal repair foci in the removal of interstrand crosslinks and slower kinetics of pyrimidine dimer repair in HDACi treated cells following exposure to UV-A (296). This ability to inhibit DNA damage responses also leads to an enhanced apoptotic response when HDACi are combined with other DNA-damaging agents such as bleomycin, doxorubicin, and etoposide (291, 297-300).

HDACi can also enhance the therapeutic effect of other anti-cancer drugs through modulation of target proteins. For example, acquired resistance to the commonly used drug 5-fluorouracil (5-FU) has been shown to result from an increased expression of the target protein thymidylate synthase (TS); treatment of tumor cells with HDACi results in a reduction of TS mRNA and protein levels through decreased TS transcription and inhibition of TS-HSP90 interaction (126, 301). This activity translates into enhanced apoptotic effect of 5-FU (302-304). The specific timing of HDACi administration in combination with other chemotherapeutic agents has been clearly demonstrated to be essential in obtaining enhanced therapeutic effect. In many cases, chemosensitization

requires pre-treatment with the HDACi allowing for chromatin decondensation and/or changes in target gene expression before exposure to traditional cytotoxic agents (305). Combinations of HDACi and hormonal therapy are also being evaluated because of the ability of HDACi to induce expression of hormone receptors. In breast cancer, the importance of developing strategies to overcome hormone therapy resistance is clear; in many of these tumors estrogen-mediated signaling plays a pivotal role in pro-growth and pro-survival pathways by inducing genes such as IGF-1 and cyclin D1 (306, 307). Thus, targeting the signaling pathways through inhibition of estrogen receptor expression or by blocking estrogen production in peripheral tissues is a meaningful targeted therapy; however, resistance of cells to anti-estrogen therapy is well-documented and correlates with a decrease in the expression of estrogen and progesterone receptors (308, 309). It has been shown that the downregulation of estrogen receptors is, in part, due to recruitment of HDAC1 to the promoter by DNMTs and MBPs (310). Treatment with HDACi eliminates this interaction and, when combined with DNA-methyltrasferase inhibitors, results in re-expression of estrogen receptor and re-establishment of sensitivity to hormone therapy (311, 312).

The essential role of HDAC, particularly HDAC6, in the control of protein degredation has led to the evaluation of combinations involving proteosome inhibitors as well. The ability of tumor cells to respond to accumulations in misfolded proteins is critical in their survival, and this is done through the chaperone function of HSP90 as well as through the formation of aggresomes to remove larger complexes of misfolded proteins that are not degraded through the ubiquitin-proteosome pathway (313). Numerous studies have shown that the combination of HDACi and molecules that

regulate proteosomal degredation pathways can be synergistic (314-318). Additional links between HDACi and the proteosome pathway have been made that implicate the proteosome pathway in regulating the efficacy of HDACi, whereby deletion of specific proteosome components appear to render cells insensitive to HDACi induced apoptosis (319).

Resistance to histone deacetylase inhibitors

The clinical utility of HDACi depends upon rational design of combination therapies as well as an understanding of the mechanisms leading to resistance. Resistance to HDACi therapy has been suggested to arise from several different mechanisms in different cell lines. First, HDACi have been shown to increase the trans-activating potential of the anti-apoptotic transcription factor NF-κB by relieving HDAC-induced transcriptional repression as well as through acetylation of NF-κB leading to upregulation of anti-apoptotic BCL-X_L in non-small cell lung cancer cells (320). This ability to upregulate NF-κB dependent transcription correlated with an ineffectiveness to induce apoptosis. This provides additional rationale for combining HDACi with proteosome inhibitors as one of the actions of proteosome inhibition is to stabilize I-κB which sequesters NF-κB and prevents its movement to the nucleus (321). Another cytoprotective protein, clusterin, has been shown to protect cells against apoptosis and is induced following exposure to cytotoxic chemotherapy and radiation therapy (322). This protein has been shown to be induced following treatment of numerous HDACi and protects cells against the effects of HDACi; down-regulation of clusterin has also been shown to sensitize cells to the apoptotic effects of HDACi (323).

A mechanism for cross-resistance to HDACi and cytotoxic chemotherapy has also been discovered in drug-resistant OS and Ewing's sarcoma, where upregulation of the drug-efflux pump P-glycoprotein also results in resistance to cyclic tetrapeptide HDACi (324). Mutation of HDAC enzymes has also been implicated in resistance to inhibition, particularly in colon cancer where a truncating mutation in HDAC2 results in resistance to HDACi therapy (123).

Clinical studies with histone deacetylase inhibitors

With the wealth of information implicating aberrant HDAC expression or activity in cancer promotion and progression, as well as *in vitro* demonstration of anti-tumor activity as single-agents and in combinations, numerous clinical studies have been undertaken to evaluate the translational relevance of these findings. Thus far, responses to single-agent HDACi therapy have been limited to hematologic malignancies and have led to FDA approval of SAHA (Vorinostat®) for use in relapsed or refractory cutaneous T-cell lymphoma (325). Recently, FK228, or Istodax®, has also been approved as single-agent therapy for CTCL. For the most part, these drugs seem to have a general class toxicity profile that includes gastrointestinal disturbance, myelosuppression and thrombocytopenia, and electrocardiographic disturbances (QT interval prolongation) although the route of administration, oral versus I.V., may also dictate the spectrum of adverse effects (326, 327). Many of the clinical studies undertaken to date have evaluated pharmacodynamic effects (i.e. induction of histone hyperacetylation) of HDACi in normal peripheral blood mononuclear cells (PBMC) and tumor tissues and most have reported that there is no correlation between the level of histone

hyperacetylation and response, although it appears to be a useful biomarker to determine if HDACi are modulating their target (325, 328-334). Response rates in these studies approach 30% or better with generally mild or moderate side-effects. A few correlative studies have identified possible biomarkers predictive of response for select HDACi in select hematologic malignancies; nuclear accumulation of STAT1 and high levels of phosphorylated STAT3, along with elevated expression of ROS scavenger genes and overexpression of p21 and p53-responsive genes in CTCL correlate with a lack of clinical response to vorinistat (244, 335). In addition, clinical responses in Hodgkin's Lymphoma (HL) to vorinostat and the class I-specific HDACi MGCD0103 correlate with decreased expression of thymus and activation regulated chemokine (TARC) (336). The overexpression of MDR1 gene in patients with CTCL and PTCL also correlate with a decreased response to Romidepsin (Istodax)(337). Although promising single-agent anti-tumor activity has been seen in hematologic malignancies, numerous studies have, or are currently, evaluated combinations of HDACi with other targeted agents such as proteosome inhibitors and demethylating agents (338-343).

Responses to single-agent HDACi therapy in solid tumors are much less promising. The documented, and previously discussed, overexpression of some HDAC enzymes in a number of solid tumors along with in vitro evidence of growth arrest and apoptosis induction gave initial hope that single-agent activity would be seen, but that has not been the case. While Vorinostat shows promising anti-tumor activity in MM, leukemias, and some lymphomas, the single-agent responses in solid tumors are much more disappointing with only stable disease being achieved in a percentage of patients with no objective responses in head and neck (H&N), glioblastoma, NSCLC, breast and

prostate cancers (327). In addition, negative clinical trial results have been witnessed for melanoma, GBM, renal, prostate, NSCLC, colorectal, and breast cancers (327, 344). This has not halted the investigation of HDACi in solid tumors because much of the original proof-of-concept information came from studies using HDACi in combination with other cytotoxic or targeted agents and radiotherapy. In fact, some clinical trials in solid tumors using HDACi in combination with cytotoxic chemotherapy have shown promising results.

In one study evaluating Vorinostat in combination with doxorubicin (Topoisomerase II inhibitor), objective responses were seen in breast and prostate as well as melanoma, a tumor not typically sensitive to anthracycline-based therapies (345). In another study evaluating a combination of valproic acid and epirubicin (Topoisomerase II inhibitor) in advanced solid tumors, partial responses were seen in 22% of patients and stable disease or minor responses were seen in 39% of these patients, many of whom were heavily pre-treated prior to this study (346). In addition, enhanced anti-tumor activity was seen in a study evaluating valproic acid in combination with karenetecin (a Topoisomerase I inhibitor) in metatstatic melanoma; an overall response rate of 47% was seen in this study (347). The overlapping roles in chromatin condensation between topoisomerase II and HDAC enzymes as well as the physical interaction between these enzymes may explain the enhanced, often synergistic, anti-tumor activity seen in combination therapies (348, 349). The utility of HDACi and HDACi-based combination therapies has not been evaluated clinically for spontaneously occurring canine tumors, although in-vitro evidence exists that canine tumors are sensitive to the effects of HDACi

Valproic Acid

History and use

Valproic acid $(CH_3CH_2CH_2)_2CHCOOH$, or 2-propylpentanoic acid, was first synthesized in 1882 in a search for organic solvents (350). The first reports of medical use of valproic acid came from studies using it as a solvent for compounds being tested for their anti-epileptic potential that found it was an effective inhibitor of seizures by itself (351). VPA is slightly soluble in water and is easily administered in the form of sodium or magnesium salts because it exists in a dissociated form in water solutions containing alkali metals (352). The anti-epileptic mechanisms of action involve potentiating the inhibitory function of the neurotransmitter gamma-aminobutyric acid (GABA) through increased synthesis, inhibition of degradation, and decreased GABA turnover (353-356). In addition, VPA has anti-epileptic activity through modulation of neuronal firing frequency, attenuation $NMDA^+$-mediated excitation, and blockage of voltage-dependent sodium channels (355, 357). VPA is now an established drug for treating epileptic seizures, mania in bipolar disorder, and prophylactic migraine therapy (353, 358). VPA has well-documented side effects that include a 3-fold increased risk of congenital anomalies such as defects in neural tube closure when administered during early pregnancy (359-361). The teratogenic effect appears to be dependent upon HDAC inhibition activity as structure-function studies of VPA and related derivatives have shown that HDACi-induced activation of the peroxisome proliferators-activated receptors (PPAR) is necessary for teratogenicity (362, 363). In addition to teratogenicity, reported side-effects include dyspepsia, weight gain, dysphoria, fatigue, dizziness, drowsiness, hair loss, headache, nausea, sedation and tremor as well as impaired liver function and

thrombocytopenia which can prolong blood coagulation times (360). Hepatotoxicity is another reported side-effect of VPA administration and, in fact, rare cases of fatal hepatotoxicity have been reported in children under 2 years of age who are being treated with multiple drugs (364). The basis for the observed side-effects may lie in the fact that VPA interferes with important physiologic processes such as oxidative phosphorylation, gluconeogenesis, and fatty acid oxidation (365-368). The effect of hepatotoxicity may be related to VPA induced inhibition of the rate limiting step in mitochondrial β-oxidation, carnitine palmitoyl-transferase 1, and there is evidence that carnitine supplementation may be beneficial in treating cases of acute VPA-induced hepatotoxicity (369, 370). In addition, VPA has been shown to inhibit microsomal CYP2C9 activity and to induce CYP3A4 expression, which may lead to adverse drug interactions (371, 372).

In humans, valproic acid is absorbed rapidly after oral administration with nearly 100% bioavailability, has a small volume of distribution (0.13 to 0.19 L/kg) due to a high degree of ionization at physiologic pH and high degree of protein binding, reaches maximal plasma concentrations within 4 hours, and has a half-life ranging from 7-19 hours (360, 373). VPA is highly protein bound (85-90%) and is metabolized by glucuronidation and complete β-oxidation, with urinary excretion of metabolized drug as the primary route of elimination (360, 374). In addition, VPA exhibits a high degree of CNS penetration following oral administration, reaching levels that are required to inhibit HDAC in *in vivo* orthotopic CNS tumors (375).

Original forumulations of VPA have historically been used in dogs as an anti-epileptic therapy as well, however a relatively short half-life led to the suggestion that VPA would not be a good drug for maintenance therapy (376). In addition, the high

doses of drug required to maintain plasma levels also led to hepatotoxicity in dogs (personal communication with Dr. Doug Thamm). A comparative study utilizing a non-sustained release formulation of VPA has shown that valproate binding to serum proteins was much less in the dog (75%) than in humans, and given the importance of serum binding in metabolism and elimination, this correlated with a much shorter elimination half-life in the dog as only the unbound fraction of valproate is available for elimination (377, 378). However, a study performed on sustained release formulations of VPA in dogs showed a more prolonged and uniform absorption rate, sustained plasma levels, and an overall bioavailability of approximately 84% (379).

The therapeutic potential of VPA in cancer was realized upon the discovery that VPA was capable of inhibiting HDAC, first discovered through an ability to induce Wnt-dependent gene expression leading to the induction of differentiation as well as inhibit N-CoR/HDAC activity (380, 381). Further studies on VPA and structurally related analogs showed that the ability to induce differentiation paralleled the ability to cause histone hyperacetylation, leading to the conclusion that VPA-induced differentiation was due to inhibition of HDAC (382). Original studies had indicated the VPA is predominantly a class I-specific inhibitor of HDAC that, in contrast to other HDACi, was capable of inducing proteosomal degradation of HDAC2 as an additional inhibitory mechanism (383). Initial reports on isoform selectivity suggested that VPA was capable of inhibiting both class I and class IIa HDAC, although it is much more potent against class I enzymes, and had no activity against class IIb HDAC6 or HDAC10 (382). Contrary to early reports, a recent study has demonstrated that VPA is capable of inhibiting HDAC6 enzymatic activity by nearly 50% at 2mM concentration; this was accompanied by

hyperacetylation of tubulin, a target of HDAC6 activity (384). These data suggest that VPA acts more as a pan-inhibitor than a class-specific inhibitor, although much less potent than other pan-inhibitors such as TSA. As discussed earlier, the alterations in gene expression induced upon HDACi treatment are, in part, due to altered chromatin structure resulting from histone tail modifications. In addition to the effect on histone tails, valproic acid has been shown to alter chromatin structure through depletion of proteins that are required to maintain chromatin in a condensed state; the structural maintenance of chromatin (SMC) proteins 1-5, DNMT1, and heterochromatin protein-1 (HP1) (385). The effect of altered SMC proteins was not a direct effect of histone hyperacetylation, but was a downstream event. This finding of VPA-induced reductions in DNMT-1 are supported by an additional study that correlated the upregulation of genes following VPA treatment with promoter demethylation, providing further evidence for the dynamic role between DNA methylation and histone tail modifications (386). Valproic acid has been shown to induce the expression of a variety of genes in various cell systems, and the mechanism has been proposed to involve the interaction of AP-1 transcription factors with AP-1 response elements and/or Sp1 transcription factor interaction at Sp1-binding sites, depending on the promoter region of the particular gene (387-389). Interestingly, the ability of VPA to enhance the interaction of Sp1 with Sp1-binding sites and induce gene expression has been suggested to require the generation of ROS (390).

Valproic acid has been shown to modulate a number of signaling cascades. The inhibition of HDAC3, which is both nuclear and cytoplasmic, has the effect of reducing signaling through the pro-survival Akt pathway by dissociating HDAC3 from a complex containing protein phosphatase 1 (PP1) and allowing PP1 to dephosphorylate Akt (391).

The inhibition of the phosphatidylinositol-3 kinase (PI-3K)/Akt pathway can come about through an additional mechanism as VPA is capable of activating the nuclear receptor peroxisome proliferators-activated receptor gamma (PPARγ) which is involved in regulating the expression of the tumor suppressor, and Akt inhibitor, phosphatase and tensin homolog deleted from chromosome 10 (PTEN) (360). Upregulation of PTEN by HDACi has been reported for TSA as well (392). In addition, VPA has shown an ability to activate the mitogen-activated protein kinase (MAPK) pathway leading to downstream phosphorylation of extracellular-related kinase (ERK), and this activity is enhanced in VPA analoges that have less HDAC inhibitory capacity suggesting that it is a histone acetylation-independent effect (393).

Increased signaling through the Wnt/β-catenin pathway is also an effect of VPA, and elevated levels of β-catenin target genes such as E-cadherin are seen in endometrial cancer cells following VPA exposure (394). This finding provides additional evidence of the link between different types of epigenetic modifications, as loss of E-cadherin, a putative tumor suppressor gene, is linked to promoter hypermethylation and expression is partially restored upon increased histone acetylation (395, 396). In addition to upregulating Wnt-dependent differentiation pathways, VPA has been shown to lead to differentiation of some cell types through activation of Notch1 signaling. Notch1 plays a critical role in embryonic development of lung and the neuroendrocrine system (397) and acts as a tumor suppressor gene as it is found to be suppressed in neuroendocrine tumor (NET) cells including small cell lung cancer (SCLC) (398). Valproic acid has been shown to increase expression of Notch1, upregulate Notch1 signaling, and increase differentation in carcinoid, neuroblastoma, thyroid, and SCLC cells (399-402).

VPA also has anti-angiogenic properties resulting from decreased eNOS expression that was shown to inhibit proliferation, migration, and tube formation of endothelial cells at therapeutic concentrations (265). VPA also leads to reductions in the pro-angiogenic factors VEGF and FGF in colon carcinoma cells (403).

In addition to the effects on hematalogic malignancies previously discussed, VPA has demonstrated anti-tumor activity in a large number of solid tumor model systems particularly when combined with other forms of therapy. In glioma models, VPA was shown to decrease proliferation, increase cell differentiation and adhesiveness, and demonstrated synergistic anti-tumor activity when combined with etoposide (404, 405). VPA was shown to result in differentiation and increased adhesion of neuroblastoma and drug resistant neuroblastoma as well as decrease the expression of the N-myc oncoprotein and cause synergistic growth inhibition when combined with interferon alpha (IFN-α) (406-408). In breast cancer models VPA demonstrates an ability to inhibit proliferation of estrogen-sensitive cell lines through p21 induction and altering apoptotic protein expression in favor of those that promote apoptosis (409). In addition, VPA has been shown to restore estrogen receptor alpha (ERα) and antiestrogen sensitivity to (ERα)-negative cells through upregulation of the ER-related transcription factor FoxA1 (410). In xenograft models of breast cancer, VPA combined with epirubicin was shown to have superior anti-tumor activity through an increased binding of Topo II inhibitors following HDACi-induced chromatin decondensation and recruitment of topoisomerase IIβ (411). In these studies the effect of VPA on the magnitude of histone hyperacetylation in PBMC was correlated with baseline HDAC2 expression. Subsequent clinical studies evaluating VPA and epirubicin in breast cancer patients showed objective responses in 64% of

patients and demonstrated no alterations in epirubicin PK or side effects when VPA was administered for 48 hours prior colon (346, 412). In models of prostate cancer, VPA was shown to induce growth arrest, cell death, and senescence, and reduce angiogenesis in androgen receptor positive and negative cell lines (413, 414) In addition, VPA was able to sensitize androgen-refractory cells to ionizing radiation when combined with 1,25-dihydroxyvitamin D3 (415). VPA has demonstrated anti-tumor activity in models of poorly differentiated and differentiated thyroid cancer cells that involved activation of intrinsic apoptosis pathways, as well as enhancing the cytotoxicity of doxorubicin in poorly differentiated thyroid cells (416, 417). VPA has also been evaluated in melanoma, and when combined with the topoisomerase I-inhibitor kareneticin (KTN) increases the KTN-induced DNA strand breaks leading to enhanced apoptotic effect both in cell lines and xenograft models. In addition, the combination has demonstrated disease stabilization in 47% of patients in a phase I trail of metastatic poor prognosis melanoma (347). This study also showed that VPA did not enhance toxicities associated with KTN administration. VPA has also been evaluated and shown to have promising anti-tumor activity in hepatoma (418, 419), cervical, ovarian, and endometrial cancers (394, 420, 421), neuroectodermal tumors (422), bladder tumors (423), carcinoid (399), fibrosarcoma (424), and colon cancer (425, 426). In addition, VPA has recently been evaluated in skeletal tumor systems, and has shown an ability to enhance the apoptotic effect of doxorubicin in human and canine osteosarcoma tumors *in vitro* and *in vivo* (427). A similar ability to potentiate the anti-tumor effects of doxorubicin on bone sarcoma cells has also recently been reported for the novel HDACi PCI-24781 (428). Additionally, human osteosarcoma cells can be sensitized to the apoptotic effects of the Fas-mediated

extrinsic apoptosis pathway by VPA-induced decreases in soluble Fas secretion which in turn increases the binding of Fas-ligand to Fas receptors (235).

Potential pro-tumor effects of histone deacetylase inhibitors

Although multiple model systems demonstrate anti-tumor activity of HDACi, careful selection of tumor types and further research into HDACi-specific effects in those tumor types is warranted as there are also reports of potential pro-metastatic effects of VPA and other HDACi. One important mediator of tumor invasion and metastasis is the protien urokinase plasminogen activator (uPA) that is partly responsible for degredation of basement membranes allowing for tumor cell invasion (429). A study of the pan-HDACi TSA and Scriptaid showed that HDAC1 is associated with the uPA promoter in a panel of human neuroblastoma and prostate cell lines and that inhibition by TSA or Scriptaid resulted in dissociation of HDAC1 and upregulation of uPA leading to an increase in *in vitro* invasion (430). These results are in contrast to another study that evaluated the invasive potential of the same prostate cancer cell lines treated with VPA that showed no difference in invasion of treated versus control cells, but a decrease in the invasive potential of treated bladder cancer cells (423). Taken together, these results suggest that although both TSA and VPA are capable of inhibiting HDAC1, there are clearly differences in the result of treatment with these two HDACi within a single tumor type, and this may be due to the combined effect on other, non-HDAC1 targets that differ between classes of HDACi.

In addition, VPA has an effect on CXCR4 expression and, as previously mentioned, this molecule has an important role in metastatic progression of OS. Interestingly, the effects of HDACi with VPA on CXCR4 expression appear to be

dependent upon the differentiation status of the cells being treated. In a study of leukemic cells where the CXCR4/CXCL12 axis plays a crucial role in migration, proliferation, and anchorage of AML cells in the bone marrow, VPA treatment resulted in increased CXCR4 expression in more immature, undifferentiated (CD34-positive) cells while conversely resulting in decreased expression in more differentiated (CD34-negative) cells (431). It is not known whether this differential effect on CXCR4 expression correlates to other tumor types. Although the effect of VPA treatment on CXCR4 expression in OS cells has not been evaluated, OS is a tumor that is often characterized by poorly differentiated cells and an increase in CXCR4 expression may lead to an enhanced propensity to metastasize to tissues expressing the ligand. Adding another layer of complexity to the issue, solid tumors are composed of a heterogeneous mixture of cells, presumably in many different stages of differentiation, and the ratio of undifferentiated verses more differentiated cells may dictate the overall response to a particular HDACi, particularly in light of the fact that HDAC isoform expression is dynamic throughout the differentiation process.

In addition, and particularly salient to the treatment of skeletal tumors with HDACi, long-term use of VPA in epileptic patients has been associated with osteopenia or osteoporosis and an increased fracture risk (432-435). The increased fracture risk in patients receiving anti-epileptic drugs including VPA was reported as “limited” and appeared to correlate with the ability of drugs to induce liver enzymes (435). Additional, non HDACi -mediated mechanisms potentially responsible for decreased bone mineral density may relate to certain characteristics of epilepsy such as decreased physical activity or insufficient vitamin D or calcium intake (179, 433). It is important to note that

this was observed in patients on long-term therapy and this particular phenomenon has not been identified with short-term, pulsed use of VPA.

Valproic acid has also been shown to induce *MDR1* gene expression *in vitro* in hepatocyte carcinoma and colon adenocarcinoma cells, resultant from an interaction between VPA and the PXR and CAR ligand-activated nuclear receptors that dimerize with RXRα (372). However, this study used an altered version of the *MDR1* promoter to eliminate the potential interaction with other transcription factors such as Sp1, NF-κB, and NF-Y which are all altered by VPA exposure. This brings into question the clinical relevance of this upregulation as the final effect on *MDR1* expression in a clinical situation is likely to be dependent upon the culmination of the effects on each individual transcription factor and its interaction with the promoter. In addition, the clinical relevance of this study is brought into question in light of the numerous studies already mentioned that have demonstrated an ability of VPA to sensitize tumor cells to P-gp substrates such as doxorubicin.

Pan- versus isoform-specific HDACi: Is more actually better?

Currently, the fundamental question surrounding the clinical use of HDACi for cancer therapy is whether broad-spectrum inhibition, class-specific inhibition, or isoform-specific inhibition provides a better anti-tumor effect or reduced adverse events (436, 437). The emerging evidence suggests that class I HDACs are more clinically relevant to cancer as these are frequently overexpressed in cancer versus normal tissues and this overexpression is generally associated with a poor prognosis; for example the expression of all three in gastric cancer is associated with a decrease in 3-year survival from 48% (all

three negative) to 5% (all three positive) (130). Furthermore, specific knockdown of some class II HDACs (HDACs 4 and 7) in cancer cells do not result in morphologic changes or anti-proliferative effects (438, 439). Also, as previously mentioned, overexpression class II HDACs (specifically HDAC6) has been associated with better prognosis in some tumor types such as breast cancer (169, 170). In situations of combination therapy it also appears that class I HDAC may be the better target as sensitization to TRAIL-mediated apoptosis is dependent upon class I but not class II HDAC (138).

The essential role that HDACi-induced chromatin decondensation plays in providing synergy with DNA-targeting chemotherapeutics appears to be mediated through HDAC2 (412). There are also cases in which targeting of class II enzymes has proven beneficial such as HDAC6 in fusion-protein driven leukemias, and targeting of HDAC4 in combination with ionizing radiation which results in decreased levels of the key signaling protein p53- binding protein 1 (53BP1) involved in the DNA damage response pathway and enhanced sensitivity to the effects of ionizing radiation (440). As previously mentioned, the clinical toxicities from both pan inhibitors and class I specific inhibitors demonstrate similar toxicity profiles such as fatigue, nausea, vomiting, diarrhea, thrombocytopenia, neutropenia, and some cardiac irregularities. It has been proposed that the current group of class I specific inhibitors may not be selective enough to offer an improved therapeutic index of the pan inhibitors and thus targeting of specific HDAC isoforms may be the next necessary step (437). This has proven somewhat difficult because all HDAC isoforms, with the exception of HDAC8, are found in complexes with other proteins in large multisubunit complexes which hinders the ability

to crystallize and elucidate the structure of each individual isoform so that specific inhibitors can be designed (441). Furthermore, the similarity between catalytic sites of all HDACs contributes to the difficulty in designing potent isoform-specific inhibitors, which in turn has hindered the ability to delineate the biology of the individual HDAC isoforms in cancer (442).

However, there are currently a couple of isoform specific inhibitors in development. The fact that HDAC8 crystal structure has been elucidated (146) has allowed for the development of and HDAC8 specific inhibitor that has demonstrated activity in T-cell lymphoma (147). Interestingly, the HDAC8 inhibitor appears to induce apoptosis in T-cell lymphoma cells but does not induce apoptosis in cells where HDAC8 knockdown induces apoptosis, emphasizing the fact that knockdown of HDAC isoforms does not necessarily indicate the effect of inhibiting the enzymatic activity. This may be due to the interaction of HDAC8 with the telomeric protein EST1b that is may be essential for survival in tumor cells but not necessarily dependent upon deacetylase activity of HDAC8 (143). Inhibitors of HDAC8 may prove useful clinically in pediatric neuroblastoma where it is the only HDAC shown to be overexpressed and is strongly correlated with a poor prognosis (144). Another class specific inhibitor is the compound tubacin which selectively inhibits HDAC6. This compound is not in clinical development but has shown an ability to synergize with other chemotherapeutics in multiple myeloma, although it does not appear to directly induce apoptosis (443).

Despite the clinical promise of HDACi for neoplastic diseases, the toxicity profile of the current pan- and class-specific inhibitors clearly leaves room for improvement of the therapeutic index. The design of isoform-specific inhibitors may be one such avenue

for improvement that will not only allow for improved therapeutic index, but will help to elucidate the cancer specific biology of individual HDAC isoforms and contribute to a greater understanding of the epigenetic mechanisms involved in cancer initiation and progression, as well as provide essential information to make better informed decisions on the design of rational combinatorial therapeutics.

PROJECT RATIONALE

The potential of HDAC inhibitors in the treatment of cancer has only recently been recognized. Although a multitude of studies have examined the effects of HDACi in cancer cells *in vitro*, the exact mechanisms of action in all tumor types have not been fully elucidated. Nevertheless, these agents are rapidly being entered into clinical trials in human medicine. While there have been some promising reports of single-agent activity in specific forms of cancer, their full potential as anti-cancer agents is most likely to come from combination with other forms of therapy. It has been demonstrated that the current drug development pipeline for novel cancer therapies oftentimes fails to correctly identify the agents that will lead to improved survival times in humans, and the use of spontaneously occurring tumors in canines has been proposed as a model that can bridge the gap between mouse and human studies to improve drug discovery (444-446). Very few studies have evaluated the efficacy of these compounds in canine tumors and there are no published reports on the clinical safety or efficacy of these compounds in veterinary medicine. With this in mind, the basis of this dissertation is to determine the potential of combining a commonly used chemotherapeutic drug used in veterinary medicine for the treatment of OS (DOX) with a readily available HDACi that already has

a history of use in veterinary medicine, albeit for a different purpose. We hypothesized that, similar to what has been shown in human cancer cell lines, the addition of VPA to a DNA-targeting agent would show superior anti-tumor activity in canine cancer cells. This hypothesis is tested in **Chapter 2 (The Histone Deacetylase Inhibitor Valproic Acid Sensitizes Human and Canine Osteosarcoma to Doxorubicin)**. This chapter describes the *in vitro* effects on target modulation evaluated by western blot and immunofluorescence microscopy for acetylated histone H3, proliferation by fluorometric bioreductive and clonogenic assays, and apoptosis by activated caspase 3 and flow cytometry-based Annexin V staining for the combination treatment of VPA and DOX. It explains how the correct dosing schedule can have an impact on maximizing the anti-tumor effect and furthermore, describes a xenograft model of OS in nude mice used to gain preliminary information on antitumor activity *in vivo* using a treatment schedule that would recapitulate what might be seen in a clinical setting; that is, a treatment cycle that includes pre-treatment with VPA followed by DOX that is then repeated two weeks later.

Based upon the promising results obtained from the experiments in Chapter 2, we next wanted to test the clinical safety of this combination in dogs with spontaneously occurring tumors. Although previous formulations of VPA administered in dogs have been reported to cause hepatotoxicity at doses required for seizure control, we hypothesized that a sustained release form of VPA might be given safely in a pulse-dosing regimen that would achieve plasma concentrations sufficient to inhibit HDAC. The basis of pulse-dosing is that higher doses might be safely administered as a result of the washout period between treatment cycles. In addition, we wanted to ensure that the addition of VPA to DOX therapy would not result in potentiation of DOX-induced

adverse effects such as myelosuppression or GI toxicity. This hypothesis is tested in **Chapter 3 (Phase I Pharmacokinetic and Pharmacodynamic Evaluation of Combined Valproic Acid/Doxorubicin Treatment in Dogs with Spontaneous Cancer)**. This chapter explains the patient recruitment into a phase I dose-escalation trial aimed at determining a maximum tolerated dose of VPA that can be safely administered to dogs for 48 hours prior to a standard dose of DOX. We demonstrate that the addition of VPA to DOX chemotherapy protocols is well tolerated in dogs and in fact, we failed to reach a dose that was toxic before owner compliance became a limiting factor in further dose-escalation. In addition, we demonstrate that pre-treatment of dogs with VPA does not significantly alter DOX pharmacokinetics through determination of plasma concentrations of DOX and the major metabolite, DOXOL, by LC/MS/MS and comparing to previously reported values for dogs receiving DOX alone. In addition we show that VPA pre-treatment does not result in myelosuppression nor potentiate DOX-induced myelosuppression by correlating VPA dose with post-VPA and post-DOX white blood cell counts. Interestingly, we also show that dosing to toxicity may not improve chances of gaining a therapeutic effect as target modulation in PBMC and tumor tissues, evaluated by a combination of immunohistochemistry, western blot, and immunofluorescence cytochemistry, was seen across the range of doses administered. We next wanted to better understand the mechanisms by which VPA exerted its anti-tumor effect in OS through gene expression microarray followed by pathway analysis. We hypothesized that not only would we find alterations in genes involved in cell cycle control and apoptosis, based upon the results from Chapter 2, but that we might also find potential biomarkers that could potentially be used for determining which tumors might

respond favorable to HDACi-containing combination protocols and/or determine additional pathways that might be targeted along with HDACi to provide synergistic results.

While there are reports of microarray experiments performed on HDACi treated cells, they often focus on the identification of individual, differentially expressed genes and not pathways. One pitfall of this type of analysis is that the genes showing the largest change in expression are often those that are expressed at low levels prior to treatment, making them poor choices for biomarkers since they may not be easily identified in a repeatable fashion. **Chapter 4 (A Systems Biology Approach to Identify Molecular Pathways Affected by HDAC Inhibition in Osteosarcoma)** addresses this issue and describes the findings of the pathway analysis following a 48 hour treatment of canine OS cells with a clinically achievable dose of VPA. The candidate genes in our analysis were chosen based on moderate or high levels of expression in addition to being differentially expressed. We found that among the pathways most affected by VPA treatment in OS, the oxidative phosphorylation pathway was most significantly overrepresented. This pathway appears to be upregulated in response to VPA treatment in these cells, which has important implications as decreased oxidative phosphorylation has been associated with more aggressive tumors, metastasis, and resistance to apoptosis. In addition we identified cell cycle control, ubiquitin proteosome pathway, cytoskeleton and cell adhesion, and ubiquinone metabolism as being pathways that are altered by VPA. We confirmed these results by a combination of independent methods including evaluation of chymotrypsin-like activity in treated cells, NQO1 enzymatic activity, and

quantitative real time RT-PCR performed on genes selected from within the altered pathways.

This dissertation has two main overarching goals: the first is to determine the efficacy and safety of combining HDACi with conventional chemotherapy in dogs with spontaneously occurring cancer in an effort to improve anti-tumor responses and long-term outcome of these patients utilizing a combination of drugs that are readily available to general practitioners and, if proven efficacious, could be implemented relatively easily into clinical practice. The second goal is to demonstrate the similarity in the molecular responses to HDAC inhibition with VPA between canine and human OS and to develop the necessary tools for rigorous clinical evaluation of HDACi-containing chemotherapy protocols in dogs that might lead to better informed decisions on drug development and human clinical trials.

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Chapter 2

The Histone Deacetylase Inhibitor Valproic Acid Sensitizes Human and Canine Osteosarcoma to Doxorubicin

ABSTRACT

Osteosarcoma (OS) remains an incurable and ultimately fatal disease in many patients, and novel forms of therapy are needed. Improved models of OS that more closely mimic human disease would provide more robust information regarding the utility of novel therapies. Spontaneous OS in dogs may provide such a model. Pharmacologic inhibition of histone deacetylase (HDAC) enzymes has a variety of anti-tumor effects, but may demonstrate the most utility when utilized in combination with standard cytotoxic therapies. We sought to determine the *in vitro* and *in vivo* effects of the HDAC inhibitor valproic acid (VPA) on doxorubicin (DOX) sensitivity in canine and human OS. We evaluated the *in vitro* anti-proliferative and apoptotic effects of VPA/DOX combination treatment, alterations in histone acetylation and nuclear DOX accumulation resulting from VPA treatment, and the *in vivo* efficacy of combination therapy in a xenograft model. Treatment of canine and human OS cell lines with clinically achievable VPA concentrations resulted in increased histone acetylation but only modest anti-proliferative effects. Pre-incubation with VPA followed by doxorubicin

(DOX) resulted in significant growth inhibition and potentiation of apoptosis, associated with a dose-dependent increase in nuclear DOX accumulation. The combination of VPA and DOX was superior to either monotherapy in a canine OS xenograft model. These results demonstrate a rationale for the addition of HDAC inhibitors to current protocols for the treatment of OS, and illustrate the similarities in response to HDAC inhibitors between human and canine OS, lending further credibility to the canine OS model.

INTRODUCTION

A multitude of pathways have been identified as targets of aberrant gene silencing via epigenetic mechanisms, including cell cycle control, apoptosis, developmental and differentiation pathways, DNA damage repair, and cell adhesion and migration (1). Post-translational modification, including acetylation, of core histone proteins has been shown to be a major determinant of chromatin structure, thereby serving as a primary regulator of gene transcription (2). Histone acetylation is dependent upon the balance between enzymes with histone acetyltransferase (HAT) activity and those with histone deacetylase (HDAC) activity. Altered expression of genes that encode the HAT and HDAC enzymes or their binding partners has been clearly linked to carcinogenesis (3-6). Moreover, aberrant expression of HDAC enzymes has been linked to prognosis in a variety of cancers (4, 7, 8). Combination therapies utilizing HDAC inhibitors and conventional cytotoxic drugs have shown superior *in vitro* efficacy versus monotherapy in a variety of tumor types (9-13). In the case of agents that directly interact with DNA, the conformational changes in chromatin resulting from exposure to HDAC inhibitors may be partially responsible for enhancing antitumor effects (14).

Valproic acid (VPA) is a short chain fatty acid historically used for the treatment of epilepsy and bipolar disorder, and can have antineoplastic effects through inhibition of HDAC at low millimolar concentrations (15). While much of the initial work with VPA as a cancer therapy was performed on hematologic disorders such as acute myelogenous leukemia and myelodysplastic syndrome, recent evidence has shown efficacy in a number of solid malignancies, particularly when used in combination with demethylating agents, cytotoxic chemotherapy, and radiation therapy (10, 11, 13, 14, 16, 17). Recent studies on the effect of HDAC inhibition in OS have found an increased sensitivity to Fas-mediated cell death occuring through downregulation of Fas-inhibitory molecules and/or increased expression of Fas-ligand (18-20). In addition, other reports have documented the ability of various HDAC inhibitors to induce apoptosis in a caspase-dependent manner in OS cell lines (21, 22).

Osteosarcoma (OS) is the most common primary bone cancer in humans, primarily affecting pediatric patients. It typically demonstrates invasive and rapid growth with frequent occurrence of pulmonary metastasis. Current combinatorial therapies include surgery and multimodal chemotherapy, and a clear correlation between histologic necrosis following neoadjuvant chemotherapy and survival has been documented (23). While cure rates approach 65% for patients with localized disease, those presenting with metastasis have a worse prognosis, and no significant improvements in survival for these patients have been achieved in the past two decades (24).

The dog is an excellent translational model for the investigation of novel antineoplastic therapies. Unlike murine models, dogs are relatively outbred, immunocompetent animals with spontaneously occurring tumors experiencing

spontaneous metastasis and therapy resistance, representing a spectrum of tumor histotypes that have biology similar to that found in humans. The relatively large size of canine tumors, when compared with murine tumors, more closely approximates human solid tumors with respect to important biological factors such as hypoxia and clonal variation, and allows for multiple samplings of tumor tissue over time. The relatively rapid time course of disease progression, when compared with human cancer, allows for more rapid assessment of therapeutic endpoints than is possible in many human clinical trials (25, 26).

We hypothesized that treatment of canine and human OS cells with clinically achievable concentrations of VPA prior to DOX treatment would yield superior antitumor effects compared to DOX alone. Our results demonstrate that pretreatment of OS cells with VPA leads to decreased proliferation and increased apoptosis *in vitro* and an improved antitumor effect in an *in vivo* xenograft model, providing a rationale for further investigation into combination therapies involving HDAC inhibitors in the treatment of OS in humans, and in dogs as a preclinical model.

MATERIALS AND METHODS

Cell lines and conditions

The D17 canine OS cell line and the SAOS-2 human OS cell lines were purchased from the American Type Culture Collection (Rockville, MD). The Abrams canine OS cell line was kindly provided by Dr. William Dernell (Colorado State University, Fort Collins, CO). The SJSA1 human OS cells were kindly provided by Dr. Lia Gore (University of Colorado Health Sciences Center, Denver, CO). Species authentication of

canine cell lines was performed by evaluation of prepared metaphase spreads. Cells were serially passaged by trypsinization in C/10 [Minimum Essential Medium Eagle (Lonza, Walkersville, MD) supplemented with 1X MEM vitamin solution (Cellgro, Henderson, VA), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate (Cellgro), 1X non-essential amino acid solution (Cellgro), 1X antibiotic/antimycoticsolution (Cellgro), and 10% heat inactivated fetal bovine serum (FBS) (Atlas, Fort Collins, CO)]. For experimental procedures, cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Chemicals and antibodies

Valproic acid was purchased from Sigma (St. Louis, MO) and dissolved in tissue culture medium immediately prior to use. Anti acetyl-histone H3 and total histone H3 antibodies were purchased from Upstate Biotechnology (Waltham, MA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was purchased from Pierce (Rockford, IL). Doxorubicin (DOX) was purchased from Bedford Laboratories (Bedford, OH).

Growth inhibition

For single agent VPA evaluation, cells were plated in C/10 at $2x10^3$ per well in 96-well plates and allowed to adhere overnight. The following day, the plates were washed and the media replaced with C/10 containing increasing concentrations of VPA (0 to 10 mM). After 48 hours, relative viable cell number was determined using a bioreductive fluorometric assay (Alamar Blue™, Promega, Madison, WI) according to manufacturer directions. Fluorescence was determined using a microplate reader

(Synergy™ HT, Bio-Tek, Winooski, VT) with excitation/emission spectra of 530 and 590 nm, respectively. For combination DOX/VPA assays, cells were either co-incubated in 0, 0.5, or 1 mM VPA and increasing concentrations of DOX (0 to 1000 ng/mL Abrams, D17, SAOS2; 0 to 3000ng/mL SJSA1), or pre-incubated for 48 hours in VPA, followed by a 48 hour dose of DOX. Relative viable cell numbers were determined as above. For the clonogenic assay, Abrams cells were pre-incubated in 0, 0.5, or 1 mM VPA for 48 hours, and incubated in media containing 0 or 20 ng/mL DOX for 24 hours. Cells were then trypsinized and washed followed by plating in single cell suspension into 6 well plates in drug free C/10. After 7 days, colonies were stained with crystal violet and counted.

Apoptosis

Caspase activity: Apoptosis of OS cells was evaluated using the SensoLyte® Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA) according to manufacturer's directions. Briefly, canine and human OS cells were incubated in 0, 0.5, or 1.0 mM VPA for 48 hours prior to the addition of 0 or 100 ng/mL DOX (200 ng/mL for SJSA1) for an additional 48 hours. Cells were lysed in 1X lysis buffer (AnaSpec) and transferred to 1.5 mL Eppendorf tubes. Tubes containing lysates were placed on a rotating apparatus at 4°C for 30 minutes. Lysates were then centrifuged at 2500 x g for 10 minutes at 4°C. Supernatants were collected and 60 uL was added to wells of a 384 well plate, followed by 20 uL of Caspase 3/7 substrate solution (AnaSpec). Reagents were mixed by shaking on a plate shaker for 60 minutes at 200 rpm. Fluorescence was

determined at 360/460 nm and results were reported as relative fluorescence units for each treatment condition.

Annexin V/Propidium Iodide staining: Apoptosis results from caspase 3/7 activity assay were validated with a flow cytometry based assay. Treatment conditions were identical to those listed for the caspase assay. After incubation, cells were harvested by trypsinization and washed three times in PBS. Apoptosis was then determined using the BD Pharmingen™ Annexin V-FITC Apoptosis Detection Kit 1 (BD Biosciences, San Diego, CA) according to manufacturers recommendations. Results were analyzed using Summit v4.3.02 software (Beckman Coulter, Inc., Fullerton, CA).

In vitro histone acetylation

Western analysis: Human and canine OS cells were incubated in 0, 0.5, or 1.0 mM VPA for 48 hours and then harvested by trypsinization. Cells were added to lysis buffer [M-PER Protein Extraction Reagent (Pierce), 1 mM $NaVO_4$, 1 mM PMSF, Complete Mini protease inhibitor tablet (Roche, Indianapolis, IN), and 1% SDS], transferred to 1.5 mL microfuge tubes and passed through a 25 gauge needle 7-10 times before centrifugation at 10,000 x g for 10 min. Supernatants were transferred to new 1.5 mL tubes and protein concentration was determined via BCA assay (Pierce). Lysates were loaded into a denaturing 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA) and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. After three washes in TBST (40 mM Tris-HCl, pH 7.6, 300 mM NaCl, and 0.5% Tween-20), membranes were blocked with 5% non-fat dry milk in TBST and incubated in a 1:4000 dilution of rabbit polyclonal anti acetyl-H3 in blocking solution overnight at 4°C. After three

washes in TBST, membranes were incubated in a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG for 1.5 hours at room temperature. Immunoreactive proteins were detected using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and analyzed by autoradiography. Densitometry was performed using Image J software freely available online (NIH).

Doxorubicin accumulation

Abrams OS cells were plated in 150 cm^2 culture flasks and treated with 0, 0.5 or 1 mM VPA for 48 hours, followed by a 4-hour incubation in 20 ng/mL DOX. Cells were washed, harvested by trypsinization, and placed into lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl, 10 mM KCl, 0.5 mM DTT, 2 mM $NaVO_4$, 5 mM NaF, 0.2 mM PMSF, 0.1% nonidet-P40 and protease inhibitor cocktail tablet) at $2.5x10^7$ cells/mL in 1.5 mL microfuge tubes. Cells were centrifuged at 16,000 x g for 5 minutes at 4ºC. Cytosolic supernatants were removed and the nuclear pellet was solubilized in lysis buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl, 420 mM NaCl, 0.5 mM DTT, 2 mM $NaVO_4$, 5 mM NaF, 0.2 mM PMSF, 25% glycerol and protease inhibitor cocktail tablet) at 1 x 10^8 cells/mL in 1.5 mL tubes. Tubes were vortexed for 10 seconds and incubated on ice for 20 minutes, followed by centrifugation at 16,000 x g for 5 minutes at 4ºC. Supernatants were aliquotted into fresh ice-cold tubes and stored at -80ºC. Protein concentration of the nuclear extracts was determined using the Qubit Fluorometer (Invitrogen, Carlsbad, CA). Fifty μg of protein from each treatment condition was added, in triplicate, to a 96 well-plate and fluorescence intensity of DOX was assessed (excitation 480/20 nm, emission 590/30 nm).

Animal studies

All animal experiments were performed under an Institutional Animal Care and Use Committee-approved protocol and institutional guidelines for animal welfare. Four- to 6-week old *nu/nu* athymic male mice were obtained from Harlan Laboratories (Indianapolis, IN) and housed in ventilated caging. After a one-week acclimatization period, Abrams canine OS cells ($1x10^6$ in 0.9% NaCl) were injected s.c. on the right flank. Seven days after tumor inoculation, tumors were size-matched, divided into 4 groups and treated in the following cohorts: saline only, VPA only, DOX only, and VPA followed by DOX. Mice receiving VPA were injected with 500 mg/kg VPA in 0.1 mL saline i.p. twice daily for 5 doses. Doxorubicin (3 mg/kg in .05 mL saline) was injected intravenously by tail-vein once after the last dose of VPA. These treatments were repeated 2 weeks later. Tumor growth (8 mice per cohort) was monitored three times per week by measuring two perpendicular tumor diameters with a caliper. Tumor volumes were calculated as $V = (L \times W^2) / 2$. Animals were sacrificed when the largest tumor diameter reached 10 mm or when the tumor became ulcerated. In a separate experiment, groups of mice (n=3) bearing established Abrams OS tumors were treated as above and sacrificed 48 hours following DOX treatment. Tumors were removed, placed in formalin and paraffin embedded for immunohistochemistry (IHC).

Immunohistochemistry

Proliferation*:* Tissue samples were cut in 5 μm sections and mounted onto positively charged slides. Sections were deparaffinized in xylene, followed by

rehydration in graded ethanol to water. Intrinsic peroxidase activity was blocked with 3% H_20_2 in methanol for 15 minutes at room temperature. The sections were then incubated in monoclonal mouse anti-human Ki67 antibody clone MIB-1 (Dako, Carpinteria, CA) at 1:50, overnight at 4°C. Antibody binding was detected with a goat anti-mouse HRP antibody (Pierce) at 1:250 for 1.5 hours at room temperature. Immunoreactive complexes were detected using diaminobenzidine (DAB) (Vector Labs), lightly counterstained with hematoxylin, and examined under light microscope. Canine lymph node was used as a control tissue. Images were obtained using a Zeiss Axioplan 2 microscope coupled with a Zeiss AxioCam HRc camera and results were calculated by counting the number of Ki67 positive nuclei per 20x field in 7 random fields per tissue section.

Histone acetylation*:* Deparaffinized sections of VPA treated and control tumors were stained for histone H3 acetylation after antigen retrieval using DakoCytomation Target Retrieval Solution pH9 (Dako). Prepared sections were incubated with anti AcH3 at 1:50 overnight at 4°C followed by goat anti-rabbit HRP at 1:250 for 1.5 hrs at room temperature followed by DAB staining and hematoxylin counterstain.

Apoptosis*:* Slides were deparaffinized as described above and TUNEL staining was performed after antigen retrieval (low-pH) using DakoCytomation Target Retrieval Solution Citrate pH 6 (Dako). Slides were stained using the In Situ Cell Death Detection Kit (Roche, Indianapolis, IN) per manufacturer's recommendations and mounted using VectaShield plus DAPI® (Vector Labs). Images were obtained and results were reported as the number of dual-positive cells (TUNEL and DAPI) per 40x field.

Statistical analysis

In order to determine whether the addition of VPA to cytotoxic chemotherapy synergistically enhanced antiproliferative activity, the Bliss independence model was utilized. Briefly, the Bliss criterion is described by the following equation:

$$E(x,y) = E(x) + E(y) – E(x) * E(y)$$

where E(x) is the fractional inhibition of concentration x of VPA (between 0 and 1), E(y) is the fractional inhibition of concentration y of chemotherapy, and E(x,y) is the combined effect. Theoretical growth inhibition curves were constructed using this equation, and standard deviations were estimated by error propagation of experimental SD. Differences between treatment groups (Bliss theoretical vs. experimental) were assessed using 2-way ANOVA and a Bonferroni post-test. Using this model, if the experimental fractional inhibition across concentration was significantly higher than the theoretical value, the interaction was considered synergistic. Comparison of tumor volumes among groups in the xenograft study was done by two-way ANOVA. Survival curves, with maximal tumor diameter (10 mm) or tumor ulceration as endpoints, were generated using the Kaplan-Meier method, and differences between treatment groups compared using log-rank (Mantel-Cox) analysis. Ki67 and TUNEL immunoreactivity was compared between groups using one-way ANOVA with Bonferroni multiple comparison test. *In vitro* apoptotic values were compared using one-way ANOVA. Statistical analysis was performed using GraphPad Prism® (GraphPad Software, La Jolla, CA). For all comparisons, a P-value less than .05 was considered significant.

RESULTS

Valproic acid enhances osteosarcoma chemosensitivity. We first evaluated the antiproliferative effect of increasing concentrations of VPA as a single agent against human and canine OS cells. In all four cell lines, VPA was able to significantly reduce proliferation at suprapharmacologic concentrations (10 mM). However, there was only a modest anti-proliferative effect in the dose range that is considered clinically achievable (0.1 to 2 mM) **(Figure 2.1)**. We next evaluated the antiproliferative effect of VPA when combined with DOX, an intercalating agent belonging to the anthracycline class commonly utilized in the treatment of canine and human OS. While co-incubation of VPA and DOX led to measurable chemosensitization, pre-incubation with VPA for 48 hours followed by exposure to DOX clearly led to superior chemosensitization (**Table 2.1**). Proliferation results were confirmed by clonogenic assay in the Abrams cell line **(Figure 2.2)**.

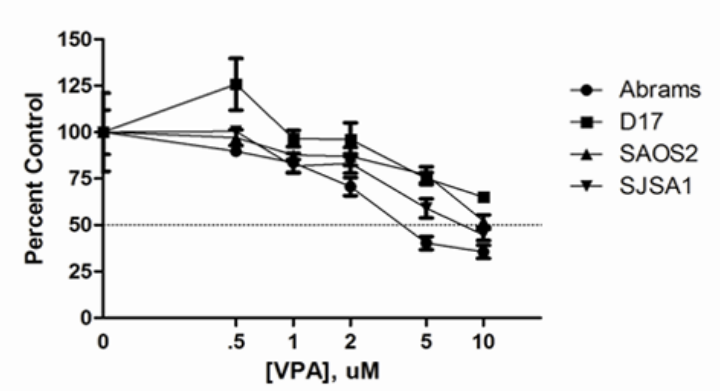

Figure 2.1 Cell viability of canine and human OS cells treated with single-agent VPA. Cells were incubated in VPA ranging from 0 to 10 mM and relative viable cell numbers determined by Alamar blue reduction with fluorescence detection. While suprapharmacologic doses of VPA resulted in reduced viability, only a modest anti-proliferative effect is seen when cells are exposed to clinically achievable concentrations (up to 2mM).

	Co-incubation			Pre-incubation		
[VPA], mM	**0**	**0.5**	**1.0**	**0**	**0.5**	**1.0**
Abrams	60	24.3	13	74.6	26.1	10.3*
D17	17	10	9	758	193*	66*
SAOS2	225.5	117.4*	120*	111	64.7*	53.4*
SJSA1	104	109.7	77.06	271.3	134.3*	92.4*

Table 1: Summary of DOX IC_{50} values for cell growth inhibition in ng/mL.

Cells were plated in 96 well plates and treated with DOX ranging from 0-1,000ng/mL (Abrams, D17, SAOS2) or 0-3,000 ng/mL (SJSA1). Relative viable cell numbers were determined by Alamar blue reduction and fluorescence detection. For co-incubation, cells were treated for 48 hours with VPA and DOX. For pre-incubation, cells were treated with VPA for 48 hours prior to 48 hour incubation in VPA and DOX. There are larger reductions in IC_{50} with VPA pre-incubation than co-incubation, with synergism seen only after pre-incubation in three of four cell lines. * indicates synergistic interaction ($P<.05$) determined by Bliss analysis as described in Methods.

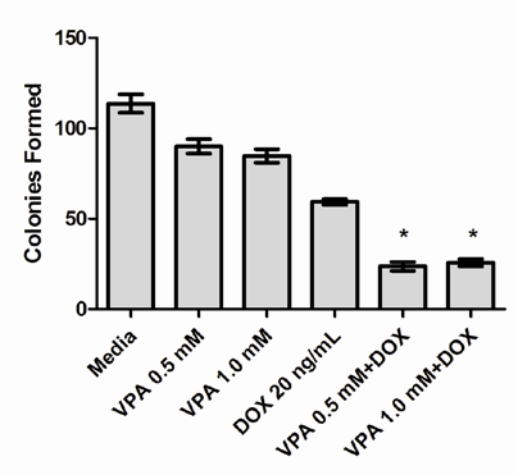

Figure 2.2 Clonogenic assays on Abrams OS cells incubated in 0, 0.5, or 1.0 mM VPA for 48 hours followed by a 24 hour exposure to VPA plus DOX (20 ng/mL). In accordance with the cell viability assays, VPA pre-treated cells have reduced colony formation ability.

HDAC inhibition enhances doxorubicin-induced apoptosis. The bioreductive methodology applied above cannot distinguish between growth arrest and cell death. To determine whether the combination treatment was inducing apoptosis, all four cell lines were pre-incubated in 0, 0.5 or 1.0 mM VPA for 48 hours followed by 48 hour exposure to DOX (100 ng/mL) and VPA, followed by apoptosis assessment by caspase 3/7 activity. An increase in apoptosis was seen in the cells pre-treated with VPA compared to DOX alone in all four cell lines (**Figure 2.3)**. These results were confirmed by apoptosis evaluation via AnnexinV/Propidium iodide staining and flow cytometry **(Figure 2.4)**.

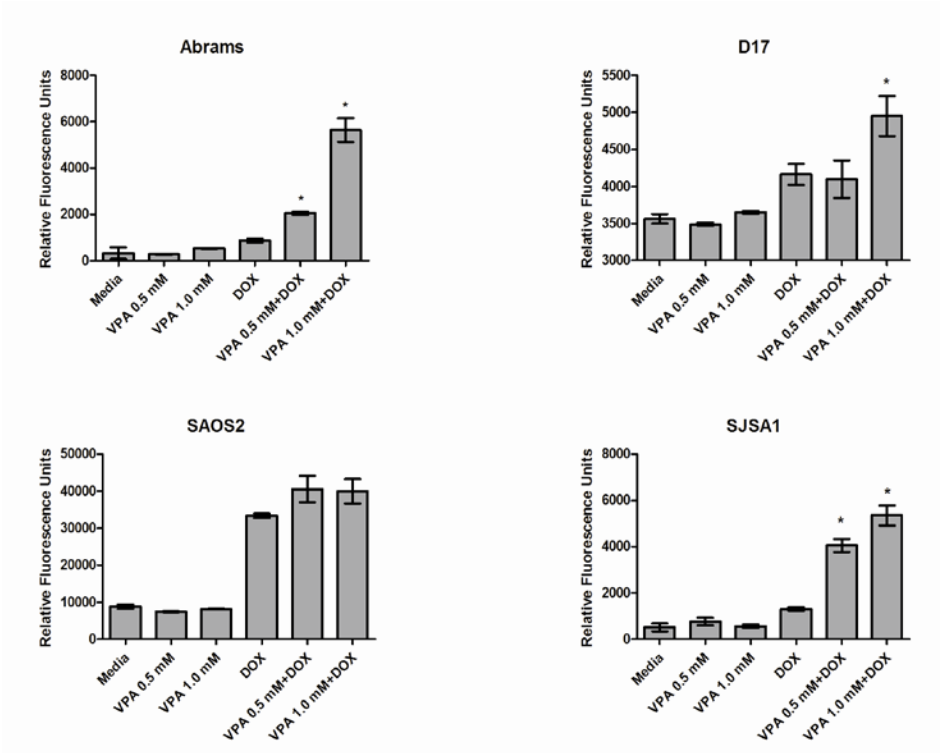

Figure 2.3 Evaluation of *in vitro* apoptosis in canine and human OS. Cells were pre-incubated in VPA for 48 hours followed by 48 hours of combination treatment using 100 ng/mL DOX (Abrams, D17, SAOS2) or 200 ng/mL DOX (SJSA1). Pre treatment with VPA results in enhanced apoptotic effect compared to DOX alone. * P<.05

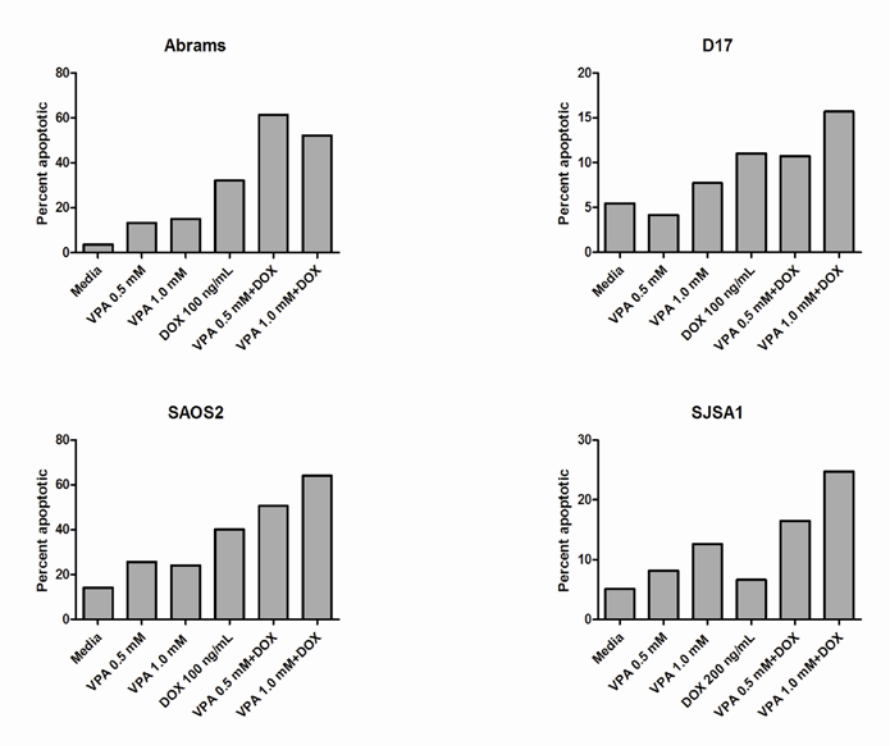

Figure 2.4 Flow cytometric evaluation of apoptosis in OS cells pre-treated for 48 hours with 0, 0.5mM, or 1.0 mM VPA and then exposed to VPA plus DOX for an additional 48 hours. Bars indicate the total number of apoptotic cells (i.e. Annexin-V positive plus Annexin- V/propidium iodide double positive). In accordance with the caspase activity assay, an increase in total apoptotic cells is seen with VPA pre-treatment compared to cells treated with DOX alone.

VPA increases histone acetylation in canine and human OS. We next compared the ability of VPA to induce histone hyperacetylation in human and canine OS cell lines treated *in vitro*. For tumor cell lines, 48 hour incubation in 0, 0.5, or 1 mM VPA was followed by cell lysis and western analysis for acetylated histones H3, using total H3 as a loading control. A dose dependent increase in acetylated histones was observed in VPA treated canine and human OS cells (**Figure 2.5)**. These results demonstrate that VPA effectively hyperacetylates histones in both canine and human OS, at concentrations that are clinically achievable.

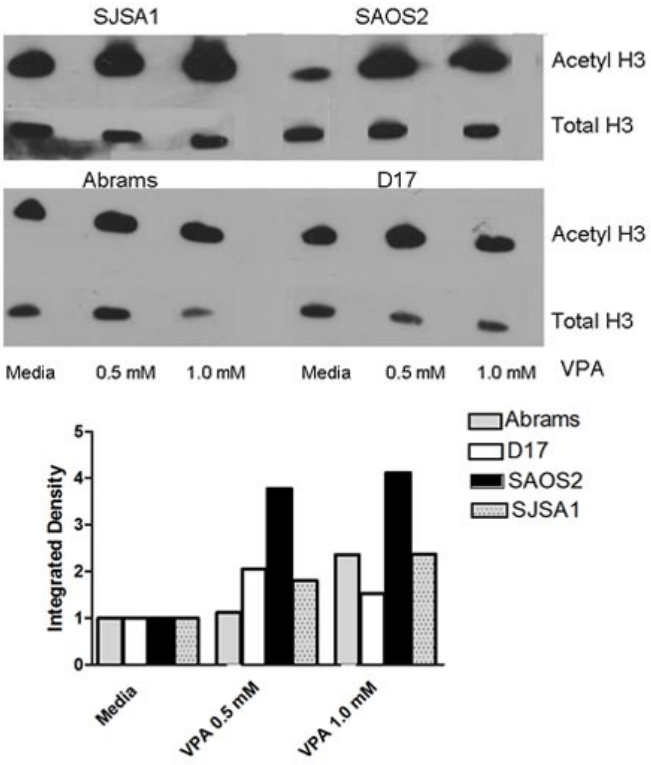

Figure 2.5 Western blot analysis of acetylated histones in human and canine OS cells treated with 0, 0.5, or 1.0 mM VPA for 48 hours. Densitometry normalized to total histone H3 reveals a dose dependent increase in acetylated histones with VPA treatment.

Valproic acid enhances nuclear doxorubicin accumulation. To address whether HDAC inhibition and the resultant alteration in chromatin structure might increase access of DNA intercalating molecules leading to enhanced activity, we next evaluated whether the VPA-induced sensitization of OS cells to DOX was associated with an increased nuclear accumulation of DOX. We measured the nuclear accumulation of DOX in VPA pre-treated Abrams OS cells after a 4-hour DOX exposure. Nuclei were extracted and DOX fluorescence measured and standardized to protein concentration as described above in Methods. The VPA pre-treatment resulted in a significant and dose-dependent

increase in nuclear DOX accumulation **(Figure 2.6)**, presumably as a result of chromatin decondensation secondary to histone hyperacetylation.

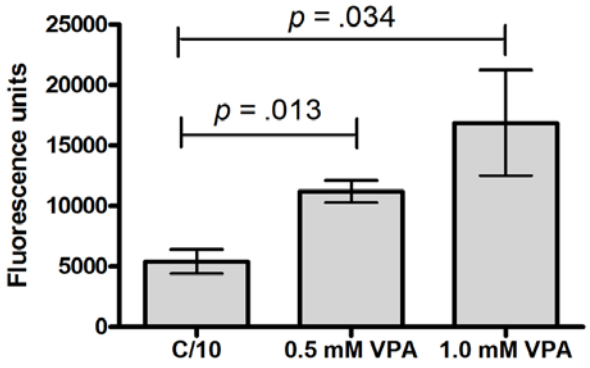

Figure 2.6 Evaluation of nuclear DOX accumulation. Abrams OS cells were incubated with 0, 0.5, or 1 mM VPA for 48 hours and then exposed to a 4 hour pulse of DOX. Fluorescence of nuclear extracts at 485/595 nM indicates VPA pre-exposure increases nuclear accumulation of DOX.

In vivo evaluation of VPA-DOX combination therapy. Based on the promising *in vitro* results obtained when combining VPA and DOX, we next evaluated the *in vivo* efficacy in a canine OS subcutaneous xenograft model. Mice bearing established subcutaneous Abrams canine OS tumors were treated with VPA alone, DOX alone, VPA followed by DOX, or saline. VPA treatment was initiated 48 hours prior to DOX administration in the combination group and the treatment cycles were given on days 7-9 and 21-23. Mice were weighed and monitored for treatment related morbidity during the entire study. Combination treated mice exhibited moderate weight loss (less than 10%) during treatment but regained weight between treatment cycles (data not shown). Tumor volumes from the VPA alone treatment were similar to saline-treated animals, indicating that short-term single agent therapy with VPA is not effective in this model. Doxorubicin treated mice exhibited a modest reduction in tumor growth, while the combination treated

mice had a significantly reduced tumor growth ($P < .05$) when compared to all other treatment groups **(Figure 2.7)**. This translated into a significant increase in survival, compared to all other groups, when growth to 10 mm or ulceration was used as the endpoint **(Figure 2.7)**. These results suggest that the addition of VPA to standard chemotherapy for OS can improve the anti-tumor effects of cytotoxic chemotherapy.

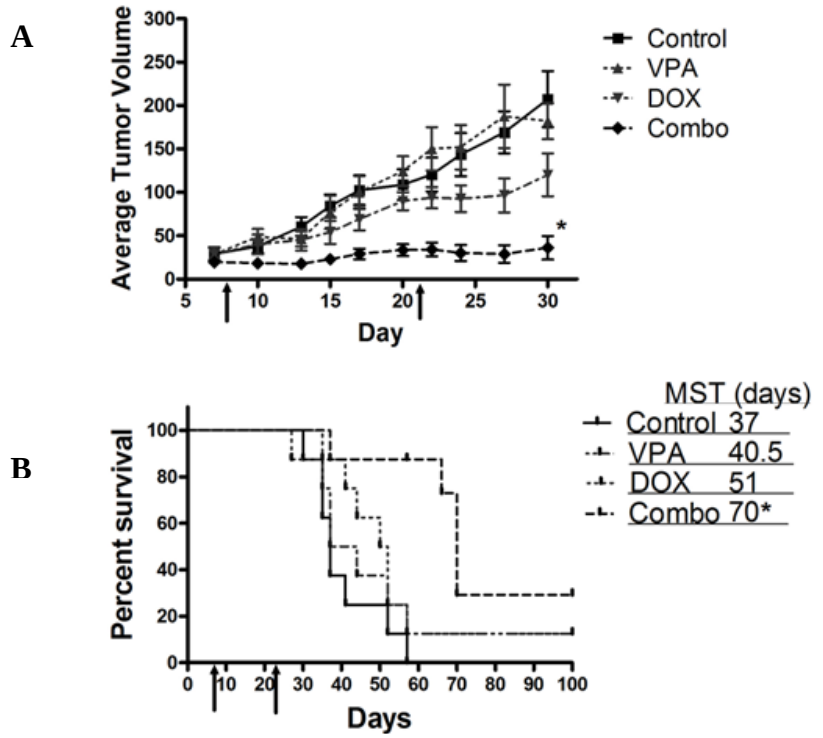

Figure 2.7 Growth of subcutaneous Abrams OS xenografts implanted into athymic mice (n=8 per group). Arrows indicate treatment cycles. **A)** Average tumor volumes were measured for each group and tumor growth curves over time were compared using ANOVA. A significant reduction in tumor growth is observed in the VPA/DOX combination treated mice when compared to all other groups; * $P<.05$ **B)** Kaplan Meier survival curve showing a statistically significant improvement in overall survival for the VPA pretreated group

Immunohistochemical analysis of OSA xenografts. We next utilized IHC to evaluate the xenograft tumors for changes in histone acetylation, apoptosis and proliferation. Mice were divided into treatment groups (n=3 per group) identical to those described above and sacrificed 24 hours after the first DOX administration. Tumors were

removed and formalin fixed prior to IHC analysis. As previously demonstrated *in vitro*, there was a statistically significant increase in apoptosis (P<.0001) and a decrease in proliferation (P=.0003) in the combination treated mice **(Figure 2.8)**. In addition, increased histone acetylation was evident by IHC in the VPA treated mice **(Figure 2.9)**. Taken together, these results indicate that the addition of VPA to standard cytotoxic chemotherapy results in tumor cell histone hyperacetylation, increased apoptosis, and decreased proliferation in OS when compared to either drug administered alone.

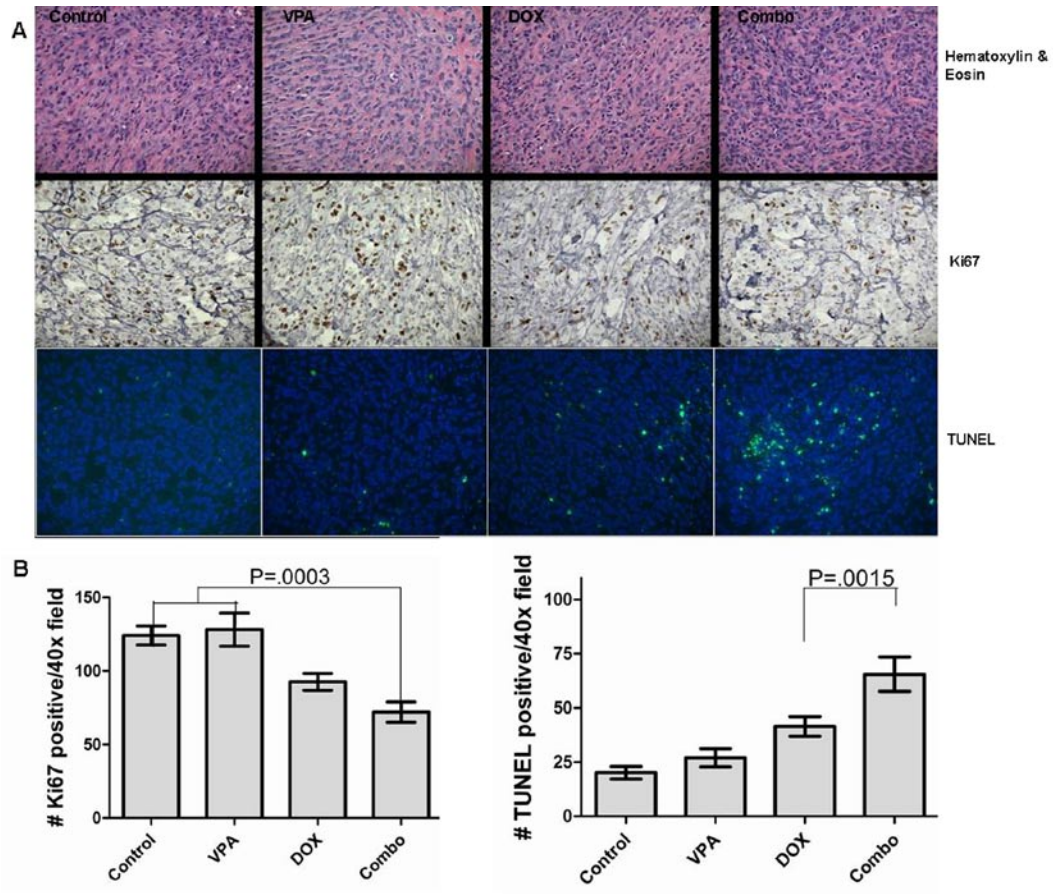

Figure 2.8 A) Photomicrographs of Abrams OS xenografts. Formalin fixed, paraffin embedded tumors were evaluated for changes in proliferation (Ki67 immunoreactivity) and apoptosis (TUNEL) between treatment groups. B) Graphical respresentation of Ki67 and TUNEL immunoreactivity. Proliferation is significantly decreased in tumors from combination treated mice (left) accompanying a significant increase in apoptotic cells in this same group (right).

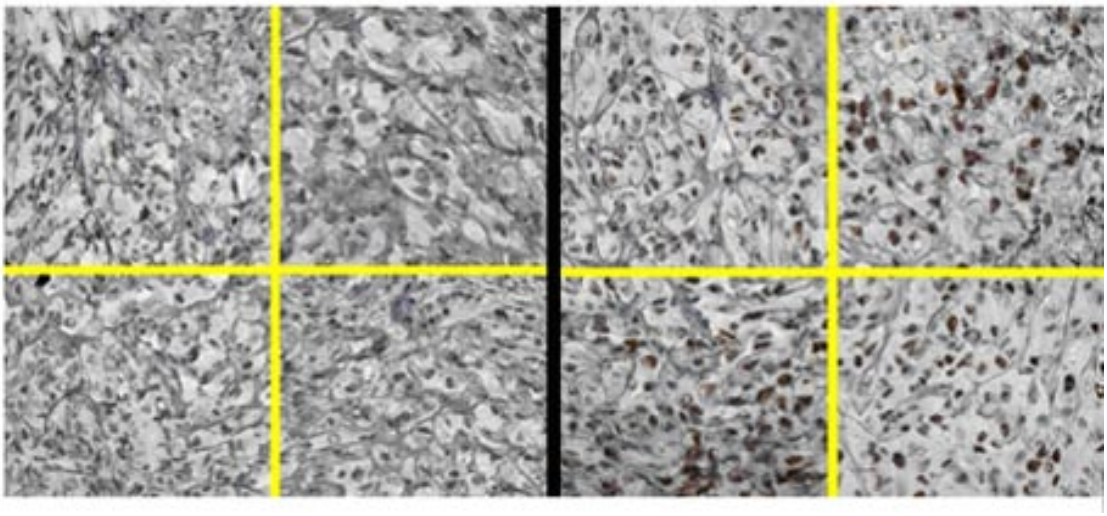

Figure 2.9 Photomicrographs depicting acetyl-H3 from control (left) and VPA treated (right) xenograft tumors. Sections from different tumors in each group were evaluated by IHC for acetyl-H3 immunoreactivity, and a clear induction of histone hyperacetylation is seen in VPA treated tumors suggesting that the dose of VPA was adequate for intra-tumoral target modulation.

DISCUSSION

Osteosarcoma remains the most common primary bone tumor, developing most commonly in the long bones during adolescence (24). The cause of death for the vast majority of these patients continues to be the development of metastases, primarily to the lungs, as local failure in patients with appendicular tumors has been minimized by improvements in primary tumor management (27). While the development of multi-modality chemotherapy protocols for OS in the 1980's provided significant improvements in long-term outcome for patients presenting with localized disease, this has not held true for patients in which metastases are present at the time of diagnosis and fewer than 30% of these patients survive (24, 27). Factors that have been correlated with

long-term survival in OS patients include the degree of necrosis of the primary tumor following an intensified, multi-modality chemotherapy protocol prior to surgical amputation or limb salvage procedure (28). Unacceptable toxicities prevent further dose intensification from providing any further improvement in long term survival. The use of agents targeting epigenetic changes is one example of the recent trend in cancer therapy to combine biologically targeted agents with cytotoxic chemotherapy.

In our present study, we found that the HDAC inhibitor VPA can sensitize human and canine OS cells to the anti-tumor effects of the topoisomerase-II inhibitor DOX. As a single agent, VPA demonstrated only modest antiproliferative effects in the concentration range that is considered achievable *in vivo*. While simultaneous treatment with both agents was able to produce an increase in DOX sensitivity, the most profound chemosensitization was seen with 48 hour VPA pre-treatment. This might be explained by decondensation of chromatin induced by VPA, allowing increased access of DOX to the DNA. This schedule dependence has also been reported by others evaluating different combinations of HDAC inhibitors and intercalating macromolecules *in vitro* and *in vivo* (29). Interestingly, the partial charge neutralization brought about by hyperacetylation of histone tails may only be partly responsible for the decondensation of chromatin following exposure to HDAC inhibitors. Alterations in expression levels of proteins that are important for maintaining the structure of chromatin (SMCs) have been implicated in this conformational change (14). The combination of decreased associations of histone tails with DNA and reduction of levels of proteins that maintain chromatin structure may explain the reductions in DOX IC_{50} in the VPA pre-treated OS cells compared to those that were co-incubated.

We also found that the decreased viable cell number observed with combination therapy was, at least in part, due to an increase in apoptosis. Caspase and Annexin V/PI assays revealed that the addition of VPA to DOX treatment significantly increased the apoptotic index of OS cells *in vitro* and this was associated with increased nuclear DOX accumulation in these same cells. We found identical results *in vivo*, with the addition of VPA providing a significant increase in the percentage of TUNEL positive tumor cells over mice treated with DOX alone. These results are particularly noteworthy in light of the fact that increased tumor cell death in primary OS tumors following neoadjuvant chemotherapy is associated with improved long-term survival in human patients (28). Similarly, a direct correlation between percentage necrosis following DOX therapy and survival time has also been described in dogs (30). In addition to enhanced apoptotic activity, our results from the evaluation of the proliferation marker Ki-67 show that the combination of VPA and DOX also has a greater antiproliferative effect than either agent alone.

We demonstrated by western analysis that *in vitro* treatment of canine and human OS cells with VPA results in similar hyperacetylation of histone H3 using concentrations that are achievable in patients. Hyperacetylation was observed *in vivo* as well, although no increased anti-tumor activity was observed with short-term single agent VPA treatment compared to controls. Although it has not been shown to be a predictor of anti-tumor activity in single agent HDACi treated cells, evaluation of tumor histone acetylation *in vivo* may be useful in evaluating pharmacodynamics of VPA when determining optimal dosing in combination with DOX, as chromatin decondensation and increased access to DNA may require histone hyperacetylation.

The relatively low incidence rate of OS in humans is a significant obstacle in developing and rigorously evaluating novel treatment combinations and designing clinical trials that will generate meaningful outcome data. In contrast, the incidence of spontaneously occurring OS in canine patients is approximately 8-12 times higher (31). These canine tumors are histologically indistinguishable from their human counterparts and share common features such as biological aggressiveness, response rates, propensity to metastasize to the lungs, anatomic site predilections, and prognostic factors (31-33). Studies in canine patients with spontaneous OS have proven useful in developing novel therapeutic strategies for humans. A randomized, double blind study in canine patients using a liposome encapsulated form of the macrophage activating compound muramyl tripeptide phoshphatidylethanolamine (L-MTP-PE) demonstrated a significant improvement in event free survival following amputation (34), the results of which led to a large, randomized phase-III trial in human OS patients (35). Our current study illustrates the similarity between canine and human OS cells in their molecular responses to HDAC inhibition by VPA, providing further evidence that spontaneously occurring OS in dogs may provide a robust model to develop novel epigenetic strategies that may further improve long-term outcomes.

In this study, we have demonstrated that VPA is capable of effective inhibition of HDACs in canine and human OS cells, resulting in histone hyperacetylation. In addition, pretreatment of these cells with VPA results in enhanced sensitivity to DOX *in vitro* and profound tumor growth inhibition *in vivo*. We also found that decreases in markers of proliferation and increases in apoptosis were sequelae of VPA-DOX combination therapy in a xenograft model of canine OS. This study provides further support to the use of

HDAC inhibitors as a means of chemosensitization in the treatment of cancer and, more specifically, the integration of HDAC inhibitors into cytotoxic chemotherapy protocols in OS. Spontaneous canine OS may serve as a novel translational bridge for the evaluation of these combinations.

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Chapter 3

Phase I Pharmacokinetic and Pharmacodynamic Evaluation of Combined Valproic Acid/Doxorubicin Treatment in Dogs with Spontaneous Cancer

ABSTRACT

Histone deacetylase inhibitors (HDACi) are targeted anti-cancer agents with a well-documented ability to act synergistically with cytotoxic agents. We recently demonstrated that the HDACi valproic acid (VPA) sensitizes osteosarcoma cells to doxorubicin (DOX) *in vitro* and *in vivo.* As there are no published reports on the clinical utility of HDACi in dogs with spontaneous cancers, we sought to determine a safe and biologically effective dose of VPA administered prior to a standard dose of DOX.

Twenty-one dogs were enrolled into eight cohorts in an accelerated dose-escalation trial consisting of pre-treatment with oral VPA followed by DOX on a three-week cycle. Blood and tumor tissue were collected for determination of serum VPA concentration and evaluation of pharmcodynamic effects by immunofluorescence cytochemistry and immunohistochemistry. Serum and complete blood counts were obtained for determination of changes in DOX pharmacokinetics or hematologic effects.

All doses of VPA were well tolerated. Serum VPA concentrations increased linearly with dose. DOX pharmacokinetics were comparable to those in dogs receiving DOX alone. Histone hyperacetylation was documented in tumor and PBMC. A positive correlation was detected between VPA dose and histone hyperacetylation in PBMC. No potentiation of DOX-induced myelosuppression was observed. Responses included 2/21 complete, 3/21 partial, 5/21 stable disease, and 11/21 progressive disease.

In conclusions, VPA can be administered to dogs at doses up to 240 mg/kg/day prior to a standard dose of DOX. In addition, we have developed the PK/PD tools necessary for future studies of novel HDACi in the clinical setting of canine cancer.

INTRODUCTION

Histone deacetylase (HDAC) enzymes, responsible for the removal of acetyl groups from N-terminal tails of histone proteins, play a crucial role in chromatin plasticity and have recently been identified as one of the most promising therapeutic targets in cancer therapy (1). The rationale behind the use of HDAC inhibitors stems from the discovery of epigenetically silenced tumor suppressor genes in a number of model systems resulting in tumor cell addiction to altered signaling pathways (2, 3). In addition, an imbalance in the activity of histone acetyltransferases, the enzymes responsible for lysine acetylation, and HDAC plays a crucial role in the development and progression of some tumors (4). A number of HDAC inhibitors have demonstrated anti-tumor activity *in vitro* and *in vivo*, either as single agents or in combination with other chemotherapeutics, and have shown efficacy in hematologic and solid tumors through inhibition of proliferation by cell cycle arrest, effects on terminal differentiation, and

apoptosis (5-13). A few of these agents have entered into clinical trials, and of the HDAC inhibitors, SAHA (vorinostat) and romidepsin (Istodax®) have received FDA approval for use in humans as single agents in the treatment of cutaneous T-cell lymphoma (CTCL). For the most part, these agents are well tolerated, demonstrate target modulation in tumor tissues, and show some single-agent efficacy in solid and hematologic malignancies (8, 14, 15). However, these agents are likely to provide the most benefit when combined with other treatment modalities such as chemotherapy or radiotherapy.

While early-phase studies in humans provide promising results and describe some biological parameters for determining potential susceptibility to HDACi containing treatment protocols, it has also been clearly demonstrated that the current drug development pipeline for novel anti-cancer therapies in many cases fails to properly identify agents that provide long-term improvements in patient survival. The use of spontaneously occurring tumors in pet dogs has the potential to improve current drug development and modeling strategies (16-18). This realization has led to the completion and publication of the first canine clinical trial conducted by the National Cancer Institute's Comparative Oncology Trials Consortium (COTC) evaluating safety, toxicity, tumor specificity, and efficacy of a novel tumor endothelium targeting agent (RGD-A-TNF) being developed as a therapy for human cancers (18). The translational utility of the canine cancer model stems from the greater similarities to human cancers when compared to murine models. Unlike murine models, dogs are relatively outbred, immunocompetent animals with spontaneously occurring tumors experiencing spontaneous metastasis and therapy resistance, representing a spectrum of tumor

histotypes that have biology similar to that found in humans. When compared with murine tumors, the relatively large size of canine tumors more closely approximates human solid tumors with respect to important biological factors such as hypoxia and clonal variation, and allows for multiple samplings of tumor tissue over time. The relatively rapid time course of disease progression, when compared with human cancer, allows for more rapid assessment of therapeutic endpoints than is possible in many human clinical trials (19, 20). In addition, the lack of a meaningful standard of care for many types of canine cancer alleviates the obligation to start with single-agent Phase I trials and allows immediate evaluation of novel agents in combination protocols, where they are most likely to show a benefit.

While there have been some reports on the *in vitro* sensitivity of canine cancer cells to HDAC inhibitors (21, 22), there have been no reports on the clinical use of HDAC inhibitors in canine cancer patients.

The anti-epileptic drug valproic acid (VPA), belonging to the short-chain fatty acid class of HDACi, has shown efficacy in a variety of tumor models, especially when combined with other forms of therapy (5, 6, 15, 23-26). We previously showed that pre-treatment of canine and human osteosarcoma cells with VPA results in sensitization to doxorubicin (DOX), partially via increasing nuclear DOX accumulation, resulting in decreased proliferation and increased apoptosis. This was also demonstrated in an *in vivo* xenograft model of canine osteosarcoma (22). Here we report the pharmacokinetic and pharmacodynamic results of a Phase I trial of oral VPA in tumor-bearing dogs given prior to a standard dose of DOX. We hypothesized that VPA could be safely administered to

dogs for 48 hours prior to a standard dose of DOX, would demonstrate target modulation in PBMC and tumor tissues, and would have no effects on DOX PK.

MATERIALS AND METHODS

Chemicals and antibodies

Divalproex sodium extended-release tablets (Depakote® ER) were purchased from Cardinal Health (Dublin, OH). Doxorubicin (DOX) was purchased from Bedford Laboratories (Bedford, OH). Controls for the VPA assay were purchased from Cliniqa (San Marcos, CA). Anti acetyl-histone H3 and H4 and total histone H3 and H4 antibodies were purchased from Upstate Biotechnology (Waltham, MA). A FITC-conjugated goat anti-rabbit antibody was purchased from Bethyl Laboratories (Montgomery, TX). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was purchased from Pierce (Rockford, IL). Daunorubicin hydrocholoride was purchased from Sigma (St. Louis, MO), and doxorubicinol hydrochloride from Toronto Research Chemicals, Inc. (North York, Ontario, Canada). All HPLC reagents were of analytical grade.

Patient recruitment

All dogs in this study were pet dogs presenting as patients to the Colorado State University Animal Cancer Center. Study participation was offered in cases where standard therapy had failed or had been declined by the dog's owner, or in cases of advanced disease where no meaningful standard therapy exists. Dogs were treated in accordance with the "NIH Guidelines for Care and Use of Laboratory Animals".

Protocol approval was obtained from the Institutional Animal Care and Use Committee and the Colorado State University Veterinary Teaching Hospital Clinical Review Board. Signed informed consent and consent to necropsy were obtained from all owners.

This study was open to patients with histologically or cytologically confirmed neoplasia. Patients with regional or distant metastasis were included if a survival time of greater than 6 weeks was expected. Patients were required to be free of other severe complicating concurrent disease conditions, and required to have adequate laboratory and clinical indices to safely undergo therapy (specifically: total bilirubin not exceeding 1.5x normal; creatinine not exceeding 2x normal; at least 2,500 neutrophils/µL, 75,000 platelets/µL, and a hematocrit of at least 28%). Treatment related adverse events were graded based on guidelines set forth in the Veterinary Comparative Oncology Group-Common Terminology Criteria for Adverse Events (VCOG-CTCAE) (27). A VCOG performance status of 0-1 was required for study inclusion; (0, normal activity; 1, restricted activity [decreased activity from pre-disease status]; 2, compromised [ambulatory only for vital activities, consistently defecates and urinates in acceptable areas]; 3, disabled [dog needs to be force-fed, is unable to confine urination and defecation to acceptable areas], and; 4, dead). Prior chemotherapy and radiation therapy were allowed with a 3-week or 6-week washout period, respectively. In addition, a 72-hour washout from prednisone was required if being used as an antineoplastic drug. No concurrent anti-neoplastic therapy was allowed, and prior DOX exposure could not exceed 90 mg/m^2.

Pretreatment procedures and evaluations

A complete blood count (CBC), serum biochemistry profile, and urinalysis were performed prior to enrollment in the study and staging was performed as appropriate for specific tumor type. 10 mL of heparinized whole blood was collected for PBMC separation, and 14-Ga needle core biopsies were obtained from accessible tumors using local anesthesia or brief sedation.

Treatments

All dogs were given oral divalproex (VPA) for 48 hours prior to DOX administration with an initial dose of 2X the intended maintenance dose. Patient doses were escalated according to an accelerated dose-escalation protocol whereby one patient was enrolled in each cohort and the cohort was expanded to six only if toxicity was encountered in the first patient. All dogs received a standard dose of DOX (30 mg/m^2, or 1 mg/kg if <15 kg) as initial treatment on day 3, between the morning and evening VPA doses. DOX dose was reduced by 20% for subsequent treatments if grade 3 or 4 toxicities were observed after the first dose.

Patient monitoring procedures and evaluations

A CBC and blood chemistry were obtained 48 hours after initiation of VPA therapy. Serum, plasma, and heparinized whole blood were collected immediately prior to the morning VPA dose on day 3 for determination of VPA trough concentrations and VPA pharmacodynamic evaluation. Serum was collected after DOX administration at 0, 10, 20, 30, 60, 120, 240, 360 minutes, and 24 hours for evaluation of DOX

pharmacokinetics. Tumor biopsies were obtained again 48 hours after initiation of VPA therapy. A CBC and blood chemistry were obtained 7 and 21 days following DOX administration. Owners were asked to fill out Quality of Life/Pain questionnaires prior to study, after 48 hours of VPA therapy, and at 7 and 21 days following DOX administration. The VPA/DOX combination was continued on an every 3 week basis until disease progression or maximal cumulative DOX dose was reached. Patient removal from the study resulted from either disease progression, decreased quality of life (VCOG score >2), cumulative DOX dose > 180 mg/m^2, or owner request.

Valproic acid analysis in serum

Serum trough VPA concentrations were determined using the Cedia® Valproic Acid II Assay (Microgenics; Fremont, CA) on a Hitachi 917 System Analyzer (Roche; Indianapolis, IN). Results were graphed as serum trough VPA concentration vs. maintenance dose.

Doxorubicin and doxorubicinol analysis in plasma by LC/MS/MS

Doxorubicin and doxorubicinol were measure in patient plasma using an LC/MS/MS assay. Positive ion electrospray ionization (ESI) mass spectra were obtained with a MDS Sciex 3200 Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems, Inc., Foster City, CA) with a turbo ionspray source interfaced to an Agilent 1200 Series Binary Pump SL HPLC system (Santa Clara, CA). Samples were chromatographed with a Phenomenex Prodigy, 5μm, C18 100 Å, 150 x 2.00 mm column (Phenomenex, Torrance, CA). An LC gradient was employed with mobile phase A

consisting of 10 mM ammonium acetate plus 0.1% acetic acid (pH 4.4) and mobile phase B consisting of acetonitrile. Chromatographic separation was achieved holding mobile phase B steady at 10% from 0-2 min, increasing mobile phase B linearly from 10% to 90% from 2-9 min, holding steady from 9-11 minutes, and decreasing linearly from 90% to 10% from 11-13 minutes, followed by re-equilibration from 13-15 minutes. The sample injection volume was 50 μL and the analysis run time was 15 minutes. The mass spectrometer settings were optimized as follows: turbo ionspray temperature, 350°C; ion spray voltage, 5500 V; declustering potential (DP), 20 V; entrance potential (EP), 5.0 V; collision energy (CE), 40 V; collision cell exit potential (CXP), 5.0 V; curtain gas, N_2, (CUR), 50 units; collision gas, N_2, (CAD), medium. Samples were quantified by internal standard reference method in the MRM mode monitoring ion transitions *m/z* 544→361 amu for DOX, *m/z* 546→363 amu for doxorubicinol, and *m/z* 528→321 for the internal standard, daunorubicin. Scan times were 200 ms and Q1 and Q3 were both operated in unit resolution mode.

Analytical standards (2.5-500 ng/mL), quality control (QC) and unknowns were all prepared by adding 500 μL of unknown or spiked blank plasma samples to 1.5 mL microcentrifuge tubes containing 5 μL of 10 μM daunorubicin solution followed by brief vortexing. Plasma proteins were then precipitated by the addition of 500 μL of acetonitrile followed by 10 min vortex mixing. Samples were then centrifuged at 18,000 RCF for 10 min and the supernatant collected and transferred to autosampler vials for analysis. The lower limit of quantitation for the assay was 2.5 ng/mL for both DOX and doxorubicinol with accuracy and precision (CV%) of 91.7% ± 5.7% and 95.3% ± 3.8%, respectively.

Immunohistochemistry

Deparaffinized sections of pre- and post-VPA tumor biopsies were stained for histone H3 acetylation after antigen retrieval using DakoCytomation Target Retrieval Solution pH 9 (Dako, Carpinteria, CA). Prepared sections were incubated with anti AcH3 at 1:50 overnight at 4°C followed by goat anti-rabbit HRP at 1:250 for 1.5 hrs at room temperature followed by diaminobenzidine (DAB) (Vector Labs, Burlingame, CA) staining and hematoxylin counterstain. Images were obtained using a Zeiss Axioplan 2 microscope coupled with a Zeiss AxioCam HRc camera. For blinded comparison of acetylated histones between pre- and post-VPA samples, an overall H-score was given to each image, obtained by multiplying the percent staining (0-100) by an intensity score (0-3). Seven images were obtained for each pre-and post-treatment sample and results were averaged.

Immunofluorescence

Isolated peripheral blood mononuclear cells (PBMC) from pre- and post-VPA blood samples were stained for acetylated histones H3 and H4 using total histone H3 and H4 as controls. PBMC were cytospun onto slides and fixed in 95% ethanol/5% glacial acetic acid at -20°C for 5 minutes prior to permeabilization using 0.2% Tween 20 in PBS. Nonspecific binding was blocked with 1% BSA in PBST for one hour at room temp, followed by overnight incubation with polyclonal rabbit anti-acetyl histone H3 (1:200) or anti-total H3 (1:50) then 1.5 hours with goat anti-rabbit-FITC (1:250). Slides were then rinsed three times and mounted using VectaShield plus DAPI® (Vector Labs). Images were obtained using a Zeiss Axioplan 2 microscope coupled with a Zeiss AxioCam HRc

camera. For comparison of acetylated histones between samples, a semi-automated computerized image analysis program was developed using AxioVision Rel. 4.5 software (Zeiss) that measured fluorescence intensity of each cell within a field and then averaged the intensity of the entire field. Seven fields were evaluated for each patient treatment condition and results were averaged and graphed.

Western analysis

For evaluation of tumor tissue histone acetylation, snap-frozen pre- and post-VPA biopsy samples were lysed in buffer containing T-PER Protein extraction reagent (Pierce), 1 mM $NaVO_4$, 1 mM PMSF, Complete Mini protease inhibitor (Roche), and 1% SDS, transferred to 1.5 mL microfuge tubes and passed through a 25 gauge needle 7-10 times before centrifugation at 10,000 x g for 10 minutes. Protein concentration of lysates was determined via BCA assay (Pierce). Lysates were loaded into a denaturing 4-12% Bis-Tris gel (Invitrogen, Carslbad, CA) and electrotransferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% non-fat dry milk in TBST (40 mM Tris pH 7.6, 300 mM NaCl, and 0.5% Tween-20) for one hour at room temperature and incubated in a 1:2500 dilution of rabbit polyclonal anti-acetyl H3, or total H3, in blocking solution overnight at 4°C. After three washes in TBST, membranes were incubated in a 1:20,000 dilution of HRP-conjugated goat anti-rabbit IgG for 1.5 hours at room temperature. Immunoreactive proteins were detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce) and analyzed by autoradiography. Densitometry was performed using Image J software available online from the NIH (http://rsb.info.nih.gov/ij/index.html).

Statistical analysis

Determination of correlations between serum trough VPA levels and dose, WBC parameters and VPA dose, and fold induction of PBMC histone hyperacetylation versus dose were performed using linear regression. DOX pharmacokinetic parameters were calculated by non-compartmental analysis as previously described (28) and comparisons between patient data and historical controls were made by unpaired two-tailed T-test. Comparison of H-scores between pre and post treatment samples was done by two-tailed T-test. Statistical analysis was performed using GraphPad Prism® (GraphPad Software, La Jolla, CA). For all comparisons, a P-value less than 0.05 was considered significant.

RESULTS

Dose-escalation trial

An accelerated dose-escalation design was used to govern dose escalation toward a MTD for oral administration of VPA. In this design, one patient was enrolled in each cohort and followed for a full treatment cycle before beginning enrollment into the next highest cohort. If toxicity was noted or reported by the owner, an additional five dogs were enrolled in that cohort. If no further toxicity was noted, escalation to the next highest dose was begun. If two toxicities were reported then dose escalation would be stopped and the MTD reached. In all, 21 dogs met the inclusion criteria and were enrolled in the study. All dogs underwent pre-treatment evaluation of blood chemistry and CBC, and pre-treatment biopsies were obtained from accessible tumors. Age, weight, sex, and breed, and tumor type was recorded for each patient **(Table 3.1)**. At enrollment, 10 of 21 patients (47%) had documented metastatic disease, and 8 of 21

(38%) had received prior chemotherapy and/or radiation or had been enrolled in a previous clinical trial.

Table 3.1 Patient Characteristics

Characteristic	No. of Patients (N=21)	%
Sex		
Male	11	52.4
Female	10	47.6
Age, years		
Median	9	
Range	6.2-13.4	
Weight, kg		
Median	30.1	
Range	4.7-42.4	
Breed		
Purebred	16	76.2
Mixed	5	23.8
Tumor Histology		
Lymphoma	5	23.8
Melanaoma	4	19
Osteosarcoma	3	14.3
Carcinoma	3	14.3
Soft Tissue Sarcoma	3	14.3
Hemangiosarcoma	1	4.8
Mast Cell	1	4.8
Cutaneous T-cell Lymphoma	1	4.8
Dose Cohort, mg/kg (loading/maintenance)		
30/15	2	9.5
60/30	1	4.7
90/45	1	4.7
120/60	1	4.7
150/75	1	4.7
180/90	6	28.6
210/105	6	28.6
240/120	3	14.3

Oral VPA was well tolerated in all dose-escalation cohorts with only a single Grade 3 metabolic event reported (ALKP > 5x ULN) at the 180/90 mg/kg cohort in a patient with Stage 5 lymphoma. In addition, a single Grade 2 anorexia was reported in a patient at the 210/105 mg/kg cohort. Other owner-documented side effects were reported as mild and included lethargy, decreased appetite, and diarrhea. A total of 69 treatment cycles were administered with an average of 3.3 cycles per patient (range 1 to 5). No maximum tolerated dose (MTD) was reached, as the highest dose failed to produce any dose-limiting toxicity. Escalation was halted due to compliance issues with the number of tablets required to be administered.

Responses were evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) (29) and included 2/21 (10%) complete responses (both lymphoma), 3/21 (14%) partial responses (lymphoma, melanoma, lung carcinoma), 5/21 (24%) dogs with stable disease through 5 treatment cycles (osteosarcoma, renal cell carcinoma, apocrine gland adenocarcinoma, melanoma, soft-tissue sarcoma), and 11/21 (58%) progressive disease.

Pharmacokinetics

For determination of serum trough VPA concentrations, blood was obtained 48 hours after initiation of therapy, immediately prior to administration of the fifth dose. A linear correlation between the administered maintenance dose and serum trough VPA concentration existed in the patient population (**Figure 3.1**). In addition, we evaluated DOX pharmacokinetic parameters including AUC, clearance, and half-life in three patients in the highest dose cohort. To determine if administration of VPA altered DOX

pharmacokinetics, our PK results were compared to those of two previously reported studies evaluating DOX PK in dogs receiving single agent therapy (30, 31). As shown in **Figure 3.2**, there were no significant differences in any of the evaluated PK parameters between our study patients and historical controls.

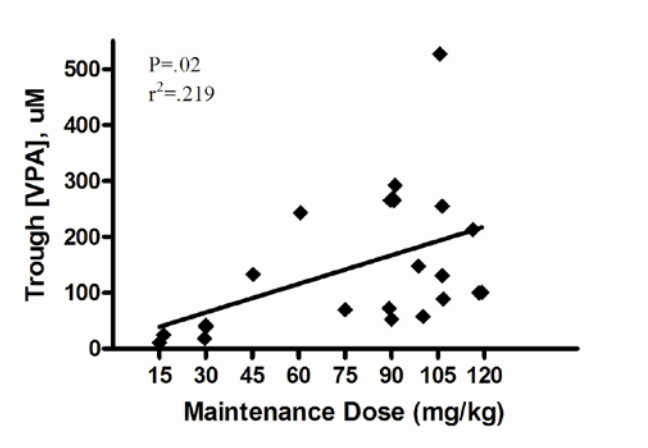

Figure 3.1 Evaluation of trough VPA levels in dogs. Serum was collected after 48 hours of divalproex (VPA) therapy for determination of trough VPA levels by serum chemistry analyzer. Results were plotted against the actual maintenance dose received and demonstrate that trough VPA level increases linearly with dose.

Pharmacodynamics

In order to determine if VPA therapy was associated with any myelosuppression, or potentiated DOX-induced myelosuppression, we evaluated patient complete blood counts (CBC) prior to and 48 hours after initiation of VPA therapy as well as 7 days after DOX administration. By examining the ratio of total WBC as well as neutrophil counts from day three to day one, we observed no correlation between dose administered and myelosuppression (**Fig. 3.3A**). The WBC nadir following DOX is typically around day 7 post administration, and a comparison of nadir WBC and neutrophil count with VPA

dose revealed no potentiation of DOX induced myelosuppression (**Fig. 3.3B and C**). Taken together, these data indicate that VPA administered at doses up to 210 mg/kg/day does not induce any significant myelosuppression or potentiate DOX induced myelosuppression.

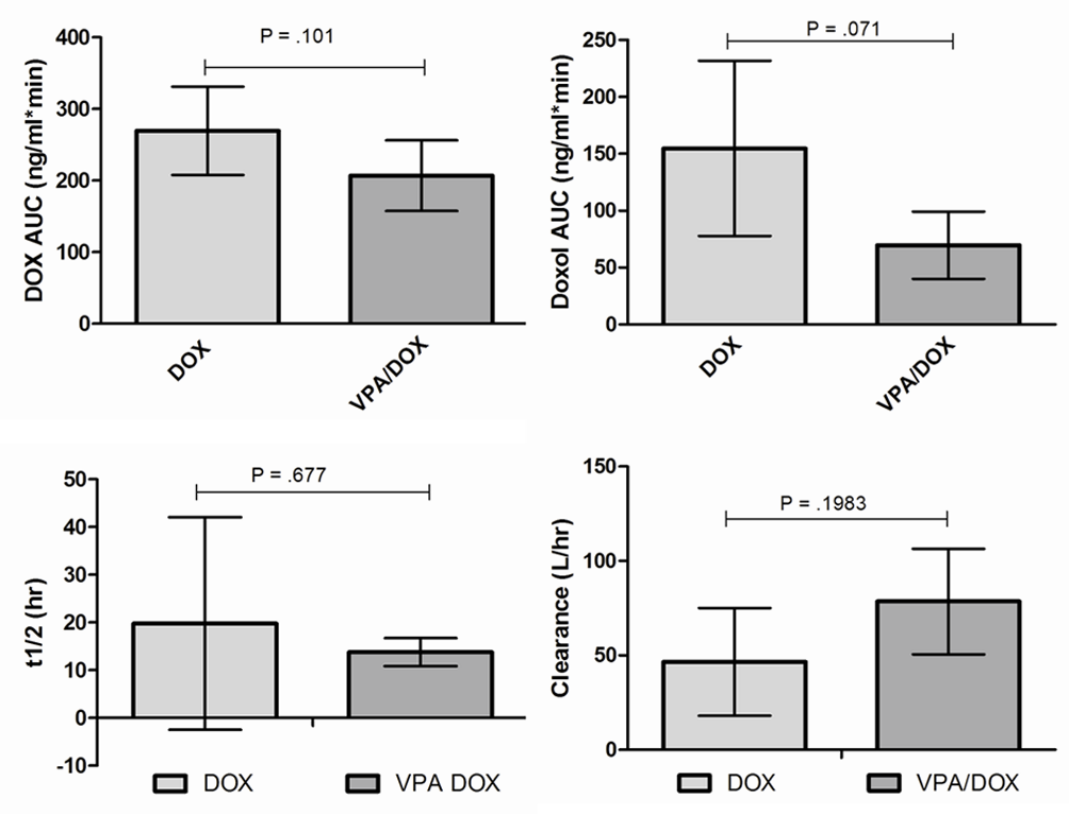

Figure 3.2 Serial serum samples were collected after IV doxorubicin (DOX) administration in three patients in the highest VPA dose cohort (240 mg/kg/day) for determination of AUC, half-life, and clearance as well as AUC of the major metabolite, doxorubicinol by HPLC tandem M/S. Results were compared to those previously published for dogs receiving DOX alone and show no significant alterations in DOX PK parameters for VPA pre-treated dogs.

Immunofluorescence

As described in the Methods section, blood was collected prior to and 48 hours after administration of VPA for separation of PBMC and evaluation of histone hyperacetylation. Fourteen matched patient samples were available for comparison of pre- and post-VPA histone acetylation by fluorescence immunocytochemistry. The results were recorded as fold change induction of acetylated histone H3, with total histone or total histone H3 as a control. As shown in **Figure 3.4**, there was a significant correlation between the fold induction of acetyl H3 and the administered VPA maintenance dose, suggesting that histone hyperacetylation is useful as a PD marker to evaluate 48 hour VPA exposure in future clinical trials.

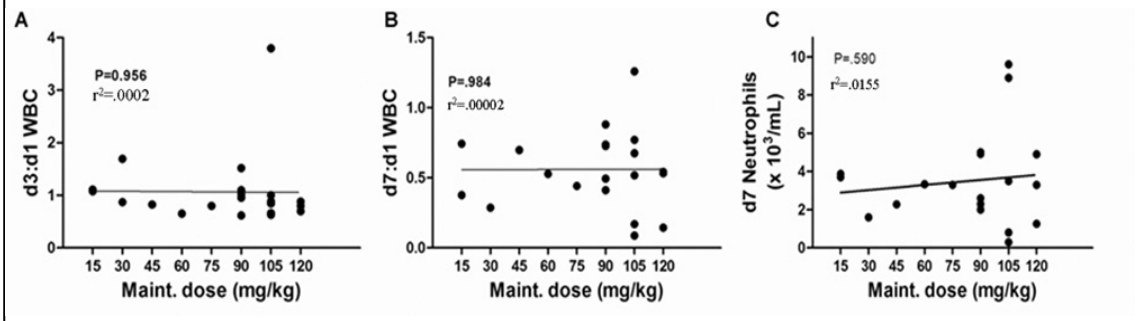

Figure 3.3 Complete blood counts were obtained prior to and 48 hours after initiation of VPA as well as on day 7 post DOX. A) Ratio of WBC on day 3 to pre-treatment shows no correlation between VPA dose and myelosuppression. B) Ratio of WBC on day 7 (DOX nadir) to pre-treatment and C) absolute neutrophil count on day 7 demonstrate that VPA pre-treatment does not potentiate DOX-induced myelosuppression.

Immunohistochemistry and Western blot

Nine patients had tumors that were amenable to biopsy pre-and post-VPA treatment. For eight of these patients, formalin fixed paraffin embedded samples were evaluated for induction of histone hyperacetylation by immunohistochemistry, while snap-frozen biopsy samples were used for detection of target modulation by western blot in eight patients. As shown in **Figure 3.5**, histone hyperacetylation in tumor tissues could be detected by IHC, and Western blot confirmed similar induction in these same patients **(Figure 3.6)**. There was no direct correlation between administered VPA dose and magnitude of tumor histone acetylation by IHC, nor was there a positive correlation between tumor and PBMC histone hyperacetylation **(Figure 3.7 A and B)**.

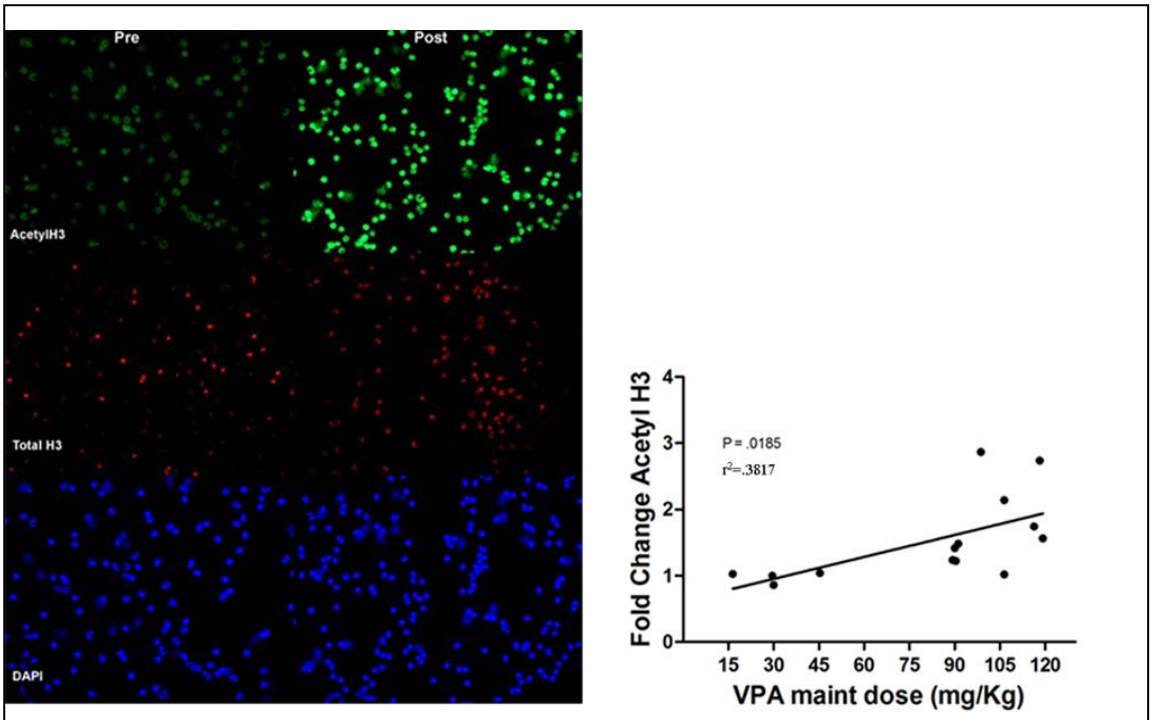

Figure 3.4 Left: Photomicrograph of PBMC in a dog before and 48 hours after initiation of VPA (240/120 mg/kg cohort) demonstrating an increase in acetylation of histone H3 (top) with no change in staining intensity of total histone (middle); nuclei are stained with DAPI (bottom). Right: Comparison of fold change in histone H3 acetylation vs maintenance dose received shows a positive correlation between dose and target modulation in PBMC.

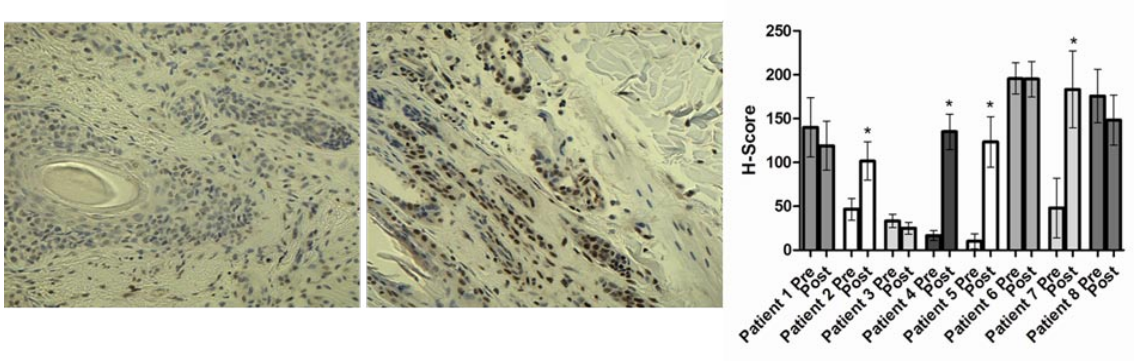

Figure 3.5 Accessible tumors were biopsied before and 48 hours after initiation of VPA. Left: Photomicrograph of IHC for acetylated histone H3 in a patient with cutaneous T-cell lymphoma pre and post VPA (210/105 mg/kg cohort) demonstrating significant induction of hyperacetylation. Right: Graphical representation of IHC comparisons for all evaluable biopsy samples. Patient dose increases from left to right. * indicates significant increase in histone H3 acetylation (P<.05).

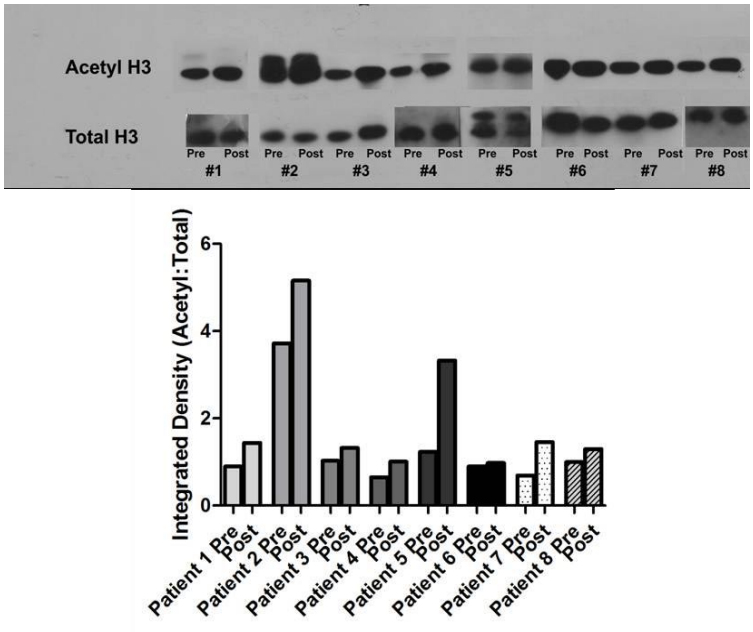

Figure 3.6 Western blot analysis of acetyl histone H3 in patient tumor samples. Top: Western blot of patient samples pre and post VPA treatment. Bottom: Graphical representation of integrated density demonstrating similar results to those seen by IHC in the same patients.

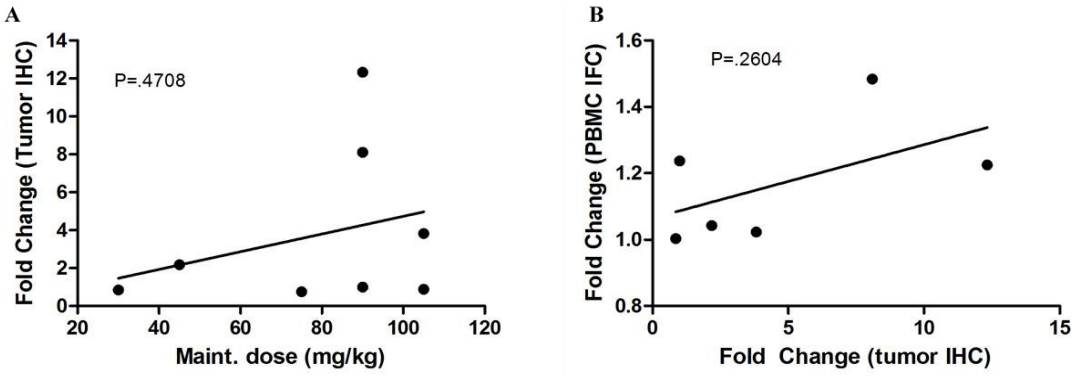

Figure 3.7 A) Comparison of fold change in acetyl H3 of tumor samples and maintenance dose; there is no significant correlation between the magnitude of VPA-induced histone H3 hyperacetylation by IHC and administered maintenance dose. B) Comparison of fold change in acetyl H3 of patient PBMC by IFC and fold change in acetyl H3 of tumor samples by IHC; there is no significant correlation seen between target modulation in PBMC (by IFC) and target modulation in tumor tissues (by IHC).

DISCUSSION

Here we report the results of a Phase I clinical trial evaluating the use of combined VPA/DOX therapy in dogs with spontaneously occurring tumors, aimed at defining a safe and biologically effective dose of VPA to use in future efficacy trials, as well as validating a toolbox of pharmacodynamic assays that can be applied to future studies of novel HDACi. The dosing scheme of a 48 hour pre-treatment period with an HDAC inhibitor prior to DOX was based upon previous findings by our own group as well as others of superior *in vitro* chemosensitization when compared to co-administration in a number of cell types, as well as *in vivo* activity in a canine osteosarcoma xenograft model (6, 22, 32). Additional benefits of pulse-dosing of VPA compared to continuous administration include the ability to give higher doses, because

of the washout period between treatment cycles, as well as ease of compliance and reduced cost.

Eight dose cohorts, ranging from 30 mg/kg/day up to 240 mg/kg/day, were evaluated. An MTD was not reached with the examined doses; however, treatment at higher doses was not feasible because of the tablet size of VPA and its local toxicity. Consistent with Phase I reports of VPA as well as other HDACi in humans, the toxicities encountered were generally mild (8, 14, 33-35). The most commonly reported adverse events were Grade 1 anorexia and lethargy and Grade 1 diarrhea. A single Grade 2 metabolic toxicity (increase in serum ALP > 5x ULN) was reported in a patient with Stage V lymphoma in the 180 mg/kg/day cohort and was most likely reflective of disease progression during the 48 hour VPA pre-treatment time period. This was only observed in the first treatment cycle for this patient, with ALP levels returning to near normal after the first cycle and remaining stable for the remainder of the study. In addition, a single Grade 2 anorexia was reported in a patient in the 210 mg/kg/day cohort but no further toxicity was reported after expansion of that cohort. In all, two patients required dose reductions in DOX after the first treatment cycle because of neutropenia; one patient at the 60 mg/kg maintenance dose exhibited febrile grade 3 neutropenia (neutrophil count 500-999/μL) and another at the 105 mg/kg maintenance dose with non-febrile grade 3 neutropenia. The number of patients requiring DOX dose reduction in our study does not exceed that expected for patients receiving DOX alone (36). Although no MTD was reached as there was no reported dose limiting toxicity in the highest dose cohort, the number of tablets required and owner compliance became a limiting factor in further dose escalation. However, it has been suggested that the traditional MTD dosing of anti-

cancer agents may not be optimal when using targeted agents, as the determination of biologically effective dose is a more relevant endpoint and these agents may have the most efficacy with doses below MTD (37-40). It is interesting to note that human Phase I studies have identified a much lower dose of 140 mg/kg/day as the MTD for VPA (14), while we observed no dose limiting toxicities in dogs at 240 mg/kg/day. This is likely due to PK differences (i.e. volume of distribution or bioavailability) between species as 48 hour trough serum concentrations in human patients appear to be much higher than those seen in dogs given comparable doses on a mg/kg basis. This may also be explained by interspecies allometric scaling parameters that would suggest dogs require higher administered doses to achieve the same drug exposure. However, our data may also suggest that dogs require lower overall VPA exposure for biologic efficacy as, in spite of lower serum VPA concentrations compared to human patients, objective responses were observed in our Phase I study with a few responses observed in traditionally anthracycline-resistant tumors. It is possible that higher doses could be administered to dogs that would result in plasma concentrations more indicative of those seen in human patients, and novel oral formulations of VPA that allow higher doses to be administered with fewer tablet numbers could help to further elucidate the relationships between dose, exposure, and response in dogs. We did not observe any obvious cardiotoxicity in any patients, although this was not directly evaluated in this study and the total cumulative DOX dose was low.

Serum trough VPA concentrations increased linearly with the administered maintenance dose. The relative weakness of this correlation could easily be explained by discrepancies in the times that VPA was actually administered to patients at home and

those reported by owners. Pharmacodynamic evaluation of fold change in histone H3 acetylation in nine evaluable patient PBMC samples also correlated with administered dose, but did not correlate with trough VPA levels. This would suggest that the use of a pharmacodynamic endpoint, in this case direct target modulation, may give a better estimate of overall drug exposure and activity during the 48 hour treatment period than determining VPA concentrations at a single time point. However, this assessment may be limited by the small sample size in this study.

We measured DOX pharmacokinetics in three patients to ensure that VPA pre-treatment had no effects on DOX elimination. Since VPA PK was linear in our patient population, it would be safe to assume that any changes to DOX PK would be manifest in the highest dose cohort, and these three patients were used for DOX PK comparisons. We did not anticipate any alterations in DOX PK as the mechanisms of metabolism are non-overlapping; VPA is primarily metabolized by complete beta-oxidation, while DOX metabolism occurs primarily via reduction by aldo-keto reductase to form doxorubicinol (30, 41). There were no significant changes in PK of DOX or the major metabolite, doxorubicinol, when compared to two separate historical control populations receiving DOX alone, suggesting that no alterations in DOX dosing are required in patients receiving combination therapy. Our results of a lack of potentiation of anthracycline-induced side effects are consistent with a report in humans evaluating a combination of VPA and epirubicin, although this report did not specifically evaluate AUC, clearance, or half-life of epirubicin in VPA pre-treated patients (14).

Treatment responses were evaluated by RECIST criteria. Two complete responses were observed, both in patients with lymphoma. Responses in lymphoma with

DOX alone are not unexpected as reported remission rates for treatment-naïve lymphoma in dogs range from 60-85% (42). For this reason, the number of lymphoma patients in the study was limited to five. Three patients experienced partial responses; these included one lymphoma, one melanoma, and one pulmonary carcinoma. In five patients, stable disease persisted until treatment was stopped after five DOX cycles as opposed to disease progression. Eleven patients were removed from the study because of progressive disease. No patients were removed from study due to decreased quality of life (VCOG > 2) or owner request. Although a portion of the objective responses measured may be attributable to DOX alone, it is interesting to note that antitumor activity (one PR and one prolonged SD) was observed in melanoma, a tumor that is generally resistant to anthracycline therapy in dogs (43).

In addition to demonstrating target modulation in patient PBMC following VPA treatment, we also found histone H3 hyperacetylation in 4/8 (50%) evaluable tumor samples by IHC, and western blot of these samples confirmed histone hyperacetylation. There was no apparent correlation between administered dose and target modulation in tumor tissue, supporting the idea that dosing to toxicity does not increase likelihood of enhanced therapeutic effect. The lack of correlation between dose and tumor target modulation could also be explained by sampling error; obtaining small biopsies from large heterogeneous tumors may result in sampling of areas with different basal acetylation levels or varying blood flow resulting in differences in drug exposure.

In conclusion, this is the first study to evaluate the safety and clinical utility of HDACi in dogs with spontaneous cancer. We used a sustained-release formulation of VPA given for 48 hours prior to a standard dose of DOX and demonstrated that serum

trough VPA level increased linearly with the administered dose. In addition, we found no evidence that the administration of VPA altered AUC, half-life, or clearance of DOX. VPA administration did not result in significant myelosuppression nor did it potentiate DOX-induced myelosuppression at VPA doses up to 240 mg/kg/day. We were able to demonstrate target modulation, specifically histone H3 hyperacetylation, in both normal and tumor tissues after administration of VPA, and the magnitude of PBMC hyperacetylation correlated positively with the administered dose of VPA. Serum trough VPA concentrations did not correlate with magnitude of histone hyperacetylation, suggesting that this particular PD marker may be a better overall determinant of 48 hour VPA exposure than trough VPA concentrations. In this study, we have demonstrated the safety of HDACi in dogs with cancer and have developed the tools necessary for rigorous PK/PD evaluation in future trials of VPA or other HDACi.

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Chapter 4

A Sytems Biology Approach to Identify Molecular Pathways Altered by HDAC Inhibition in Osteosarcoma

ABSTRACT

Osteosarcoma (OS) is the most common tumor in humans and canines affecting the skeleton, and spontaneously occurring OS in canines serves as an extremely useful model to study the human disease. Unacceptable toxicities using current combination treatment protocols prevent further dose-intensification from being a viable option to improve patient survival and thus, novel treatment strategies must be developed. Histone deacetylase inhibitors (HDACi) have recently emerged as a promising class of epigenetic cancer therapeutics demonstrating an ability to enhance the anti-tumor activity of chemotherapeutics, radiation, and immune-based therapies. While a number of HDACi have entered into clinical trials, some with encouraging results, the exact mechanisms by which they exert their effect remain unclear in many tumor types. To date, gene expression analysis of OS cell lines treated with HDACi has not been reported. We attempted to elucidate the mechanism of HDACi action in OS using a systems approach relying on gene interaction networks and molecular pathways. Following a 48 hour treatment with clinically achievable concentrations of the HDACi sodium valproate (VPA), cDNA from the D17 canine OS cell line was hybridized to the Affymetrix Canine

v2.0 gene chip, and a set of differentially expressed genes was selected using the University of Pittsburgh Gene Expression Data Analysis suite (GEDA), utilizing a standard J5 metric to provide a list of genes deviating from the expected average value and enriched with differential genes. We then applied DAVID web-based tools to provide functional annotation of all potentially differential genes based on homology of probe targets to human genes determined by online BLAST and/or BLAT (UCSC genome browser) search tools. Subsequent analysis of pathways was performed with MetaCore software and Ingenuity Pathways Analysis. Results of the analysis reveal that regulation of oxidative phosphorylation, cytoskeleton remodeling, and cell cycle are among those pathways most affected by HDACi treatment in the OS cell line. In addition, pathways involved in osteoblast lineage determination and differentiation such as Wnt and Hedgehog show changes in response to VPA treatment. Further analysis and validation of these pathways will lead to a better understanding of the mechanisms by which HDACi exert their effect in OS, and has the potential to identify biomarkers that may serve as novel targets and/or predictors of response to HDACi-containing combination therapies in OS.

INTRODUCTION

Osteosarcoma (OS) is a high-grade primary bone neoplasm of mesenchymal origin and represents the most common malignant tumor of the bone in both humans and canines (4, 5). These tumors demonstrate remarkable similarities between humans and canines which include a biphasic peak incidence, a slight male predilection, similar anatomic site predilections (metaphases of long bones), aggressive biological behavior,

hematogenous metastasis to the lungs early in the course of disease, similar sensitivity to chemotherapeutics, a relative resistance to radiotherapy, and a nearly identical reported incidence of occult metastasis at the time of diagnosis at 80-90% (4-7). In both cases, the addition of adjuvant chemotherapy results in an improved overall survival, although this does not hold true for patients presenting with metastatic disease at the time of diagnosis, and in humans the 5 year survival for these patients remains around 30% (5, 8, 9). Unacceptable toxicity prevents further dose intensification of current multimodal chemotherapy protocols from being a viable option to further improve survival, and thus there is a need to develop novel therapeutics for this disease. Generating meaningful data from clinical studies in humans with OS can be difficult because of the relatively low incidence; approximately 900 new cases reported per year (4). In contrast, the high incidence of OS in canines (approximately 8,000 cases per year) presents a unique opportunity to not only study the biology of OS but also rigorously evaluate novel therapeutics (5).

Histone deacetylase inhibitors (HDACi) have emerged as a very promising novel class of therapeutics in cancer treatment. There are currently a number of these inhibitors in clinical evaluation for hematologic and solid tumors, and two (orinostat and romidepsin) have received FDA approval as single agents in the treatment of relapsed or refractor T-cell lymphoma (10-13). The exact mechanisms by which these drugs act in cancer therapy is not fully understood but they appear to have pleiotropic anti-tumor effects including induction of differentiation, growth arrest, initiation of senescence, enhanced apoptosis, decreased angiogenesis, immuno-modulatory activities, and an ability to synergize with traditional cytotoxic chemotherapies and radiation (10, 14-24).

The clinical utility of agents that target histone deacetylase (HDAC) enzymes is, in part, based upon the aberrant expression and/or function of HDACs in cancer initiation and progression, with overexpression of some HDACs correlating with poor prognosis in a number of tumor types including gastric, prostate, breast, pancreatic, lung, and hepatocellular carcinomas (25-30).

The specific role of HDAC enzymes in the pathogenesis of OS has not been elucidated; however, HDAC enzymes play crucial roles in the normal development of bone cells including osteoblasts and osteoclasts (31-33). It has been shown that the treatment of normal osteoblasts by HDACi results in accelerated maturation and differentiation, in part due to the inhibition of the interaction between HDAC3 and the transcription factor Runx2 which controls expression of osteocalcin, a marker of osteoblast differentiation (33). In addition, the HDACi-induced effect on osteoblast differentiation has been shown to act through the regulation of Wnt/β catenin pathway (34). The canonical Wnt pathway plays a role in early developmental osteoblast lineage determination from a precursor common to both osteoblasts and chondrocytes (35). Given that greater than 80% of OS tumors display a lack of normal bone differentiation, it is not surprising that the majority of these tumors also have demonstrable inactivity of the Wnt pathway, determined by a lack of nuclear β catenin staining in OS biopsies (4, 7). However, there are conflicting reports on the activity of the Wnt pathway in OS, and in some cases inhibition of the pathway in OS has been shown to reduce tumorigenesis and metastatic potential of some cell lines (36). In fact, one decoy receptor for the Wnt pathway, secreted frizzled-related protein 3 (SFRP3) has been suggested to have a potential tumor suppressor function in OS showing an ability to reduce osteoblast

proliferation and increase osteoblast differentiation. Furthermore, high- grade OS tumors appear to lack or have downregulated expression of SFRP3 (37, 38).

In addition to the effects on differentiation processes of osteoblasts, HDAC inhibition also results in alterations of chromatin structure, a property that has been utilized to sensitize cells to chemotherapeutic agents that target DNA. The chromatin decondensation resulting from HDACi has been shown to increase sensitivity to topoisomerase I and II inhibitors such as doxorubicin, epirubicin and keranotecin in a variety of models of human and canine solid tumors, including OS (39-43).

Although microarray data exist on the effect of HDACi in osteoblasts, to our knowledge there are no reports of microarray data utilizing pathway analysis in HDACi treated OS cells. Here we attempt to further elucidate the molecular sequelae of HDAC inhibition in OS cells through pathway analysis of a canine OS cell line treated with the HDACi valproic acid (VPA), and use a combination of quantitative PCR and biochemical/functional assays in both human and canine OS cells to validate the results of our microarray analysis.

MATERIALS AND METHODS

Cell lines. The D17 canine OS cell line and the SAOS-2 and MG63 human OS cell lines were purchased from the American Tissue Culture Collection (Rockville, MD, USA.) The Abrams canine OS cell line was kindly provided by Dr. William Dernell (Colorado State University, Fort Collins, CO). MG63 and SAOS-2 cells were all below passage 20. Cells were cultured in Minimum Essential Medium Eagle (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (Atlas, Fort Collins, CO)

and penicillin/streptomycin (Invitrogen, Carlsbad, CA). For experimental procedures, cells were incubated in a humidified atmosphere with 5% CO2 at 37°C.

Chemicals and antibodies. Valproic acid was purchased from Sigma Chemical, Co. (St. Louis, MO). Anti acetyl-histone H3 and total histone H3 antibodies were purchased from Upstate Biotechnology (Waltham, MA). Bovine serum albumin, 2,6-dichlorophenolindophenol (DCPIP), dicumarol, and NADH were purchased from Sigma. Suc-Leu-Tyr-AMC fluorogenic substrate was purchased from AnaSpec (San Jose, CA). Bicinchoninic acid protein assay reagents and SuperSignal chemiluminescent substrate were purchased from Pierce Chemical Co (Rockford, IL). The proteasome inhibitor ONX-912 was obtained from Onyx Pharmaceuticals Inc (South San Francisco, CA). Alamar Blue™ fluorogenic substrate was obtained from AbD Serotec (Oxford, UK).

Gene Expression Microarray.

VPA treated (1 mM) and untreated D17 canine OS cells were used for analysis of differential gene expression using the Affymetrix™ Canine v2.0 gene chip, performed by the Microarray Core at the University of Colorado Health Sciences Center (Denver, CO). Briefly, cells in log-phase growth were treated in triplicate with 0 or 1 mM VPA for 48 hours, after which cells were harvested and stored in RNA*later*™ (Qiagen, Inc., Valencia, CA) for transfer to the core. RNA was extracted using the Qiashredder and RNeasy Kit from Qiagen. RNA quantity and integrity were assessed using the Nanodrop 1000 (ThermoScientific, Waltham, MA) and Bioanalyzer (Agilent Technologies, Foster City, CA) respectively. Resulting RNA was then used for first- and second-strand cDNA synthesis followed by cDNA cleanup and overnight *in vitro* transcription. Following

cRNA cleanup and fragmentation steps, samples were then hybridized to the Affymetrix chip and scanned.

Microarray Data Analysis.

Data normalization and selection of differentially expressed genes was performed as previously described by Ptitsyn et. al. (1). Briefly, normalized data were used to select a preliminary set of differentially expressed genes using the University of Pittsburgh Gene Expression Data Analysis suite (GEDA, http://bioinformatics.upmc.edu/GE2/GEDA.html). To reduce the number of false-positive differential genes and provide a shortlist of genes deviating from the expected average value and enriched with differential genes, a standard J5 metric with threshold 4 and optional 4 iteration of jackknife procedure was applied. Then we prepared a proxy set of human genes based on homology of canine probe targets to human genes determined by BLAST and BLAT search tools. We then applied DAVID web-based tools to provide preliminary functional annotation of all potentially differential genes (2). Subsequent analysis of statistically significant pathways was performed with Genego MetaCore software and Ingenuity Pathways Analysis (Ingenuity Systems Inc).

NQO1 Activity measurement.

NQO1 activity in VPA treated and untreated cells was measured as previously described (3). Briefly, canine OS cell lines were grown in the presence of 0 or 1 mM VPA and, following treatment, were washed twice with PBS and pelleted by centrifugation. Pellets were resuspended in 100 uL of 25 mM Tris (pH 7.4) and disrupted

by sonication using three 2-second bursts at 30% power; the resulting sonicates were centrifuged at 15,000 x g and the supernatant was collected and assayed for NQO1 activity and total protein content. For NQO1 activity measurement in lysates, a reaction mix containing 25 mM Tris (pH 7.4), 0.07% bovine serum albumin (w/v), 200 uM NADH, and 40 uM DCPIP was used and reactions were carried out in the presence and absence of 20 uM dicumarol. NQO1 activity is described as the dicumarol inhibitable decrease in absorbance at 600 nm with DCPIP as a substrate and is expressed in nanomoles of DCPIP reduced per minute per milligram of protein. Total protein in cell lysates was determined by the bicinchoninic acid assay using BSA as a standard.

Quantitative real time RT-PCR.

For validation of microarray results, a subset of VPA-altered genes was chosen for qRT-PCR in canine OS cell lines treated with VPA (0 or 1 mM). RNA was extracted using the Qiashredder and RNeasy Kit (Qiagen) according to manufacturer's recommendations. Reverse transcription of RNA was performed using the Omniscript® RT Kit (Qiagen) with a no-RT control for each sample. Each PCR reaction contained iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA), 100 nM forward primer, 300 nM reverse primer, 50 nM reference dye (ROX), and 25 ng template cDNA. Primer sequences for canine genes are shown in **Table 4.1**.

PCR reactions were carried out in duplicate on a Stratagene Mx3000P™ thermal cycler, programmed to run at 95°C for 10 min, 60°C for 1 minute with fluorescence monitoring (42 cycles), then ramp back up to 95°C with continuous fluorescence monitoring for dissociation curves. Average threshold values (Ct) were then used to

evaluate changes in gene expression using freely available Relative Expression Software Tool (REST) v2.0.13 (Qiagen).

Table 4.1 PCR primer sequences for selected canine genes

Gene	***Forward***	***Reverse***
Ezrin	AGC CAA TCA ACG TCC GAG TTA CCA	ACT GGA GGC CAA AGT ACC ACA CTT
Moesin	ATT GGC CAA GGA ACG TCA AGA AGC	AGA TTC GAG CTG TCA ACT CTG CCA
PSMD6	AAG GAT GGT GCT CTG ACA GCC TTT	TTA GGC GGT TTC TCC TGT CCC AAT
HSP90	TGT AAT TGC TGA CCC ACG AGG GAA	TTC TTC CAT GGG CTC CTC AAC AGT
NCAPH	ATG TGG AGC TTG CTG ACA AAG TGC	AGA CAG GCA AAG GCC AGA GGT ATT
MCM4	TCC CAG CTG ATT CCA GAG ATG CAA	AAA CAG AAG GCT CAG CAA TGC GAC
AURKA	TTG GGT GGT CAG TAC ATG CTC CAT	AGG TCT CTT GGT ATG TGC TTG CCT
CCNA2	AGC ACT CTA CAC AGT CAC AGG ACA	TCT GGT GGG TTG AGG AGA GAA ACA
THBS1	CAA TGC CAA CCA AGC TGA CCA TGA	ACA AGT CTG CAG TTG TCC CTG TCA
HPRT	ACT TTG CTT TCC TTG GTC AGG CAG	GGC TTA TAT CCA ACA CTT CGT GGG

20S proteasome chymotrypsin-like activity

Human and canine OS cells were plated in 6 well plates and allowed to adhere overnight prior to treatment with 0 or 1 mM VPA for 48 hours. After incubation, media was aspirated and cells were washed twice with PBS. Plates were then placed on ice and 90-100 uL of lysis buffer (20 mM Tris-HCl pH 8.0; 5mM EDTA) was added to wells. Cells were scraped into the lysis buffer and placed into 1.5 mL Eppendorf tubes prior to freezing at -20°C. Lysates were thawed on ice and spun down at 3,000 x G for 15 minutes at 4°C and the supernatants were transferred to fresh ice-cold tubes. Twenty µL of lysate was added, in duplicate, to wells of a black-walled 384 well plate followed by 20 µL of Assay Buffer (20 mM Tris pH 8.0; 0.5 mM EDTA) containing 120 µM Suc-Leu-Tyr-AMC substrate. Fluorescence was read on a Synergy™ HT microplate reader (BioTek, Winooski, VT) using an excitation wavelength of 340 nm and an emission wavelength of 465 nm. The fluorescence reader was programmed to read relative fluorescence units (RFU) every 3.5 minutes for 12 cycles. Mean slopes of the linear

portion of the curves were calculated as RFU/min. Protein content of the lysates was determined with the BCA assay using bovine serum albumin as a standard. Calpain activity was then determined as the RFU/min/ug of protein loaded. Results were then normalized to the untreated controls and expressed as a fold change in calpain activity.

Proliferation Assay

The canine Abrams OS cell line was plated in triplicate in a 96 well plate and allowed to adhere overnight at 37°C. ONX0912 and VPA were then made up at 2 X concentrations and 100 uL of each were added to wells of the plate followed by 48 hour incubation at 37°C. Relative viable cell number was evaluated by fluorometric bioreductive assay on a microplate reader (Synergy™ HT, Bio-Tek, Winooski, VT) with excitation/emission spectra of 530 and 590 nm, respectively and normalized to untreated control cells

Endothelin measurement

Canine and human OS cells were incubated in 0, 0.5, or 1.0 mM VPA for 48 hours and the levels of endothelin in cell culture supernatants were evaluated using the ELISA-based Endothelin-1 Assay Kit (Immuno-Biological Laboratories Co., Ltd, Gunma, Japan) according to manufacturer's recommendations. Briefly, cell supernatants were added to a precoated ELISA plate provided in the kit and allowed to incubate overnight at 4°C. Then labeled antibody solution was added to each well and incubated at 37°C for 30 minutes, followed by addition of a chromogenic substrate and

measurement of absorbance at 450 nm. Endothelin levels were calculated by comparing to a standard curve prepared simultaneously with the measurement of test samples.

VEGF measurement

Canine and human OS cells were incubated in 0, 0.5, or 1.0 mM VPA for 48 hours, and cell supernatants were collected for measurement of canine-specific VEGF by ELISA following manufacturers' recommendations (R&D Systems, Minneapolis, MN).

Proliferation assays

For the endothelin and VEGF measurement assays, parallel proliferation assays were set up to determine if changes in the levels of measured Et-1 and VEGF were a result of decreased cell viability. Cells were plated in triplicate in 96 well plates and allowed to adhere overnight at 37°C. Cells were then incubated in 0, 0.25, 0.5, or 1.0 mM VPA for 48 hours, after which the relative viable cell numbers were determined by fluorometric bioreductive assay (Alamar Blue) normalized to untreated controls.

RESULTS

Selection of genes and pathway analysis

Analysis of our data set from the microarray resulted in the selection of 1,571 genes that were differentially expressed between VPA treated and control cells. As shown in **Figure 4.1**, the genes that were selected were a balanced group between those that were moderately and highly expressed, categories that are most appropriate for selection of potential biomarkers (1). After selection of the differentially expressed

genes, a proxy set of human genes was generated using DAVID web-based tools to provide functional annotation based on sequence homology to probe targets using BLAST and BLAT web-based tools, and this proxy set of human genes was then used for subsequent pathway analysis.

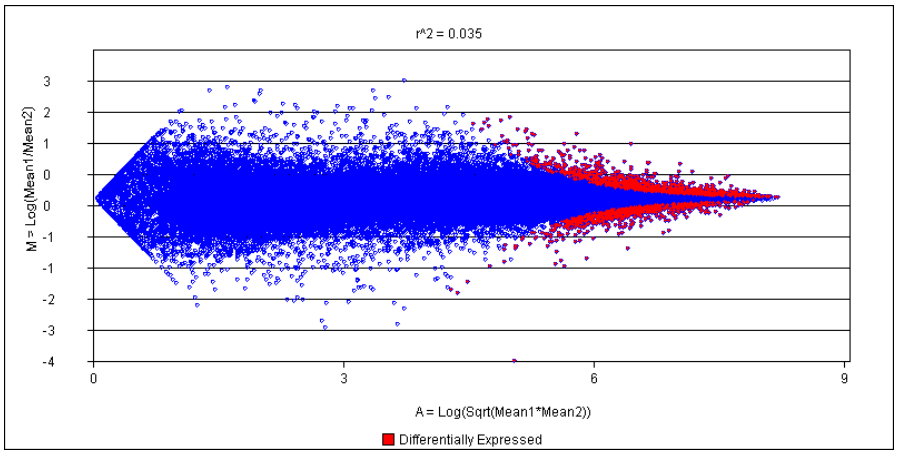

Figure 4.1 M-A plot of genes represented on the Affymetrix Canine v2.0 gene chip. The y-axis represents the log fold change and x-axis represents the average log intensity. Red dots represent genes selected for subsequent pathway analysis i.e. those deviating from the average mean signal intensity with VPA treatment and moderately or highly expressed.

Analysis of functional pathways revealed 43 that were statistically overrepresented ($P < .05$) in the list of genes; the top 15 are represented in **Figure 4.2.** The most significantly overrepresented pathway in this dataset involves oxidative phosphorylation, and as shown in **Figure 4.3**, the overall result of VPA treatment seems to be an upregulation of a number of genes involved in oxidative phosphorylation, resulting in an overall upregulation of respiratory complex I, respiratory complex II, cytochrome C oxidase, and ATP synthase. The regulation of oxidative phosphorylation may have important implications in the progression of metastasis as well as susceptibility to apoptosis in OS cells. Additional pathways with potential importance to OS initiation, progression, and

response to therapy include development through the hedgehog pathway, cytoskeleton remodeling, antigen presentation by MHC class I, cell adhesion, cell cycle, and proteolysis.

Validation by quantitative real-time RT-PCR

In order to validate the results of the microarray, a short list of genes chosen from the pathway maps as well as from a list of the most differentially expressed genes was generated for quantitative real-time PCR. These genes include the metastasis related genes ezrin and moesin, the S-phase replication associated protein MCM4, heat-shock proteins HSP70 and 90, cell cycle associated proteins Cyclin A, Cyclin B, and Aurora A, proteasome regulatory subunit PSMD6, and thrombospondin 1. The results of PCR analysis are shown in **Figure 4.4** and demonstrate the reproducibility of the pathway analysis by an independent method.

Proteasome inhibition by valproic acid

In addition to PCR analysis of the proteasomal regulatory subunit PSMD6, we also looked at the inhibition of the proteasome pathway as a whole through evaluation of chymotrypsin-like activity in VPA treated cells. As shown in **Figure 4.5**, canine OS cells treated for 48 hours with VPA exhibit an overall decrease in the chymotrypsin-like activity compared to controls, providing further validation of the pathway analysis.

Figure 4.2 Biological pathways significantly overrepresented in the list of genes differentially expressed between VPA treated and control OS cells.

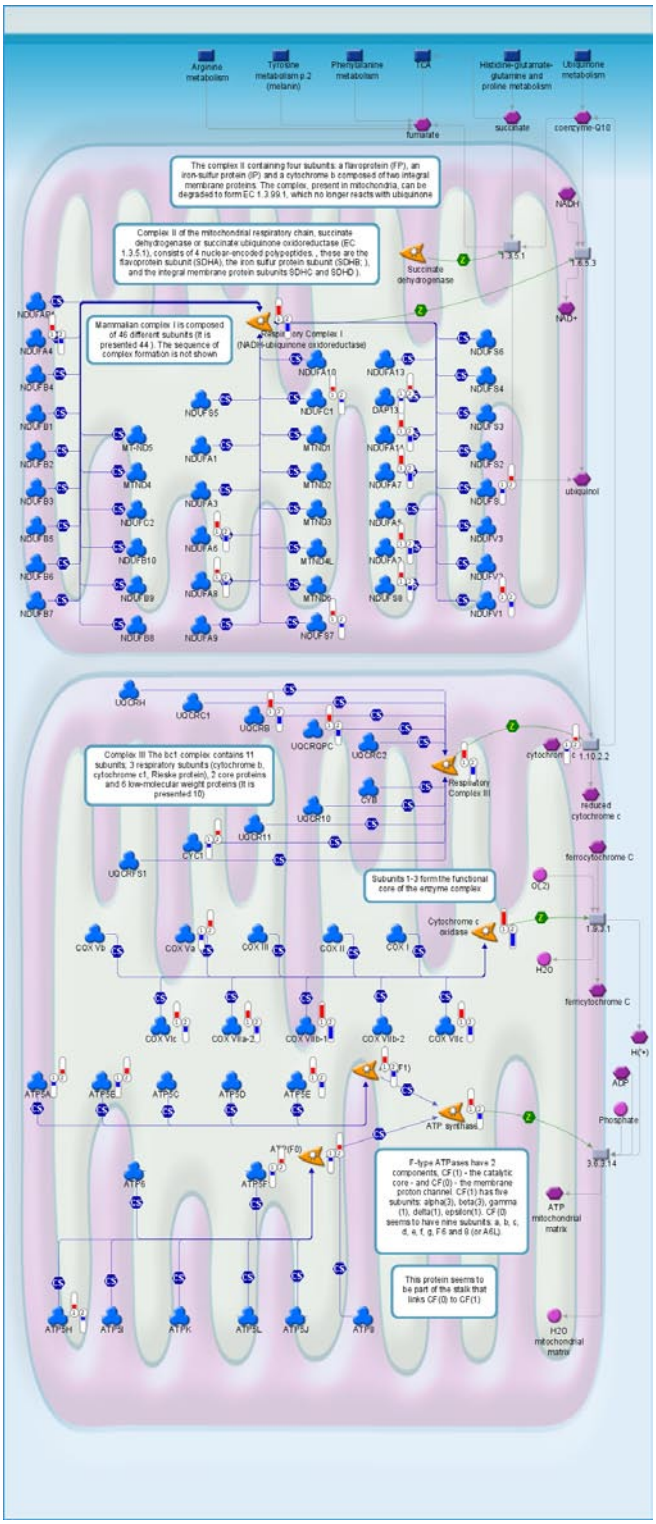

Figure 4.3 Oxidative phosphorylation genes differentially expressed in VPA treated OS cells. Relative change and direction of change are marked with color flags. Red designates higher abundance and blue designates lower abundance between (1) VPA and (2) control cells.

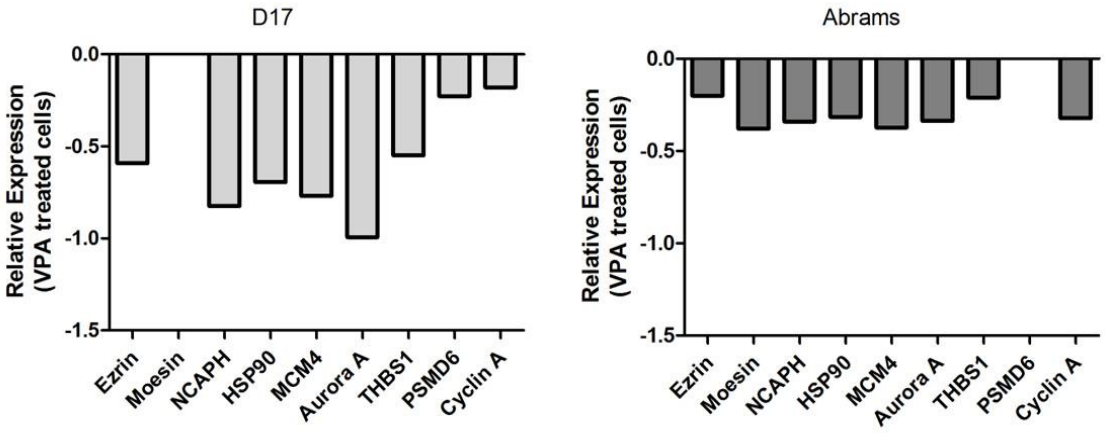

Figure 4.4 Validation of microarray by quantitative real time RT-PCR of genes selected from significantly altered pathways as well as from a list of the most differentially expressed genes in response to VPA treatment in canine OS cells. PCR results are consistent with down-reguations of genes seen in microarray pathway analysis.

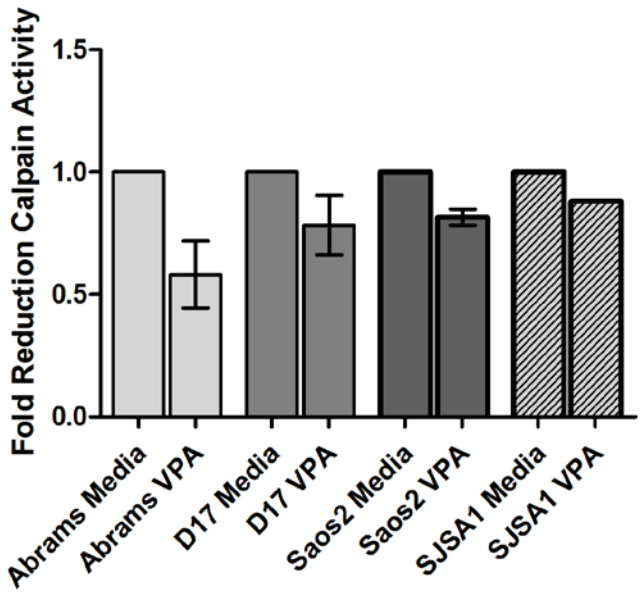

Figure 4.5 Evaluation of proteasome inhibition by VPA. Human and canine OS cells were treated with 0 or 1.0 mM VPA for 48 hours and then proteasome inhibiton was measured by evaluation of calpain activity in cell lysates. Reductions in calpain activity are seen in all four cell lines following VPA exposure.

Based upon the finding that VPA treatment appears to lead to proteasome inhibition, we evaluated the potential of a combination therapy utilizing VPA and the proteasome inhibitor ONX0912, and show that the addition of VPA leads to reduced proliferation compared to ONX0912 alone **(Figure 4.6)**.

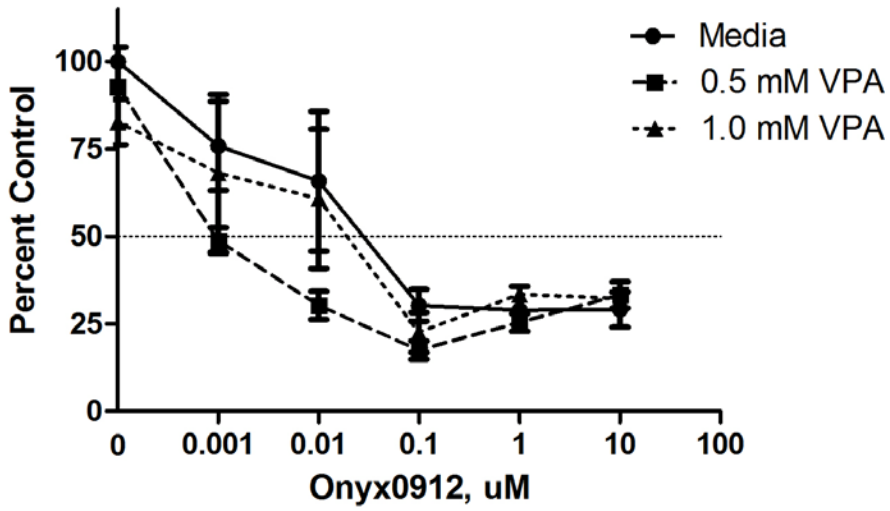

Figure 4.6 Proliferation evaluation of Abrams OS cells co-treated with 0, 0.5, or 1.0 mM VPA and increasing concentrations of the proteasome inhibitor ONX0912. The addition of VPA sensitizes these OS cells to the antiproliferative effects of a proteosome inhibitor.

Upregulation of NQO1 activity by valproic acid

One of the pathways that was statistically overrepresented in our microarray analysis is the ubiquinone metabolism pathway, and NAD(P)H: quinone oxidoreductase 1 (NQO1) was also on the list of the most highly upregulated genes in response to VPA treatment. To validate this finding, we evaluated NQO1 activity in VPA treated cells. As

shown in **Figure 4.7**, the specific activity of NQO1 is enhanced by treatment with VPA, which is consistent with upregulation of NQO1 gene activity seen by microarray.

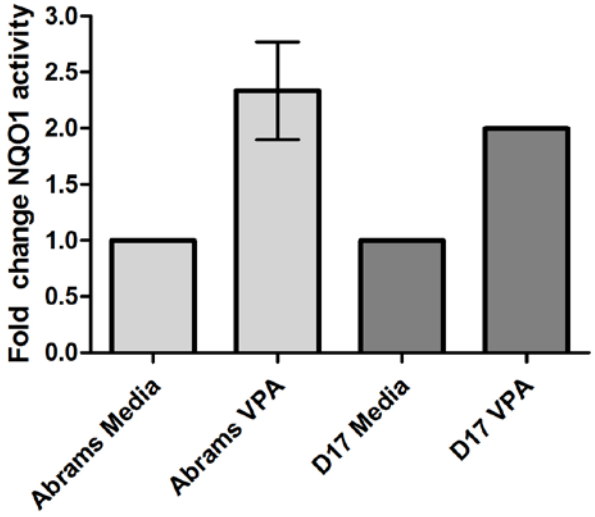

Figure 4.7 NQO1 activity measured in lysates of VPA treated (1 mM) and untreated OS cells by determination of the ability to reduce DCPIP using NADH as a substrate. Both cell lines demonstrate increased NQO1 activity in response to VPA treatment, consistent with upregulation of NQO1 gene activity.

In addition to increased NQO1 activity, we also assessed the ability of VPA to sensitize OS cells to the antiproliferative effects of the NQO1 substrate drug Mitomycin C. As shown in **Figure 4.8**, SAOS2 human OS cells pretreated for 48 hours with VPA show a trend toward an enhanced anti-proliferative effect compared to Mitomycin C alone.

Downregulation of endothelin-1 by VPA

Another gene found to be downregulated by VPA on gene expression microarray was endothelin-1 (Et-1). We used a commercially available ELISA-based kit to evaluate

the levels of endothelin in supernatants of cells treated with a clinically achievable concentration of VPA for 48 hours. As shown in **Figure 4.9**, the concentrations of Et-1 secreted by OS cells in reduced in the presence of 1 mM VPA, and this reduction is not the result of reduced cell viability as there is negligible anti-proliferative effect at that concentration of VPA.

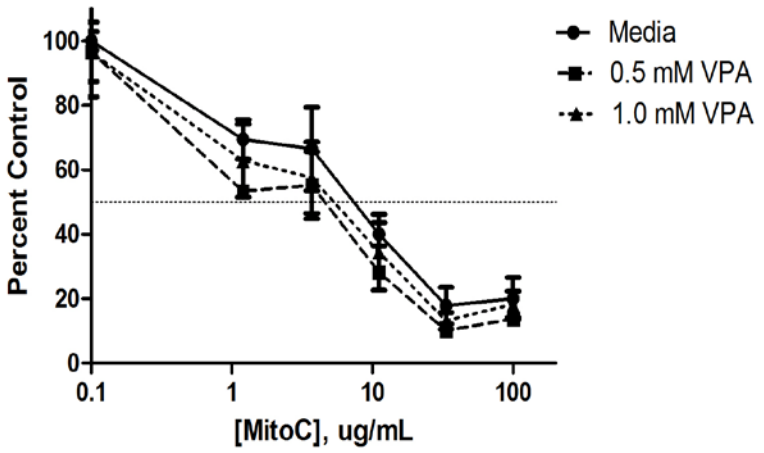

Figure 4.8 Evaluation of the anti-proliferative effect of Mitomycin C on SAOS2 human OS cells pre-treated with 0, 0.5, or 1.0 mM VPA. Pre-treatment of these cells with VPA results in a trend toward increased anti-proliferative effect when compared to cells treated with Mitomycin C alone.

We additionally evaluated the ability of VPA to affect the concentrations of VEGF secreted by OS. Cells were exposed to VPA in a similar fashion to that described for the ET-1 assay and the secreted level of VEGF in cell supernatants was evaluated by ELISA. As shown in **Figure 4.10**, a dose-dependent reduction in VEGF in supernatants

is observed for both canine OS cell lines (D17 and Abrams), while a reduction in VEGF is seen at 1mM for the SAOS2 human cell line. Only a slight change in secreted VEGF is seen in the MG63 human OS cell line in response to VPA exposure up to 1 mM.

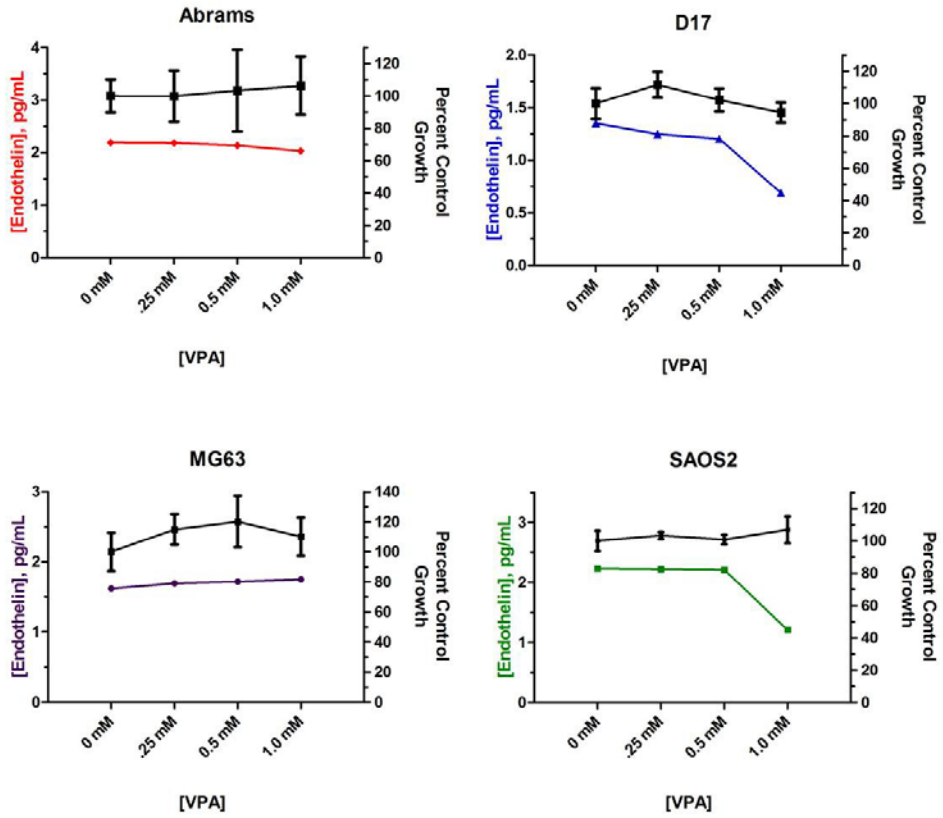

Figure 4.9 Measurement of endothelin-1 in supernatants of VPA treated OS cells by ELISA. A marked reduction in endothelin-1 (colored lines) is observed in the D17 canine OS and SAOS2 human OS cell lines upon 48 hour exposure to 1 mM VPA. This reduction in endothelin is not the result of reduced cell viability as the percent viable cells remains relatively constant across the range of VPA doses (black lines).

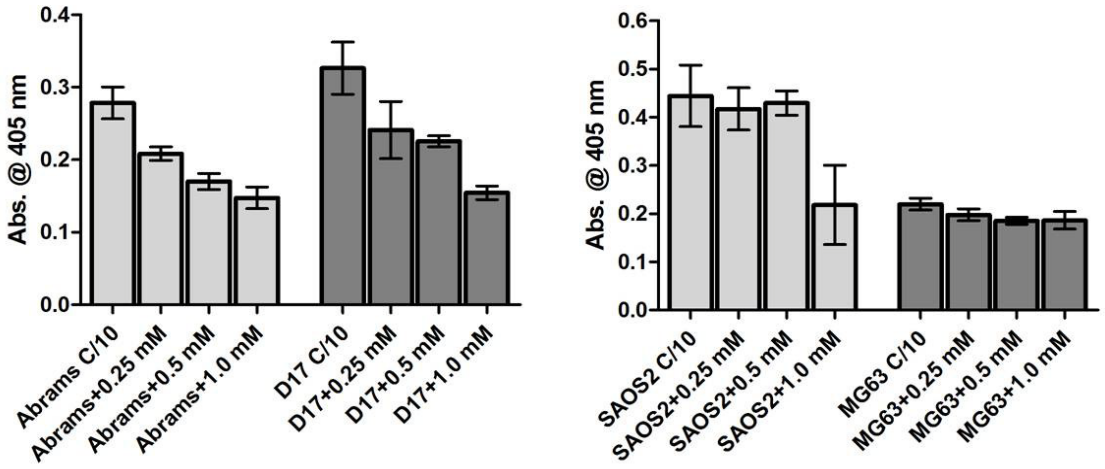

Figure 4.10 VEGF measurement in cell supernatants of VPA treated OS. Cells were treated for 48 hours in increasing concentrations of VPA and secreted VEGF was measured by ELISA of cell supernatants. A dose-dependent decrease in secreted VEGF is seen in both canine cell lines while a reduction is seen at 1 mM for the human SAOS2 cell line.

Discussion

Here we present the results of a gene expression microarray experiment performed on a canine OS cell line treated for 48 hours with the HDAC inhibitor VPA, and present a panel of experiments designed to validate the individual genes and pathways identified by microarray data analysis. While many microarray experiments focus on the identification a few "key" differentially regulated genes, we have taken the additional step of placing all of the genes that we identified as differentially expressed in response to HDAC inhibition into functional pathways. Validation of our dataset, in part,

was performed through quantitative real time RT-PCR of a few individual genes chosen from the pathways that were overrepresented, and we show in two canine cell lines that downregulation of these selected genes is reproducible by PCR.

While some of the individual genes may not be considered significantly altered when evaluated alone, functional annotation that groups them together may result in significant alteration of an entire pathway. In our study, we identified approximately 1,500 genes that fit the criteria selected for determination of differentially expressed genes, and when placed into pathways, the oxidative phosphorylation pathway was the most significantly overrepresented with a large number of genes upregulated by VPA treatment. This finding has significant importance in cancer, as it has been shown that loss of activity in this pathway, with concomitant upregulation of glucose utilization results in increased aggressiveness and metastatic potential, referred to as the Warburg effect (44-47). This may be partly explained by the finding that functional oxidative phosphorylation is required for apoptosis induced by the pro-apoptotic protein Bax in OS, providing evidence that evasion of apoptosis can be obtained by down-regulation oxidative phosphorylation (48, 49). Our results suggest that one potential mechanism by which HDACi, and particularly VPA, exert an anti-tumor effect may be through re-sensitization of intrinsic apoptotic pathways via upregulation of oxidative phosphorylation.

An additional pathway that was overrepresented in our microarray analysis involves protein degredation via the ubiquitin-proteosome pathway. While evaluation of mRNA levels by quantitative real time RT-PCR demonstrated a reduction in the level of one regulatory subunit of the 20S proteosome (PSMD6), we also performed functional

assays that looked at proteosomal activity in VPA treated cells to determine the biological relevance. VPA treated OS cells clearly demonstrate an inhibition of chymotrypsin-like activity, and this result is in agreement with another study describing a siRNA-mediated loss-of-function screen that identified the proteasome as playing an important role in HDACi-induced apoptosis (50). Interestingly, the loss-of-function study suggests that one particular protein involved in shuttling of ubiquitinated cargo proteins may govern the sensitivity to drugs that target the proteasome, and found this particular protein to overexpressed in T-cell lymphoma, a cancer that appears particularly sensitive to HDACi. Proteasome inhibition has been reported to be effective in the treatment of OS *in vitro* in a number of studies (51-53) possibly through regulation of cell-cycle associated proteins such as the cyclin-dependent kinases. This led us to test whether a combination of HDACi with VPA and proteasome inhibition with a novel compound, ONX0912, would provide an enhanced anti-tumor effect. We show that the combination of VPA and ONX912 results in a reduced proliferative potential of canine OS cells compared to cells treated with the proteasome inhibitor alone.

We also found ubiquinone metabolism to be one of the pathways altered by VPA treatment and NAD(P)H: quinine oxidoreductase 1 (NQO1) was additionally found to be one of the most significantly upregulated genes on the microarray. This could have important implications for the treatment of cancer as this particular enzyme plays an important role in the bioactivation of anti-tumor quinines such as mitomycin C (3). We validated this finding through evaluation of NQO1-specific activity in canine OS cell lysates following a 48 hour treatment with VPA. We demonstrate that the ability of NQO1 to reduce dichlorophenolindophenol (DCPIP) is enhanced following VPA

treatment, consistent with increased levels of the enzyme. To further test the significance of this we evaluated the ability of VPA to sensitize OS cells to the anti-proliferative effects of mitomycin C and show a mild sensitizing effect on the human SAOS2 cell line. This is consistent with in vitro reports of synergy between VPA and mitomycin C in colon carcinoma cells (54). However, as mitomycin C is also a DNA-binding drug (55), the effect of VPA-induced chromatin decondensation and increased access to DNA by mitomycin C cannot be ruled out as a contributing factor along with upregulation of NQO1.

An additional gene identified by microarray analysis as significantly downregulated by VPA treatment is endothelin 1. Et-1 is reported to have a variety of pro-tumorigenic properties that include acting as a mitogen for numerous human cancer cell lines as well as endothelial cells and vascular smooth muscle cells, protecting cells from Fas ligand-mediated apoptosis, and promoting metastasis through upregulation of stroma-degrading matrix metalloproteinases in OS (56, 57). In addition, elevated plasma levels of Et-1 have been found in tumor-bearing patients with the highest levels found in those with metastatic disease (56). This makes targeting Et-1 a rational target in cancer therapy. We found that incubation of OS cells with clinically achievable levels of VPA results in decreased Et-1 secretion into culture media, consistent with the downregulation seen on microarray. This may provide additional support to the use of combinations of VPA and proteasome inhibitors for the treatment of OS, as the induction of pro-metastatic genes such as the MMPs by Et-1 appears to be mediated through NF-κB, a well-documented target of proteasome inhibitors (58, 59).

We also evaluated the ability of VPA to reduce VEGF secretion into culture media by OS cells. Although this was not a gene that was found to be differentially expressed in response to VPA treatment on microarray analysis, it has been demonstrated that VEGF is prognostic in OS patients, and particularly, increased plasma levels in dogs are correlated with a poor outcome suggesting a role for VEGF in the development and progression of OS metastasis (60). We incubated canine and human OS cells with increasing concentrations of VPA for 48 hours and found that it reduced VEGF secretion into culture media in a dose-dependent manner for both canine cell lines tested. In addition, the SAOS2 human OS cell line exhibited VEGF inhibition following VPA treatment.

The results of this study shed further light onto the potential mechanisms by which HDACi with VPA exerts its effect in OS. We have identified oxidative phosphorylation as the most significantly altered metabolic pathway in response to VPA. In addition, we show that the proteasome pathway is altered through decreased chymotrypsin-like activity, leading us to test a combination of VPA and a proteasome inhibitor in OS cells. Our results suggest that this particular combination may prove beneficial in the treatment of OS. We show that VPA is capable of upregulating the oxidoreductase NQO1, enhancing its enzymatic activity and potentially leading to increased sensitivity of OS cells to the NQO1 substrate drug mitomycin C. In addition, we show that treatment of OS cells with VPA results in decreased secretion of two tumor-related molecules, both of which have been shown to correlate with poor outcome in cancer when elevated serum concentrations are detected in patients. This provides

further support to the design of VPA-containing combination protocols for the treatment of OS.

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Chapter 5

General Conclusions

The studies contained within this dissertation describe the use of valproic acid (VPA) as a histone deacetylase inhibitor (HDACi) for the sensitization of osteosarcoma (OS) cells to the anti-tumor effects of doxorubicin. Osteosarcoma is the most common primary bone tumor in both humans and canines and novel therapeutic options for are sorely needed since there has been no improvement in the last 20 years for patients who develop metastatic disease. The similarities between canine and human OS as well as the significantly increased incidence in dogs provide a unique opportunity to evaluate novel combination therapies that might be difficult to perform in human clinical trials. In addition, the relatively rapid disease progression in dogs compared to humans provides the ability to generate data on outcome in a timely manner. Although the goal of most translational studies is to find novel therapeutics that will provide a benefit to human cancer patients, the hope is that we may, in the process, discover therapeutic options that will improve survival of canine patients with cancer, thus improving quality of life for pets and their owners.

Based on the plethora of *in vitro* data on the ability of HDACi to act synergistically with traditional chemotherapeutics in a variety of tumor types, we wanted to evaluate the combination of an HDACi with the most commonly used chemotherapeutic in veterinary medicine, doxorubicin. Doxorubicin belongs to a class of

drugs, the anthracycline antibiotics, that is also commonly used for the treatment of OS in human medicine. The rationale for combining an HDACi with this drug comes from the finding that inhibition of HDAC enzymes results in chromatin decondensation and increased access of DNA targeting agents such as doxorubicin.

The antiepileptic drug VPA has a well-documented history of use in human medicine and has also been used in veterinary medicine for the treatment of epilepsy. It has recently been discovered that VPA has HDACi properties. Newer sustained-release formulations of oral VPA have demonstrated a relatively safer toxicity profile compared to early formulations, and since VPA is readily available and relatively inexpensive it serves as a very useful drug for evaluation of HDAC inhibition in canine cancer.

In Chapter 2 we demonstrated the VPA is capable of inhibiting HDAC in human and canine OS cells, but that it has relatively little antiproliferative activity when used as a single agent at clinically achievable doses. However, combination treatment of OS cells with VPA and DOX resulted in sensitization of these OS cells in a schedule dependent manner; pre-treatment with VPA resulted in greater chemosensitization, and this effect was associated with increased nuclear accumulation of DOX. This effect was tested *in vivo* in a xenograft model of OS. Pre-treatment of mice for 48 hours with VPA resulted in enhanced tumor control compared to either agent alone. Tumors from these xenograft experiments demonstrated a decreased proliferative capacity and increased apoptosis, evident by decreased Ki67 and increased TUNEL staining, respectively.

These results were quite promising and led us to proceed with a clinical evaluation of the combination in dogs with spontaneous cancer. In Chapter 3 we describe a Phase I dose-escalation trial of oral VPA given for 48 hours prior to a standard dose of

doxorubicin. The initial goal of this study was to determine a maximum tolerated dose (MTD) of VPA for use in future trials. We demonstrated that doses of VPA up to 240 mg/kg/day are well tolerated in dogs with only mild to moderate side effects reported. In fact, further dose escalation was halted due to complications with owner compliance due to the number of pills that had to be administered. We showed that VPA follows linear pharmacokinetics over the dose range tested and importantly, does not potentiate the myelosuppression or GI toxicity associated with DOX administration. In addition, we documented antitumor activity in patients with tumors that are traditionally resistant to anthracycline therapy (melanoma).

Furthermore, we detected target modulation in patient PBMC samples after 48 hours of VPA therapy, and the magnitude of this change correlated with the administered dose. Interestingly, there was no correlation between trough plasma VPA concentrations and the magnitude of target modulation in PBMC, which suggested that the biologic half-life of VPA exceeds the plasma half-life and this particular pharmacodynamic marker is a better indicator of overall 48 hour VPA exposure. We also demonstrated target modulation in tumor tissues of some patients following VPA therapy and interestingly, there did not appear to be any correlation between the dose administered and tumor tissue target modulation. This suggests that the traditional method of MTD dosing may not be applicable to HDACi therapies as increasing dose did not correlate with an increased chance of target modulation in tumor tissues.

In addition to the previously documented ability of VPA to alter chromatin structure and lead to increased nuclear DOX accumulation, we also were interested in evaluating the repertoire of genes whose expression is altered in response to VPA

administration. This was accomplished by gene expression microarray analysis of a canine OS cell line treated with VPA. In Chapter 4 we report the results of the microarray analysis. The majority of expression microarray analysis reports tend to focus on individual genes that are most significantly altered in response to a specific treatment in an attempt to identify potential targets. In addition to evaluating a few of these genes with large changes in expression, we also wanted to evaluate the molecular pathways that are altered by HDACi in OS, with the goal of not only elucidating the mechanism of action, but also identifying potential targets for combination therapies.

The most significantly altered pathway was oxidative phosphorylation; VPA treatment appeared to result in an increased expression of a number of genes within the pathway. Additional pathways identified involved cytoskeleton remodeling, antigen presentation, and cell cycle control. It is interesting to note that these pathways were also identified as the most differentially expressed in a panel of primary versus metastatic carcinoma tumors in humans. Furthermore, the ubiquitin-proteasome pathway was significantly altered following VPA treatment. We were able to validate the results with a combination of quantitative real time RT-PCR performed on select genes from altered pathways combined with functional assays for additional confirmation. We confirmed the inhibition of the proteasome pathway through demonstration that VPA treatment of human and canine OS cells results in decreased chymotrypsin-like activity (specifically calpain activity), and showed that combining HDACi with proteasome inhibitors may be a promising therapeutic option as well. We also demonstrated that VPA treatment of canine OS cells results in increased NQO1 activity and may potentially sensitize tumor cells to another chemotherapeutic agent, mitomycin C, which is partly dependent upon

NQO1 activity for activation. Finally, we demonstrated that treatment of canine and human OS cells with VPA at doses that have minimal effects on cell survival are capable of reducing the secreted levels of two important proteins in OS pathogenesis; endothelin-1 and VEGF. Both of these proteins have been found to be elevated in tumor bearing patients and are correlated with poor survival.

In conclusion, we provide a rationale for the introduction of VPA to DOX-based chemotherapeutic protocols for the treatment of OS and provide further validation of the model of canine OS for studying this disease in human patients. We have elucidated some of the potential mechanisms through gene expression and pathway analysis and demonstrate that VPA can be safely administered to dogs prior to DOX.

Future Directions

There are a number of additional studies that could be performed to further validate the use of this particular combination in the treatment of OS. In Chapter 2 we demonstrate that the combination has a superior ability to delay the growth of subcutaneous xenograft tumors in nude mice; however, primary tumor control in OS is not typically a problem as the majority of patients succumb to metastatic disease. While *in vitro* experiments that evaluate migration and invasion may shed some light onto the anti-metastatic potential of this combination, these models ignore the role that tumor stroma and the associated cells play in the metastatic process. Designing a mouse study that uses a more clinically relevant model of OS, such as orthotopic injection of a spontaneously metastasizing cell line (1) would be an important next step. The anti-metastatic potential of VPA in this case could then be evaluated by initiating treatment

with either DOX or VPA/DOX following removal of the primary tumor and then evaluating the time to metastasis in these two groups.

While this particular experiment is more clinically relevant to the case of canine OS, therapy in humans differs in the fact that there is a use of multi-modal chemotherapy prior to surgical removal of the primary tumor and the response to this therapy is prognostic for survival. Thus, a study that evaluates an orthotopic model treated with VPA/DOX prior to removal with subsequent histologic evaluation of tumor necrosis may provide better information on the potential of this combination in humans. This type of study is not easily performed in canines because of the increased risk of pathologic fracture associated with surgical delay to administer chemotherapy. In addition, increased anti-tumor activity of the combination in primary tumors could also potentially lead to increased chance of pathologic fracture; thus, these studies may be best performed in murine models.

In addition to evaluating VPA/DOX combinations in more clinically relevant models, further investigation into the specific role of HDAC enzymes in the development and progression of OS is an important step in further development of HDACi containing treatment protocols. The use of isoform-specific inhibitors of HDAC used alone and in various combinations would not only help to elucidate the specific role of HDAC in osteosarcoma but also provide a potential means to improve the therapeutic index of combinations by targeting only those isoforms that are involved in OS pathogenesis.

In Chapter 3 we demonstrate that VPA doses up to 240 mg/kg/day are safely administered to dogs prior to DOX, but we failed to reach a MTD. However, we demonstrate that there is target modulation in tumor tissues even at lower doses. An

additional study that could be implemented to determine if lower doses are just as biologically effective as higher doses would be to randomize dogs with a single tumor type into two groups: low dose and high dose VPA combined with DOX and then evaluate the magnitude of histone hyperacetylation in tumor biopsies. This would also allow us to compare objective tumor responses in the two groups. Additionally, it would be important to identify potential biomarkers that would predict the ability of VPA to hyperacetylate tumor tissues. As our clinical study included dogs with a variety of tumor types, this correlation could not be adequately performed. Evaluation of the expression of each HDAC isoform before VPA treatment would be a good marker to look at in this type of study.

Another clinical study that would be a natural progression from the work reported within this dissertation would be a randomized, placebo-controlled clinical trial of post-surgical adjuvant use of VPA/DOX in dogs with OS following amputation. This study should include dogs with no evidence of metastasis at the time of diagnosis, determined by a more rigorous methodology than thoracic radiography (potentially PET-CT) so that a true comparison of the time to metastasis could be evaluated.

In Chapter 4 we provide insight into the molecular pathways that are altered by HDAC inhibition in canine OS cells, and attempt to validate these results by PCR and functional/biochemical assays. Validation of human cell lines by qPCR still needs to be performed. Also, repeating the PCR and functional assays with a structurally distinct class of HDACi such as TSA should be performed to determine if our results are specific to VPA or can be generalized to HDAC inhibitors as a whole.

The importance of the oxidative phosphorylation pathway in tumor progression and metastasis certainly deserves some attention in studies using VPA. Although the end result of our pathway analysis showed upregulation of ATP synthase, simply measuring ATP levels in treated versus untreated cells is not likely to validate this finding as there are multiple means of generating cellular ATP. Isolated mitochondria could be used for this type of study, but then the interaction between other cytosolic and/or nuclear factors is omitted. Since the regulation of oxidative phosphorylation is generally associated with regulation of mitochondrial membrane potential then measuring the membrane potential of VPA treated cells could provide some validation of alterations in this pathway. Additionally, since oxidative phosphorylation has been shown to play a role in Bax-mediated apoptosis in OS, we could evaluate the effect of Bax overexpression in control and treated cells, along with measurements of mitochondrial membrane potential.

With the demonstration that combinations of VPA and proteasome inhibition may be promising in the treatment of OS, studies should be implemented that determine if there is a specific schedule dependence of this particular combination. We demonstrate that co-incubation appears to provide an enhanced anti-proliferative effect but this may be enhanced or antagonized with pre-treatment of either drug. Based upon the preliminary findings of antiproliferative activity of VPA and proteasome inhibition, this would be a good combination to administer prior to DOX.

Finally, it would be interesting to evaluate some of the intrinsic differences between hematologic and solid tumors in regard to their sensitivity to HDAC inhibition. Although HDAC enzymes have traditionally been described as having zinc-dependent catalysis, it has recently been shown that other divalent metals can function in the place

of zinc and, in fact, the enzymatic activity is actually higher when iron (Fe^{2+}) is in the catalytic site (2). In addition, this same study discovered that HDAC8 activity is oxygen sensitive with the highest activity seen in an environment of relative hypoxia. This was suggested to be partly due to the oxidation of Fe^{2+} to Fe^{3+} when oxygen is present; Fe^{3+} has a lower affinity for the HDAC8 catalytic site and is replaced by Zn^{2+}. Furthermore, the current class of inhibitors appears to have the highest affinity for Zn-HDAC, with a reduced affinity for Fe-HDAC.

This brings into question not only the *in vivo* metal that is present in these enzymes, but also the design of inhibitors of HDACs. If Fe^{2+} is actually the metal that is present *in vivo*, then design of drugs that have a higher affinity for Fe-HDAC might provide more anti-tumor effect. In addition, it might be possible that the increased responses to hematologic tumors with HDACi relate to relatively higher levels of Zn than Fe within the catalytic site. It is possible that solid tumors, with the relatively higher levels of hypoxia compared to hematologic tumors, retain iron in the Fe2+ state which has a lower affinity for current inhibitors. This could also lead to more tumor-specific inhibitors with reduced side-effects, as the solid tumor will be relatively more hypoxic than normal tissues.

Measuring the *in vivo* metal in HDAC enzymes has a number of potential problems as oxygen introduced into the experiment in any step could potentially result in the oxidation of Fe^{2+} to Fe^{3+} and replacement within the catalytic site by Zn^{2+}. However, by sparging all solutions with argon to remove dissolved oxygen and working in an anaerobic environment (Nitrogen-Hydrogen box) it may be possible to measure the difference between the metal content of HDACs in hematologic versus solid tumors. An

immunoprecipitation of a specific HDAC from isolated tumor samples, followed by inductively coupled plasma-mass spectrometry to determine the levels of iron versus zinc might reveal a difference that could correlate to HDAC inhibitor sensitivity.

In addition, screening of compounds that have a high affinity for Fe-HDAC might uncover novel therapeutics that could be combined with the current class of Zn-HDAC inhibitors to enhance therapeutic effect through a greater inhibition of HDAC activity within a tumor.

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