IDENTIFICATION OF NOVEL TREATMENT APPROACHES FOR HUMAN AND CANINE OSTEOSARCOMA

By

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ABSTRACT

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Osteosarcoma (OSA) is an aggressive neoplasm, characterized with high level of heterogeneity, high metastatic potential and poor prognosis in both humans and dogs. In this study, I used drug screening studies including existing therapeutic agents and novel compounds to identify more effective approaches to treat human and canine osteosarcoma.

One of the challenges in the field of OSA is to identify optimal tools for study. A limited number of human and canine OSA cell lines are available. In this study, I established and characterized a new cell line, BZ, derived from a German shepherd dog with OSA and studied key oncogenic pathways in BZ. Our findings revealed activation of STAT3 and ERK pathways in BZ, as well as in a number of other cell lines, indicating that these two pathways are critical for cell survival and proliferation in OSA and the potential of using STAT3 and ERK inhibitors.

Furthermore, I screened ten tyrosine kinase inhibitors (TKIs) on two dog and one human OSA cell lines. Among the selected TKIs, sorafenib showed promising results in effectively inhibited cell growth and migration *in vitro* studies. In addition, the effects of combing sorafenib with current chemotherapeutics (cisplatin, carboplatin, and doxorubicin) for OSA were investigated. Data from the combination index pointed to synergistic effects of sorafenib combined with doxorubicin and resulted in profound cell arrest at G2/M phase. In contrast, combination of sorafenib with cisplatin or carboplatin in both human and canine OSA cell lines proved to be antagonistic.

In addition to sorafenib, two other novel drugs with different mechanism of action were identified from the drug screening: the proteasome inhibitor bortezomib and the bromodomain inhibitor JQ1. Both drugs showed IC_{50} concentrations in achievable plasma ranges in all 3 human and 4 dog cell lines studied. In addition to inhibition on cell growth both drugs inhibited migration, and invasion properties of the cell lines. The co-incubation of bortezomib and JQ1 induced synergistic on both hOSA and cOSA cell lines, suggesting the use of this combination for future studies.

These studies also further revealed the heterogeneity among different cell lines. The cell lines differed in their sensitivity for each drug, as well as key tumorigenic pathways that were activated. While only some showed activation of AKT, more cell lines showed ERK and STAT3 activation. This heterogeneity was present in both human and canine cell lines. While there is heterogeneity in OSA in both species, there share similar variations, and studying a wide range of cell lines and canine patients should lead to translatable findings to the human OSA patients. Our studies point out to the need and the possibility of molecular characterization of the cell lines and of the patient populations. The drugs and drug combinations identified in our studies should form the basis for trial designs that are informed by the molecular characteristic of each tumor in patients enrolled. The proteasome inhibitor bortezomib has a significant adverse effect profile, and JQ1 is still under development. However, clinical trials of sorafenib and sorafenib combined with doxorubicin in canine OSA are warranted and should yield findings translatable to the human.

This dissertation is dedicated to my family:
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KEY TO ABBREVIATIONS

Bor	bortezomib
CDDP	cisplatin
CDK	cyclin-dependent kinase
CTCs	circulating tumor cells
cOSA	canine osteosarcoma
DFI	disease-free interval
DFS	disease-free survival
DMSO	dimethyl sulfoxide
DOX	doxorubicin
EGF	epidermal growth factor
EOD	every other day
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
GD2	disialoganglioside
HTS	high throughput screening
hOSA	human osteosarcoma
MST	median survival time
PBS	phosphate buffered saline
qRT-PCR	quantitative real time reverse transcription PCR
OC	osteocalcin

OSA	osteosarcoma
OS	overall survival
PD-1	programmed death 1
PDGFR	platelet-derived growth factor receptor
PFS	progression Free Survival
TKIs	tyrosine kinase inhibitors
VEGF	vascular endothelial growth factor

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Osteosarcoma (OSA)

1.1.1 OSA in humans and dogs

Osteosarcoma is the most common bone tumor in both humans and dogs. The diagnosis of osteosarcoma is based on the physical presentation (pain, limited joint movement, and swelling), radiological examination, and the definitive diagnosis is based on histological evaluation of fine needle biopsy or resected specimen [1, 2]. In humans, around 1000 cases were diagnosed with OSA (hOSA) in 2016 in the United States [3], with a major peak in adolescents (14-19 years of age) and in those over 70 years of age [4]. In canine OSA, besides an small incidence peak in 1–2-year-old dogs, the largest incidence is in older dogs from 6-10 years of age, with around 80% of cases occurring in dogs over 7 [4, 5]. Compared to the OSA incidence rate of 1/100,000 in humans [6], canine osteosarcoma (cOSA) has a much higher incidence rate with more than 10,000 cases diagnosed with osteosarcoma in the United States per year [7] and an estimated incidence rate of about 14/100,000 [8, 9].

In both species, the survival rate varies depending on whether the tumor is localized or has metastasized. In humans, around 20% of patients are diagnosed with metastatic disease at the time of presentation [10]. In hOSA, if the tumor is localized when diagnosed and treated, the overall 5-year survival rate is around 60% to 80%, but if the patient presents with metastatic disease, the 5-year survival is only 15 to 30% [6, 11]. In cOSA, the one-year survival rate with dogs receiving both surgery and chemotherapy ranges from 45-60% [10, 12, 13]. Similar to hOSA, metastatic disease in cOSA causes a difference in survival time. A study which reviewed OSA dogs that underwent amputation of the affected limb found that those with lymph node metastasis at surgery had a median survival time (MST) of 59 days, significantly short than 318

days in dogs without metastasis at the time of presentation [14]. In previous studies, although only a few of dogs (10%) presented with metastases at the time of presentation, around 90% of cOSA cases with surgery alone die of metastatic disease after one year of diagnosis of OSA [15, 16]. This aggressiveness is presumed to be associated with micro metastases, which are not detected by the diagnostic imaging techniques at the time of presentation [17-20]. Therefore, a canine OSA patient is considered to have high metastatic potential. In both species, metastatic OSA patients are difficult to treat, they are resistant to drugs and have very limited treatment options, thus novel therapeutic agents are urgently needed.

1.1.2 Epidemiology of OSA

The etiology of OSA in both humans and canines is complicated. In addition to the genetic factors that may be predisposing as described below in section 1.1.4, there are other environmental factors that may contribute to risk. These include prior bone injuries, history of implants in the bone, other trauma and prior radiation exposure [21, 22]. Nonetheless, most of the OSA cases are sporadic.

In cOSA, large breed dogs have a higher risk for osteosarcoma [5]. A survey from Norway, listed the OSA incidences rates ranging from 0.2 to 8.9% in four large breed dogs (8.9% in Irish wolfhound, 5.8% in Leonberger, 0.9% in Newfoundland, and 0.2% in Labrador retriever) among 4380 dogs [5]. Another study which contained data on 120 dogs from Poland reported that the OSA incidences were between 2.7 and 10.7%, and the most predisposed breed was the Rottweiler. In addition, among dogs affected with OSA, more than 80% were large and giant breed dogs [23]. A study which used data from 400,000 Swedish dogs, found that the Irish

wolfhound, St. Bernard, Leonberger, Great Dane and the Rottweiler, flat coated retriever, greyhound dogs, in this order, had the highest incidence rates among 56 breeds [24].

1.1.3 Dog as a translational model

Canine osteosarcoma is viewed as a good translational model for human OSA for several reasons. First, canine OSA closely resembles human OSA in histopathological characteristics and responses to conventional chemotherapeutic agents. Second, canine OSA occurs spontaneously [7], which is an good system to study the progression of this tumor. Moreover, cOSA has high metastatic potential, which provides a source to evaluate the responsiveness of novel therapeutic agents for the most challenging human counterpart [25]. Also, the similarity of genomic and transcriptomic findings provide the rationale of using canine OSA as a model for studying human disease [26].

1.1.4 Genetics of osteosarcoma

Osteosarcoma is a genetically diverse and karyotypically complex cancer in both species and in nearly all cases examined, it is characterized by significant somatic copy number alteration, structural variations, and chromosomal instability [27-29]. While most cases are sporadic, there are inherited mutations in tumor suppressor loci that predispose to OSA.

1) Genetic predisposition

In hOSA, there is an increased incidence of primary OSA associated with several genetic syndromes. Two of the most common germline mutations are Li-Fraumeni syndrome (germline mutation of the *TP53* gene) and hereditary retinoblastoma (germline mutation of the Rb gene)

[30, 31]. Patients with Li-Fraumeni syndrome have high risks of having wide range of cancers such as osteosarcoma, soft tissue sarcoma, breast cancer, brain tumors, acute leukemia, adrenal cortical cancers, and gonadal giant cell tumors [32, 33]. Other germline mutations associated with risk of osteosarcoma in humans include Rothmund Thomson syndrome type 2 (germline mutation of the *RECQL4* gene), Werner syndrome (germline mutation of the *RECQL3* gene), Bloom syndrome (BS, germline mutation of the *RECQL2* gene), RAPADILINO syndrome, and Diamond Blackfan anemia [34-36].

In cOSA, a recent study used whole exome sequencing to compare matched tumor and normal tissues with three breeds (golden retrievers, Rottweilers, and greyhounds) predisposed to OSA [37]. The authors reported that the commonly seen germline variants in humans such as *RB, P53,* and *NFIB* are not as common in dogs [37], nonetheless, the most common affected genes are *CDKN2A/B* (31.8%) and *GRM4* (18.2%) within the examined cases. A genome-wide association study revealed 33 risk loci for osteosarcoma, including *CDKN2A/B, AKT2*, and *BCL2* [38] in three high-risk breeds (greyhound, Rottweiler, and Irish wolfhound), but no specific mutations were identified.

2) Somatic Mutations

Most of the somatic mutations associated with this disease are seen in both species and include *CDKN2A/B, DLG2, MYC, TP53, PTEN, SETD2, and RUNX2* [7, 36, 37, 39, 40].

In dogs, OSA patients have similarities of several top somatic mutations or copy number alterations in *TP53, RB1, PTEN,* and *MYC* [29, 40].

Another common somatic mutation in cOSA is in tumor suppressor gene *SETD2*. One study documented that mutated *SETD2* was found in 21% of examined samples whereas other

reports of hOSA had less than 2% in *SETD2* mutations [37]. Moreover, a similar finding was reported earlier this year, where besides somatic point mutations in *TP53* (71%), mutations in are *SETD2* (42%) and *DMD* (50%) were reported [40]. The inactivation of tumor suppressor gene *SETD2* was described in renal carcinoma and lymphoma in humans [41, 42]. While the loss of function of *SETD2* in cOSA is not clear, *SETD2* as a potential oncogenic driver was found to regulate *p53*; with mutations in these two genes being concurrent in cOSA [37, 40]. Although loss of DMD has not been reported in human OSA [40], DMD deletions were associated with enhanced cell migration, invasion, cell growth in several human cancer cell lines (gastrointestinal stromal tumor, rhabdomyosarcoma, and leiomyosarcoma), suggesting it may function as a tumor suppressor [43].

1.2. Prognostic factors for OSA

Traditional prognostic factors in osteosarcoma include clinical factors like age, gender, tumor histological grade, tumor site, and stage. However, different techniques and endpoints used for evaluation make comparisons between studies difficult [44]. In addition, group size and specific target groups might also alter the conclusion of prognostic determinants. For instance, it is believed that young age at diagnosis is a negative prognostic factor, however, a research group pointed out that compared to younger patients, older patients (>65) presented with a worse prognosis. These studies are complicated by the variable use of chemotherapies in some of the studies and the variable presence or absence of metastatic disease at diagnosis, and variable inclusion/exclusion criteria of the studies [45].

Another potential prognostic indicator shared by both species is post-surgical infection. In dogs, deep infection is common after limb-sparing surgery [46-48]. In two small cohorts of cOSA studies, 11 dogs with surgical infection had a 685 days of median survival time (MST) compared to 289 days in the control groups (9 dogs) [49]. In another studies of 47 dogs with limb-sparing surgery, the researcher reported that 69% of the dogs that had infection post-surgery, had a median survival time of 480 days, whereas patients with no infections had a median survival time of 228 days [47]. Later in 2018, a retrospective study reported that the MST between 15 dogs that had surgical site infection and 134 non infected dogs was not significant different. In human OSA, the effect of post-surgery infection is also controversial. A retrospective analysis of 412 patients reported 10% of patients with infection; the 10 year survival rate of the patients with infections was 85% as compared to the 62% of those without infections [50]. In another study of 79 patients, however, the one-year and two-year survival rate showed no difference between those 13 patients who developed infection as compared to the other 66 patients that did not have an infection [51]. The positive effect of post-surgical infection on patient survival might seem unexpected as infection control is part of standard operating procedures in all surgeries both human and veterinary medicine and infection is an unwanted complication. However, most tumors also deploy immunosuppressive signals to their surroundings and recent insights into the effects of immune activation on combatting tumor growth [52, 53] may explain these paradoxical findings.

In dogs, different osteosarcoma locations result in different prognosis and survival times. Dogs with OSA of maxilla, mandible, or calvarium who received surgery had a median survival time of 329 days [54], while others with tumors in ulna had an overall survival (OS) time of 277 days

[55]. When tumor location was divided into radius or non-radius sites, 14 out of total 48 dogs with tumors at radius reached 596 days of OS time whereas dogs with non-radius OSA resulted in a shorter OS time of 232 days [20]. Although these studies reported a significant difference on disease free and survival time based on the locations of tumors, the conclusions should be interpreted with caution as the authors did not report which dogs in each category received chemotherapy.

OSA is very aggressive disease and recurrence is common. Despite improvements in diagnostic imaging, micro metastases are frequently not detected. Therefore, developing a highly sensitive diagnostic method to detect early micro-metastasis or monitor high risk OSA patients would benefit the patients. For example, additional treatments or change of regiment may be included earlier if the progression of OSA can be monitored closely, and this will potentially improve the survival.

Besides traditional prognostic factors, other studies have focused on the correlations between molecular biomarkers, such as protein markers like hypoxia-inducible transcription factor (HIF-1), VEGF, c-FOS, with prognosis outcome in clinic (**Table 1.1**). Nonetheless, with the improvement of technologies and the need for real-time monitoring, other methods are being developed to meet the need. Three types of potential biomarkers are currently being studied: these are a) circulating tumor cells, b) analysis of circulating tumor derived DNA for mutations, and c) circulating miRNAs as will be discussed in detail below. Compared to traditional tissue biopsy, these sampling methods are known as liquid biopsy. Liquid biopsies have the advantage of relative simplicity, being noninvasive, and offering real time monitoring, and providing key tumor related information.

1.2.1 Circulating tumor cells (CTCs)

The CTCs are cells shed from a tumor site and enter the blood circulation and are considered as the major cause of tumor metastasis and recurrence in many different types of tumor, such as breast and prostate cancers [56-58]. In the peripheral blood, the CTCs are isolated from whole blood after erythrocyte lysis and flow cytometric analysis [59] or other techniques like dielectrophoresis and CTC enrichment technique [60, 61], or use of microfiltration in two and three dimensions [62]. The liquid biopsy of circulating tumor cells can be used to monitor the changes in an animal during chemotherapy. A preclinical model mouse model demonstrated that CTCs increased with the chemotherapy, ifosfamide, whereas the lung metastasis were decreased [60]. The author interpreted this observation as these cancer cells were released from the primary tumor site due to ifosfamide, and not reflecting more metastases. So, careful studies will need to be carried out when interpreting CTC numbers.

Utilizing CTC numbers as diagnostic and prognostic have been explored in hOSA clinical studies. A study demonstrated CTC counts are higher in metastatic patients and associated in patients with poor response of neoadjuvant chemotherapy [63]. In another study of small numbers of hOSA patients, researchers found that CTCs changes can be detected earlier than the appearance of lung metastases, an increase of mesenchymal CTCs are associated with shorter disease free survival [61]. Thus, the CTC studies have to be interpreted with caution and caution should be exercised when extrapolating from mouse models to the clinic.

In canine OSA, circulating tumors cells can also be detected. One study used flow cytometry to detect circulating tumor cells with positive staining of intracellular collagen 1 and osteocalcin during different period of treatments in three canine patients [59]. The CTCs decreased soon

after limb amputation and increased prior to metastases, suggesting the potential of using CTCs to monitor cOSA disease progression. However, some limitations might decrease the strength of this study, such as small number of canine patients and the lack of methods to verify the sensitivity of the liquid biopsy techniques. In our preliminary studies, we have been able to detect CTCs in canine osteosarcoma using CellSieve^{TM} Microfilters. OSA cells were isolated from peripheral blood by using this low pressure filtration equipment to filter with membranes contain 7 μM pores, and confirmed with biomarkers osteocalcin, vimentin, and negative for normal blood cells marker CD45 [64]. Additional studies are needed to effectively assess the utility and interpretation of CTCs in both human and canine OSA.

1.2.2 Circulating microRNAs

MicroRNAs (miRNAs) are a class of small non-coding RNA, showed strong association with oncogenesis through post-transcriptionally. They are involved in controlling critical biological processes such as cell proliferation, differentiation, apoptosis, and metastasis. In some types of cancer, cancer related miRNAs are released and can be detected in the blood, also reflecting the pathologic state of the tumor [65]. Since MicroRNAs are stable, RT-qPCR techniques can therefore measure the status of these circulating miRNAs and study the correlation between these potential biomarkers and the progression of disease. One study measured plasma levels of four potential circulating miRNAs, miR-547-3p, miR-214, miR-355-5p, and miR-205-5p in twenty OSA and fifteen healthy samples [66]. Among them, miR-205-5p was statistically significantly decreased whereas miR-547-3p, miR-214, miR-355-5p were statistically significantly increased in OSA plasma samples. In addition, miR-214 showed potential as OSA

prognostic factor since low plasma levels of miRNA-214 at diagnosis was associated with better outcomes in metastatic patient group. Other miRNAs that were found in plasma or in tumor samples that's are associated with OSA prognosis are summarized **in Table 1.2**.

In the canine OSA, clinical studies of circulating miRNAs are very limited. Only one clinical report focused on circulating miRNA and its clinical significance. This report suggested high circulating miR-126 predicted prolonged disease free survival and overall survival and high circulating miR-214 was corelated with short disease free survival and overall survival in canine osteosarcoma patients [67]. Despite the limited information of circulating miRNAs in canine osteosarcoma, several miRNAs were found to play a role in tumor samples, which can be viewed as potential candidates for future studies. Among them, miR-1 and miR-133b were found to be drown regulated in cOSA and associated with higher levels of MET and MCL1 [68].

1.3. Treatments of osteosarcoma

1.3.1 Standard of care therapeutic agents in hOSA

The current standard of care treatment for hOSA patients is surgery with wide excision of the primary tumor (unless the tumor is unresectable), combined with radiation and chemotherapies. With surgery alone, the overall 5-year survival rate is less than 20% [69, 70]. Chemotherapies for hOSA patients was started in the 1970s. With the advance of chemotherapy, either adjuvant or neoadjuvant treatment, the overall 5-year survival rate improved to around 75-80% [6, 71]. While systemic therapy has improved disease-free interval (DFI) and overall survival, there has been limited progress in developing more effective chemotherapies over the past three decades [72, 73]. In human OSA patients, doxorubicin,

cisplatin, carboplatin, ifosfamide, and high-dose methotrexate are the main components of chemotherapy regimens [2, 74, 75].

1.3.2 Standard of care therapeutic agents in cOSA

The standard of care for canine osteosarcoma is surgery, amputation or limb-sparing surgery (if the tumor locates at distal radius, and the tumor is affecting less than 50% of the limb [49]), followed by adjuvant chemotherapy. In cOSA, limb amputation is the current standard of care for local management [16]. Clinical outcomes between different treatment approaches from different studies are summarized in **Table 1.4**. Median survival time of amputation alone has been reported to be 119 to 175 days, with one-year survival rates range from 11 to 21% [16, 76-78]. Adjuvant use of chemotherapy was shown to result in a) increase in disease-free interval from 160 days to 226 days, b) nearly two-fold increase in median survival time and c) increase in one-year survival rate from 11-21 % to 33-62% with the addition of chemotherapy as compared to groups with surgery alone. These advantages were seen either with single chemotherapeutic agent [13, 76-82] or combinations of two [12, 17, 20, 83-85] in many studies. As summarized in **Table 1.4**, the most widely used chemotherapeutic regimens in cOSA dogs, cisplatin, carboplatin, and doxorubicin resulted in significantly prolonged overall survival time compared to dogs receiving surgery alone as the main treatment [8, 17, 48].

1) Cisplatin: Many studies provided evidence that cisplatin used in OSA treatment prolonged the overall survival time. With amputation only, the median survival time was around 134 days and the one-year survival rate was 11.5% in a report of 162 canine OSA cases [16]. There are two subsequent studies that used this amputation report [16] as a historical control, and

compared the outcomes of the dogs that received both surgery and cisplatin [77, 80]. One study of 16 dogs that received cisplatin after amputation reported a median survival time of 413 days with one-year survival rate of 62% of dogs (10 dogs) [80]. Another study of 22 dog cases reported a median survival time of 325 days and 45.5% of one-year survival rate with cisplatin [77]. Likewise, significant differences were noted in other two studies where cOSA cases treated with surgery alone or with additional cisplatin were directly compared [76, 78]. In one of these studies, among 30 dogs that presented with OSA, 15 dogs that were treated with surgery alone had a median survival time of 168 days, and 15 others treated with surgery and cisplatin treatment had a longer time of 290 days [76]. Another study indicated similar clinical benefit of cisplatin, 36 canine OSA patients treated with cisplatin resulted in significantly longer median survival time (262 days) than 35 patients treated with amputation alone (119 days) [78]. The duration and timing of cisplatin use was also evaluated in a study where 17 out of 36 dogs who received 2 cycles of cisplatin after amputation surgery were compared with 19 dogs that received one dose before and after surgery. The outcomes from two groups were similar (262 days versus 282 days), indicating the effects of the time to start chemotherapy induction did not have a significant effect [78].

2) Doxorubicin: Some studies indicate a significant longer median survival time for cOSA with doxorubicin as compared to surgery alone. A research group reported that the median survival time was prolonged with the doxorubicin treatments in 35 appendicular OSA patients, 366 days versus 134 days in the control group [16, 81]. In another study, researchers reported results of 303 dogs with appendicular OSA and treated with doxorubicin after amputation. The median survival time of dogs received doxorubicin was 240 days [13].

3) Carboplatin: Carboplatin as a single agent to treat cOSA was evaluated in a study with 48 cases, and these dogs reached a median survival time of 321 days and disease-free interval of 257 days [86]. This clinical benefit was also documented in another study which included 155 cases of cOSA. The dogs had a median survival time of 307 and DFI of 256 days after 3 cycles of carboplatin [82]. These two clinical studies showed that carboplatin is similarly effective as cisplatin, suggesting carboplatin can be use as alternative option for cisplatin. Instead of traditional IV administration, a single subcutaneous infusion of carboplatin was given after limb amputation in two studies. One of the reports showed a comparable median survival time of 365 days in 17 dogs [87], however, another report published in 2019 reported a median survival time of 196 days in 45 dogs treated with subcutaneous injection [46], which suggest this subcutaneous infusion protocol cannot replace IV administration.

4) Cisplatin + doxorubicin: Using combinations of chemotherapeutic agents is also validated in cOSA patients. In a study of 102 cOSA cases, 94 dogs that received three cycles of cisplatin and doxorubicin had a median survival time of 330 days [88].

5) Carboplatin + doxorubicin: Another commonly use combination is carboplatin and doxorubicin. Thirty-two dogs that received three cycles of carboplatin and doxorubicin after surgery had a median survival time of 320 days [84]. Another retrospective study reported 29 dogs that completed three cycles of this combination reached a median survival time of 258 days [20]. The results of carboplatin and doxorubicin indicated that this combination had a significant effect when compared to dogs treated with surgery alone. The combination reached effect similar with cisplatin plus doxorubicin, indicating this combination can be an alternative option for cOSA.

6) High-dose methotrexate: While high-dose methotrexate is used in the first line chemotherapy in hOSA, there is very little experience with this drug in canine patients. In the literature, only two clinical reports more than thirty years ago used high-dose methotrexate, one reported 4 dogs developed metastatic disease at an median time of 4 months after amputation [89], and another report used bone cement that contained methotrexate in 6 dogs and reported the time of local tumor recurrence to be 1-6 months [90]. A more recent study documented methotrexate inhibited tumor cells growth on a canine patient-derived cell line [91]. In veterinary medicine, high-dose methotrexate is not a major chemotherapeutic agent for cOSA, and further evaluation is needed.

1.3.3 Targeted therapies

With understanding of osteosarcoma biology, genomic and signaling transduction pathway analyses, and drug screenings, targeted therapies have shown potential in clinical trials. Several novel strategies have been proposed to use in osteosarcoma, such as 1) tyrosine kinase inhibitors like mTOR (mammalian target of rapamycin) inhibitor, IGF1-R (insulin-like 1 receptor) inhibitors, sorafenib, dasatinib [92-94], or 2) mTOR inhibitors like rapamycin, 3) monoclonal antibodies like denosumab and dinutuximab, 4) cyclin-dependent kinase (CDK) inhibitors.

1.3.3.1 Tyrosine kinase inhibitors

Tyrosine Kinase Inhibitors in Human OSA

In humans, tyrosine kinase inhibitors (TKIs) such as Apatinib and sorafenib have been tested in clinical trials for hOSA. In preclinical models, sorafenib inhibits cell growth, angiogenesis and metastasis through the inhibition of VEGF and MAPK/ERK pathway [95, 96]. The potential of

using sorafenib combined with cisplatin [96] and everolimus (mTOR inhibitor) [97] has been explored in preclinical models, and the latter combination was used in a human clinical trial [92]. Based on the guideline published by The European Organization for Research and Treatment of Cancer (EORTC) soft tissue and bone sarcoma group, in second line therapy, a 3-months progression-free survival in 40% patients suggested drug activity [98]. This clinical trial which combined sorafenib and everolimus reported 6-month progression-free survival in 45% of recruited OSA patients[92]. Therefore, this finding provided rationale for further investigation of using sorafenib with mTOR inhibitor. Apatinib is another TKI that has been used in advanced OSA patients who failed with standard care drugs, high-dose methotrexate, doxorubicin, cisplatin, and ifosfamide [99]. In this Phase 2 clinical trial, a total of 37 patients were included and 57% had progression free survival of 4 months [99]. In the case report, an OSA patient with lung metastases received apatinib as the only therapy and reached partial response after 11 months of treatment [100].While the number of patients involved in these two clinical trials are small , the outcome of improved survival time brought further insights into the treatment of OSA. Understanding the complex biology of osteosarcoma will benefit the patients by identifying the targets for individual patient and offering more individualized therapeutic options.

Tyrosine Kinase Inhibitors in Canine OSA

Despite the use of nearly a dozen tyrosine kinase inhibitors in human clinical trials for hOSA, the knowledge is very limited [101]. A few TKIs, masitinib [102], erlotinib [103], dasatinib [104] have been used in a limited way in cell culture studies in cOSA and showed some anti-tumor effects. Dasatinib, a multi-kinase which was used a first line drug in one cOSA case [105] and

used as an adjuvant therapy after amputation and carboplatin treatment in four dogs with cOSA[104]. Both of these studied showed promise of these approaches, with prolong survival times ranging from 1.5 to 3.2 times longer than median survival time when compared to historical controls. Three TKIs have been approved in the veterinary medicine to date: toceranib, masitinib and oclacitinib [101]. Toceranib and masitinib both target c-KIT. Masitinib had conditional approval for mast cell tumors in the US, but was not extended beyond 2015, although it is still available in Europe. The main target of palladia and masitinib, c-KIT, is not a major oncogenic driver in cOSA. Palladia and masitinib may also have effects on other similar TK receptors, such as the VEGF receptors, and may find some use in cases where overexpression of these receptors are involved, but no definitive evidence of their usefulness has been documented. The other approved TKI, oclacitinib, targets JAK, which is not relevant in cOSA. Thus, the potential of use of TKIs is an understudied area in cOSA and other veterinary cancers.

1.3.3.2 Targeting MYC pathway in OSA

The c-MYC oncogene is documented to be a major driver of OSA. Amplification of the MYC gene has been reported in OSA [106], and high-level of MYC is associated with poor prognosis in osteosarcoma patients [107]. While there are no established drugs that have successfully targeted MYC, there are some novel agents under development that show some promise. Among those novel therapeutic agents targeting MYC, one class of drugs is bromodomain and extra-terminal domain (BET) inhibitors. BETs regulate various genes involved in the cell cycle, cell proliferation, and inflammation [108]. To date, the most common pharmacological inhibitors of BET protein family are JQ1, I-bet151, and I-bet762. JQ1 is a selective small

molecular inhibitor of BRD4 (bromodomain-containing protein 4) [109, 110]. However, the effects of JQ1 and related mechanisms in OSA remain controversial. While the first study in humans OSA cell lines reported JQ1 regulates MYC expression [111], two other studies showed that JQ1 alone failed to inhibit MYC expression [110, 112]. While JQ1 as single therapeutic agent failed to inhibit MYC, JQ1 synergized with a mTOR inhibitor rapamycin and downregulated MYC and reduced tumor volume in a mouse model [112]. Other inhibitors in the literature involved in the suppression of MYC expression in OSA are Cyclin-dependent kinases inhibitors. Cyclin-dependent kinases are members of a family of protein kinases, involved in essential cellular processes such as cell cycle and cell proliferation. CDK4/6 inhibitor Palbociclib and CDK9 [106, 113] inhibitors have been considered as promising therapeutic agents for OSA. A study used CDK9 (cyclin-dependent kinase-9) to suppress MYC expression successfully inhibited tumor growth in a preclinical model [106].

1.3.4 Immunotherapies for OSA

The observation that post-surgery infections correlated with prolonged survival time and metastasis-free interval in certain patients indicated that the immune activation can have a major positive role in curtailing osteosarcomas [114]. Several immunotherapeutic strategies have been explored to improve the OSA treatments. These include activation of the immune system with specific antibodies and removal of immunosuppressive signals as discussed below.

1.3.4.1 Monoclonal antibody therapy

Prospective targeted therapies in hOSA also Treatments with humanized monoclonal antibody Denosumab has been reported in hOSA patients [115, 116]. Denosumab binds to nuclear factor

kappa-β ligand (RANKL) and prevents the interaction of RNAKL and RANK, which leads to the inhibition of osteoclast activation [117]. Preclinical animal model demonstrated that anti-RANKL agents have an effect on inhibition of tumor growth as well as metastatic disease [118]. Denosumab is currently phase II clinical trial with relapsed or refractory OSA in humans. Another promising monoclonal antibody is dinutuximab (mAb ch14.18), which was first developed to target disialoganglioside (GD2), a cell surface antigen expressed in human neuroblastomas and also expressed in osteosarcomas [119]. An OSA patient was include in a phase I clinical trial [120] and the use of dinutuximab is currently in phase II study in humans.

1.3.4.2 HER2-targeting *Listeria* vaccine

One of the novel strategies to improve the treatment efficiency or prevent metastatic disorder in cOSA is a cancer vaccine strategy. HER2 is a tyrosine kinase receptor belonging to the EGFR family and is found to be overexpressed in breast, prostate and pancreatic cancers [121, 122]. HER2 expression is often associated with activation of tumor growth, tumor cells survival, and thus with poor prognosis. In OSA, overexpression of HER2 was observed in 32% of human OS patients and corelated with worse clinical outcome and resistance to chemotherapies [123]. A recent Phase I trial documented the first Listeria-based vaccine approved for clinic use in veterinary medicine, named as ADXS31-164 [124]. ADXS31-164, established to induce HER2-specific immunity, was given in 18 dogs with a protocol of every 3 weeks for 3 doses after 3 cycles of carboplatin. Fifteen dogs out of 18 vaccinated dogs developed T-cell response against HER2 with a delay in disease progression and improve overall survival. The MST in this trail is 956 days, with 56% of survival rate at 3 years after the treatments, compared to 423 days and

22% respectively to the control group. In humans, a Phase I/II trial has tested this strategy in human patients (NCT02386501) [125, 126] but the results of the trial have not been published. However, the possibility of *Listeria* infections in anti-cancer vaccines was reported in both human [127] and dogs [128]. Recently, a case report of dogs developed *Listeria* abscess located at the rib after 3 doses of vaccine [128], and in January 2020, the trial in dogs was suspended. The results of the human clinical trial have not yet been announced. Regardless, these studies further support that canine OSA is a useful platform to improve the treatment options for hOSA but also provides informative information for parallel studies.

1.3.4.3 Anti PD-1/PD-L1

Immune checkpoint inhibitors such as anti-programmed death (PD-1) and anti-PD-ligand 1 (PD-L1) antibodies showed potential for several types of cancers in humans, including non-small cell lung carcinoma, advanced melanoma, renal cell carcinoma. The investigation of PD-L1 and PD-1 is limited in osteosarcoma. One study reported high expression of PD-L1 is associated with shorter survival time in hOSA [129]. Another study indicated the correlation between high and low PD-L1 (refer to normal bone) expression and the clinical outcome; high expression of PD-L1 patients had a longer survival time compare to patients with low PD-L1 expression [130]. In veterinary field, a study investigated PD-L1 expression in various cancer types reported that 7 out of 10 tested cOSA samples showed positive PD-L1. This finding indicated the potential of PD-1/PD-L1 as therapeutic targets for canine OSA , but, further studies are need [131]. Immune based therapies discussed above can be combined with chemotherapy to enhance anti-tumor effects. With the success of immune therapies in other cancer types, and as the field

of immunotherapy expands, more treatment approaches that have an immune activation component hold potential for OSA patients.

1.3.5 Current clinical trials in hOSA

As compared to improvements in survival in various other tumor types, the survival rate of OSA patients has not improved for 30 years [6, 73, 132]. Even with current treatment options, patients with such aggressive tumors still have many challenges. Challenges include the development of resistance to conventional chemotherapies, recurrence and treatment of metastatic disease. Therefore, further research is needed to develop effective therapeutic agents for advanced osteosarcoma patients.

The current clinical trials for hOSA are summarized in **Table 1.5.** There are a number of small molecules that target tyrosine kinases and oncogenic signaling pathways, as well as monoclonal antibodies that target tumor antigens (RANKL and GD2) and PD-1.

1.4 Conclusion

Osteosarcomas are naturally occurring cancers in dogs and humans. In both species, the etiology of OSA remains unclear. Osteosarcoma is a heterogenous tumor with a variety of cell populations, therefore, revealing knowledge of the molecular and cell-signaling pathways involved in OSA will contribute to the identification of biomarkers, lead to discovery of potential drug candidates, improve the recognition of potential patients who will benefit from different therapeutic strategies and ultimately improve patient survival.

Most OSA patients are now treated with conventional chemotherapies, however, many patients with relapsed or metastatic disease will not benefit from the current therapeutic strategies. Preclinical animal models play a critical role in the drug discovery process. Using canine patients as a platform will not only benefit canine OSA patients but also will provide possibility of using novel therapeutic strategies in the humans because of the similarity in responses to chemotherapy in two species. Given this background, we wanted to explore novel therapeutics on cOSA and hOSA. In the following chapters, we present establishment and characterization of a patient-derived cell from a canine OSA, explore repurposing existing drugs by screening for cytotoxicity against OS cell lines as well as novel drugs and investigate the potential mechanisms involved in tumorigenesis and treatment response.

APPENDIX

Table 1.1. Comparison of characteristics between human and canine OSA.
The table is modified from Morello E, Martano M, Buracco P. Biology, diagnosis and treatment of canine appendicular osteosarcoma: similarities and differences with human osteosarcoma. *Vet J.* 2011;189(3):268-277 [133].

	Human OSA	Canine OSA
Age	Major peak: young adolescent (14-19 years old) elder >70 years old	Major peak: elder dogs 6-10 years old 1–2 years old
Race/breed	Not applicable	Large purebreds [5]: Irish wolfhound, Leonberger, Newfoundland, Labrador retriever
Incidence per 100,000	1	14
Gender	Male predominance [134] Female tend to present at earlier age	Male predominance [10, 134]
Locations of common tumor sites	80-90% long bones [10] Distal femur> proximal tibia> proximal humerus	75-77% long bones Distal radius> proximal humerus> distal femur>tibia
Survival rate	70% at 5 years with chemotherapy	60% at 1 year with chemotherapy
Metastasis rate without chemotherapy	80% before 2 years	90% before 1 year
Most common metastatic sites	lung	
Associated molecular and genetic factors [10, 40, 44, 135]	*TP53*: mutated and/or overexpressed IGF-1/IGF1-R: over expressed*, poor outcome HGF/c-Met: over expressed erbB-2/HER-2: over expressed*, poor outcome *PTEN*: mutated or down regulated Ezrin: detected Matrix metalloproteinases: expressed Sis/PDGF: expressed VEGF: expressed *Rb*: mutated or down regulated MMP-2, MMP-9: up regulated	*TP53*: mutated and/or overexpressed DMD: somatic point mutations, deletions, and chromosomal translocations SETD2: mutated IGF-1/IGF1-R: over expressed, poor outcome HGF/c-Met: over expressed erbB-2/HER-2*: over expressed, poor outcome *PTEN*: mutated or down regulated Ezrin: detected Matrix metalloproteinases: expressed Sis/PDGF: expressed VEGF: expressed COX-2 Angiogenetic factors Telomerase reverse transcriptase gene
IHC markers	osteocalcin, alkaline phosphatase [136]	osteocalcin, vimentin, osteonectin [137] Alkaline Phosphatase [138]

*Denotes that overexpression of the gene in the tumor was correlated with poor outcome

Table 1.2. Comparison of prognostic markers between human and canine OSA

	Human OSA	Canine OSA
Positive prognostic indicators	Post-operative limb sparing infection [47, 49, 50] High percentage of necrosis induced by chemotherapy or radiotherapy	
Negative prognostic indicators	Tumor volume, grade Metastasis at diagnosis: lung, bones, lymph nodes High serum ALP, LDH activities Young age at diagnosis	
Prognostic markers	microRNAs (miR-214, miR-382, miR-134, miR-544) DNA methylation analysis Circulating tumor cells (CTCs)	microRNAs (miR-214, miR-126, miR-34a)

Table 1.3. Circulating miRNAs found in patients (serum or plasma)

miRNA	Expression in human	Expression in Canine	Target	reference
miR-9	If high: short OS		TGFBR2	[139]
miR-34a	If low: short DFS	decreased	hOSA: P53 cOSA: KLF4 and VEGFA	[140-142]
miR-34b	down		hOSA: P53	[143]
miR-92a	up		cOSA: PTEN/AKT	[144, 145]
miR-126		If high: longer DFS and longer OS		[67]
miR-133b	If low: short DFS and OS			[146]
miR-192	If low: short DFS		hOSA: P53	[141]
miR-205-5p	down in OSA patients			[66]
miR-206	If low: short DFS and OS			[146]
miR-214		If high: short DFS and short OS		[66, 67]
miR-335-5p	up in OSA patients			[66]
miR-574-3p	up in OSA patients			[66]

DFS: disease-free survival; OS: overall survival

miRNAs as a prognostic marker found in tumor sample

	Expression in human	Expression in Canine	Target	reference
miR-1		decreased when compared with normal bone	MET	[68]
miR-133b		decreased when compared with normal bone	MCL1	[68]
miR-34a		decreased when compared with osteoblasts	KLF4 and VEGFA	[140]
miR-34b	decreased when compared with normal tissue		P53	[143]

Table 1.4. Comparison between no chemotherapy, or with chemotherapies, or chemotherapies and immunotherapy after surgery in cOSA

Surgery only							
Treatment	Reference	Median survival times (days)	Disease-free interval (DFI)	one-year survival (%)	Two-year survival (%)	Surgery type	Study sizes
Surgery alone	Shapiro 1988 [79]	102	NA	NA	NA	amputation	Total 19 n= 8 in surgery alone group
	Spodnick 1992 [16]	134	NA	11	2	amputation	162
	Mauldin 1988 [83]	175	160	21	0	amputation	19
	Thompson 1991 [76]	168	NA	20	NA	amputation	15
	Straw 1991 [78]	119	NA	11	4	amputation	35
Surgery + chemotherapy							
Treatment	Reference	Median survival times (days)	Disease-free interval (DFI)	one-year survival (%)	Two-year survival (%)	Dosage	Study sizes
Cisplatin	Shapiro 1988 [79]	301	NA	NA	NA	2-6 cycles of 40-50 mg/m^2 every 4 weeks	Total 19 n= 11 in surgery +cisplatin group
	Thompson 1991 [76]	290	NA	33	NA	2 doses of 60 mg/m^2 given at 2 and 7 weeks after surgery	15
	Straw 1991 [78]	262 282	226 177	38 43	18 16	1 or 2 doses of 70 mg/m^2 Group 1 (n=17): two cycles of cisplatin after surgery Group 2 (n=19): one cisplatin before and one after surgery	36

Table 1.4. (cont'd)

	Kraegel 1991 [80]	413	NA	62	NA	6 cycles of 50 mg/m^2 Given 1 or 2 weeks after amputation (n=5 received <6 cycles; n=3 received 6 cycles, and n=3 received > 6 cycles)	16
	Berg 1992 [77]	325	NA	46	21	1-6 cycles of 60 mg/m^2 Given 1 or 2 weeks after amputation	22 n=17 with amputation n=5 with limb=sparing surgery
Doxorubicin	Berg 1995 [81]	366	NA	51	10	5 cycles of 30 mg/ m^2 for every two weeks Surgery was given after second (n=18) or third (n=17) dose	35
	Moore 2007 [13]	240	NA	35	17	5 cycles of 30 mg/m^2 for every two weeks started 2 weeks after amputation	303
Carboplatin	Bergman 1996 [86]	321	257	31	NA	Up to 4 cycles of 300 mg/ m^2 carboplatin (n=34), others discontinued because of metastases (n=11) or unanticipated death (n=2)	48
	Philips 2009 [82]	307	256	37	19	3 cycles (n=25) or 4 cycles (n=128) Average dose <270 mg/m^2 (n=17) or > 270 mg/m^2 (n=138)	155
	Simcock 2012 [87]	365	NA	41	0	Single subcutaneous infusion of carboplatin (dosage 300 mg/m^2)	17

Table 1.4. (cont'd)

	Santamaria 2019 [46]	196	197	20*	13*	Single subcutaneous infusion of carboplatin (dosage 300 mg/m^2)	45
Cisplatin + Doxorubicin	Berg 1997 [88]	345 330	NA	48 46	28 28	Chemotherapy given two days after surgery (n=47) or 10 days after surgery, 3 cycles of 60 mg/m^2 cisplatin and 15-20 mg/ m^2 doxorubicin (1-2 hours prior to cisplatin)	94
	Mauldin 1988 [83]	300	210	37	26	Total 2 cycles of each drug 30 mg/ m^2 of doxorubicin was given 2 weeks after surgery Cisplatin were given at 60 mg/ m^2 3 weeks after doxorubicin was given	19
	Chun 2005 [85]	540 (2/16 still alive at 24.1 and 75 months after diagnosis)	471	69	25	4 cycles of 50 mg/ m^2 of Cisplatin and 15 mg/ m^2 of Doxorubicin were given with 24 hours of surgery (Doxorubicin was given 24 hours after Cisplatin)	16
Carboplatin+ Doxorubicin	Kent 2004 [84]	320	227	48	18	2 cycles of 300 mg/ m^2 carboplatin and 30 mg/ m^2 doxorubicin	32
	Bacon 2008 [20]	258	202 (187, n=30) (239, n=20)	NA	NA	n=29 completed 3 cycles of 300 mg/ m^2 carboplatin and 30 mg/ m^2 doxorubicin n=30 received carboplatin first n=20 received doxorubicin first	50

*Number inferred from graph presented in the publication [46]

Table 1.4. (cont'd)

	Frimberger 2016 [12]	317	NA	43	14	3 cycles of doxorubicin (30 mg/ m^2 for dogs greater than 15kg or 1mg/kg instead) every two weeks and then 3 cycles of carboplatin (300 mg/m^2 for dogs greater than 15kg or 300 mg/m^2 instead) every three weeks	38
Limb-sparing surgery+ Cisplatin, Carboplatin, and/or Doxorubicin	Liptak 2006 [49]	430	NA	NA	NA	n=4 received 4 cycles of carboplatin (300 mg/ m^2 every 3 weeks) n= 3 received 5 doses of doxorubicin (30 mg/ m^2 every 3 weeks) n=12 received 1 cycle of doxorubicin (30 mg/m^2) and carboplatin (300 mg/m^2) n=1 received 6 cycles of cisplatin (70 mg/m^2 every 3 weeks).	20 n=10 used cortical allograft n=10 used endoprosthesis in limb-sparing surgery
HER-2 targeting *Listeria* vaccine	Mason 2016 [124]	956	615	78	67	4 cycles of 300 mg/ m^2 carboplatin every three weeks and 3 doses of ADXS31-164 every three weeks	18

Table 1.5. Current targeted therapies for hOSA in clinical trials

	TKIs	Molecular targets	Trial number	Phase	References
Receptor tyrosine kinase inhibitors	Apatinib	VEGFR	NCT02711007	2	[99]
	Apatinib + anti PD-1	VEGF-2	NCT03359018	2	
	Bevacizumab	VEGF	NCT00667342	2	[147]
	Cabozatinib	MET, VEGFR2, RET, c-KIT, TIE2	NCT02243605	2	
	Figitumuamb	IGF1R	NCT00474760	1	[148]
	Imatinib	ABL, c-Kit, PDGFR	NCT00030667 NCT00031915	2 2	[149]
	Pazopanib	VEGFR1-3, PDGFR-α, PDGFR-β, FGFR, c-Kit, CSF-1	NCT02048371 NCT01759303	2 2	
	Regoragenib	VEGFR1-3, PDGFR-β, TIE2, FGFR, c-KIT, RET, RAF	NCT02048371 NCT02389244	2 2	
	Sorafenib	VEGFR2-3, PDGFR-β, CRAF, BRAF, c-Kit, FLT3	NCT00889057 NCT01804374	2 2	[150] [92]
	Sunitinib ($\pm$ Nivolumab)	VEGFR1-3, PDGFR-β	NCT03277924	1/2	
Monoclonal antibody	Pembrolizumab	PD-1	NCT02301039	2	
	Denosumab	RANKL	NCT02470091	2	https://clinicaltrials.gov/ct2/show/NCT02470091
	Dinutuximab	GD2	NCT02484443	2	https://clinicaltrials.gov/ct2/show/NCT02484443
	Nivolumab ($\pm$ ipilmumab)	PD-1 ($\pm$ CTLA-4)	NCT02304458	1/2	[130]

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CHAPTER 2

ESTABLISHMENT AND CHARACTERIZATION OF A NOVEL CANINE OSTEOSARCOMA CELL LINE

2.1 Introduction

Osteosarcoma (OSA), the most common bone tumor, is a highly aggressive tumor and occurs naturally in both humans and dogs. In humans, OSA (hOSA) is a rare tumor compared to other tumor types, and the incidence rate is around 1/100,000 [1]. Canine OSA (cOSA), however, has a 14 times higher incidence rate compared to hOSA, around 14/100,000 [2, 3]. In canine OSA, several large breeds have higher incidence rates, including Irish wolfhound, Leonberger, greyhound, and German shepherd, as the lifetime risk is as high as 8% in Irish wolfhound, 3% in Leonberger in a published population-based studies contained 11350 dogs [4].

Canine osteosarcoma is a unique translational model for human OSA for several reasons. First cOSA is a naturally occurring disease and is similar in clinical presentation and histopathology to the human with the long bones being common sites of presentation in both species. Second, cOSA presents with higher incidence rate and high metastatic potential, which makes cOSA a useful tool to investigate treatments for the most challenging disease state. Importantly, heterogeneity, a common and challenging feature of most cancers, also seen in OSA in both species. Current strategies to treat metastatic OSA are still not effective. Therefore, cell lines generated from patients, especially from a metastatic site are useful tools in experimental and drug studies [5].

For the past thirty years, despite advances in treatment of other cancers, the overall survival rate for OSA has stagnated. This is in part due to the low number of OSA cases in humans, the high cost of drug development and the difficulty in carrying out clinical trials with novel agents on a small patient population dispersed across large distances. In order to gain a better understanding into OSA biology, and to facilitate drug development studies have used

commercially available human OSA cell lines, established novel patient derived OSA cell lines from canine [6-9] and human osteosarcoma [5, 10-13], and evaluated OSA xenograft models [10, 12]. In the current study, we established a novel canine OSA cell line, BZ, from a metastatic site in a patient that was previously treated with chemotherapy before tumor recurrence. Further study of the BZ Cell line will provide an additional tool and an *in vitro* model for studying cellular behavior, differential gene expression, and tumorigenesis.

2.2 Material and Methods

2.2.1 Origin of primary tumor and cell culture

The cell line was established from a fine needle aspirate of a metastatic mass under the right eye on the zygomatic arch from an eight-year-old male German shepherd dog with osteosarcoma which presented to the Michigan State University Veterinary Teaching Hospital. The patient had received two cycles of carboplatin after the primary osteosarcoma was found on right femur. The diagnosis of OSA was based on the histopathology findings. Cell line was established, cells were pelleted, fixed in formalin and embedded in paraffin, and examined by histopathology. Positive staining for osteocalcin (OC) and vimentin also confirmed OSA. The BZ cell line is currently in its 56^{th} passage.

2.2.2 Preparation and maintenance of cell culture

The cells were isolated from a fresh fine needle aspirate, first treated with RBC lysis buffer for 10 minutes, and pelleted. Cells were then resuspended in Minimum Essential Medium α (αMEM medium, Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum and

antibiotics (0.1% gentamycin, Life Technologies, Carlsbad, CA), and seeded to a 100mm cell culture dish. The BZ cell line was passaged using 0.025% trypsin when cells reached 80% confluence, and now is currently in the 56th passage. NIH-3T3 is a mouse fibroblast cell line which we included as a positive control for PTEN expression studies. NIH-3T3 and all human OSA cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM medium, Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum, and gentamicin. Canine osteosarcoma cell lines were maintained in αMEM medium, supplemented with 10% fetal bovine serum, and gentamicin. All cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.2.3 Immunohistochemistry

For immunolabeling, the histochemistry staining was performed using CellSieve™ CTC Enumeration Kit (Creav MicroTech) according to manufacturer's protocol. OSA cells were first seeded into chamber slide™ (Lab-Tek®) then fixed with CellSieve™ Prefixation Buffer for 15 minutes at room temperature and washed with PBS. Next, fixed cells were incubated with CellSieve™ Postfixation Buffer for 20 minutes and changed to CellSieve™ Permeabilization buffer for 20 minutes. Cells were washed with PBS after each incubation. Later, two fluorescent dye-conjugated antibodies (osteocalcin/FITC and vimentin/EF615) were added to the chamber and incubated with cells for 2 hours. After washing with PBS, a drop of CellSieve™ Mounting Solution with DAPI (4′,6-diamidino-2-phenylindole), was applied on the top of fixed cells. Images were captured by Nikon C2 confocal microscope.

2.2.4 Cell viability assay

The MTS assay (Promega Corp.) was used to determine IC_{50} values for conventional chemotherapy drugs and other compounds on OSA cells. Briefly, OSA cells (2,500-3500/well) were seeded on 96-well plates, then treated with compounds for 72 hours. 20 μl of MTS tetrazolium compound was added to 100 μl culture media. Cell viability was determined by the amount of colored formazan dye produced by live cells. The absorbance of formazan dye was measured at 490 nm, and IC_{50} values were calculated by PRISM Statistical Software.

2.2.5 Protein expression analysis

For western blotting, 500,000 cells from cOSA and hOSA cell lines were collected, cells were palleted by centrifugation at 1000 rpm , the supernatant removed and the cells lysed with 250 μl of CelLytic M lysis buffer (C2978, Sigma-Aldrich) with 2 μl of protease inhibitor (P8340, Sigma-Aldrich) and 2 μl of phosphatase cocktail inhibitor B (sc-45045, Santa Cruz.) according to manufacturer's protocol. Protein concentrations were quantified with *Qubit™ Protein Assay* Kit. After gel electrophoresis on an 8-12% Bis-Tris Plus Gel run at 100 volts for about 90 minutes the proteins were transferred to PVDF membranes using an iBlot 2 Dry Blotting System apparatus. The membranes were blocked with 5% BSA for 2 hours at room temperature and incubated with the following primary antibodies at 4 °C overnight to detect antigen: ERK (1:1,000), PTEN (1:500), p-ERK (1:500), p-STAT3 (1:500), STAT3 (1:1,000), β-actin (1:4,000) and β-tubulin (1:4000) (CST, Cell Signaling Technology). After 3 washes in tris-buffered saline with 0.05% Tween 20, the membranes were incubated with the appropriate secondary antibody (donkey anti-mouse (1:15,000) or goat anti-rabbit (1:15,000)) for 1 hour at room temperature. The

membranes were examined, and the bands visualized by Odyssey Infrared Imaging System (LI-COR Biosciences).

2.3 Results

2.3.1 Characteristics of BZ cell line

As seen in **Figure 2.1**, BZ cells present a spindle-shaped cell morphology and disorganized cell growth pattern at different passages. As shown in **Figure 2.2**, the immunohistochemical analysis confirmed that BZ cell line maintained the osteoblast specific marker osteocalcin and mesenchymal marker vimentin after the cell line was established. These two markers were found to continue to be expressed at the 30^{th} passages of the BZ cell line. Other OSA cell lines, three canine OSA (D17, Abrams, and Gracie) and three human OSA (SAOS2, U2OS, and the MG63) also showed positive staining for osteocalcin and vimentin.

2.3.2 The IC_{50} values (concentration of drug inhibiting growth by 50%) for conventional chemotherapeutics on BZ and other OSA cell lines

The IC_{50} values for conventional drugs, cisplatin, carboplatin, and doxorubicin were determined on the BZ cell line. As seen in **Table 2.1**, two drugs, cisplatin and carboplatin were found to have higher IC_{50} values when compared to the published achievable plasma concentration, which are 4 and 72 μM. The IC_{50} values for doxorubicin, another commonly used chemotherapeutic agent, is below the achievable plasma concentration. The IC_{50}s for carboplatin on BZ cell line is 273 μM, along with another cOSA Abrams, are the highest among all cell lines.

2.3.3 BZ showed limited AKT activation

The status of PTEN was analyzed by western blot. Two canine OSA cell lines D17 and Abrams as well as human OSA cell line SAOS2 showed PTEN loss whereas BZ had limited PTEN expression. Three cell lines (D17, Abrams, and SOAS2) showed an activation of AKT pathway while BZ had limited activation of AKT pathway.

2.3.4 ERK and STAT3 pathway

In protein analysis, two cOSA (Abrams and BZ) and two hOSA (MG63 and U2OS) showed upregulated of ERK signaling pathway compared to other OSA cell lines. All OSA cell lines displayed STAT3 activation, which is reflected by the expression of phospho-STAT3.

2.4 Discussion

In the current study, we report the establishment of a new canine OSA cell line, BZ, derived from a metastatic OSA lesion of a German shepherd dog, a breed that has a high frequency of OSA. BZ cells have been cultured for more than 56 passages, and undergone freeze-thaw cycles. Moreover, we used two biomarkers, osteocalcin and vimentin to characterize the BZ cell line along with 6 other OSA cell lines we use in our lab, including 4 canine OSA (D17, Abrams, Gracie, and BZ) and 3 human OSA (SAOS2, U2OS, and MG63). The immunohistochemistry analysis confirmed that BZ as well as the other cell lines maintained the osteoblast-specific marker osteocalcin as well as mesenchymal maker vimentin after all passages. The IC_{50} value for carboplatin on BZ cell line is 273 μM, along with another cOSA Abrams, are the highest among

all cell lines. This might reflect to the fact that BZ was from a patient that failed to respond carboplatin treatment and may be appropriate for study of carboplatin resistant OSA.

The most frequently studied OSA commercial cell line is D17 (CCL-183), which was derived from a standard poodle [14]. However, large breeds like Irish wolfhound, Leonberger, greyhound, and German shepherd have a much higher incidence rate of OSA compared to other breeds. The BZ cell line was derived from a German shepherd, one of breeds that have a high incidence of OSA [15]. In humans, the widely studied cell lines like SAOS2, MG63, and U2OS were all derived from Caucasian patients. Other studies tried to establish OSA cell lines from different ethnic groups , such as Chinese [13] and Japanese [16]. The individual and ethnic differences in humans and breed differences in dogs may point out different genetic backgrounds and result in different clinical outcomes. Thus, it is necessary to develop more cell lines for research use, as well as to expand the knowledge of OSA between different ethnic groups and breeds.

For OSA diagnosis, biomarkers such as osteoblastic markers (Alkaline Phosphatase (ALP), osteocalcin, and RUNX2), vimentin, osteonectin have been used [10, 17-19]. A key hallmark of OSA is the ability to produce osteoid [20]. Osteocalcin (OC) is a protein that binds to hydroxyapatite crystals in bone matrix and is considered as a marker for late osteoblastic differentiation. As seen in Figure 2.1, two hOSA cell lines SAOS2 and MG63 showed positive staining for OC, which is in consistent with a previous study [21]. In cOSA samples, osteocalcin showed a highly sensitivity and specificity compared to osteonectin, suggesting that OC can be used as a diagnostic tool for osteoblastic OSA [7, 18, 22].

Osteosarcoma is a heterogenous disease, the diversity of oncogenic drivers within this cancer makes improving the overall survival rate with current standard of care challenging. Despite

advances in understanding underlying drivers of osteosarcoma, more effective treatments for OSA has not been identified, suggesting a clear need for developing novel therapeutic agents. Also, patient derived cell lines that reflect different clinical cancer characteristics are needed for potential evaluation of drugs. BZ, derived from a metastatic lesion, and showing carboplatin resistance, will serve as an additional tool in OS research. We need to further evaluate the tumorigenic and the metastatic potential of the current patient-derived cell line in a mouse model. Common routes to induce tumor growth in mice models include subcutaneous, orthotopic and tail vein injection. Previously in our lab, we established two models with canine OSA D17 cell line, subcutaneous and orthotopic injection to the tibia (data not show). One way to monitor tumor cells in live animals is to transduce cells with luciferase and tracked cells by bioluminescent signals under IVIS spectrum [23]. We have generated a D17 cell line stably transfected with a luciferase reporter plasmid, and we can do so with BZ cell line. With this system, we can closely monitor the OSA progression, such as metastatic sites within the lung in a live animal.

The analysis of proteins from key pathways in the BZ and other cell lines demonstrated potential therapeutic strategies. Importantly, the activation of ERK pathway was upregulated in this new cell line, BZ as well as in Abrams, and in two hOSA cell lines, MG63 and U2OS, suggesting the potential to target this pathway with inhibitors like sorafenib or sunitinib. The activation of AKT pathway was observed in three OSA cell lines D17, Abrams, and SAOS2, indicating these OSA might benefit from PI3K/AKT inhibitors such as rapamycin. Moreover, the activation of STAT3 pathway was shown in all OSA cell lines we tested, which is consistent with a previous report [24], suggesting STAT3 pathway plays a role for the survival and proliferation

in BZ cell line as well as other OSA cell lines. In one study, a STAT3 inhibitor (LLL3) was shown to successfully downregulate STAT3 transcriptional activities and reduced OSA cell proliferation and viability in two human and two dog cell lines, including D17, revealing that the inhibition of this pathway holds promise for therapeutics application [24].

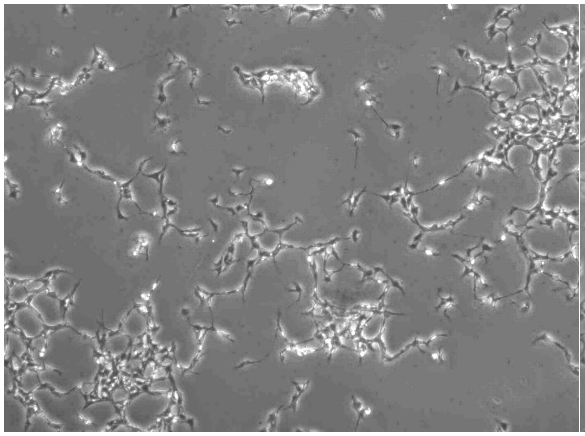
PTEN loss in OSA is documented in both cOSA and hOSA. A positive PTEN expression is associated with higher 5-year survival rate compared to negative PTEN patients [25]. Besides being a clinical predicator, PTEN is also a potential therapeutic target in invitro studies. Activators of PTEN such as tepoxalin [26], evodiamine [27], or celecoxib [28] have been tested and confirmed its effect of increase PTEN on human OSA cells [29]. Furthermore, PTEN loss is accompanied by AKT activation and as discussed above, AKT can also be targeted.

In summary, we describe here the establishment of a novel canine OSA cell line, BZ, a canine OSA cell line derived from a metastatic mass from a German shepherd. To our knowledge, there are no other OSA cell lines established from this breed. Further evaluation of the BZ cell line in xenograft mouse model are needed. However, the BZ cell line can be a useful tool for further understanding of OSA and in drug development. In addition, genomic and transcriptomic studies can be carried out in this cell line to fully characterize it at the molecular level.

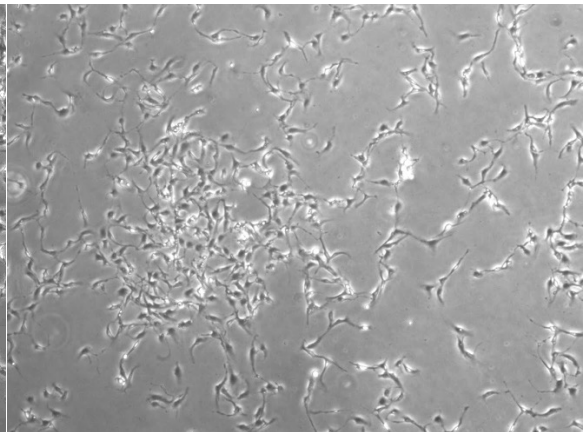
APPENDIX

Figure 2.1. Images of BZ cell lines during culture. Phase contrast images showed growth morphology of BZ at different passages, X 4 **(A)** 5 weeks after isolation, passage 5 **(B)** passage 16, **(C)** passage 50 in cell culture. **(D, E)** Confocal images presented IHC staining of DAPI (blue), osteocalcin (green), and vimentin (red) with OSA cells. For (**D, E**), calibration bar: 50μM.

A



B



C

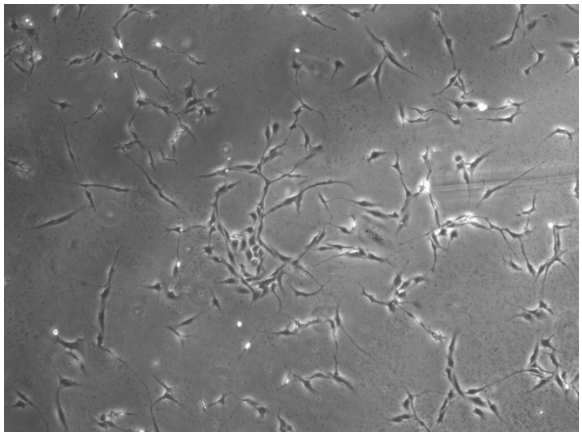


Figure 2.1. (cont'd)
(D) BZ and other cOSA cell lines were confirmed as osteosarcoma by IHC staining. Calibration bar: 50μM.

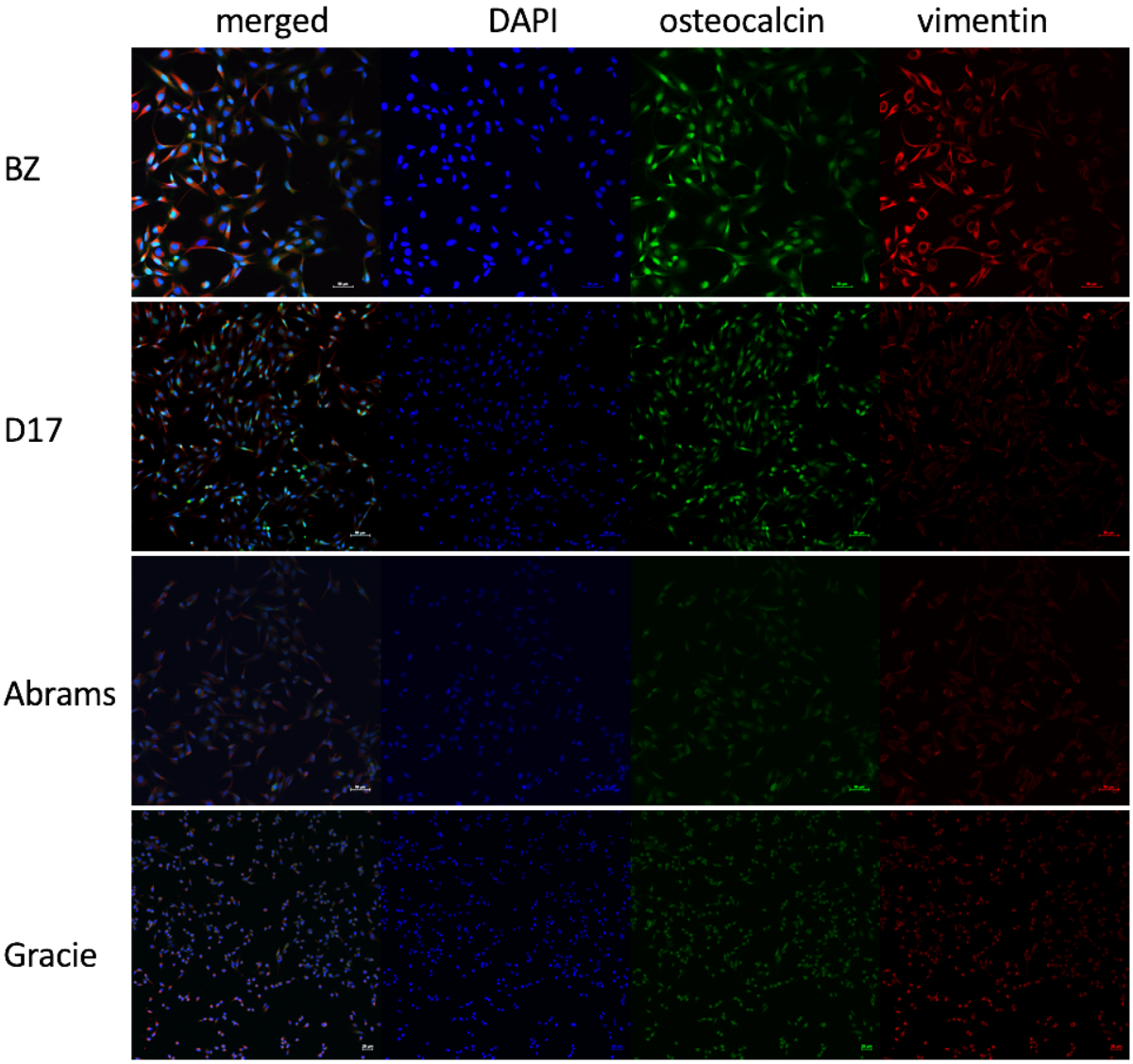


Figure 2.1. (cont'd)
(E) Human OSA cell lines SAOS2, U2OS, and MG63 were confirmed as osteosarcoma by IHC staining. Calibration bar: 50μM.

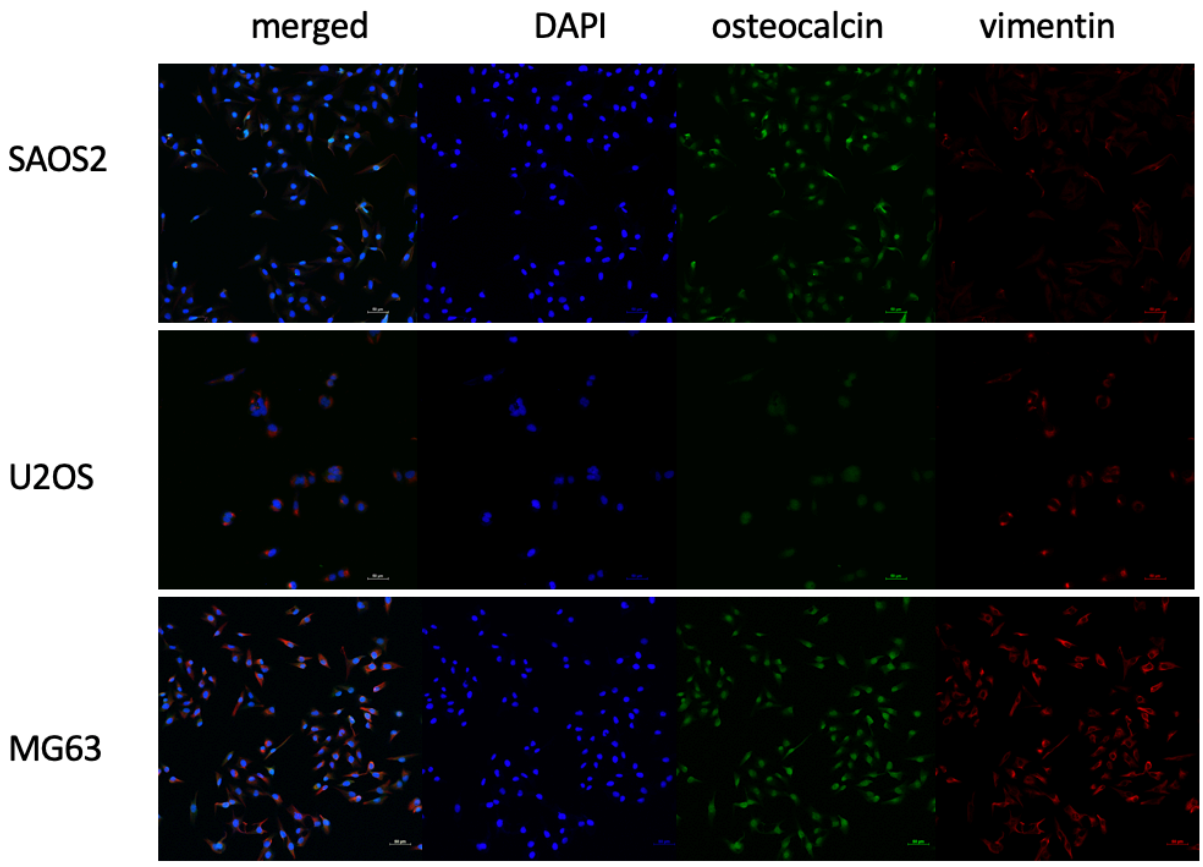


Table 2.1. IC_{50} values for cisplatin, carboplatin, and doxorubicin on OSA cell lines

	Cisplatin (μM)	Carboplatin (μM)	Doxorubicin (μM)
D17	4	45	0.2
Abrams	12	263	0.06
Gracie	2	41	0.06
BZ	12	273	0.05
SAOS2	3	47	0.07
U2OS	7	57	0.06
MG63	5	70	0.05
Reported Maximum plasma concentration	4 μM (dog)	72 μM (dog)	1.13 μM (dog)
reference	[30]	[31]	[32]

Figure 2.2. Protein analysis in cOSA and hOSA cell lines.

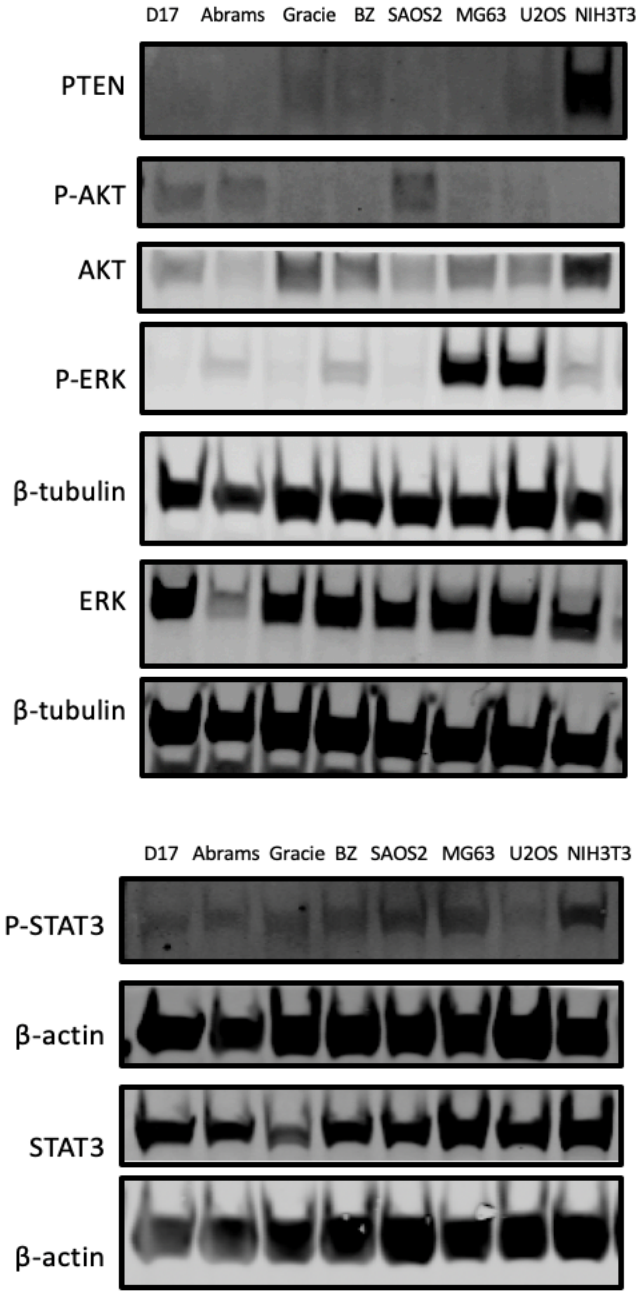


Table 2.2. Canine and human osteosarcoma cell lines in this study

source	Cell line	Race/ Breed	Age	Gender	Type	Source
cOSA	D17	poodle	11	Female	Lung metastasis	ATCC
cOSA	Abrams	N/A	N/A	N/A	N/A	*Colorado State University
cOSA	Gracie	N/A	N/A	N/A	N/A	*Colorado State University
cOSA	BZ	German shepherd	8	Male, Neutered	Mass under right eye Right femur OSA, second chemotherapy of carboplatin	MSU
hOSA	SAOS2	Caucasian	11	Female	Morphology: epithelial	ATCC
hOSA	U2OS	Caucasian	15	Female	Morphology: epithelial Original from tibia	ATCC
hOSA	MG-63	Caucasian	14	Male	Morphology: fibroblast	ATCC

*Cell lines Abrams and Gracie were kindly shared by Dr. Doug Thamm, Colorado State University.

Table 2.3. Antibodies used in immunohistochemistry (IHC) staining and western blot analysis

Primary antibodies						
Protein	Catalog #	Lot#	Clone	Host	Manufacturer	Concentration used
DAPI	30-804931				Abbot	
Osteocalcin	Ab13418	GR2624639	OC4-30	Mouse	abcam	1:100
Vimentin	EF615	0410-2017			Creatv	1:6 (0.3 μg/ml)
Secondary antibody						
Protein	Catalog #	Lot#	Host	Target	Manufacturer	Concentration used
IRDye680RD	925-68072	C81107-03	Donkey	Mouse	LI-COR	1:10,000
Conjugation kit						
FITC-conjugation	Ab102884				abcam	

Primary antibodies						
Protein	Catalog #	Lot#	Clone	Host	Manufacturer	Concentration used
AKT	9272	9	D9E	Rabbit	Cell Signaling	1:1,000
β-actin	3700	13, 14	8H10D10	Mouse	Cell Signaling	1:4,000
β-actin	4970	12	13E5	Rabbit	Cell Signaling	1:4,000
β-tubulin	86298	1	D3U1W	Mouse	Cell Signaling	1:4,000
ERK1/2	4695	14	137F5	Rabbit	Cell Signaling	1:1,000
p-AKT (Ser473)	9271	12	D9E	Rabbit	Cell Signaling	1:500
p-ERK (Thr202/Tyr204)	4370	15	D13.14.4E	Rabbit	Cell Signaling	1:500
p-STAT3 (Y705)	9145		D3A7	Rabbit	Cell Signaling	1:500
PTEN	9188	0006	D4.3XP	Rabbit	Cell Signaling	1:500
STAT3	4904		79D7	Rabbit	Cell Signaling	1:1000
Secondary antibody						
Protein	Catalog #	Lot#	Host	Target	Manufacturer	Concentration used
IRDye 680RD	925-68072	C81107-03	Donkey	Mouse	LI-COR	1:10,000
IRDye 800CW	925-32211	C80118-01	Goat	Rabbit	LI-COR	1:10,000

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CHAPTER 3

EVALUATING THE POTENTIAL SYNERGISTIC EFFECTS OF COMBING SORAFENIB AND DOXORUBICIN IN HUMAN AND CANINE OSTEOSARCOMA CELL LINES

3.1 Introduction

Osteosarcoma (OSA) is the most common primary bone tumor of both humans and dogs, about 1000 OSA cases are reported in humans and 10,000 cases in dogs every year in the United States [1]. Similar to humans, companion animals such as the dog, can spontaneously develop cancer. Canine osteosarcoma is an aggressive cancer, around 80%-90% of the dogs present with micro metastasis disease when diagnosed with osteosarcoma in clinics. Canine OSA (cOSA) closely resembles human OSA (hOSA) including histopathological appearance, molecular markers and the response to conventional chemotherapies. As humans and dogs share similarity in their genome and living environments, dog disease is an excellent parallel disease to study osteosarcoma. Current first line chemotherapies in cOSA are: cisplatin, carboplatin, and doxorubicin [2-4]. With the current standard care, human patients show a high recurrence rate and develop resistance to chemotherapy. To overcome these clinical challenges, effective and well-tolerated therapeutic agents are needed for both humans and dogs.

One of the most important classes of novel anti-tumor drugs developed in recent years are tyrosine kinase inhibitors (TKIs). The receptors tyrosine kinase (RTKs) are a family of cell surface receptors that play an essential role in mediating cell to cell communication and key signaling transduction pathways involved in cell proliferation, differentiation, and survival [5]. These molecules control and alter many biological processes in the cells and deliver downstream signals when bound to their cognate ligand. However, many oncogenic mutations have been documented in RTKs, such as over expression of MET (receptor for the hepatocyte growth factor) in canine osteosarcoma [6] and human osteosarcoma [7-9]. Another example is activating mutations in the juxtamembrane domain c-KIT in canine mast cell tumors, which

causes constitutive activation of receptor in the absence of ligand binding [10]. RTKs and their tyrosine kinase domains are potential targets for tyrosine kinase inhibitors (TKIs). Many TKIs compete with ATP binding site of catalytic domain, or inhibit the catalytic activity of the kinase in other ways and prevent auto-phosphorylation, block the transfer of growth signals downstream from the receptor, and thereby inhibit cancer cell proliferation [11, 12].

Sorafenib (BAY 43-9906, Nexavar®), an oral small molecular inhibitor, inhibits a variety of signal transduction pathways. Sorafenib was primarily developed as a RAF inhibitor but showed inhibition of other targets, including VEGFR-2 (vascular endothelial growth factor), VEGFR-3, PDGFR (platelet-derived growth factor receptor), RAF-1, B-RAF, and c-KIT [13-15]. Sorafenib is indicated for the treatment of patients with hepatocellular carcinoma [16, 17], renal [18, 19], hepatic [20], and thyroid [21] cancer in humans. In the literature, there were limited reports of using sorafenib to treat osteosarcoma. These studies include several clinical trials, two that used sorafenib as single agent [22, 23] and one which combined it with an mTOR inhibitor [24], as well as a case study which combined sorafenib with a RANKL inhibitor denosumab [25]. In dogs, however, there have not been any clinical trials. There was one recent study that reported the tolerable dosage of sorafenib in a small group of dogs with various cancers [26], which showed that sorafenib was well tolerated, up to 3 mg/kg, given from 3 to 8 doses. While sorafenib has been studied in other solid tumors, evaluation of the potential for sorafenib in cOSA and hOSA has been limited.

So far, most studies on the anti-tumor activity of sorafenib were carried out in hepatocellular carcinoma (HCC). Sorafenib alone has been used as standard of care for HCC patients since 2007, and the combination of sorafenib and doxorubicin have been proposed in clinical trials to

treat advanced hepatocellular carcinoma (HCC) [16, 17]. Doxorubicin, a topoisomerase II inhibitor, causes DNA damage by disruption topoisomerase II mediated DNA repair and generation of free radicals and is one of first line chemotherapies for human and canine osteosarcoma [27, 28]. However, there is very limit knowledge of using this combination in osteosarcoma.

The purpose of this study was to evaluate whether a tyrosine kinase inhibitor, sorafenib, has an effect on canine and human osteosarcoma cells alone and in combination with current chemotherapeutics agents for OSA. We report here that sorafenib alone showed growth inhibition effects in both human and canine OSA cell lines. Furthermore, we show that sorafenib and in combination with doxorubicin had synergistic effects on canine and human OSA cells. These preclinical findings suggest the multi kinase inhibitor, sorafenib should be considered for use in future clinical trials alone as well as in combination with doxorubicin and that dogs can serve as proof of concept studies for such trials.

3.2 Material and Methods

3.2.1 Cell culture

Canine OSA cell line D17 and human OSA cell line (SAOS2, U2OS, and MG63) were purchased from ATCC. Canine OSA cell line Abrams was provided by Dr. Elizabeth McNeil, and originally established and shared by Dr. Doug Thamm. Canine BZ OSA cell line was established from a German shepherd dog by our laboratory. For cell culture maintenance, human OSA cell lines were incubated with DMEM medium and canine OSA cell lines were maintained with α-MEM

medium, all cells were supplemented with 10% fetal bovine serum and incubated in a humidified incubator at 37°C with 5% CO_2.

3.2.2 Compounds used in Drug Screening

Sorafenib was purchased from LC laboratories (Woburn, MA). Sorafenib was dissolved in DMSO while cisplatin was dissolved in PBS, and carboplatin was dissolved in water. Other TKIs, including cladribine, dasatinib, erlotinib, gefitinib, masitinib, nilotinib, sorafenib, sunitinib, toceranib, and tozasertib were all purchased from Sigma-Aldrich and dissolved in DMSO.

3.2.3 Cell viability assay (MTS assay) and small panel drug screening with TKIs

The MTS assay (Promega Corp.) was used to determine IC_{50} values of sorafenib and other compounds on OSA cells. Cells were seeded to 96-well plates at a density of 2,500-350,0/well. After 24 hours, cell culture medium was replaced by complete medium with each compound at the designated concentrations. Cells were treated for 72 hours. The cell viability was analyzed by CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) and determined by the amount of colored formazan dye produced by live cells. The absorbance of the formazan dye produced was measured at wavelength 490 nm, and IC_{50} values were calculated by PRISM Statistical Software. Each concentration for each drug was assayed in triplicate for the IC_{50} calculations.

In this study, we treated three OSA cell lines (D17, Abrams, and SAOS2) with a small panel of compounds including ten tyrosine kinase inhibitors (cladribine, dasatinib, erlotinib, gefitinib, masitinib, nilotinib, Sorafenib, Sunitinib, toceranib, and tozasertib) for 48 hours. Percent growth

inhibition was calculated for each treatment with the MTS assay with the vehicle control treatment containing 1% DMSO. For all TKIs, we used a high drug dose of 100 μM.

3.2.4 Wound healing assay

The wound healing assay was used to examine migration capacity of cells in a monolayer. Briefly, 100,000 cells/well were plated overnight and allowed to reach 70-80% confluence in 6-well plates. Then, scrapes were made on the plates using a 1-mL pipette tip. The cells were then incubated with cell culture medium with or without sorafenib. Each scrape was photographed after being made and at each specific time points thereafter.

3.2.5 Combination Index (CI)

Cells were simultaneously incubated with two compounds at a fixed ratio (sorafenib: doxorubicin= 20:1, 50:1, or 100:1, sorafenib: cisplatin= 4:1, and sorafenib: carboplatin= 1:5) for 72 hours. Synergistic effects of two drugs were determined by isobologram and combination index (CI) analysis by the CompuSyn software (Combosyn, Paramus, NJ). The analysis was adapted from the median-principle methods of Chou and Talalay [29], the results of CI <1, CI=1, and CI >1 indicate synergism, addition, and antagonism, respectively.

3.2.6 Cell cycle analysis

Cells were treated with 1% DMSO (control), sorafenib (1.25, 2.5, or 5 μM), doxorubicin (25, 50, or 100 nM), or combination (ration 50:1) for 24 hours, then collected by centrifugation and fixed with 70% ethanol at 4 °C overnight. Ethanol was removed by centrifugation, and cellular

DNA was stained with propidium iodide (50 μg/ml) containing RNase (1 mg/ml). After cells were stained for at least 4 hours, the PI fluorescence of individual nuclei was recorded with FACScan. The quantitative assessment of cell cycle phase and apoptosis were then determined by Modfit Lt™ software after correction for debris and aggregate cell populations.

3.2.7 Protein analysis using western blots

Abrams OSA cells (500,000/well in 6-well plate) were treated with wither vehicle (0.1% DMSO) or sorafenib for 24 hours. Cell were lysed with 250 μl of CelLytic M lysis buffer (C2978, Sigma-Aldrich) with 2 μl of protease inhibitor (P8340, Sigma-Aldrich) and 2 μl of phosphatase cocktail inhibitor B (sc-45045, Santa Cruz.) according to manufacturer's protocol. Protein concentration were quantified with Qubit™ Protein Assay Kit.

A total of 60 μg of protein per well was loaded on Bolt Bis-Tris 4-12% polyacrylamide gels (Thermo Fisher Scientific Inc.) and transferred to polyvinylidene difluoride membranes. The membranes were incubated with 5% bovine serum albumin (BSA) for 2 hour at room temperature then incubated with the following primary antibodies at 4 °C overnight to detect antigen: AKT (1:500), p-AKT (1:500), ERK (1:500), p-ERK (1:250), STAT3 (1:500), p-STAT3 (1:500), PARP (1:250), β-tubulin (1:4000) (Cell Signaling Technology). After three washes in tris-buffered saline with 0.05% Tween 20, the membranes were incubated with appropriate secondary antibody (donkey anti-mouse (1:15,000) or goat anti-rabbit (1:15,000)) for 1 hour at room temperature. The membranes were visualized by Odyssey Infrared Imaging System (LI-COR Biosciences), analyzed by Image Studio™ Lite software (LI-COR).

3.2.8 Statistical analysis

Results represent at least three separate experiments. Statistical analysis was performed with Graph Pad Prism (8.0.0, Graph Pad Software Inc., San Diego, CA), differences between categories were analyzed with one-way ANOVA, p-values of < 0.05 were considered to be significant.

3.3 Results

3.3.1 Sorafenib, gefitinib, and sunitinib showed growth inhibition potential

As seen in **Figure 3.1A**, after 48 hours of incubation, the cell viability was measured across 10 tyrosine kinase inhibitors. Among these, only sorafenib, sunitinib, and gefitinib were capable of reducing cell viability to below 10% at 100 μM. Thus, we selected these three TKIs for further studies.

3.3.2 Cytotoxicity assay with sorafenib and conventional chemotherapeutics for OSA treatment

Three TKIs, gefitinib, sorafenib, and sunitinib were selected for further evaluation.

The effect of sorafenib and other drugs on cell growth inhibition of four cOSA cell lines (D17, Abrams, BZ, and Gracie) and three hOSA cell lines (SAOS2, U2OS, and MG63) was examined by MTS assay. After 72 hours of incubation, dose response curves were generated based on the cell viability at a range of concentrations, and IC_{50} values were calculated by Graph Pad Prism software (**Figure 3.1B**). Two TKIs, gefitinib [30] and sunitinib [31], which displayed IC_{50} values above the achievable plasma concentration s reported in the literature were not included in further studies.

The IC_{50} values for conventional chemotherapeutics, cisplatin, carboplatin, and doxorubicin, were also determined in our cell lines (**Table 3.1.**). Cisplatin [32] and carboplatin [33] were found to have IC_{50} values larger than the reported achievable plasma concentration in the literature. However, the IC_{50} values for doxorubicin ranged from 58-226 nM in our cell lines, which were lower than a documented achievable plasma concentration of 1130 nM in dog. Also, the IC_{50} values for sorafenib were from 3-9 μM, whereas a study reported achievable plasma concentration of 10 μM in human. These comparisons with reported achievable plasma concentration provided the rationale to include sorafenib and doxorubicin for further evaluation, in order to select combinations of drugs each with potential to reach therapeutic effects within clinical relevant doses [34, 35].

3.3.3 Cell migration ability was inhibited by sorafenib

As shown in **Figure 3.2**, the effect of sorafenib on the migration capacities of on OSA cells was determined by a wound-healing assay. D17, Abrams, and SAOS2 cells were treated with or without sorafenib, for up to 48 hours of incubation time, after performing a scratch at the center of a 6-well plate. In this assay, sorafenib effectively suppressed the migration on SAOS2 but not the two other OSA cell lines. To avoid cell cytotoxic effects, we used a dose of sorafenib lower that the IC_{50}s in this study. After 24- and 48-hours treatment, SAOS2 cells incubated with sorafenib migrated less than control group (**Figure 3.2**).

3.3.4 Sorafenib inhibited STAT3 and ERK phosphorylation in the Abrams and D17 cell lines.

Two major signal transduction pathways activated by RTKs are the PI3K/AKT (phosphatidylinositol 3-kinase/ serine-threonine protein kinase) and the MAPK/ERK (mitogen-activated protein kinases/extracellular signal- regulated kinases) pathways [12, 36]. As shown in **Figure 3.3**, the potential mechanisms of sorafenib treatments were investigated by western blot analysis of key pathways. In canine Abrams cell line, p-ERK and p-STAT decreased at 10 μM or higher concentrations of sorafenib. However, sorafenib did affect the PI3K-AKT pathway as p-AKT remained unchanged, even in highest dose (20 μM). Cleaved PARP, indicator of apoptosis, was detected at 0.1 μM and higher concentrations of sorafenib. The D17 cell line also displayed complete loss of p-ERK upon treatment with sorafenib at 10 μM, while in SAOS2 this pathway appeared unaffected by this treatment.

3.3.5 Sorafenib and doxorubicin showed synergistic effects

We next assessed the possibility of improving anti-tumor effects through combinations of drugs with different modes of action. We examined three drugs (cisplatin, carboplatin, and doxorubicin) in combination with sorafenib. Three OSA cell line (D17, Abrams, and SAOS2) were exposed to various concentration of sorafenib, one of the other three drugs, or their combination for 72 hours. Later, cell viability was examined by MTS assay and the effects were calculated by the CompuSyn software (Combosyn, Paramus, NJ). As seen in **Figure 3.4 A and B**, sorafenib combined with doxorubicin resulted in combination index values are less than 1, indicating synergistic effects. Cisplatin (**Figure 3.4C**) and carboplatin (**Figure 3.4D**) showed an antagonistic effect with sorafenib on the D17 cell line.

3.3.6 Sorafenib and doxorubicin induced cell cycle arrest

We studied the effects of cell cycle changes with sorafenib and doxorubicin use on three OSA cell lines and found cell arrest at G2/M phase in all cell lines with their combined use. In the cell cycle assay, we compared three different concentrations (1.25, 2.5, and 5 μM) of sorafenib. Sorafenib alone from 1.25 μM to 5 μM did not cause an effect on cell cycle phase distribution, which is correlated with previous findings [37]. The distributions are very similar after treatment with 5 μM sorafenib alone **(Figure 3.5A and B)**, whereas G2/M arrest was clearly augmented when 5 μM sorafenib and 100 nM doxorubicin added jointly **(Figure 3.5D) (changing from 10 to 91 %)**. As shown in **Figure 3,5**, the combination of sorafenib and doxorubicin resulted in a cell arrest at G2/M phase. This observation was consistent among all three OSA cell lines (D17 **(Figure 3.5E)**, Abrams **(Figure 3.5F)**, and SAOS2 **(Figure 3.5G)**). Interestingly, the apoptosis fraction induced by doxorubicin alone was reduced in the presence of sorafenib **(Figure 3.5C and D)**.

3.4 Discussion

In this present study, we demonstrated that sorafenib, a multi kinase inhibitor, acts as an effective drug against both human and canine osteosarcoma cells. We provided evidence of anti-proliferation and migration inhibition effects *in vitro*. In canine Abrams cell line, the activation of ERK and STAT3 pathways was inhibited by sorafenib at 10 μM. However, in the PI3K-AKT pathway, p-AKT remined unchanged with the treatments, even at the highest dose. Cleaved form PARP was detected at 0.1 μM and higher doses indicating apoptosis was induced by sorafenib. As we explored the potential of drug combinations, sorafenib and doxorubicin

showed synergistic effect and resulted in a cell arrest at G2/M phase in D17 and Abrams. These findings indicating sorafenib alone and in combination with doxorubicin can be used as novel therapeutic strategies for treatment of certain canine and human OSA which show activation of the ERK pathway.

In bone cancers, targeting MAPK/ERK pathway as a therapeutic strategy has been studied *in vitro* [11, 14, 38] and recently put into clinical trials [39-42]. The inhibitory effects of sorafenib on the MEK/ERK signaling pathway have been documented [11, 14, 43]. In one study, around 67% (20/30 samples) of OSA tumors in human patients showed immunopositivity in histopathology for p-ERK, indicating that ERK pathway is highly activated among hOSA and the blocking p-ERK may be a potential therapeutic option [43]. STAT3 activation also plays a critical role in OSA cells as it supports cells survival and proliferation [44-46]. A previous study demonstrated that STAT3 activation contributes to the survival and proliferation of human and canine OSA cells and thus suggested that STAT3 is a potential target for therapeutic strategy [44]. Previous studies reported sorafenib as well as its derivative sc-1 were capable of downregulating the activation of STAT3 and ERK pathways and reducing tumor volumes [46]. Our study on canine Abrams OSA cell line shows similar results in that sorafenib downregulated the expression of p-STAT3 and p-ERK. Besides MAPK/ERK and STAT3 signaling pathway, another target for sorafenib is VEGFA. A recent study investigated somatic copy-number alternations (SCNA) as the approach to identify potential targeted drugs to treat different amplifications in osteosarcoma patient derived xenografts model (PDTX) [47]. In the VEGFA- amplified PDTX model of OSA, sorafenib resulted in reduced tumor volume compared to vehicle, whereas in another PDTX model without VEGFA- amplified OSA, there was no benefit from sorafenib

treatments. This finding suggests that sorafenib could be used in OSA patients with VEGFA amplified tumors as well. The observation of induction of apoptosis by sorafenib is consistent with a previous study where an increase of caspase-3 activity in sorafenib treated cells was documented in D17 cell line [37].

In the current study, cisplatin showed an antagonistic effect with sorafenib (**Figure 3.4C and D**), which is consistent with a previous study of combing sorafenib and carboplatin (a derivative of cisplatin) in the D17 cell line [37]. Although sorafenib and doxorubicin as single treatments have been studied extensively, the present study is the first to show the further efficacy of combing sorafenib and doxorubicin in inhibiting OSA cell growth. The combination of sorafenib and doxorubicin has been studied and carried out in a phase III clinical trial in hepatocellular carcinoma (HCC). An earlier clinical trial in 2010 reported HCC patients receiving sorafenib and doxorubicin together had a longer survival time compared to sorafenib alone [48], but a more recent clinical trial published this year had opposite results [49]. It is likely that molecular heterogeneity among the HCC patients contributes to the different results observed. Thus, including further characterization of the tumors in various groups should be undertaken in new clinical trials to determine the best treatments.

In humans, sorafenib had been used in clinical trials for osteosarcoma. Two clinical trials in osteosarcoma patients were reported in recent years. One phase ll non-randomized trial explored sorafenib treatment in 35 patients with relapsed and unresectable OSA and reported that the median survival was 7 months [23]. In another non-randomized phase 2 clinical trial, researchers used sorafenib and everolimus to treat a group of 38 high-grade osteosarcoma patients. Among these patients, a subgroup of patients with overexpression of both P-ERK1/2

and P-RPS6 responded better than double negative patients, where the progression-free survival was 7 months and 2 months, respectively [24]. These small cohorts indicate that using sorafenib alone or with other drugs to treat OSA has potential to improve outcomes, yet more robust studies are needed to determine a more personalized approach to identify the optimal drug for each patient. One limitation in these clinical trials is that the recruited patients all had advanced and relapsed osteosarcoma. There is therefore lack of the knowledge of using sorafenib alone or in combination with other chemo therapeutic agents to treat early stage patients. For using sorafenib in clinical studies, a dosage of 400 mg twice a day was the maximum-tolerated dose and recommended in a preclinical model [15]. In another phase I clinical trial, used administration dosage for sorafenib, with 400 mg twice a day for 21 days with 7 days off in one cycle for repeated cycles, in patients with advanced, refractory solid tumors [50]. The adverse effects mentioned in these reports such as lymphopenia and hypophosphatemia (16%) hand and foot syndrome (13%), fatigue (5%), oral mucositis (5%), diarrhea (5%), and anemia thrombocytopenia (11%) were consistent with previous phase I clinical trials [42, 50-52]. These toxic effects lead to dose reductions, short interruptions, or discontinuation for these patients. Some adverse effects were noted in sorafenib clinical trials, including mucositis (20%), rash (19-40%), alopecia (27%), xerosis (16%), xerostomia (11%), and hand foot skin reaction (20-30%) [24, 42, 53]. One strategy to reduce these toxicity effects is to use combination therapies. Therefore, involving sorafenib and other therapeutic agents such as doxorubicin can be an effective approach to reach effects but also avoid intolerable adverse effects. Combined chemotherapies are with drugs utilizing different mechanisms of action,

have the potential to decrease the possibility of drug resistance and reduce the dosage of drugs, while remain the optimal treatment efficiency.

In dogs, there are very limited reports on sorafenib. One report published pharmacokinetic studies on sorafenib on healthy dogs, and showed that 60 mg/kg per day was tolerable in a 4-week study [54]. To date, there is only one recent report of tolerable dosage in a small group of dogs with different types of cancer [26]. In this report, 12 dogs were separated into two different doses (2 mg/kg and 3 mg/kg per week). The dogs tolerated the drug up to a dose of 3 mg/kg for 3 to 8 weeks of treatment period. Thus, clinical studies with sorafenib can be undertaken in dogs but therapeutic doses for cOSA have not determined. Dogs can be used as preclinical models before chemotherapies are applied in human clinical trials [32, 33, 55]. The reference dosages used in these studies will be helpful to identify initial treatment dosages for future clinical trials in cOSA and hOSA patients. Also, due to the current regulations in human clinical trials, the recruited patients are those who have failed to respond or relapsed after first line treatments [23, 24], which rise the difficulties to evaluate the sorafenib in earlier stage patients. However, in canine patients, we can initiate studies in both naïve and in relapsed cases, and hopefully such information can facilitate human clinical trials.

In the canine oncology field, dasatinib [56, 57] and toceranib [58-61] use has been reported in a small numbers of osteosarcoma patients for oral tolerability and safety verification. The major aspects of these studies are summarized in **Table 3. 3.**. Two clinical studies reported the potential of dasatinib for treating cOSA. One of studies was a drug screening containing 86 small molecule kinase inhibitors on cells derived from one canine patient which reported the IC_{50} values for dasatinib and sorafenib were 0.15 and 9.5 μM respectively, suggesting that

dasatinib was the most promising therapeutic agent. The patient survived for more than 730 days after the initial diagnosis after completed amputation and 5 cycles of carboplatin and followed by dasatinib treatments [56]. Another study reported four cOSA patients with reported survival times ranging from 456-1003 days with treatment of dasatinib at 0.5-0.75 mg/kg every day or every other day for 6.5 to 25 months and one dog was still alive at the time of publication of the paper, at which time it was 1003 days post treatment [57]. These two reports indicate potential clinical benefit of dasatinib as adjuvant treatment for cOSA, but larger scale studies are needed. Toceranib phosphate, which is the first TKI approved for veterinary use, is the most utilized molecularly targeted agent in the United States. Toceranib targets receptor tyrosine kinases, including C-KIT, VEGFR-2, PDGFα/β, CSF-1 (Colony stimulating factor-1) and is used as a first-line treatment for dogs with mast cell tumor [62]. The first clinical study used toceranib in cOSA, reported that it contributed to clinical benefit on eleven out of a total of twenty-three cOSA patients, as these 11 patients presented with either partial response or stable disease [63]. Later on, two small cohorts contained 20 [58] and 22 [59] cOSA patients with lung metastasis reported with a different conclusion, as canine patients treated with amputation and adjuvant chemotherapies and toceranib had median survival times of 90 and 89 days respectively, vs 76 [64] and 95 [65] days in reported studies of metastatic cOSA patients, indicating limited clinical benefit. While those two studies showed that using toceranib did not have a therapeutic effect on canine patients with metastatic osteosarcoma [58, 59], other studies explored combing toceranib with other therapeutic agents in cOSA without metastatic disease. A clinical study that reported on 10 cOSA patients treated with toceranib and carboplatin resulted in overall survival times of 253 days [60], which was lower

than the previous published median survival 321 days from a study of 48 cOSA treated with amputation and 4 cycles of carboplatin [66]. In another study of 126 cOSA patients compared the outcomes of addition toceranib to treatments with carboplatin/piroxicam/cyclophosphamide [61]. In the treatment group with the addition of toceranib, the overall survival times was 318 days, while the overall survival times in control group was 242 days, but, the statistical analysis showed no clinical benefit with toceranib [61]. These findings are not surprising, since toceranib mainly targets c-KIT, and c-KIT is not a major driver in OSA. In addition, the effects of toceranib on other targets VEGFR-2, PDGFα/β, CSF-1 are not fully studied in canine OSA. One clinical trial with 10 cOSA reported the levels of VEGF did not change over time in cOSA patients with toceranib treatments [60]. Also, a recent study documented that toceranib treatments did not change the expression of VEGFR-2, PDGFα/β and c-KIT from control group in *in vivo* study [67]. The expression of VEGFR-2 varied in different OSA cell lines and tumors [47, 68], which suggest the potential of using toceranib with VEGFR-2 overexpressed patients and the significance of individual targeted therapy. Sorafenib and dasatinib, on the other hand, inhibit multiple receptor tyrosine kinases that are relevant in OSA. The data from our study also support use of sorafenib alone and in combination with doxorubicin in canine clinical trials.

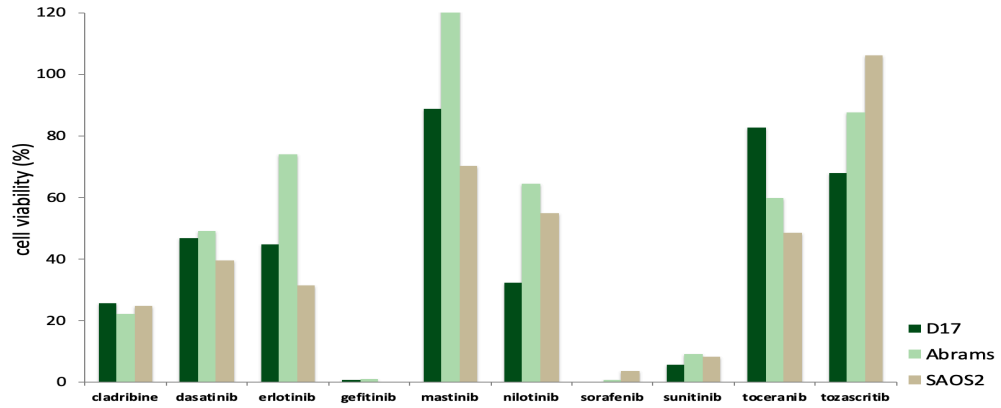
In conclusion, the findings from our studies suggest that, tyrosine kinase inhibitor sorafenib alone exhibits anti-tumor activity through inhibition of proliferation, induction of apoptosis via inhibition of ERK/MAPK, and STAT3 pathway in OSA cells. Sorafenib is a comparatively novel drug in osteosarcoma, therefore, information on large scale clinical data and other drug combinations are limited [69]. In addition, when we combined sorafenib with doxorubicin to

treat osteosarcoma cells, these two drugs induced cell arrest in the G2/M phase. Based on current findings, clinical trials using combination of doxorubicin and sorafenib in proof of concept studies in dogs are warranted. These studies can be carried out relatively quickly in dogs where case load is high, and in turn, provide useful information for initiation of clinical trials in humans.

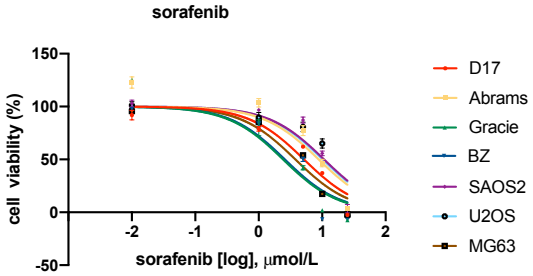
APPENDIX

Figure 3.1. Cell viability was measured by CellTiter-Glo assay on all seven OSA cell lines, including four canine OSA (D17, Abrams, Gracie, and BZ) and three human OSA (SAOS2, U2OS, and MG63) cell lines. All cell lines were treated with drugs for 72 hours. (**A**) 10 tyrosine kinase inhibitor showed different levels of inhibition on OSA cells viability. **(B**) OSA cell lines were treated with sorafenib for 72 hours. **(C)** OSA cell lines were treated with doxorubicin.

A



B



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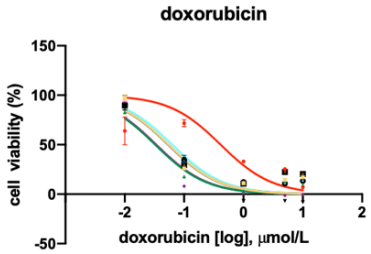


Table 3.1. The effects of sorafenib on cell viability and IC_{50}s for sorafenib and three first line OSA chemotherapy agents (cisplatin, carboplatin, and doxorubicin)

	cisplatin (µM)	carboplatin (µM)	doxorubicin (µM)	sorafenib (µM)
D17	4	45	0.2	6
Abrams	12	263	0.06	9
Gracie	2	41	0.06	4
BZ	12	273	0.05	3
SAOS2	3	47	0.07	7
U2OS	7	57	0.06	5
MG63	5	70	0.05	4
Reported Maximum plasma concentration	4 µM (dog)	72 µM (dog)	1.13 µM (dog)	13 µM (human) 7.6 µM (dog) *
reference	[32]	[33]	[55]	[54, 70]

*A single 400 mg dose was given to dogs (n=3) as tablets in a PK study.

Figure 3.2. Photomicrographs taken with Nikon camera at 40X magnification, compared wound healing in three OSA cell line with and without sorafenib for up to 48 hours. (D17: 3 µM, Abrams: 4 µM, SAOS2: 3 µM)

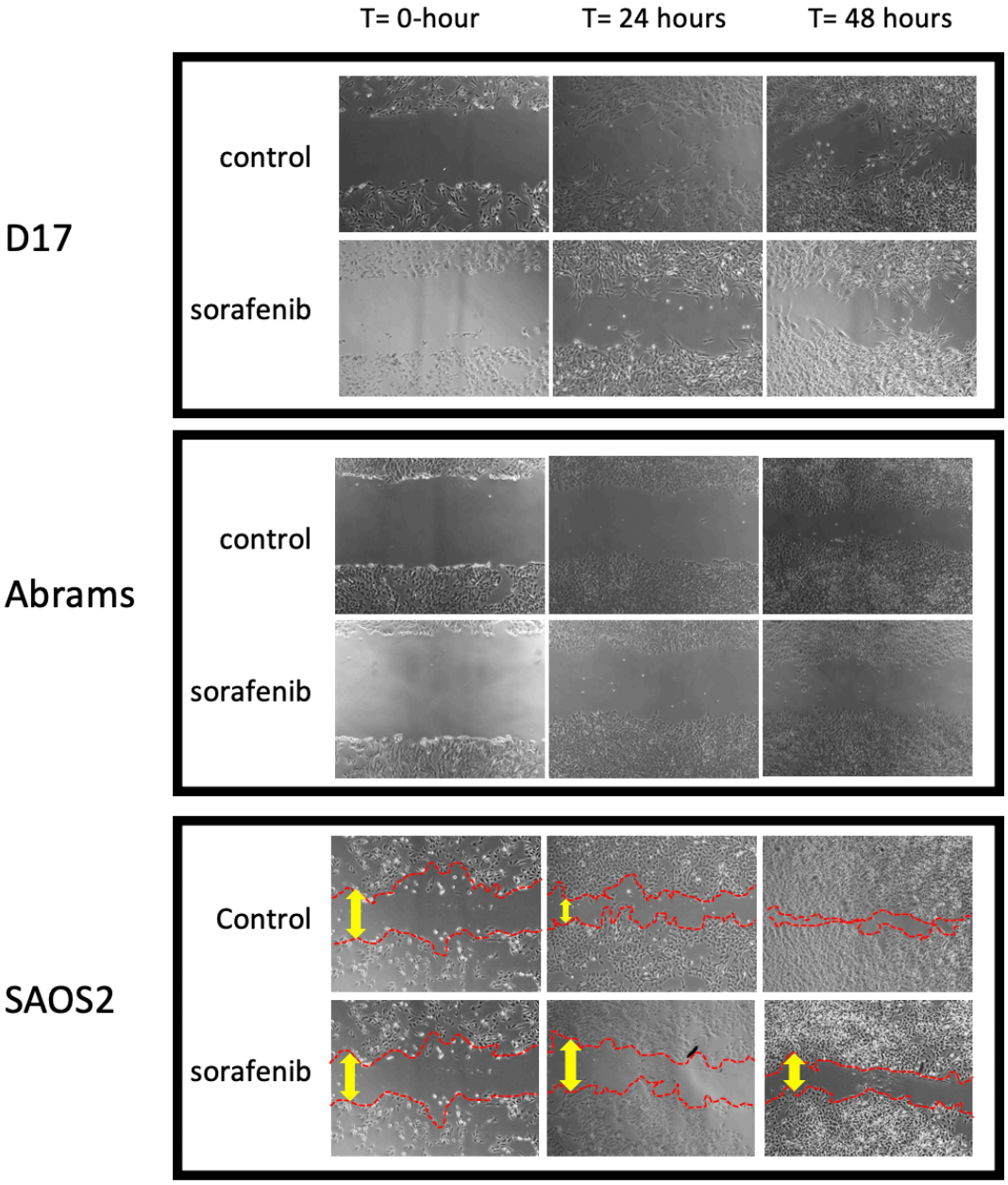
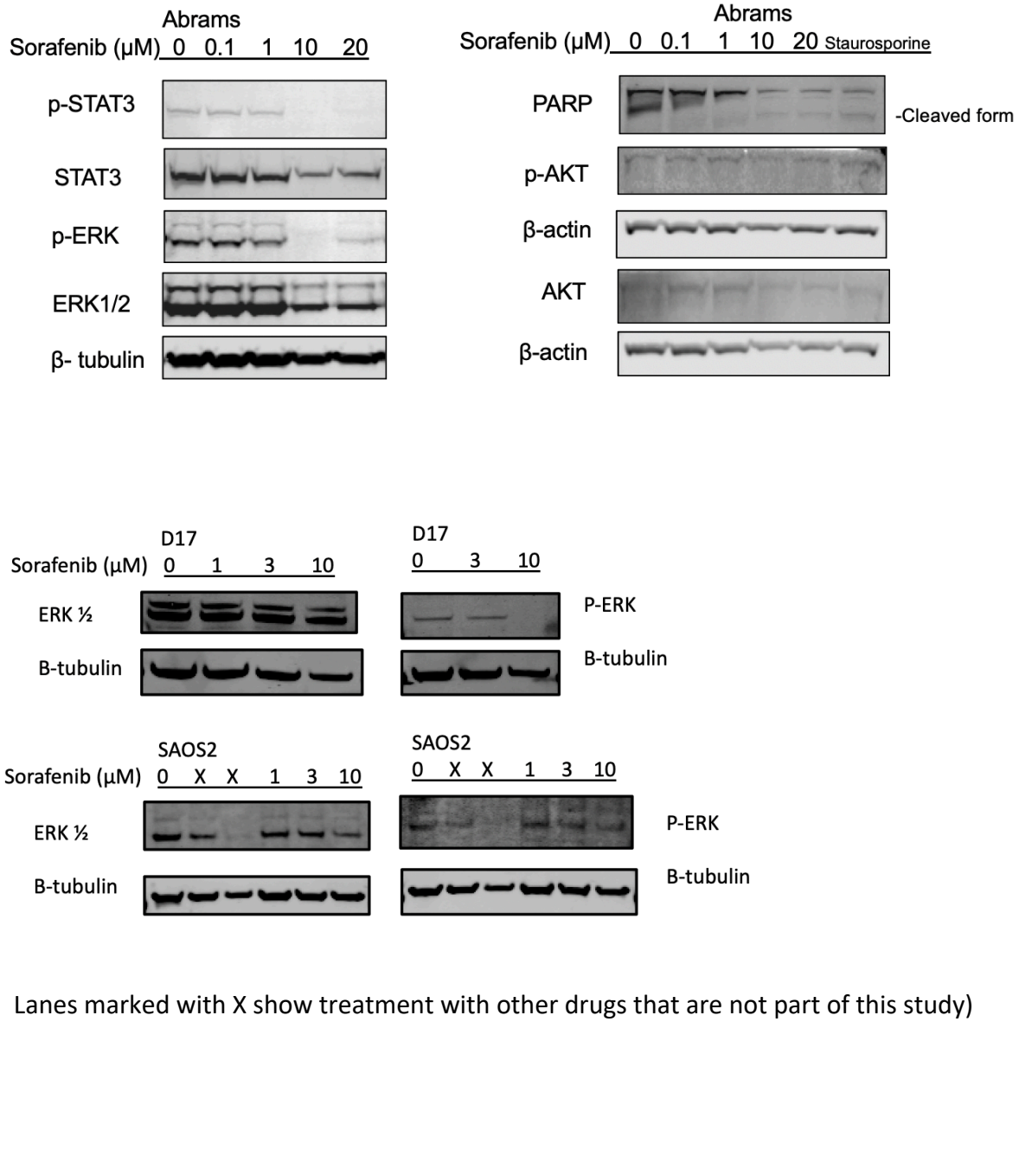


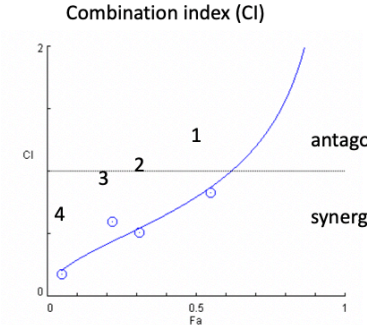
Figure 3.3. Sorafenib decreased expression of p-STAT3 and p-ERK but not p-AKT in protein analysis. Canine osteosarcoma Abrams cells were treated with either DMSO (control) or various concentration of sorafenib (0.1, 1, 10, 20 µM) for 24 hours then subjected to western blot analyses. β-actin and β-tubulin were used as loading controls. Cells incubated with 1 µM staurosporine for 3 hours were used as a positive control for PARP expression.



* Lanes marked with X show treatment with other drugs that are not part of this study)

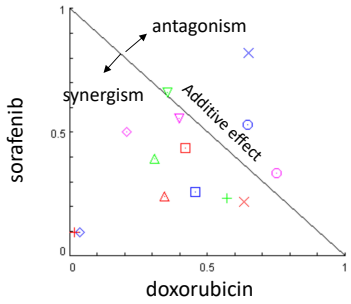
Figure 3.4. (A and B) Three different ratios of sorafenib and doxorubicin were examined with Combination index (CI) assay on SAOS2 cells, CI values less then 1 suggest synergistic effects (CompuSyn software). **(A)** a representative chart showed four different combination doses and CI values indicating these combinations are all synergism. **(B)** in Normalized isobologram, we included three different rations of sorafenib and doxorubicin (20:1, 50:1, and 100:1).
(C) The combination of sorafenib and cisplatin was examined on D17 cells at a ratio of 4:1.
(D) The combination of sorafenib and carboplatin was examined on D17 cells at a ratio of 1:5.

A



	CI values
1	0.83
2	0.52
3	0.6
4	0.18

B



CI values	sorafenib (μM)	doxorubicin (nM)	ratio
1.18	1.25	25	50:1
0.86	2.5	50	
0.7	5	100	
0.96	10	200	
0.13	20	400	
0.86	0.63	31.3	20:1
0.8	1.25	62.5	
1.09	2.5	125	
0.72	5	250	
0.59	10	500	
1.02	2.5	25	100:1
0.71	5	50	
1.47	10	100	
0.12	20	200	

C

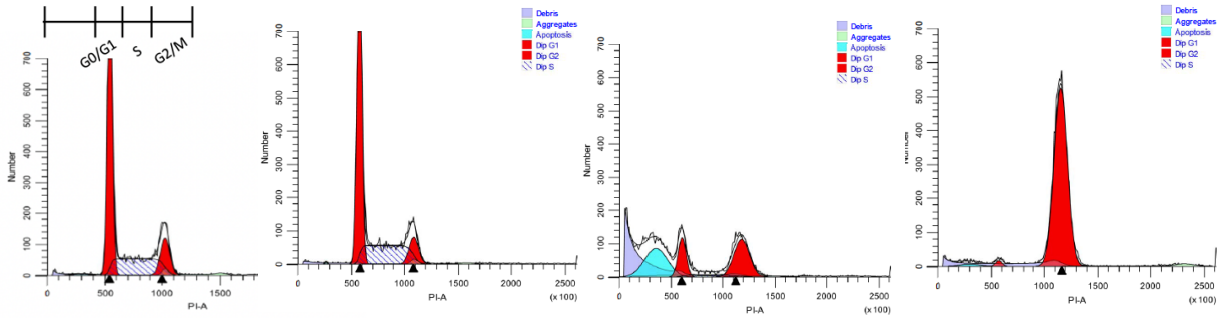
CI values	sorafenib (μM)	cisplatin (μM)	ratio
1.1	1.25	0.3	4:1
1.6	2.5	0.6	
1.5	5	1.3	
1.3	10	2.5	
0.9	20	5	

D

CI values	sorafenib (μM)	carboplatin (μM)	ratio
1.1	1.25	6.25	1:5
1.2	2.5	12.5	
1.3	5	25	
1.6	10	50	
0.9	20	100	

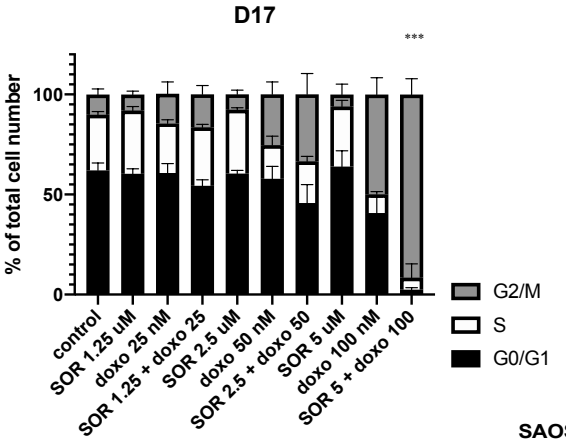
Figure 3.5. The combination of sorafenib and doxorubicin caused cell cycle progression in D17 cells. Cell cycle distribution of D17 OSA cells treated with wither **(A)** DMSO (control), **(B)** sorafenib 5μM, **(C)** doxorubicin 100 nM, or **(D)** both sorafenib 5μM plus doxorubicin 100nM for 24 hours. Representative flow histograms demonstrating changes in the cell cycle progression on canine OSA D17 cell line.

A **B** **C** **D**

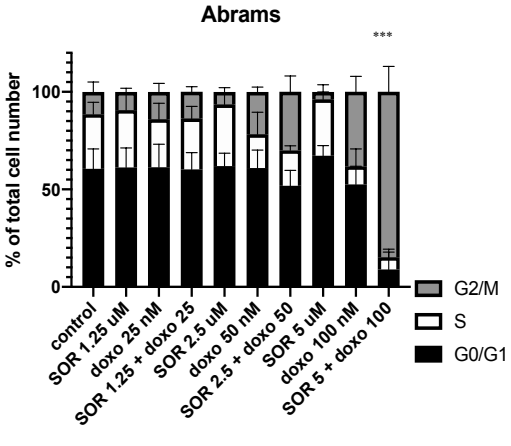


The combination of sorafenib and doxorubicin resulted in a cell arrest at G2/M phase. Representative cell cycle distribution graphs showed a G2/M cell arrest in **(E)** D17, **(F)** Abrams and human OSA **(G)** SAOS2 cell lines. ***P<0.001 for G2/M arrest compared to the combined treatment (5uM sorafenib and 100nM doxorubicin) as determined by one-way ANOVA with Dunnett's multiple comparison test.

E



F



G

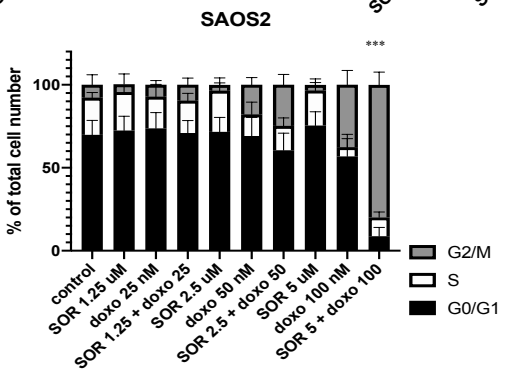


Table 3.2. Summary information and comparison of studies using TKIs in clinical studies for canine OSA (EOD every other day)

TKI	Survival time (days)	Treatment	Study size	Reference
Dasatinib	Alive 730 days after diagnosis	Amputation with carboplatin (5 cycles 275-300 mg/m^2, dose reduction due to grade 1 neutropenia) Followed by: Dasatinib dose: 0.5 mg/kg every other day (EOD) for a week, 0.5 mg/kg daily for first week and increased to 1mg/kg daily for 26 weeks	1, case report (golden retriever)	Davis 2013 [56]
	Dog1: 913 days Dog2: 882 days Dog3: > 1003 days Dog4: 456 days	n=3 amputation and 5 cycles of carboplatin (300 mg/m^2 every 3 weeks) n=1 (dog 3) amputation and 3 cycles of carboplatin (300 mg/m^2 every 3 weeks), discontinue due to detectable pulmonary metastases Followed by: Dasatinib dose: Starting dose was 0.5 mg/kg, dose was increased to 0.75 mg/kg after 2 weeks. Dog1: daily for 6.5 months Dog2: EOD for 10 months Dog3: daily for 6.5 months for 25 months Dog 4: EOD for 7 months	4 Dog1 (golden retriever) Dog2 (Labrador retriever) Dog3 (German shepherd mix) Dog4 (great Pyrenees)	Marley 2015 [57]
Toceranib (TOC)	48% of the patients had clinical benefits. One dog had partial remission and 10 dogs had stable disease, the median duration for the 11 dogs with clinical benefit was 24 weeks (ranging from 10-42 weeks)	n=21 had surgery followed with chemotherapy n=1 no treatment n=1 palliative radiation therapy only Followed by: TOC dose at 2.7 mg/kg (ranging 2.5-3.3), three times/week	23 metastatic OSA 4 mixed breeds, 4 golden retrievers, 3 Labrador retrievers, 3 greyhounds, and 9 other primarily large breed dogs	London 2011 [63]

TKI	Median survival times (days)	Disease-free interval (days)	Treatment	Study size	Reference
Toceranib (TOC)	89	57	Amputation, followed with carboplatin (n=19 received carboplatin, n=1 received carboplatin+ doxorubicin, and n=2 had no chemotherapy) Followed by: TOC dose at 2.7 mg/kg (ranging 2.5-2.9, due to tablet sizes) EOD	22 metastatic OSA 5 mixed breeds, 5 Labrador retrievers, 2 greyhounds, and 10 other breeds	Laver 2018 [58]

Table 3.2. (cont'd)

	90	36	1. surgery (n=15 limb amputation, n=1 scapulectomy, n=1 acetabulectomy, n=1 ulnar ostectomy) 2. chemotherapy (n=13 carboplatin, n=1 doxorubicin, n=3 metronomic cyclophosphamide), and/or radiation therapy (*n* = 2) 3. n=1 had no previous treatment Followed by: TOC dose: 2.52 mg/kg (ranging 2.12- 2.72 mg/kg) 3 times/week for a duration of 60 days (ranging 17 to 231 days)	20 metastatic OSA 3 Rottweilers, 2 Labrador retrievers, 2 Doberman pinschers, 2 golden retrievers, 2 mixed breeds, and 1 each of 9 other breeds	Kim 2017 [59]
	253	238	Amputation followed with 4 cycles of carboplatin (n=8 received the first dose of carboplatin at 300 mg/m^2, n=1 received 239 mg/m^2 (unknown reason), n=1 received 150 mg/m^2 (due to renal insufficiency) every three weeks Followed by: TOC doses, EOD starting at day 14 post carboplatin: n=8 received TOC at doses ranging from 2.2 to 2.9 mg/kg (median dose: 2.7 mg/kg) n=1 had two 1-week drug holidays related to grade I diarrhea n=1 had dose reductions and an extended dosing interval due to persistent, non-progressive grade I neutropenia.	10 (3 golden retrievers, 1 mixed breed dog, and 1 each of 6 other primarily large breed dogs	Ginger 2016 [60]
	242 control 318 TOC	215 control 233 TOC	Amputation followed with 4 cycles of carboplatin (300 mg/m^2 IV) every 3 weeks within 14 days of amputation, piroxicam at 0.3 mg/kg EOD Cyclophosphamide at 10 mg/m^2 EOD Followed by: Control Group: Amputation followed by carboplatin and piroxicam/cyclophosphamide Treatment Group: Amputation followed by carboplatin and piroxicam/cyclophosphamide and carboplatin and then by TOC (median dose of toceranib administered was 2.73 mg/kg EOD, and n=27 had dose reduction due to adverse events)	126 cOSA (Control n=63, Treatment n=63)	London 2015 [61]

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CHAPTER 4

EVALUATING THE POTENTIAL SYNERGISTIC EFFECTS OF BROMODOMAIN INHIBITOR JQ1 AND PROTEASOME INHIBITOR BORTEZOMIB ON HUMAN AND CANINE OSTEOSARCOMA CELL LINES

4.1 Introduction

Osteosarcoma is the most common bone tumor in both humans and dogs [1, 2]. In the United Sates, around 1,000 cases of human osteosarcoma (hOSA) [3] and 10,000 cases of canine osteosarcoma (cOSA) [4] are diagnosed each year. Humans patients have higher incidences in young adolescents (14-19 years old) and those over 70 years old compared to other age distribution. Similar to humans, there are two peaks of the incidences in the dogs, 1–2-year-old and from 6-10 years old [5]. Compared to humans, dogs have a much higher occurrence rate, the estimated incidence rate is about 14/100,000 in dogs [6, 7] and 1/100,00 in humans [8]. The standard of care for human OSA patients are cisplatin, doxorubicin, and high-dose methotrexate [9, 10]. Before the era of chemotherapy, the overall 5-year survival rate was around 20-25% with surgery as main treatment. With chemotherapy, either adjuvant or neoadjuvant treatment, the overall 5-year survival rate improved to around 75-80% [8, 11]. Canine OSA patients also benefit from chemotherapies. As described in Chapter 1 (1.3.2), one year survival rate in cOSA patients increased from 11-21% with surgery alone [12-15] to 33-62% with the chemotherapy regimens, including cisplatin [12, 13, 16, 17], carboplatin [18], and doxorubicin [18-20]. However, although systemic therapy has improved event-free period and overall survival in both species, there has been little progress in developing more effective therapies over the past three decades. In addition, around 80-90% of dogs and 20% of human patients are diagnosed with metastatic disease in the clinic [2, 21]. Patients with metastatic disease are difficult to cure, and show drug resistance to standard chemotherapies [22]. When metastatic disease occurs, the 5-year survival is only 15% to 30% in human OSA patients [8]. Similarly in dogs, the median survival time for dogs with lymph node metastasis was 59 days,

which is significantly shorter than patients without metastasis, 318 days [23]. Moreover, canine OSA are considered to have high metastatic potentials, with 90% of cOSA patients die of metastatic disease after one year with surgery alone [14, 24]. Thus, novel therapeutic agents are in urgent need.

Among the challenges of developing new drugs, one important fact is that OSA is a rare tumor type among all cancer in humans, and the high cost of developing new drugs for small patient populations makes funding limited for both basic research as well as drug development. Repurposing existing FDA-approved drugs can improve the situation, as it will decrease the period of time to bring new therapies for a given disease [25-27]. In the current study, we used a library of FDA-approved drugs to screen for anti-tumor activity on OSA cell lines. Among all the compounds we screened, a proteasome inhibitor, bortezomib showed the highest anti-proliferative effect. Bortezomib uses a novel mechanism, as compared to conventional chemotherapies that target DNA damage. Bortezomib inhibits both 20s and 26s proteasome function, binds to the β subunits at 20s catalytic core [28], causes the accumulation of ubiquitinated target proteins and amino acids, increases hypoxic stress, and leads to cell death. Bortezomib is the first in class of proteasome inhibitors that was approved by FDA for treating relapsed multiple myeloma patients in 2003 and has now been in clinical use for more than 10 years [29-31]. In other cancer type such as sarcomas, bortezomib was reported to induce cell apoptosis through inhibition of both the MAPK pathway [32] as the STAT3 pathway [33] in preclinical trials. In the current study, we show that bortezomib inhibits growth of canine and human OS cell lines in a concentration dependent manner at levels demonstrated to be achievable in plasma.

Another class of compounds that has been identified with promising results in recent research that employ yet another mechanism of action is the bromodomain and extra-terminal domain (BET) inhibitors. BET family proteins are epigenetic modulators that read acetylated lysine residues and regulate gene expression. The BETs regulate various genes involved in the cell cycle, cell proliferation, and inflammation. In recent studies, targeting BETs has become a compelling focus in diverse therapeutic areas [34, 35]. To date, JQ1, I-bet151, and I-bet762 are the most potent pharmacological inhibitors of BET protein family, demonstrating anti -tumor activity in a range of cancer types [36-44]. JQ1 is a selective, small molecular inhibitor of bromodomain developed by Bradner and colleagues [45]. The compound showed the ability to affect bromodomain and extra-terminal domain (BET) protein family and effective inhibition of BRD4 (bromodomain-containing protein 4), compared to other components in the family including BRD1, BRD2, BRD3, BRD4, and BRDT. Furthermore, studies have shown that JQ1 antagonized BET bromodomain proteins during MYC-dependent transcription among different cancers, including lung cancer [41], breast cancer [43, 46], multiple myeloma [38], acute myeloid leukemia [47], lymphoma [48], and osteosarcoma [42].

The oncogene *c-MYC* (also referred as *MYC*) is one of the major oncogenes that has been identified as a key driver of osteosarcoma [49]. MYC proteins are transcription factors composed of a basic helix domain, promotes oncogenic transcriptional amplification program in cancers [50]. *MYC* gene has been reported to be amplified in OSA, and high-level of MYC is associated with poor prognosis in osteosarcoma patients [51-53]. Moreover, some studies have reported that JQ1 inhibits the cancer cells through the suppression of MYC driven transcriptional amplification in human osteosarcoma cell lines [42, 51, 52].

In this study, we first investigated the effects of JQ1 on canine and human OSA cells. We found that JQ1 alone suppressed the *MYC*, *RUNX2*, and *FOSL1* expression at mRNA levels in D17 osteosarcoma cell line. However, FOSL1 and RUNX2 protein levels in JQ1 treated cells remained unchanged, and MYC protein levels were decreased with 10 μM of JQ1. Interestingly, when OSA cells treated with JQ1 and with bortezomib, the combination caused a significant *MYC* suppression at both mRNA and protein level. Taken together, we showed that JQ1 in combination with bortezomib has potential to be considered among the new chemotherapeutic strategies for OSA patients.

4.2 Material and Methods

4.2.1 Human and canine osteosarcoma cell line maintenance

Four canine osteosarcoma (D17, Abrams, Gracie, and BZ) and three human osteosarcoma (SAOS2, MG63, U2OS) cell lines were used in this study. D17, SAOS2, MG63, and U2OS cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA), two canine OSA cell lines Abrams and Gracie were gifts from Dr. Thamn, Colorado State University. The BZ cell line was established from a metastatic site from an eight-year-old male German shepherd dog with osteosarcoma presented to the Michigan State University Veterinary Teaching Hospital. The cells were isolated from a fresh fine needle aspiration sample, first treated with RBC lysis buffer for 10 minutes, the cells were palleted, resuspended in Minimum Essential Medium α (αMEM medium, Gibco, Carlsbad, CA), and later seeded to 100mm cell culture dish. The diagnosis of OSA was based on the histopathology findings. Positive staining of osteocalcin and vimentin from of cells cultured in the cell line also confirmed OSA.

Canine osteosarcoma cell lines were maintained in αMEM medium, supplemented with 10% fetal bovine serum, gentamicin. Human OSA cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM medium, Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum, gentamicin. All cell lines were incubated at 37°C containing 5% CO_2.

4.2.2 Chemical compounds

Bortezomib was purchased from LC laboratories, and initially was dissolved in DMSO. JQ1 was purchased from Cayman Chemicals, initially dissolved in DMSO. Both compounds were stored at -20°C until use. Final concentration of DMSO in all treatments was 1%.

4.2.3 High throughput drug screening (HTS)

High throughput drug screening was carried out at Michigan State University Assay Development and Drug Repurposing Core (ADDRC). OSA cell lines SAOS2 (750/well), D17 (1000/well), and Abrams (1000/well) were seeded into 384-well plates and screened with Approved Oncology Drug Set (NCI Developmental therapeutics Program, containing 114 antitumor drugs). Drugs were delivered to the wells by a Biomek FX Workstation liquid handling system (Beckman Coulter) at a single concentration of 1 μM. After 48 hours of incubation with drugs, cell viability was determined by CellTiter-Glo® Assay (G7570 Promega). The luminescence signal was read by a Synergy Neo (Bio Tek) detection platform. The Level of cell viabilities 3 standard deviations above control were identified as potential drugs to inhibit OSA cells.

4.2.4 Cell viability assay

OSA cells were treated with either 1% DMSO (as vehicle control), bortezomib, JQ1, or co-treatment of JQ1 and bortezomib at the indicated drug concentrations. For cell viability assay, cells were seeded to 96-well plates with 3,500 cells/well (for SAOS2, we used 2,500 cells/well). After overnight incubation, the culture medium was replaced with 100 μl of complete medium containing the designated drug concentrations. The OSA cells were incubated with the drugs for another 72 hours. In the end of incubation period, the viability of cells was analyzed using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation assay (MTS) according to the manufacturer's instructions (Promega Corp.). The formazan product was measured by EnVision Multimode Plate Reader (PerkinElmer) at 490 nM wavelength, and the percent growth inhibition was calculated for each treatment with the vehicles control containing 1% DMSO. The IC_{50} values (concentration of drug necessary to inhibit the cell growth by 50%) was then determined by PRISM Statistical Software (8.0.0, Graph Pad Software Inc., San Diego, CA). Each experiment was run in triplicate.

4.2.5 Caspase 3/7 assay

Apoptosis induced by bortezomib or JQ1 on three cell lines (D17, Abrams, and SAOS2) was determined by Caspase-Glo 3/7 assay kit (Promega). The caspase assay was carried out in white 96-well plates (so as not to interfere with the luminescent signal); 10,000 OSA cells were plated in each well, treated with either 1% DMSO (vehicle control), or bortezomib (0.001, 0.01, 0.1, and 1μM) or JQ1 (0.1, 1, 5, and 10 μM) for 24 hours. After incubation for 24 hours, caspase reagent was added to the cells and re-incubated at 37°C for 30 minutes. The luminescent signal

was detected by EnVision plate reader (PerkinElmer) and analyzed by Graph Pad Prism (8.0.0, Graph Pad Software Inc., San Diego, CA). Staurosporine (81590, Cayman), as an apoptosis inducer, was used as a positive control at 10μM concentration [54].

4.2.6 Wound healing assay

In this study, the scrape motility assay was used to test migration ability of cells in a monolayer. Briefly, 100,000 cells/well were plated overnight and allowed to reach confluence in 6-well plates. Then, scrapes were made using a 1-mL pipette tip. The cells were incubated with cell culture medium with or without compounds (bortezomib or JQ1). Each scrape was photographed after being made and at the specific time points thereafter.

4.2.7 Matrigel assay

Cell invasiveness was determined using Matrigel-coated invasion chambers. A total number of 50,000 cells incubated with or without the drug (bortezomib or JQ1) were added to the upper compartment of the chamber, and 10% FBS was placed in the bottom compartment as chemo-attractant. Cells incubated with 1% of DMSO were used as vehicle control. After 21 hours, cells that traversed the insert and reached the bottom of the membrane insert were fixed and stained with DiffQuick according to the manufacturer's instructions, photographed, and counted.

4.2.8 mRNA isolation

Total RNA was extracted from human or canine OSA cell pallets with *mirVana*™ miRNA isolation kit (Life Technologies, Thermo Fisher Scientific Inc.), then treated with DNase I to remove contaminating DNA. The concentrations of RNA were quantified by Qubit™ RNA HS Assay (Life Technologies, Thermo Fisher Scientific Inc.), a fluorescence-based RNA quantification method. cDNA was synthesized from total RNA using random hexamer primers and Superscript III reverse transcriptase (Invitrogen, CA).

4.2.9 Quantitative real-time PCR analysis

PCR was performed with Taq polymerase (Invitrogen, CA), using cycling conditions as follows: 94°C for 4 min; 40 cycles at 94°C for 1 min, optimal annealing temperature for 1 min, 72°C for 1 min; followed by 72°C for 5 min. *MYC*, *RUNX2*, and *FOSL1* expression at mRNA level were evaluated by using TaqMan primers (Life Technologies, Thermo Fisher Scientific Inc.) and analyzed by StepOnePlus Real-Time PCR system (Life Technologies, Thermo Fisher Scientific Inc.). All reactions were run in triplicate with GAPDH used as the endogenous control, water was included each time as negative control.

4.2.10 Drug combination and Combination Index (CI) assays

OSA cells were simultaneously incubated with two compounds at a fixed ratio (JQ1: bortezomib= 100:1, or 160:1) for 72 hours. Synergistic effects of two drugs were determined by isobologram and combination index (CI) analysis by the CompuSyn software (Combosyn, Paramus, NJ). The analysis was adapted from the median-principle methods of Chou and

Talalay [55], the results of CI <1, CI=1, and CI >1 indicate synergism, addition, and antagonism, respectively.

4.2.11 Cell Cycle assay

Cells were treated with DMSO (control), bortezomib (10nM), JQ1 (1μM), or combination for 24 hours, then collected by centrifugation and the cell pallet was fixed with 70% ethanol at 4 °C overnight. Ethanol was then removed after centrifugation, and cellular DNA in the remaining cell pallet was stained with propidium iodide (50 μg/ml) and RNase (1 mg/ml) in a total volume of 300 μl. After cells were stained for at least 4 hours, the PI fluorescence of individual nuclei was recorded with FACScan. The quantitative assessment of cell cycle phase and apoptosis were then determined by Modfit Lt™ software after correction of debris and aggregate population.

4.2.12 Protein analysis

For western blotting, a total number of 500,000 cells from D17, Abrams, and SAOS2 cell lines were treated with either vehicle (1% DMSO), bortezomib, JQ1, or combinations for 24 hours. Cell were lysed with 250 μl of CelLytic M lysis buffer (C2978, Sigma-Aldrich) with 2 μl of protease inhibitor (P8340, Sigma-Aldrich) and 2 μl of phosphatase cocktail inhibitor B (sc-45045, Santa Cruz.) according to manufacturer's protocol. Protein concentration were quantified with *Qubit™ Protein Assay* Kit.

A total of 50 μg of protein per well was loaded on Bolt Bis-Tris (4-12%) gel (Life Technologies, Thermo Fisher Scientific Inc.) and were transferred to polyvinylidene difluoride (PVDF)

membranes. The membranes were incubated with 5% bovine serum albumin (BSA) for 2 hour at room temperature then incubated with the following primary antibodies at 4 °C overnight to detect antigen: MYC (1:500), FOSL1(1:500), PARP (1:1000), β-actin (1:4000), β-tubulin (1:4000), (Cell Signaling Technology). After three washes in tris-buffered saline with 0.05% Tween 20, the membranes were incubated with appropriate secondary antibody (donkey anti-mouse (1:15,000) or goat anti-rabbit (1:15,000)) for 1 hour at room temperature. The membranes were visualized by Odyssey Infrared Imaging System (LI-COR Biosciences), and the protein band intensities were quantified by Image Studio™ Lite software (LI-COR).

4.2.13 Statistical Analysis

Results represent at least three separate experiments. Statistical analysis was performed with Graph Pad Prism (8.0.0, Graph Pad Software Inc., San Diego, CA), differences between categories were analyzed with one-way ANOVA, p-values of < 0.05 were considered to be significant.

4.3 Results

4.3.1 Drug screening identified several FDA-approved drugs as potential treatments for hOSA and cOSA

As seen in **Table 4.1**, several classes of drugs showed growth inhibition at 1 μM on two canine OSA (D17 and Abrams) and one human OSA (SAOS2) cell lines. These drugs include a purine analog drug (cladribine), protein translation inhibitor (methotrexate), DNA polymerase inhibitor (omacetaxine), DNA topoisomerase II alpha inhibitors (mitoxantrone, idarubicin, teniposide,

doxorubicin, daunorubicin), tubulin inhibitors (paclitaxel, ixabepilone, docetaxel, cabazitaxel, vincristine), and proteasome inhibitors (bortezomib and carfilzomib).

Among all the candidates, bortezomib was the most effective compound that inhibited cell viability on all three OSA cell lines (D17, Abrams, and SAOS2) examined in the initial high throughput screening. We selected bortezomib as potential compound for further studies. JQ1 was also effective against OSA cells at the 10 µM concentration.

4.3.2 Both bortezomib and JQ1 inhibit proliferation of human and canine OSA cells in a dose dependent manner

The effect of JQ1 and bortezomib alone on the proliferation was examined *in vitro* with a panel of human and canine osteosarcoma cell lines by MTS assay. As seen in **Table 4.2**, both bortezomib and JQ1 showed dose-dependent inhibition of proliferation of all OSA cell cultures. The IC_{50} values of bortezomib ranged from 11- 215 nM on cOSA and 16- 32 nM on hOSA cell lines. For JQ1, the IC_{50} values ranged from 0.5- 8 µM on cOSA and 1- 8 µM on hOSA cell lines. Both JQ1and bortezomib are effective against OSA at concentrations demonstrated to be achievable in plasma in the literature, suggesting these two drugs have therapeutic potential.

4.3.3 Bortezomib and JQ1 alone decreased migration and invasion ability of D17, Abrams and SAOS2 cell lines

As shown in **Figure 4.2A**, bortezomib decreased OSA cells migration ability. Similarly **Figure 4.2B**, when OSA cells were treated with a dose lower than its IC_{50}s, JQ1 inhibited the migration ability. We also evaluated the invasion ability in canine and human OSA cells with the

treatments of these two compounds separately. In **Figure 4.3C**, we demonstrated that both bortezomib and JQ1 caused an inhibitory effect on OSA invasion ability. In these two assays, we used concentrations below the IC_{50} values to avoid toxicity effects.

4.3.4 Bortezomib and JQ1 induced apoptosis in OSA cells

To further study the cellular mechanisms related to the inhibition of OSA cell growth, we evaluated apoptosis by caspase 3/7 activity when cells incubated with bortezomib **(Figure 4.3A)** or JQ1 **(Figure 4.3B)**. After 24 hours, bortezomib induced apoptosis in a dose-responsive manner. As seen in **Figure 4.3A,** in all three OSA cell line we examined, a significant increase of 3/7 activity was noticed at the two concentrations, 100 nM and 1000 nM, compared to vehicle control (1% DMSO). In **Figure 4.3B**, JQ1 also induced apoptosis at concentrations above 1uM.

4.3.5 Effect of JQ1 on *MYC* and *RUNX2* expression

In previous reports, JQ1 has been reported to downregulate *MYC* expression at mRNA levels in various cancer [41, 42, 47, 48, 56]. In the current study, seven different OSA cell lines showed different levels of *MYC* expression, as quantified by qPCR and compared to canine MSCs (adipose derived mesenchymal stem cells) **(Figure 4.4A)**. To evaluate inhibition of *MYC* expression by JQ1 alone or with bortezomib, we treated canine OSA cell lines Abrams and D17 with JQ1 at different concentrations (0.1 to 10 μM) for 24 hours and monitored changes at mRNA levels by qPCR **(Figure 4.4B, C)**. JQ1 treatment decreased *MYC* expression in a dose-dependent manner in both Abrams and D17 cells. In D17 cell line, JQ1 treatment also decreased *RUNX2* and *FOSL1* expression at mRNA level **(Figure 4.5C)**. In protein analysis **(Figure 4.5D)**,

MYC expression was downregulated in a dose-dependent manner while and FOSL1 level was not changed with the treatments of JQ1 for 24 hours.

4.3.6 Synergistic effects of JQ1 and bortezomib on canine and human OSA

Bortezomib and JQ1 were identified by our drug screening as the two most efficient compounds to inhibit OSA cell growth. To test the possibility of combining these two drugs with different mechanisms of action, SAOS2 cells were exposed to various concentrations of JQ1 and bortezomib for 72 hours, then their cell viability was examined by MTS assay. In a constant ratio of 100:1 and 160:1, average combination index (CI) values were below 1 at all doses **(Figure 4.5A),** suggesting synergism. In **Figure 4.5B**, we examined the migration capacity with either one drug, or in combination by wound healing assay. In D17 cell line, when we used a low concentration, we can see cells migrated less in co-inhibition groups (JQ1+bor) compared to control group. But this was not observed in the other two cell lines, Abrams and SAOS2.

4.3.7 Changes of Cell Cycle

When OSA incubated with 1 μM JQ1, D17 and Abrams cells were arrested at G2/M phase **(Figure 4.6A)**. Also, 10 nM bortezomib alone increased cells arrested at G2/M phase cells in D17 and SAOS2 cell lines whereas Abrams showed an increase of G1 phase arrested cells. However, co-treatments of JQ1 and bortezomib resulted in a significant increase of G2/M phase cell arrest in all three OSA cell lines we tested **(Figure 4.6B, C)**.

4.3.8 Co-inhibition of JQ1 and bortezomib induced significantly suppression of MYC

When D17 cells incubated with 1 μM JQ1 combined with a low dose 10 nM bortezomib, the *MYC* and *RUNX2* expression was significantly downregulated at mRNA level, whereas no effect of *FOSL1* gene expression were observed **(Figure 4.7A)**. Interestingly, we saw a decrease of FOSL1 protein expression with the two compounds combined **(Figure 4.7A)**. In western blotting, no change of MYC or FOSL1 expression were noted when cells incubated with JQ1 or bortezomib only, a decreased of MYC, RUNX2, and FOSL1 protein expression were showed in JQ1 (1 μM) plus bortezomib (10 nM) treatment cells **(Figure 4.6B)**. Next, we examined apoptosis in Western blot to detect PARP cleavage. Single treatment of JQ1 or bortezomib caused an increase in cleaved PARP and, combined treatments resulted an elevated cleaved PAPR expression in all three cell lines **(Figure 4.6C)**.

4.4 Discussion

Our findings provide rationale of using JQ1 to target MYC pathway in canine osteosarcoma and point out the potential of using JQ1 in combination with bortezomib for future studies. Bortezomib and JQ1 showed different mechanisms of action in OSA cells, and they caused an add-on effects in cell growth inhibition, cell cycle arrest, and inhibition on MYC expression. The synergistic effects pointed to a prospective way to treat OSA.

The conventional chemotherapeutic agents including cisplatin, carboplatin, and doxorubicin are the major agents in the OSA treatment since the 1970s. Since then, no novel therapeutic agents have been found to improve survival compared to these chemotherapies. Therefore, we wanted to use high throughput drug screening to identify potential therapeutic agents to treat

this disease. This approach have been tested in preclinical models [25, 57] or in canine patients [26]. In the present study, we used HTS to identify a highly effective compound, bortezomib. Our data show that bortezomib is capable of inhibiting OSA cell growth with an IC_{50} value below plasma achievable concentration in the literature [58].

Bortezomib is a selective proteasome inhibitor which has been approved by FDA as the treatment of multiple myeloma patients after failure of first line therapies [59]. Bortezomib affects multiple signaling pathways, including decreasing invasion and increasing G2/M cell cycle arrest [60], inhibiting MAPK pathway activity and increasing apoptosis [32, 61], or increasing apoptosis through RUNX2 stabilization [62]. However, while bortezomib shows great growth inhibition in cell culture, it also causes severe side effect in mice models [63] and human patients [58]. Therefore, utilizing combinations of bortezomib at lower doses with other therapeutic agents may be potentially useful. Using combination is a way to enhance the therapeutic effects and decrease the adverse effects for both drugs [44, 64, 65]. When bortezomib was evaluated in a histiocytic sarcoma models from our research group (data not shown), the toxicity was dramatic to mice and thus dosage had to be decreased within the first week of treatment. This resulted in difficulties to evaluate drug efficiency. The toxicity to animal models urged us to consider drug combinations, in order to decrease the dosages but maintain equal effect. In the current study, we explored the potential of combination dosages and found that JQ1 and bortezomib had synergistic effects at certain doses; one of the doses we used is 10 nM bortezomib and 1μM JQ1, these two doses are below or close to IC_{50} values. The IC_{50} values as shown in Table 4.1 were measured after 72 hours of incubation, the IC_{50} values of 24 hours (which is the incubation period we used for most of *in vitro* studies) are higher.

In osteosarcoma, transcription factor c-MYC is one of the critical oncogenic drivers. Another focus in the study is whether JQ1 caused a decrease in MYC expression. Although etiology of osteosarcoma is still unclear, several genes have been identified as drivers of OSA development. Among them, *MYC* is one of the most highly amplified oncogenes in cancer, involved in cell proliferation, apoptosis, metabolism, and angiogenesis [66-69]. In osteosarcoma, MYC amplification has been detected in canine OSA samples [70, 71], and the overexpression of MYC were associated with poor prognosis and malignancy in both human [51, 72] and canine OSA. Therefore, targeting MYC has been proposed to treat many cancers. Due to difficulties to target MYC directly since it lacks of a specific active site or a specific confirmation where a small molecule can bind to inhibit function, alternative approaches are used to indirectly target MYC [47, 66, 73]. One of the approaches is to inhibit MYC transcription. JQ1 was developed as a BET inhibitor, and was shown to suppress MYC expression [37, 38, 42, 47], and this is confirmed in our study.

The effects of JQ1 in human osteosarcoma cells has been reported in three studies using established OSA cell lines [42, 44, 65], and the results suggest that MYC plays a critical role in some cases [42]. In some cases, JQ1 alone did not induce changes in *MYC* expression, but the effects of JQ1 was enhanced when combined with other drugs, including rapamycin [65], bortezomib [64] or CDK inhibitor [44]. Among these studies, only one show that JQ1 alone significantly inhibited the growth in xenograft mice model, but the other two groups proposed to use a combined therapy such as rapamycin or CDK inhibitors to enhance the antitumor activity of JQ1 [44, 65]. These previous studies focused on human osteosarcoma cell lines, the effects of JQ1 on cOSA has not previously been reported. To our knowledge, the current study

is the first to investigate JQ1 treatment in cOSA. Our study showed *MYC* expression was downregulated in both mRNA and protein levels at a high dose at 24 hours of exposure time. Besides *MYC*, recent studies demonstrate alternative gene targets for JQ1, such as *FOSL1* and *RUNX2*, which may be involved in anti-tumor activity of BET inhibitors [42, 44]. RUNX2 is a key transcription factor associated with osteoblast differentiation [42] and an oncogenic driver in osteosarcoma [70]. The overexpression of RUNX2 was also reported to correspond with poor response to chemotherapy [74]. A previous study revealed that RUNX2 expression is significantly increased in OSA tumors compared to normal tissues [70]. In our study, we also demonstrated that JQ1 causes significant decrease in RUNX2 expression in D17 and SAOS2 cell lines. These findings suggest that RUNX2 may be important to OSA disease progression in both species.

In our studies, we showed that the combination of JQ1 and bortezomib induced a significant decrease of *MYC* expression at mRNA and protein levels, which is similar to a recent study in colorectal cancer cell lines [64]. In this colorectal study, other mechanisms involved in this synergism include the FOXM1, NFκB (receptor activator of nuclear factor kappa B) pathway, G2/M arrest related cell cycle genes, including GADD45A, GADD45B, and GADD45G genes [64]. The induction of GADD45 proteins was correlated with MYC inhibition with JQ1 and bortezomib and led to cell death [64]. Although the authors proposed that MYC and FOSL1 suppression is critical for the synergistic antitumor effects of JQ1 and bortezomib, there are still other mechanisms such as through RUNX2. One possible mechanism involved in the synergism of these combination is NFκB pathway, a key pathway implicated in osteoclast activation. As reported by Lamoureus et. al., JQ1 inhibited NFκB activity but without affecting its transcription

in human OSA cells [42]. Interestingly, bortezomib is known for its function of block the activation of NFκB through stabilization of the NFκB inhibitor (IκB) [75, 76]. This might be a potential mechanism to explain the growth inhibition of OSA cells with co-treatments.

To our knowledge, the current study is the first study to demonstrate that JQ1 suppresses MYC expression and shows synergistic effects with bortezomib in canine osteosarcoma. In the current study, we showed that JQ1 treatments suppressed *MYC* expression at mRNA and protein levels, which is similar to hOSA findings [42]. Also, our data demonstrated that JQ1 and bortezomib in combination downregulated MYC, RUNX2 and FOSL1 protein expression and induced apoptosis *in vitro*. The combination of the two compounds showed positive results in *in vitro* studies, however, evaluation of utilizing these two drugs, or these two classes of drugs *in vivo* are needed. In addition, the next generation of proteasome inhibitor, carfilzomib, was one of other compounds identified from the drug screening. Carfilzomib is a second-generation proteasome inhibitor that is orally bioavailable and was also identified as an effective against OSA cells in our screening. Other BET inhibitors have also been developed after JQ1, such as IBET-151 and IBET-762, which have improved oral bioavailability. The improvement on bioavailability of these drugs might also decrease the needed dose and might reduce adverse effects. In summary, our findings provide a rational for the use of this combination of two different mechanisms for further xenograft studies, clinical trials and ultimately to treat canine and human OSA patients.

APPENDIX

Table 4.1. Protential drugs identified from Hight Throughput Screening (HTS)

Name (trade name)	Percent canine fibroblast growth inhibition	Percent of OSA growth inhibition in different cell lines			Mechanism of Action
		D17	Abrams	SAOS2	
Cladribine (Leustatin)	< 9	29	68	55	purine analog inhibitor
Methotrexate (Trexall)	<6	64	49	13	dihydrofolate reductase (DHFR) inhibitor
Omacetaxine (Synribo®)	31	80	86	90	protein translation inhibitor
Gemcitabine (Gemzar®)	<5	22	82	62	DNA polymerase (alpha/delta/epsilon) inhibitor
Mitoxantrone (Novantrone®)	<10	34	66	54	DNA topoisomerase II alpha inhibitor
Idarubicin (Idamycin®)	23	79	93	87	DNA topoisomerase II alpha inhibitor
Teniposide (Vumon®)	27	26	82	63	DNA topoisomerase II inhibitor
Doxorubicin (Adriamycin®)	43	75	73	73	DNA topoisomerase II alpha inhibitor
Daunorubicin (Cerubidine)	53	74	84	80	DNA topoisomerase II alpha inhibitor
Paclitaxel (Taxol®)	20	73	71	71	Tubulin inhibitor
Ixabepilone (Ixempra™)	20	79	74	74	Tubulin inhibitor
Docetaxel (Taxotere®)	27	63	74	73	Tubulin inhibitor
Cabazitaxel (JEVTANA®)	28	63	64	70	Tubulin inhibitor
Vincristine	30	62	73	76	Tubulin inhibitor
Bortezomib (Velcade®)	70	97	96	96	proteasome inhibitor
Carfilzomib (Kyprolis®)	89	88	95	94	proteasome inhibitor
JQ1	NA	71	52	78	Bromodomain inhibitor

*JQ1 result was using 10μM

Table 4.2. IC_{50} values for bortezomib and JQ1 on OSA cell lines
Cell viability was measured by CellTiter 96® MTS assay on all seven OSA cell lines, including four cOSA (D17, Abrams, Gracie, and BZ) and three hOSA (SAOS2, U2OS, and MG63) cell lines. All cell lines were treated with drugs for 72 hours.

	bortezomib IC_{50}s (nM)	JQ1 IC_{50}s (μM)
D17	13.5	0.5
Abrams	215	8
Gracie	11	0.5
BZ	15	0.7
SAOS2	32	4
U2OS	30	8
MG63	16	1
Reported achievable plasma concentration	200-300 nM (human) [58]	22 μM (mice) [77]

Figure 4.1. Cell viability was measured using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation assay MTS assay on four cOSA (D17, Abrams, Gracie, and BZ) and three hOSA (SAOS2, MG63, U2OS) cell lines after treated with JQ1 or bortezomib for 72 hours.

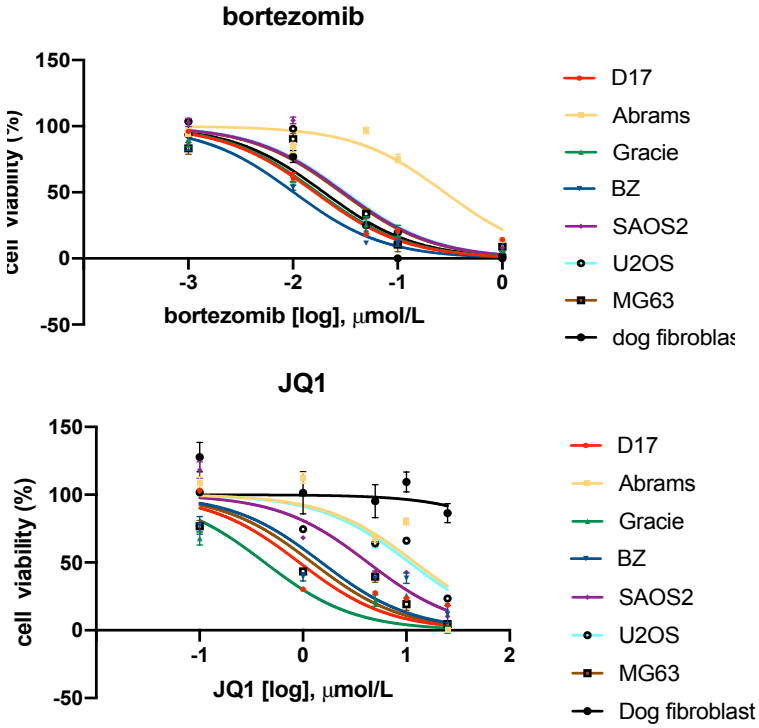
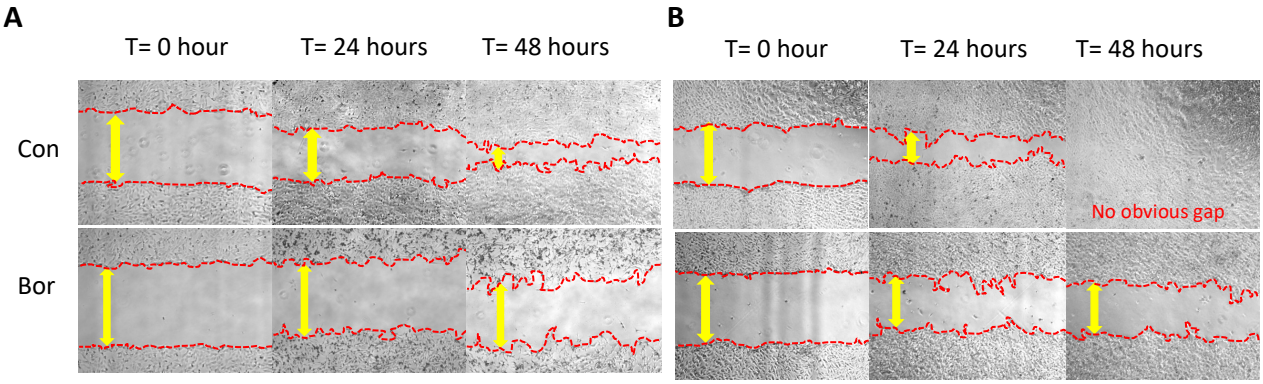


Figure 4.2. OSA cell migration and invasion ability were inhibited by bortezomib and JQ1. **(A)** Pictures comparing wound healing in SAOS2 cell line with and without bortezomib at 0.015 µM concentration (IC_{50}= 0.03 µM). **(B)** Pictures comparing wound healing in D17 cell line with and without JQ1 at 0.4 µM concentration (IC_{50}= 0.5 µM).



(C) Invasion of Matrigel by OSA cell lines with and without JQ1 (D17: 0.25, Abrams: 4, SAOS2: 2 µM) or bortezomib (D17: 0.05, Abrams: 0.1, SAOS2: 0.02 µM). **(D)** Cells invaded through the Matrigel membranes were quantified.

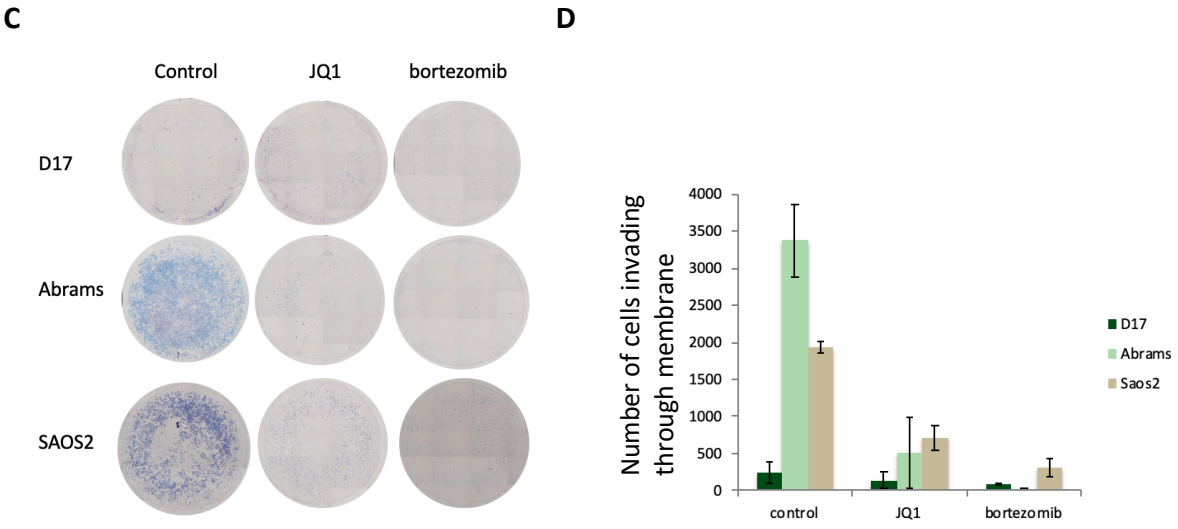
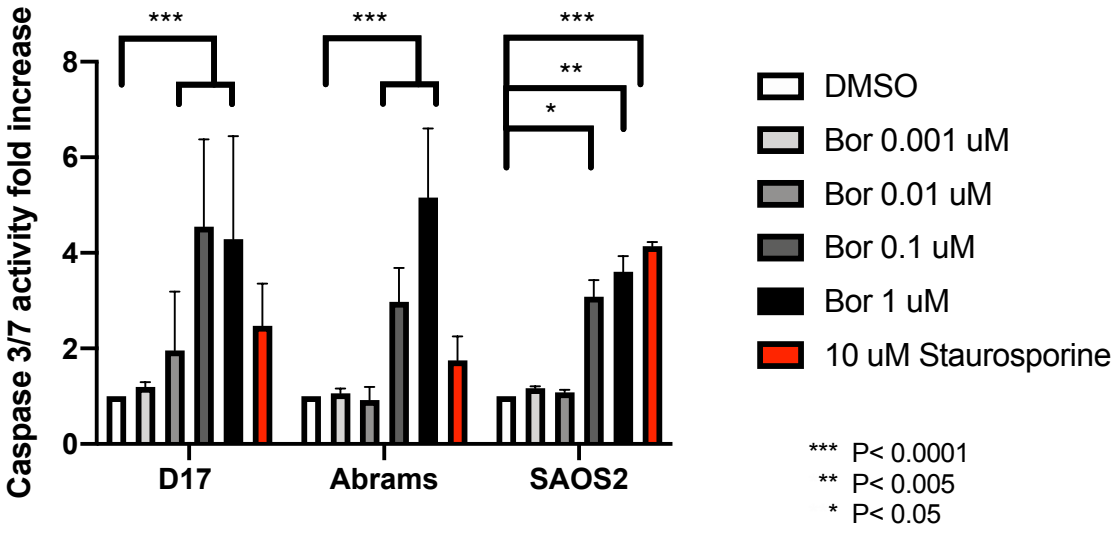


Figure 4.3. JQ1 induced apoptosis in canine OSA cell lines D17, Abrams, and SAOS2. **(A)** OSA cells were incubated with bortezomib, concentration ranged from 0.001μM to 1μM. **(B)** D17, Abrams, and SAOS2 cell line were treated with JQ1, concentrations ranged from 0.1 to 10μM for 24 hours. For all caspase 3/7 assays, levels of apoptosis were evaluated by Caspase-Glo 3/7 assay on cells treated with DMSO (as control), bortezomib (0.001μM, 0.01μM, 0.1μM, 1μM), JQ1 (0.1μM, 1μM, 5μM, or 10μM), or Staurosporine for 24 hours. 10 μM staurosporine (81590, Cayman) was used as a positive control. Values correspond to averages and error bars to standard deviations generated from three separate experiments.

A



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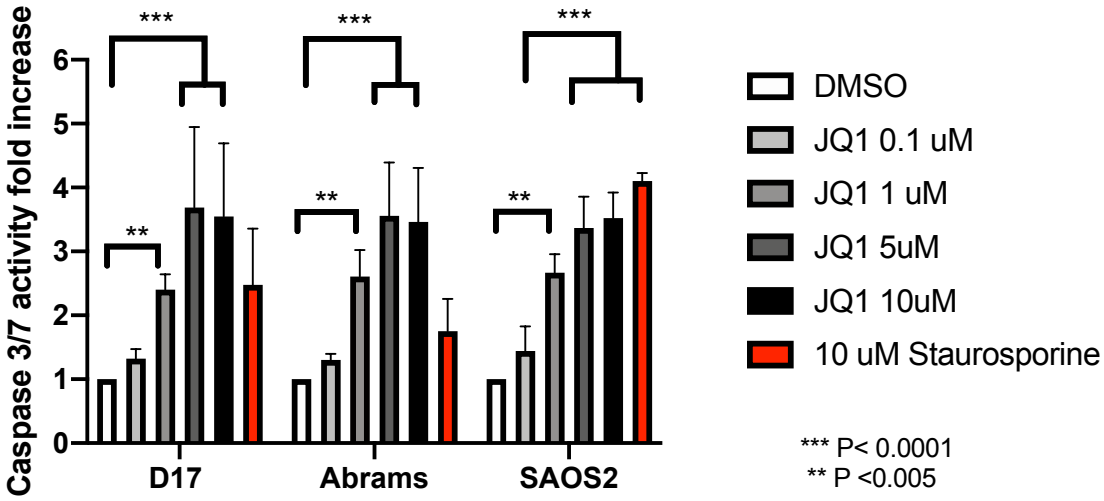


Figure 4.4. JQ1 decreaed *MYC* expression at mRNA and protein level.
(A) *MYC* expression was examined among all cell lines, Abrams and BZ were upregulated comapred to other cell lines and MSCs were used as control. **(B)** Abrams cells were incubated with different JQ1 concentrations for 24 hours. **(C)** D17 cells were incubated with different JQ1 concentrations for 24 hours. **(D)** Western blot analysis of D17 and SAOS2 cells treated with vehicle (DMSO), JQ1 (0.5 μM, 1 μM, and 10 μM) for 24 hours. Protein band intensities were quantified by Image Studio™ Lite software (LI-COR).

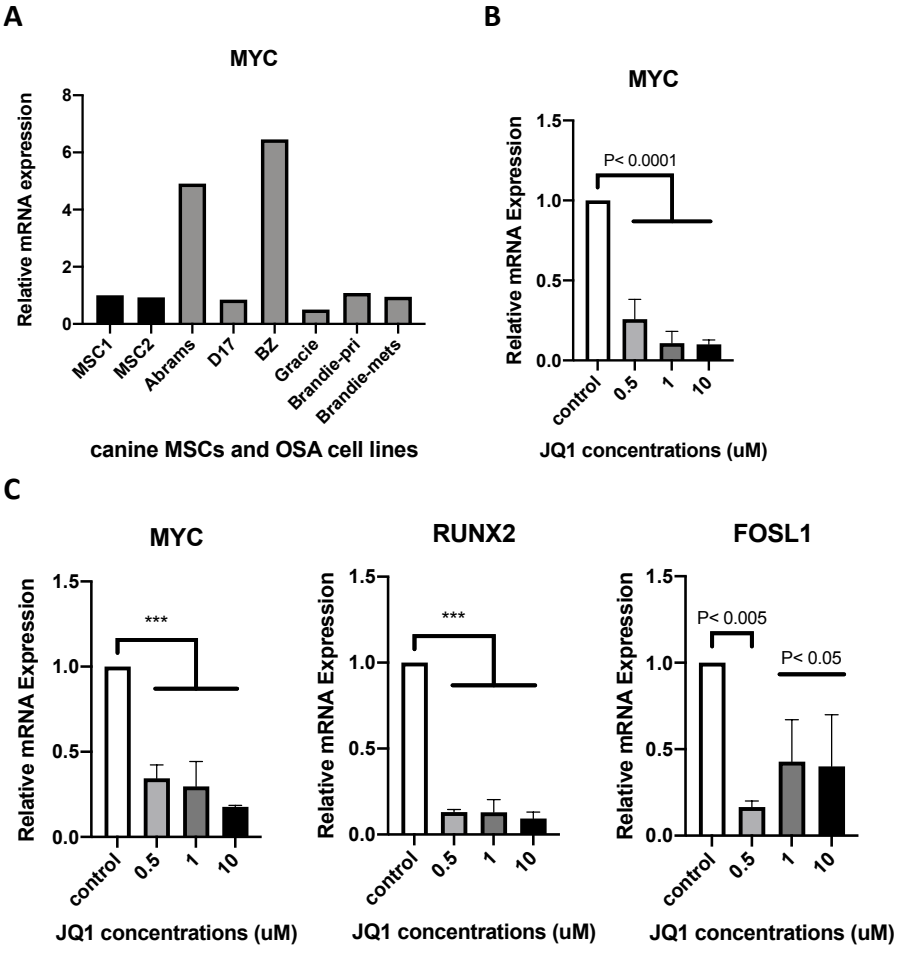
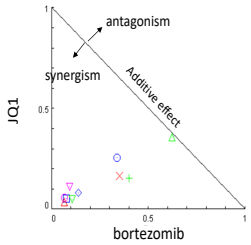


Figure 4.5. Co-treatment of JQ1 and bortezomib show synergistic effects **(A)** Combination index (CI) results showed a synergistic effect of JQ1 and bortezomib in ration 100:1. Date shown here are in D17 cell line. **I**n Normalized isobologram, we included two different rations of JQ1 and bortezomib (100:1 and 160:1). Data showed that the different combinations are all synergistic. (B) Migration capacity was evaluated by wound healing assay. OSA cells were incubated with 1% DMSO (control), JQ1 (0.5 μM), bortezomib (10 nM), or combination (JQ1 0.25 μM + bortezomib 5 nM) for up to 24 hours.

A



CI values	JQ1 (μM)	bortezomib (nM)	ratio
0.59	1.25	12.5	100:1
0.98	5	50	
0.2	0.1	1	
0.22	1	10	
0.52	5	50	
0.55	10	100	
0.12	1	0.63	160:1
0.13	2.5	1.56	
0.1	5	3.13	
0.16	10	6.25	

B

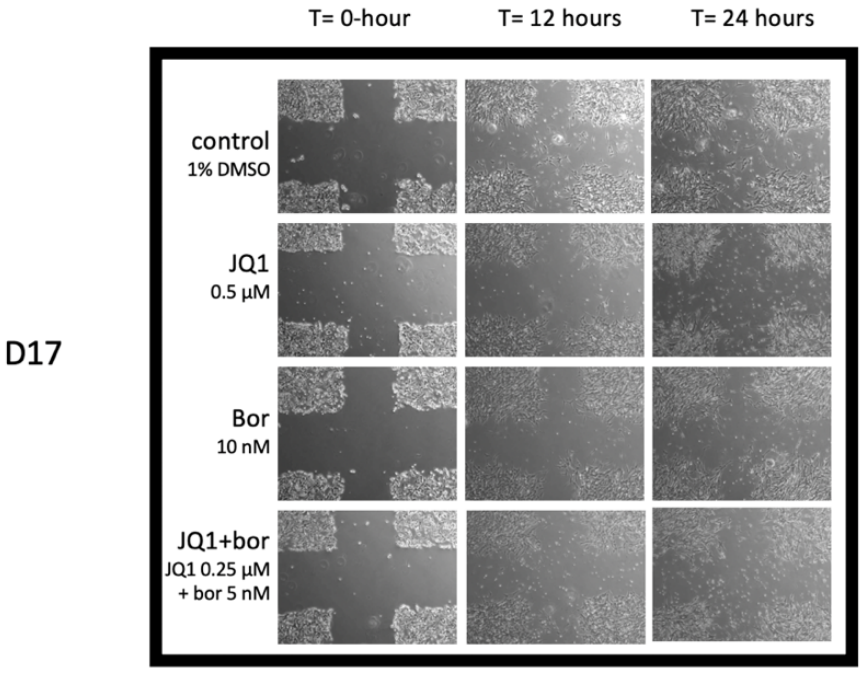
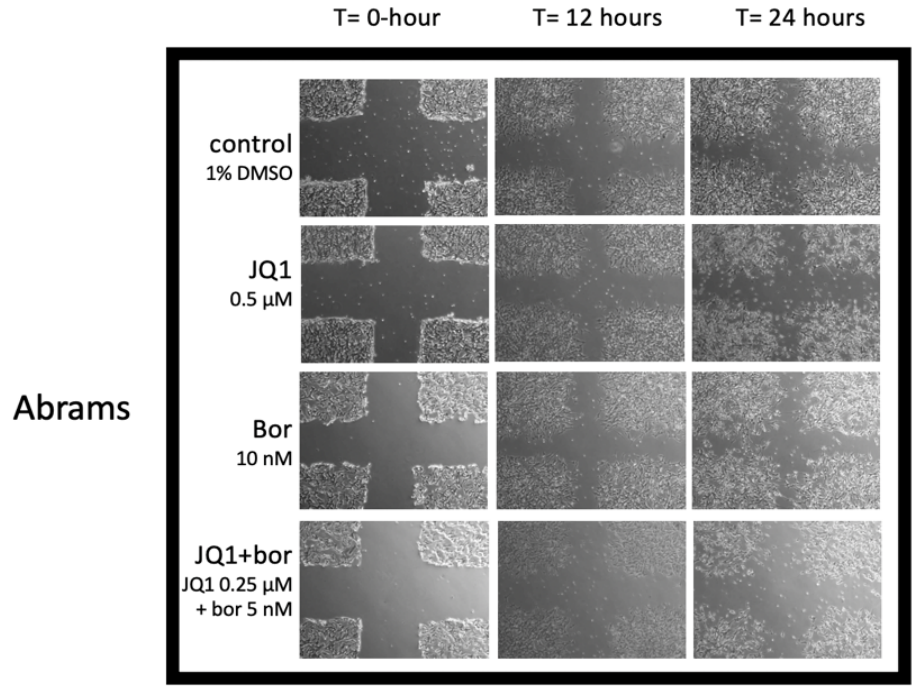


Figure 4.5. (cont'd)



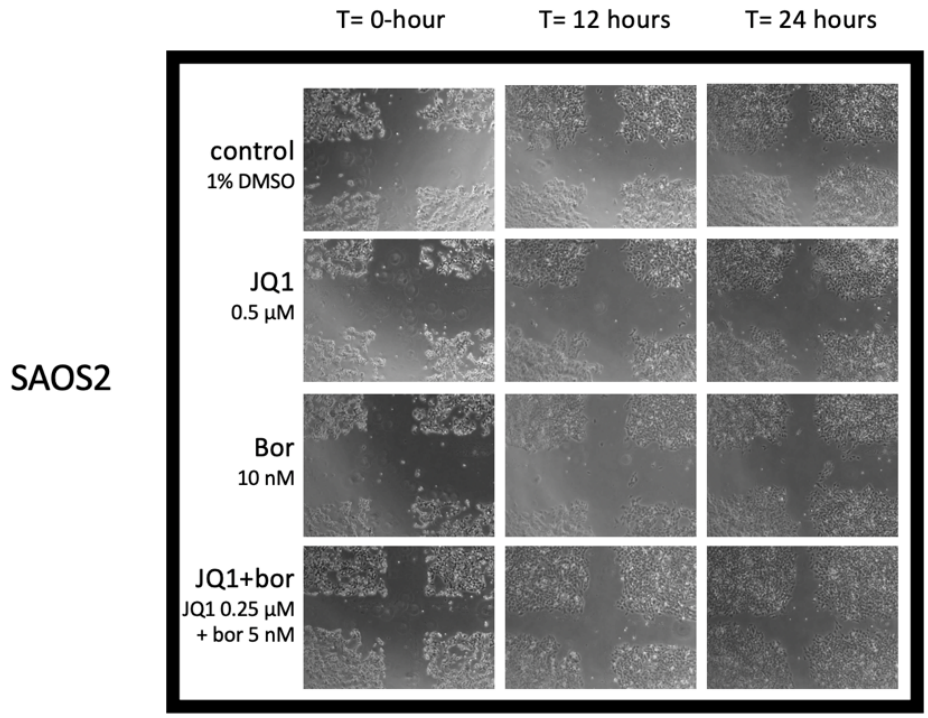


Figure 4.6. Combination of bortezomib and JQ1 induced cell arrest in G2/M phase. D17, Abrams, and SAOS2 cells were treated with JQ1 (1μM), bortezomib (10 nM), or JQ1 + bortezomib for 24 hours. Cell cycles were analyzed by PI staining and Modfit Lt™ software. The results showed an increase of G2/M cell arrest with JQ1 and bortezomib on **(A)** D17, **(B)** Abrams, and **(C)** SAOS2 cell lines. ***P<0.001, **P<0.01, *P<0.05 for G2/M arrest compared to vehicle control (1% DMSO) or JQ1+bortezomib as determined by one-way ANOVA with Dunnett's multiple comparison test.

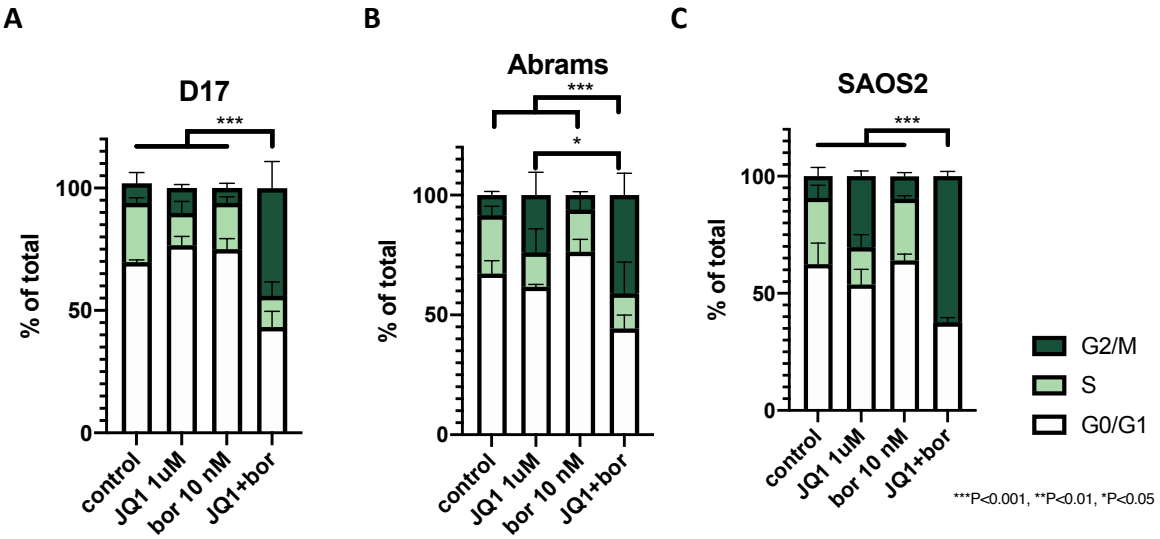
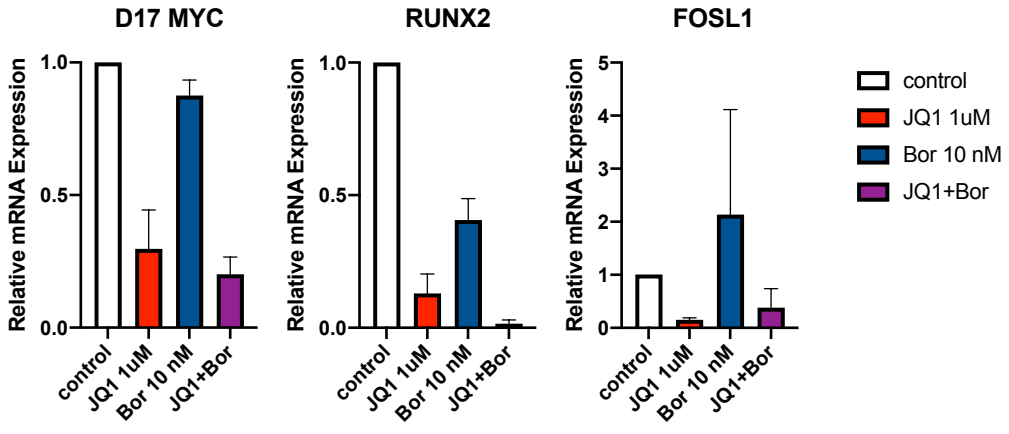


Figure 4.7. Co-treatment of JQ1 and bortezomib show synergistic effects, driven by MYC, RUNX2 and FOSL1 suppression at mRNA and protein levels.
(A) mRNA alternation of *MYC, RUNX2, and FOSL1* expression in D17 cells treated with vehicle (DMSO), JQ1 (1 μM), bortezomib (10 nM), or JQ1+bortezomib for 24 hours. **(B)** Protein expression of MYC after 24 hours were also examined by Western blot Analysis, OSA cells were treated with JQ1 (1 μM) or bortezomib (10 nM), or the combination of these two drugs for 24 hours, a decrease of MYC was noted when two drugs were added to the cells. **(C)** co-treatment of JQ1 and bortezomib caused an increase of cleaved PARP expression, indicating apoptosis. Protein band intensities were quantified by Image Studio™ Lite software (LI-COR).

A



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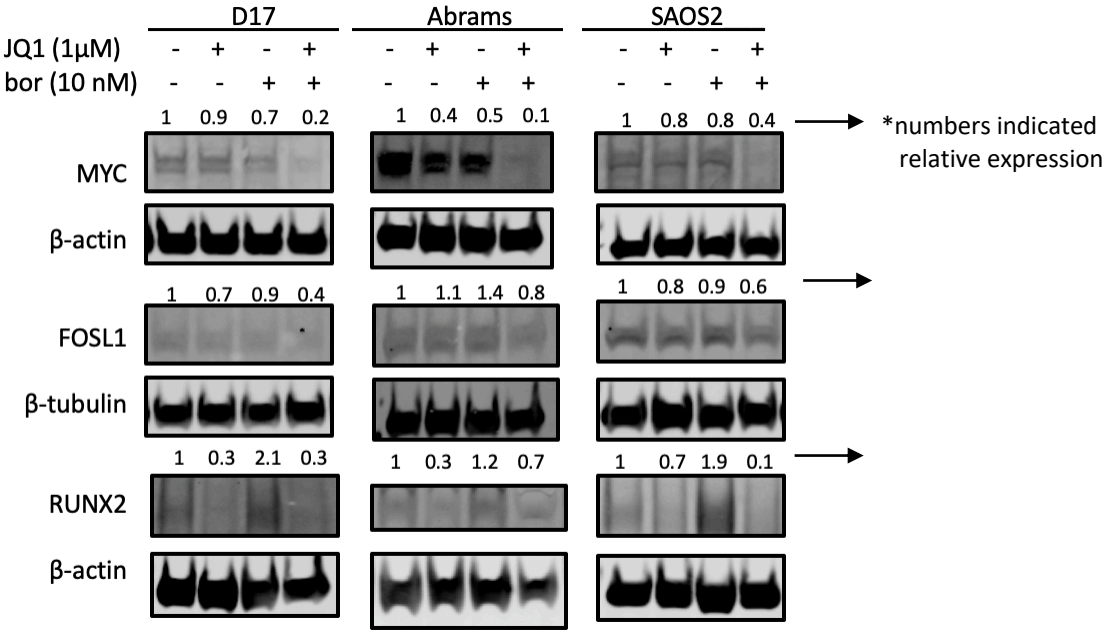

Figure 4.7. (cont'd)

C Realtive cleaved PARP protein expression when OSA cells incubated with drugs for 24 hours.

	D17	Abrams	SAOS2
DMSO (control)	1	1	1
JQ1 (1 μM)	1.6	2.2	3.5
Bor (10 nM)	2.2	1	1.4
JQ1 (1 μM) and bor (10nM)	4	3.5	6.4

A representative image of SAOS2 cells treated with DMSO, JQ1, bortezomib, or co-incubated with JQ1 and bortezomib for 48 hours.

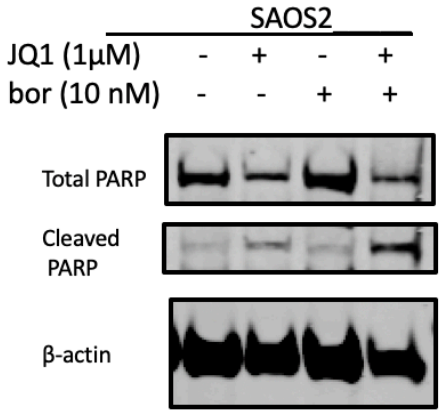

Table 4.3. Antibodies used in western blot analysis

Primary antibodies						
Protein	Catalog #	Lot#	Clone	Host	Manufacturer	Concentration used
β-actin	3700	13, 14	8H10D10	Mouse	Cell Signaling	1:4,000
β-actin	4970	12	13E5	Rabbit	Cell Signaling	1:4,000
β-tubulin	86298	1	D3U1W	Mouse	Cell Signaling	1:4,000
c-MYC	5605	8	D84C12	Rabbit	Cell Signaling	1:250
FRA1 (FOSL1)	Ab124722		EP4711	Rabbit	abcam	1:500
PARP	611038		SC99		BD	1:1,000
RUNX2	12556		D1L7F	Rabbit	Cell Signaling	1:500
Secondary antibody						
Protein	Catalog #	Lot#	Host	Target	Manufacturer	Concentration used
IRDye 680RD	925-68072	C81107-03	Donkey	Mouse	LI-COR	1:10,000
IRDye 800CW	925-32211	C80118-01	Goat	Rabbit	LI-COR	1:10,000

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CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

Osteosarcoma is one of the most heterogeneous tumor types, which leads to the challenge of defining optimal standard of care for OSA patients. Therefore, it is important to establish and characterized models for finding more effective therapeutic agents [1]. Patient derived cell lines [2-4] or patient derived tumor xenografts (PDTX) are useful and informative tools for this approach. We also proposed that utilization of proof of concept studies in dogs spontaneously presenting with osteosarcoma provide an excellent translational system for testing new therapies. In the present study, we established a canine OSA cell line from a metastatic site and characterized with different tumorigenesis associated signaling pathways. We also searched for more effective drugs to improve the current status in both canine and human OSA using drug screening approaches. Screening and repurposing compounds already approved by the FDA may further speed up the drug development process. In addition, we utilized different drug combinations and examined the associated mechanism of action.

Canine OSA is a unique translational model for human OSA because dogs spontaneously develop osteosarcomas at similar sites to humans and have similar response to treatments. In addition, the ten times higher incident rate and high metastatic potential of the dogs also provide compelling rationale to compare both species. Another translational potential of canine OSA is that the drug development process in dogs is similar to humans but simpler and faster, and there are fewer limitations for clinical trials in dogs, such as the ability to carry out clinical trials in the untreated patient [5]. Therefore, these novel drugs can be evaluated in dog patients before being used in humans. Using canine studies can provide the basic understanding of efficiency, pharmacokinetics and toxicity/adverse effects, and reduce the failure rate and de-risk human studies. In short, dogs not only provide foundational results to support the use of

chemotherapeutic agents for human patients, but also benefit from the chemotherapy agents that was previously used in human cancers.

So far, the most active conventional chemotherapy drugs for OSA in the clinics are drugs that cause DNA damages in cancer cells. For instance, platinum-based chemotherapy drugs cisplatin and carboplatin bind to DNA, induce oxidative stress, interfere with the repair system, and damage DNA [6, 7]. Another conventional chemotherapy drug doxorubicin inhibits cancer cells by inhibiting topoisomerase II activity and inducing DNA double-strand breaks [8]. However, these drugs have not improved much on OSA survival for the past thirty years, and the relapsed patients have even less options for effective chemotherapeutic agents. Also, the heterogeneity in osteosarcoma suggests the need for different and multiple approaches to target OSA.

In the current study, we identified drugs with different mechanism of action that were effective in suppressing tumor proliferation. Among them, the first candidate we selected is sorafenib (BAY 43-9006, Nexavar®), an oral multiple tyrosine kinase inhibitor. Sorafenib alone has been used in human clinical trials for diverse cancers, including hepatocellular carcinoma and hOSA [9-12] but not in cOSA. We demonstrated sorafenib inhibited OSA cells growth and inhibited the activation of ERK and STAT3 pathways. Our data from combination index analysis revealed the synergistic effects of sorafenib and doxorubicin and thus provide the rationale of combining these two compounds for treating OSA. In hepatocellular carcinoma, the combination of sorafenib and doxorubicin verses sorafenib treatment alone indicated controversial results in phase II/III clinical trials [13-15]. Two randomized clinical trials addressed whether sorafenib plus doxorubicin would improve the outcome of patients than doxorubicin [15] or sorafenib [14] alone. Despite the first study reported a two times longer median survival time of control

group (13.7 verses 6.5 months), the patients in the latter group had a 4 months of median survival time. Therefore, canine clinical trials need to be carried out to assess if this combination is safe and more effective than current first line chemotherapies to treat cOSA or hOSA.

The other novel therapeutic approach we selected include the proteasome inhibitor bortezomib and bromodomain inhibitor JQ1. Bortezomib blocks cancer cell proliferation with a novel approach through the inhibition of proteasome and causes accumulation of unwanted proteins. JQ1 works through an epigenetic approach, targets especially BRD4 and regulates MYC transcription. Our findings provided evidence that of JQ1 inhibited MYC expression alone or in combination with bortezomib in OSA. Co-treatment with JQ1 and bortezomib showed synergistic effect on three OSA cell lines, suggesting the potential of this combination for further studies. Although MYC is considered classically as an undruggable target, more strategies are being introduced for MYC regulation [1, 16, 17], such as PLK1 and AURKA inhibitors that regulate the stabilization of MYC protein, mTOR inhibitor rapamycin which blocks MYC translation, and inhibitors of CDK7/CDK9 block MYC at the transcriptional level. Besides exploring repurposing existing FDA-approved drugs, we also screened our cells with a GSK published Kinase Inhibitor library (developed by GlaxoSmithKline, contain 558 drugs). As shown in **Table 5.1**, we identified several classes of compounds with promising anti-tumor effect, including CDK2/CDK4 inhibitors, CLK2 inhibitors, PLK1 (polo-like kinase 1) inhibitors, or IGF1-R (insulin-like growth factor 1) inhibitors. These inhibitors have been evaluated in preclinical models and clinical trials. PLK1 contributes to the MYC stabilization in human OSA cell lines, whereas the inhibition of PLK1 resulted in MYC protein degradation and a delay in

tumor growth in mice model [18]. The first selective PLK1 inhibitor, BI-2536, has been used in the clinical trials with advanced solid tumors which included colorectal, melanoma, liver cancer, breast cancer, and sarcomas [19, 20]. In a preclinical model, second generation PLK1 inhibitors such as BI-6727 (volasertib) and GSK461364 were shown to suppress growth of OSA cells and enhance radiation sensitivity [18]. In a study across murine, canine, and human OSA cells, PLK1 pathway was identified with potential for therapeutic targeting as BI-6727 was effective to inhibit OSA cells that were derived from the most aggressive tumors[21]. In addition, the synergism of BET inhibitors and PLK1 inhibitors was evaluated in prostate cancer [22], and acute myeloid leukemia [23]. Interestingly, the BI-2536 and BI-6727 are dual PLK1/ bromodomain inhibitors [24, 25], indicating PLK1 and BETs may play important roles in regulating cancer progression**.**

Osteosarcoma is a heterogeneous disease genetically and clinically and using one therapeutic approach for all patients is not the best way to treat this disease. Our cell lines showed different levels of activation on ERK, STAT3, AKT pathways or PTEN expression, indicating they may respond differently to the same drug. In patients, these differences also lead to diverse response to the same set of chemotherapies. For example, a previous clinical trial indicated hOSA patients with positive p-ERK and P-RPS6 tumors benefit from the treatment of combined sorafenib and everolimus compared to patients that were negative for these two biomarkers [11]. Our findings show that the ERK pathway is activated in some cOSA cell lines. Thus, studying the efficacy of sorafenib in a clinical trial on canine patients, especially those showing ERK activation, with and without chemotherapy would be a logical next step.

Although the great clinical benefit of the functional immune activation in post-surgery infections in OSA patients was reported as early as the 1890s [26], the immunotherapeutic approaches in OSA are still in the early stages. Clinical investigations of monoclonal antibody and immune checkpoint inhibitors (PD-1 and PD-L1) still require large scale study and further understanding in order to identify effective and safe therapeutic approaches for OSA. In this process, canine OSA is a useful platform to select appropriate treatment options for hOSA, one example is the *Listeria*-based HER2 expressing vaccine (ADXS31-164). While the incidence of a few cases of active *Listeria* infections caused by *Listeria*-based vaccine in cOSA has resulted in the discontinuance of this study in both species, remarkable response was seen in some cOSA patients, with some dogs achieving very long survival times, up to 3 years while the median OS was 14.4 months in this vaccine trial vs the median of 9.8 months with surgery and chemotherapy [27]. Despite the failure of this vaccine, studies of other osteosarcoma vaccines are still ongoing including one in our group using mutant Qβ particles with tumor-associated carbohydrate antigens, expressed in OSA.

In this work, we provide evidence supporting the use of sorafenib, bortezomib, and JQ1 in human and canine OSA. While JQ1 is a first in class drug and is used mostly to study mechanism of action, additional and more orally available bromodomain inhibitors are being developed that can be tested in proof of concept studies in canine OSA patients. Understanding the molecular mechanisms involved in both species will bring more insights of possible strategies to treat human and canine OSA patients. Importantly, suitable therapies targeting the key lesions in each case can be selected through precision medicine and/or by engaging power of the

immune system to identify and destroy tumor cells to improve the survival rate and the quality of life for the patients.

APPENDIX

Table 5.1. Protential drugs identified from GSK published Kinase Inhibitor library

Name	Percent canine fibroblast growth inhibition	Percent of OSA growth inhibition in different cell lines			Mechanism of Action
		D17	Abrams	SAOS2	
GW780056A	<1	84	70	87	CDK2/CDK4 inhibitor
GW778894X	<1	75	74	82	CDK2/CDK4 inhibitor
GW779439X	<5	73	71	88	CDK2/CDK4 inhibitor
GW801372X	<1	78	68	85	CLK2 inhibitor
GSK994854A	<10	56	14	40	IGF-1R inhibitor
GSK2186269A	17	91	99	98	IGF-1R inhibitor
GSK237700A	<1	46	20	22	PLK1 inhibitor
GW852849X	<1	52	58	56	PLK1 inhibitor
GSK978744A	<3	80	67	61	PLK1 inhibitor
GSK237701A	<6	89	64	76	PLK1 inhibitor
GSK641502A	<3	93	67	88	PLK1 inhibitor
GSK580432A	<10	88	74	81	PLK1 inhibitor

BIOBLIOGRAPH

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