DISSERTATION

EFFECTS OF SURVIVIN AND SURVIVIN INHIBITION IN CANINE MODELS OF LYMPHOMA AND OSTEOSARCOMA

Submitted by

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EFFECTS OF SURVIVIN AND SURVIVIN INHIBITION IN CANINE MODELS OF LYMPHOMA AND OSTEOSARCOMA

Canine lymphoma (LSA) and osteosarcoma (OS) have high mortality rates and remain in need of more effective therapeutic approaches. Survivin, an IAP (inhibitor of apoptosis) family member protein that inhibits apoptosis and drives cell proliferation, is commonly elevated in human and canine cancer. Survivin expression is a negative prognostic factor in both dogs and humans with LSA and OS, and canine LSA and OS cell lines express high levels of survivin. Due to the strong similarities between canine and human LSA and OS, canine LSA and OS are excellent models for the human disease. In the following research, we illustrate the potential of the canine LSA and OS models as a translational tool for evaluating survivin-directed therapies, owing to the striking similarities in gross and microscopic appearance, biologic behavior, gene expression and signaling pathway alterations compared to the respective human forms of these diseases.

In this research we sought to determine the effects of survivin inhibition in canine OS and LSA cell lines in vitro, and in vivo in canine OS, and to evaluate a correlation between survivin expression and outcome in canine OS patients. We hypothesized, as observed in human OS and LSA, that survivin inhibition would decrease cell proliferation and increase apoptosis and chemosensitivity in canine OS and LSA cell lines. We further hypothesized that we would observe inhibition of survivin and reduced tumor growth in murine models of canine OS treated with EZN-3042, an inhibitor of survivin. We additionally hypothesized, as observed
in human OS, that increased survivin expression would correlate with a poor prognosis in canine OS patients.

Survivin attenuation in canine OS cells via siRNA was confirmed by RT-PCR and western blot analysis. Cell number and viability was assessed via manual cell counting with trypan blue. Cellular apoptosis was confirmed via caspase-3/7 and TUNEL assays. Cell cycle analysis was performed with propidium iodide staining followed by flow cytometry. Chemosensitivity to doxorubicin (DOX) was also assessed with caspase-3/7 assay. We determined that survivin inhibition via siRNA in canine OS cells inhibited cell cycle progression, and increased apoptosis, mitotic arrest and chemosensitivity.

Next we inhibited survivin using EZN-3042, a locked nucleic acid oligonucleotide targeting survivin, in two canine LSA and two canine OS cell lines. Survivin inhibition was confirmed by qRT-PCR and Immunofluorescence. Percent dead and total cell number were assessed by manual cell counting with trypan blue. Growth inhibition was confirmed with a bioreductive fluorometric assay. A caspase-3/7 assay was used to determine levels of apoptosis and chemosensitivity. Survivin inhibition in vitro using EZN-3042 resulted in decreased total and viable cell numbers and increased apoptosis and chemosensitivity to DOX. In vivo, nude mice with subcutaneous and orthotopic OS xenografts were given 100 mg/kg EZN3042 intraperitoneally. Survivin inhibition was confirmed with immunohistochemistry and qRT-PCR analysis. EZN-3042 treatment in vivo in subcutaneous and orthotopic canine OS xenografts decreased tumor survivin expression. Mice treated with EZN-3042 in combination with DOX had significantly decreased tumor growth when compared to single agent treatment and control groups.

Lastly, we evaluated survivin expression in archived paraffin embedded canine OS tissue samples. Survivin expression was studied via immunohistochemistry in 67 canine OS
Elevated survivin protein immunoreactivity in primary canine OS tissue samples correlated with increased histologic grade and mitotic index and a decreased disease free interval (DFI).

These findings strongly suggest that survivin-directed therapies may be highly effective in treatment of both canine and human LSA and OS, and spontaneous canine cancer may be a valuable model for the evaluation of survivin-targeted treatment.
ACKNOWLEDGEMENTS

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DEDICATION

This dissertation is dedicated to the Flint Animal Cancer Center patients and their families.
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CHAPTER ONE

Literature Review

Osteosarcoma:

Overview, Comparative Biology and Molecular Pathogenesis

Osteosarcoma (OS) in humans has a 5 year survival rate of 65%. It is the most common bone tumor in children and adolescents, usually occurring in the tibia, femur, or humerus. Osteosarcoma represents 3% of the cancer in children, with a rate of 4.4 per million in children and adolescents age 0-24. Rates of OS then drop off substantially until age 60 or older, when rates of OS again increase to 4.2 per million (1). Osteosarcoma is considered a primary neoplasm in the young, but in the elderly it is often a secondary neoplasm caused by malignant transformation of Paget’s disease or another benign bone lesion. The majority of OS cases are primary cases in the young; only 10% of cases are in the elderly population (2).

Osteosarcoma is derived from primitive bone-forming mesenchymal cells, and can be classified as high, intermediate, or low-grade disease (3). Most OS in children and adolescents is high grade. Histologic and anatomic subtypes of high-grade OS include: osteoblastic, chondroblastic, fibroblastic, mixed, small cell, telangiectatic, juxtacortical, Pagetoid, extraskeletal, and post-radiation. Intermediate grade are uncommon and are periosteal. Low-grade OS include parosteal, intramedullary, and intraosseous. Typical symptoms are pain and swelling around the affected bone. Pain may be worse at night or with increased activity, swelling or a lump may be felt in the affected area. Fractures due to the cancer are possible, but relatively rare. Suspect OS can usually be presumptively diagnosed with a radiographic
image, but other modalities can also be used. A bone biopsy is required for a definitive
diagnosis. Radiographs and a bone scan are performed to determine if there is metastatic
spread at the time of diagnosis, which is important for staging disease.

Osteosarcoma staging is based on the TNM system: primary tumor (T), regional lymph
nodes (N), distant metastasis (M), and histologic grade (G) as shown in Table 1.1 (4).

<table>
<thead>
<tr>
<th>Primary tumor (T)</th>
<th>Regional lymph nodes (N)</th>
<th>Distant metastasis (M)</th>
<th>Histologic grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unable to assess (Tx)</td>
<td>Unable to assess (Nx)</td>
<td>Unable to assess (Mx)</td>
<td>Unable to assess (Gx)</td>
</tr>
<tr>
<td>No evidence of presence (T0)</td>
<td>No regional lymph node metastasis (N0)</td>
<td>No distant metastasis (M0)</td>
<td>Well differentiated-low grade (G1)</td>
</tr>
<tr>
<td>8 cm or less in size (T1)</td>
<td>Regional lymph node metastasis (N1)</td>
<td>Distant metastasis (M1)</td>
<td>Moderately differentiated-low grade (G2)</td>
</tr>
<tr>
<td>Larger than 8 cm (T2)</td>
<td></td>
<td>To the lung (M1a)</td>
<td>Poorly differentiated-high grade (G3)</td>
</tr>
<tr>
<td>Discontinuous tumors in the primary bone site (T3)</td>
<td></td>
<td>To other sites (M1b)</td>
<td>Undifferentiated-high grade (G4)</td>
</tr>
</tbody>
</table>

Osteosarcoma staging is divided up into four stages and further subdivided as shown in Table 1.2.
Canine OS has a median survival of 235-366 days with surgery and chemotherapy. The 2 year survival rate is only 20%, and as many as 90% of dogs have micro-metastasis in the lungs upon time of diagnosis. Canine OS can be appendicular or axial, but it is primarily appendicular.

Canine OS accounts for 85% of malignancies of the bone, usually occurring in middle aged to older, larger breed dogs (5). Typical symptoms of appendicular OS include pain and swelling at the site, lameness, and sometimes fracture can occur, due to the weakened state of the bone. The pain can cause irritability, aggression, loss of appetite, weight loss, whimpering, sleeplessness, and reluctance to exercise (6). In late stage disease there may be difficulty breathing and coughing due to lung metastasis. When dogs present in the clinic, typically a radiograph is taken of the affected limb to determine cause of lameness. Preliminary diagnosis of OS can be made with the radiograph, followed by a bone biopsy if necessary for confirmation. If regional lymph nodes are enlarged they are typically aspirated or biopsied to further determine disease spread. All these diagnostics combined will help determine stage of disease (7).

Staging of canine OS is very similar to staging human OS, using the TNM system (6). However, the TNM system hasn’t been found to correlate stage with survival in humans, so the Japanese Orthopedic Association proposed the addition of alkaline phosphatase to the TNM classification.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Primary tumor (T)</th>
<th>Regional lymph nodes (N)</th>
<th>Distant metastasis (M)</th>
<th>Histologic grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage IA</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>G1/2-low grade</td>
</tr>
<tr>
<td>Stage IB</td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
<td>G1/2-low grade</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T1</td>
<td>N0</td>
<td>M0</td>
<td>G3/4-high grade</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T2</td>
<td>N0</td>
<td>M0</td>
<td>G3/4-high grade</td>
</tr>
<tr>
<td>Stage III</td>
<td>T3</td>
<td>N0</td>
<td>M0</td>
<td>Any grade</td>
</tr>
<tr>
<td>Stage IVA</td>
<td>Any classification</td>
<td>N0</td>
<td>M1a</td>
<td>Any grade</td>
</tr>
<tr>
<td>Stage IVB</td>
<td>Any classification</td>
<td>N1 or any</td>
<td>M1b or any</td>
<td>Any grade</td>
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</tbody>
</table>
system. They found that when alkaline phosphatase levels were added to the TNM classification system, they were able to correlate stage and survival rates (8). Alkaline phosphatase has also been found to be a useful prognostic factor in dogs with OS (9) and it has been suggested the classification system would benefit with its addition (10).

Canine and human OS are very similar, in fact, they are nearly identical, from clinical presentation to histology and genetics (11). Some general similarities include: increased incidence in males, large patient size, 75% or more are appendicular, typical metaphyseal location, unknown etiology, less than 10% of patients have clinically identifiable metastasis on presentation, over 90% of tumors have high-grade histology, 75% of tumors show aneuploidy, the metastatic rate is 80% or more with amputation alone, the lung is the most common site of metastasis, and survival is improved with adjuvant chemotherapy. A few differences include: increased relative age of onset and greater frequency of occurrence in dogs. (12). Although the exact etiology of OS is unknown, some OS has been shown to cluster in families (or litters) in both species (13, 14).

Canine and human OS are not only similar on the clinical level, but on the genetic (15, 16) and histologic (12, 17) levels as well. In a study comparing the gene expression signatures of canine and human OS, the two diseases could not be distinguished by hierarchical clustering. It was found that both interleukin-8, and solute carrier family 1 member 3 gene, were expressed uniformly in dogs and a subpopulation of pediatric OS patients that had a significantly poorer prognosis compared to the rest of the population (15). It has also been shown that mutations in the P53 gene occur in both dogs and humans with OS, and roughly the same frequency (16). The proto-oncogene erbB-2 encodes the human epidermal growth factor receptor 2, which induces cell transformation and growth. It has been found to be expressed in human OS and is associated with poor outcome (18). ErbB-2 is also overexpressed in canine OS (19). Myc (also c-Myc) is another oncogene that is overexpressed in both human and canine OS (20, 21). The
Myc gene codes for a transcription factor, that when constitutively expressed leads to the unregulated expression of genes involved in cell proliferation and many other functions, which lead to the formation of cancer.

Upon histologic examination, both canine and human OS are most commonly high grade (22) and share many of the same characteristics (12). Many of the same proteins that are up-regulated in human OS upon histologic analysis are also up-regulated in dogs, such as cyclooxygenase-2 (COX-2) and STAT3. COX-2 converts arachidonic acid to prostaglandin endoperoxide H2. COX-2 is expressed during inflammation, which can be associated with cancer development. COX-2 has been shown to be an indicator of poor prognosis in both humans and dogs with OS (23, 24). STAT3 is known to be involved in cancer growth and metastasis and has been associated with a more malignant phenotype. Both canine and human canine OS cells have been shown to up-regulated STAT3, which aids in cell survival and proliferation (25).

The molecular pathogenesis of OS has only recently started to become clear. We still do not know the exact cell of origin or have a consistent precursor lesion. Despite these obstacles much progress has been made in understanding the etiology of OS. We know OS originates in the bone, from a mesenchymal cell that can produce osteoid (26). Bone growth is believed to play a role in OS tumorigenesis. Rapidly growing bone, such as the rapid bone growth experienced during puberty, is at the highest risk of developing an OS lesion (27, 28). It has also been noted that children who grow taller than average seem to have an increased risk of OS compared to their average height peers (29). This may also explain why peak incidence of OS is earlier for girls than boys, as girls grow taller at an earlier age than boys (30).

Multiple chromosomal abnormalities have been found in OS, including amplifications of 6p21, 8q24, and 12q14, with loss of heterozygosity at 10q21.1 (31). In addition to specific
alleles being lost or amplified, whole chromosome losses (chromosomes 9, 10, 13, and 17) or gains (chromosome 1) have also been observed in OS tumor cells. No specific chromosomal alteration has been found consistently in all OS, suggesting these chromosomal alterations can aid, but are not required for OS pathogenesis.

Tumor suppressor genes are genes that oversee DNA repair, or if DNA is too badly damaged, cause induction of apoptosis. However, if these tumor suppressor genes become mutated, these checkpoints are lost and the cell can further undergo somatic mutations, potentially resulting in a highly proliferating malignant cell. The Rb and p53 genes are both tumor suppressor genes that are often mutated in many cancer types. Rb and p53 gene mutations have both been shown to be involved in OS pathogenesis (30). P53 is mutated in 22% of OS (32). Mutations in p53 prevent cell cycle arrest and activation of pro-apoptotic Bax (33). They also impair DNA repair mechanisms and disrupt anti-angiogenesis activity (34). Rb is mutated in 39-42% of OS cases (35, 36). Mutations of Rb remove cell cycle regulation so the cell may continuously divide (33). Loss of the Rb gene may even explain familial risk for OS (37).

Transcription factors initiate transcription of DNA to RNA. Amplification or silencing of certain transcription factors can allow cells to become malignant. Myc is a transcription factor that stimulates cell growth and division. Myc expression has been found in 85.7% of OS cases and correlates with a poor prognosis (38). Myc has been implicated in OS pathogenesis, as overexpression of myc in bone marrow stromal cells leads to OS development (39).

It has been suggested that the environment also has a role in OS pathogenesis, especially in older patients who have had longer exposure to the environment. The first connection made between the pathogenesis of OS and the environment was with radiation exposure. Radium dial workers who painted watch faces with radium so they would be
luminescent often succumbed to OS later in life (40). However only 2% of OS cases have been linked to radiation exposure (41). Radiotherapy is sometimes used in children as treatment for solid tumors. Of these, 5.4% develop a secondary neoplasm, 25% being some type of sarcoma (42). Besides radiation there are also other chemical agents in our environment that have been linked to OS, including: methylcholanthrene and chromium salts (43), beryllium oxide (44), zinc beryllium silicate (45), asbestos and aniline dyes (46). The environment is thought to have a minor impact overall on incidence of OS (47). Further understanding of the molecular pathogenesis of OS would allow for alternative and possibly improved treatment options for this disease.

**Treatment of OS**

Treatment of OS usually involves a combination of surgery and chemotherapy. Radiation is typically used if the tumor is inaccessible with surgery. Some of the most common chemotherapy drugs used to treat OS include doxorubicin (DOX), carboplatin, cisplatin, cyclophosphamide, epirubicin, etoposide, gemcitabine, ifosfamide, methotrexate, and topotecan. Doxorubicin (Adriamycin) (48) is a chemotherapy agent used in numerous types of cancer, in both dogs and humans. It is used to treat both canine and human OS. Common side effects in people include: low white blood cell (WBC) count (increased risk of infection), low platelet count (increased risk of bleeding), loss of appetite, darkening of nail beds and skin creases of hands, hair loss or hair thinning, nausea, and vomiting. Doxorubicin is isolated from a bacteria and functions by intercalating into the DNA, preventing DNA replication. It also inhibits topoisomerase II, thereby interfering with double-strand break repair. It additionally forms oxygen free radicals resulting in increased cytotoxicity. As rapid DNA replication is abundant in the cancer cell, DOX is most effective against it specifically and isn't as destructive
to normal body cells. However, normal rapidly dividing cells, although they do not have as many DNA replications, can be severely affected. Skin cells, hair cells, white blood cells, platelets and cells in the GI tract are all rapidly dividing cells and account for the more common side effects of DOX.

Carboplatin (49) is used to treat a variety of cancer in both dogs and humans. In humans it is used to treat OS, lung cancer, ovarian cancer and more. In dogs it is primarily used to treat OS, but works on other cancer types as well (50). Common side effects in humans include: low WBC count, low platelet count, low RBC count, brittle hair, altered kidney function, and fetal abnormalities if pregnant. Carboplatin is a second generation platinum compound. Carboplatin is more stable but less toxic than cisplatin. It forms reactive complexes that bind to GC-rich sites in DNA, causing DNA-DNA crosslinks and DNA-protein crosslinks. These DNA crosslinks cause cellular apoptosis and growth inhibition. Carboplatin targets rapidly dividing cells’ DNA, making cancer cells its primary target. It also effects normal rapidly dividing cells, causing the above mentioned side effects.

Before the development of chemotherapy for the treatment of human OS, the 5-year survival rate was 17-20% (51, 52). In dogs with OS, only 10% survived a year after removal of the affected limb (53). With the development of neoadjuvant and adjuvant chemotherapy, the 5-year survival rate is now 60% in humans, and the 2-year survival rate in dogs is 20% (54). While the improved outcome with the use of chemotherapy is substantial, the outlook for patients with metastatic disease is still very grim.

Surgical options for OS are typically limb amputation or limb sparing surgery, usually done in combination with chemotherapy. Limb amputation is complete removal of the affected limb. A limb spare or limb salvage surgery is aimed at preserving the function of a limb without increasing risk to the patient (55). Dogs typically undergo limb amputation, unless there is a
history of arthritis or other lameness issues that make amputation unfeasible. Then limb sparing
or radiation therapy are alternative options.

Currently there are 88 clinical trials listed by the National Institutes of Health for human
patients with OS (56). These trials include new combinations of the treatment options listed
above, as well as new treatment protocols and novel chemotherapeutic agents, immunotherapy,
targeted agents, gene therapies and more. Very similar studies are being conducted in canine
medicine. The Veterinary Cancer Society currently has 11 clinical trials listed for dogs with OS,
including immunotherapies and targeted agents similar to those currently being studied in
humans (57).

**Prognostic Factors in OS**

Prognostic factors help determine outlook for patients with disease, and can identify
patients that have a poor prognosis. These patients, when identified, could benefit from a more
aggressive treatment regimen, often by specifically targeting the factor that gave them the poor
prognosis.

Traditional prognostic factors in humans with OS include age and sex of the patient,
tumor size, site, stage, and metastatic status, and histologic response to chemotherapy. Older
patients have an overall worse prognosis (58). In those over 40 years of age this has been
linked to higher rates of axial tumors, more frequent metastasis at presentation, and decreased
tolerance of high dose chemotherapy (59). Other research has shown children under 5 years of
age that develop OS (60), or children under 14 years of age (61) have a worse prognosis
compared to older patients. So it would seem the very old and the very young have the worst
outcomes. A number of studies have determined that male patients have a worse outcome than
female patients, and that female patients have better treatment responses (62-64). More recent
studies however, have been unable to confirm these results (58, 59, 65). Increased tumor size or volume is another negative prognostic indicator for OS patients (59, 61, 65), as is presence of metastasis at time of diagnosis (58, 65). Location of the tumor has prognostic significance as well. The most common sites of OS are the distal femur and proximal tibia, which have a more favorable prognosis. Axial locations, and those proximal to the body such as proximal humerus have a considerably worse outcome (62, 66). Proximal tibia has a 5 year survival rate of 77.5%, and distal femur 66%. Pelvic OS, which are 3rd most common, only have a 5 year survival rate of 27-47% (62). Tumor staging can also give clues to patient prognosis. Stage I-A is the uncommon low grade OS, with a nearly 100% survival rate (67). Conversely, Stage II-B and Stage III, which are high grade OS, have survival rates of 47-68% (68, 69). Histologic response to chemotherapy is also a prognostic factor in human OS. Increased apoptosis in response to chemotherapy correlates with a positive outcome. An absence of response to chemotherapy upon histologic analysis correlates to a poor outcome (70, 71). The serological marker alkaline phosphatase (ALP) is another prognostic factor. Patients with normal serum ALP levels upon presentation with OS have a significantly higher 5-year disease free interval compared to patients with high serum ALP levels at presentation (72).

Recently, molecular markers have become yet another way to evaluate prognosis in human OS. Although there is a lot of contradictory data surrounding molecular markers (59), the most promising ones are listed below in Table 1.3.
Table 1.3: Molecular markers as prognostic indicators in Human OS.

<table>
<thead>
<tr>
<th>Negative prognostic indicator</th>
<th>Positive indicator</th>
<th>Reference data</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Matrix metalloproteinases</strong> (MMP-2 and MMP-9)</td>
<td>X</td>
<td>(75, 78)</td>
</tr>
<tr>
<td><strong>Urokinase plasminogen activator (uPA)</strong></td>
<td>X</td>
<td>(83)</td>
</tr>
<tr>
<td><strong>P-glycoprotein</strong></td>
<td>X</td>
<td>(84, 85)</td>
</tr>
<tr>
<td><strong>CXCR4 (chemokine receptor type 4)</strong></td>
<td>X</td>
<td>(86)</td>
</tr>
<tr>
<td><strong>Loss of p53 expression</strong></td>
<td>X</td>
<td>(41, 87)</td>
</tr>
<tr>
<td><strong>ErbB-2</strong></td>
<td>X</td>
<td>(75)</td>
</tr>
<tr>
<td><strong>Down reg. of HLA class I</strong></td>
<td>X</td>
<td>(88)</td>
</tr>
<tr>
<td><strong>Ezrin</strong></td>
<td>X</td>
<td>(89, 90)</td>
</tr>
<tr>
<td><strong>Rb gene down reg.</strong></td>
<td>X</td>
<td>(91, 92)</td>
</tr>
<tr>
<td><strong>c-Fos</strong></td>
<td>X</td>
<td>(93, 94)</td>
</tr>
<tr>
<td><strong>c-Myc</strong></td>
<td>X</td>
<td>(41)</td>
</tr>
<tr>
<td><strong>Vascular endothelial growth factor (VEGF)</strong></td>
<td>X</td>
<td>(95)</td>
</tr>
<tr>
<td><strong>Bcl-2 (B-cell lymphoma 2)</strong></td>
<td>X</td>
<td>(41)</td>
</tr>
<tr>
<td><strong>Survivin</strong></td>
<td>X</td>
<td>(96)</td>
</tr>
</tbody>
</table>

The few known prognostic factors for canine OS are very similar to those in human OS. The traditional prognostic factors seen in human patients are nearly identical for the traditional factors in canines. Age, sex, tumor size, site, stage, and metastatic status, and histologic response to chemotherapy have all been shown to be prognostic indicators in canine OS (66). As in human OS, there have been conflicting results on the impact of age on prognosis. In
dogs, older age at diagnosis has shown to be a negative prognostic indicator in some studies, but it is confounded by other medical problems (66). In other studies of canine OS, younger patients have a worse prognosis (73, 74), which is similar to what is seen in childhood OS, but opposite of what is seen in human OS overall. Historically it was believed that sex might be a prognostic factor in canine OS (12), however more recent studies have been unable to confirm its relevance (75). Large tumor size, as in human OS, has been correlated with a negative outcome in canine OS (76-78). Location of canine OS also has prognostic significance as in human OS. Specifically, proximal humerus is the location with the worst prognosis in the appendicular skeleton for canine OS, just as it is in the human disease (66). Histologic grade, is a negative prognostic indicator in canine OS (10). Presence of metastasis at the time of diagnosis is also a negative prognostic indicator shared by canine (6) and human OS (16). Histologic response to chemotherapy is important prognostic indicator that canine and human OS share. Decreased tumor necrosis in response to chemotherapy has been found to be a negative prognostic indicator in canine OS (79). The serological marker alkaline phosphatase (ALP) has been recognized as a prognostic indicator in human osteosarcoma for the past 50 years (80). High serum ALP is also a strong negative prognostic indicator for canine OS, decreasing survival time by half (9, 81).

Molecular markers found to be prognostic indicators in human OS may also have potential in evaluating prognosis in canine OS. MMPs, ErbB2/HER2, and c-Myc are up-regulated in canine OS tumors (19, 20, 82) and all are prognostic indicators in human OS as mentioned previously. P53 expression has been shown to be correlated with negative prognostic indicators in canine OS (83), which is similar to results in some studies mentioned above in human OS. Ezrin, which drives metastasis in human OS, has also been found to be associated with early development of metastasis in canine OS (84). COX-2 (cyclooxygenase-2) is involved in production of PGE₂ (prostaglandin E₂), which has been found to be up-regulated in
human cancer (85). Over expression of COX-2 in canine OS has been found to be a negative prognostic indicator for survival. VEGF, mentioned previously as a negative prognostic marker in human OS is also a negative prognostic indicator in canine OS (86). Survivin expression in dogs with OS is associated with a negative prognosis (87) and is discussed in chapter #4.

**Lymphoma:**

*Overview, Comparative Biology and Molecular Pathogenesis*

Lymphoma (LSA) in humans can be divided up into two main types: Hodgkin LSA (HL) and Non-Hodgkin LSA (NHL). Hodgkin LSA has a 5 year survival rate of 85.1%. Non-Hodgkin LSA has a 5 year survival rate of only 69.0%. While only 0.2% of men and women will develop HL, 2.1% of men and women will develop NHL at some point in their lifetime. In 2013, there will be an estimated 9,290 new cases and 1,180 deaths from HL, and an estimated 69,740 new cases of NHL and 19,020 deaths from the disease (88). Hodgkin LSA is determined by the presence of Reed-Sternberg cells, which are derived from B-cells that have undergone unfavorable genetic mutations. The two major subtypes of HL are classical HL and nodular lymphocyte-predominant HL. Typical symptoms of HL are painless enlarged lymph nodes, enlarged spleen, fever, weight loss, fatigue, and night sweat.

Non-Hodgkin LSA has many subtypes. It can be divided into fast growing aggressive and slow growing types, and is formed from B-cells, T-cells, or NK (natural killer)-cells. Non-Hodgkin LSA includes Burkitt LSA, small lymphocytic LSA, diffuse large B-cell LSA (DLBCL), follicular LSA, immunoblastic large cell LSA, precursor B-lymphoblastic LSA, mantle cell LSA, mycosis fungoides, anaplastic large cell LSA, and precursor T-lymphoblastic LSA. DLBCL makes up 40% of NHL and can further be divided into germinal center B-cell like (GCB) and
activated B-cell like (ABC) subtypes (88). Typical symptoms are the same as HL, with the addition of skin rash and pain in the chest, abdomen or bones.

Staging of HL and NHL is very similar, with both being divided up into four stages (Table 1.4) (88, 89).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Lymphatic area (lymph node, tonsil, thymus, spleen)</th>
<th>Extra-nodal (E), organ or area</th>
<th>Crosses Diaphragm</th>
<th>Splenic Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IE</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIE</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2+</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IIIE</td>
<td>2+</td>
<td>1</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>IIIS</td>
<td>2+</td>
<td></td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>IIIE,S</td>
<td>2+</td>
<td>1</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>IV</td>
<td>2+</td>
<td>1+, lung, liver, bone marrow, cerebrospinal fluid</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Canine LSA has a 2 year survival rate of 10% with treatment. Only 1% of dogs will survive to 5 years (90). Canine LSA accounts for up to 24% of all canine neoplasms. Most dogs treated for LSA relapse within 6-9 months. Their overall average survival rate is 1 year(91). Essentially all canine LSA are classified as NHL. There are only a handful of HL cases reported in dogs. Lymphoma in dogs can further be divided up into five main anatomic types: multicentric, cutaneous, gastrointestinal, mediastinal, and extra-nodal. Canine LSA are typically either B-cell or T-cell in origin, and can be further classified as either low grade or intermediate/high grade disease. Multicentric LSA is the most common canine LSA. Symptoms of multicentric LSA include painless enlarged lymph nodes, loss of appetite, lethargy, weight
loss, edema, and sometimes increased thirst and urination. If there is CNS involvement then CNS signs may be present. Cutaneous LSA starts out as dry, red, itchy, flaky areas of skin which later become very red, wet, ulcerated, and thickened. Masses may be found on the skin and it can also occur in the mouth. Gastrointestinal LSA usually presents with vomiting, watery diarrhea, and weight loss. In mediastinal LSA the dogs usually have dyspnea. This can be from the mass itself or from pleural effusion. Swelling of the face and legs may be seen as well as increased thirst and urination due to hypercalceimia. Extra-nodal LSA is rare and can be found in the breast, liver, eye, bone, and mouth. The affected organ is usually enlarged.

Canine LSA is staged according to the World Health Organization’s human lymphoma staging system (Table 1.5). (91, 92).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Enlarged lymph node</th>
<th>Crosses Diaphragm</th>
<th>Splenic/Liver Involvement</th>
<th>Bone marrow/other organ Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>2+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>2+</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage IV</td>
<td>2+</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Stage V</td>
<td>2+</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

The incidence of canine and human NHL is very similar. Dogs commonly develop an aggressive high grade multicentric LSA that is comparable to human NHL (93). Both humans and dogs present with painless enlarged lymph nodes and a human LSA classification system can be used to grade canine LSA (94). Dogs share their environment with their human counterparts and are exposed to the same environmental contaminants that humans are exposed to. It has been suggested that by studying the dogs that share our environment, we might learn a great deal about what contributes to human disease (95). A study in France
showed a correlation between the incidence of canine NHL and human NHL, and additionally reported an association between the canine LSA and proximity to radioactive waste and waste incinerators (96). In a study on Naples Italy and a waste management crisis, it was found that canine NHL was increased 2.39 fold and similar increases were found in people residing there (97, 98).

Canine and human NHL appear similar not only on the clinical level, but on the histologic and genetic levels as well (99-102). Many of the genetic mutations, copy number aberrations, and chromosomal instabilities are similar across both species (102-104). At the 12th International Conference on Malignant Lymphoma, the potential value of the canine model of NHL in humans was shown at the level of NF-kB/p65 pathway, the Bcl-2 family of proteins, Ki67 and the S-phase fraction, as well as the matrix metalloproteinases (MMPs), VEGF, and platelet derived growth (PDGF) (105). NF-κB is similarly up-regulated in both dogs and humans with DLBCL (106). Upon histological analysis, both human and canine LSA have similar up-regulated Bcl-2 family proteins, such as the well-known cancer protein survivin (107, 108).

B-cell NHLs, are derived from a variety of mechanisms. Specific types B-cell NHL are developed from distinct subpopulations of B-cells that have undergone specific genetic alterations (109). VDJ recombination, somatic hypermutation, and class switch recombination are all steps in the B-cell life cycle where cells can become predisposed to malignancy. Follicular LSA and some DLBCL are known to arise from unintended translocations during VDJ recombination. Aberrant somatic hypermutation is another way DLBCL arise. Errors in regulation of class switch recombination lead to Burkitt LSA, multiple myeloma, and other types of LSA (110).

T-cell malignancies are relatively rare, and the molecular pathogenesis of most is not well understood. Peripheral T-cell LSA and angioimmunoblastic T-cell LSA appear to be
derived from CD4+ T-cells. Adult T-cell LSA is known to be connected with HTLV-1 infection. The virus appears to activate multiple oncogenes and inhibit mitotic checkpoints within the T-cells. Chromosomal translocations have been shown to cause other T-cell LSA, which could take place during rearrangement of TCR genes (111).

Treatment of LSA

Standard of care treatments in humans with LSA are dependent on the LSA type and the stage of disease. Generally though there are five different standard of care options: chemotherapy, radiation therapy, immunotherapy, stem cell transplants, and combinations of the above. In addition to standard of care therapies there are clinical trial options with novel drugs for patients who either fail standard treatment options, or would like to have additional treatment in the hopes of increased chance for a cure.

Some of the most common chemotherapy drugs used in treating human LSA include: doxorubicin, bendamustine, bleomycin, chlorambucil (nitrogen mustard), cisplatin, cyclophosphamide, cytarabine, dacarbazine, dexamethasone, etoposide, fludarabine, gemcitabine, ifosfamide, mechlorethamine (nitrogen mustard), methotrexate, mitoxantrone, pralatrexate, prednisone, procarbazine, vinblastine, and vincristine. Some of these are also used in the treatment of canine LSA. A typical combination of some of the above chemotherapy agents for treatment of human LSA is the CHOP protocol. ‘C’ stands for cyclophosphamide, ‘H’ stands for hydroxydaunorubicin (aka doxorubicin), ‘O’ stands for Oncovin (aka vincristine), and ‘P’ stands for prednisone. The CHOP protocol is also commonly used in dogs to treat canine LSA.
The earliest forms of treatment for human LSA allowed a 3 year survival rate of 7% for HL and 10% for NHL (112). Before the development of combination chemotherapy, the 5-year survival rate was 38-39% for HL (113, 114), and 9-24% for NHL (115, 116). Dogs with LSA survive only 1-2 months without treatment (92). With the development of multi-drug chemotherapy regimens, the 5-year survival rate has risen to 85% for HL (89), and 69% for NHL (88). The 2-year survival rate for dogs with LSA is now 25% (117). While the improved outcome with the use of current chemotherapy treatments is considerable, the outlook for both dogs and humans with these diseases could still be significantly improved.

Radiation therapy (RT) can be delivered both externally and internally. External beam RT is most common, where high powered x-ray beams are either carefully aimed at areas of high cancer burden, called Involved Field RT (IFRT), or x-ray beams are targeted over larger areas of the body, called Extended Field RT (EFRT). Internal radiation involves ingesting or injecting radioactive particles that must travel to their intended site. Radio-immunotherapy is one such example used in LSA treatments. In stage I aggressive localized NHL, half of patients are cured by RT alone. The addition of chemotherapy does seem to improve outcome in this type of disease. Radiation therapy is also used to treat canine LSA. In studies where chemotherapy was combined with RT, dogs had longer remission rates when compared to a study where dogs received the same chemotherapy regimen without RT (118-120). Low-dose RT in dogs with LSA has also been found to be comparable to chemotherapy treatment alone (121).

Immunotherapy or biologic targeted therapy, is the use of the immune system to target the cancer directly, or to bring drugs or RT directly to the tumor cells via the immune system. Rituximab is a monoclonal antibody used to treat some types of B-cell NHL. It is directed against the CD20 antigen found on normal pre-B and mature B-cells. Once rituximab binds to
B-cells, it targets a host immune response against all CD20 positive cells, killing both normal and neoplastic B-cells (122).

Antibody-drug conjugates are antibodies conjugated to cancer drugs, so that the drug can be targeted to the lymphocytes and cancer cells. Upon finding its target the conjugate is internalized by lysosome into the cell, where the drug is released into the cytoplasm inducing cell death. Brentuximab vedotin is antibody to CD30 conjugated to monomethyl auristatin E, an antimicrotubulin agent. CD30 is a TNF receptor with a possible role in apoptosis, highly expressed on both Hodgkin and some types of NHL. Brentuximab is the only antibody-drug conjugate currently approved for treatment of human LSA, and has had some success in relapsed and refractory anaplastic large cell LSA (123), relapsed or refractory HL (124), and CD30 positive T-cell and NK-cell LSA (125).

Stem cell transplants are not the first treatment option for LSA patients, but are used in an attempt to cure disease, either while patient is in remission or after relapse. It allows use of higher doses of chemotherapy or radiation that would otherwise not be possible. In a study of relapsed NHL comparing patients who received chemotherapy and RT to patients who received intensive chemotherapy, RT, and bone marrow transplant, overall survival was significantly improved when bone marrow transplant was added (126).

According to the National Cancer Institute, there are currently 231 clinical trials in adult HL and 88 trials in pediatric HL. There are also 465 currently listed trials for adult NHL and 126 clinical trials for children with NHL. Clinical trials in LSA include all of the above treatment options, including different combinations of treatment, new treatment protocols, and new chemotherapy drugs (127).
The Veterinary Cancer Society currently lists 23 clinical trials in canine LSA. These trials include treatment with novel agents, RT, immunotherapy, bone marrow transplant, and combinations of the above (128).

**Prognostic Factors in LSA**

Prognostic factors help determine outlook and prognosis for patients with disease. New prognostic indicators help reduce overtreatment of patients with a good prognosis, and help identify patients that have a poor prognosis that would benefit from a more aggressive treatment regimen. An international prognostic index has been developed that helps doctors determine outlook for patients with LSA. It is dependent on five factors: the patient’s age, the stage of disease, how far the LSA has spread, how well the person functions in daily life, and the blood serum level of lactate dehydrogenase (LDH). The younger the patient, and the better they function in daily life, the better the prognosis. Increased stage of disease and LSA spread are indicators of poor prognosis. LDH levels increase with increasing LSA spread, so high LDH levels are also indicators of poor prognosis (129). The Ann Arbor classification scheme is another prognostic index that has very similar clinical factors considered (130). In addition to clinical characteristics we can look at gene expression and protein levels in LSA cells to determine prognosis (131, 132). See **Table 1.6**, and **Table 1.7**.
<table>
<thead>
<tr>
<th></th>
<th><strong>Negative prognostic indicator</strong></th>
<th><strong>Positive prognostic indicator</strong></th>
<th><strong>Reference data</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 antigen</td>
<td>X</td>
<td></td>
<td>(133, 134)</td>
</tr>
<tr>
<td>Proliferating cell nuclear antigen (PCNA)</td>
<td>X</td>
<td></td>
<td>(135)</td>
</tr>
<tr>
<td>VCAM-1 and ICAM-1 adhesion molecules</td>
<td>X</td>
<td></td>
<td>(136, 137)</td>
</tr>
<tr>
<td>CD20</td>
<td>X</td>
<td>X</td>
<td>(138) (139, 140)</td>
</tr>
<tr>
<td>Abnormally high CD30</td>
<td>X</td>
<td></td>
<td>(141, 142)</td>
</tr>
<tr>
<td>MAL</td>
<td>X</td>
<td></td>
<td>(143)</td>
</tr>
<tr>
<td>Topoisomerase II α</td>
<td>X</td>
<td></td>
<td>(144)</td>
</tr>
<tr>
<td>IL-10</td>
<td>X</td>
<td></td>
<td>(145, 146)</td>
</tr>
<tr>
<td>MHC class II antigens</td>
<td></td>
<td>X</td>
<td>(147)</td>
</tr>
<tr>
<td>Retinoblastoma (Rb) protein</td>
<td></td>
<td></td>
<td>(133)</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
<td>X</td>
<td>(148) (149)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>X</td>
<td></td>
<td>(135, 150)</td>
</tr>
<tr>
<td>P53</td>
<td>X</td>
<td></td>
<td>(151) (150) (135)</td>
</tr>
</tbody>
</table>
### Table 1.7: Prognostic Indicators in Non-Hodgkin’s Lymphoma

<table>
<thead>
<tr>
<th>Negative prognostic indicator</th>
<th>Positive indicator</th>
<th>Reference data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 antigen</td>
<td>X</td>
<td>(152, 153)</td>
</tr>
<tr>
<td>Abnormally high CD44</td>
<td>X</td>
<td>(154) (155-157)</td>
</tr>
<tr>
<td>Anemia</td>
<td>X</td>
<td>(158)</td>
</tr>
<tr>
<td>Mutated P53</td>
<td>X</td>
<td>(159)</td>
</tr>
<tr>
<td>Karyotypic abnormalities</td>
<td>X</td>
<td>(160, 161)</td>
</tr>
<tr>
<td>Chromosomes 17 &amp; 7 deletions/abnormalities</td>
<td>X</td>
<td>(162, 163)</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>X</td>
<td>(164-168) (169)</td>
</tr>
<tr>
<td>chromosomal translocation t(14;18)(q32;q31)</td>
<td>X</td>
<td>(170, 171) (172)</td>
</tr>
<tr>
<td>P-glycoprotein-1</td>
<td>X</td>
<td>(173)</td>
</tr>
<tr>
<td>Survivin</td>
<td>X</td>
<td>(174)</td>
</tr>
</tbody>
</table>

DLBCLs are the most common type of non-Hodgkin LSA in humans and dogs, making up 30-40% of all non-Hodgkin lymphoma cases (Table 1.8).
Table 1.8: Prognostic Indicators in DLBCL

<table>
<thead>
<tr>
<th>Negative prognostic indicator</th>
<th>Positive prognostic indicator</th>
<th>Reference data</th>
</tr>
</thead>
<tbody>
<tr>
<td>activated B-cell like (ABC) subtype</td>
<td>X</td>
<td>(175)</td>
</tr>
<tr>
<td>germinatal center B-cell (GCB) subtype</td>
<td></td>
<td>X (175)</td>
</tr>
<tr>
<td>Bcl-6</td>
<td></td>
<td>X (176)</td>
</tr>
<tr>
<td>CD5</td>
<td>X</td>
<td>(177, 178)</td>
</tr>
<tr>
<td>CD43</td>
<td></td>
<td>X (179)</td>
</tr>
<tr>
<td>MHC class I</td>
<td>X</td>
<td>(180, 181)</td>
</tr>
<tr>
<td>MHC class II</td>
<td></td>
<td>X (180, 181)</td>
</tr>
<tr>
<td>P21</td>
<td></td>
<td>X (182)</td>
</tr>
<tr>
<td>Constitutively expressed MYC</td>
<td>X</td>
<td>(183, 184)</td>
</tr>
<tr>
<td>Low expression of LMO2</td>
<td>X</td>
<td>(185)</td>
</tr>
<tr>
<td>Skp2</td>
<td>X</td>
<td>(186)</td>
</tr>
<tr>
<td>VEGFR2</td>
<td></td>
<td>X (187)</td>
</tr>
<tr>
<td>Hif-1α</td>
<td>X</td>
<td>(188)</td>
</tr>
<tr>
<td>CCND2</td>
<td>X</td>
<td>(189)</td>
</tr>
<tr>
<td>Hypercalcemia</td>
<td>X</td>
<td>(190)</td>
</tr>
</tbody>
</table>

Follicular LSAs are the second most common type of NHL and make up 20% of all NHL cases. Follicular LSAs are known for progressing to DLBCLs, and are monitored for characteristic changes in MYC (191). The chromosomal translocation t(14;18)(q32;q31) is characteristic of follicular LSA, up-regulating Bcl-2. Deletions of chromosome 6q or 17p also occur, which results in deletion of tumor suppressor genes and leads to an unfavorable prognosis (192, 193). TNFRSF14 protein is a receptor involved in lymphocyte activation. Mutations of the TNFRSF14 gene on chromosome 1 are linked to a worse prognosis (194). MUM1/IRF4 is an interferon protein regulator of lymphoid differentiation. MUM1/IRF4 is an indicator of high grade follicular LSA with poor prognosis (195).
Many of the prognostic factors found in human LSAs are also shown to be useful determinants of prognosis in canine LSA. Stage and substage of disease at time of diagnosis, just as in human LSA, is a useful prognostic indicator (196). Clinico-morphological subtypes of canine NHL also showed significant prognostic differences, similar to the differences previously mentioned in subtypes of human NHL (197). Anemia, as in human NHL, is also a negative prognostic factor for dogs with LSA (198). Hypercalcemia was found to correlate with decreased survival and remission time in dogs with LSA, similar to findings in human DLBCL (196). Unlike, human LSA, it has been argued that canine LSA prognosis is dependent on the sex of the patient. Female canines with LSA have been shown to have a significantly prolonged remission and survival time compared to males (196, 199). Conversely, another study found that males had a significantly prolonged remission compared to female dogs with LSA (200). P-glycoprotein, as mentioned above as a poor prognostic indicator in human NHL, is also negative predictor of overall survival in canine LSA (201). P53, previously mentioned as a predictor of worse overall survival in human NHL, is also correlated with poor survival in canine LSA, in the 22% of cases that were positive for P53 expression (202). Survivin, as mentioned above as a negative prognostic factor in NHL, is also a negative prognostic indicator in canine LSA (107).

**Cancer Development: Apoptosis, Cell Cycle, Immortality, Invasion, and Metastasis**

In order for cancer to develop, the following must occur: sustained proliferative signaling, evasion of growth suppressors, evasion of cell death, enabled replicative immortality, induction of angiogenesis, activation of invasion and metastasis (203), reprogrammed energy metabolism, evasion of immune destruction, promotion of genomic instability, and tumor promoting inflammation (204). Survivin, to be discussed next, functions in evasion of cell death
(specifically, apoptosis) and growth suppression (specifically, cell cycle arrest), as well as enabling immortality, invasion, and metastasis.

The most common method for evasion of apoptosis is the loss of p53 tumor suppressor function. The p53 gene is responsible for identifying DNA damage and chromosomal abnormalities, and inducing apoptosis (205). Downstream of p53, suppression of apoptosis via up-regulation of anti-apoptotic Bcl-2 proteins and IAP molecules such as survivin can occur (206, 207). Many cancers have loss of, or suppression of, the retinoblastoma (RB) gene (208) which is involved in cell cycle arrest. One of the most commonly mutated and up-regulated proteins in cancer is Ras, an oncogene that promotes growth factor-independent cell cycle entry, allowing the cell to further evade cell cycle checkpoints (209). Another similarly functioning oncogene is Myc, which also enhances progression through the cell cycle (210) and is upregulated by survivin (211). A rare mutation of p53 in Epstein-Barr virus induced nasopharyngeal carcinoma also causes survivin up-regulation which contributes to cell cycle progression (212).

The key to evading senescence and achieving immortality is to lengthen a cell’s telomeres via telomerase. TERT, a protein subunit of telomerase, controls telomerase activity (213) and is up-regulated by c-Myc and Sp-1 (214, 215). Both c-Myc and Sp-1 have been shown to be up-regulated by survivin (211). Overexpression of survivin is one mechanism by which a cancer cell can up-regulate both integrin $\alpha_5$ and the Akt signaling pathway, which induces invasion and epithelial-mesenchymal transition (216, 217). Survivin and XIAP form a complex and have been shown to coordinate NF-\(\kappa\)B activation of fibronectin gene expression, $\beta_1$ integrin signaling, and activation of FAK and Src kinases, all of which are required for metastatic dissemination (218).
Survivin:

Function and Relevance to Cancer

The canine survivin gene is located on chromosome 9 and is 5,154 base pairs long from base 5927501 to 5932654. In humans the survivin gene is located on chromosome 17, at 17q25. The survivin gene was discovered through hybridization screening of a human P1 genomic library with the cDNA of effector cell protease receptor-1 (EPR-1). Survivin is located on the opposite strand to EPR-1, and contains four exons and three introns (219, 220). The survivin gene is conserved across humans and dogs, as well as the chimpanzee, cat, cow, pig, rat, mouse, chicken, zebrafish, and fruit fly. The survivin mRNA is 2655 bp in humans and 1630 bp in canines. The open reading frame for both human and canine cDNA is 429 bp see Table 1.9, and both human and canine mRNA code for a 142 amino acid (aa) chain (91.5% similarity)(Table 1.10), resulting in a 16.5 kD protein (219, 221).
Five other survivin splice variants have been confirmed in humans, termed Survivin-2B (165 aa), Survivin-ΔEx3 (137 aa), Survivin-3B (120 aa), Survivin-2α (74 aa) and Survivin 3α (78 aa) (222). Splice variants of survivin have not been confirmed in dogs.

Survivin (BIRC5) is made up of four exons composing a single N-terminal zinc-binding fold, called a baculoviral IAP repeat (BIR), and an alpha-helix coiled-coil for its body and C

**Table 1.9:** *Human survivin cDNA vs Canine survivin cDNA*

<table>
<thead>
<tr>
<th>human</th>
<th>ATGGGTGCCCCGACGTTGCCCCTGCCTGGCAGCCCTTTTCTCAAGGACCCACGACATCTCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>canine</td>
<td>ATGGGGCCCTTCTCCTGGCTGCCCTGCCCTGCCCTGCCCTGCCCTGCCCTGCCCTGCCCTGTCT</td>
</tr>
<tr>
<td>human</td>
<td>ACATTCAAAAGACTGCAGGAGCCAGAGGACTGTTGGGAGCGCCCATAGAGGAACATAAAAAGCAT</td>
</tr>
<tr>
<td>canine</td>
<td>GCCTGTCTCCTATCCTGGCGCCACTTGAGAAGAGCGAGCCAGATGACCCCTAGATGACCCCATAGGAGATAAAAAACAT</td>
</tr>
<tr>
<td>human</td>
<td>TCAAGGAAGGCTGGAAGGCCTGCGGAGCCAGATGACGCACGCCCATAGAGGAACATAAAAAGCAT</td>
</tr>
<tr>
<td>canine</td>
<td>TCATCTGGTTGGCTTCTGTTGTAAGAGACAGTTTTGAAAGATTAACCCCTTGCTGAA</td>
</tr>
<tr>
<td>human</td>
<td>TTTTGAAGACTGGACAGAAAGGACCCAGAAACAAAATTGCAAGGAAACGAAACTAAAGAG</td>
</tr>
<tr>
<td>canine</td>
<td>TTTTGAAGACTGGACAGAAAGGACCCAGAAACAAAATTGCAAGGAAACGAAACTAAAGAG</td>
</tr>
<tr>
<td>human</td>
<td>AAGAAAGAATTGGAAACTGGGCAAGGAAAGGACCCAGAAACAAAATTGCAAGGAAACGAAACTAAAGAG</td>
</tr>
<tr>
<td>canine</td>
<td>CAGAAAGAATTGGAAACTGGGCAAGGAAAGGACCCAGAAACAAAATTGCAAGGAAACGAAACTAAAGAG</td>
</tr>
<tr>
<td>human</td>
<td>ATGGATGGA</td>
</tr>
<tr>
<td>canine</td>
<td>GCAGAATAG</td>
</tr>
</tbody>
</table>

**Table 1.10:** *Human survivin aa seq vs Canine survivin aa seq*

human: MGAPLTPLVPNQFLKDHRSTFKNWPFLGCACPTERMAEAGFIHCPTENPDAPCQFFCFFELEGWEPDD 
canine: MGASSPLPNQRPFLKHVSFGKNWPFLGCACPTDRMAEAGFIHCPTENPDAPCQFFCFFELEGWEPDD 

human: DPLLHKHSQGSAFLSVKKQFEELTGEFLKDRERAKNIKAKTNKKEFETAKVRRRAIEQLAAAMD 
canine: DPLLHKHSQGSAFLSVKKQFEELTGEFLKDRERAKNIKAKTNKKEFETAKVRRRAIEQLAAAMD
Survivin lacks a carboxyl terminal RING (really interesting new gene) finger and a CARD (caspase-associated recruiting domain) found in other IAP molecules (218). The BIR domain is thought to function in inhibition of apoptosis, and the coiled-coil domain in regulating cell division in the G2/M phase (223). The survivin gene is positively regulated by b-catenin/TCF-Lef, HIF1a (hypoxia-inducible factor 1-a), Sp1 (specificity protein 1), and Stat3 transcription factors. It is negatively regulated by p53, Rb, and PTEN (phosphatase and tensin homolog), all of which are tumor suppressor genes (224). Survivin is post-translationally modified by PLK-1 (polo-like kinase-1), aurora B kinase, p34cdc2/cyclin B, and ubiquitination (225). The survivin protein is present in the nucleus, mitochondria and cytoplasm (226, 227). Both mitochondrial and cytoplasmic survivin are exported from the nucleus via a Crm-1 dependent nuclear export signal (228).

Nuclear survivin localizes to kinetochores during metaphase and to the central spindle midzones at anaphase (226). It functions in regulation of mitosis, as a part of the chromosomal passenger complex aligning chromosomes, in spindle formation, and kinetochore microtubule attachment (229). Survivin is found as a monomer in the chromosomal passenger complex (CPC) and binds via its C terminal to the microtubules of the mitotic spindle during mitosis (230). Nuclear survivin also functions in transcription complex formation with Stat3 in its acetylated form (231). Mitochondrial survivin is dispersed to the cytosol under apoptotic stimulation (226). Survivin localized to the mitochondria is thought to prevent apoptosis upon release as well as block the release of apoptosis-inducing factor (AIF) (232). Cytosolic survivin is associated with microtubules during interphase and centromeres during prophase and prometaphase (226). Cytosolic survivin also localizes to spindle poles and mitotic spindle microtubules during metaphase and anaphase. Additionally, it is found at the midplate during telophase, and finally the midbody at the cleavage furrow during cytokinesis (233).
Survivin can be dimeric in its role as a cytosolic IAP molecule (223) and is thought to have targets upstream of effector caspases or target effector caspases themselves (234, 235). Evidence suggests that survivin plays a role in inhibiting both caspase-dependent and caspase-independent apoptosis (232, 236). Survivin has been found to interact with another IAP molecule, XIAP (X-linked inhibitor of apoptosis protein), to inhibit caspase-3 cleavage (237). It also interacts with HBXIP (hepatitis B X-interacting protein) to inhibit caspase-9 which additionally inhibits caspase-3 cleavage (238). Survivin may also indirectly inhibit apoptosis, via binding to the pro-apoptotic protein Smac (second mitochondria-derived activator of caspases)/DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with a low isoelectric point), preventing it from binding to and inhibiting other IAP molecules (239).

Survivin-ΔEx3 lacks survivin’s third exon due to alternative splicing. Survivin-ΔEx3 also has a truncated BIR domain as a result of this lost exon; however, it continues to function in inhibition of apoptosis, possibly by alternative binding sites or different molecular targets (240). The loss of exon 3 in Survivin-ΔEx3 also causes a frame shift, resulting in an extension of the reading frame in to the open reading frame of the 3’ untranslated region of the survivin gene. Survivin-ΔEx3 also retains its coiled-coil domain, and functions in regulation and promotion of cell division (240). Survivin-ΔEx3 has been shown to be found predominantly in the nucleus (241, 242). However, other researchers with other cell lines have found it localized to both the nucleus and mitochondria (243), and even some in the cytoplasm (244). Survivin-ΔEx3 has been found to be up-regulated in glioma (245), astrocytoma (246), oral squamous cell carcinoma (247), thyroid carcinoma (248), acute myeloid leukemia (249), non-small cell lung cancer (250), colorectal carcinoma (251), bladder cancer (252), soft tissue sarcoma (253), breast cancer (254) cervical carcinoma (255) and prostate cancer (256). SurvivinΔEx3 has been evaluated in at least 13 different types of cancer in numerous studies for prognostic significance. With the exception of one breast cancer study looking at chemosensitivity to
docetaxel and epirubicin (257), increased SurvivinΔEx3 expression correlated with a poor prognosis (222) and in some studies was also correlated with increased tumor invasiveness (258) and metastasis (256).

Survivin-2B differs from survivin because it has an additional exon from intron 2, exon 2B, resulting in a modified BIR domain (240). Survivin-2B may not function in inhibition of apoptosis, possibly due to this modification. Survivin-2B retains its coiled-coil domain. Survivin-2B may actually function in induction of apoptosis, possibly via interaction with survivin’s molecular targets (240). Survivin-2B has been found to localize to the cytoplasm (241, 244) and contains a Crm-1 dependent nuclear export signal which when inhibited, allows nuclear accumulation (228). Other researchers have also shown Survivin-2B localizes to the mitochondria where it is thought to block other survivin variants from being released (259).

Survivin-2B has been shown to decrease as the stage of cancer increases, indicating it may be unfavorable to cancer progression (260, 261). Survivin-2B has been shown to have a positive impact on cancer outcome in studies on colorectal cancer (262, 263), breast cancer (254), glioma (245), renal cell carcinoma (260), gastric cancer (264), and bladder cancer (252). Survivin-2B up-regulation has been correlated to poor prognosis in cervical carcinoma (255), astrocytoma (246), and in some additional studies done on colorectal cancer (251).

Survivin-3B, like survivin 2B, also has an additional exon but from intron 3, exon 3B. This additional exon however, adds in an early stop codon, causing a truncation resulting in a smaller protein (265). Because Survivin-3B retains its BIR domain, it is believed it continues to function in inhibition of apoptosis. However, due to its early truncation, it lacks a tubulin-interacting carboxy-terminal coiled-coil region, which had been thought could inhibit its function in regulation of the G2/M phase of the cell cycle (265). It has since been found that Survivin-3B is capable of functioning as a chromosomal passenger complex protein as well as functioning in cytoprotection (inhibition of apoptosis) (228), essentially capable of everything wild type survivin
can do. Survivin-3B localizes to the cytoplasm, and also contains a Crm-1 dependent nuclear export signal which when inhibited, allows nuclear accumulation (228). Survivin 3B has been found to be up-regulated in acute myelogenous leukemia (265), oral squamous cell carcinoma (247), colorectal cancer (251), and breast cancer (266); however, it has only been associated with poor prognosis in breast cancer (254, 257, 267).

Survivin-2α is composed of survivin’s exon 1 and exon 2, as well as a 197 bp region of intron 2 contains an early stop codon. Survivin-2α contains the first two alpha helices of the BIR domain, but lacks the third alpha helix of this domain (268). Survivin-2α, like Survivin-3B, completely lacks a carboxy-terminal coiled-coil domain. It has been demonstrated that survivin-2α, like survivin-2B, functions in induction of apoptosis. Survivin-2α directly antagonizes the anti-apoptotic effects of survivin, and is present in the nucleus and cytoplasm (268). Survivin-2α’s up-regulation had been correlated with increased apoptosis in vitro (268). Survivin-2α has been found present in breast cancer (266), medulloblastoma (268), astrocytoma (246), colorectal cancer (251), lung cancer, osteosarcoma, and acute lymphoblastic leukemia (268). Despite studies showing survivin-2α’s ability to induce apoptosis, it has been linked to poor prognosis in breast cancer (254) and astrocytoma (246).

Survivin-3α is also composed of survivin’s exon 1 and 2, but it includes 207 bp from intron 2. Survivin-3α has been identified in acute myeloid leukemia (269) and more recently has been found to be upregulated in breast cancer (266). However further studies on Survivin-3α and its function are lacking.

The survivin protein is present during fetal development, but is undetectable in terminally differentiated adult tissues (233). Survivin is also present in low levels in highly proliferative cells, such as hematopoietic progenitor cells, thymocytes, basal colonic epithelium, endothelial cells, endometrial cells and T-cells (270-272). Most types of cancer express survivin at very
high levels and depend on it for continued proliferation (219, 233). In dogs, survivin is found in many normal adult organs, but at much lower levels than observed in canine osteosarcoma and lymphoma, as well as other types of canine cancers (87, 107, 221, 273, 274). Survivin functions primarily in regulation of cell division and inhibition of apoptosis (220). Survivin may also play a role in enhancing telomerase activity via up-regulation of specificity protein 1- (Sp1) and c-Myc-mediated human telomerase reverse transcriptase (hTERT) gene transcription (211). It also plays roles in tumorigenesis (226, 275, 276), invasion (217), and metastasis (218, 277, 278).

Survivin is known to increase chemotherapy resistance (279-281), and has also been found to increase radiation therapy resistance (282-284). Survivin is prominently expressed in transformed cell lines, and in all the most common human cancers of the lung, colon, pancreas, prostate, and breast in vivo, as well as approximately 50% of all high-grade non-Hodgkin’s lymphomas (219). The up-regulation of survivin is known to correlate with a poor prognosis in acute myeloid leukemia (276), acute lymphoblastic leukemia (285), diffuse large cell B-cell lymphoma (108), T-cell lymphoma (286), astrocytoma (246), neuoblastoma (287), head and neck squamous cell cancer (288), melanoma (289), esophageal squamous cell carcinoma (290), gastric cancer (291), colorectal cancer (278), bladder cancer (292), soft tissue sarcoma (253), lung adenocarcinoma (293), non-small cell lung cancer (294), breast cancer (254), ovarian cancer (295), cervical cancer (296), Ewing sarcoma (297), osteosarcoma (298), Wilms tumor (299), pancreatic endocrine tumors (300) and pancreatic adenocarcinoma (301).

Survivin has been targeted on ultimately every level of its biogenesis, at the promoter and survivin gene, mRNA, protein stability, folding, and secondary modifications, even immunologically (302).

Survivin Inhibition: small molecule inhibitors, siRNAs, vaccines, etc.
At the promoter and survivin gene level, the transcription factors ILF3/p54(nrb), Sp1, Stat3, NF-κB, Sox2, and others where the exact target is unknown, have been targeted. The ILF3/p54(nrb) complex binds to the survivin promoter and regulates survivin expression.

YM155 (sepantronium bromide) induces disruption of the ILF3/p54(nrb) complex, which is required for survivin expression (303). The Sp1 transcription factor also mediates transcription of survivin. M4N (tetra-O-methyl nordihydroguaiaretic acid or terameprocol) is an inhibitor of Sp1-mediated transcription of survivin and other genes (304). Stat3 dimerization is required for its nuclear translocation, DNA binding, and transcription of downstream target genes such as survivin. S31-1757 is a small molecule capable of disrupting Stat3 dimerization (305). FLLL32 is a stable derivative of curcumin with superior targeting of Stat3, preventing phosphorylation and DNA binding, consequently reducing survivin expression (306). NF-κB transcribes genes including survivin, and must be activated by RIP1 (nuclear factor NF-κB activator). Endogenous caspase 2, a cell death effector, causes proteolytic cleavage of RIP1, which prevents transcription of survivin and other NF-κB target genes (307). Another way to target NF-κB is through thymoquinone treatment. Thymoquinone is derived from the medicinal spice *Nigella sativa*, also known as black cumin. Thymoquinone has been shown to inhibit the binding of NF-κB to DNA, thereby downregulating XIAP, survivin, and VEGF in human osteosarcoma cells (308). The transcription factor Sox2 has been found to directly up-regulate survivin expression. When Sox2 is inhibited, survivin expression is also decreased (309). TGFβ signaling down-regulates survivin transcription. Belinostat, an HDAC (histone deacetylase) inhibitor can reactivate the TGFβ signaling pathway, thereby down-regulating survivin (310). Another survivin promoter and gene inhibitor is FL118, which not only inhibits survivin expression, but also other cancer-associated survival genes (311).

At the mRNA level, survivin mRNA can be bound and destabilized, through antisense oligonucleotides, siRNA, and miRNAs. Antisense oligonucleotides are single stranded DNA and
are very stable *in vivo*. They work by either inducing degradation of target mRNA, or by binding to them and blocking and inhibiting their translation (312). LY2181308 is an antisense oligonucleotide that has been shown to down-regulate survivin in lung, colon, pancreas, liver, breast, prostate, ovary, cervical, skin, and brain cancer cells. Inhibition in many of these cell lines cause increased apoptosis, cell cycle arrest, and increased chemosensitivity to a number of chemotherapeutics (313). The antisense oligonucleotide 4003 has been show to down-regulate survivin in lung adenocarcinoma cells, and increase apoptosis and chemosensitivity to etoposide (314). EZN-3042 is a locked nucleic acid antisense oligonucleotide that targets survivin mRNA in both human and canine species (Figure 1.1).

![Figure 1.1: Human and canine survivin mRNA compared to EZN-3042 complement. Query represents the human survivin mRNA sequence, Sbjct represents the canine survivin mRNA. The orange highlighting represents the EZN-3042 complement in human survivin mRNA sequence, the yellow highlighting represents the EZN-3042 complement in the canine survivin mRNA sequence. EZN-3042 is not known to target survivin mRNA in other species. The complement to EZN-3042 has 100% homology in 8 of its 16 nucleotides to the mouse survivin mRNA sequence. It has been shown that as few as 5 of 17 nucleotides in 100% homology can have an antisense effect.](image-url)
effect (315), so it is theoretically possible that EZN-3042 could down-regulate survivin mRNA in the mouse. EZN-3042 has been shown to down-regulate survivin in prostate cancer cells, as well as increase apoptosis, cell cycle arrest, and chemosensitivity to paclitaxel. It also downregulates Bcl-2, but the mechanism is as of yet, unknown (316). EZN-3042 has additionally been shown to downregulate survivin in lung tumor xenograft models. Tumor growth inhibition was doubled when EZN-3042 was combined with paclitaxel, compared to EZN-3042 as a single agent treatment (317). EZN-3042 inhibits survivin in acute lymphoblastic leukemia (ALL) cells, and when combined with chemotherapy it can eliminate drug resistant ALL cells (318). EZN-3042 also inhibits survivin in neuroblastoma cell lines, and induces apoptosis (319).

Small interfering RNAs (siRNAs) are double stranded RNA designed for *in vitro* and short term *in vivo* studies, and work by binding to mRNA and directing its degradation. One siRNA targeting survivin caused increased radiosensitization in a wt-p53 sarcoma cell line when combined with irradiation treatment (320). Another siRNA targeting survivin reversed drug resistance when combined with cisplatin in non-small cell lung cancer (321).

MicroRNAs (MiRNAs) are single stranded RNAs that can occur naturally in animals and plants. We can artificially up-regulate and down-regulate them, but they are less specific than siRNA and will bind to multiple targets. It has been shown that miR-34a is down-regulated in cisplatin-resistant gastric cancer cell lines via the PI3K/AKT/survivin signaling pathway, and that over expression of miR-34a could increase sensitivity of these cell lines to cisplatin (322), as miR-34a negatively regulates survivin expression (323). MiR-203 has been shown to target survivin, causing decreased proliferation, increased apoptosis, and cell cycle arrest in pancreatic cancer cells when enhanced with a MiR-203 mimic (324). Another study found that an miRNA construct with the target sequence (5'-GCAGGTCATAGTTTTGGCCACTG-3') to the
hypoxia-inducible factor-1α (HIF-1α) down-regulated survivin, inhibiting human adenocarcinoma cell line growth when cells were transfected with HIF-1α miRNA (325).

At the protein level, protein stability, protein folding, and secondary protein modifications can be targeted. The HDAC inhibitor belinostat was shown to decrease survivin protein stability through survivin’s decreased half-life in treated cells (310). In another study, the depletion of K-Ras promoted the proteosomal degradation of survivin (326). HSP90 folds survivin, as well as many other proteins, and is up-regulated in many malignancies. Geldanamycin is a drug used to inhibit HSP90 (327). Another drug used to inhibit HSP90 is shepherdin, which is a novel peptidyl antagonist of the interaction between HSP90 and survivin (328). Secondary protein modification targets include acetylation and phosphorylation, which control survivin subcellular localization and complex formation. Acetylation of survivin on lysine-129 by CBP (CREB binding protein) results in survivin binding to Stat3, inhibiting its ability to activate target genes, thereby suppressing Stat3’s oncogenic activity while preventing survivin from binding to Crm1 which facilitates nuclear export of survivin (231). Deacetylation of survivin by HDAC6 (histone deacetylase 6) at this same site allows nuclear export of survivin so it can resume its anti-apoptotic function (329). Up-regulation of CBP, or down-regulation of HDAC6 could target survivin in this manner. The phosphorylation of survivin by PLK1 (polo-like kinase 1) is required for survivin to bind to and activate Aurora B in the chromosomal passenger complex, which is required for correct spindle microtubule attachment in cell division (330). Thus, pharmacologic inhibition of PLK1 may interfere with survivin function as well.

To target survivin immunologically, survivin vaccines have been evaluated. A DNA vaccine targeting survivin and co-expressing secretory chemokine CCL21 cause both apoptosis and suppression of angiogenesis while eradicating lung tumors in a mouse model (331). A Fowlpox based survivin vaccine was found to slow tumor growth and improve survival in mice given malignant mesothelioma (332). Additionally, a survivin peptide vaccine induced a very
efficient cytotoxic T cell response against primary gastric adenocarcinoma cells (333). All these studies demonstrate the potential efficacy of vaccines against survivin. Dendritic cell loading involves harvesting dendritic cells, loading them with survivin mRNA or protein, and returning them to their host. In a study of pancreatic cancer, survivin mRNA transfected dendritic cells were shown to induce a cytotoxic T cell response, which was amplified when the dendritic cells were co-transfected with another tumor associated antigen (334). Another study showed that dendritic cells that were pulsed with survivin peptide allowed mice to reject an otherwise lethal inoculation of B-cell lymphoma (335). Finally, a study using dendritic cells transduced with full length dominant-negative survivin gene were able to induce a potent cytotoxic T cell response to prostate cancer cells (336).

Clinical Studies with Survivin Inhibitors

Transcriptional repressors, antisense oligonucleotides, HSP90 inhibitors, and immunotherapies against survivin have all made it into clinical trials in human cancer. YM155 and terameprecol are transcriptional repressors currently in clinical trials. LY2181308 and EZN-3042 are antisense oligonucleotides in clinical trials. For HSP90 inhibitors, currently 17 have entered into the clinical trial phase (337). Finally, there are currently 20 clinical trials registered with the NIH for survivin vaccines, with others already completed, some with positive patient responses (338).

YM155 has gone through both phase I and phase II clinical trials. In phase I clinical trials in Japan and the US, the trials included prostate, colorectal, non-Hodgkin’s lymphoma, head and neck, sarcoma, breast, liver, non-small cell lung, melanoma, ovarian, small-cell lung, endometrial, thyroid, esophageal, pancreatic, renal, thymus, esophageal, thyroid, malignant fibrous histiocytoma, pleural mesothelioma, thymoma, synovial sarcoma, duodenal, double
cancer of hypopharynx and thoracic esophageal, paranasal sinus, pancreatic, and esophageal leiomyosarcoma cancer (339, 340). In the US phase I clinical trial, 3 of 5 patients with recurrent and refractory NHL had “major durable responses” and 2 of 9 patients with hormone-refractory prostate cancer had a “response by PSA criteria”. The maximum tolerated dose (MTD) was determined to be 4.8 mg/m²/d by 168-hour constant intravenous infusion every 3 weeks (340). In the phase I clinical trial in Japan, 9 of 33 patients achieved stable disease, in malignant fibrous histiocytoma, thymoma, non-small cell lung, synovial sarcoma, thymus, thyroid, and esophageal leiomyosarcoma. The MTD was determined to be 8.0 mg/m²/d by 168-hour constant intravenous infusion every 3 weeks (339). The difference between the US MTD and Japan’s MTD has been theorized to be due to decreased renal function in the US patients who failed at the higher dose (341). A second phase I clinical trial in the US in advanced non-small cell lung cancer patients gave a MTD of 10 mg/m²/d by 72-hour constant intravenous infusion every 3 weeks (342).

The phase II clinical trials have been completed in the US and the Netherlands. Most studies went with the lowest MTD determined, 4.8 mg/m²/d by 168-hour constant intravenous infusion every 3 weeks. The phase II clinical trial conducted in the Netherlands was in patients with non-small cell lung cancer. Patients received 4.8 mg/m²/d by 168-hour constant intravenous infusion every 3 weeks. Of the 37 patients receiving treatment, 2 had partial responses and 14 achieved stable disease. The median duration of progression free survival was 1.7 months (343). A similar trial was conducted in the US with advanced non-small cell lung cancer patients, but YM155 was combined with paclitaxel and carboplatin. YM155 was given at 10 mg/m²/d by 72-hour constant intravenous infusion every 3 weeks, and although it had a favorable safety profile, it failed to demonstrate an improved response rate over paclitaxel and carboplatin without YM155 (342). Another phase II clinical trial in the US was in castration-resistant taxane-pretreated prostate cancer. It was found to prolong stable disease in 25% of
patients treated and is currently being evaluated for combination treatment with docetaxel in patients with castration-resistant prostate cancer (344). In a phase II clinical trial in patients with unresectable stage III or IV melanoma, YM155 was given at 4.8 mg/m$^2$/d by 168-hour constant intravenous infusion every 3 weeks. Only 1 of 29 patients had a partial response to treatment (345). A phase II clinical trial was also completed in patients with refractory DLBCL. YM155 was given at 5.0 mg/m$^2$/d by 168-hour constant intravenous infusion every 3 weeks. A total of 41 patients were treated with YM155. 1 patient had a complete response and 2 other patients also responded (346).

Terameprocol (tetra-O-methyl nordihydroguaiaretic acid or M4N), has also gone through both phase I and phase II clinical trials. A phase I clinical trial was completed in patients with recurrent high grade glioma. Of the 32 patients treated, 9 achieved stable disease, and a dose of 1,700 mg/day was deemed safe for future studies (347). Two phase I clinical trials were completed with terameprocol used as a vaginal ointment. The first was in healthy volunteers, results showing that 90 mg daily for 7 days was safe (348). The second study was both a phase I and phase II clinical trial, and was done in patients with HPV-linked cervical squamous intraepithelial neoplasia. Of the 7 patients enrolled in the study, 2 had responses to treatment (349).

LY2181308 has completed phase I clinical trials and has very recently undergone a phase II clinical trial. In the first study, 4 of 22 patients with a variety of cancer types achieved stable disease with a safe dose of 750 mg determined (350). In the second phase I clinical trial in patients with advanced solid tumors, 1 of 12 achieved stable disease, with the 750 mg dose having manageable toxicity (351). In the phase II clinical trial, LY2181308 was combined with doxorubicin in patients with castration resistant prostate cancer. In this trial they found that adding LY2181308 to doxorubicin treatment in these patients did not improve outcome when compared to doxorubicin treatment alone (352).
EZN-3042 has completed two phase I clinical trials, one as a single agent and one in combination with docetaxel. In both studies patients included had advanced solid tumors or lymphomas. In the first study, the maximum tolerated dose for EZN-3042 was determined to be 6.5 mg/kg. Of the 24 patients treated, 5 achieved stable disease (353). In the second study, the maximum dose of EZN-3042 in combination with docetaxel given was 6.5 mg/kg. Of the 16 patients treated, 1 had a partial response and 5 achieved stable disease (354). Currently a phase I clinical trial in dogs with LSA is being conducted with EZN-3042.

HSP90 inhibitors have completed numerous phase I and phase II clinical trials. A phase I clinical trial with 17-AAG (17-allylamino-demethoxy-geldanamycin) found a maximum tolerated dose to be 220 mg/m\(^2\) twice weekly. Of the 13 patients with advanced cancer treated, 3 achieved stable disease (355). Another phase I clinical trial with alvespimycin (17-DMAG; 17-dimethylaminoethylamino-17-demethoxygeldanamycin) revealed a dose of 80 mg/m\(^2\) weekly IV. Of the 25 patients with advanced solid tumors that received treatment, 1 had a complete response, 1 had a partial response, and 3 achieved stable disease (356). Another phase I clinical trial with ganetespib (STA-9090) in patients with solid malignancies was found to recommend a clinical dose of 200 mg/m\(^2\). Of the 53 patients treated, 1 had a partial response and 23 achieved stable disease (357). A phase II clinical trial has also been completed for tanespimycin (17-AAG). The trial was conducted in patients with advanced trastuzumab-refractory HER2-positive metastatic breast cancer. The patients received 450 mg/m\(^2\) tanespimycin weekly IV in combination with trastuzumab. Of the 27 patients evaluated, 6 had a partial response and 10 achieved stable disease (358). In another phase II clinical trial, retaspimycin hydrochloride (IPI-504), was evaluated in patients with castration-resistant prostate cancer. Unfortunately, at the dose of 400 mg/m\(^2\), they saw unacceptable toxicity and no response to therapy (359). A phase II clinical trial conducted with BIIB021 in patients with
gastrointestinal stromal tumors, however had much better results. Of the 23 patients treated, 5 had partial responses and 10 achieved stable disease (360).

Survivin vaccines have also completed numerous phase I and II clinical trials. Multiple phase I clinical trials have been completed with the survivin-2B80-88 vaccine. In a study of patients with advanced or recurrent breast cancer, 2 out of 10 achieved stable disease (361). In a study of patients with advanced or recurrent urothelial cancer, 1 of 46 had a slight reduction in tumor volume (362). In a study of patients with advanced or recurrent oral cancer, 1 out of 11 had a partial response (363). In a study of patients with advanced or recurrent colorectal cancer, 1 of 15 patients had a minor response and 3 patients achieved stable disease (364). Finally, a study in advanced pancreatic patients with the survivin-2B80-88 vaccine, 4 of the 6 patients achieved stable disease (365). In a phase II clinical trial a survivin vaccine targeting HLA-restricted peptide epitopes was used in patients with metastatic melanoma. Of the 55 patients evaluated, 1 had a complete response, 3 had a partial response, and 7 achieved stable disease (366). In another phase II clinical trial with metastatic melanoma patients, a dendritic cell vaccine was used. The dendritic cells were pulsed with survivin and other tumor peptides. Of the 28 patients treated, 16 achieved stable disease (367). In another phase II clinical trial, a vaccine against three survivin peptides restricted to HLA A1, A2 and B35 was used in patients with therapy-resistant advanced cancers. Of the 79 patients enrolled, 3 had complete responses and 3 had partial responses, with 50% of patients having a vaccine-specific immune response (368). Survivin vaccines currently in clinical trials are for patients with malignant melanoma, brain tumors, breast cancer, ovarian cancer, cervical cancer, pancreatic cancer, colon cancer, prostate cancer, multiple myeloma, renal cell carcinoma, and soft tissue sarcoma.

In phase I human clinical trials of LSA, there are two current trials looking specifically at survivin. In the phase I clinical trial: Alisertib, Bortezomib, and Rituximab in Treating Patients With Relapsed or Refractory Mantle Cell Lymphoma or B-Cell Low Grade Non-Hodgkin
Lymphoma, they are looking to determine the best dosing regimen and possible side effects of this treatment. They are also looking at apoptosis and cell cycle proteins such as survivin, pretreatment and post treatment to see if treatment affects their expression (369). In the phase I clinical trial: Administration of TAA-Specific CTLs; Hodgkin or Non-Hodgkin Lymphoma; TACTAL, they use a new experimental therapy which uses the patients cytotoxic T-cells and programs them to target tumor associated antigens. Survivin is one such antigen that these cytotoxic T-cells are programed to target (370). There are currently no clinical trials looking at survivin in OSA.

**Project Rationale**

The primary overarching goal of this dissertation was to determine the effects of survivin inhibition in canine LSA and OSA with consideration of relevance to the human forms of these diseases. Both canine LSA and OSA have poor prognoses and short survival times. Survivin is up-regulated in both of these diseases and is correlated with a worse prognosis with decreased survivial time (87, 107). Inhibiting survivin may prove to be a novel treatment option and may improve outcome in these patients. Additionally, human LSA and OSA also demonstrate up-regulated levels of survivin, also correlating with a poor prognosis (108, 271). If survivin inhibition is found to be a beneficial treatment option in canine medicine, it may very well be a beneficial treatment option for human patients too.

In Chapter #2 (Survivin inhibition via siRNA in canine osteosarcoma cell lines) we established that survivin can be inhibited in canine OSA cell lines with siRNA targeting survivin. We found that survivin inhibition with siRNA causes cell cycle arrest, increased apoptosis, and increased chemosensitivity to carboplatin and doxorubicin in canine OSA cell lines. This
information allowed us to move forward, to look for *in vivo* treatment options for inhibiting survivin, and to look at survivin expression in canine OSA tumors and how it affected outcome.

In **Chapter #3 (Survivin inhibition via EZN-3042 in canine lymphoma and osteosarcoma)** we utilized a locked nucleic acid anti-sense oligonucleotide molecule, EZN-3042, to inhibit survivin. We inhibited survivin in both canine LSA and OSA cell lines, and found that inhibition of survivin via EZN-3042 caused growth inhibition, increased apoptosis, and increased chemosensitivity to doxorubicin in all cell lines. We also looked at xenograft models using canine OSA cell lines, and found that we could inhibit survivin expression with EZN-3042 *in vivo*, and reduce tumor growth when combining EZN-3042 with doxorubicin.

In **Chapter #4 (Expression and function of survivin in canine osteosarcoma)** we looked at survivin expression in archived canine OSA tissue samples. As had been similarly demonstrated in canine LSA (107), we found that increased survivin expression in canine OSA correlated with increased histologic grade, increased mitotic index and decreased disease free interval. These findings are consistent with the findings in human LSA (108, 371) and OSA (298).

In the following chapters, we determined the effects of survivin inhibition in canine LSA and OS with consideration of relevance to the human forms of these diseases. By inhibiting survivin *in vitro*, we caused growth inhibition, increased apoptosis, and increased chemosensitivity to doxorubicin in all cell lines. Additionally, when inhibiting survivin *in vivo*, we found we could decrease tumor growth when combining EZN-3042 treatment with doxorubicin. We also found that increased survivin expression corresponds with a poor prognosis in canine LSA, which has been previously confirmed with canine LSA and in human disease. Thus, we conclude that survivin inhibition could be a novel therapeutic for improving outcome in both canine and human OSA and LSA.
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353. Tolcher AW, Patnaik A, Papadopoulos KP, Jimenez I. EZN-3042 (E), a novel survivin messenger ribonucleic acid (mRNA) antagonist, administered as a single agent (SA) or with docetaxel (D): results of a Phase 1, first-in-human, pharmacokinetic (PK), combination after single agent (CASA) dose-escalation study. American Association for Cancer Research; 2011; San Francisco, CA; 2011.


CHAPTER TWO

SURVIVIN INHIBITION VIA siRNA IN CANINE OSTEOSARCOMA CELL LINES

Summary

Osteosarcoma (OS) has a high mortality rate and remains in need of more effective therapeutic approaches. Survivin is an IAP family member protein that blocks apoptosis and drives proliferation in human cancer cells where it is commonly elevated. In this study, we illustrate the superiority of a canine OS model as a translational tool for evaluating survivin-directed therapies, owing to the striking similarities in gross and microscopic appearance, biologic behavior, gene expression and signaling pathway alterations. Survivin attenuation in canine OS cells inhibited cell cycle progression, and increased apoptosis, mitotic arrest and chemosensitivity. Our findings illustrate the utility of a canine system to accurately model human OS and suggest that survivin-directed therapies might be highly effective in its treatment.

1 Support: This study was supported in part by the Colorado State University College Research Council and American Kennel Club Canine Health Foundation grant # 1064-A
**Introduction**

The dog is a well-established model for spontaneous OS in humans, owing to striking similarity in biology and gene expression (1, 2). The dog’s large size, relative outbreeding and immunocompetence increase their model potential. Furthermore, dogs with spontaneous tumors naturally develop therapy resistance and metastasis. Additionally, tumor burdens in spontaneously arising cancers of dogs are more similar to humans than the experimentally-induced tumors found in murine models, which may be important with regard to biologic factors such as hypoxia and clonal variation. The size of canine tumors also allows for serial imaging and tissue collection over time (1, 2).

As previously mentioned, survivin is a 16.5 kD protein belonging to the Inhibitor of Apoptosis (IAP) family (3). Survivin has two known functions in cells: regulation of cell division and inhibition of apoptosis (4). More recent evidence suggests that survivin also enhances telomerase activity, and may play a role in chemotherapy resistance and metastasis (5-8). Normal cells do not require survivin for survival (9). In cancer cells, however, survivin is critical for its roles in cell division, inhibition of apoptosis (3), tumorigenesis (10, 11), and drug resistance (6, 7). Survivin is only expressed at very low levels in normal osteoblasts (12). Most types of cancer express survivin at very high levels and depend on it for continued proliferation (3, 9).

Survivin expression has prognostic significance in many types of human cancer (13). Small studies in human OS have suggested that survivin may be useful in determining prognosis and degree of malignancy (14-16); however, definitive studies regarding the role of survivin in human OS are lacking. Survivin expression is a negative prognostic factor in dogs with B-cell lymphoma (17), as has been demonstrated in human B-cell lymphoma (18), and survivin expression has been identified in select other canine neoplasms (17).
In this study, we sought to determine the effects of survivin inhibition in canine OS cell lines. We hypothesized, as observed in human OS, that survivin inhibition would decrease cell proliferation and increase apoptosis and chemosensitivity in canine OS cells. If antitumor effects are observed in canine OS, survivin targeted therapies may prove effective in treating canine OS.

**Materials and Methods**

**Cell lines and conditions**

The Abrams canine OS cell line was provided by Dr. William Dernell, and the D17 canine OS cell line was purchased from American Type Culture Collection (Rockville, MD). Both cell lines were serially passaged by trypsinization, and maintained in C/10 media [Minimum Essential Medium (Lonza, Walkersville, MD) supplemented with 1X MEM vitamin solution (Cellgro, Henderson, VA), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate (Cellgro), 1X non-essential amino acid solution (Cellgro), 1X antibiotic/antimycotic (Cellgro), and 10% heat inactivated fetal bovine serum (FBS) (Atlas, Fort Collins, CO)]. Cells were grown in a humidified incubator, with 5% CO$_2$ at 37°C. Both cell lines were confirmed to be of canine origin by multispecies multiplex PCR and identified by short tandem repeat analysis as described (19).

**siRNA Transfection**

A custom siRNA against canine survivin (430) and a scrambled siRNA control were designed using an online resource (BLOCK-iT™ RNA Designer, Invitrogen, Carlsbad, CA) and purchased from Invitrogen (Table 2.1).
For a comparison of canine survivin cDNA and the 430 siRNA see Table 2.2.

<table>
<thead>
<tr>
<th>Canine surv</th>
<th>430 siRNA</th>
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<tr>
<td>5'- AGAAUUCGAAAGAGCCGCAAAGAAA -3’ 5'- UUUCUUGCGUGCCUCUUUCGAAUUUCU -3’</td>
<td>Scrambled (Sham) survivin knockdown 5'- UACGAUCGAAAGCAAGAAAGACG -3’ 5'- GCUCUUUUCUUUGCUUUCGAUCGA -3’</td>
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Transfection was accomplished using HiPerFect transfection reagent (Qiagen, Valencia, CA) in 100 μL Opti-mem media (Invitrogen, Carlsbad, CA). Cells were plated at a density of 1x10^4 – 1x10^5 in 2 mL C10 in 2-well chamber slides or 6-well plates and incubated overnight at 37°C.
The siRNA was diluted to 2 μM and 6 μL was complexed to 5 μL of HiPerFect transfection reagent (Qiagen, Valencia, CA) in 100 μL Opti-mem media (Invitrogen, Carlsbad, CA), in a 10-minute incubation at room temperature. The complexes were then added to the cells (100-200 μL per well) for 12-24 hours at 37°C. The complexes were then removed from the wells and fresh media was added. Cells were then incubated an additional 24-72 hours, then harvested for analysis.

**Survivin Expression**

qRT-PCR – We evaluated survivin mRNA in the survivin siRNA transfected, sham transfected and control cells at 48 hours post transfection using real-time RT-PCR. Primers for canine survivin and the housekeeping gene, HPRT, were designed using Integrated DNA Technologies’ (IDT) website and purchased from IDT (Coralville, IA) (Table 2.3).

<table>
<thead>
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<th>Table 2.3:</th>
<th>PCR Primers</th>
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<td><strong>Survivin</strong></td>
<td></td>
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<tr>
<td>Forward: 5’- TCG AAG AGA CCG CAA AGA AAG TGC -3’</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5’- GAA TTG TGG CCG TTC TCC TTT CCT -3’</td>
<td></td>
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<tr>
<td><strong>HPRT</strong></td>
<td></td>
</tr>
<tr>
<td>Forward: 5’- TGC TCG AGA TGT GAT GAA GG -3’</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5’- TCC CCT GTT GAC TGG TCA TT -3’</td>
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</table>

Cells were harvested via trypsinization from 6-well plates. The mRNA was extracted from pooled supernatant and adherent cells and purified using an RNaseFree DNase Set (Qiagen). The mRNA quantification was performed with a Nano Drop 1000 spectrophotometer (Thermo Scientific, Rockford, IL), followed by conversion to cDNA using an Omniscript® Reverse Transcription (RT) Kit (Qiagen). With the use of Brilliant® SYBR® Green
qPCR Master Mix (Stratagene, Cedar Creek, TX), qRT-PCR was then performed on the cDNA with primers for survivin and HPRT using an MX3000P real-time PCR thermal cycler and MX3000P software (Stratagene). Fold change was determined using the standard $2^{-\Delta\Delta CT}$ method.

**Survivin Antibody** – Survivin antibody (NB500-201) was purchased from Novus Biologicals (Bloomington, MN). It is a polyclonal rabbit antibody targeted to full-length recombinant human survivin. It has been confirmed to recognize human, mouse, rat, cat, and dog survivin. The antibody recognizes a band at ~16.5 kDa. It has been used in chromatin immunoprecipitation, immunocytochemistry, immunofluorescence, immunohistochemistry-paraffin, immunoprecipitation, and western blot assays.

**Immunofluorescence** – Transfected cells were washed in 1x PBS and air-dried on 2-well chamber slides for 24 hours. The slides were then fixed for 15 seconds in room temperature methanol, and air-dried for at least an hour. Slides were then placed in Target Retrieval Solution Citrate pH 6 (Dako Cytomation, Via Real Carpinteria, CA), and heated to 125°C in a pressure cooker. Slides were slowly brought down to 90°C, then transferred to de-ionized water and cooled to room temperature. The slides were washed in 1x TBST, blocked in Background Sniper (Biocare Medical, Concord, CA) for 10 minutes, then washed again. Incubation in rabbit polyclonal anti-survivin antibody (Novus Biologicals) diluted 1:600 in antibody diluent (Dako), occurred overnight at 4°C, then the slides were washed three times in 1x TBST. The slides were then incubated in Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Invitrogen) diluted 1:500 in antibody diluent, for 30 min at 4°C in the dark. The slides were washed again three times in 1x TBST, then mounted using VectaShield plus DAPI mounting medium (Vector Laboratories, Burlingame, CA). Images were acquired in random 20x fields using a Zeiss Axioplan 2 imaging microscope and Axio Vision Release 4.6 software (Carl Zeiss Micro-Imaging Inc, Thornwood, NY).
**Western blot:** At 48 hours post transfection, the cells were harvested from 6-well plates via trypsinization, then washed once in 1x PBS before being lysed in lysis buffer [M-PER Protein Extraction Reagent (Pierce), 1 mM NaVO₄, 1 mM PMSF, Complete Mini protease inhibitor tablet (Roche, Indianapolis, IN), and 1% SDS]. Next, the protein lysates were pulled through a 25 gauge needle 5 times, and centrifuged at 10,000 x g for 10 minutes. The lysates were assessed for protein quantity using a BCA assay (Pierce) and the NanoDrop 1000 spectrophotometer. Lysates were heated with loading buffer to 95°C for 5 minutes, and loaded onto a 4-12% Bis-Tris gel (Invitrogen). The lysates were electrophoresed at 200 V, 100 mA, for 20 minutes followed by transfer to a polyvinylidene difluoride (PVDF) membrane at 30 V, 170 mA, for 45 minutes. The membrane was then blocked in 5% non-fat dry milk in 1x TBST for 1 hour. Rabbit polyclonal anti-survivin (Novus) was then added at 1:1,000 dilution in blocking buffer and incubated overnight at 4°C. The membrane was then washed 3 times in 1x TBST then incubated with HRP goat anti-rabbit IgG (Pierce, Rockford, IL), in blocking buffer at 1:30,000 dilution. The membrane was again washed 3 times in 1x TBST and rinsed for 2 minutes in de-ionized water. Protein bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) using radiographic film (Kodak, Rochester, New York). The membranes were stripped and re-blocked, then incubated in rabbit polyclonal antibody to beta-actin (Abcam) at 1:2,000 dilution in blocking buffer and incubated overnight at 4°C. The membranes were then treated exactly as above after the overnight incubation. Using image J analysis the integrated density of the survivin and beta-actin protein bands was measured for three separate survivin transfections. The integrated density of each survivin band was divided by the integrated density of the corresponding beta-actin band. The three survivin transfections were then normalized to their controls (control = 100%), and averaged together.
Cell Number and Viability

To determine cell numbers, total and live/dead cell numbers were counted in triplicate at 24, 48, 72 hours post transfection using trypan blue.

Apoptosis

Caspase-3/7 Assay – To determine levels of apoptosis, we used a SensoLyte Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA). At 48 hours post transfection, the cells and their supernatants from each individual well were harvested from 6-well plates, and centrifuged at 1500 rpm for 7 minutes. The media was aspirated off and the cells were lysed with 1x lysis buffer (AnaSpec). Next the lysates were transferred to Eppendorf tubes and inverted for 30 minutes at 4°C. Then the lysates were centrifuged at 2,500 g for 10 minutes at 4°C. The supernatant was collected from each sample and transferred at 60 μL per well to a 384-well black-walled plate in duplicate. Twenty μL of caspase-3/7 assay reagent mix was added to each well and the plate was put on a plate shaker for ~60 minutes at 100-200 rpm in the dark at room temperature. The plate was then read on a microplate reader (Synergy HT, Bio-Tek, Winooski, VT) to measure fluorescence intensity at 360/460 nm. Fluorescence intensity measurements were termed ‘Relative Fluorescent Units’ or RFUs in graphical presentations.

TUNEL Assay – For further analysis of apoptosis, we used a commercial TUNEL kit (In Situ Cell Death Detection Kit, Roche Diagnostics, Mannheim, Germany). At 48 hours post transfection, 2-well chamber slides were rinsed in PBS and air-dried overnight followed by fixation (4% paraformaldehyde in 1x PBS) for 1 hour at room temperature. The slides were rinsed in 1x PBS and incubated in permeabilization solution (0.1% Triton X 100 and 0.1% sodium citrate in 1x PBS) for 2 minutes at 4°C. The slides were rinsed 2 more times in 1x PBS,
then 200 μL of TUNEL reaction mixture was added. Slides were incubated for 60 minutes in the dark at 37°C, rinsed 3 times in 1x PBS, and mounted using VectaShield plus DAPI mounting medium. Microscopic images of random 20x fields were obtained.

Cell Cycle Analysis

Propidium iodide staining and flow cytometry was used to evaluate changes in cell cycle distribution following siRNA transfection. The cells were trypsinized, resuspended in 1.5 mL 1x PBS and 3.5 mL ice-cold 100% EtOH was added dropwise while vortexing slowly. The cells were then placed on ice for 30 minutes or overnight at -20°C. After this incubation, the cells were centrifuged at 1500 rpm for 7 minutes, washed once in PBS, then resuspended in 250 μL 1x PBS. Two hundred μL of extraction buffer (192 μL of 0.2 M Na₂HPO₄, 8 μL of 0.1 M citric acid), followed by 500 μL PI-RNase reagent (50 μg/mL propidium iodide, 125 Worthington U/mL RNAse) was added. Cells were filtered through a 40 μm nylon cell strainer and incubated at 37°C for 30 minutes. Samples were then run on a FACscan flow cytometer (BD Biosciences, Durham, NC). Cell cycle analysis on samples was performed using FlowJo Software (Tree Star, Ashland, OR).

Chemosensitivity

To determine sensitivity to carboplatin (CPT, Amatheon, Miami, FL) or doxorubicin (DOX, Bedford Laboratories, Bedford, OH), cells were incubated with no drug, 21.55 μM CPT, or 125 nM DOX. Drug was added for 36-48 hours directly after a 24 hour transfection. Cells were then harvested from their individual wells with their supernatants and total cell count for each well was determined. Cells were lysed and processed according to the SensoLyte Homogenous
AMC Caspase-3/7 Assay Kit as above. RFUs for each sample were normalized to cell count (RFUs/cell) and results expressed as fold-change versus control (untreated cells).

**Statistical analysis**

Statistical analysis of *in vitro* data was performed using GraphPad Prism for Macintosh Version 5.0b (GraphPad Software, La Jolla, CA). Survivin expression levels were summarized by standard descriptive statistics in terms of means and standard deviations. The comparisons of survivin expression, cell numbers, caspase activity and apoptosis levels between experimental conditions was performed using a two-sample t-test with a two-sided significance level.

**Results**

siRNA-mediated knockdown decreases survivin expression. After preliminary validation of survivin as a viable target by confirming high levels of survivin expression in 4 canine OS cell lines by western analysis ([Fig. 2.1](#)), we proceeded with survivin knockdown experiments.
Survivin expression was found in Abrams, D17, McKinley, and Moresco, all canine osteosarcoma cell lines.

To verify the efficiency of the survivin knockdown in our survivin siRNA transfection, we harvested mRNA from the survivin knockdown, sham knockdown, and control cells at 48 hours post siRNA transfection. Analysis of the qRT-PCR data revealed ~20 fold and ~9 fold decreases in survivin mRNA expression in Abrams and D17 respectively in the survivin knockdown cells when compared to control and sham knockdown cells (Fig. 2.2a).
Figure 2.2: *Efficiency of survivin gene knockdown in canine osteosarcoma cells.* A. qRT-PCR for survivin mRNA in Abrams and D17 cells showed a ~20 fold and ~9 fold decrease, respectively, in survivin siRNA when compared to the control and sham knockdown groups. B: Western blot analysis confirms survivin knockdown in both cell lines. C: Using image J analysis we measured the integrated density of the survivin protein bands following three separate survivin siRNA transfections. There was ~85% reduction in survivin protein expression in both cell lines. 430 = survivin siRNA, scrm = sham knockdown (scrambled) siRNA. Error bars in C represent standard deviation. *P < 0.05 vs. control and sham transfection.

Since inhibition of survivin mRNA expression does not confirm decreased survivin protein, we performed further experiments to verify decreased protein expression. Western blot analysis of Abrams and D17 cells confirmed approximately 85% reduction in protein expression in survivin siRNA transfected groups compared to the sham transfected and control groups (Fig.
2.2b, 2.2c). Immunofluorescence cytochemistry further confirmed reduced survivin protein expression. Based on immunofluorescence assessment, knockdown efficacy was 80.2 +/- 7.1% for Abrams and 76.2 +/- 9.4% for D17 (Fig. 2.3).

**Figure 2.3:** siRNA mediated knockdown decreases survivin expression in canine osteosarcoma cells. Abrams and D17 canine osteosarcoma cells were sham transfected (scram) or transfected with siRNA against survivin (430), followed by assessment of survivin expression 48 hours later by immunofluorescence. Five images were taken of each slide and densitometric mean value of survivin immunofluorescence was averaged from these images for each treatment group. Survivin immunoreactivity (Texas Red) was significantly reduced in the siRNA-transfected cells.
Survivin inhibition decreases total cell number and cell viability. Total and live/dead cell counts were performed at 24, 48, and 72 hours post survivin knockdown in both cell lines. At all three time points, there were significantly decreased total cell numbers and significantly higher percent dead cells in the survivin siRNA transfected cells compared to both the sham transfected and control groups (Fig. 2.4).

\[\text{Figure 2.4: Survivin knockdown reduces both total cell numbers and cell viability.}\]

Calculating total cells and percent dead cells for both Abrams (upper panel) and D17 (lower panel) cell lines at 24, 48, and 72 hrs post transfection revealed significantly reduced cell numbers and increased percentage of dead cells in survivin knockdown cells when compared to respective controls according to a 2-tailed, unpaired student T test. Error bars indicate standard deviation. *P < 0.05 and **p < 0.01 compared with control and sham-transfected cells.

Survivin inhibition increases apoptosis. To determine if the cell death observed was via apoptosis, we evaluated caspase-3,7 activity in the survivin siRNA transfected, sham transfected, and control cells 48 hours post transfection. There was a significant increase in
caspase activity in survivin siRNA transfected cells compared to their respective sham transfected and control cells (Fig. 2.5a). This was confirmed by observation of increased DNA fragmentation 48 hours post siRNA transfection, as assessed via TUNEL (Fig. 2.5b).

**Figure 2.5:** *Survivin knockdown induces apoptosis in canine osteosarcoma cells.* A. 48 hours following survivin siRNA knockdown, activated caspase-3,7 activity (A) and TUNEL staining (B) were evaluated using ELISA and immunofluorescence respectively. A significant increase in
caspase activity and TUNEL reactivity was observed following survivin knockdown. Error bars indicate standard deviation. *P < 0.0001 vs. control and sham transfection.

Survivin inhibition results in failure of normal mitosis. We used flow cytometry to analyze cell cycle distribution 48 hours post siRNA transfection. In addition to an increase in the sub-G1 population consistent with previous observations regarding apoptosis, we observed an increase in a 4N population of cells, consistent with failure of mitosis, in survivin siRNA transfected cells. Compared with control transfected cells, the sub-G1 population increased an average of 3.0 and 1.9-fold in Abrams and D17 respectively, and the super-G2 (4N) population increased an average of 3.5 and 2.5-fold in Abrams (Fig. 2.6a,b) and D17 (Fig. 2.6c) respectively. The increased sub G1 peak in the D17 cells treated with the 430 siRNA was not significant compared to controls, possible due to gating done for FlowJo analysis. In order for FlowJo to properly analyze results, debris or sub G1 is typically gated out, reducing sub G1 population available for analysis. Despite this gating, the sub G1 peak in the Abrams cells treated with the 430 siRNA was still significant compared to controls.
Figure 2.6: Cell cycle changes following survivin knockdown in canine osteosarcoma cells. Cell cycle analysis was performed 48 hours following survivin knockdown. A representative histogram for the Abrams cell line is shown in panel A, demonstrating increases in both the sub-G1 (apoptotic) and super-G2 (4N) populations, as indicated by arrows. B: Means (+/- SD) of 4 independent transfections demonstrating significantly increased sub-G1 and super-G2 populations following survivin knockdown in the Abrams cell line. Error bars indicate standard deviation. *P < 0.05 compared with control and sham-transfected cells. C: Means (+/- SD) of 4
independent transfections demonstrating significantly increased super-G2 population following survivin knockdown in D17 cell line. There is also a trend toward increased sub-G1 populations following survivin knockdown. Error bars indicate standard deviation. *P < 0.05 compared with control and sham-transfected cells.

Survivin inhibition increases chemosensitivity. Doxorubicin and platinum drugs form the mainstays of medical therapy for the treatment of both canine and human OS. To determine if survivin was important in mediating resistance to chemotherapy in canine OS, we incubated canine OS cells with CPT or DOX, with or without concurrent survivin or sham siRNA transfection. Survivin knockdown increased caspase activity in both cell lines in the presence of DOX and CPT (Fig. 2.7).

Figure 2.7: Survivin knockdown increases canine osteosarcoma chemosensitivity. Abrams and D17 cells were treated for 48 hours with 21.55 μM carboplatin or 125 nM doxorubicin, +/-
survivin knockdown. Caspase-3,7 activity was then determined by ELISA. Survivin knockdown significantly enhanced caspase activity in cells exposed to carboplatin and doxorubicin. Error bars indicate standard deviation. *P < 0.05 compared with control and sham-transfected cells.

**Discussion**

To assess the utility of canine OS as a potential model for survivin-directed therapeutics, we sought to determine the impact of survivin inhibition on canine OS cell lines *in vitro*. Survivin inhibition in Abrams and D17 canine OS cell lines induced apoptosis, cell cycle arrest, and increased caspase activity in the presence of CPT and DOX. The effects of survivin inhibition in canine OS cell lines had been previously unknown. Other research groups have reported similar results when indirectly and directly inhibiting survivin in human OS. In one recent paper, inhibition of STAT3 activity (which down regulated survivin expression) in canine and human OS decreased cell proliferation and viability, and induced caspase-3/7 mediated apoptosis in treated cells (20). Another group inhibited survivin in HeLa cells and observed caspase-dependent cell death as well as mitotic failure, resulting in multinucleated cells, up to 8 and 16N (21). This observed increase in 4N(+) population could be attributed to the importance of survivin in the chromosomal passenger complex (22) and its association to the mitotic spindle during mitosis (4, 9). Additionally, siRNA-mediated survivin inhibition in human MG-63 OS cells and shRNA-mediated survivin inhibition of human SAOS2 OS cells enhanced sensitivity to cisplatin and DOX (23, 24).

We observed significantly increased apoptosis in survivin knockdown compared to the sham knockdown and control cells in both canine OS cell lines in the absence of any pro-apoptotic stimulus (e.g. serum withdrawal or chemotherapy). There was also modestly increased apoptosis in the sham knockdown compared to the control for both cell lines. We
speculate that the enhanced basal apoptosis observed in the survivin knockdown was possibly
due to the cellular stress imparted by the siRNA transfection process combined with the survivin
inhibition. The modest increase in apoptosis observed in the sham knockdown cells supports
this observation.

Abrams cells had noticeably increased apoptosis when DOX was combined with survivin
siRNA inhibition compared to the D17 cells with the same treatment. Conversely, D17 cells had
noticeable increased apoptosis when CPT was combined with survivin siRNA inhibition
compared to the Abrams cells with the same treatment. DOX intercalates into DNA and inhibits
topoisoamerse II. CPT alkylates DNA and forms DNA crosslinks. Both drugs prevent
successful DNA replication, however it seems each cell line is more sensitive to a specific drug.
The Abrams cell lines seems to better resist the effects of DNA alkylation and cross links,
whereas the D17 cell line handles DNA intercalation and inhibition of topoisomerse II more
easily. Different mutations allow individual cells to become mutated and cancerous. It is
possible that an up-regulation of topoisomerse II happened in the D17 cell line, and perhaps
the Abrams cell line up-regulated glutathione which can bind and inhibit CPT (25).

Survivin is a viable target for therapy. YM155, a small-molecule suppressor of survivin,
is currently in phase II clinical trials in human cancer. Single-agent objective responses have
been observed in patients with melanoma and non-small cell lung cancer (26, 27) as well as
regression of established human hormone-refractory prostate cancer in xenograft models (28).
Studies in combination with chemotherapy are ongoing. EZN-3042, an antisense locked nucleic
acid anti-sense oligonucleotide, is capable of inhibiting survivin expression and tumor growth in viv0 (29) and improves chemotherapeutic response in vitro (30). EZN-3042 is currently in
phase I clinical trials in human cancer. Survivin is also being considered as an immunotherapy
target (31, 32). Phase I and phase II clinical trials of survivin-targeted vaccines are currently
under way.
In conclusion, we have demonstrated that transient survivin knockdown in canine OS cells results in decreased total and viable cell numbers, increased apoptosis and mitotic arrest, and enhanced sensitivity to carboplatin and doxorubicin. These findings are consistent with those in human OS, and indicate that survivin may be a viable therapeutic target for evaluation in canine OS as a preclinical model for human OS. There remains substantial room for improvement in the medical therapy for OS, and canine OS may provide a novel translational model for the investigation of survivin-directed therapeutics.


CHAPTER THREE

SURVIVIN INHIBITION VIA EZN-3042 IN CANINE LYMPHOMA AND OSTEOSARCOMA

Summary

Canine lymphoma (LSA) and osteosarcoma (OS) have high mortality rates and remain in need of more effective therapeutic approaches. Survivin, an IAP family member protein that inhibits apoptosis and drives cell proliferation, is commonly elevated in human and canine cancer. Survivin expression is a negative prognostic factor in dogs with LSA and OS, and canine LSA and OS cell lines express high levels of survivin. In this study, we demonstrate that survivin inhibition in canine LSA and OS cells using a locked nucleic acid antisense oligonucleotide (EZN-3042) inhibits growth, induces apoptosis and enhances chemosensitivity in vitro. Additionally, EZN-3042 inhibits survivin transcription and protein production in vivo, and cooperates with chemotherapy to significantly improve tumor control in mice with canine OS xenografts. Our findings strongly suggest that survivin-directed therapies might be highly effective in treatment of canine LSA and OS.

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**Introduction**

Osteosarcoma (OS) is the most common primary bone tumor in dogs and humans (1-3), and is characterized by both aggressive local tissue infiltration and a very high metastatic rate. Despite the use of neoadjuvant and adjuvant chemotherapy, the 5-year survival rate is only 60% in humans and the 2-year survival rate is only 20% in dogs, illustrating that new therapeutics are needed (1).

Lymphoma (LSA) is one of the most common neoplasm in dogs (4). Non-Hodgkin's lymphoma (NHL), a type of LSA common to dogs and humans, is a leading cause of cancer death in humans (5, 6). The 5-year survival rate for NHL in humans is 68%, and the 2-year survival rate for LSA in dogs is 25% (7). Clearly, new therapy options are also needed for this disease.

Survivin is a 16.5 kD protein belonging to the Inhibitor of Apoptosis (IAP) family (8). The survivin gene has five known mRNA splice variants: Survivin, Survivin-2B, Survivin-ΔEx3, Survivin-3B, and Survivin-2α (9). Unlike other IAP family members, survivin has two known functions in cells: regulation of cell division and inhibition of apoptosis (10). Survivin is found as a monomer in the chromosomal passenger complex (CPC) and binds via its C terminal to the microtubules of the mitotic spindle during mitosis (11). Survivin is dimeric in its role as an IAP molecule (12) and is thought to have targets upstream of effector caspases or target effector caspases themselves (13, 14). Evidence suggests that survivin plays a role in inhibiting both caspase-dependent and caspase-independent apoptosis (15, 16). Survivin may also indirectly inhibit apoptosis, via binding to the proapoptotic protein Smac/DIABLO, preventing it from binding to and inhibiting other IAP molecules (17). Some survivin is localized to the mitochondria and is thought to prevent apoptosis upon release as well as block the release of apoptosis-inducing factor (AIF) (16). Both cytoplasmic and mitochondrial survivin are thought to inhibit apoptosis via binding to other proteins, possibly the effector caspases or other associated proteins (13, 18).
More recent evidence suggests that survivin also enhances telomerase activity, and may play a role in chemotherapy resistance and metastasis (19-22).

Most normal cells do not require survivin for survival (23). In cancer cells, however, survivin is critical for its roles in cell division and inhibition of apoptosis (8). It also appears to have roles in tumorigenesis (18, 24), and drug resistance (20, 21). Hematopoietic progenitor cells, thymocytes, and T-cells express survivin at low levels for cell proliferation (25-27); however, differentiated cells do not express survivin. Additionally, survivin is only expressed at very low levels in normal osteoblasts (28). Most types of cancer express survivin at very high levels and depend on it for continued proliferation (8, 23).

Survivin expression has prognostic significance in many types of human cancer (29). Small studies in human OSA have suggested that survivin may be useful in determining prognosis and degree of malignancy (30-32); however, definitive studies regarding the role of survivin in human OSA are lacking. Survivin expression in Ewing sarcoma, another type of human bone sarcoma, has been shown to be a poor prognostic marker (33). Survivin expression is a negative prognostic factor in both dogs with B-cell lymphoma (34) and in human B-cell lymphoma (35, 36). Survivin expression has been identified in select other canine neoplasms (34). Survivin expression correlates with a poor prognosis in canine OS patients, and inhibition of survivin with small interfering RNA in canine OSA cell lines has been shown to decrease cell proliferation and increase apoptosis and chemosensitivity (37).

EZN-3042 (Enzon Pharmaceuticals, Piscataway Township, NJ) is a locked nucleic acid antisense oligonucleotide (LNA-AsODN) that down regulates survivin mRNA and protein (38, 39). LNA-AsODNs are single-stranded nucleic acids with locked nucleic acid structures attached, providing protection against degradation and enhancing mRNA binding (40). EZN-3042 has been previously reported to down-regulate survivin in two different mouse lung
xenograft models and a mouse subcutaneous canine OS xenograft model (37, 38). SPC-3836, a mouse EZN-3042 analog has also been shown to down-modulate survivin mRNA in a mouse liver regeneration model (38). EZN-3042 has also down-regulated survivin in prostate cancer cells, inducing cell cycle arrest and increased apoptosis and sensitivity to paclitaxel, both *in vitro* and *in vivo* (41). Additionally, EZN-3042 has completed a phase I clinical trial in humans and was generally well tolerated with a few patients achieving stable disease with EZN-3042 as a single agent (42).

In this study, we sought to determine the effects of survivin inhibition in canine LSA and OS cell lines using the survivin-targeting clinical candidate drug EZN-3042. We hypothesized that inhibition of survivin via EZN-3042 in canine LSA and OS cell lines would increase growth inhibition, apoptosis and chemotherapy sensitivity. We additionally hypothesized that we would observe inhibition of survivin in a murine intra-tibial OS model treated with EZN-3042.

**Materials and Methods**

**Cell lines and conditions**

The Abrams canine OS cell line was provided by Dr. William Dernell, and the D17 canine OS cell line was purchased from American Type Culture Collection (Rockville, MD). The 1771 canine B-cell LSA cell line was provided by Dr. K. A. Jeglum of the Wistar Institute (43). The OSW canine T-cell LSA cell line was provided by Dr. W. Kisseberth of Ohio State University (44). Adherent cell lines were serially passaged by trypsinization, and maintained in C/10 media [Minimum Essential Medium (Lonza, Walkersville, MD) supplemented with 1X MEM vitamin solution (Cellgro, Henderson, VA), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate (Cellgro), 1X non-essential amino acid solution (Cellgro), 1X antibiotic/antimycotic (Cellgro), and 10% heat inactivated fetal bovine serum (FBS) (Atlas, Fort Collins, CO)]. Non-adherent cell lines
were passaged by density gradient centrifugation and maintained in C/10 media. Cells were
grown in a humidified incubator, with 5% CO$_2$ at 37°C. All cell lines were confirmed to be of
canine origin by multispecies multiplex PCR and identified by short tandem repeat analysis as
described (45).

**LNA-antisense oligonucleotide treatment**

EZN-3042 is comprised of 16 monomeric units, 7 of which are replaced with LNAs (42). It has the sequence $5'$-CTCAatccatggCAGc-3'$ with capital letters representing LNAs and lower case letters representing DNA monomers (42). EZN-3042 and a scrambled control oligonucleotide (EZN-3046), supplied by Enzon Pharmaceuticals (Piscataway, NJ), were delivered via gymnosis, longer-term cell incubation without use of a transfection reagent (46). Abrams and D17 cells were seeded in T25 or T75 flasks to a concentration of 1x10$^4$-1x10$^5$ cells/flask and incubated 24 hours at 37°C. EZN-3042 or EZN-3046 were then added to individual flasks at concentrations of 5-10 μM and the cells incubated an additional 3-7 days. Cells were then harvested for appropriate analyses.

**Survivin Expression**

$qRT$-$PCR$: We evaluated survivin mRNA expression after 72-96 hours of exposure to 5-10 μM EZN-3042 or EZN-3046 using real-time RT-PCR. Primers for canine survivin and the housekeeping gene, HPRT, were designed using Integrated DNA Technologies’ (IDT) website and purchased from IDT (Coralville, IA) (Table 2.3). Cells were harvested via trypsinization from 6-well plates. The mRNA was extracted from pooled supernatant and adherent cells and purified using an RNeasy® Kit and an RNase-Free DNase Set (Qiagen). The mRNA quantification was
performed with a Nano Drop 1000 spectrophotometer (Thermo Scientific, Rockford, IL), followed by conversion to cDNA using an Omniscript® Reverse Transcription (RT) Kit (Qiagen). With the use of Brilliant® SYBR® Green qPCR Master Mix (Stratagene, Cedar Creek, TX), qRT-PCR was then performed on the cDNA with primers for survivin and HPRT using an MX3000P real-time PCR thermal cycler and MX3000P software (Stratagene). Fold change was determined using the standard 2-ΔΔCT method (47).

**Immunofluorescence** – Treated adherent cells were washed in 1x PBS and air-dried on 2-well chamber slides for 24 hours. Non-adherent cells were washed in 1x PBS and pipetted onto slides to air-dry for 24 hours. The slides were then fixed for 15 seconds in room temperature methanol, and air-dried for at least an hour. Slides were then placed in Target Retrieval Solution Citrate pH 6 (Dako Cytomation, Via Real Carpinteria, CA), and heated to 125°C in a pressure cooker. Slides were slowly brought down to 90°C, then transferred to de-ionized water and cooled to room temperature. The slides were washed in 1x TBST, blocked in Background Sniper (Biocare Medical, Concord, CA) for 10 minutes, then washed again. Incubation in rabbit polyclonal anti-survivin antibody (Novus Biologicals Bloomington, MN) diluted 1:600 in antibody diluent (Dako), occurred overnight at 4°C, then the slides were washed three times in 1x TBST. The slides were then incubated in Alexa Fluor 594 goat anti-rabbit IgG (H+L) (Invitrogen) diluted 1:500 in antibody diluent, for 30 min at 4°C in the dark. The slides were washed again three times in 1x TBST, then mounted using VectaShield plus DAPI mounting medium (Vector Laboratories, Burlingame, CA). Images were acquired in random 20x fields using a Zeiss Axioplan 2 imaging microscope and Axio Vision Release 4.6 software (Carl Zeiss Micro-Imaging Inc, Thornwood, NY).
**Cell Number and Viability**

To determine cell numbers, total and live/dead cell numbers were counted in triplicate at 7 days post transfection using trypan blue.

**Cell Growth Inhibition**

Cell lines were plated in 96-well plates in C/10 at a density of 250 cells per well in quintuplicate and then incubated overnight at 37°C. Medium was aspirated and replaced with 5-10 μM EZN-3042 or EZN-3046 or media alone as a control. Cells were incubated for 5 days at 37°C. Relative viable cell number was determined using a bioreductive fluorometric assay (Cell Titer Blue; Promega, Madison, WI) according to manufacturer directions, using a Synergy HT plate reader (Bio-Tek, Winooski, VT). Relative viable cell number was then expressed as a percentage of control-treated cells. Each experiment was repeated a minimum of three times and mean (±SD) calculated.

**Apoptosis**

To determine levels of apoptosis, we used a SensoLyte Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA). At 5-7 days post treatment with 5-10 μM EZN oligonucleotides, the cells and their supernatants from each individual well were harvested from 6-well plates, and centrifuged at 1500 rpm for 7 minutes. The media was aspirated off and the cells were lysed with 1x lysis buffer (AnaSpec). Next the lysates were transferred to Eppendorf tubes and inverted for 30 minutes at 4°C. Then the lysates were centrifuged at 2,500 g for 10 minutes at 4°C. The supernatant was collected from each sample and transferred at 60 μL per well to a 384-well black-walled plate in quadruplicate. Twenty μL of caspase-3/7 assay reagent...
mix was added to each well and the plate was put on a plate shaker for ~60 minutes at 100-200 rpm in the dark at room temperature. The plate was then read on a microplate reader (Synergy HT, Bio-Tek, Winooski, VT) to measure fluorescence intensity at 360/460 nm. Fluorescence intensity measurements were termed ‘Relative Fluorescent Units’ or RFUs in graphical presentations.

**Chemosensitivity**

To determine sensitivity to doxorubicin (DOX, Bedford Laboratories, Bedford, OH), cells were incubated with 250 nM DOX. Drug was added for 24 hours directly after a 4 or 6 day treatment with EZN-3042 or EZN-3046, depending on the assay. After a 4 day treatment, 250 nM DOX was added directly to half of a 96-well plate and the plate was incubated for 24 hours at 37°C. Relative viable cell number was determined with a bioreductive fluorometric assay using a Synergy HT plate reader as above. Relative viable cell number was then expressed as a percentage of control-treated cells. Each experiment was repeated a minimum of three times and mean (±SD) calculated. After a 6 day treatment, 250 nM DOX was added directly to half of a 6-well plate and the plate was incubated for 24 hours at 37°C. Cells were then harvested from their individual wells with their supernatants and total cell count for each well was determined. Cells were lysed and processed according to the SensoLyte Homogenous AMC Caspase-3/7 Assay Kit as above. RFUs for each sample were normalized to cell count (RFUs/cell) and results expressed as fold-change versus control (untreated cells).
Murine Intra-tibial Xenograft

Female 6-8 week old nu/nu mice were purchased from the National Institutes of Health. After a 1-week acclimatization period, mice were implanted intra-tibially with Abrams OS cells. Mice were anesthetized with isoflurane, then the surgical site was prepared by shaving and then cleaning with ethanol. A 23 gauge needle was used to drill an injection site into the proximal tibia. Abrams cells were then injected into the site at $2 \times 10^6$ concentration suspended in 50 µL 1x HBBS through a 25 gauge needle. Mice then received 0.05 mg/kg buprenorphine every 12 hours for 72 hours post surgery for pain management. Tumors were grown to 10 mm diameter, size-matched and then allocated into 2 groups (n=4-5 per group). Group 1 received EZN-3046, group 2 received EZN-3042. EZN-3042 and EZN-3046 were administered intraperitoneally at 100 mg/kg in 100 µL normal saline, every three days starting day 0 until the end of the study (day 18). Mice were all sacrificed when the first mouse reached a tumor size of 15 mm tumor diameter. Tumors were harvested and snap-frozen or formalin fixed and paraffin embedded for analysis of survivin expression by qRT-PCR as described above and immunohistochemistry as described below.

Murine Subcutaneous Xenograft

Female 6-8 week old nu/nu mice were purchased from the National Institutes of Health. After a 1-week acclimatization period, mice were injected subcutaneously with $2 \times 10^6$ Abrams OS cells while anesthetized with isoflurane. Tumors were grown to 7 mm diameter, size-matched and then allocated into 4 groups (n=8 per group). Group 1 received saline and EZN-3046, group 2 received DOX (Bedford) and EZN-3046, group 3 received saline and EZN-3042, and group 4 received DOX and EZN-3042. EZN-3042 and EZN-3046 were administered
intraperitoneally at 100 mg/kg every three days starting day 0 until the end of the study (day 55). Doxorubicin or an equivalent volume of saline was administered intravenously at 3 mg/kg on day 4 and repeated every two weeks until the end of the study. Mice were weighed and tumors measured every three days. Mice were sacrificed at 15 mm tumor diameter or at the end of the study if they were still alive. A separate group of tumor-bearing mice were randomized similarly at 10 mm tumor diameter. They received EZN-3042 or EZN-3046 on days 0 and 3 (AM) and DOX or saline on day 3 (PM) and sacrificed on day 4. Tumors were harvested and snap-frozen or paraffin embedded for analysis of survivin expression by qRT-PCR as described above and immunohistochemistry as described below.

Survivin RNA extraction

Tumors snap-frozen for RNA extraction were freeze fractured to a fine powder then transferred to Trizol (Life Technologies, Carlsbad, CA). The samples were then homogenized and centrifuged at 2,000 rpm for 30 seconds and incubated at room temperature for 5 minutes. Collect supernatant and add 0.2 mL chloroform per mL of Trizol. Samples are shaken for 15 seconds, then incubated at room temperature for 5 minutes and shaken again. Samples are then centrifuged at 12,000 x g at 4°C for 15 minutes. The top clear aqueous phase is then transferred to a new tube and RNA is precipitated with 0.5 mL 100% isopropanol per original mL or Trizol. Samples are then gently rocked and incubated at room temperature for 10 minutes. The samples are then centrifuged at 12,000 x g at 4°C for 10 minutes. Supernatant was decanted and RNA pellet is washed with 1 mL 75% ethanol, then centrifuged for 12,000 x g at 4°C for 5 minutes. The supernatant was decanted and the pellet was dried for 5-10 minutes before re-suspension in nuclease free water. The samples were then cleaned up using an RNeasy® Kit and an RNase-Free DNase Set (Qiagen) per manufacturer’s instructions.
**Survivin Immunohistochemistry**

Canine OS orthotopic and heterotopic xenografts were prepared from paraffin blocks. Slides were put through a hydration process of xylene baths to graded alcohol, then placed in Target Retrieval Solution Citrate pH 6 (Dako Cytomation, Via Real Carpinteria, CA), and heated to 125°C in a pressure cooker. Slides were slowly brought down to 90°C, then transferred to de-ionized water and cooled to room temperature. The slides were washed in 1x TBST, blocked in Background Sniper (Biocare Medical, Concord, CA) for 10 minutes, then washed again. Incubation in rabbit polyclonal anti-survivin antibody (Novus Biologicals Bloomington, MN) diluted 1:600 in antibody diluent (Dako), occurred overnight at 4°C. The slides were washed 3 times before a 15-minute incubation in 3% hydrogen peroxide at room temperature and washed 3 additional times. Incubation in secondary antibody, Envision+ Dual Link System Peroxidase (Dako) for 30 minutes occurred at 4°C. The slides were washed 3 more times, chromogen stained for 10 minutes using DAB Peroxidase Substrate Kit, (Vector) washed once more and lightly counterstained with hematoxylin. The slides were graded based on survivin stain intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong, 4 = intense) and proportion of cells with positive survivin staining (0 = 0%, 1 = 1-10%, 2 = 10-25%, 3 = 25-50%, 4 > 50%). A final immunoreactivity score for each tissue sample was calculated by multiplying the percentage score by the intensity grade (possible score of 0-16). Scoring was performed by 2 individuals blinded as to treatment allocation and the final survivin score averaged across the 2 raters. This scoring system has previously been used in immunohistochemical scoring of survivin intensity in canine OS (48) and lymphoma tissue sections (34).
Statistical analysis

Statistical analysis of \textit{in vitro} data was performed using GraphPad Prism for Macintosh Version 5.0b (GraphPad Software, La Jolla, CA). Survivin expression levels were summarized by standard descriptive statistics in terms of means and standard deviations. The comparisons of survivin expression, cell numbers, caspase activity and apoptosis levels between experimental conditions was performed using a two-sample t-test with a two-sided significance level. The Shapiro Wilk test was used to verify the normality assumption. P-values of less than 0.05 were considered statistically significant.

Bliss Analysis

Briefly, the Bliss criterion is described by the following equation: $E(x,y) = E(x) + E(y) - E(x)E(y)$ where $E(x)$ is the fractional growth inhibition with survivin knockdown, $E(y)$ is the fractional growth inhibition with chemotherapy, and $E(x,y)$ is the theoretical combined effect if additive. Standard deviations were estimated by error propagation of experimental SD. Differences between treatment groups (Bliss theoretical vs. experimental) were assessed using a 2-tailed unpaired T test. Using this model, if the experimental combined fractional inhibition was significantly higher than the theoretical value, the interaction was considered synergistic.

\textbf{Results}

\textit{EZN-3042 treatment decreases survivin expression.} To verify the efficiency of the survivin inhibition in our EZN-3042 treatment, we harvested mRNA from the 5 and 10 μM EZN-3042 treated and EZN-3046 (scrambled EZN-drug) treated control cells at 72 and 96 hours post treatment. Analysis of the qRT-PCR data revealed 3.4, 2.2, 1.3, and 1.3 fold decreases in survivin
mRNA expression in Abrams, D17, 1771, and Oswald cells, respectively, in the EZN-3042 treated cells when compared to scrambled EZN-drug (scrm) treated cells (Fig. 3.1).

**Figure 3.1:** Survivin mRNA inhibition in canine OS and LSA cell lines. qRT PCR of Abrams, D17, 1771 and OSW 5-10 μM 3-4 day EZN-3042 treated cells showed a 3.4, 2.2, 1.3, and 1.3 fold decrease in survivin mRNA when compared to control and scrambled oligonucleotide treated cells. Error bars represent standard deviation. * = P < 0.05

Since inhibition of survivin mRNA expression does not confirm decreased survivin protein, we performed further experiments to verify decreased protein expression. Immunofluorescence analysis of survivin expression of cells confirmed approximately 72%, 35%, 34%, and 34% reduction in survivin protein expression in Abrams, D17, 1771, and Oswald respectively, in the 5-
10 μM, 72-96 hours post treatment EZN-3042 treated groups compared to the scrm treated and control groups (Fig. 3.2).

**Figure 3.2:** *Survivin protein inhibition in canine OS and LSA cell lines.* Immunofluorescence images (A) and analysis (B) of Abrams, D17, 1771 and OSW cell lines demonstrates 72, 35, 34, and 34% survivin protein inhibition, respectively, in the 5-10 μM 3-4 day EZN-3042 treated cells, compared to the control and scrambled oligonucleotide treated cells. Five images were taken per treatment group. Densitometric mean value of survivin immunofluorescence for all five images was averaged for each treatment group. Error bars represent standard deviation.

** = P < 0.001, *** = P < 0.0001
Survivin inhibition decreases total cell number and cell viability. Total and percent live/dead cell counts were performed at 5 and 7 days post 10 and 5 μM EZN-3042 treatment in the LSA and OS cell lines, respectively. There were significantly decreased total cell numbers and significantly higher percent dead cells in the EZN-3042 treated cells compared to both the scrm treated and control groups (Fig. 3.3).

**Figure 3.3:** Calculating total cells and percent dead cells. Treating Abrams and D17 cells for 7 days at 5 μM EZN-3042 treatment, and 1771 and OSW cell lines for 4 days at 10 μM EZN-3042 treatment, revealed significantly reduced cell numbers and increased cell death in EZN-3042 treated cells compared to respective controls. Error bars represent standard deviation.

** = P < 0.05 for both percent dead and total cell count.
This was further confirmed through cellular metabolic activity via bioreductive fluorometric assay in 10 μM EZN-3042 treated, scrm treated and control cells 5 days post treatment in both LSA and OS cell lines. There was a significant increase in growth inhibition in all EZN-3042 treated cells compared to their respective scrm treated and control cells (Fig. 3.4).

![Graphs showing growth inhibition analysis following EZN-3042 treatment.](image)

**Figure 3.4: Growth inhibition analysis following EZN-3042 treatment.** In bioreductive growth inhibition assays performed on EZN-3042 treated Abrams, D17, 1771 and OSW cell lines, there was significantly decreased growth in 5 day 10 μM EZN-3042 treated cells when compared to the control and scrambled treated cells. Error bars represent standard deviation.

*** = P < 0.0001

**Survivin inhibition increases apoptosis.** To determine if the cell death observed in live/dead counts was via apoptosis, we evaluated caspase-3,7 activity in 5 μM EZN-3042 treated,
scrm treated, and control cells 5 (LSA) and 7 (OS) days post treatment. There was a significant increase in caspase activity in EZN-3042 treated cells compared to their respective scrm treated and control cells (Fig. 3.5).

**Figure 3.5:** Activated caspase-3/7 analysis following EZN-3042 treatment. In the caspase-3/7 assays performed on EZN-3042 treated Abrams, D17, 1771 and OSW cell lines, there was significantly increased activated caspase-3 and caspase-7 activity in the 7 day 5 μM EZN-3042 treated cells when compared to the control and scrambled treated cells. Error bars represent standard deviation. *** = P < 0.0001

Survivin inhibition increases chemosensitivity to DOX. To determine if survivin inhibition via EZN-3042 would enhance cell susceptibility to chemotherapy in canine OS and LSA cell lines,
we incubated all cell lines with 250 nM DOX, with or without concurrent 5 μM EZN-3042 or scrm treatments for 5-7 days. Survivin inhibition via EZN-3042 significantly increased growth inhibition and caspase activity in all cell lines in the presence of DOX, which we defined as increased chemosensitivity (Fig. 3.6 and 3.7). The effect of combining EZN-3042 with DOX on growth inhibition was additive according to Bliss analysis.

![Figure 3.6](image)

**Figure 3.6:** *Growth inhibition analysis following EZN-3042 and DOX treatment.* Abrams, D17, 1771 and OSW cells were treated with 5-10 uM EZN-3042 for 5 days +/- 250-500 nM DOX for 24 hours in growth inhibition assays. Cells that received both the chemotherapy drug and the
EZN-3042 oligonucleotide had significantly decreased growth when compared to the EZN-3042 treated cells without DOX. The above graphs represent the means of four independent experiments. Error bars represent standard deviation. Significance was determined with Student’s two-tailed T tests. ** = P < 0.001, *** = P < 0.0001

Figure 3.7: Activated caspase-3/7 analysis following EZN-3042 and DOX treatment. Abrams, D17, 1771, and OSW cells were treated with 5 uM EZN-3042 for 5-7 days +/- 250-500 nM DOX for 24 hours. Cells that received both the chemotherapy drug and the EZN-3042 oligonucleotide had significantly increased activated caspase-3 and caspase-7 when compared to the EZN-3042 treated cells without DOX. The above graphs are one independent
experiment, representative of 3 independent experiments performed. Error bars represent standard deviation. Significance was determined with Student’s two-tailed T tests.

*** = P < 0.0001

**EZN-3042 treatment in canine OS intra-tibial xenografts results in decreased survivin expression.** Survivin knockdown in vivo was accomplished using the locked nucleic acid antisense inhibitor EZN-3042. Mice bearing established intra-tibial xenografts of Abrams canine OS cells were randomized to receive EZN-3042 or the scrambled control oligo EZN-3046. Knockdown was confirmed in vivo using both qRT-PCR and immunohistochemistry following 7 intraperitoneal oligo treatments (Fig. 3.8).

![Figure 3.8: Intra-tibial tumor survivin mRNA and protein.](image)

**Survivin inhibition in canine OS xenografts results in increased doxorubicin sensitivity.** Mice bearing established subcutaneous xenografts of Abrams canine OS cells were randomized...
to receive DOX or saline, +/- EZN-3042 or the scrambled control oligo EZN-3046. Knockdown was confirmed \textit{in vivo} using both qRT-PCR and immunohistochemistry following 19 oligo treatments and 4 intravenous DOX treatments (\textbf{Fig. 3.9}).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig39.png}
\caption{Subcutaneous tumor survivin mRNA and protein. EZN-3042 treatment in nude mice with subcutaneous Abrams xenografts resulted in decreased survivin mRNA (A) and protein expression (B) upon qRT-PCR and IHC analysis. Error bars represent standard deviation. Significance was determined with one- and two-tailed Student’s T tests. * = P < 0.05, ** = P < 0.001}
\end{figure}

Notably, there was a significant increase in survivin expression in the DOX/EZN-3046 group compared to the control (saline/EZN-3046) (\textbf{Fig. 3.9b}), suggesting possible survivin induction as a DOX response mechanism. Tumor growth rate was significantly reduced in the combined EZN-3042/DOX treated group compared to the other treatment groups (\textbf{Fig. 3.10}).
Figure 3.10: Subcutaneous tumor growth. Mice were treated with 100 mg/kg EZN-3042 or EZN-3046 (scrm) via IP injection every three days, and 3 mg/kg DOX or saline via IV injection every two weeks. EZN-3042 treatment in nude mice with subcutaneous Abrams xenografts resulted in significantly decreased tumor growth when combined with DOX. Significance was based on analysis of overall curve using a one-way ANOVA. * = P < 0.05

In both intra-tibial and subcutaneous xenograft models, the EZN-3042 treated mice suffered significant weight loss from effects of the drug, which were presumably unrelated to its survivin inhibiting properties (EZN-3042 does not target mouse survivin, and the murine homolog antisense product has not been reported to induce weight loss in mice) (Fig. 3.11). The intra-tibial study model had to be ended early because of this phenomenon.
Figure 3.11: Mouse weight following treatment. Weight loss was severe enough to stop EZN-3042 treatment after seven doses in the intra-tibial mouse model, but did not reach statistical significance. However, weight loss in EZN-3042 treated mice in the subcutaneous mouse model was statistically significant. Error bars represent standard deviation. Significance was determined with a two-tailed Student’s t-test. *** = P < 0.0001

Discussion

To determine the efficacy of EZN-3042 as a potential treatment for canine OS and LSA, we sought to determine the impact of survivin inhibition via EZN-3042 on canine OS and LSA cell lines *in vitro*, as well as the ability of EZN-3042 to inhibit survivin expression *in vivo*. Survivin inhibition via EZN-3042 in canine OS and LSA cell lines inhibited cell growth, increased apoptosis, and enhanced DOX sensitivity *in vitro* and *in vivo*. Furthermore, EZN-3042 treatment successfully inhibited survivin expression in subcutaneous and orthotopic canine OS xenografts.
Other research groups have reported similar results when indirectly and directly inhibiting survivin in OS and LSA. In one recent paper, inhibition of STAT3 activity (which caused down regulation of survivin expression) in canine and human OS decreased cell proliferation and viability, and induced caspase-3/7 mediated apoptosis in treated cells (49). Additionally, siRNA-mediated survivin inhibition in human MG-63 OS cells and shRNA-mediated survivin inhibition of human SAOS2 OS cells enhanced sensitivity to cisplatin and DOX (50, 51). In human LSA, a group inhibited survivin with the small molecule YM155 in diffuse large B-cell lymphoma (DLBCL) and a STAT3 inhibitor and saw significant apoptosis in single agent treatments, and a synergistic effect when the two were used in combination (52).

We observed significantly increased apoptosis in EZN-3042 treated cells compared to control cells in both canine OS and LSA cell lines in the absence of any pro-apoptotic stimulus (e.g. serum withdrawal or chemotherapy). There was also modestly increased apoptosis in the scrambled oligo EZN-3046 treated cells compared to the control for all cell lines. We speculate that the enhanced basal apoptosis observed in the EZN-3042 treatments was possibly due to the cellular stress imparted by drug toxicity. The modest increase in apoptosis observed in the EZN-3046 treated cells supports this observation. This toxicity could also explain the discrepancy between the survivin knockdown via siRNA and the reduced knockdown with EZN-3042. With siRNA knockdown, HPRT levels in qRT-PCR and beta-actin levels in western blots were not affected. However, when cells were treated with EZN-3042, HPRT levels were reduced as were beta-actin levels, which made analysis difficult. These reductions in normal cell mRNA and protein could be due to the increased cell death observed in the EZN-3042 treatments, making survivin inhibition less apparent.

In our initial treatments of our canine OS and LSA cell lines we were treating for up to 6-10 days. This was due to initial thought that it would take up to 6-10 days before LNA-AsODNs could be taken up by cells in efficacious concentrations via gymnotic delivery (46). We found in
order to maintain the cells for this time period we had to reduce EZN-drug concentrations by half in the OS and by dilutions of 1:5 and 1:10 for the LSA. After new data was published revealing gymnotic delivery could be effective as early as 2 days after treatment initiation, we revised our treatment protocols (53).

It is interesting to note that the growth curves in the murine xenografts did not begin to diverge until after the second DOX dose. It is possible that multiple EZN-3042 treatments enhanced gene knockdown beyond what was observed at the time we evaluated expression (following 2 injections), leading to a more pronounced effect on survivin expression. It is also worth noting that co-treatment with EZN-3042 and DOX resulted in tumor stabilization rather than regression in the Abrams xenograft model. This could be a function of the DOX dose intensity (supported by the lack of single-agent effect of DOX in this experiment), or dosage and scheduling of EZN-3042 treatments relative to DOX.

The DOX dose we chose (3 mg/kg) is a lower dose than the 10-15 mg/kg DOX dose often used in mice. This dose allows for plasma concentrations of DOX that are very similar to the concentrations seen in humans and dogs on CHOP protocols (54). Additionally the peak plasma concentration attainable at this dose level, ~2,800 ng/mL, is well above the concentration of DOX used to kill Abrams cells in vitro, an IC_{50} of 20 ng/mL with 72 hour DOX treatment.

The subcutaneous xenograft model requested by Enzon was an excellent model for determining effect of EZN-3042 on tumor volume. However it is important to note the significance of the orthotopic model to OS research. By demonstrating that EZN-3042 could cause survivin inhibition in the tumor implanted in the bone, we demonstrate its potential to inhibit survivin in an OS tumor in an actual patient.
EZN-3042 was delivered through what is called “gymnotic” delivery. Antisense oligonucleotides have been delivered for decades using lipidic or particulate transfection reagents. However this older method of delivery has little relevance in vivo and is rather ineffective on suspension cells. Locked nucleic acid antisense oligonucleotides (LNA-As-ONs) are a more modern version of ONs, with extra modifications providing exceptionally high binding affinity for mRNA and resistance to nucleases which stabilizes them in both cell culture media and plasma, making them much more reliable in vivo. Gymnotic delivery employs normal cellular metabolism for LNA-As-ON uptake into cells. Using this delivery method LNS-As-ONs easily enter both adherent and suspension cells, and have been found to be effective both in vitro and in vivo (46, 55, 56).

Both mouse studies had to be stopped early due to severe weight loss. The EZN-3042 dose recommended by Enzon was somewhat higher than what was used in other studies. A earlier study used a dose of 20 mg/kg IP in mice, every day for 4 days, and then decreased to every other day for 16 days. No weight loss was observed in this study after 19 days of treatment (41). In another study, mice were treated with 25 mg/kg IP 3 times weekly, however the dose was reduced to 12.5 mg/kg IP after 7 treatments (14 days) due to weight loss. They reported the weight loss was due to decreased intake and dehydration, with 5-15% body weight lost. The study ended after 28 days treatment (57). In our orthotopic model we were able to treat up to 21 days and up to, 57 days in the subcutaneous model, both at 100 mg/kg IP treatment every 3 days. Considering the 100% homology in 8 of the 16 nucleotides in the EZN-3042 sequence, it is possible that this weight loss is caused by survivin inhibition in the mice. Another possibility is off target effects. For example, Mus musculus dephospho-CoA domain containing (Dcakd) mRNA is100% homologous with 12 of the 16 nucleotides of the EZN-3042 sequence, and would be targeted more effectively than mouse survivin (Figure 3.12). Dephospho-CoA kinase domain containing protein functions in ATP binding and dephosphorylation. Using lower doses of EZN-
Survivin is a viable target for therapy. In human OS, high survivin mRNA expression has been correlated with both presence of metastasis and overall survival (32). In human LSA, the five year survival rate of patients expression survivin in diffuse large B cell lymphoma (DLBCL) is significantly lower than those that do not express survivin (35). YM155, a small-molecule suppressor of survivin, is currently in phase II clinical trials in human cancer. Single-agent objective responses have been observed in patients with melanoma, non-small cell lung cancer, and castration resistant prostate cancer, with a complete response observed in one patient with DLBCL (58-61). Studies in combination with chemotherapy are ongoing. EZN-3042, the antisense oligonucleotide utilized in these in vivo experiments, is capable of inhibiting survivin expression and tumor growth in vivo (38) and improves chemotherapeutic response in vitro (53). EZN-3042 has completed a phase I clinical trial in adults with solid tumors and lymphoma. One
patient had a partial response, five patients had stable disease, and the remaining six that were evaluated did not respond (42). Survivin is also being considered as an immunotherapy target (62, 63). Most notable was a clinical trial in which one patient had complete remission of liver metastasis of pancreatic cancer (64). Phase II clinical trials of survivin-targeted vaccines are currently under way.

In conclusion, we have demonstrated that survivin inhibition via EZN-3042 in canine OS and LSA cells results in decreased total and viable cell numbers, increased growth inhibition and apoptosis, and enhanced sensitivity to DOX. Furthermore, EZN-3042 treatment in vivo resulted in decreased survivin expression in xenografted canine OS tumors and decreased tumor growth when combined with DOX. These findings are consistent with those in human OS and LSA, and indicate that survivin may be a viable therapeutic target for evaluation in canine OS and LSA, and as a preclinical model for the human disease. There remains substantial room for improvement in the medical therapy for both human and canine OS and LSA. Canine OS and LSA may provide a novel translational model for the investigation of EZN-3042 and other survivin-directed therapeutics. Phase-I clinical investigation of EZN-3042 is underway in canine lymphoma patients.
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42. Tolcher AW, Patnaik A, Papadopoulos KP, Agnew J, Lokiec FM, Rezaï K, et al. Results of a Phase 1, open-label, dose-escalation study evaluating the safety and tolerability of EZN-3042, a survivin messenger ribonucleic acid (mRNA) antagonist, administered with or without docetaxel in adult patients with advanced solid tumors or lymphoma. American Association for Cancer Research; 2011; San Francisco, CA; 2011.


CHAPTER FOUR

EXPRESSION AND PROGNOSTIC VALUE OF SURVIVIN IN CANINE OSTEOSARCOMA

Summary

Osteosarcoma (OS) has a high mortality rate and remains in need of more effective therapeutic approaches. Survivin is an IAP family member protein that blocks apoptosis and drives proliferation in human cancer cells where it is commonly elevated. In this study, we determined the role of survivin in canine OS outcome, and illustrate the potential of a canine OS model as a translational tool for evaluating survivin-directed therapies in humans. Elevated survivin expression in primary canine OS tissue correlated with increased histologic grade and mitotic index and a decreased disease free interval (DFI). Our findings illustrate the utility of a canine system to more accurately model human OS and strongly suggest that survivin-directed therapies might be highly effective in its treatment.

1 Support: This study was supported in part by the Colorado State University College Research Council and American Kennel Club Canine Health Foundation grant # 1064-A
**Introduction**

The dog is a well-established model for spontaneous OS in humans, owing to striking similarity in biology and gene expression (1, 2). The dog’s large size, relative outbreeding and immunocompetence increase their model potential. Furthermore, dogs with spontaneous tumors naturally develop therapy resistance and metastasis. Additionally, tumor burdens in spontaneously arising cancers of dogs are more similar to humans than the experimentally-induced tumors found in murine models, which may be important with regard to biologic factors such as hypoxia and clonal variation. The size of canine tumors also allows for serial imaging and tissue collection over time (1, 2).

Survivin expression has prognostic significance in many types of human cancer (3). Small studies in human OS have suggested that survivin may be useful in determining prognosis and degree of malignancy (4-6); however, definitive studies regarding the role of survivin in human OS are lacking. Survivin expression is a negative prognostic factor in dogs with B-cell lymphoma (7), as has been demonstrated in human B-cell lymphoma (8), and survivin expression has been identified in select other canine neoplasms (7).

In this study, we sought to evaluate a correlation between survivin expression and outcome in canine OS patients. We hypothesized, as observed in human OS, that increased survivin expression would correlate with a poor prognosis in canine OS patients.

**Materials and Methods**

**Canine Osteosarcoma Patient Population**

The population of canine appendicular OS patients studied was a subset of patients from a previously reported randomized, prospective clinical trial (9). The study was approved by the
Institutional Animal Care and Use Committees of the participating institutions. All dogs underwent amputation followed by 5 cycles of adjuvant doxorubicin (DOX), with or without an investigational matrix metalloprotease inhibitor and had decalcified primary tumor tissue blocks available for analysis. Adult dogs with stage IIB osteosarcoma were considered eligible. Dogs with preexisting evidence of metastasis, concurrent disease that required additional treatment or was likely to prevent the dog from living 1 year, or previous chemotherapeutic treatment were excluded from the study. Patients were staged by complete blood count, serum biochemical profile, urinalysis, radiographs, history, and complete physical examination. When metastasis was detected, dogs were withdrawn from the study. Study dogs were followed out up to 3 years (9). Histologic grading (from 1 to 3) was performed in a subset of cases by one author (BEP) utilizing a published schema incorporating amount of matrix, percent necrosis, nuclear pleomorphism, nucleolar size/number and mitosis score (9). Mitotic index was also calculated by counting the number of mitotic figures per 10 random 400X fields.

**Survivin Immunohistochemistry**

Slides of canine OS tissues were prepared from paraffin blocks. Samples had previously been decalcified. Slides were put through a hydration process of xylene baths to graded alcohol, then immersed in Target Retrieval Solution (DakoCytomation) and put through a pressure cooker cycle and cooled to room temperature. The slides were then washed in TBST, blocked with Biocare Sniper (Biocare Medical) for 10 minutes, then washed again. Incubation in primary rabbit polyclonal anti-survivin antibody, at 1:600 dilution occurred overnight at 4°C. The slides were washed 3 times before a 15-minute incubation in 3% hydrogen peroxide at room temperature and washed 3 additional times. Incubation in secondary antibody, Envision+ Dual Link System Peroxidase (Dako) for 30 minutes occurred at 4°C. The slides were washed 3 more times, chromogen stained for 10 minutes using DAB Peroxidase Substrate Kit, (Vector)
washed once more and lightly counterstained with hematoxylin. The slides were graded based on survivin stain intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong, 4 = intense) and proportion of cells with positive survivin staining (0 = 0%, 1 = 1-10%, 2 = 10-25%, 3 = 25-50%, 4 > 50%). A final immunoreactivity score for each tissue sample was calculated by multiplying the percentage score by the intensity grade (possible score of 0-16). Scoring was performed by 2 individuals blinded as to patient outcome and the final survivin score averaged across the 2 raters. This scoring system has previously been used in immunohistochemical scoring of canine OS samples (10), and for survivin scoring in canine lymphoma (7).

**Statistical analysis**

The Intra-Class Correlation Coefficient (ICC) was used to evaluate the reproducibility of the survivin immunoreactivity scores between the two independent raters. The ICC can be interpreted as follows: 0 – 0.2 (slight), 0.2 – 0.4 (fair), 0.4 – 0.6 (moderate), 0.6 – 0.8 (substantial) and 0.8 – 1.0 (almost perfect) (11).

Statistical analysis of survival data was performed using a combination of Prism and SAS software version 9.2 (SAS Institute, Cary, NC, USA). Correlations between survivin expression levels and other markers on a continuous scale were evaluated using linear regression analysis. Analysis of variance was used to evaluate the association between survivin expression levels and categorical markers. The median disease free interval (DFI) was estimated using the Kaplan-Meier method. Markers were categorized into a low risk and high risk group (with respect to predicting DFI) using the recursive portioning method (12). This method selects the best predictor variables using recursive splitting. It starts with the best possible predictor from the data set and successively splits the data into categories predicted to observe the event or not. As a splitting method, the exponential scaling method was used.
splitting process stopped when a minimum of five patients per group was reached or when there was no further decrease in prediction error. The associations between the categorized markers and DFI were evaluated using the log-rank test. Multivariate Cox proportional hazard regression analysis was used to determine the prognostic significance of the markers for predictive value of DFI. Predictive markers were selected by backward selection procedures with a p-value cut off of <0.10. A previously deleted variable was allowed to re-enter the final model if its p-value was <0.05. The likelihood ratio test was used to compare various models. The proportional hazard assumption was verified using plots of the log(-log) survival curves and Schoenfeld residuals.

**Results**

Survivin protein expression in canine OS tissues correlates with histologic features and clinical outcome. Survivin expression was studied via immunohistochemistry in 67 primary canine OS tissues from dogs that underwent standardized staging, treatment and follow-up as part of a previously reported prospective clinical trial (9). The Intra-Class Correlation Coefficient of the final survivin immunoreactivity score between the two independent raters was 0.90 (95% CI: 0.84 – 0.93) indicating a very high level of reproducibility. Demographic information regarding the patient population is reported in Table 4.1.
Survivin was expressed in 65 of 67 cases evaluated, with expression intensity ranging from modest to heavy (Fig. 4.1).
Figure 4.1: Evaluation of survivin protein via IHC in canine OS tissue. Survivin expression was studied via immunohistochemistry in 67 primary canine osteosarcoma (OS) tissues from dogs that underwent standardized staging, treatment and follow-up as part of a prospective clinical trial. Images were taken at 400 magnification. Survivin immunoreactive complexes were visualized with DAB substrate, hematoxylin counterstain. **A:** Negative control, normal canine lymph node. **B:** Positive control, normal canine lymph node. **C:** Canine OS tissue with a low survivin immunoreactivity score. **D:** Canine OS tissue with a high immunoreactivity score.

Survivin immunoreactivity score was based on a grade (0 = negative, 1 = weak, 2 = moderate, 3 = strong, 4 = intense) of the intensity of the stain multiplied by a grade (0 = 0%, 1 = 1-10%, 2 = 10-25%, 3 = 25-50%, 4 > 50%) of the percentage of cells stained. Median survivin
immunoreactivity score was 5 (range, 0 to 12). Staining was predominantly nuclear, although a combination of nuclear and cytoplasmic staining was observed in most samples. Survivin immunoreactivity score correlated positively with both histologic grade and mitotic index (Figs. 4.2a and b).

Figure 4.2: *Survivin immunoreactivity correlates with grade and mitotic index in canine osteosarcoma.* A. Primary canine appendicular osteosarcomas of histologic grade 2 or 3 had higher survivin immunoreactivity scores than did grade 1 tumors. Brackets = P<0.05. B. There was a weak, but significant correlation between survivin immunoreactivity and mitotic index in primary canine osteosarcomas.

The overall median DFI in the studied patient population was 211 days (range 43 – 1,393+ days). Upon univariate analysis, histologic grade (1/2 vs. 3), bone-specific alkaline phosphatase (BALP) activity (≤ 48 vs. >48) and survivin immunoreactivity score (≤ 2.75 vs. >2.75) were identified as significant predictors of DFI (Fig. 4.3, Table 4.2).
Survivin immunoreactivity correlates with outcome in canine osteosarcoma. Canine osteosarcoma patients treated with amputation and doxorubicin with high survivin immunoreactivity scores (>2.75) had a significantly inferior disease-free interval on univariate analysis. The survivin score of 2.75 was determined as the cutoff point through statistical analysis of the data as previously detailed in the Methods.

<table>
<thead>
<tr>
<th>Table 4.2: Univariate Analysis of Factors Associated with Outcome in Dogs with Appendicular Osteosarcoma Treated with Amputation and Doxorubicin.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Univariate Analysis</strong></td>
</tr>
<tr>
<td>-------------------------</td>
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<tr>
<td><strong>Survivin score</strong></td>
</tr>
<tr>
<td>≤2.75</td>
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<tr>
<td>&gt;2.75</td>
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<tr>
<td><strong>BALP</strong></td>
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<tr>
<td>≤48</td>
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<tr>
<td>&gt;48</td>
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<tr>
<td><strong>Histologic Grade</strong></td>
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<tr>
<td>1/2</td>
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<td>3</td>
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The median DFI in patients with a lower survivin immunoreactivity score (≤ 2.75) was 331 days versus 173 days in patients with a higher survivin immunoreactivity score (>2.75) (p = 0.046).

As observed in the original clinical trial (9), drug assignment did not impact DFI. Upon multivariate analysis, BALP and histologic grade retained prognostic significance (Table 4.3).

Table 4.3: Multivariate Analysis of Factors Associated with Outcome in Dogs with Appendicular OS Treated with Amputation and Doxorubicin.

<table>
<thead>
<tr>
<th>Multivariate Analysis</th>
<th>Hazard Ratio</th>
<th>p-value</th>
<th>95% CI</th>
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<tbody>
<tr>
<td>BALP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤48</td>
<td>0.272</td>
<td>0.0052</td>
<td>0.11 – 0.69</td>
</tr>
<tr>
<td>&gt;48</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Histologic Grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>0.275</td>
<td>0.0088</td>
<td>0.11 – 0.72</td>
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<td>3</td>
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</table>

Survivin immunoreactivity was not identified as an independent significant predictor of DFI in the multivariate analysis, owing in large part to the strong positive correlation between survivin immunoreactivity and histologic grade.

Discussion

To assess the utility of canine OS as a potential model for survivin-directed therapeutics, we sought to determine the prognostic significance of survivin expression in primary canine OS treatment with surgery and chemotherapy. To our knowledge, the effects of survivin expression in canine OS have been previously unknown and unreported.
Elevated survivin protein immunoreactivity in canine OS tissue samples correlated with increased histologic grade and mitotic index as well as decreased DFI upon univariate analysis. Survivin immunoreactivity lost prognostic significance upon multivariate analysis owing to a strong correlation between survivin score and histologic grade. The correlation of increased survivin protein immunoreactivity to increased mitotic index is not surprising considering our *in vitro* findings that survivin inhibition caused failure of mitosis and cytokinesis and survivin’s known roles in mitosis and the cell cycle (13).

Our results in canine OS are comparable with the limited information regarding survivin expression and outcome in human OS. One group has reported that nuclear localization of survivin correlated with a positive outcome, but did not report whether overall survivin expression had an impact on DFI or survival (4). Perhaps lack of survivin in the cytoplasm indicates limited functionality in cell cycle regulation, and lack of evasion of apoptosis. In our own patient samples, most had both cytoplasmic and nuclear survivin although we did not distinguish between the two. Another group associated survivin expression with histologic grade, differentiation and proliferation index (5). Most recently, high survivin mRNA expression has been correlated with both presence of metastasis and overall survival (6).

Inhibition of survivin with other modalities has further demonstrated that survivin is a viable target. YM155, as mentioned previously, targets survivin at the genetic level, and is currently in phase II clinical trials in human cancer. Its success as a single agent in melanoma, non-small cell lung cancer (14, 15), and prostate cancer (16) demonstrate the potential of therapies targeting survivin. Studies in combination with chemotherapy are ongoing. EZN-3042, the antisense oligonucleotide utilized in these *in vivo* experiments, is capable of inhibiting survivin expression and tumor growth *in vivo* (17) and improves chemotherapeutic response *in vitro* (18). EZN-3042 is currently in phase I clinical trials in human cancer. Survivin is also being considered as an
immunotherapy target (19, 20). Phase I and phase II clinical trials of survivin-targeted vaccines are currently under way.

In conclusion, we have demonstrated that elevated survivin expression in canine OS tissue correlates with increased histologic grade, increased mitotic index and decreased DFI. These findings are consistent with those in human OS, and indicate that survivin may be a viable therapeutic target for evaluation in canine OS as a preclinical model for human OS. There remains substantial room for improvement in the medical therapy for OS, and canine OS may provide a novel translational model for the investigation of survivin-directed therapeutics.


CHAPTER FIVE

General Conclusions

This dissertation explored the effects of survivin inhibition in canine lymphoma and osteosarcoma. Both canine LSA and OS have poor long-term prognoses and short survival times. Survivin is up-regulated in both malignancies and correlates with a worse prognosis (1, 2). Therefore, Inhibition of survivin may represent a new treatment option and ultimately improve outcome in dogs with these diseases. Human LSA and OS also have up-regulated levels of survivin, also correlating with a poor prognosis (3, 4). Based on the results of this research, survivin inhibition may indeed be a beneficial treatment option in canine medicine, with translational relevance to human cancer.

In Chapter #2 we demonstrated that transient survivin knockdown via siRNA in canine OS cell lines resulted in decreased total and viable cell numbers, increased apoptosis and mitotic arrest, and enhanced sensitivity to carboplatin and doxorubicin. These findings are consistent with those in human OS, and further support canine OS as a preclinical model for the human disease. Based on our findings in this chapter we were encouraged to move forward, to look for more clinically applicable survivin-directed treatment options, and to evaluate survivin expression in canine OS tissue with respect to patient outcome.

In Chapter #3 we demonstrated that survivin inhibition via EZN-3042 (a locked nucleic acid anti-sense oligonucleotide molecule) in canine OS and LSA cell lines resulted in decreased total and viable cell numbers, increased growth inhibition and apoptosis, and enhanced sensitivity to doxorubicin. Furthermore, EZN-3042 treatment in vivo resulted in decreased survivin expression in xenografted canine OS tumors, and reduced tumor growth when EZN-3042 was
combined with doxorubicin. These findings are also consistent with those in human OS and LSA, and indicate that survivin may be a viable therapeutic target for further evaluation in both diseases.

In Chapter #4 we looked at survivin expression in archived canine OS tissue samples. As had been similarly demonstrated in canine LSA (1), we found that elevated survivin expression in canine OS tissue correlated with increased histologic grade, increased mitotic index and decreased disease free interval. Currently, the outlook for most patients with canine OS is very grim, however, knowing which patients have a worse prognosis will help us give owners a better understanding of outlook, so they can make informed decisions about how to proceed, and if treatment is a viable option for their pet.

This dissertation has accomplished its purpose. The effects of survivin inhibition in canine LSA and OS were determined with consideration of relevance to the human forms of these diseases. By inhibiting survivin in vitro in canine OS, we decreased total and viable cell numbers, increased apoptosis and mitotic arrest, and enhanced sensitivity to carboplatin and doxorubicin. By inhibiting survivin in vitro in canine LSA, we decreased total and viable cell numbers, increased growth inhibition and apoptosis, and enhanced sensitivity to doxorubicin. Additionally, we inhibited survivin in vivo in canine OS mouse xenografts, and reduced tumor growth in EZN-3042 and doxorubicin combination treatments. We also demonstrated that elevated survivin expression in canine OS tissue correlates with increased histologic grade, increased mitotic index and decreased disease free interval. Survivin inhibition could be a novel therapeutic for improving outcome in both canine and human OS and LSA.
This dissertation demonstrates that survivin is involved in cell cycle progression, inhibition of apoptosis, and chemotherapy resistance in canine LSA and OS, which has been confirmed in other cancer types (7-10). In still other research, survivin has been found to enhance telomerase activity via up-regulation of specificity protein 1- (Sp1) and c-Myc-mediated human telomerase reverse transcriptase (hTERT) gene transcription (11), increase radiation therapy resistance (12-14), and have additional roles in tumorigenesis (15-17), invasion (18), and metastasis (19-21). These functions of survivin were not looked at in this dissertation, and could be further described in canine LSA and OS, with respect to the EZN-3042 drug or other survivin inhibitors.

When nude mice were treated with EZN-3042 they suffered severe weight loss, presumably due to off target effects, or due to mouse survivin being partially targeted. The exact mechanism for the weight loss remains unidentified. It would be interesting to find out what was being targeted in those mice, to understand why they suffered such severe weight loss. Additionally treating mice with an oligonucleotide drug that specifically targets their survivin might be useful in determining definitive side effects of survivin inhibition. In clinical trials survivin inhibitors are used in combination with other drugs so it can be difficult to determine which drug is causing which reaction. Collecting blood & tissues from mice treated with survivin inhibitors specific to the mouse and analyzing them for survivin expression, drug levels and tissue damage would give us a starting point to extrapolate from with regards to dogs and people. A Ki-67 stain on rapidly growing tissues like the gastrointestinal (GI) tract and skin would be a useful indicator of cell proliferation, and whether or not survivin inhibition is severely affecting those tissues. Evaluating the crypts in the small intestine would be an additional method of analysis to determine the effects of survivin inhibition in the GI tract, and whether or not it is severe enough to cause weight loss due to decreased absorbance.
Currently a phase-I clinical investigation of EZN-3042 is underway in canine LSA patients. These dogs only receive 3 infusions of EZN-3042 over the course of 7 days. Unfortunately, such a short time frame does not allow us to determine if dogs experience the same severe weight loss as seen in the mouse studies. No studies have yet been conducted with survivin inhibitors in canine OS.

In human phase 1, open-label, dose-escalation study of EZN-3042 in adult patients with advanced solid tumors or lymphoma, single agent EZN-3042 caused at least one treatment-emergent adverse event (AE) in all 24 patients who received it. The most common AEs were fatigue (46%), tumor pain and increased AST (aspartate) (42% each), increased ALT (alanine aminotransferase) (38%), anorexia (29%), and diarrhea, nausea, and rash (21% each). Patients received weekly doses of 2.5-8 mg/kg EZN-3042 until progressive disease or unacceptable toxicity. Weight loss as a side effect was not mentioned. The best response for single-agent treatment was stable disease in 5 of the 24 patients, including one patient with thymic carcinoma treated for 16 weeks. The best response for EZN-3042 combined with docetaxel was a confirmed partial response in a patient with prostate cancer, treated for 27 weeks (22). In a phase I study of EZN-3042 administered in combination with chemotherapy in six children with relapsed acute lymphoblastic leukemia, two dose-limiting toxicities were observed. One patient developed a grade 3 (5)-glutamyl transferase elevation and another had a grade 3 gastrointestinal bleed. The study was ended with the conclusion that the combination of EZN-3042 with, “intensive reinduction chemotherapy” was not tolerable at doses that could consistently down-regulate survivin (23).

EZN-3042 targets survivin at the mRNA level. However, there are some very promising survivin inhibitors that target survivin in other ways. At the gene level, YM155 (sepantronium bromide) induces disruption of ILF3/p54(nrb), a transcription factor complex that binds to the survivin promoter and up-regulates survivin expression (24). YM155 has additional targets
resulting in widespread DNA damage, furthering its effectiveness as an anti-cancer agent (25).

At the protein level, secondary protein modifications to survivin can be targeted. Acetylation and phosphorylation, which control survivin subcellular localization and complex formation can be disrupted. As previously mentioned, acetylation of survivin on lysine-129 by CBP results in survivin binding to Stat3, inhibiting its oncogenic activity while preventing survivin from binding to Crm1 which facilitates nuclear export of survivin (26). Deacetylation of survivin on lysine-129 by HDAC6 (histone deacetylase 6) allows nuclear export of survivin so it can resume its anti-apoptotic function (27). Up-regulation of CBP, or down-regulation of HDAC6 could target survivin in this manner. Again, we know the phosphorylation of survivin by PLK1 (polo-like kinase 1) is required for survivin to bind to and activate Aurora B in the chromosomal passenger complex, which is required for correct spindle microtubule attachment in cell division (28). Recently we have demonstrated in unpublished data that the pharmacologic inhibition of PLK1 may interfere with survivin function as well.

Recall that survivin primarily functions in cell cycle progression and evasion of apoptosis (29) (30, 31), with possible roles in metastasis, telomerase expression, and drug resistance (8-11, 19-21). Investigations into combining survivin inhibition with drugs that have similar preventative roles may demonstrate its ability to enhance the efficacy of such drugs. Survivin inhibition could be also combined with drugs that target other hallmarks of cancer cells, such as sustained angiogenesis, self-sufficiency in growth signals, and insensitivity to anti-growth signals (32). Additionally, survivin inhibitors could be added into treatment regimens to potentially prevent or prolong time to drug resistance, in cancers where developed resistance is a common problem.

There remains substantial room for improvement in the medical therapy for both human and canine OS and LSA. Canine OS and LSA may provide a novel translational model for the investigation of EZN-3042 and other survivin-directed therapeutics, as single agents and in combination with other chemotherapy agents. If studies of survivin inhibitors in canine LSA and
OS show promise, there will be a basis for more studies with survivin inhibitors in human LSA and OS.


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