Biologic Activity of the Novel Small Molecule STAT3 Inhibitor Against Canine Osteosarcoma Cell Lines

THESIS

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Abstract

Signal transducer and activator of transcription 3 (STAT3) plays an important role in cancer cell proliferation, survival and metastasis. We have previously shown that STAT3 is dysregulated in canine and human osteosarcoma (OSA) and have evaluated several potential small molecule inhibitors (FLLL32, LLL3, among others) of STAT3 in OSA cell lines, although these all have several limitations. LLL12, a novel analog of LLL3, exhibits significantly improved solubility, better predicted oral bioavailability, and greater potency over previously tested inhibitors, making it a potentially more viable candidate for future in vivo evaluation.

The purpose of this study was to first characterize the biologic activity of LLL12 in canine OSA tumor cell lines and then establish OSA cell lines resistant to LLL12 for future evaluation. Canine OSA lines (OSA 8, OSA 16, D17 and Abrams) were treated with LLL12 and effects on proliferation and apoptosis were using CyQuant, Caspase 3/7 assays, and Annexin-V/PI staining. Evaluation of STAT3 transcriptional targets modulated by LLL12 was performed using Western
blotting and qRT-PCR. To generate drug resistant cell lines, cells were cultured continuously or pulsed with LLL12 at increasing concentrations until biologic activity of drug (i.e., cell death) was no longer apparent.

LLL12 inhibited proliferation of canine OSA cell lines in a dose dependent manner and induced apoptosis of canine OSA lines as evidence by Annexin-V/PI double staining and Caspase 3/7 activity. STAT3 phosphorylation was inhibited by LLL12 in all cell lines evaluated, resulting in subsequent downregulation of survivin expression. qRT-PCR confirmed downregulation of survivin gene expression, in addition to Cyclin D1, VEGF, and MCL-1. Lastly, LLL12 exhibited synergistic inhibitory effects on OSA cell line proliferation in the presence of the chemotherapeutic doxorubicin. Our results show that LLL12 is a promising, highly soluble small molecule inhibitor of STAT3 that exhibits biologic activity against canine OSA cell lines.
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Chapter 1: Literature Review

Chapter 1.1: STAT3 Structure, Activation and Signaling

STAT3 is a signal transducer and activator of transcription [1] protein responsible for relaying extracellular signals from cytokines and growth factors to the nucleus, where it then act as a transcription factor and regulates gene expression related to a multitude of normal cellular processes, including growth, cell survival and apoptosis. The molecule consists of a conserved N terminus involved in protein-protein interactions, a DNA binding domain, a C-terminal transactivation domain and, most importantly, an Src-homology 2 domain (SH2) [2]. Upon stimulation of the cytokine and/or growth factor receptors by their respective ligands, STAT3 is recruited via its SH2 domain from the cytoplasm and phosphorylated by activated tyrosine kinases (JAKs) at a specific tyrosine residue (Y705) in the C-terminal domain. The pSTAT3 molecule is then released from the receptor, where it dimerizes to another pSTAT3 molecule and translocates into the nucleus, forming a STAT-DNA complex and actively regulating gene transcription. This process is rapid and transient, allowing for quick, controlled responses from a variety of different molecules and signals.

STAT3 can be activated via a number of different receptor/growth factor pairs (i.e, Met/HGF, Kit/SCF) as well as cytokines (IL-6, oncostatin M) and by non-receptor
tyrosine kinases, most notably the Src family kinases (SFKs) [3]. While originally discovered in the context of IFN-signaling, STAT3 is now known to be a key regulator of genes involved in apoptosis, proliferation, angiogenesis, and immune responses.

Given its diverse roles in cell signaling and regulation, STAT3 requires a large network of tightly controlled endogenous protein regulators, in order to prevent excessive activation and associated potential cellular dysregulation. The constitutively expressed protein inhibitors of activated STAT (PIAS) are a family of proteins with a distinct, highly conserved structure to recognize STAT molecules. PIAS3 specifically binds to and blocks STAT3 DNA binding and therefore transcriptional activation of STAT3 target genes. Other STAT3 regulators like SOCS, which acts as classical feedback inhibitor and is activated by STAT3, and several protein tyrosine phosphatases monitor and regulate aberrant STAT3 activation to limit the duration and effect of STAT3 signaling.

Chapter 1.2: STAT3 Dysregulation and Inhibition in Cancer

Although originally discovered as a signal transducer in involved in cytokine signal transduction, the link between STAT3 and cancer was made when researchers showed that constitutive STAT3 activity is required for oncogenic Src (v-Src) induced cellular transformation. The link was strengthened after showing that transfection of a synthetic constitutively activated STAT3 molecule was sufficient to transform normal
fibroblasts and epithelial cells from several tissues. Subsequent studies found that STAT3 was dysregulated in a number of human cancers, and this aberrant activation was often linked with a malignant phenotype and poor prognosis. Importantly, though STAT3 knockout mice display embryonic lethality, inhibition of STAT3 signaling has little effect on fully developed normal cells yet markedly inhibits proliferation in transformed cells, suggesting that STAT3 has the potential to be a relatively specific target for therapy in many cancers.

The only naturally occurring STAT3 mutations characterized to date are associated with T-cell and NK cell large granular lymphocytic leukemia (LGL). These activating mutations are all located in exon 21, which encodes the SH2 domain of the STAT3 protein. The resulting amino acid change creates a more hydrophobic protein surface and is associated with phosphorylation and translocation of STAT3 to the nucleus, leading to increased gene transcription.

Despite the identification of these novel mutations, the majority of cancers likely express constitutive pSTAT3 secondary to upstream drivers. Elevated EGFR/MET or other growth factor receptor or IL-6 expression, hyperactive JAKs or dysregulated SFKs could all theoretically lead to constant STAT3 activation. Conversely, inactivation of endogenous protein regulators like PIAS and SOCS could easily allow for abnormal and sustained activation of STAT3. Despite the fact that the mechanism of STAT3 activation
likely varies among tumor types, it is clear that dysregulation of STAT3 plays a critical role in tumorigenesis.

Inhibition of STAT3 was initially considered challenging for several reasons. As the protein does not contain an active catalytic site, blocking STAT3 activation would necessitate an inhibition of dimerization through allosteric interactions. Additionally, any inhibitor would need to be specific for STAT3 which is difficult due to the homology of the STAT3 family members and the possibility for heterodimerization. Despite this, several small molecule inhibitors of STAT3 have been developed and tested in vitro and now in mouse models. The first approach uses knowledge of STAT3 structure to target and disrupt specific domains within the molecule and the subsequent protein-protein interactions. One of the more attractive targets on the STAT3 molecule to date is its SH2 domain, where dimerization takes place. Inhibition of dimerization acts to block nuclear translocation of STAT3, thereby disrupting the action of STAT3 dimers on gene transcription. Peptides, due to their potential for immunogenicity and poor cell permeability, are not optimal for this approach, but the use of peptidomimetics and small molecules is generally considered more viable. Screening of vast chemical libraries and the use of computational models to evaluate binding ability have revealed a number of compounds that inhibit STAT3 dimerization and exhibit biologic activity against tumor cell lines in vitro.
Besides disrupting protein interactions with small molecules and peptidomimetics, recently discovered tyrosine kinase-selective inhibitors are being utilized to target tyrosine kinase activity upstream of the STAT3 pathway. While originally a daunting task due to the multitude of inputs that lead to STAT3 activation, JAK inhibitors and Src inhibitors have been shown to block STAT3 activation and inhibit proliferation in a number of cancer cell lines. These inhibitors are may have induce clinical toxicities \textit{in vivo} due to the blockade of multiple signaling pathways, but are currently being investigated in clinical trials.

Disruption of the DNA binding domain via synthetic oligonucleotides has also shown promise in inhibiting STAT3 activity, as the anti-apoptotic and survival genes normally upregulated by STAT3 activation are left untouched. By competing for active STAT3 dimers, double-stranded oligonucleotides can block STAT3 associated gene expression and therefore tumor cell growth. Chemical modifications are needed to improve the half-life and delivery method of these synthetic oligonucleotides, but the proof of principle for STAT3 inhibition is sound, and has even been tested in primates.

While there is still much work to be done in the field, it has become clear that STAT3 represents a promising anticancer target for pharmacologic intervention, due to its central position in numerous signaling pathways and relatively indispensable expression in fully developed organisms.
Chapter 1.3: Osteosarcoma

Osteosarcoma (OSA) is a malignant bone neoplasia of mesodermal origin, characterized primarily by anatomical location and the production of a bone or osteoid-like matrix. The tumor cells are commonly spindle shape with hyperchromatic, pleomorphic nuclei and easily visible mitotic figures. The type of matrix produced has given rise to 3 typical classifications of OSA; osteoblastic, chondroblastic and fibroblastic. In practice, most tumors create varying amounts of all 3 matrices, so the subdivisions are meant only to reflect tumors where one matrix is produced significantly more than the others. Traditionally, OSA is thought to arise from an osteoblast, due to the production of osteoid. But recent evidence points to a more primitive cell with pluripotent abilities, for example a mesenchymal stem cell that acquires osteoblastic characteristics during transformation, or perhaps an osteoblast that has acquired, through de-differentiation, pluripotent capacity. This theory helps explain the mixed patterns of osteoid production and histologic subtypes found within the same tumor, and the many diverse clinical manifestations of the same disease. Osteosarcoma stem cells have been identified through a number of methods, including cell sorting and tumor sphere creation. These cells are rich in stem cell markers (CD 133, CD117, Notch), efficiently form serially transplantable tumors in mice after orthotopic injection, and lead to classic lung metastasis.
1.3.1: Human Osteosarcoma

Osteosarcoma is the most common primary malignant bone neoplasm found in humans. Its estimated worldwide incidence rate is 4 per year per million persons.

Conventional chemotherapy has dramatically increased overall survival rates of patients with OSA, as prior to 1970 surgical amputation was the only viable treatment option. The chemotherapy mainly acts on preexisting metastatic disease since over 80% of patients present with microscopic pulmonary metastasis not visible using standard imaging techniques. With the use of adjuvant chemotherapy, 5 year survival rates have improved to 60-70%.

OSA is most commonly a cancer of young children and adolescents, and like most childhood cancers, osteosarcoma incidence rates have been traditionally shown to be higher in males than in females. The disease follows a bimodal age distribution, with the largest, initial peak representing adolescence, and the latter peak representing adults over the age of 65. The first peak appears to closely relate to periods of bone growth and puberty, while the second peak is most likely related to a second malignancy or previous chemotherapy treatment for an unrelated disease or transplant.

Regardless of the tumor classification, patients with OSA present with nonspecific clinical symptoms such as localized pain and a firm, tender mass, with possible signs of hypervascularity and even a decreased range of motion. Imaging studies and radiographs are subsequently used to reveal a poorly defined, radiodense mass, most
commonly in the metaphyseal region of a long bone, like the femur. Ultimately, fine needle aspirates are utilized to diagnose OSA in both human and canine patients. The most successful treatment option for patients with OSA is neoadjuvant therapy followed by surgical resection and adjuvant chemotherapy. Chemotherapy protocols using doxorubicin, cisplatin and methotrexate are considered the standard of care for this disease. No definitive set of events have been linked to initiation, maintenance and tumor progression in OSA. The complex, heterogeneous karyotypes of osteosarcoma make it difficult to link chromosomal aberrations with phenotypes and characteristics of the tumor. Indeed, research has shown a complex interaction between a multitude of genetic factors and environmental stresses. Age, ethnicity, size and gender have all been shown to pre-dispose a patient to OSA, but the molecular mechanisms behind this have yet to be elucidated. A host of genetic abnormalities have been associated with OSA development, including Li-Fraumeni, Rothmund-Thomson syndrome and Pagets Disease. The relationship between these genetic disorders and elevated levels of osteosarcoma is most likely due to germline alterations in tumor suppressor genes, most notably p53 and retinoblastoma susceptibility gene (RB1). The p53 gene codes for a nuclear phosphoprotein that regulates normal cell cycle progression, and is estimated to be defective in over 50% of all human cancers, while in OSA specifically this number ranges from 20-60%. Li-Fraumeni syndrome is a disease characterized by early bone and soft tissue sarcomas in children, and is the result autosomal dominant mutation in the p53
gene, providing researchers with a strong link between p53 and OSA development. The evidence for RB1 involvement in OS initiation is strong, with a 12.1% chance of developing OS after bilateral retinoblastoma diagnosis.

1.3.2: Canine Osteosarcoma

Osteosarcoma is the most common primary bone tumor in the pet dog population, with over 10,000 cases a year in the US alone. Unlike humans, canine OSA is primarily a disease of older dogs, with a median onset age of 7 years. While the sex distribution is equal, the disease clearly affects large breeds at a much higher rate, with only 5% of OSA occurring in dogs that weigh less than 15 kg.

Canine patients typically present with acute lameness and/or swelling of the affected limb. As with humans, radiographs and fine needle aspiration cytology are typically used to diagnose and evaluate the mass. As with the human disease, most of the tumors are high-grade, osteoblastic malignancies localized to the appendicular skeleton with aggressive metastatic potential.

The etiology of osteosarcoma in canine patients remains to be defined. A host of environmental risk factors have been associated with the disease, for example size, local radiation treatment, heavy weight bearing, and metallic implants. Similar to the human population, p53 and RB1 have been shown to be inactivated in canine OSA. While large
and giant breed dogs are at increased risk for the development of OSA, a clear breed predisposition exists. For example Greyhounds, Rottweilers and Scottish Deerhounds have been shown to have substantial increased risk for development of disease. Studies are ongoing to determine the factors underlying these breed predispositions.

In dogs, the standard therapy for OSA is surgical resection of the primary tumor typically through amputation followed by several cycles of a doxorubicin- or platinum based chemotherapy protocol, to address pre-existing microscopic metastatic disease. Unfortunately, despite this treatment, the one year survival rate is only 40-50%, with less than 15% of dogs living beyond 2 years.

Canine spontaneous OSA represents a good model of the human disease, and offers a variety of advantages over similar in vivo mouse models. First, dogs represent an extremely large outbred, genetically diverse population that live in the same environment as humans. Second, and perhaps most importantly, these are spontaneous tumors in dogs, as opposed to mice where the disease is induced artificially. Dogs also possess a comparable body size and therefore opportunities exist for repeat tumor collection and analysis. Lastly, given the rapid disease progression and relatively short survival times of dogs with OSA following amputation and adjuvant chemotherapy assessment of outcome following evaluation of novel therapies can be accomplished in a relatively short period of time.
1.3.3 STAT3 and Osteosarcoma

STAT3 has been shown to be constitutively activated in both human and canine OSA tissue samples, as well as human and canine OSA cell lines. STAT3 protein levels in human OSA tissue samples correlate with tumor differentiation and status of metastasis, and have a significantly negative impact on disease-free survival and overall survival when compared to patients with low STAT3 expression. Downregulation of STAT3 activity via small molecule inhibitors, dominant-negative forms of the molecule and siRNA inhibits tumor cell growth and induces apoptosis in both human and canine OSA tumor cell lines validating its potential anticancer activity in this disease.

Although the mechanisms that lead to constitutive STAT3 activation have yet to be demonstrated, there is a strong and well-documented relationship between STAT3 and IL-6 [4], the latter of which has been shown to be overexpressed in many cancer types, including OSA. Importantly, no STAT3-activating mutations have been found in OSA. However, given the central role STAT3 plays in cellular signaling processes, there are numerous upstream effector molecules that could potentially activate STAT3. Oncostatin M (OSM), IFN, and leukemia inhibitory factor (LIF) can all bind to STAT3 and induce phosphorylation and activation of the molecule under a variety of different settings [5].
Chapter 2: Methods and Materials

2.1 Cell Lines and Reagents

Canine OS cell lines OSA 8 and OSA 16 were provided by Jaime Modiano (University of Minnesota, Minneapolis, MN), the canine D17 OS cell line was purchased from American Type Cell Culture Collection (ATCC, Manassas, VA), and the canine Abrams OS cell line was provided by Doug Thamm (Colorado State University, Fort Collins, CO). OSA 8, OSA 16 and D17 were maintained in RPMI-1640 supplemented with 10% FBS, non-essential amino acids, sodium pyruvate, penicillin, streptomycin, L-glutamine, and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at 35°C, supplemented with 5% CO₂. The Abrams cell line was cultured in DMEM medium with 10% FBS and L-glutamine. Normal canine osteoblasts (Cell Applications Inc, San Diego, CA) were cultured in canine osteoblast medium (Cell Application Inc). LLL12 was synthesized and purified as described previously [6]. The following antibodies were used for Western blotting experiments: pSTAT3 (Y705, Cell Signaling Technology, Danvers, MA), total STAT3 (Cell Signaling Technology), survivin (Novus Biologicals, Littleton, CO) and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA).
2.2 Cell Proliferation

OSA cells (2.5 x 10³) were seeded in triplicate in 96-well plates overnight in 10% FBS supplemented medium and incubated with DMSO or increasing concentrations of LLL12, doxorubicin, or both for 24 hours. The medium was removed and the plates were frozen at -80°C overnight before processing with the CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. Cell proliferation was calculated as a percentage of the DMSO-treated control wells and IC₅₀ values derived after plotting proliferation values on a logarithmic curve. Each experiment was repeated 3 times.

2.3 Detection of Apoptosis

OSA cells (1.1x10⁴) were seeded in triplicate in 96-well plates overnight in 10% FBS supplemented medium and incubated with medium only, DMSO or LLL12 at increasing concentrations for 24 hours. Caspase 3/7 activity was determined using the SensoLyte® Homogeneous AMC Caspase 3/7 Assay kit (Anaspec Inc, San Jose, CA) according to manufacturer’s instructions. To further assess apoptosis, 2x10⁶ cells were plated in a T175 plate and allowed to grow overnight before being treated with DMSO or LLL12 (0.5 µM) for 24 hours. The cells were then harvested and incubated with FITC conjugated Annexin V and propidium iodide dye [7] following the manufacturer’s
protocol (BD Biosciences, San Jose, CA) before evaluation by flow cytometry (FACS Caliber, BD Biosciences). CellQuest software (BD Biosciences) was used to analyze the samples for early and late apoptosis.

2.4 Western Blotting

OSA cells or canine osteoblasts (2x10^6) in 1% FBS medium were treated with DMSO or 0.5 µM LLL12 for 12 hours. Normal canine osteoblasts were serum starved for 2 hours prior to identical treatment. Protein lysates were prepared and quantified, separated by SDS-PAGE, and Western blotting was performed using previously described methods [8]. The membranes were incubated overnight with anti-pSTAT3 (Y705, Cell Signaling Technology, Danvers, MA) or anti-survivin (Novus Biologicals, Littleton, CO) antibodies, then incubated with appropriate horseradish peroxidase linked secondary antibodies, washed, and exposed to substrate (SuperSignal West Dura Extended Duration Substrate, Pierce, Rockford, IL). Blots were stripped, washed, and reprobed for total STAT3 (Cell Signaling Technology) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), respectively.
2.5 RT-PCR and qRT-PCR

Total RNA was extracted from canine OSA cells in 10% FBS supplemented medium following 12 hours of treatment with DMSO or 0.5 µM LLL12 using RNeasy Mini Kits (Qiagen, Valencia, CA) according to the manufacturer’s instructions. After RNA extraction, samples were treated with DNase I using RQ1 Rnase-Free DNase (Promega, Madison, WI). cDNA was generated from 2 µg of total RNA using Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. 1/20 of the resultant cDNA was used for each PCR reaction in a total volume of 25 µl. Primers designed and utilized for canine survivin, cyclin D1, BCL-2, VEGFA, MCL-1 and 18s are listed in Table 1. Standard PCR was performed with all primer sets and amplicon length verified through agarose gel electrophoresis and visualization of products using the Alpha Imager system (Alpha Innotech Corp, San Leandro, CA).

To quantitatively measure the effect of LLL12 treatment on STAT3 downstream targets, total RNA was collected as described above. Real-time quantitative PCR was performed using Applied Biosystem’s StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA). Canine survivin, cyclin D1, BCL-2, VEGFA, MCL-1 and 18s mRNA were detected using Fast SYBR green PCR master mix (Applied Biosystems) according to the manufacturer’s protocol. All reactions were performed in triplicate and included non-template controls for each gene. Relative expression was calculated using
the comparative threshold cycle method [9]. Experiments were repeated 3 times using samples in triplicate.

2.6 Drug Combination Analysis

Experiments were performed in 96-well plates. OSA cells were seeded at a density of 2.5×10⁴ cells per well in RPMI medium containing 10% FCS. Stock solutions of LLL12 and doxorubicin were generated and serial dilutions (2-fold) for each compound were prepared, with the concentration range from .0625X to 4X the IC₅₀ value of each drug. To assess potential synergistic interactions, the treatment regimen involved simultaneous treatment of cells with LLL12 and doxorubicin for 24 hours, in addition to controls consisting of cells treated with the individual compounds alone for 24 hours. All treatments were performed in triplicate wells. Following drug treatment, the number of viable cells in each well was determined using CyQUANT® as described previously. Drug interactions were analyzed using CompuSyn 3.0.1 (CompuSyn, Inc.,Paramus, NJ), which is based on the median effect model of Chou and Talalay [10]
2.7 Generation of Resistant Cell Lines

To generate OSA lines resistant to LLL12, two different approaches were used. OSA 8P and OSA 16P were generated by pulse treatment of cells with 5 µM LLL12 for 1 hour. Cells were then washed and cultured in drug-free complete medium until they returned to their exponential growth phase, at which point they were pulsed again. OSA 8R and OSA 16R were generated by continuous exposure to LLL12 in the culture medium. The initial concentration of LLL12 used was 50 nM with medium changed every 48 hours. When the cells became confluent, they were detached, washed and replated in drug-free medium for 24 hours before the next concentration of LLL12 was added. LLL12 concentration was escalated from 50 nM to 2.5 µM.

2.8 Statistical Analysis

All the values reported are mean ± SD. Delta CTs from qRT-PCR were compared using two sample t-tests and Holm’s method [11] was used to control type-I error across tests of multiple genes. The Jonckhere-Terpstra (JT) test [12, 13] was used to test for a monotone trend in cell proliferation and caspase activity with dose of drug. If the JT test was insignificant, the Mack-Wolfe test [14] was performed for a non-monotone, or umbrella, dose-response. All analyses were performed using SAS Version 9.2 (SAS Inc.,
Cary, NC). The Mack-Wolfe test was performed using the MWUSPU and MWUSPK SAS macros developed by Juneau [15].
Chapter 3: Results

3.1 LLL12 Inhibits Proliferation in Canine OSA Lines

Canine OSA cell lines were treated with increasing concentrations of LLL12 (0.05 µM- 5 µM) for 24 hours and effects on cell proliferation were assessed. LLL12 significantly reduced cell proliferation at concentrations as low as 0.1 µM with the calculated IC$_{50}$ concentrations in the nanomolar range (231-411 nM) for all cell lines (Figure 1). Normal canine osteoblasts were comparatively resistant to the anti-proliferative effects of LLL12, with an approximately 7-fold higher calculated IC$_{50}$ of 1.780 µM (Figure 1).

3.2 LLL12 Promotes Apoptosis of Canine OSA Lines

To determine if LLL12 growth inhibition was mediated via apoptosis, canine OSA cell lines were treated with DMSO or LLL12 for 24 hours, and caspase 3/7 activity was measured. In all cell lines, caspase 3/7 activity was increased at 24 hours post treatment with LLL12 at concentrations of 0.4-0.8 µM (Figure 2A). OSA cells were also stained with annexin V-FITC/PI and analyzed by flow cytometry to assess the percentage of early and late apoptotic cells in the population. After a 24 hour exposure to 0.5 µM LLL12 there was an increase in the proportion of early apoptotic (annexin V positive)
and late apoptotic (annexin V/PI positive) cells. This correlated with data generated from the caspase assay (Figure 2B). Normal canine osteoblasts were treated and analyzed by flow cytometry as described above, and were far less sensitive to the apoptosis inducing effects of LLL12 (Figure 2C).

3.3 LLL12 Decreases pSTAT3 and Survivin Expression

Canine OS A cells and normal canine osteoblasts were treated with DMSO, 0.1 µM LLL12 or 0.5 µM LLL12 for 4, 8 or 12 hours to determine the time and dose dependence of its effect on STAT3 phosphorylation and survivin expression. Western blot analysis revealed pSTAT3 was completely downregulated following treatment with 0.5 µM LLL12 for only 4 hours, with a concomitant downregulation of survivin expression (Figure 3A). As expected, these results were time- and dose-dependent. Importantly, normal canine osteoblasts treated identically to their OS counterparts had significantly lower pSTAT3 expression and demonstrated no change in survivin expression (Figure 3B) following 0.5 µM LLL12 treatment at 12 hours.

3.4 LLL12 Treatment Decreased STAT3-Mediated Gene Transcription

To assess the effects of LLL12 on transcriptional targets of STAT3 the expression of cyclin D1, BCL-2, MCL-1 and survivin was assessed using quantitative RT-PCR.
Standard PCR was run with all primer sets and amplicon length verified prior to quantitative analysis. Expression of the STAT3 regulated genes evaluated was downregulated in all 4 OS cell lines after 12 hours of treatment with 0.5 µM LLL12 when compared to DMSO treated cells (Figure 4) supporting the notion that inhibition of pSTAT3 by LLL12 affects its transcriptional activity.

3.5 LLL12 Enhances the Effect of Doxorubicin in Canine OSA Lines

To assess whether inhibition of pSTAT3 would enhance the biologic activity of chemotherapy in OS cell lines, Abrams and OSA 16 cells were treated with LLL12 (0.016 µM-1 µM) and doxorubicin (0.022 µM-1.4 µM) over a range of doses reflecting multiple concentrations of their respective IC$_{50}$ concentrations ranging from 0.0625x to 4x. Dose-response curves and combination index (CI) graphs were generated and analyzed using Compusyn software (Figure 5). The CI values were <1 in 12/14 dose combinations in both OS cell lines tested demonstrating that LLL12 exhibits synergistic anti-proliferative effects with doxorubicin in these lines (Table 2).
Chapter 4: Discussion

Despite advances in our understanding of the underlying molecular biology of OSA, treatment for the disease has not changed significantly over the last 20 years in dogs or people [16]. Surgical resection and aggressive chemotherapy protocols are effective, but have failed to improve the 5-year overall survival rate past 60-70% in humans [17]. Similarly, in dogs, limb amputation followed by adjuvant chemotherapy with doxorubicin, carboplatin or both results in a 1-year survival rate of less than 50% and a 2-year survival rate of approximately 10-20%[18]. Treatment with doxorubicin and platinum based compounds, the current standards of care in the field, also comes with the potential for significant toxicities including myelosuppression, gastrointestinal toxicity, cardiotoxicity and in humans ototoxicity and secondary malignancies also occur[19]. This is particularly relevant for pediatric patients in whom late toxicities can substantially affect quality of life. Clearly, new drugs and new therapeutic targets are needed to both improve the outcome of patients suffering from OS and to reduce the long-term toxicities associated with the current standard of treatment.

Our laboratory previously characterized constitutive STAT3 activation in primary canine OS tumor samples and canine OS cell lines, and showed that direct downregulation of STAT3 protein expression in OS lines using siRNA induced loss of cell viability and apoptosis [4,19]. We similarly demonstrated that two earlier STAT3 small molecule inhibitors (LLL3 and FLLL32, both developed at OSU) also impacted OS
cell viability and induced cell death in all cell lines evaluated [4,19]. In concordance with our work, STAT3 dysregulation has been demonstrated in OS in humans, where high levels of STAT3 correlated with metastasis and lower rates of overall survival [13,32]. Together, these data define STAT3 as an important target for therapeutic intervention in OS, particularly given the fact that STAT3 function is dispensable in many normal cells.

The mechanism(s) of persistent STAT3 phosphorylation remain to be elucidated in OSA. Activating mutations in STAT3 are relatively rare in human cancers and have not been identified in canine cancers, and thus, do not appear to be responsible for the constitutive STAT3 activation found in most tumors [22]. However, there are a multitude of ligands (e.g. IL-6, OSM, EGF, HGF, IGF) and kinases (RTKs, JAKs, SRC family members) that initiate STAT3 activation and thus there are many potential upstream drivers that could contribute to the observed dysregulation [1]. In our prior studies we identified OSM as a potential driver of STAT3 phosphorylation in canine OSA tumor cells and found that inhibition of STAT3 signaling disrupted OSM induced biologic activities [23]. It is also possible that a loss of STAT3 regulatory mechanisms may play a role in sustained STAT3 pathway signaling.

STAT3 appears to be an important mediator of chemoresistance in a number of cancer types, including osteosarcoma [24, 25]. The mechanisms through which this may occur are not well understood, although available data suggest that upregulation of the drug-resistance and anti-apoptotic STAT3-regulated genes survivin, MCL-1 and MDR1
may play a part. Indeed, research has shown that the expression of P-gp, the product of the MDR1 gene, can be modified by STAT3 [26], providing a possible mechanism for STAT3-mediated resistance to certain chemotherapeutics such as doxorubicin. 

Experimental evidence generated by our laboratory and by others has clearly demonstrated that disruption of STAT3 signaling inhibits the survival and proliferation of OSA cell lines and decreases the growth of OSA in mouse models of disease [20, 27-29]. However, the challenge has been to develop a STAT3 inhibitor that has good potential for future clinical application. LLL12 is an optimized analog of LLL3, a novel small molecule allosteric STAT3 inhibitor that has been shown to inhibit proliferation and induce apoptosis in various cancer cell lines in vitro and in several mouse xenograft models, including OSA [6, 30, 31]. LLL12 works by binding to STAT3 monomers at the phosphorylation site on Y705 and thereby preventing STAT3 dimerization and translocation into the nucleus. Similarly, anti-STAT3 therapies, such as dominant negative STAT3 molecules, RNA interference and antisense oligonucleotides have been shown to be effective against a number of tumor types in vitro, but have yet to be tested in clinical trials, due in part to drug delivery issues including cell permeability, stability and solubility of the DNA, RNA and small molecules. With respect to the small molecule inhibitors previously tested in canine OS, FLLL32 suffered from lower activity than LLL12 and solubility issues that precluded its further use in vivo. LLL3, while potent, did not bind directly to the pY705 binding site of the STAT3 monomer, unlike its
optimized analog LLL12, which has a 10-fold increase in simulated binding energies to STAT3 [31].

Our current work shows that LLL12 inhibits cell viability while inducing apoptosis in canine OS cell lines expressing elevated levels of pSTAT3. LLL12 is quite potent, with IC50s for the 4 canine OS lines between 0.23 μM and 0.41 μM. Importantly, the IC50 generated for normal canine osteoblasts was 1.78 μM, which demonstrates minimal toxicity in cells that lack constitutive activation of STAT3. With respect to the concentrations of drug used in these studies, preliminary pharmacokinetic data generated in mice indicate that exposures above 1 μM occur following intravenous and intraperitoneal administration of LLL12 (J. Lin, data not shown). Doxorubicin administration to dogs results in peak plasma levels of drug ranging from 1.3-1.5 μM, with drug concentrations above 0.2-0.4 μM lasting for 10–12 hours following a single IV bolus of drug given over 20 minutes [42].

For the agents in combination, the IC50 of LLL12 is reduced from 0.23-0.4 μM to 0.08-0.11 μM which are concentrations that are achievable in vivo; the IC50 of doxorubicin is reduced from 0.42-0.43 μM to 0.11-0.16 μM, which are also concentrations achievable in vivo. Therefore, we believe the drug concentrations used in this body of work are reflective of exposures obtainable in vivo. STAT3 transcriptional targets were all downregulated after only 12 hours of 0.5 μM LLL12 treatment, showing clear, rapid effects at biologically relevant concentrations. Protein expression of pSTAT3
and survivin were similarly downregulated under identical conditions. While the timing of survivin downregulation lagged somewhat behind the loss of pSTAT3, this was expected as STAT3 is a transcriptional activator of survivin and existing transcript and protein would need to turn over first before a loss of surviving protein would be observed. Additionally, normal canine osteoblasts which have little to no pSTAT3 exhibit no loss of survivin protein at 12 hours of treatment, supporting the notion that STAT3 and not other transcription factors are linked to the loss of survivin. Together, these results show that LLL12 is more potent at inhibiting cell proliferation and decreasing pSTAT3 protein expression than both LLL3 and FLLL32.

The synergy experiments in combination with doxorubicin show promise, with obvious clinical implications. LLL12 has strong activity against cells with constitutive pSTAT3 expression, but little effect on normal cells. The significant dose reduction index seen when LLL12 is used with doxorubicin could permit its use in the setting of lower doxorubicin doses, thereby potentially limiting some of the acute and long-term toxicities associated with dose intense doxorubicin. This has particular relevance for dogs where cardiotoxicity limits the cumulative dose of doxorubicin to 180 mg/m² (typically 6 doses) and the pediatric population where cognitive deficiencies, secondary neoplasia, and/or cardiac disease occur as long-term consequences following dose-intense treatment with doxorubicin.
4.1 Conclusions

LLL12, a novel allosteric STAT3 inhibitor, inhibited proliferation and promoted apoptosis in canine OSA cell lines. LLL12 decreased pSTAT3 and survivin expression and downregulated the STAT3-mediated gene transcription of survivin, cyclin D1, BCL-2 and MCL-1 within 12 hours of drug exposure in the nanomolar range. These data support the clinical development of LLL12 for the treatment of OSA and other cancers in which STAT3 is known to be constitutively activated.
Figure 1: Effects of LLL12 on the proliferation of canine OS cell lines and normal osteoblasts.

Canine OS cell lines (Abrams, OSA 8, OSA 16 and D17) and normal canine osteoblasts were treated with vehicle or LLL12 for 24 hours. Proliferation was analyzed using the CyQUANT® cell proliferation assay kit. Proliferation values are listed as a percentage of DMSO control. Experiments were performed in triplicate and repeated three times. For each cell line, there was a significant decreasing trend in cell proliferation with dose of LLL12 (p < 0.001).
**Figure 2**: Detection of apoptosis after 24 hr LLL12 treatment

Canine OS cell lines treated with vehicle or LLL12 for 24 h. Apoptosis was assessed by measuring activated caspases 3 and 7 (A) using the Sensolyte® Homogeneous AMC Caspase-3/7 Assay kit. Experiments were performed in quadruplicate and repeated three times. The same canine OS cell lines were treated under identical conditions as above and stained with Annexin V-FITC/PI and analyzed by flow cytometry (B). Normal canine osteoblasts were treated under identical conditions and stained as above (C). There was a significant increasing trend in caspase activity for all lines except OSA 8 (p<0.01).
Figure 2

A

B

Continued
Figure 2 cont.
Figure 3: Analysis pSTAT3, STAT3 and survivin in canine OS cell lines and normal osteoblasts following LLL12 treatment

Canine OS cell lines were treated with DMSO or LLL12 for 4, 8 or 12 hours prior to collection. Normal canine osteoblasts were treated with DMSO or LLL12 for 12 hours prior to collection. Protein lysates were generated and separated by SDS-PAGE and Western blotting for pSTAT3, STAT3, survivin and β-actin were performed. Experiments were repeated two times.
Figure 3

A

Abrams

<table>
<thead>
<tr>
<th>LLL12 treatment (μM)</th>
<th>4 hr</th>
<th>8 hr</th>
<th>12 hr</th>
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<tbody>
<tr>
<td>DMSO 0.1</td>
<td></td>
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</tr>
<tr>
<td>DMSO 0.5</td>
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</tr>
<tr>
<td>DMSO 0.1</td>
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</tr>
<tr>
<td>DMSO 0.5</td>
<td></td>
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</tr>
</tbody>
</table>

**pSTAT3**

**Total STAT3**

**Survivin**

**β-Actin**

**OSA 8**

**pSTAT3**

**Total STAT3**

**Survivin**

**β-Actin**

**OSA 16**

**pSTAT3**

**Total STAT3**

**Survivin**

**β-Actin**

**D17**

**pSTAT3**

**Total STAT3**

**Survivin**

**β-Actin**

Continued
Figure 3 cont.

**B**

**Canine Osteoblasts**

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>0.1</th>
<th>0.5</th>
<th>LLL12 treatment (μM)</th>
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</thead>
<tbody>
<tr>
<td>pSTAT3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>STAT3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Survivin</td>
<td></td>
<td></td>
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<tr>
<td>β-Actin</td>
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</tbody>
</table>
Figure 4: Evaluation of STAT3-related gene expression using qRT-PCR after LLL12 treatment

RNA was collected and qRT-PCR was performed for survivin, cyclin D1, BCL-2 and MCL-1 and 18s. Data was analyzed via the Delta C(T) method [9], and graphed as percent fold change. Experiments were performed in triplicate and repeated three times. For each cell line, all four STAT3-mediated gene products were down regulated after LLL12 treatment when compared to untreated controls. Delta C(T)’s from qRT-PCR were compared using two sample t-tests and Holm’s method was used to control type-I error across tests of multiple genes (**p<0.02, *p<0.001).
Figure 4

![Graphs showing expression levels of Survivin, Cyclin D1, MCL-1, and BCL-2 in different groups: Abrams, OSA 8, OSA 16, and D17. The graphs indicate significant differences marked by asterisks.](image-url)
**Figure 5:** LLL12 synergizes with doxorubicin in canine OS cell lines

Proliferation curves (CyQUANT) and combination index graphs of canine (Abrams and OSA 16) OS cell lines after 24 hours of treatment with LLL12, doxorubicin, or both are shown. For each cell line and treatment (LLL12 alone, DOXO alone, LLL12 + DOXO) there was a significant decreasing trend in cell proliferation with dose (*p < 0.001).
<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer Sequences</th>
<th>Tm˚</th>
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<tbody>
<tr>
<td>Canine Survivin F</td>
<td>5’- GAA GGC TGG GAG CCA GAT GAT G -3'</td>
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<td>Canine Survivin R</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>Canine 18s R</td>
<td>5’- AAT ATA CGC TAT TGG AGC TGG A -3'</td>
<td>58.9</td>
</tr>
</tbody>
</table>

**Table 1:** Canine RT-PCR Primer Sets
References


13. Terpstra TJ: The asymptotic normality and consistency of Kendall's test against trend, when ties are present in one ranking. Indagationes Mathematicae 1952, 14:327-333.


