Evaluation of Two Novel Therapeutics Against Human and Canine Osteosarcoma

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Research article

Keywords: Osteosarcoma, canine, human, MLN9708, SH4-54, proteasome, STAT3

Posted Date: August 24th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-51104/v1>

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Abstract

Background: Osteosarcoma (OS) is the most common primary bone tumor in both humans and canines. This tumor has an aggressive course leading to the development of metastatic lesions in most patients diagnosed with this disease. Two new novel agents, MLN9708 and SH4-54, work as a proteasome inhibitor and a STAT3 inhibitor, respectively. Targets of these drugs have been shown to be overexpressed in OS in both species.

Methods: Two human and two canine OS cell lines were exposed *in vitro* to both drugs alone and in combination. The number of cells undergoing apoptosis, as well as the number of cells capable of invasion through a matrigel basement membrane was evaluated after exposure to the drugs. Additionally, PCR and Western blots of downstream targets were evaluated. Finally, both drugs were tested against each cell line in an *in vivo* murine xenograft model.

Results: All four cell lines were sensitive to MLN9708, one of the human cell lines and both canine cell lines were resistant to SH4-54. MLN9708 was also better at inhibiting invasion in three of the four cell lines. In the murine xenografts MLN9708 inhibited growth and metastasis in 143B (human OS) and the combination inhibited growth in the canine OS cell line (MCKOS).

Conclusions: Though SH4-54 demonstrated robust cell killing in 143B *in vitro*, MLN9708 demonstrated broader activity across species for the treatment of OS. Further investigation into this drug is warranted as a treatment for OS. Combination of this drug with a STAT3 inhibitor may be worthwhile in canine OS.

Background:

Osteosarcoma (OS) is the most common malignant primary bone tumor of children and pet dogs[1]. Approximately 800 new cases of OS are diagnosed in people each year and at least half of those are in children or adolescents. The incidence is approximately 8–10 times higher in pet dogs than in children[1]. Genetically, these two diseases are nearly identical in humans and dogs[2–4]. They share dysregulation of many of the same pathways that lead to metastasis in over 80% of affected individuals treated with surgery alone[2–4]. The biggest risk to patients with OS is the threat of metastatic disease that often develops after the primary tumor has been removed[5]. Therefore, new therapies aimed at the prevention or delay of metastases are needed just as desperately as therapies directed at the primary tumor itself[5]. Additionally, the higher incidence and rapid disease progression seen in pet dogs allows for faster and more cost effective data collection making them an excellent model for studying this disease to the mutual benefit of both species[5].

SH-4-54 is a potent pan STAT small molecule inhibitor, that has strong activity against STAT3 and is a moderate inhibitor of STAT5[6]. STAT3 has been shown to be dysregulated and constitutively activated in both canine and human osteosarcoma[4, 7–11]. In human OS in particular, STAT3 activity has been associated with enhanced invasion and metastasis [12, 13]. STAT3 is an important mediator of cell growth and plays a role in tumor growth as well as treatment resistance[14–16]. Due to its broad activity,

STAT3 has a regulatory role in many important cellular pathways including survival, proliferation, angiogenesis, and metastasis[15, 16]. SH-4-54 blocks the SH2 binding domain of STAT3 by preventing phosphorylation and disrupting transcriptionally active dimerization through mimicry of its naïve phosphopeptide binding [6]. Without the ability to dimerize, STAT3 is prevented from initiating cancer cell proliferation, migration, invasion and motility[15]. This compound has been shown to knockdown both total STAT3 and phosphorylated STAT3 levels in brain tumor stem cells[15]. Additionally, normal human fetal astrocytes were exposed to SH-4-54 at concentrations of up to 5 μ M with little to no toxicity[15].

MLN9708 (ixazomib) is a next generation proteasome inhibitor currently under investigation against resistant multiple myeloma (MM) in humans[17]. The myeloid cell leukemia-1 (Mcl-1) gene encodes a protein of the BCL-2 family with strong anti-apoptotic activity when the protein is cleaved through the proteasome. Mcl-1 has been shown to play a key role in tumor cell survival and drug resistance in MM, breast cancer, neuroblastoma, melanoma, lymphoid malignancies and numerous others[18–23]. However, when Mcl-1 undergoes caspase-induced cleavage it activates a pro-apoptotic pathway from the resulting cleavage fragments, 28 kDa Mcl-1 (128–350) and 17 kDa Mcl-1 (1-127) fragments[17]. These Mcl-1 (128–350) fragments trigger Bax and Bak dependent mitochondrial mediated apoptosis in the cell[17]. When the proteasome is inhibited by MLN9708 it shunts Mcl-1 towards the caspase cleavage pathway favoring apoptosis over cell survival[17]. Inhibition of MCL-1 proteosomal cleavage has been shown to enhance apoptosis in human OS[24, 25]. MLN9708 induces the generation of Mcl-1 (128–350) in MM cells with nuclear accumulation of drug induced Mcl-1 (128–350) suggesting a potential regulatory role in cell cycle control[17]. MLN9708 is essentially a prodrug that hydrolyzes to the biologically active compound MLN2238 in the body[26]. MLN2238 preferentially and reversibly inhibits the β 5 chymotryptic-like subunit of the proteasome[17]. A partial remission rate of 94% was seen in drug resistant MM patients in a phase I/II clinical trial after four cycles and a complete remission rate of 32% after eight cycles[27]. Common toxicities included neutropenia, thrombocytopenia, skin rash, fatigue, vomiting, diarrhea, and peripheral neuropathy[17, 26, 27].

The present study investigates the therapeutic potential of SH-4-54 and MLN9708 alone and in combination against human and canine OS both *in vitro* and *in vivo*.

Results

Time Lapse Videography

Apoptosis curves generated for each cell line showed that both human OS cell lines were sensitive to SH4-54 and MLN9708 in combination. In the HOS cell line 50% of the cells were undergoing apoptosis by 12.2 hours for the combination however it took 15.4 hours of exposure for 50% of the cells to die with MLN9708 and SH4-54 had very little effect on the HOS cell line. One hundred percent cell killing was seen with the combination at 24.5 hours and with MLN9708 at 26 hours. The human OS cell line 143B was more sensitive than HOS. Fifty percent of the cells were undergoing apoptosis with the combination and SH4-54 alone by 5.5 hours. One hundred percent of the cells were undergoing apoptosis in the SH4-54

group within 16.7 hours. The highest level of apoptosis measured in the combination group was 95% and was seen at 16 hours. MLN9708 induced apoptosis in 50% of the 143B cells by 13.6 hours and 95% apoptosis by 25.5 hours (Fig. 1).

Apoptosis curves generated for the canine OS samples demonstrated resistance to SH-4-54 in both cell lines. Though both canine OS cell lines were sensitive to MLN9708 and the combination. The combination was the most effective for both cell lines. The combination induced 50% cell killing in Abrams by 14.1 hours and in MCKOS by 12 hours. SH-4-54 alone did not induce 50% cell killing in either cell line. MLN9708 alone induced 50% cell killing for Abrams at 21.3 hours and for MCKOS at 26 hours. The combination induced its highest level of cell killing (90% for MCKOS and 86% for Abrams) by 25 hours (Fig. 2).

Quantitative PCR in vitro

MLN9708 induced significant increases in *BAK* expression levels in all 4 cell lines evaluated. HOS and 143B had 2.5 (p = 0.0015, SD 0.1) and 3.47 (p = 0.0003, SD 0.06) fold increases in *BAK* expression respectively. MCKOS and Abrams had 1.2 (p = 0.03, SD 0.04) and 2.48 (p < 0.0001, SD 0.01) fold increases in *BAK* expression. MLN9708 also significantly induced the pro-apoptotic factor *BAX* in both human OS cell lines (HOS 1.66-fold increase, p = 0.004, SD 0.07 and 143B 2-fold increase, p = 0.01, SD 0.3). However, it significantly decreased *BAX* expression in both of the canine OS cell lines (MCKOS 3.14-fold decrease, p < 0.0001, SD 0.01 and Abrams 1.81-fold decrease, p < 0.0001, SD 0.02) (Table 2).

Table 1
MLN9708 Downstream Target Alterations

Cell Line	Bak (Fold change/p/SD)	Bax (Fold change/p/SD)
MCKOS	1.2/0.03/0.04	-3.14/<0.0001/0.01
Abrams	2.48/<0.0001/0.1	-1.81/<0.0001/0.02
HOS	2.5/0.0015/0.1	1.66/0.0039/0.07
143B	3.47/0.0003/0.06	2.00/0.01/0.3

SH-4-54 induced no significant changes in expression of *BCL2* or *Cyclin D1* in the HOS cell line, which was expected given the lack of response in the apoptosis assay. Mild but significant changes were seen in both genes in the 143B cell line (*BCL2* 1.4-fold increase, p = 0.006, SD 0.06 and *Cyclin D1* 2.64-fold increase, p = 0.002, SD 0.12). Unexpectedly, *BCL2* expression was induced in both canine OS cell lines after exposure to SH-4-54 (MCKOS 81.6 fold increase, p = 0.07, SD 3.3 and Abrams 21 fold increase, p < 0.0001, SD 0.02), though this was not significant in the MCKOS cell line due to wide variability between samples. *Cyclin D1* was significantly decreased in the MCKOS cell line (17.3-fold decrease, p = 0.0004, SD 0.01) but was virtually unaffected in the Abrams cell line (1.03-fold increase, p = 0.07, SD 0.02) (Table 3).

Table 2
SH4-54 Downstream Target Alterations

Cell Line	Bcl-2 (Fold change/p/SD)	Cyclin D1 (Fold change/p/SD)
MCKOS	81.6/0.07/3.3	-17.3/0.0004/0.01
Abrams	21/<0.0001/0.02	1.03/0.07/0.02
HOS	1.03/0.94/0.01	-1.12/0.88/0.005
143B	1.4/0.006/0.06	2.64/0.002/0.12

The combination of the two drugs significantly increased *Bak* in all cell lines except MCKOS (6.75-fold decrease, $p = 0.01$, SD 0.17). However, the combination decreased *Bax* expression in all cell lines except for 143B where it was not significantly altered (1.37-fold increase, $p = 0.14$, SD 0.02). Both canine cell lines had a significant increase in BCL2 after exposure to the combination of SH4-54 and MLN9708 (MCKOS 181-fold increase, $p < 0.0001$, SD0.08 and Abrams 15.5-fold increase, $p = 0.001$, SD 0.06). However, HOS expression of BCL2 was not significantly affected by the combination (1.62-fold decrease, $p = 0.34$, SD 0.02). 143B had a mild decrease in expression after being treated with the combination (1.37-fold decrease, $p = 0.001$, SD 0.02). All cell lines had a significant decrease in *cyclin D1* expression after exposure to the combination. The largest decreases were in the two less aggressive cell lines (HOS 3.1-fold decrease, $p = 0.0001$, SD0.01 and MCKOS 3.9-fold decrease, $p = 0.04$, SD 0.09). Abrams and 143B had mild decreases in expression of *cyclin D1* (1.47-fold decrease, $p < 0.0001$, SD0.06 and 1.34-fold decrease, $p = 0.003$, SD 0.06, respectfully) (Table 4).

Table 3
Combination Therapy Downstream Target Gene Expression

Cell Line	Bak (Fold change/p/SD)	Bax (Fold change/p/SD)	Bcl-2 (Fold change/p/SD)	Cyclin D1 (Fold change/p/SD)
MCKOS	-6.75/0.01/0.17	-8.7/0.02/0.08	181.2/<0.001/0.08	-3.9/0.04/0.09
Abrams	1.87/0.02/0.12	-2.03/<0.0001/0.05	15.5/0.001/0.06	-1.47/<0.0001/0.06
HOS	1.65/<0.0001/0.02	-2.63/<0.0001/0.01	-1.62/0.34/0.02	-3.1/0.0001/0.01
143B	2.14/0.0003/0.03	1.37/0.14/0.02	-1.37/0.001/0.02	-1.34/0.003/0.06

Invasion Assays

The human OS cell line HOS was the least inhibited in the invasion assay of all the cell lines tested. There was a significant decrease in invasion after exposure to SH4-54 (mean invasion 36.85%, SD 6.2%, $p = 0.03$), however, MLN9708 alone and the combination did not result in a significant decrease in invasion. For 143B MLN9708 had a significant effect on the cells' ability to invade (MLN9708 alone mean invasion 8.9%, SD 3.4%, $p < 0.0001$) and this effect carried over into the combination treatment group (mean invasion 16.5%, SD 2.5%, $p = 0.0004$). SH4-54 had no effect on the ability of 143B cells to invade (mean invasion 81.31%, SD 12.3%, $p = 0.56$) (Fig. 3 panels A and B).

Abrams responded in a similar fashion to 143B with a significant decrease in invasion after incubation with MLN9708 (mean invasion 41.03%, SD 2.5%, $p = 0.0007$) that carried over into the combination group (mean invasion 22.16%, SD 2.5%, $p < 0.0001$). Abrams ability to invade through the Matrigel membrane was not significantly affected by SH4-54 (mean invasion 76.45%, SD 4.7%, $p = 0.88$). MCKOS was the most sensitive of the 4 cell lines tested. MLN9708 had the largest effect on invasion (mean invasion 28.38%, SD 5.7%, $p = 0.0002$) and this also carried over to the combination group (mean invasion 17.02%, SD 6.4%, $p = 0.0001$). SH4-54 caused moderate inhibition of invasion (mean invasion 51.52%, SD 21.88%, $p = 0.0016$) (Fig. 3 panels C and D).

Western Blots

Western blots were performed on cell pellets from both human OS cell lines using anti-STAT3, anti-pSTAT3, and anti-MCL-1 antibodies. Interestingly, the highest STAT3 levels were seen in both the control and the SH4-54 only groups with the lowest levels of STAT3 seen in the combination groups (Fig. 4). In the HOS cell line, pSTAT3 was lowest in the combination group and actually increased in the SH4-54 and MLN9708 individual treatment groups. For the 143B cell line pSTAT3 was decreased in all the treatment groups but lowest in the combination group. As expected, we were able to detect the MCL1 subunit in all the cell lines treated with MLN9708 either alone or in combination (Fig. 4).

For the canine cell lines, we found similar results. STAT3 was the lowest in the MLN9708 treated groups of both cell lines, though SH4-54 did seem to decrease STAT3 expression in Abrams to some degree. pSTAT was unaffected by treatment in the MCKOS cell line and even increased above baseline in the combination. pSTAT was decreased in all of the Abrams treatment groups with the lowest level being detected in the combination group. We were able to detect low levels of the smaller MCL-1 subunit in the MLN9708 treatment groups of both cell lines and the amount of the larger subunit was increased in all MLN9708 treated cells as proteasome degradation was blocked (Fig. 5).

Murine Xenografts

The two most sensitive cell lines based on *in vitro* data (apoptosis curves, invasion assays and western blots results) were selected for murine studies (one from each species), 143B (human) and MCKOS (canine). Athymic nude female mice purchased from Charles River were used to evaluate how effectively SH4-54 and MLN9708 might work alone and in combination for the treatment of human and canine OS *in vivo*. There were 4 mice in each group (vehicle control, SH4-54 alone, MLN9708 alone and the combination). Mice received bilateral flank injections of 1 million tumor cells in matrigel and were monitored over 21 days while receiving treatment or vehicle control. In the 143B group, all 4 mice in the control (vehicle only) group developed tumors bilaterally (mean tumor volume 2129 mm³, SD 1357 mm³). This was also true in the combination group where the tumors were not significantly smaller than the control group (mean tumor volume 925 mm³, SD 694 mm³, $p = 0.15$). The MLN9708 only group developed the fewest tumors (6 of 8 possible tumors) and they were the smallest tumors of the group (mean tumor volume 325.5 mm³, SD 331 mm³, $p < 0.0001$). The SH4-54 only group developed 7 tumors (of a possible 8) and they were slightly smaller than the combination group (mean tumor volume 847.9

mm³, SD 985.3 mm³, $p = 0.009$). Regarding lung metastasis, all groups of mice developed detectable lung metastasis during the study period. Lung metastases were quantified using whole lung DNA to determine the amount of human DNA present. In the control group there was a mean of 9.35 ng (SD 3.11 ng) of human DNA in the lungs. For the SH4-54 group there was a mean of 7.56 ng human DNA (SD 1.47 ng, $p = 0.78$) and this was not statistically different from the control group. In the combination group there was a mean of 8.61 ng of human DNA (SD 1.89 ng, $p > 0.99$) and this was also not significantly different from the control group. However, in the MLN9708 there was a significant lower amount of human DNA found in the lungs of this group (mean 5.63 ng, SD 1.84 ng, $p = 0.004$) (Fig. 6).

For the canine cell line, MCKOS had more predictable results with the combination therapy group having the smallest and fewest tumors. All 4 mice developed 2 tumors each in the control and MLN9708 groups. The control tumors were the largest with a mean of 566 mm³ (SD 344.7 mm³) and the MLN9708 treated mice did not have significantly smaller tumors than the control group with a mean of 349.9 mm³ ($p > 0.99$, SD 156.3 mm³). The SH4-54 treated group form a total of 6 tumors out of the possible 8 injected sites. The mean tumor size was 281.1 mm³ ($p = 0.33$, SD 209.4 mm³) and this was not statistically smaller than the control group. The best response was seen in the combination therapy group where only 3 tumors grew on 3 mice ($p = 0.006$) and they were small with a mean tumor volume of 36.5 mm³ (SD 56.55mm³, $p = 0.0005$), which was significantly smaller than the other groups. No mice in this cohort developed detectable lung metastasis.

Discussion

The data presented here describe the efficacy of two novel agents, MLN9708 and SH4-54, against human and canine OS cell lines *in vitro* and *in vivo*. Overall, the human cell lines were more sensitive to the two drugs in the apoptosis assays than the canine cell lines. Both Abrams and 143B are considered highly aggressive and metastatic. The HOS and MCKOS cell lines are the less aggressive cell lines of this cohort. This demonstrates more similarities between the species from which the cell lines were derived rather than similarities between tumor behavior for this particular drug combination. Even though 143B was the most sensitive to SH4-54 *in vitro*, MLN9708 had more broad activity across the species and may be a more useful therapy for OS in humans and canines.

MLN9708 was able to inhibit invasion in 3 of the 4 cell lines studied. Interestingly, in the invasion assays HOS was most sensitive to SH4-54 while the other drugs and the combination did not significantly affect the ability of this cell line to invade through the matrigel basement membrane. Overall, however, this particular cell line was resistant to the drugs and their combinations in both the apoptosis and invasion assays. Given the fact that the majority of patients with OS, both canine and human, succumb to metastatic lesions rather than the primary tumor, it may be more important to focus on treatments that inhibit metastasis rather than those that kill the primary tumor again supporting MLN9708 over SH4-54 for the treatment of OS in humans and canines.

When exposed to MLN9708 all tumor cell lines had an expected increase in the expression of the pro-apoptotic gene *Bak*. The two human cell lines likewise had modest increased expression of *Bax*. Unexpectedly, the two canine cell lines had decreased expression of the pro-apoptotic gene *Bax*. It is likely that yet undefined targets of MLN9708 may be leading to enhanced proteasome degradation of an upstream activator of *Bax* gene expression leading to decreased expression in the face of MLN9708 in canine OS. SH4-54 also had an unexpected up regulation of *Bcl-2* in both canine cell lines. However, both canine cell lines were resistant to SH4-54 and enhanced expression of *Bcl-2* may explain that inherent resistance. For the human cell lines, 143B demonstrated the most significant response in the apoptosis assay to SH4-54, though gene expression of both *Bcl2* and *Cyclin D1* were increased rather than decreased. Explanations for this may include evidence of an emerging resistant subclone within the cell line as so many of the cells in 143B die so quickly after being exposed to SH4-54 *in vitro* or an alternate mechanism for this drug's activity in this cell line.

The combination of the two drugs showed a different pattern of gene expression than either drug alone. Interestingly, the down regulation of *Bax* that was missing from the MLN9708 treated canine cell lines was evident in the combination treated cells. MCKOS had decreased expression of *Bak*, however the other cell lines continued to up regulate it, though only modestly. The two canine cell lines continued to demonstrate enhanced *Bcl2* expression at an even higher level in the combination treated cells and all cell lines maintained the modest amount of *Cyclin D1* down regulation seen in the SH4-54 treated group. The inherent resistance of 3 of the 4 cell lines to SH4-54 may be explained by this upregulation.

The western blot results for both human and canine cell lines were more in line with what was predicted with the exception that all 4 cell lines demonstrated lower protein levels of STAT3 in the MLN and combination treated groups rather than in the SH4-54 treated groups. However, the amount of pSTAT3 in the SH4-54 treated 143B was predictably lower than the control which may explain, at least partly, why this cell line was so sensitive to this drug. As predicted, we saw very few alterations in the protein expression of the SH4-54 treated HOS cells, though there was a significant decrease in pSTAT3 in the combination treated group which may explain why HOS was more sensitive to the combination than to either drug alone. The canine cell lines both had expected decreases in the protein levels of pSTAT3 in the SH4-54 treated groups. The MLN9708 treated human OS cells all expressed the smaller MCL1 subunit as expected in both the MLN9708 and combination treated cells. The canine cell lines had much fainter bands for the Mcl1 smaller subunit, suggesting that caspase degradation of Mcl-1 may occur less commonly in the dog than the human when the proteasome is inhibited.

The two most sensitive cell lines from each species were chosen for the murine xenografts. Surprisingly, the MLN9708 group had the best responders in the 143B xenografts. Due to the sensitivity of this cell line to SH4-54 *in vitro*, we expected a more robust inhibition of tumor growth in this group. The tumor microenvironment or altered drug metabolism of SH4-54 in the mice may have played a part in these results. The MCKOS cell line did not metastasize during the study period, but as predicted by the *in vitro* data, the combination of the two drugs produced the best results. Neither drug alone performed well against this cell line *in vivo*.

Conclusion:

These two drugs demonstrated moderate efficacy against many of the cell lines tested *in vitro* but those responses were less obvious in the *in vivo* model with the exception of the combination in the canine cell lines. Future studies in human and canine OS should focus more on MLN9708 and its analogs rather than SH4-54. For canine OS, a combination of MLN9708 with a different STAT3 inhibitor may be worth further investigation.

Methods:

Osteosarcoma cell culture

Two human cell lines, HOS and 143B as well as 2 canine OS cell lines, MCKOS and Abrams were cultured in monolayers at 37° in 5% CO2 incubator in RPMI 1640 media supplemented with 10% fetal bovine serum (R10). HOS was kindly provided by Dr. Michael Bittner with Center for Bioinformatics and Genomic Systems Engineering, Texas A&M Engineering Experiment Station. 143B was purchased directly from ATCC. MCKOS was generously provided by Ms. Christina Mazcko with the Comparative Oncology Trials Consortium at the National Cancer Institute and Abrams was generously provided by Dr. David Vail at the University of Wisconsin-Madison School of Veterinary Medicine.

Cell Viability

Cells were grown in T175 culture flasks (Corning, Corning, NY). Cells were treated with 10 μ M concentrations of either SH-4-54 or MLN9708 alone or in combination. Controls from each cell line were suspended in the media alone. Cells were incubated with the drug for 24 hours and then tested for percent of cells undergoing apoptosis as described below. RNA was also collected and stored for the future evaluation of expression levels of genes of interest.

Time lapse-videography for Apoptosis Analysis

Human and canine OS cell lines were each treated with 10 μ M of SH-4-54 alone, MLN9708 alone, and both drugs in combination at the same concentrations. After cell culture and sample preparation, cells were plated in 384-well plates and treated with the different drugs. Each well was scanned every hour for 2 days using an ImageXpress Micro (Molecular Devices) robotic scanner sampling at 3 different imaging sites within each well. The images were then sent into an in-house pipeline developed in Matlab (Mathworks) using SDC morphological toolbox²⁸. After image segmentation on both a nuclear channel and a green fluorescent channel²⁸, a large number of measurements were collected for each individual cell. The following morphological features were used to determine apoptosis on each cell in dead/alive callings: Nucleus size (S_{nuk}), Nucleus mean intensity (μI_{nuk}) and CellTox™ Green mean intensity in nucleus ($\mu I_{CTG/nuk}$). Cells are declared “apoptotic” if the following conditions are met:

$(S_{nuk} < S_{0,nuk})$ OR $(\mu I_{nuk} > \mu I_{0,nuk})$ OR $(\mu I_{CTG/nuk} > \mu I_{0,CTG/nuk})$ where $S_{0,nuk}$, $\mu I_{0,nuk}$ and $\mu I_{0,CTG/nuk}$ are the corresponding thresholds derived from images. For each cell line, drug and time point, we computed the

percentage of dead cells (%apop) as:

%Apop = *Number of Cells that meet the conditions/Number of total cells*[28]. The data is then plotted on a curve using ImageJ software.

Quantitative real time PCR

Real time reverse transcriptase PCR (qRT-PCR) was used to determine fold changes in expression levels of the downstream targets of the Mcl-1 (128–350) fragment, *Bax* and *Bak*, as well as downstream targets of the STAT3 pathway, *cyclin D1* and *Bcl-2* (Table 1). RNA was isolated from human and canine OS cells using the RNeasy Mini RNA isolation kit (Quiagen, Dusseldorf, Germany) according to the manufacturer’s instructions. RNA was further purified using the Turbo DNA-free kit by Ambion (Life Technologies, Carlsbad, CA). RNA quality was confirmed using a spectrophotometer.

Table 4
A. Human PCR primers

Gene of Interest	Sequence 5’-3’	Amplicon Size	Gene ID (<i>Homo Sapiens</i>)
<i>Bax</i> Forward	CCTGTGCACCAAGGTGCCGGA	99 bp	581
<i>Bax</i> Reverse	CCACCCTGGTCTTGGATCCAGCCC		
<i>Bak</i> Forward	CCGAAGCCATTTTTCAGGT	94 bp	578
<i>Bak</i> Reverse	GACCTCCATCTCCACCCTG		
<i>Cyclin D1</i> Forward	CAGATCATCCGCAAACACGC	143 bp	595
<i>Cyclin D1</i> Reverse	AAGTTGTTGGGGCTCCTCAG		
<i>Bcl-2</i> Forward	GACTGAATCGGAGATGGAGACC	179 bp	596
<i>Bcl-2</i> Reverse	GCAGTTCAAACGTCGCCT		
<i>GAPDH</i> Forward	AATCCCATCACCATCTTCCA	82 bp	2597
<i>GAPDH</i> Reverse	TGGA		

Table 4
B. Canine Primers

Gene of Interest	Sequence 5'-3'	Amplicon Size	Gene ID (CanFam3.1 genome)
<i>Bax</i> Forward	GCTGTGGACACAGACTCTCC	165 bp	403523
<i>Bak</i> Reverse	GATGGTCCTGATCAGCTCGG		
<i>Bak</i> Forward	GGTCACCTTGCCCCTAGAAC	188 bp	481744
<i>Bak</i> Reverse	TGCCGCTCTCAAATAGGCTC		
<i>Cyclin D1</i> Forward	TTAAGGCGGAGGAGACTTGC	111 bp	449028
<i>Cyclin D1</i> Forward	CGCAGACCTCTAGCATCCAG		
<i>Bcl-2</i> Forward	CAGAGAAAAGGGGGTGGTGA	77 bp	403416
<i>Bcl-2</i> Reverse	ACCCGTGGGCTCTTTTGA		
<i>RPS19</i> Forward[29]	CCTTCCTCAAAA/GTCTGGG	95 bp	476450
<i>RPS19</i> Reverse[29]	GTTCTCATCGTAGGGAGCAAG		

Invasion Assays

Coating buffer was made from 0.7% NaCl and 0.1M tris in distilled water, filtered using a 0.2 µm sterile syringe filter and stored at 4° C. Matrigel Matrix (Corning, Tewksbury, MA, USA) was stored at 4° C. The coating solution was made for a final concentration of 250 µg/mL. Fifty-four µL of matrigel was mixed into 1946 µL of coating buffer. The experimental, positive control and negative control wells were completed in triplicate and designated + 1, +2, and 0 respectively. Three thinerts (Greiner Bio-one, Kremsmünster, Austria) per cell line were each placed in well of a 24 well plate (VWR, St. Louis, MO, USA). These were coated in 100 µL of coating solution. The thinerts were placed in the incubator at 37° C for 2 hours. A cell suspension was prepared with 2×10^6 cells in 3 mL PBS and was added to each tube. The solution was centrifuged for 3 minutes at 1300 RPM. After decanting the supernatant, 2 mL of serum free media was added to the cell pellet and mixed with a pipette. On the 24 well plate, 600 µL of serum free media was placed in wells marked 0. Six hundred microliters of media containing serum was placed in wells marked + 1 and + 2. Thinerts were placed in wells designated 0 and + 2, while the coated thinerts were placed in the + 1 wells. 200 µL of the cells in serum free media were added to the top of the thinerts. The 24 well plate was placed in the incubator for 20 hours at 37° C. A new 24 well plate was prepped with 450 µL of serum free media with 8 µM calcein-AM added to each well. The thinerts were moved to the new plate containing the calcein-AM solution. The plate was incubated for 45 minutes at 37° C. Each thinert was removed and placed in a new well of a 24 well plate. The thinert was aspirated and material on top of the thinert was discarded. On a new 24 well plate 500 µL of trypsin-edta was added to each well. The thinerts were placed into the trypsin-edta wells. This plate was incubated for 15

minutes at 37° C. Thinserts were discarded. The cells and trypsin were mixed and 200 µL were pipetted into new wells of a black 96 well plate in duplicate. The plate was read by the fluorescent plate reader (excitation 485 nm/emission 520 nm) (BioTek Synergy 2, Winooski, VT, USA). Four replicates of each condition were performed.

Invasion index percentage was calculated using the formula:

$$\text{Invasion index \%} = \frac{(\text{experimental average} - \text{negative control average})}{(\text{positive control average} - \text{negative control average})} \times 100\%.$$

Western Blot Analysis

Human and canine OS cells were treated with 10 µM SH-4-54, 10 µM MLN9708 and the combination for 24 hours. Cells were trypsonized and collected and protein was isolated using acetone precipitation of proteins according to the manufacturer's instructions (ThermoScientific, Rockford IL, USA). Briefly, Cells were collected, washed and resuspended in M-PER (300 uL). Four times the sample volume of cooled acetone (-20° C) was applied to sample in an acetone compatible tube. The sample was vortexed and incubated at -20° C for 60 min. The sample was then centrifuged for 10 min at 13,000 x g. The supernatant was discarded and the acetone was allowed to completely evaporate from the uncapped tube at room temperature for 30 min. The pellet was resuspended in 200uL of M-PER for further analysis. The concentration of protein from each sample was determined using the Pierce BCA Protein Assay Kit (ThermoScientific, Rockford IL, USA) according to the manufacturer's protocol.

Western blot analysis was performed by Raybiotech, Inc., using the automated Capillary Electrophoresis Immunoassay machine (WES™, ProteinSimple Santa Clara, CA) according to the manufacturer's protocol[30, 31]. In brief, 0.6ug of samples were mixed with a master mix (ProteinSimple) to a final concentration of 1x sample buffer, 1x fluorescent molecular weight markers, and 40 mM dithiothreitol (DTT) and then heated at 95 °C for 5 min. The samples, blocking reagent, wash buffer, primary antibodies, secondary antibodies, and chemiluminescent substrate were dispensed into designated wells in the manufacturer provided microplate. Human anti-STAT3 (Cell Signaling D3z2G) – this antibody was also used for the dog cells based on demonstrated reactivity in the literature[32], human and canine anti-pSTAT3 (phosphor S727)(Abcam ab30647), human and canine anti-Mcl-1 (s-19) (Santa cruz sc-819). After plate loading, the separation electrophoresis and immunodetection steps took place in the capillary system and were fully automated. Simple Western analysis was carried out at room temperature, and instrument default settings were used. The data was analyzed with inbuilt Compass software (Proteinsimple).

Xenograft mouse model

Thirty-two 490 athymic nude female mice (42 days) (Charles River Laboratories, Houston, TX) were maintained in specific pathogen free conditions based on guidelines established by Texas A&M University Institutional Animal Care and Use Committee. Human 143B cells and canine MCKOS cells were suspended in Matrigel at a concentration of 1×10^6 cells per mL and injected subcutaneously into both right and left flanks of each mouse. Mice were divided into 4 groups of 4 mice each: control (vehicle only), SH-4-54 treated, MLN9708 treated, and combination therapy. Therapy was initiated 24 hours after the injection of tumor cells. SH-4-54 was suspended in 100 μ L 50% polyethylene glycol 300 (PEG 300, Sigma-Aldrich) in water and dosed at 10 mg/kg. It was injected intraperitoneally [33] in groups 2 and 4, for four consecutive days with three consecutive rest days. MLN9708 was suspended in 5% 2hydroxypropyl- β cyclodextrin and dosed at 10.7 mg/kg. Mice were orally gavaged in groups 3 and 4 two times per week. Control mice were treated with drug carrier agents only, using the same volume, route of administration, and treatment schedule as the combination mice. Tumors were allowed to grow for 3 weeks before mice were euthanized. Mice were weighed and tumors were measured with calipers twice weekly throughout the entire course of treatment. The mice were humanely euthanized according to AVMA standards and the tumors as well as lungs were collected. Tumor tissues from mice were flash frozen. Whole lung DNA was isolated and evaluated for total quantity of human or canine DNA present as evidence of micro-metastatic disease. The Femto Human DNA Quantification kit (Zymo Research Corporation, Irvine, CA) was used to quantify the amount of human DNA present in the mouse lungs. For mice with canine xenografts lung tissue from mice injected with no tumor were used to generate the control and a standard curve for comparison using known quantities of canine DNA. Each sample was run in triplicate using 45 ng of template per well (SsoAdvanced™ Universal SYBR® Green Supermix #1725271 and CFX Connect™ Real-Time PCR Detection System #1855201, BioRad, California, USA). Canine specific primers that target a short interspersed element, or SINE, were used to target DNA within the mouse lung DNA samples (forward: 5'-AGGGCGCGATCCTGGAGAC-3', reverse: 5'-AGACACAGGCAGAGGGAGAA-3')[34]. A standard curve for each species was created using known amounts of human or canine DNA in normal mouse lung DNA. This curve was used to interpolate the percentage of foreign species DNA present, ranging from 45 fg to 45 ng per reaction. The cycling conditions are three minutes at 98°C, followed by 40 cycles of 15 seconds at 98°C, 30 seconds at 55°C, 20 seconds at 60°C, and 15 seconds at 72°C. The results are plotted on the standard curve and converted to a percent canine DNA in the total lung DNA. This percentage is then plotted in a graph.

Statistical Analysis

All *in vitro* experiments were performed in biologic and temporal triplicates providing a minimum of 9 data points for each condition. Results were imported into Graph Pad Prism 7 (2017) for analysis. Viability data were analyzed using a 2-way ANOVA with Tukey's multiple comparisons test. For qPCR data results the relative fold change between DMSO-control and treated cells, standard error, and statistical significance via a Pair Wise Fixed Reallocation Randomization Test© was calculated using REST© software. For the time-lapse videography, a two-way ANOVA was used to calculate significance using the recorded percentages of apoptotic cells over time. Significance was set at a p value of 0.05 and confidence intervals were set at 95%. The total DNA in the mouse lungs was calculated using a standard

curve generated using standards of known quantities of human or canine DNA and mouse lung tissue from a control mouse that received no tumor. The standard sigmoidal curve was generated to calculate the percent of canine DNA in the unknowns (murine xenograft lungs). The level of significance for positive micrometastasis was set at 0.01% canine DNA or higher [21]. Two-way ANOVAs or Mann-Whitney tests were used to compare treated groups xenograft comparisons and invasion assays. A chi-squared (Fischer's exact test) was used to compare treatment groups for the incidence of metastasis. Significance was set at a p value of 0.05 and confidence intervals were set at 95%.

List Of Abbreviations

OS- Osteosarcoma, MM- Multiple Myeloma, Mcl-1- Myeloid Cell Leukemia 1, STAT3- Signal Transducer and Activator of Transcription 3, pSTAT3- Phosphorylated Signal Transducer and Activator of Transcription 3

Declarations

Ethics approval and consent to participate: All animal experiments were approved by Texas A&M University Animal Care and Use Committee (AUP #2015-0813).

Consent for publication: Not Applicable

Availability of data and materials: All data generated or analyzed during this study are included in this published article.

Competing interests: The authors declare that they have no competing interests.

Funding: Funding for this study was provided by the Fred and Vola Chair for Comparative Oncology held by HMWR.

Authors' contributions:

HMWR: made substantial contributions to the conception and design of the work, interpretation of data and drafted and revised the manuscript.

TM: Made substantial contributions through the acquisition and analysis of the work.

CS: Made substantial contributions through the creation of new software used in the work and analysis of the data.

JH: Made substantial contributions through the creation of new software used in the work and analysis of the data.

MC: Made substantial contributions to the design of the work and the acquisition of data.

MB: Made substantial contributions to the conception and design of the work, interpretation of the data and revision of the manuscript.

Acknowledgements: The authors would like to acknowledge RayBiotech, Inc for their services in producing the western blot data and chemiluminescence curves.

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Figures

Human OS Apoptosis Curves

Figure 1

Apoptosis curves for the two human cell lines. Two human OS cell lines were evaluated against MLN9708 and SH4-54. The aggressive and highly metastatic 143B cell line was most sensitive to SH4-54 (purple line) and this sensitivity carried over into the combination samples (brown line). This cell line was less sensitive to MLN9708 (blue line) however all three treated groups reached near 100% apoptosis within 24 hours. The HOS cell line was resistant to treatment with SH4-54 (lime green line) but was sensitive to MLN9708 (dark green line) and the combination of the two drugs appeared to be additive.

Canine OS Apoptosis Curves

Figure 2

Apoptosis curves for the two canine cell lines. Two canine OS cell lines were tested against MLN9708 and SH4-54 alone and in combination. Similar to the human HOS cell line, both Abrams (purple line) and MCKOS (lime green line) were resistant to SH4-54. Both Abrams (blue line) and MCKOS (dark green line) had similar levels of sensitivity to MLN9708 alone. The two cell lines had the best response to the combination of the two drugs, even though SH4-54 showed very little activity along against either cell line (Abrams- brown line, MCKOS- pink line).

Invasion Assays

Figure 3

Invasion Assays: A. The ability of the human OS cell line, HOS, to invade through a matrigel membrane was only mildly inhibited by SH4-54 exposure. No other drug or drug combination affected HOS. B. The invasive capabilities of the human OS cell line, 143B, was significantly inhibited by MLN9708 both alone and in combination with SH4-54. C. The invasive abilities of the canine OS cell line was similar to 143B in that MLN9708 exerted the greatest inhibition both alone and in combination with SH4-54. D. The ability of MCKOS to invade through the matrigel membrane was significantly inhibited by both SH4-54 and MLN9708, though MLN9708 exerted a greater effect than did SH4-54 on this particular cell line.

Human Osteosarcoma Western Blot

Figure 4

Western Blot Analysis of Human Cell Lines: Western blots were performed on both human cell lines to evaluate the effects of the drugs on protein expression. In all cell lines STAT3 was decreased in the MLN9708 and combination treated groups. pSTAT3 was decreased in the HOS combination cohort and all treatment groups of the 143B cell line. The smaller MCL1 subunit was identified in both cell lines in the MLN9708 and combination treated cohorts.

Canine Osteosarcoma

Figure 5

Western Blot Analysis of Canine Cell Lines: Western blots were performed on both canine cell lines to evaluate the effects of the drugs on protein expression. Similar to the human cell lines, STAT3 was lowest in the MLN9708 treated groups and was virtually unaffected by SH4-54. pSTAT3 seemed to increase in the MCKOS cell line after exposure to MLN9708 and was virtually unaffected by either drug in the Abrams cell line. Very faint bands for the MCL1 small subunit can be detected in MCKOS in the combination cohort, however, an increase in the larger MCL1 subunit suggested decreased proteosomal degradation in these cell lines.

Figure 6

Murine Xenografts of human OS cell line, 143B, treated with MLN9708, SH4-54 or the combination: A. Mean tumor volumes of all mice treated with either vehicle (control), SH4-54, MLN9708 or the combination. B. Number of mice in each group that developed bilateral tumors while undergoing treatment. C. The total amount of human DNA (ng) identified in the mouse lungs at the end of the experiment.

Figure 7

Murine Xenografts of canine OS cell line, MCKOS, treated with MLN9708, SH4-54 or the combination: A. Mean tumor measurements at the end of the 21 day study period. B. Number of tumors that developed in each group over the study period. C. Images of the mice in each group at the end of the study. In the combination group one mouse was found dead in the cage 2 nights before the end of the study with no tumors on either side. No image of this mouse was captured.