In vitro Evaluation of the Effects of Combining Bisphosphonates and Radiation Therapy in Canine Osteosarcoma Cells

by
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ABSTRACT

IN VITRO EVALUATION OF THE EFFECTS OF COMBINING BISPHOSPHONATES AND RADIATION THERAPY IN CANINE OSTEOSARCOMA CELLS

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Osteosarcoma (OSA) continues to be a devastating disease in veterinary medicine. One palliative treatment option currently being employed is the combination of radiation therapy and bisphosphonates despite a limited body of literature to support its use. Investigation of this combined therapy has been performed in human and murine OSA cells in vitro, however this is the first report of their evaluation in canine OSA cells in vitro. Dose dependent effects of pamidronate, zoledronate and radiation were identified in two canine OSA cell lines: D17 and Dharma. Some combinations of bisphosphonates and radiation resulted in decreased clonogenic survival and cell viability compared to single agent therapies. Evaluation of the combined effects of these two modalities via combination index identified antagonistic relationships between pamidronate + radiation and zoledronate + radiation. Furthermore, timing of administration of bisphosphonates in relation to radiation indicated post-radiation treatment with bisphosphonates resulted in an increased response to treatment under our laboratory conditions.
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To my co-advisors Dr. Michelle Oblak and Dr. Tony Mutsaers, I would like to extend my sincerest gratitude for their continued guidance and support, even when I doubted myself. Without them I would never have made it to the end of this journey. I have learned so much from this process, as well as from working by your sides in the clinic.

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DECLARATION OF THE WORK PERFORMED

I declare that, with the exception of the works listed below, all work in this thesis was performed by me, Katie Hoddinott.

With my guidance and support, Stephanie Lovell and Sarah Laliberte performed experimental replicates of clonogenic survival and viability assays for both D17 and Dharma cell lines, while attempting to determine appropriate bisphosphonate doses and timing of administration of bisphosphonates in relation to radiation therapy.

I, Katie Hoddinott, performed all writing, graphing and table formatting in this thesis with editorial comments made by Dr. Michelle Oblak, Dr. Anthony Mutsaers, Dr. Geoff Wood and Dr. Sarah Boston.

The statistical analysis was performed by Dr. Gabrielle Monteith, of the Department of Clinical Studies, Ontario Veterinary College, University of Guelph, Guelph, Ontario.
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<td>18F-FDG-PET-CT</td>
<td>18F-fluorodeoxyglucose-positron emission tomography-computed tomography</td>
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<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>BP</td>
<td>aminobisphosphonates</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>CI</td>
<td>combination index</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>CTx</td>
<td>C-terminal telopeptide</td>
</tr>
<tr>
<td>DEXA</td>
<td>dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagles medium</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Gy</td>
<td>Grey</td>
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<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>MST</td>
<td>median survival time</td>
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<tr>
<td>NTx</td>
<td>N-terminal telopeptide</td>
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<tr>
<td>OSA</td>
<td>osteosarcoma</td>
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<tr>
<td>PAM</td>
<td>pamidronate</td>
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<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
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<tr>
<td>PS</td>
<td>penicillin streptomycin</td>
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<tr>
<td>PET</td>
<td>positron emission tomography</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>µL</td>
<td>microliter</td>
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CHAPTER I: Literature Review

1.1 Overview of osteosarcoma

Osteosarcoma (OSA) is the most common primary bone tumour in dogs (1,2), and humans (3), accounting for 85-98% (1,2) and 56% (3) of all primary bone tumours respectively. It is thought to arise from primitive transformed cells of mesenchymal origin that exhibit osteoblastic differentiation and produce malignant osteoid (1,2). In dogs, the primary tumour occurs in either the appendicular or axial skeleton, with 75% of cases occurring in the appendicular skeleton (1,4) Initially, the tumour is aggressive in the local bone microenvironment but also metastasizes early in the disease process (1–3).

This disease shares many similarities between dogs and humans, including a large/giant breed association in dogs and tall adolescents (3), a male predilection, and a predisposition for the appendicular skeleton in the metaphyseal region (4). It has also been shown that as the height of a dog increases, so does its risk for developing OSA (5). A main point of differentiation between dogs and humans is that despite a bimodal age distribution in both species, OSA more commonly affects adolescents in humans, whereas it more commonly affects the middle aged to older population in dogs (3,4). Additionally, there is a 2-fold increased risk for neutered dogs to develop OSA compared to intact dogs (5). OSA itself appears to be more prevalent in the canine population than in humans, however due its similar biologic behavior and histologic appearance it may serve as an excellent source for research into this devastating disease (4,6).
1.1.1 Clinical signs

The majority of patients presenting with appendicular OSA will have a history of acute or progressive lameness, with a palpable boney mass and varying degrees of localized pain (1,2). The distal radius and proximal humerus are the most frequent sites of metaphyseal bone that are affected, with the proximal and distal femur and tibia being diagnosed less frequently (1,2). Bone pain is typically the first sign of disease in these patients. In humans, bone pain may initially begin as a dull but on-going ache, which progressively worsens over time, as more bone is destroyed by the tumour and results in significant pain associated with movement (7,8). Other sources of bone pain and lameness may be associated with localized periosteal inflammation, microfractures and pathologic fractures of the affected bone (2,7,8). A mouse model for bone cancer pain has shown that there is peripheral and central sensitization of the nervous system that accompanies bone destruction secondary to local osteoclasts, thus proving bone pain to be more than localized pain (9).

1.1.2 Diagnosis

A tentative diagnosis may be made based on the combination of history, signalment and radiographic evidence of an aggressive bone lesion (1,2). A definitive diagnosis may also be attempted via fine needle aspirates and cytology or biopsy and histopathology. The major benefits of a cytological diagnosis compared to a histological diagnosis are a less invasive procedure, reduced cost and quicker turn-around time (10). The patient requires sedation only and the procedure can be done with minimal equipment. However, in order to provide the greatest chance of diagnosis, it is important
to use diagnostic imaging (radiographic landmarks or ultrasound) to guide the aspiration (10,11). Bone tumours will have a reactive region where the body is attempting to stabilize the abnormal portion of bone, therefore aspirates should be collected from the center of the lesion and not at its periphery (10). A recent human pathology report has shown cytology of malignant neoplasms to agree with histologic diagnoses in 77 out of 78 cases(12), while cytology specific for OSA agreed with histology 83% of the time (13). In the veterinary literature, ultrasound guided fine needle aspirates of appendicular OSA lesions have a 97% sensitivity for diagnosing a sarcoma, with a 100% sensitivity for diagnosing OSA when an alkaline phosphatase stain is applied (11). While cytology may be able to provide a reliable diagnosis, histology remains important for grading purposes and aiding in determination of the patient’s prognosis (14). A pretreatment biopsy can provide a correct diagnosis and grade prediction 7/9 times, as demonstrated by Kirpenstein et al (14).

1.1.3 Staging

Before considering treatment options, complete oncologic staging is required to determine the extent of the patient’s disease. This should include complete blood work, urinalysis, regional radiographs, thoracic screening (radiographs or computed tomography), abdominal ultrasound, local lymph node aspirates, orthopedic and neurologic examinations and nuclear scintigraphy to evaluate for other areas of metastatic bone disease (2,15).
Complete bloodwork and urinalysis allow for assessment of the overall patient health and may help in deciding treatment options. An elevated alkaline phosphatase (ALP) at the time of diagnosis has been correlated to those patients with a shorter survival time, typically by less than 50% survival time achieved by those with normal ALP at the time of diagnosis (16–18). Regional radiography allows for local assessment of bone, to rule out other orthopedic diseases that may be the cause for lameness.

Thoracic screening is important, to assess for pulmonary metastasis, as this is the most common site for metastasis of OSA, with bone and lymph nodes being less common (1,2,17,19). Although appendicular OSA is a highly metastatic tumour, only approximately 15% of patients will have gross pulmonary metastasis at the time of initial presentation (2,15). Even with this low initial gross metastatic rate, it is estimated that 90% of patients have micrometastatic disease at the time of presentation, due to development of pulmonary metastasis despite treatment of the primary tumour (2,15). Thoracic screening can be performed either by 3-view radiographs or by computed tomography (CT). However, up to 14% of patients with negative thoracic radiographs may have identifiable metastases on a thoracic CT due to the ability of CT to identify smaller lesions (20).

Abdominal ultrasonography can be considered to assess for concurrent but unrelated disease, as most patients are geriatric and additional findings may alter decision-making regarding treatment of OSA. However, two veterinary studies evaluating the utility of abdominal ultrasound for staging of OSA patients, found
metastatic lesions in 2.5% of one study population (21) while no metastatic lesions were identified in the second study (22). Although few cases of abdominal metastases were identified, the utility of abdominal ultrasound in those cases likely played an important role in therapeutic decision-making. Local lymph node aspirates should be a routine part of pretreatment staging, as 4.4-25% of patients with OSA have metastatic disease diagnosed histologically within the lymph nodes (14,23). Identifying pre-treatment lymph node metastases is important as this significantly alters prognosis (23).

Orthopedic and neurologic examinations are important to rule out concurrent orthopedic or neurologic disease that may alter the patient’s quality of life or response to treatment, but may also help to localize any regions of possible bone metastases. Nuclear scintigraphy can subsequently be considered, to assess for sites of possible bone metastases however any regions of concern should be followed up with regional radiography to further assess the site. This is because nuclear scintigraphy can identify sites of abnormal bone activity but cannot differentiate between primary and metastatic lesions or malignant and benign lesions. Bone metastases have historically been reported to be identified in ~8% of canine patients with OSA (24,25). More recently, a higher rate of bone metastases was identified when using a combination of imaging modalities, with a rate of 27% (20). A combination of survey radiographs, whole body CT scan and bone scintigraphy were evaluated. While no definitive lesions were identified on radiographs or CT, these were considered adjunctive modalities that allowed for further evaluation of equivocal lesions found on bone scintigraphy (20).
A novel diagnostic is now considered in place of nuclear scintigraphy - 18F-fluorodeoxyglucose-positron emission tomography–computed tomography (18F-FDG-PET-CT) (26). When comparing bone scintigraphy to PET-CT, PET-CT is significantly more sensitive at diagnosing distant metastatic lesions, with a sensitivity of 79% compared to a sensitivity of 32% for bone scintigraphy alone (26). 18F-FDG-PET-CT can be combined with thoracic computed tomography in a single study, for complete assessment of the two main regions of metastasis – lung and bone (26).

### 1.1.4 Prognosis

Overall disease prognosis for osteosarcoma is poor, however prognosis can vary depending on certain host and tumour factors, as well as treatment sought. Increasing age, increasing body weight, elevated ALP, and tumour location are all prognostic indicators for mortality and metastases (27,28). Patients with preoperative metastases have a median survival time (MST) of 76 days, however patients with bone metastases have increased survival times compared to those with soft tissue metastases (29). Lymph node metastasis specifically holds a worse prognosis with a MST of 59 days, compared to a MST of 318 days for patients without these metastases (23). Patients treated with amputation alone have a shorter MST (134-175 days) than those treated with amputation and chemotherapy (258-366 days), using either a single agent or double agent protocol or being treated pre or post amputation (30–33). Patients treated with palliative radiation have a MST between 125–312 days, with pain relief lasting on average from 54-129 days (34,35).
1.1.5 Treatment

Treatment options for dogs diagnosed with OSA are divided into either curative-intent or palliative protocols. Curative-intent treatment options include limb amputation, limb-sparing surgery, stereotactic radiosurgery or curative-intent radiation and adjuvant chemotherapy (1,2,36,37). The gold standard treatment for osteosarcoma is limb amputation followed by adjuvant chemotherapy (38–44). Limb sparing techniques include allografts, pasteurized tumoral autograft, stereotactic radiosurgery, metal prosthesis, bone transport osteogenesis and ulna transposition autograft (45–51,37).

The goal of palliative treatment is the relief of bone pain associated with the primary tumour (2). Palliative options are typically sought when there is concurrent orthopedic or neurologic disease rendering the patient a poor candidate for amputation, or in the face of metastatic disease. Alternatively, palliative options may be considered if the owners do not accept amputation for moral, religious or financial reasons. These options typically include amputation alone, palliative radiation therapy, analgesics and aminobisphosphonates for management of bone pain and metronomic chemotherapy (1,2).

1.2 Overview of Radiation Therapy

External beam radiation, or teletherapy, is the method of delivery of radiation that is most commonly used in veterinary medicine. It involves radiation delivered at a distance from the patient (52). The most common types of external beam radiation used
are megavoltage systems, including cobalt 60 and linear accelerators. These machines provide radiation via photons with energy greater than 1 million volts (52).

Ionizing radiation is applied directly to the tumour via an external beam, which results in either direct DNA damage of the tumour cells or indirectly causes damage secondary to the formation of free radicals which may cause enough biologic damage to kill the cell or prevent further cell reproduction (53,54). To limit damage to the surrounding normal tissues, multiple beams can be used to target the tumour more specifically and reduce the radiation dosing that affects the non-neoplastic tissues (52).

1.2.1 The four R’s

There are four main factors that determine a cell’s sensitivity to radiation. These are known as the four R’s: 1. Repair of DNA damage 2. Redistribution into the cell cycle 3. Repopulation and 4. Re-oxygenation (53–55). Once a cell has become damaged either directly or indirectly via radiation, there are two courses it can follow: death or recovery. Either a cell can repair its DNA damage and recover, or the damage is too severe and the cell dies. Other cells, which are not damaged during radiation may continue along their normal cell cycle and may become more sensitive to radiation once they are in a different part of their cell cycle (redistribution), resulting in their death. Those cells that do survive radiation continue to repopulate the tumour burden and this process tends to be accelerated by radiation. This is typically identified after 4 weeks of treatment, but the exact cause remains unclear (55). This repopulation effect may affect the outcome of radiation therapy, if the tumour continues to proliferate in spite of treatment, therefore
alteration to treatment may be required. Tumour cells that are oxygenated are more radiation sensitive than those that are hypoxic; therefore in the intervals between radiation doses these hypoxic cells may become re-oxygenated and thus become more radiation sensitive (54).

1.2.2 Side effects

Despite the goal of killing only the tumour cells, radiation therapy cannot target tumour cells specifically, therefore some normal tissues may be affected by treatment as well (53,54). Because of this lack of ability to target only tumour cells, radiation can cause both early and late side effects. Early side effects are those effects that develop during the radiation period and resolve within several weeks of completion of the radiation protocol (53,54,56). Tissues affected by early side effects are those tissues that are renewing or rapidly dividing and include the oral mucosa, intestinal epithelium and epithelial structures of the skin and eyes. Generally these side effects are self-limiting and symptomatic management is the only treatment required (52–54). Late side effects, on the other hand, do not develop until months or years after radiation therapy has terminated (52–54). These side effects develop in tissues that are non-renewing or have very slow turnover and include bone, lung, heart, kidney and nervous system (52–54). These side effects are more devastating, and can result in tissue fibrosis, necrosis, loss of function, or death (54).
### 1.2.3 Fractionation

One way to reduce the likelihood of developing late side effects is to deliver radiation therapy in fractions. Fractionation, is when a smaller fraction of radiation is delivered at one time, allowing a larger total dose of radiation to be delivered over an extended period of time (54). The amount of radiation delivered to a tissue is described in Greys (Gy), which is the amount of energy absorbed by the tissue (1Gy = 1J/kg). By dividing the total dose of radiation into fractions, there is a greater tissue response with fewer effects to the local normal tissues (54). Dosing can be delivered in several ways: hyperfractionation, hypofractionation and accelerated fractionation. Hyperfractionation occurs when the dose per fraction is reduced, allowing a larger total dose overall in a designated period of time. Hypofractionation or coarse fractionation occurs when the dose per fraction is larger, resulting in a smaller total dose overall in a designated period of time. Accelerated fractionation occurs when the total treatment time is reduced, but the dose per fraction and total dose remain unchanged (54). Fractionation can only be determined based on the 4 R’s of cell sensitivity; therefore fractionation can differ between tumour types, between patients and even within an individual patient (54). The fraction size is determined based on the local tissues and specifically the tissue that has the least ability to tolerate the radiation delivered (53). A time interval is subsequently determined based on the speed at which the local tissues are dividing (53). With each radiation dose administered a constant proportion of cells will die, however this means that it is unlikely that every tumour cell will be killed despite the total dose of radiation (53).
1.2.4 Curative & palliative protocols

Based on these different ways of fractionating radiation, curative-intent versus palliative protocols can be developed. Curative-intent protocols for soft tissues typically involve hyperfractionation, with smaller fractions provided frequently over an extended period of time, allowing a large total dose of radiation to be delivered to the site (53,54). However, curative-intent protocols for bone can range from a single high dose of radiation to a daily protocol delivered over a few weeks (47,37,57–59).

Palliative radiation, typically involves coarse fractionation with larger fractions of radiation provided infrequently, resulting in a lower total dose of radiation. These patients are receiving radiation to relieve some sort of discomfort from their local tumour, such as bone pain, or obstruction of a passageway, without the goal of complete tumour destruction or cure (52). Tumour type, extent and location all play a role in determining if a tumour can receive radiation and which type of radiation would be considered best.

Most curative intent radiation is directed at tumours that are locally invasive, but not highly metastatic, diseases with no surgical treatment option or in the setting of adjunct radiation following incomplete surgical excision of disease (53). These patients therefore receive smaller fractions of radiation at one time, to limit the late side effects that can be life threatening (53). Tumours often receiving curative intent radiation include oral tumours (acanthomatous ameloblastoma, squamous cell carcinoma, fibrosarcoma), nasal tumours, brain tumours, thyroid tumours and incompletely excised mast cell tumours and soft tissue sarcomas (60–72).
Patients that receive palliative radiation typically are patients who have a disease that is causing discomfort, a high metastatic potential or lack of surgical treatment options (53). These patients receive larger fractions of radiation at one time, because they are unlikely to live long enough to be affected by late side effects. Tumours that often receive palliative radiation protocols include malignant melanomas, histiocytic tumours, high grade mast cell tumours, subcutaneous hemangiosarcoma and osteosarcomas (73–81). Most of these tumours have such high metastatic rates that the patient is unlikely to live long enough to experience the late side effects of radiation but can benefit from the analgesic effect of radiation (53).

1.2.5 Radiation for bone pain palliation

In human medicine, external beam radiation therapy is one treatment modality that can be used for management of bone pain from metastatic bone lesions, including breast, prostate and lung cancer (82). However, multimodal pain management may still be required, even when radiation therapy is used (82). Many different local and wide field protocols exist, including hemi-body and whole body radiation to address focal or diffuse bone pain (82–87).

Approximately 60-90% of patients with single site bone metastases have shown improvement with local radiation therapy (85). Using as low a dose as 4Gy, pain relief may be achieved as early as 48 hours post-radiation (85). Many single site protocols, including high dose, low dose, single and multiple fractions have been used with success
1.2.6 Radiation and OSA in dogs

Dogs with OSA that are unable to have a limb amputation for treatment of their disease will experience significant bone pain (7,88). This bone pain may be treated with combinations of oral analgesics, however despite these oral medications often the pain is not adequately treated (2). With oral analgesics alone, MST time is expected to be approximately 1-3 months (2). If oral analgesics doses are increased to a level that can control the patients pain alone, this often results in significant side effects, such as marked sedation and gastrointestinal signs (89,90).

1.2.7 Mechanisms of bone pain

Some proposed mechanisms of this bone pain include local release of chemical mediators, increased pressure within the bone, microfractures, stretching of the periosteum, reactive muscle spasm, nerve root infiltration and compression of nerves due to collapse of the bone (34,91). Approximately 74-92% of patients with osteosarcoma that receive palliative radiation experience some amount of pain relief (35,78–80). Radiation may help to reduce the number of cells within the bone marrow, thereby

(83,84,86,87). However, single fraction radiation, with either high or low doses, is often sufficient to achieve pain relief, without additional hospitalization or prolonged treatment courses (83,84,86,87). However, when bone lesions are multifocal or diffuse, hemi-body and whole body radiation therapies are considered. Although pain relief can be achieved, this comes at the cost of increased side effects including radiation pneumonitis and myelosuppression (85).
reducing the pressure within the bone, however this is unlikely the only mechanism of pain relief, as many patients experience rapid pain relief following radiation therapy (73,91,92). Other causes for its pain relief may result from direct killing of tumour cells and inflammatory cells or by reducing osteoclastic bone destruction (91–94).

1.2.8 Palliative radiation for OSA in dogs

There have been many studies evaluating the benefits of different palliative radiation therapy protocols for patients with appendicular OSA. Despite this, there is no consensus on an ideal dosing or timing protocol. Comparison of pain relief achieved 3 months post radiation with short course versus long course radiation, for treatment of bone pain associated with bone metastases in humans, has shown equivalent pain relief and requirement for narcotics (95). In that study, the short course treatment group received 8-Gy in one treatment and the long course treatment group received a total of 30-Gy over 10 treatments. Despite equivalent pain relief, the short course group required more repeat treatment but had fewer acute toxicity events than the long course treatment group (95).

Specific to canine appendicular OSA, palliative radiation protocols typically include 16-32Gy total, administered over two, three or four fractions (35,73,78–81,96). Pain relief response rate ranged from 50-93% of patients depending on the protocol (35,73,78–81,96), with the highest response rates achieved during a 16-Gy protocol administered in two fractions separated by 24 hours (81), and in a 32-Gy protocol administered in four fractions separated by 7 days each (79). Aside from the 16-Gy
protocols administered over two consecutive days, all other treatments were administered 7 days apart. This expedited, two day protocol, is an attractive protocol for many patients with OSA, as this limits the number of episodes of general anesthesia and reducing the amount of time in hospital overall (81). However, with the overall goal of palliative radiation being pain relief and the majority of patients achieving this goal within 7-14 days post radiation, additional doses may not be required until the patients pain returns (35,78,79,96). Following the protocols to completion however has provided a median duration of pain relief from 53-130 days, with the longest pain relief associated with higher total dose protocols (35,79). However, duration of pain relief was determined from the first day of clinical improvement until the first day of clinical worsening, such as recurrent lameness (35,79). Therefore, the duration of pain relief may be perceived as longer, due to lack of clinical evidence of pain. If a more subjective measure of pain was assessed, this may provide a more realistic duration of pain relief for these patients.

1.3 Overview of Bisphosphonates

Bisphosphonates are a unique group of drugs that share the same pharmacologic effects, resulting in reduced bone resorption and direct antitumor effects (97–100). To understand the antiresorptive effects of bisphosphonates, one must first understand the basic function of cells found within bone. Osteoblasts are responsible for bone deposition, osteoclasts are responsible for bone resorption by secreting hydrogen ions and proteolytic enzymes and osteocytes are mainly involved in the homeostasis of calcium (99).
The first bisphosphonate was developed in Germany in 1865 (101) after identifying that an inorganic pyrophosphate was able to bind very strongly to calcium phosphate and inhibit calcium phosphate precipitation in plasma and urine (98,99,101). Unfortunately, pyrophosphates were ineffective in vitro due to its rapid enzymatic hydrolysis prior to reaching a target site (99,101).

1.3.1 Chemical composition

Bisphosphonates are synthetic analogs of inorganic pyrophosphates. All bisphosphonates chemically consist of two phosphate groups covalently bound to a carbon atom, instead of two phosphate groups covalently bound to an oxygen molecule, as an inorganic pyrophosphate (98). This carbon substitution results in an innate resistance to enzymatic hydrolysis (98–100). Metabolites from various bisphosphonates have failed to be identified in animals or humans (102–104), indicating a very metabolically stable compound.

In addition to this carbon substitution, bisphosphonates contain two variable chains called R1 and R2 groups (98). The R1 group is most commonly made up of a hydroxyl group, allowing for high binding affinity with calcium crystals and bone matrix. The R2 group however, plays the major role in relative antiresorptive potency of the bisphosphonate. Based on the differences in the R2 group, two main groups of bisphosphonates exist. The first generation bisphosphonates have less antiresorptive ability and they are non-nitrogen containing bisphosphonates, including etidronate and clodronate. Second and third generation bisphosphonates have a greater antiresorptive
ability secondary to a nitrogen atom present within the R2 group; this group is subsequently referred to as aminobisphosphonates, including pamidronate, alendronate, risedronate, ibandronate and zoledronate (98).

1.3.2 Pharmacokinetics

There are many different bisphosphonates that have been developed over the last several decades, each with a conceived improvement upon the last. Despite the large variety of bisphosphonates in existence, they all share some general pharmacokinetic traits. Bisphosphonates generally are poorly bioavailable (<5%) in an oral form in all species evaluated – human, monkeys, dogs, rats and mice, with an increased absorption associated with being fasted (99,104–108). Despite this low bioavailability, even small volumes that get absorbed can exert effects due to their marked affinity for hydroxyapatite; therefore oral bisphosphonates have remained on the market. However, these oral bisphosphonates are only approved for use in non-malignant bone disorders in humans (98).

1.3.3 Distribution

Bisphosphonates have a wide distribution around the body, including non-calcified and calcified tissues. However, drug concentrations decline rapidly from the non-calcified organs and in parallel with plasma concentrations (98,99). Drug concentrations in non-calcified tissues initially as high as 63% rapidly decline to 5% after 1 hour, whereas drug concentrations within the bone rapidly increase and peak at 1 hour post intravenous administration in rats (99). This is the result of bisphosphonates being
redistributed rapidly to bone due to its marked affinity for hydroxyapatite or to the kidneys for renal excretion due to its high water solubility (98,102,109). This rapid redistribution also means that non-calcified tissues are only exposed to bisphosphonates for short periods of time (99). Only when high concentrations are administered intravenously do bisphosphonates accumulate in non-calcified organs (110–113).

Despite bisphosphonates being taken up preferentially by calcified tissues, their distribution within these tissues is not homogenous (98). Bisphosphonates accumulate preferentially in metaphyseal and epiphyseal regions of bone, with two to three times as much in these regions as in diaphyseal bone (102,113,114). Metaphyseal and epiphyseal bone are made up of cancellous bone, which has a greater blood flow, higher basal resorption rate and a larger surface-to-volume ratio than diaphyseal bone, which is made up of cortical bone (98). This increased absorption in cancellous bone only exists to a point of saturation; at which time increased volumes of bisphosphonates remain in plasma and non-calcified tissues (113,115). Bisphosphonates remain within bone until said bone resorbs, therefore the half-life of bisphosphonates in bone is variable and depends on the rate of turnover. In normal bone, this can mean years, whereas in diseased bone these bisphosphonates may be released much more rapidly (113,115).

1.3.4 Antiresorptive role of BP

Bisphosphonates are most commonly used for treatment of primary or metastatic bone tumours, due to its antiresorptive effects and direct anti-tumour effects. Many \textit{in vitro} and \textit{in vivo} studies have shown that bisphosphonates inhibit resorption of bone
The main theory is that bisphosphonates are released during bone resorption as the osteoclasts dissolve the hydroxyapatite crystals they are bound to. The osteoclasts endocytose the bisphosphonates, which subsequently results in disruption of intracellular signaling and metabolism and leads to cell death via apoptosis (121,122). Although all groups of bisphosphonates can result in apoptosis of osteoclasts, there are two main avenues by which this is achieved. The non-nitrogen containing bisphosphonates substitute phosphate groups in ATP, resulting in a non-hydrolysable cytotoxic compound and interruption of intracellular signaling pathways. The nitrogen containing bisphosphonates however, target the mevalonate pathway by inhibiting farnesyl pyrophosphate synthase, which interferes with prenylation of GTP-binding proteins and causes abnormal intracellular signaling (100,123–127). The nitrogen containing bisphosphonates are 100-fold more potent due to an increased effectiveness at binding calcium (124). Historically, another theory existed that the antiresorptive action of bisphosphonates was secondary to its effects on osteoblasts that resulted in reduction of their osteoclast promoting activity (119).

### 1.3.5 Anti-tumour effects of BP

Direct anti-tumour effects of bisphosphonates include inhibition of proliferation of neoplastic cells, induction of apoptosis, prevention of bone metastases via inhibition of cell adhesion and invasion, reduction in secretion of growth factors and cytokines, and inhibition of angiogenesis (97,98,100).
After first identifying that bisphosphonates could induce apoptosis in macrophages via the mevalonate pathway, as previously described in osteoclasts, it was subsequently proven that bisphosphonates had both an antiproliferative and pro-apoptotic effect in human myeloma cells via the same pathway (125,127). Furthermore, these same effects were repeatable in human tumour cells from the breast, prostate and pancreas (128–131). Different bisphosphonates, however, had different potency with zoledronate and ibandronate being the most potent and clodronate being the least potent (129). Zoledronate was identified to have a predominant antiproliferation effect in prostate cells compared to pamidronate, which had a predominant pro-apoptotic effect (130). A synergistic effect was also identified when these bisphosphonates were combined with other antineoplastic drugs on myeloma and breast cancer cells (132–134).

Prevention of bone metastases via inhibition of cell adhesion and invasion has been demonstrated in both breast and prostate cancer cell lines (135–137). In these studies, either cancellous and cortical bone or breast and prostate cancer cells were pretreated with bisphosphonates, prior to inoculation of bone with breast and prostate cancer cells. In each of these experiments, a dose dependent effect was identified that resulted in decreased adhesion and subsequent invasion of cancer cells into bone. Despite lack of adhesion to bone, this effect was not mirrored in adjacent non-skeletal tissues, therefore suggesting disruption of a specific bone-tumour adhesion molecule (136). Doses required to achieve interruption in cell adhesion were below cytotoxic doses (137), therefore indicating that bone death may not be the cause for lack of adhesion. Furthermore, 60-90% invasion of breast and prostate cancer cells was prevented by
zoledronate, again at doses below cytotoxicity (138). The mevalonate pathway was once again targeted by bisphosphonates, resulting in prevention of prostate cell migration and invasion across an artificial membrane (139).

Reduction of secretion of growth factors and cytokines plays an important role in reducing the self-perpetuating cycle of tumour proliferation caused by on-going osteoclastic activity. During bone resorption, growth factors are constantly being released from the bone resulting in promotion of tumour proliferation. By decreasing osteoclastic activity through alteration of the mevalonate pathway, fewer growth factors and cytokines are released thus retarding tumour growth (100). Additionally, bisphosphonates may inhibit secretion of growth factors and cytokines by osteoblasts, bone marrow, monocytes and macrophages (119,140).

Bisphosphonates have been proven to have anti-angiogenic effects both in vitro and in vivo. Initially zoledronate and pamidronate were proven to have potent anti-angiogenic effects by comparing capillary growth of rat aortic rings treated with bisphosphonates compared to controls treated with EDTA, with complete inhibition of capillary sprouting in the treatment group (141). Additionally, angiogenesis was inhibited in a murine model in a dose dependent fashion when pamidronate or zoledronate were administered to mice receiving subcutaneously administered growth factors (basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF)) to encourage angiogenesis (141). In another murine model, mice were inoculated with myeloma cells. All mice developed bone lesions, however these bone lesions could be
reduced by administration of zoledronate. In those mice that developed a myeloma bone lesion, treatment with zoledronate resulted in significantly lower microvessel density, indicating a direct anti-angiogenic effect (142). Castrated rats treated with both testosterone and bisphosphonates versus testosterone alone, resulted in a 50% decrease in revascularization of the prostate gland, again proving the anti-angiogenic effects of bisphosphonates (143). Pro-angiogenic growth factors such as platelet derived growth factor (PDGF) and VEGF in the serum of cancer patients has also been proven to be decreased by both pamidronate and zoledronate, with VEGF more significantly affected (144,145).

1.3.6 Biologic effect of BP

The biologic effect of bisphosphonates can be measured in several ways, although this remains a difficult task. The major biologic effect that can be evaluated are the bone specific effects, through radiographic evaluation of the target lesions and via measurement of bone biomarkers (98,146). Radiographic bone evaluation may be performed using traditional radiography, bone scintigraphy, computed tomography (CT), magnetic resonance imaging (MRI), dual energy x-ray absorptiometry (DEXA) or positron emission tomography (PET). Traditional radiography although quick and readily available, is relatively insensitive to small or early bony lesion changes and often lags behind clinical changes (147,148). Scintigraphy, on the other hand, is too specific and fails to be able to distinguish healing bone from progression of disease (147,148). CT and MRI, while becoming more readily available are markedly expensive and require general anesthesia, which makes their use impractical, despite their excellent ability to assess
bony lesions in detail. DEXA scans are used to assess bone mineral density and have proven capable of determining changes in bone density associated with metastatic breast and prostate lesions in patients treated with bisphosphonates (149–151). PET scans are becoming more popular for cancer patient evaluation due to their highly metabolically active cells, which improve uptake of PET tracers and allow for easier lesion localization. PET scans are considered a sensitive diagnostic test and also provide good spatial resolution (98). Bone biomarkers can directly assess bone turnover, by measuring end-products of bone resorption such as terminal derived fragments of type I collagen – N-terminal telopeptide (NTx) and C-terminal telopeptide (CTx) (152). These biomarkers can be identified in both blood and urine, with the most accurate marker for pathologic skeletal diseases being NTx (153–155).

### 1.3.7 Adverse effects

Despite the many benefits of bisphosphonates in treatment of primary and metastatic bone disease, adverse effects must be considered. As previously mentioned, bisphosphonates are metabolically stable, meaning that they do not get metabolized by the body and therefore have a less chance of developing toxicity due to lack of active intermediates or metabolites in circulation. Orally available bisphosphonates can result in diarrhea, abdominal pain, esophagitis and esophageal ulceration (156–159). Intravenous bisphosphonates on the other hand can result in acute systemic inflammatory reactions, conjunctivitis, uveitis and other ocular effects, renal disease, electrolyte abnormalities and osteonecrosis of the mandible or maxilla (98,160–169). Acute systemic inflammatory reactions can occur in 15-30% of patients, but is generally self-limiting. Typical clinical
signs include flu-like symptoms such as fever, muscle and joint pain, nausea and vomiting (160). The cause of this inflammatory reaction is due to an increase in number of circulating cytokines, most importantly interleukin-6 and tumor necrosis factor (161,162). Renal side effects include both acute and chronic renal failure, along with nephrotic syndrome. Zoledronate is more likely to cause renal tubular injury and result in renal failure, whereas pamidronate is more likely to cause glomerular injury and cause a protein losing nephropathy (165–167). Osteonecrosis of the jaw appears to be a newly emerging adverse effect of intravenous bisphosphonates, with an unknown etiology (168,169). Patients receiving bisphosphonates frequently or for prolonged periods of time appear to be at greatest risk. Zoledronate appears to result in more osteonecrosis and sooner than pamidronate (170,171).

1.4 Bisphosphonates and OSA

In veterinary medicine, bisphosphonates continue to be evaluated for use in canine patients with appendicular osteosarcoma and the literature remains scarce at this time.

1.4.1 In vitro studies

Several studies have evaluated the effect of bisphosphonates on canine and human OSA cells in vitro. The bisphosphonates alendronate and zoledronate were evaluated in vitro by assessing growth inhibition and apoptosis in both human and canine OSA cell lines. It was determined that both bisphosphonates inhibit cell growth and have a dose dependent ability to cause apoptosis in these cells lines (172). Although this study
provides more information to understand the mechanisms of action of different bisphosphonates, the doses used in vitro may not be directly applicable to in vivo dosing. Additionally, the use of bisphosphonates in a single agent setting for OSA is unlikely, therefore combining bisphosphonates with other treatment modalities, such as radiation therapy or chemotherapy may be more applicable to our veterinary patients.

Alendronate’s effects on canine OSA and fibroblast cell viability was evaluated, revealing both a time and concentration dependent effect on OSA cell viability but no significant effect on fibroblast cell viability (173). These findings together support the concept of bisphosphonate’s direct anti-tumor effects on OSA cell lines in vitro (173). Similar results were found when the effects of pamidronate on canine OSA and fibroblast cell viability were assessed. However, it was also determined that under these in vitro circumstances, pamidronate’s cytotoxic effect was not secondary to apoptosis (174). Despite many studies evaluating the effects of bisphosphonates and their mechanisms of action, a lot remains unknown about these drugs.

1.4.2 Murine model

Recently, the effects of zoledronate in combination with amputation were assessed in a murine model by Wolfe et al. Mice tibiae were inoculated with osteosarcoma cells, resulting in development of an artificially induced tibia bone tumour and spontaneous pulmonary metastases. Treatment groups included amputation and saline, amputation and zoledronate in combination, zoledronate alone or saline placebo. Zoledronate alone revealed evidence of reduced bone lysis, however zoledronate alone or
in combination with amputation did not affect development of pulmonary metastasis. Amputation alone however, reduced the development of pulmonary metastases, but this effect was negated when zoledronate was added to the treatment. These results help to support bisphosphonates antiresorptive role in treatment of OSA, however bring to question their negative impact on development of distant metastases (175). As all mice were euthanized on day 78 of the experiment, the long-term effects of bisphosphonates on survival could not be assessed. However, if the addition of bisphosphonates resulted in increased pulmonary metastases, one could hypothesize that this could result in a shorter survival time compared to those patients who did not receive bisphosphonates.

1.4.3 Clinical studies

The use of bisphosphonates in clinical cases of canine appendicular OSA has become more commonplace, despite the paucity of veterinary literature to support its use. Reports of bisphosphonate use in veterinary medicine have proven their safety in both patients with malignant osteolysis and healthy patients (176–178). Pamidronate is the most reported bisphosphonate used in veterinary medicine due mainly to its cost effective nature. However, zoledronate, a more potent aminobisphosphonate used frequently in the human medical field is beginning to gain popularity due to quicker administration and more rapid response. Currently, pamidronate is administered intravenously over 2 hours, compared to a 15-minute intravenous infusion for zoledronate (179–181).

Both pamidronate and zoledronate administration for canine appendicular OSA have been proven to result in decreased pain, increased relative bone mineral density as
measured by DEXA and decreased urine concentrations of CTx and NTx (176–178). All of these findings clinically correlate with improved use of the affected limb as well. However, despite the benefits that these bisphosphonates can provide, adverse events can and do occur. A single report of zoledronate-associated osteonecrosis of the jaw has been identified in a patient with appendicular OSA (182). As bisphosphonates continue to gain use, supported by on-going literature, more adverse events are likely to be seen.

1.5 Radiation Therapy & Bisphosphonates

Both radiation therapy and bisphosphonates can improve quality of life and overall pain associated with malignant osteolysis. Review of the human literature for individual treatment with radiation or bisphosphonates for treatment of bone lesions from multiple myeloma and metastatic breast and prostate cancer show excellent individual response rates to each modality (183). Logically, the combination of treatments should result in even more pain relief for these patients. However, minimal research into the combination of radiation therapy and bisphosphonate use has been performed to date, with regard to canine appendicular OSA.

One in vitro study evaluated the combined effect of radiation therapy and bisphosphonates in a murine OSA cell line, revealing significant inhibition of growth when compared to each modality individually (184). Furthermore, a double-blinded placebo-controlled trial in canine patients with appendicular OSA treated with radiation therapy and doxorubicin +/- pamidronate continued to identify decreased bone resorption via DEXA and urine NTx evaluation, but failed to prove an increase in pain relief when
pamidronate was combined with radiation therapy (185). Another retrospective study, evaluated the effects of the combination of radiation therapy, chemotherapy and bisphosphonate use on canine appendicular OSA. This study identified an increased MST for all patients who had chemotherapy as part of their protocol, however MST significantly decreased when pamidronate was added to the protocol and was worst when radiation therapy and pamidronate were combined without chemotherapy (186). Therefore, it was recommended that pamidronate not be combined with radiation therapy due to its antagonistic type effect on survival. Lending further support to these findings, evaluation of the combination of carboplatin and pamidronate in canine OSA revealed similar disease free interval and MST when carboplatin was administered alone or in combination with pamidronate (187). Therefore, the combination of pamidronate does not appear to affect the efficacy of carboplatin, which further supports the decrease in MST in the previous retrospective study to be an effect of pamidronate with radiation therapy.

1.6 Rationale, Hypotheses, Objectives

Much of our knowledge regarding the benefits of radiation therapy and bisphosphonates is adapted from the human medical field. Although radiation therapy has been more widely used, bisphosphonates are gaining popularity in veterinary medicine. With the limited research on bisphosphonates and radiation therapy in combination, there is conflicting information making palliative treatment recommendations for dogs with appendicular OSA difficult.
Although we may know the overall mechanisms of action of bisphosphonates and the benefits of the newer nitrogen-containing generations, appropriate dosing, timing and compatibility with other treatment modalities remains unknown. Therefore, further investigation of the combination of bisphosphonates and radiation therapy, at varying doses and timing of administration is required, to guide further palliative treatment recommendations for our canine appendicular OSA population.

Based on the current body of literature, our hypotheses were:

1. Treatment of canine OSA cells with either pamidronate (PAM) or zoledronate (ZOL) would inhibit clonogenic outgrowth and cell viability
2. Treatment of canine OSA cells with single agent PAM or ZOL would inhibit clonogenic outgrowth and cell viability more than treatment with PAM or ZOL in combination with RT
3. Timing of administration of PAM or ZOL in relation to RT would not alter clonogenic outgrowth or cell viability of canine OSA cells.

Therefore, the objectives of this study were to evaluate the in vitro clonogenic survival and viability of canine OSA cells when subjected to PAM, ZOL or RT compared to the combination of either PAM or ZOL with RT, and how timing of administration of PAM or ZOL in relation to RT may alter these outcomes.
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CHAPTER II

Evaluation of the combined effects of radiation therapy and either pamidronate or zoledronate on canine osteosarcoma cells

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2.1 Abstract

Canine osteosarcoma is a devastating disease, with an overall poor prognosis. Radiation therapy and bisphosphonates are currently used in combination for palliative treatment, despite a paucity of literature to support their combined use. The objectives of this study were to assess the in vitro effects of radiation therapy and bisphosphonates on canine osteosarcoma cells alone and in combination. Canine osteosarcoma cell lines D17 and Dharma were treated with radiation, pamidronate and zoledronate alone and in combination. The effects of these treatments were assessed using clonogenic survival and cell viability assays. Dose dependent decreases in clonogenic survival and cell viability were observed for both radiation and bisphosphonate treatment. Combination index analysis revealed an overall antagonistic interaction when radiation and bisphosphonates were used in combination for both D17 and Dharma osteosarcoma cells. Further clinical investigation of the combined use of radiation and bisphosphonates for the palliative treatment of canine osteosarcoma is warranted prior to changing recommendations for current clinical practice.
2.2 Introduction

Radiation therapy (RT) and aminobisphosphonates (BP) are common palliative treatment approaches for osteosarcoma (OSA) in dogs that are not undergoing standard of care treatment (1–8). One of the main goals of using RT in patients with OSA is to provide pain relief (9–13). External beam radiation is the most common form of RT utilized and coarse-fractionated protocols that deliver higher doses over a short time period are typically used in a palliative treatment setting (7,14–17). Radiation therapy relieves pain in dogs with OSA in several ways. It reduces the number of cells (including inflammatory cells) within the bone marrow leading to decreased pressure within the affected bone, inhibits osteoclastic bone destruction, and may reduce tumour burden by directly killing OSA cells (4,15,18–20).

In addition to RT, BPs have gained popularity in veterinary medicine over the past decade for the palliative treatment of OSA (1,21). These drugs are often used in combination with RT, despite a limited understanding of the possible effects of combining these treatment modalities. BPs are synthetic analogues of inorganic pyrophosphates that inhibit osteoclast function to reduce bone resorption and have direct anti-tumour effects including inhibition of tumour cell proliferation, adhesion and invasion, as well as promotion of apoptosis (22–30). These drugs have been used for decades to treat osteoporosis, as well as primary and metastatic bone tumours in humans. The goal of BP use in OSA is to provide analgesia through bone antiresorptive properties, to prevent development of bone metastases and to cause direct anti-cancer effects (22–26).
Pamidronate (PAM) is a widely used BP in veterinary medicine. Pamidronate is a second generation BP that, in addition to its conventional mechanisms of action, has also demonstrated anti-angiogenic effects in human cancer patients by reducing serum VEGF levels (31). In canine OSA, PAM has been shown to inhibit OSA growth through a non-apoptotic mechanism in vitro and reduce pain and pathological bone turnover in a clinical trial (2,32).

Zoledronate (ZOL), a third generation BP, is the most potent intravenous BP approved for human use (33). In veterinary medicine, use of ZOL for the management of canine OSA may be increasing, although evidence for its improved benefit over other BPs in this context is currently limited. In vitro studies have shown anti-tumour effects of ZOL in both human and canine OSA cell lines, including decreased cell growth, a dose dependent increase in apoptosis, alteration of cell cycle distribution, inhibition of tumour cell invasion and anti-angiogenic effects (34–38). Zoledronate has also been shown to reduce OSA induced bone lysis in a nude mouse canine OSA orthotopic xenograft model (39). However, while ZOL may have improved bone pain, it was also associated with an increased risk for pulmonary metastases in these mice.

Independently, RT and BP each have the potential to improve cancer-related bone pain. There remains a paucity of literature investigating the combined use of RT and BP, despite the increasing clinical use of this combination treatment approach in veterinary patients. Three studies in canine OSA patients have evaluated the combined use of PAM with RT and/or chemotherapy (1,21,40). In each of these studies, PAM failed to improve
pain relief or medial survival time (MST) when combined with RT and/or chemotherapy. However, in one retrospective study, it was noted that chemotherapy either with or without PAM had a similar MST, whereas combined RT and PAM resulted in a decreased MST (1). Taken together, these studies suggest a lack of benefit, and perhaps a potential negative impact when PAM is combined with RT.

There are currently no reports evaluating the in vitro effects of the combination of PAM and RT on OSA cells. However, with ZOL, Ryu et al demonstrated that the combination of ZOL and RT resulted in significantly more growth inhibition of murine and human OSA cell lines in vitro when used in combination than when used alone (41). Zoledronate appears to be a radiosensitizer in murine and human OSA cells, which may result in a different effect when ZOL is combined with RT, compared to the combination of PAM and RT (41,42). Due to the limited in vitro evaluation of the combination of either PAM or ZOL with RT in canine OSA cell lines, the objectives of this study were to evaluate the in vitro proliferation and viability of canine OSA cells when subjected to PAM, ZOL and RT alone, compared to the combination of either PAM or ZOL with RT. The null hypothesis of this study was that the combination of either PAM or ZOL with RT would not provide a significantly increased inhibition of proliferation and viability compared to single agent treatment of canine OSA cells.
2.3 Materials and Methods

2.3.1 Cell culture

D17 and Dharma canine OSA cell lines were used for all experiments. D17 cells (43) were derived from a lung metastatic lesion and obtained from Sigma-Aldrich/European Collection of Cell Cultures (ECACC) and Dharma cells were isolated from a primary appendicular lesion in a clinical case and adapted to culture by Dr. Anthony Mutsaers (44). Cells were grown in standard cell culture dishes with Dulbecco’s modified Eagles medium (Hyclone DMEM - Fisher Scientific- Ottawa, ON, Canada) with 10% fetal bovine serum (FBS; Life Technologies, Burlington, ON, Canada) and 1% penicillin-streptomycin (PS; BioWhittaker, Mississauga, ON, Canada). Cells were incubated at 37°C in a 5% CO₂ humidified incubator.

2.3.2 Cell count

Cells were washed with phosphate buffered saline (PBS; Fisher Scientific- Ottawa, ON, Canada), and then trypsin/EDTA (Fisher Scientific- Ottawa, ON, Canada) was added. Trypsinized cells were added to standard culture media prior to counting. A 1:1 mixture of cells and trypan blue 0.4% (Fisher Scientific- Ottawa, ON, Canada) was used to manually count cells using a hemocytometer.

2.3.3 Clonogenic survival assay

For each cell line and treatment condition, six, 6-well cell culture plates were plated at 500 cells/well, in 3 mL of media for the D17 cell line and 2000 cells/well, in 3 mL of media for the Dharma cell line and plates were incubated overnight. Cells were then treated with PAM (pamidronate disodium salt hydrate; Sigma-Aldrich, Oakville, ON,
Canada) or ZOL (zoledronic acid; Sigma-Aldrich, Oakville, ON, Canada). Two dose levels of PAM or ZOL and one vehicle control were used, with 2 wells/plate for each dose. Given the potency differences between the two BP drugs, doses were chosen following initial optimization studies. All doses used fall within previously reported ranges used in in vitro studies (32,34,41,42). For D17 cells, PAM doses were 10 µM and 20 µM and for Dharma cells, PAM doses were 10 µM and 30 µM. For D17 cells, ZOL doses were 0.5 µM, and 2 µM and for Dharma cells, ZOL doses were 0.4 µM and 2 µM. Media was removed from each well and was replaced by 3mL of BP containing media or standard culture media for the control wells. Plates were subsequently placed in the incubator prior to receiving RT later that same day. One plate from each experiment received 2 Gy, 4 Gy, 6 Gy, 8 Gy or 10 Gy of RT using a 6-MV linear accelerator (Clinac IX System, Varian Medical Systems, Inc., Palo Alto, CA, USA). Control plates (0Gy) remained outside of the radiation vault during cell treatment.

Plates were returned to the incubator, and colony formation was monitored daily. The experiment was terminated before the control colonies became confluent (7 days) then cells were fixed and stained with 0.5% crystal violet in 20% methanol. Colonies were counted using light microscopy. A colony was defined as an aggregate of ≥ 50 cells. Each experiment was performed in triplicate.

2.3.4 Viability assay

For each cell line and treatment condition, six, 96-well cell culture plates were plated at 500 cells/well, in 150 µL of media for the D17 cell line and 2000 cells/well, in 150 µL of media for the Dharma cell line. Plates were placed in the incubator overnight. Cells were
subsequently treated in quadruplicate with BP the following morning. Doses were chosen following initial optimization studies. All doses fell within previously reported ranges used in \textit{in vitro} studies (32,34,41,42). Five doses of PAM or ZOL and one control were used. For D17 cells, PAM doses were 5 µM, 10 µM, 20 µM, 50 µM and 100 µM and for Dharma cells, PAM doses were 1 µM, 2 µM, 4 µM, 8 µM, 10 µM or 16 µM. For D17 cells, ZOL doses were 0.5 µM, 1 µM, 5 µM, 10 µM, 20 µM, 50 µM or 100 µM and for Dharma cells, ZOL doses were 0.2 µM, 0.4 µM, 0.8 µM, 1.6 µM, 3.2 µM or 6.4 µM. Plates were subsequently placed in the incubator prior to receiving RT later that day. One plate from each experiment received 2 Gy, 4 Gy, 6 Gy, 8 Gy or 10 Gy of RT using a 6-MV linear accelerator. Control plates (0Gy) remained outside of the radiation vault during cell treatment.

Plates were returned to the incubator for 7 days. On day 7 post RT, cell viability was assessed using the Resazurin Cell Viability Kit (Sigma-Aldrich, Oakville, ON, Canada). Twenty microliters of the resazurin solution was added to each well, and absorbance readings were obtained 6 hours later using a Synergy 2 spectrophotometer (BioTek, Winooski, VT, USA), at an excitation wavelength of 570nm and emission wavelength of 600nm. Overall, each treatment had quadruplicate wells and each experiment was performed in triplicate.

\textbf{2.4 Statistical Analysis}

A general linear mixed model (2-way ANOVA) was used to test the fixed effects of PAM or ZOL and RT and their interactions. The random effect of the plate was accounted for
in the model. A Shapiro-Wilk test and examination of the residuals assessed the data for normality. If the data was not normally distributed, a log transform was applied. A value of p<0.05 was considered significant.

After calculating the IC50’s for the viability assays, analysis of the effects of combining PAM or ZOL and RT were assessed using the combination index (CI) method of Chou and Talalay (45). This was performed using the program CompuSyn (CompoSyn, Inc., Paramus, NJ) to quantify the CI. Only combinations resulting in <50% cell growth inhibition were included in this assessment to allow for potentially equal contributions from PAM or ZOL and RT. The combined effects can be additive (effects of individual treatments added together, CI = 1.0), synergistic (effects of combination greater than individual effects added together, CI < 1.0) or antagonistic (effects of combination less than individual effects added together, CI > 1.0) (45).

2.5 Results

2.5.1 Pamidronate

Treatment of D17 cells resulted in a dose dependent reduction in clonogenic survival (Fig 1A) and cell viability (Fig 2) for both PAM and RT. Statistically significant differences were only identified during the clonogenic survival experiments.

When comparing single agent RT at 2 Gy, 4 Gy and 6 Gy to the combination of 20 μM PAM and the same RT doses, significantly fewer colonies were formed when PAM and RT were combined. However, these combinations were not significantly different to cells treated with 20 μM PAM alone (Fig 1C). One other significant combination was
Fig 2.1. Clonogenic survival for D17 OSA cells treated with PAM + RT. A) Dose dependent decrease in clonogenic survival. (N= 2, mean +/- SEM). B) A significant decrease in clonogenic survival of D17 cells was observed following treatment with 10 µM PAM + RT. (N= 6, mean +/- SEM), and C) 20 µM PAM + RT. (N= 6, mean +/- SEM). * = p<0.05
Fig 2.2. Viability of D17 OSA cells treated with PAM + RT. A dose-dependent decrease in viability was observed. (N= 4, mean +/- SEM).

identified when comparing cells treated with 10 µM PAM alone to cells treated with 10 Gy RT + 10 µM PAM. This combination was not significantly different however, when compared to the cells treated with 10 Gy RT alone (Fig 1B).

Treatment of Dharma OSA cells with RT + 10 µM PAM resulted in a trend toward more colony formation compared to RT alone (Fig 3). These differences were not significant. In contrast, the addition of 30 µM PAM significantly inhibited colony formation (Fig 3) compared to RT alone. Treatment of Dharma cells resulted in a dose dependent reduction in viability for both PAM and RT (Fig 4).
Fig 2.3. Clonogenic survival for Dharma cells treated with PAM + RT. A dose dependent decrease in clonogenic survival was observed. (N= 2, mean +/- SEM).
Fig 2.4. Viability for Dharma cells treated with PAM + RT. A dose dependent decrease in viability was observed. (N= 4, mean +/- SEM).

2.5.2 Combination Index

When the combination index was calculated using the viability results of drug combinations, antagonism was identified for all dose combinations of PAM and RT assessed in the D17 OSA cells (Table 1). However, for Dharma cells, dose-dependent effects were observed, with some combinations resulting in a CI of less than 1.0, while others displayed an antagonistic CI of greater than 1.0 (Table 2). However, the mean CI of all dose combinations was approximately 1.0, revealing a potentially additive effect of combining PAM and RT in this OSA cell line.
Table 2.1: Combination Index for D17 cell viability assay, PAM + RT

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Table 2.2: Combination Index for Dharma cell viability assay, PAM + RT

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2.5.3 Zoledronate

Treatment of D17 cells resulted in a dose dependent reduction in clonogenic survival (Fig 5A) and viability (Fig 6A) for both ZOL and RT. Significant results were identified in both assays.

When comparing single agent ZOL at 0.5 µM or 2 µM concentrations to the 6 Gy, 8 Gy or 10 Gy RT doses treated with the same ZOL concentrations, significantly fewer colonies were formed when ZOL and RT were used in combination (Figs 5B and 5C).
Fig 2.5. Clonogenic survival for D17 cells treated with ZOL + RT. A) Dose dependent decrease in clonogenic survival. (N= 2, mean +/- SEM). B) A significant decrease in clonogenic survival of D17 cells was observed following treatment with 0.5 µM ZOL + RT. (N= 6, mean +/- SEM), and C) 2 µM ZOL + RT. (N= 4, mean +/- SEM). * = p<0.05

However, these combinations were not significantly different when compared to single agent RT at 6 Gy, 8 Gy or 10 Gy (Figs 5B and 5C).

When comparing single agent RT irradiated at 2 Gy, 4 Gy, 6 Gy and 8 Gy to the 5 µM, 7.5 µM, 10 µM and 20 µM ZOL concentration irradiated at the same doses, significantly less cell viability was identified when ZOL and RT were used in combination, with the exception of the combination of 5 µM ZOL and 8 Gy RT (Figs 6B-E). However, these combinations were not significantly different when compared to single agent ZOL at 5 µM, 7.5 µM, 10 µM and 20 µM concentrations (Figs 6B-E).
Fig 2.6. Viability for D17 cells treated with ZOL + RT. A) Dose dependent decrease in cell viability. (N= 4, mean +/- SEM). B) A significant decrease in viability of D17 cells was observed following treatment with 5 µM ZOL + RT. (N= 6, mean +/- SEM), C) 7.5 µM ZOL + RT. (N= 8-12, mean +/- SEM), D) 10 µM ZOL + RT. (N=12, mean +/- SEM), E) 20 µM ZOL + RT. (N= 8-12, mean +/- SEM), F) 0.5 µM ZOL + RT. (N= 8, mean +/- SEM), G) 1 µM ZOL + RT. (N= 16, mean +/- SEM), H) 2.5 µM ZOL + RT. (N= 8-16, mean +/- SEM), and I) 5 µM ZOL + RT. (N= 16, mean +/- SEM). * = p<0.05

When comparing single agent ZOL at 0.5 µM, 1 µM, 2.5 µM or 5 µM concentrations to the 6 Gy, 8 Gy or 10 Gy RT doses treated with the same ZOL concentrations, significantly less cell viability was identified when ZOL and RT were used in combination, with the exception of the following combinations: 0.5 µM + 6 Gy, 0.5 µM + 8 Gy, and 5 µM + 6 Gy (Figs 6F-I). However, none of these combinations were
significantly different when compared to single agent RT at 6 Gy, 8 Gy or 10 Gy (Figs 6F-I).

Treatment of Dharma cells with RT + 0.4 µM ZOL resulted in more colony formation compared to RT alone (Fig 7). These differences were not significant. Treatment of Dharma cells with RT + 2 µM ZOL resulted in less colony formation compared to RT alone (Fig 7). These differences were not significant. Treatment of Dharma cells resulted in a dose dependent reduction in cell viability (Fig 8) for both ZOL and RT.

**Fig 2.7. Clonogenic survival for Dharma cells treated with ZOL + RT.** A dose dependent decrease in clonogenic survival was observed. (N= 2, mean +/- SEM).
Fig 2.8. Viability for Dharma cells treated with ZOL + RT. A dose dependent decrease in viability was observed. (N= 8, mean +/- SEM).
2.5.4 Combination Index

Antagonism was identified for all dose combinations of ZOL and RT assessed using the cell viability results in both D17 (Table 3) and Dharma (Table 4) cells, with a stronger antagonistic result identified in the Dharma cells.

**Table 2.3: Combination Index for D17 cell viability assay, ZOL + RT**

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**Table 2.4: Combination Index for Dharma cell viability assay, ZOL + RT**

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2.6 Discussion

Despite the increasing use of the combination of RT and BP for the palliative treatment of canine OSA, limited information exists regarding the effects of their combined use. Since
each modality alone has demonstrated analgesic effects for dogs with OSA, a positive interaction has been assumed when BP and RT are combined. By evaluating the use of PAM or ZOL in combination with RT on canine OSA cells in vitro, we have shown that their interactions may be more complex than anticipated and that synergistic or even additive effects cannot necessarily be assumed. Similar to what has previously been reported, we found that single agent PAM, ZOL and RT treatment resulted in dose-dependent decreases in OSA cell viability and clonogenic survival (32,34). However, in Dharma cells, a low dose of both PAM or ZOL actually resulted in increased clonogenic outgrowth compared to controls, possibly demonstrating a mild protective effect of low dose BP administration in this cell line. The significance of this result is unknown, but may warrant further investigation. The same effect was not observed with the D17 cell line, which highlights the fact that, despite their similar classification, all OSA cell lines and tumours do not have an identical response to the same treatment protocol. A contributory factor to this result may be the differing growth rates of D17 and Dharma cells in vitro, with Dharma cells having a longer doubling time than D17. In addition, D17 cells were derived from a metastatic lung lesion, while Dharma cells were derived from a primary bone tumour. This fact may be worthy of consideration, as the clinical use of BP and RT in canine OSA is directed at the primary bone tumour rather than lung metastases. Although, significant differences in cell growth inhibition were seen when comparing different BP doses or different RT doses, few combinations of BP and RT resulted in significantly more cell growth inhibition than any individual treatment. Significant differences were only identified in the D17 cell line, again highlighting the potential for heterogeneity of OSA treatment response.
It is noteworthy that the significant combinations identified in the D17 cell line only resulted in significantly fewer colonies or significantly less viability when compared to one of the treatments in the combination, but not the other treatment. In other words, from the significant ZOL combinations it is clear that one treatment (either ZOL or RT) played a greater role in a particular combination. For the significant combinations of ZOL and RT in the clonogenic survival assays, RT played a greater role in decreasing colony outgrowth, whereas ZOL made a greater contribution to reducing viability, except at high RT doses (Figs 5 and 6). These results suggest differential contributions of these two modalities to the overall anti-cancer and/or analgesic effects in the palliative treatment of OSA.

It can be difficult to discern from the data alone the relative contribution of each treatment effect when delivered in combination, aside from specific combinations where one treatment modality clearly resulted in increased cell growth inhibition on its own compared to the other modality. Relative contributions are better measured through combination index (CI) analysis. The combination index is a mathematical model that analyzes the effects of multiple drugs or treatments, in order to determine if their relationship is additive (effects of individual treatments added together, CI = 1.0), synergistic (effects of combination greater than individual effects added together, CI < 1.0) or antagonistic (effects of combination less than individual effects added together, CI > 1.0) (45).
For PAM, an overall antagonistic effect was identified with CI analysis. For the D17 cell line, the CI values were antagonistic in all combinations evaluated, whereas the results with the Dharma cell line appeared to be more additive when taken as a whole. Lower CI values in Dharma cells were identified with low doses of PAM, or a combination of higher dose PAM with higher dose RT. It is worth mentioning that higher dose RT (8-10 Gy) is commonly applied clinically for palliative treatment of canine OSA. The identification of an overall antagonistic interaction when PAM and RT are combined in vitro supports the results of previous clinical studies that suggest either a negative impact or lack of improvement when these treatment modalities are combined (1,21,40).

Similar to PAM, an overall antagonistic effect was identified by CI analysis with ZOL. These results contrast those reported by Ryu et al, who identified ZOL as a radiosensitizing agent producing decreased cell viability when combined with RT (41). The use of different OSA cell lines and/or experimental conditions may have contributed to these contradictory results (41,42). However, as with any in vitro study, other intrinsic biologic effects present in vivo, such as the tumour microenvironment, are not accounted for, which may contribute significantly to the overall response of tumours to BP and RT.

Based on these results, while a dose dependent anti-growth response to the individual treatments was confirmed, the effects of combining BP and RT remain variable amongst canine OSA cell lines tested in vitro. Additionally, results suggest the possibility of an antagonistic relationship between BP and RT. Further study is required to investigate potential contributory factors to these results, such as whether the timing of
administration of each treatment contributes to the potential for a negative interaction. In our study, cells were exposed to BP for 2-4 hours prior to radiation, which may have produced results that differ from BP treatment after RT. In most clinical patients, BPs are given at the same visit as RT, which is why this treatment sequence was chosen. Finally, clinical recommendations regarding the combined use of BP and RT in the palliative management of canine OSA cannot be made without further clinical investigation, which would ideally require a randomized controlled trial.

2.7 Acknowledgements

The authors would like to acknowledge Jodi Morrison for her guidance and support in the lab, Sarah LaLiberte and Stephanie Lovell for their assistance in pilot study data collection and Gabrielle Monteith for her assistance in data analysis.
2.8 References


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34. Poirier VJ, Huelsmeyer MK, Kurzman ID, Thamm DH, Vail DM. The


3.1 Introduction

For decades, the human medical field has exploited their knowledge of how RT affects individual cells, by investigating other therapies that may create a cell that is more sensitive to the effects of RT or has decreased potential to recover from the effects of RT. By combining RT with other therapies that will result in a weakened tumour state, potential for greater local response to therapy may be achieved (1).

Timing of administration of these secondary therapies is ultimately determined based on how they function at a cellular level. Therapies that cause alterations in cell cycle (2–4), induce DNA damage (3,5), reduce inherent radioresistance (4,6) or target hypoxic regions of tumours (7–10) will provide their best effects if present immediately prior to RT. Other therapies that may alter a cell's ability to recover from the effects of RT, by reducing the tumours ability to repopulate (2,11) or by inhibiting radiation damage repair (1,3,11), must be present after RT has been administered.

While minimal investigation has been performed to date on the combined therapy of RT and BP for treatment of canine OSA, one in vitro study has identified ZOL as a possible radiosensitizing agent for treatment of human OSA (12). In this study, ZOL was administered 24 hours prior to RT and ZOL was found to promote apoptosis, cause direct DNA damage, impair DNA repair, alter cell signaling pathways and alter the proportion...
of cells in each phase of the cell cycle (12). Because of these effects, administration of ZOL before RT lead to enhancement of RT effects.

With a variety of other treatment modalities targeting the effects of RT to improve local outcomes and evidence of ZOL as a possible radiosensitizing agent (12), investigation into timing of administration of BP and RT for the treatment of canine OSA should be considered. The potential radiosensitizing effects of PAM have not been studied but this remains an important drug combination with palliative RT for canine patients. The objective of this study was to determine if pre or post RT treatment of canine OSA cells with BP affects clonogenic survival or cell viability when compared to treatment with BP and RT administered concurrently. Our hypothesis was that timing of administration of BP (PAM or ZOL) in relation to timing of administration of RT would not affect inhibition of canine OSA cell growth in vitro.

3.2 Materials and Methods

3.2.1 Cell culture

D17 and Dharma canine OSA cells lines were used for all experiments. D17 cells (13) were derived from a pulmonary metastatic lesion and were obtained from Sigma-Aldrich/European Collection of Cell Cultures (ECACC) and Dharma cells (14) were isolated from a primary appendicular lesion from a clinical case and adapted to culture by Dr. Anthony Mutsaers. Cells were grown in standard cell culture dishes with Dulbecco’s modified Eagles medium (Hyclone DMEM - Fisher Scientific- Ottawa, ON, Canada) with 10% fetal bovine serum (FBS; Life Technologies, Burlington, ON, Canada) and 1%
penicillin-streptomycin (PS; BioWhittaker, Mississauga, ON, Canada). Cells were incubated at 37°C in a 5% CO₂ humidified incubator.

3.2.2 Cell count
Cells were washed with phosphate buffered saline (PBS; Fisher Scientific- Ottawa, ON, Canada), and then trypsin/EDTA (Fisher Scientific- Ottawa, ON, Canada) was added. Trypsinized cells were added to standard culture media prior to counting. A 1:1 mixture of cells and trypan blue 0.4% (Fisher Scientific- Ottawa, ON, Canada) was used to manually count cells using a hemocytometer.

3.2.3 Clonogenic survival assay
For each cell line and treatment condition, two, 6-well cell culture plates were plated at 500 cells/well, in 3 mL of media for the D17 cell line and 2000 cells/well, in 3 ml of media for the Dharma cell line and incubated overnight. Cells were treated with either PAM or ZOL at one of three time points: pre treatment (48 hours before radiation), concurrent treatment (same day, 2-4 hours before radiation) and post treatment (24 hours following radiation). Two doses of PAM and two doses of ZOL, along with one vehicle control were used, with 2 wells/plate for each dose. Given the potency differences between the two BP drugs, doses were chosen following initial optimization studies. All doses used fall within previously reported ranges used in in vitro studies (12,15–17). For D17 cells PAM doses were 10 µM and 20 µM and for Dharma cells PAM doses were 10 µM and 30 µM. For D17 cells ZOL doses were 0.5 µM and 2 µM and for Dharma cells ZOL doses were 0.4 µM and 2 µM. At the predetermined timing interval, cell media was removed from each well and was replaced by 3 mL of BP containing media or standard culture media for control wells. One plate from each experiment received 4 Gy of
radiation using a 6-MV linear accelerator (Clinac IX System, Varian Medical Systems, Inc., Palo Alto, CA, USA). Control plates (0Gy) remained outside of the radiation vault during cell treatment. All time points were irradiated at the same time.

Plates were returned to the incubator, and colony formation was monitored daily. The experiment was terminated before the control colonies became confluent (7 days) then cells were fixed and stained with 0.5% crystal violet in 20% methanol. Colonies were counted using light microscopy. A colony was defined as an aggregate of ≥ 50 cells. Each experiment was performed in triplicate.

3.2.4 Cell viability assay

For each cell line and treatment condition, eighteen, 96-well cell culture plates were plated at 500 cells/well, in 150 µL of media for the D17 cell line and 2000 cells/well, in 150 µL of media for the Dharma cell line. Plates were placed in the incubator overnight. Cells were treated with either PAM or ZOL at one of three time points: pre treatment (24 hours before radiation), concurrent treatment (same day, 2-4 hours before RT) and post treatment (24 hours following radiation). Doses were chosen following optimization studies. All doses used fall within previously reported ranges used in in vitro studies (12,15–17). Five doses of PAM or ZOL and one vehicle control were used, with each dose replicated in quadruplicate. For D17 cells PAM doses were 5 µM, 10 µM, 20 µM, 50 µM and 100 µM and for Dharma cells PAM doses were 2 µM, 4 µM, 8 µM, 10 µM and 16 µM. For D17 cells ZOL doses were 1 µM, 2.5 µM, 5 µM, 7.5 µM and 10 µM and for Dharma cells ZOL doses were 0.4 µM, 0.8 µM, 1.6 µM, 3.2 µM and 6.4 µM. Plates were incubated between treatments. One plate from each experiment underwent 2 Gy, 4 Gy, 6 Gy, 8 Gy or 10 Gy of radiation using a 6-MV linear accelerator. Control plates
(0Gy) remained outside of the radiation vault during cell treatment. All time points were irradiated at the same time.

Plates were returned to the incubator for 7 days. On day 7 post RT, cell viability was assessed using the Resazurin Cell Viability Kit (Sigma-Aldrich, Oakville, ON, Canada). Twenty microliters of the resazurin solution was added to each well, and absorbance readings were obtained 6 hours later using a Synergy 2 spectrophotometer (BioTek, Winooski, VT, USA), at an excitation wavelength of 570nm and emission wavelength of 600nm. Overall, each treatment had quadruplicate wells per BP dose and each experiment was performed in triplicate.

### 3.3 Statistical Analysis

A general linear mixed model (3-way ANOVA) was used to test the fixed effects of BP (PAM or ZOL), RT, timing and their interactions. Random effect of plate was accounted for in the model. A Shapiro-Wilk test and examination of the residuals assessed the data for normality. If the data was not normally distributed, a log transform was applied. A value of p<0.05 was considered significant.

### 3.4 Results

Significantly fewer colonies grew for both D17 and Dharma cell lines treated with either PAM or ZOL when the cells were treated post RT compared to pre RT (Fig 3.1). Additionally, for D17 cells treated with ZOL, all cells treated post RT grew significantly fewer colonies than those cells treated on the concurrent day of RT (Fig 3.1).
No significant differences in cell viability were identified between the different timing groups using D17 cells treated with either PAM or ZOL and Dharma cells treated with PAM (Fig 3.2). Whereas, a significant decrease in cell viability was identified when Dharma cells were treated with ZOL on the concurrent day of RT compared to those cells being treated post RT (Fig 3.2).

Fig 3.1 Clonogenic survival results for canine OSA cells treated with PAM or ZOL + RT, comparing timing of administration of BP in relation to RT. A) D17 OSA cells treated with PAM + RT. B) Dharma OSA cells treated with PAM + RT. C) D17 OSA cells treated with ZOL + RT. D) Dharma OSA cells treated with ZOL + RT. (N= 36 +/- SEM). * = p<0.05
3.5 Discussion

Timing of administration of BP in relation to RT may be an important factor when evaluating the combined effects of treatment on canine OSA cells in vitro. Based on our previous and current work evaluating the combination of BP and RT, results vary between OSA cell lines, BPs, dosing combinations of BP and RT, laboratory assays and timing of administration of each modality, therefore suggesting a multifactorial response to these treatments (personal communication Hoddinott et al.).
Decreased clonogenic survival was identified for both canine OSA cell lines when treated with either BP 24 hours post RT compared to those treated with either BP 48 hours pre RT. These effects may be due to the weakened state of the OSA cells following RT as DNA damage secondary to RT has been shown to last at least 24 hours (1,12). However, post RT clonogenic survival was not significantly different than the concurrent treatment group that received BP on the same day as RT, but in advance of RT. These results together bring to question the previously suggested radiosensitizing effect of ZOL (12).

The significant decrease in clonogenic survival identified for D17 (ZOL + RT) post treatment group compared to concurrent treatment group and the significant decrease in cell viability identified for Dharma (ZOL + RT) concurrent treatment group compared to post treatment group, may be outliers or may represent the increased potency of ZOL compared to PAM (18,19), as the same findings were not identified for the D17 (PAM + RT) or Dharma (PAM + RT) groups in their respective laboratory assay. A difference inherent in the cell lines should also be considered, as the same findings were not identified in the other cell line. Direct comparisons between PAM and ZOL can be considered for the colonization assay only due to similar dosing across cell lines.

While timing of administration of BP in relation to RT was significant for the clonogenic survival experiments, timing was not significant for the cell viability assays, aside from the Dharma (ZOL + RT) group. This may be due to the ability of BP to have a greater impact on RT treated OSA cells biologically, such that a greater inhibition of cell
replication can be achieved. However, under the same circumstances, alteration of the cell’s metabolic activity is not significant.

While post RT treatment with BP resulted in increased inhibition of clonogenic outgrowth on canine OSA cells, it failed to result in decreased cell viability, measured by metabolic activity. Therefore, further investigation into the cellular effects of ZOL and PAM on canine OSA cells is warranted, which may help to determine an optimal timing for administration in relation to RT (12).

Despite both cell lines behaving similarly in this study, other biologic effects, such as the tumour microenvironment may play a larger role in canine OSA response to BP and RT treatments (20–24). Therefore, translation of this work into an orthotopic model of canine OSA may provide additional information regarding the combination of BP and RT and their optimal timing of administration in relation to one another.

Based on the results of this study, consideration may be given to administration of BP 24 hours after RT, as this may have a more beneficial effect on reducing OSA cell replication, thus potentially prolonging the analgesic effects of RT and BP.
3.7 References


CHAPTER IV: Discussion & Conclusions

4.1 Overview of Background for Combination Therapy

Canine appendicular OSA represents the majority of all documented bone tumours in dogs (1). Standard of care treatment involves limb amputation, to treat the local disease and adjuvant chemotherapy, to treat the systemic disease secondary to circulating tumour cells, as this combination has resulted in increased median survival times compared to other treatment options (2–8). However, not every patient is a candidate for amputation, therefore alternative treatment options such as RT and BP treatment must continue to be investigated.

To date, in vitro evaluation of human, murine and canine OSA treatment with BP has proven time and dose dependent effects of alendronate, clodronate, pamidronate and zoledronate on cell viability and proliferation (9–11). These effects have been secondary to both apoptotic and non-apoptotic mechanisms (10–13). Two further in vitro studies, have subsequently evaluated the effects of combining ZOL and RT for treatment of human and murine OSA (14,15). Both studies identified that the combined treatment with ZOL + RT resulted in decreased cell viability compared to single agent treatment with ZOL. Kim et al, also concluded that ZOL acts as a radiosensitizing agent under their specific conditions (15). In addition to these in vitro studies, a single retrospective study by Oblak et al identified a decreased MST when canine patients with OSA were treated with PAM + RT +/- chemotherapy, compared to those patients treated with RT +/- chemotherapy (16).
4.2 Rationale & Objectives for Evaluating Combination Therapy

Despite limited literature to support the combined use of BP and RT for treatment of canine OSA, this combination therapy has become increasingly utilized by veterinary oncologists. Therefore, the purpose of our study was to investigate the in vitro clonogenic survival and viability of canine OSA cells when subjected to PAM, ZOL or RT compared to the combination of either PAM or ZOL with RT.

4.3 Justification for Experimental Design

4.3.1 Justification for cell lines

In vitro evaluation of canine OSA cells has been performed on a variety of cell lines to date, including D17 (17–20), HMPOS (12,13,18), POS (12,13,18), COS 31 (12,18), Dharma (19,20) and Abrams (17,20). As D17, a cell line derived from a canine pulmonary metastasis and Dharma, a cell line derived from a primary canine appendicular bone tumour, were accessible in our laboratory, previously characterized and confirmed as OSA, and are well established cell lines, we elected to use these cell lines for our study (17,19). D17 cells were obtained from Sigma-Aldrich/European Collection of Cell Cultures (ECACC), while Dharma was historically obtained from a clinical case and adapted to culture by Dr. Anthony J. Mutsaers. Cell-plating densities were determined following initial optimization studies, along with previous work performed in our laboratory that revealed D17 to be a faster growing cell line than Dharma, therefore requiring a smaller cell-plating density (20).
4.3.2 Justification for assays

Previous *in vitro* OSA studies have evaluated the response of cells to a variety of treatments, including BP and RT, through many different assays. Clonogenic survival is a common assay used to assess a single cell's ability to replicate following exposure to RT (21). Not all cells will have the ability to replicate, but this does not mean those cells are dead. A cell viability assay will assess for metabolically active cells by having the cells reduce a chemical compound which results in a colour change that can be measured with a spectrophotometer (22). The combination of these assays provides the ability to assess both cells that are able to replicate as well as those viable cells that have either slow replication rates or an inability to replicate.

4.3.3 Justification for BP

Both PAM and ZOL are nitrogen-containing BPs which have increased anti-resorptive properties and are significantly more potent compared to non-nitrogen containing BP (23,24). We elected to use PAM in our study as there has been no *in vitro* work evaluating the combination of PAM and RT on canine OSA to date. Additionally, PAM was the most commonly used BP in our hospital at the time of study initiation. ZOL had yet to be introduced to clinical use in our hospital, but was being used by other veterinary oncologists at the time of study initiation. ZOL is the most potent BP being used in human medicine (25), therefore despite previous *in vitro* evaluation of ZOL and RT on human OSA cells, we felt that ZOL warranted further investigation as it continues to gain popularity in the field of veterinary oncology (14,15). As ZOL has increased potency compared to PAM, this could potentially lead to an increased risk of side effects,
however a recent human meta-analysis identified similar rates of side effects between ZOL and PAM (26).

PAM is administered to our canine OSA patients intravenously over 2 hours, whereas ZOL is administered intravenously over 15 minutes (25,27,28). Clinically, differences in administration time between these two BP, make ZOL a more attractive treatment option, as this could minimize hospitalization time for patients, as well as decrease stress for patients, owners and staff. In addition, ZOL’s increased potency, supported by Yang’s meta-analysis on PAM and ZOL for treatment of human bone metastases revealed that ZOL is more effective than PAM which further perpetuates the desire to investigate ZOL use in treatment for canine OSA (26).

As PAM and ZOL have a difference in potency, dosing could not be consistent across experiments. BP doses were determined after initial dose optimization studies and were altered as needed throughout the pilot phase of the study. We elected for BP doses that would result in reduction of clonogenic survival and cell viability, but doses that would not result in complete loss of clonogenic survival or cell viability. All BP doses fell within previously reported ranges (11,12,14,15).

4.3.4 Justification for RT

RT for canine OSA can be used with a palliative or curative intent. Clinically, palliative RT doses range from 16-32Gy, divided over two to four fractions (29–35), whereas curative intent RT can include intraoperative RT with a single dose of 70Gy
delivered (36,37), SRT with a single dose of 20-30Gy delivered (38) or a full-course external beam RT with ~3Gy delivered per fraction for 16-19 fractions (8). Unfortunately, most of these clinically relevant doses of RT are too high to administer directly to canine OSA cells in culture as they result in destruction of all cells and leave no ability to assess their response to treatment. Therefore, lower doses of RT must be used to achieve results. We elected to use doses ranging from 2-10Gy, to encompass previously reported doses of RT in vitro (14,15,18,20), with lower doses representing individual fractions of a curative intent protocol and higher doses representing individual fractions of a palliative intent protocol.

4.4 Results for Combination Therapy

After evaluating D17 cells treated with either PAM, ZOL or RT alone, dose dependent decreases in clonogenic survival and cell viability were identified for all treatments. Dharma cells treated with PAM, ZOL or RT, resulted in dose dependent decreases in cell viability, however only RT resulted in dose dependent decreases in clonogenic survival. These results are in agreement with previous in vitro studies (9–13). For Dharma cells treated with either PAM or ZOL, low dose BP treatment resulted in increased clonogenic survival compared to the control, and high dose BP treatment resulted in decreased clonogenic survival compared to the control. With these findings, a radiation protective effect of low dose treatment must be considered. Additionally, this finding suggests an inherent difference in response between D17 and Dharma cells which may be due to their differences in origin; metastatic versus primary lesion respectively. These differences in origin may result in different subtypes of OSA, with one having a
greater ability to induce osteoclastic activity, than the other.

Differences in response between clonogenic survival and viability assays in the Dharma cell line, may be due to differences in age of the cells during experimentation resulting in different proportions of cells within each stage of the cell cycle. Additionally, a limitation of the viability assay is that it accounts for cells that are metabolically active, but does not indicate the cell's ability to replicate. This limitation may have been significant in this situation due to the fact that cells were assessed at a single time point for both experiments. While fewer cells were metabolically active when exposed to increasing doses of BP, this represents a single moment in time. Dharma cells may have been more or less metabolically active in the days leading up to their viability assessment. Considering this possibility, if Dharma cells were more metabolically active in the first few days following RT, this could possibly allow for rapid individual cell replication and therefore colony formation, leading to increased clonogenic survival yet decreased cell viability at the time of colony and viability assessment.

While significant decreases in clonogenic survival and cell viability were identified in a variety of combinations of PAM + RT and ZOL + RT, compared to single agent treatment, a greater number of combinations of ZOL + RT were identified compared to PAM + RT, possibly indicating that ZOL is more effective than PAM as was previously identified by Yang et al (26). However, as previously mentioned, doses could not be consistent between PAM and ZOL due to their potency differences and therefore make these types of direct comparisons difficult.
When assessed by the CI, both PAM and ZOL had antagonistic effects when combined with RT for both D17 and Dharma cell lines. These findings are different than what one would assume, when both treatments have positive effects individually. The cause of this antagonistic relationship remains unknown at this time but dosing of BP and RT, along with cell line differences may play a role. While antagonistic effects were identified for the Dharma cell line for both PAM and ZOL when combined with RT, synergistic effects were also identified for PAM + RT. These synergistic combinations were identified when using both low and high doses of PAM. When taken as a whole, the effects of PAM + RT on the Dharma cell line are additive, which is likely more representative of their combined effects than assessing individual combinations. Again, differences inherent in the cell lines must be considered as a cause for such varied results between D17 and Dharma.

4.5 Overview of Background for Timing Study

The human medical field has been investigating many therapies that may sensitize a cell to the effects of RT or reduce a cell’s ability to recover from the effects of RT, with the goal of improving the local response to treatment (39).

Timing of administration of these secondary therapies ultimately is determined based on how they function at a cellular level. Therapies that result in a better radiation target such as promoting more cells to be in the G2 or M phase of the cell cycle (20,40,41), causing direct DNA damage (42), causing reduction in cellular
radioresistance (20,43) or improving oxygenation in regions of relative hypoxia (44–47) will provide their best effects if administered prior to RT, either sequentially or concurrently. Therapies that target the post RT recovery phase will be more beneficial if they are present between fractions of RT (20,40,41,48).

A single in vitro study to date has identified ZOL as a possible radiosensitizing agent for treatment of human OSA (15). No reports of PAM and RT used in combination for treatment of canine OSA exist at this time.

4.6 Rationale & Objectives for Evaluating Timing

With a variety of other treatment modalities targeting the effects of RT to improve local outcomes and evidence of ZOL as a possible radiosensitizing agent, we elected to investigate the timing of administration of BP and RT for the treatment of canine OSA. The objective of this study was to determine if pre or post RT treatment of canine OSA cells in vitro with PAM or ZOL affects clonogenic survival or cell viability when compared to treatment with PAM or ZOL + RT administered concurrently.

4.7 Justification for Timing

As previously stated, appropriate timing of administration of secondary therapies in conjunction with RT depends on the cellular effects achieved. Clinically, PAM and ZOL are administered once every 3-4 weeks, both in human medicine and veterinary oncology (16,27,49). BP used for treatment of canine OSA are most often administered on the first day of RT and then subsequently after RT has finished (14,16,50).
In the Kim et al study, ZOL was administered to human OSA cells 24 hours prior to RT (15). ZOL was found to promote apoptosis, cause direct DNA damage, impair DNA repair, alter cell signaling pathways and alter the proportion of cells in each phase of the cell cycle (15). Because of these effects, administration of ZOL before RT lead to enhancement of RT effects, when compared to single agent treatments.

In light of historical clinical use of BP on the concurrent day of RT, evidence of ZOL acting as a radiosensitizing agent and lack of information on the effects of BP used post RT, it was elected to compare the effects of pre, concurrent and post RT, BP treatments. For pre-RT treatment, Kim et al had evaluated a 24-hour pre-RT incubation period (15); therefore we elected to assess a longer pre-RT incubation period of 48 hours. Concurrent BP-RT treatment occurred within 2-4 hours of one another, with BP being administered prior to RT. Post-RT BP treatment occurred 24 hours following RT, as effects of RT can last at least as long as 24 hours (15) and we wanted to ensure BP were present to manage the post-RT recovery effects. All timelines chosen were considered to be reasonable clinical timelines for canine OSA patients to receive combination therapy.

4.8 Results of Timing Study

Significantly less clonogenic survival occurred when D17 or Dharma cells were treated with PAM or ZOL 24 hours post-RT, compared to 48 hours pre-RT. However, there was no significant difference between 48-hour pre-RT treatment and concurrent BP/RT treatment. These findings contradict those of Kim et al, who found ZOL to be a
radiosensitizing agent resulting in decreased cell survival when administered 24 hours before RT (15). A difference to note was that we evaluated canine OSA cells, whereas Kim et al were evaluating human OSA cells. Additionally, pre-treatment timing was different between our studies, with Kim et al treating with ZOL 24 hours pre-RT, compared to our 48-hour pre-RT treatment. These differences alone may explain the contradictory results, although Kim et al did not compare results for any other time points.

The only significant decrease in cell viability was identified when Dharma cells were treated with ZOL and RT concurrently compared to 24 hours post-RT treatment. There was no significant difference between Dharma cells treated with ZOL 48-hours pre-RT and concurrent or post-RT treatments. This may represent an outlier or alternatively, may represent ZOL's increased efficacy compared to PAM (26) or differences in OSA cell lines.

Clonogenic survival assays had more significant differences between time points, compared to cell viability assays. While we know clonogenic survival is assessing a single cell's ability to replicate (21), cell viability is assessing metabolic activity of cells (22). These findings may indicate that BP have a greater effect on a cell's replication potential than on their metabolic abilities.
4.9 Limitations

General limitations to these studies originate from the decision to perform in vitro work. By working with cells in culture, innate complicating factors arise such as continued cell passaging. This means that the cells utilized during the initial phases of experimental design, will be “younger” or will have undergone fewer passages. Ideally, in vitro work would be performed continuously, so that cells are being passaged minimally throughout the data collection period. Due to scheduling, this was not possible for our studies, which lead to differences in cell behavior from the beginning to the end of data collection. Dharma, as previously mentioned, required a higher plating density due to the innate slower growth, compared to D17. By the end of data collection, Dharma was growing at a more rapid rate, however altering cell-plating density would not allow for direct comparison of previously collected data. Additionally, intermittent lab work can lead to increased errors in calculations, performance and interpretation of data collected, due to the requirement to “re-train” or “re-learn” skills that have not been performed regularly. While loss of data and requirement to repeat experimental work in an in vitro setting is not uncommon, working within fixed time constraints can lead to lack of complete data sets.

Although an opportunity to evaluate different cell lines and different BP may be beneficial, innate differences between cell lines and BP can lead to an inability to compare results and may lead to difficulty reaching a general conclusion, as often both cell lines and BP will respond or act differently. While this information may be important in the long term, it can make data assessment more complex.
The biggest limiting factor in all in vitro work is the simplicity of the design. While minimizing confounding variables allows for ease of interpretation, it does not take all factors into consideration that would exist in an in vivo setting, which ultimately is where these results will eventually be translated. Cells in vitro are homogenous replicates of one another, which is not realistic of a true tumour, as tumour cells may differ within an individual tumour (51). In addition to tumour cell heterogeneity, the local tumour microenvironment and how a tumour interacts with the body as a whole will significantly impact not only the response to therapies, but can offer additional therapeutic targets that cannot be assessed appropriately in vitro (51–55).

4.10 Overall Impact

While these studies may not directly affect clinical decision-making, the overall impact on veterinary oncology is considerable. Our combination therapy results add to the minimal body of literature that exists to date. While we cannot refute those studies that have come before us, we can provide an alternative theory of an antagonistic relationship between BP and RT when used in combination for the treatment of canine OSA. Additionally, we have identified a possible confounding factor of timing of administration of BP in relation to RT that may contribute to the effectiveness of this combination therapy.
4.11 Future Investigations

After completing our investigations into the combination therapy of BP and RT for treatment of canine OSA cells, and timing of their administrations in relation to one another, there are many future avenues in which we can begin to translate our results. The first steps to continue our work would include comparing single agent therapy to the combined use of BP and RT in an orthotopic model of canine OSA. Further *in vitro* assessment of PAM and ZOL cellular effects, to determine an optimal timing for administration in relation to RT should continue to be investigated, followed by subsequent translation to an orthotopic model of canine OSA.

Beyond direct extensions from our work, combining multiple modalities that affect different cellular activities and promote a greater local response to RT should be considered. This could include EGFR inhibitors, topoisomerase inhibitors, nucleoside analogs, chemotherapeutic agents and many other therapies, used in combination with BP and RT.

Another interesting avenue to consider would be risk assessment for pathologic fractures associated with RT for canine OSA patients and how the risk may be altered when BP or other agents, such as parathyroid hormone (56,57), are combined with RT.

4.12 Conclusions

Combination therapy for treatment of canine OSA using BP and RT should be further scrutinized before continuing their clinical use in veterinary oncology. Combining
BP and RT is more complex than previously assumed and may result in antagonistic
effects. Timing of administration of BP in relation to RT should be further evaluated to
optimize the treatment sequence when used in combination for treatment of canine OSA.
4.13 References


8. Walter CU, Dernell WS, LaRue SM, Lana SE, Lafferty MH, LaDue T a, et al. Curative-intent radiation therapy as a treatment modality for appendicular and...


CHAPTER V: Data Appendix

Fig 5.1 Photograph of the radiation set up, with cell culture dishes placed between two solid water-equivalent blocks. A 6-MV linear accelerator was used to irradiate all experiments.
Fig 5.2 Photograph of representative clonogenic survival assays in 6-well cell culture plates. Cells were fixed and stained with 0.5% crystal violet in 20% methanol. Colonies were counted using light microscopy.
Fig 5.3 Representative photograph of a D17 colony (>50 cells aggregated) under light microscopy, following fixation and staining with 0.5% crystal violet in 20% methanol.
Fig 5.4 Clonogenic survival results for D17 treated with PAM and RT, Rep #2.
Fig 5.5 Clonogenic survival results for D17 treated with PAM and RT, Rep #3.
Fig 5.6 Clonogenic survival results for D17 treated with ZOL and RT, Rep #2.
**Fig 5.7** Clonogenic survival results for D17 treated with ZOL and RT, Rep #3.
Fig 5.8 Representative photograph of a Dharma colony (>50 cells aggregated) under light microscopy, following fixation and staining with 0.5% crystal violet in 20% methanol.
Fig 5.9 Clonogenic survival results for Dharma treated with PAM and RT, Rep #2.
Fig 5.10 Clonogenic survival results for Dharma treated with PAM and RT, Rep #3.
Fig 5.11 Clonogenic survival results for Dharma treated with ZOL and RT, Rep #2.
Fig 5.12 Clonogenic survival results for Dharma treated with ZOL and RT, Rep #3.
Fig 5.13 Photograph of representative cell viability experiment using 96-well cell culture plates. Standard media and cells in 48 wells (pink), 24 on the left for PAM treatment and 24 on the right for ZOL treatment. One dose of BP per row with 4 wells per dose. PBS (clear) fills the remaining 48 wells, creating a buffer zone between treatment groups.
Fig 5.14 Photograph of a representative cell viability assay following the addition of Resazurin to the left half of the plate. Note the immediate color change due to the addition of Resazurin.
**Fig 5.15** Photograph of a representative cell viability assay 6 hours following addition of Resazurin. Note how the color has changed secondary to the reduction of Resazurin (blue-purple) to resorufin (pink).
Fig 5.16 Cell viability results for D17 treated with PAM and RT, Rep #2.
Fig 5.17 Cell viability results for D17 treated with PAM and RT, Rep #3.
Fig 5.18 Cell viability results for D17 treated with ZOL and RT, Rep #2.
Fig 5.19 Cell viability results for D17 treated with ZOL and RT, Rep #3.
Fig 5.20 Cell viability results for Dharma treated with PAM and RT, Rep #2.
Fig 5.21 Cell viability results for Dharma treated with PAM and RT, Rep #3.
Fig 5.22 Cell viability results for Dharma treated with ZOL and RT, Rep #2.
Fig 5.23 Cell viability results for Dharma treated with ZOL and RT, Rep #3.