EFFECT OF BISPHOSPHONATES
ON GROWTH OF CANINE OSTEOSARCOMA CELLS
IN VITRO

By

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Osteosarcoma is one of the most malignant tumors in both dogs and man. Current treatment options for dogs include amputation and limb-sparing surgeries with or without adjuvant chemotherapy, and palliative radiation therapy. Adjuvant bisphosphonate therapy has been shown to decrease skeletal-related events such as hypercalcemia and bone pain; to decrease the tumor burden; and to decrease further spread of metastasis in humans. Bisphosphonates such as alendronate and pamidronate are an important class of drugs for the treatment of bone diseases. It is widely recognized that these aminobisphosphonates antagonize the mevalonate pathway, thereby disrupting osteoclast activity and inhibiting bone resorption. The molecular mechanism of the bisphosphonates is still under investigation. Formulated theories include a direct effect on osteoclasts, an indirect effect by production of an inhibitor of osteoclast recruitment by the osteoblast, or a mediated indirect action on the osteoblasts. The goal of this study was to determine the in vitro effects of alendronate and pamidronate disodium on two
canine osteosarcoma cell lines: parent osteosarcoma (POS) and highly metastasizing 
POS (HMPOS). Bisphosphonate effects were also examined using a canine fibroblast 
cell line, derived from a canine trachea.

Growth curves were established on the three cell lines to determine growth rates 
for plating purposes. The MTT assay was used to determine cell viability in the presence 
of varying bisphosphonate concentrations and a DNA fragmentation analysis was 
performed to determine if the decrease in cell viability seen in the MTT assay was due to 
apoptosis. A cell count was performed on alendronate-treated POS cells to determine if 
cell death was the mechanism through which growth rate inhibition was occurring. 
Statistical analysis comparing mean optical density between treated wells and control 
wells was performed on the MTT assay data using a Kruskal-Wallis one-way ANOVA 
on ranks with a significant value of p < 0.01. Results of the DNA analysis were analyzed 
for signs of fragmentation indicating apoptosis. Cell counts compared the number of 
viable cells versus the number of dead cells.

Alendronate exerted a significant dose- and time-dependent effect on the POS and 
HMPOS cell lines, with pamidronate possibly exhibiting the same effect. Neither 
bisphosphonate affected the viability of the normal fibroblast cells. The 72-hour 
incubation period produced the greatest decrease in cell viability, with pamidronate 
concentrations of 100 uM to 1000 uM and alendronate concentrations of 50 uM to 
1000 uM producing the greatest inhibition of cell growth. The growth inhibition seen in 
the MTT assay may have resulted from a cytotoxic effect, rather than cell-mediated 
apoptosis, on the osteosarcoma cells.
Osteosarcoma is one of the most malignant tumors in both dogs (Berg 1996) and man. Current treatment options for dogs include amputation and limb-sparing surgeries, with or without adjuvant chemotherapy; and palliative radiation therapy. Adjuvant bisphosphonate therapy has been shown to decrease skeletal-related events such as hypercalcemia and bone pain (Body and Abt 2001, Tse et al. 1996); to decrease the tumor burden; and to decrease further spread of metastasis in humans (Fromigue et al. 2000, Garcia-Moreno et al. 1998, Shipman et al. 1997). Bisphosphonates (such as alendronate and pamidronate) are an important class of drugs for treating bone diseases. It is widely recognized that these aminobisphosphonates antagonize the mevalonate pathway, thereby disrupting osteoclast activity and inhibiting bone resorption. The molecular mechanism of the bisphosphonates is still under investigation. Formulated theories include a direct effect on osteoclasts; an indirect effect (by production of an inhibitor of osteoclast recruitment by the osteoblast); and a mediated indirect action on the osteoblasts.

In humans, bisphosphonates are used clinically to treat diseases characterized by excessive osteoclast bone resorption. However, the effect of bisphosphonates on primary bone tumors remains undetermined. Several studies (Diel et al. 2000, Green 2003, Sonneman et al. 2001) have examined the effects of bisphosphonates on human and murine osteosarcoma cell lines. These investigations found that bisphosphonates had an inhibitory effect on human and murine cells in a dose- and time-dependent manner. Our investigation is the first study to focus on canine osteosarcoma cell lines. Although there
is interest in treating dogs with osteosarcoma, a controlled study has not been performed. The objectives of this study focused on determining the in vitro effects of alendronate and pamidronate disodium on two canine osteosarcoma cell lines; parent osteosarcoma (POS) and highly metastasizing POS (HMPOS). Bisphosphonate effects were also examined using a canine fibroblast cell line derived from a canine trachea. Alendronate was chosen because it is easily administered; was reported in one case study (Tomlin et al. 2000) to potentially aid in pathologic fracture repair and extend the life of two osteosarcoma dogs; and clinically in dogs appears to elicit no side effects (unpublished data). Pamidronate was chosen because it has been used sporadically to treat dogs with osteosarcoma (unpublished data). Our hypothesis is that alendronate and pamidronate disodium will elicit a time- and dose-dependent effect on POS and HMPOS cell lines, while exerting no effect on the canine fibroblast cell line.

Two osteosarcoma cell lines from an osteoblastic lineage were used in the current study including parent osteosarcoma (POS) and highly metastasizing primary osteosarcoma (HMPOS) cell lines. A normal canine fibroblast cell line established from the trachea of a 1-year-old yellow Labrador was also examined to determine the effect of bisphosphonates on a normal cell line. The POS cell line was established from a naturally occurring osteosarcoma located on the left femur of a 1.5-year-old male mongrel dog (Kadosawa et al. 1994). The spontaneous osteosarcoma was surgically removed from the dog; and cell suspensions were made from the tumor cells. The cells were routinely cultured during a 4-month period to establish a spontaneously occurring cell line. The POS cells comprise five morphologically different cells: spherical cells, fibroblast-like cells, large or small polygonal cells, and multinucleated giant cells. The
HMPOS cell line was established by injecting cells from the POS canine osteosarcoma parent cell line at the 14th passage into the back of 5-week-old irradiated BALB/cAJc1 nude mice (Barroga et al. 1999). The subcutaneous tumors were grown for 12 weeks to allow for spontaneous metastasis to the lungs. Cells were then selected in vivo by harvesting metastatic tumor deposits in the lungs, which were immediately reimplanted into new nude mice. The selection process continued for 5 cycles, 12 weeks for each cycle, totaling 15 months. After completing of the selection process, cells with greater than 90% viability were selected for expansion in culture, thus establishing the HMPOS cell line. Morphologically, HMPOS cells are medium sized and polygonal. In vivo, the HMPOS cells formed an osteoblastic type of cellular differentiation when grown subcutaneously; whereas the POS tumors consisted of an osteoblastic type of osteosarcoma with osteoid trabeculae and partial amounts of chondroblastic type, fibroblastic type, and undifferentiated type tissues (Kadosawa et al. 1994).

Alendronate exerted a significant dose- and time-dependent effect on the POS and HMPOS cell lines, with pamidronate possibly exhibiting the same effect. Neither bisphosphonate affected the viability of normal fibroblast cells. The 72-hour incubation period produced the greatest decrease in cell viability; with pamidronate concentrations of 100 uM to 1000 uM and alendronate concentrations of 50 uM to 1000 uM producing the greatest inhibition of cell growth. The growth inhibition seen in the MTT assay may have resulted from a cytotoxic effect, rather than cell-mediated apoptosis, on the osteosarcoma cells.
CHAPTER 2
REVIEW OF LITERATURE

Incidence and Risk Factors

Osteosarcoma is defined by the World Health Organization as an aggressive, malignant tumor, characterized by the direct formation of bone or osteoid tissue by the tumor cells (Misdorp and Hart 1979). Osteosarcoma is both a local and systemic disease described as one of the most malignant tumors in both dogs (Berg 1996) and man. Canine osteosarcoma closely resembles human osteosarcoma in its biological behavior, pathogenesis and histological appearance; hence it may be a valuable model for studying human osteosarcoma (Barroga et al. 1999, Brodey and Abt 1976, Brodey and Riser 1969, Kadosawa et al. 1994, Misdrop and Hart 1979, O’Brien et al. 1993,). Osteosarcoma (OSA) is the most common canine primary bone tumor, accounting for 85% of all malignant skeletal neoplasms (Brodey and Riser 1969, Dernell et al. 2001, Morello et al. 2001, Spodnick et al. 1992, Thompson and Fugent 1992). The number of reported cases of canine OSA in the United States is 8,000 to 10,000 per year (Dernell et al. 2001, Mauldin 2002, Spodnick et al. 1992); however, this most likely is an underestimation, since not all cases are confirmed nor recorded. The demographics of canine osteosarcoma have been well documented. There is a wide range in onset of the disease; however it is commonly a disease of middle-aged to older canines with a median age of 7 years (Brodey and Riser 1969, Dernell et al. 2001). Large and giant breeds have a sixty times greater risk of developing osteosarcoma as compared to smaller breeds (Berg 1996,
Mauldin 2002). Osteosarcoma rarely occurs in dogs weighing less than 20 kg (Berg 1996). The Saint Bernard, Great Dane, golden retriever, Irish setter, German shepherd, and Doberman pinscher are the breeds most at risk; although size appears to be a more important factor than breed. The most predictive factors for canine osteosarcoma appear to be increasing weight and height (Dernell et al. 2001). Canine osteosarcoma appears to develop more frequently in males than females at a 1.2:1.0 ratio, with the Saint Bernard as the exception. More specifically, intact males and females have a higher risk for developing OSA than neutered canines (Ru et al. 1998). Approximately 75% of canine osteosarcoma tumors occur in the appendicular skeleton, while the remaining 25% occur in the axial skeleton (O’Brien et al. 1993). Weight-bearing long bones of the appendicular skeleton are more frequently affected than non-weight-bearing bones (Misdorp and Hart 1979). Within the appendicular skeleton, twice as many tumors occur in the forelimbs (bearing approximately 60% of the body weight) than in the rear limbs with the metaphyseal region of long bones being the most common site of occurrence. The primary locations in the front limbs are the distal radius and proximal humerus. Tumors rarely occur in sites adjacent to the elbow, whereas in the hind limbs, the tumors are evenly dispersed between the distal femur, distal tibia and proximal tibia (Berg 1996, Brodey and Riser 1969, Dernell et al. 2001, O’Brien et al. 1993). Primary osteosarcoma in extraskeletal sites is rare, but it has been reported in the mammary tissue, subcutaneous tissue, spleen, bowel, liver, kidney, testicle, vagina, eye, gastric ligament, and adrenal gland (Dernell et al. 2001).

**Etiology**

Canine osteosarcoma pathogenesis is generally unknown. The accepted theory is very simplistic and is based on circumstantial evidence. Osteosarcoma tends to primarily
occur in the major weight-bearing bones adjacent to late closing physes in larger breed dogs. Sensitive cells are located in the physeal region and multiple minor trauma and subsequent injury to this area may induce mitogenic signals increasing the probability for the development of a mutant lineage, thus initiating the disease (Dernell et al. 2001). Metallic implants used for fracture repair, chronic osteomyelitis, ionizing radiation and fractures with no internal repair have all been reported caustic of canine osteosarcoma (Dernell et al. 2001). Radiation therapy rarely causes osteosarcoma in people and is even less common in dogs (Dernell et al. 2001). Molecular genetic models suggest a loss of function of either Rb gene (retinoblastoma) or p53 gene. Both act as tumor suppressor genes; therefore osteosarcoma tumorgenesis may be a loss of function of these two genes (Dernell et al. 2001). In canine osteosarcoma, p53 gene appears to be over expressed. It has a higher frequency of occurrence in appendicular than flat bones suggesting it is a factor in the more aggressive form of the disease (Sagartz et al. 1996). Other factors that may contribute to the malignant phenotype are IGF-1 and its receptor IGF-1R, and hepatocyte growth factor (HGF) and its receptor c-met (MacEwen et al. 2000).

**Presentation and Treatment Options**

Clinical characteristics of osteosarcoma include chronic, progressive lameness and/or a firm swelling over the metaphysis of a long bone (Berg 1996, O’Brien et al. 1993). Locally, osteosarcoma is productive, lytic, with the most common presentation being mixed productive and lytic. Soft tissue swelling is often present, with other common occurrences including pathological fracture of the affected bone and metastasis to the lung, bones or soft tissue sites (Dernell et al. 2001). Radiography is a diagnostic tool used to confirm a primary bone tumor; however, the procedure does not allow a distinction to be made between a primary bone tumor (fibrosarcoma, chondrosarcoma or
hemangiosarcoma) and osteosarcoma (Berg 1996), as lysis of cortical bone is most suggestive of bone neoplasia. For a definitive histological diagnosis, bone biopsies are performed as an open-incisional, trephine biopsy using a Michelle trephine, or a closed-biopsy using a Jamshidi bone marrow biopsy needle (Berg 1996, Dernell et al. 2001). Thoracic radiographs should also be obtained before treatment in an attempt to identify pulmonary metastases. Usually subclinical, ranges of 80-98% of dogs with appendicular osteosarcoma have micrometastases (less than 1 cm in diameter) that are radiographically undetectable at initial diagnosis (Berg 1996, Mauldin 2002). Within 5 to 12 months following amputation surgery for osteosarcoma, lung metastasis represents the cause of death in approximately 90% of dogs (Brodey and Abt 1976, MacEwen and Kurzman 1996, Spodnick et al. 1992). Survival of dogs with radiographically visible pulmonary metastases have an extremely guarded prognosis and their life most likely will not be extended by chemotherapy treatment such as cisplatin or doxorubicin (Berg 1996).

Pending a positive diagnosis of OSA in a dog, the tumor should be removed by surgical amputation or a limb-sparing surgery. Amputation is the most common surgical procedure for treating osteosarcoma in the appendicular skeleton (Morello et al. 2001, O’Brien et al. 1993, Spodnick et al. 1992), and is usually a complication free procedure that is well tolerated by patients if no significant neurological and/or orthopedic disease is present in the remaining limbs (Berg 1996, O’Brien et al. 1993, Straw 1996). Extremely large, heavyset dogs including Newfoundlands or Saint Bernards are poor candidates for amputation (Berg 1996). For animals that are poor candidates for surgical amputation, limb-sparing surgery offers an alternative treatment (Morello et al. 2001). Limb-sparing consists of an “en bloc” tumor resection and replacement with a fresh-frozen cortical
bone allograft, fixed to the host bone with a dynamic compression plate and screws. Arthrodesis of the nearby joint is usually required. Although allografts cannot contribute living cells for osteogenesis, they are able to allow osteoinduction, and are incorporated to some extent, in the recipient (Morello et al. 2001). The most suitable candidates for limb-sparing are dogs with radial OSA not involving more than 50% of the length of the diaphysis, since a stable host allograft system cannot be achieved if the allograft is too long and only a few screws (less than four) can be inserted in the host bone after OSA removal (Straw and Withrow 1996). Infection is a complication of an allograft surgery, however, Straw (1996) reported that dogs that develop infection have a longer survival than those that do not, perhaps due to increased immune system stimulation.

After surgical removal of the tumor, through amputation or limb-sparing surgery, adjuvant treatment such as chemotherapy or radiotherapy should be administered due to the metastatic potential of the disease and to increase the life expectancy of the animal. If chemotherapy is used as an adjuvant treatment, the type of surgery (limb sparing vs. amputation) does not appear to influence the survival time (Morello et al. 2001, O’Brien et al. 1993). The median survival time is up to one year if chemotherapy with cisplatin or doxorubicin is administered with a limb amputation or limb sparing surgery producing a one-year survival rate of about 50% (Berg 1996, Straw and Withrow 1996) and a two-year survival rate of only 20% to 25% (MacEwen and Kurzman 1996). Cisplatin and doxorubicin are the most effective chemotherapeutic agents, either given as single doses or in combination (Berg et al. 1992). However, improved post-surgical survival time has been reported following administration of cisplatin at 290 days (Thompson 1992), doxorubicin (Berg et al. 1995), cisplatin alternated with doxorubicin at 300 days (Chun et
al. 2001), and carboplatin (Liptak et al. 2001). Without adjuvant treatment following surgery, lung metastases represent the cause of death within 5 to 12 months in 90% of dogs with osteosarcoma, with a one-year survival rate of about 10% (Berg 1996, Morello et al. 2001, O’Brien et al. 1993). The goal of chemotherapy is to prolong life without compromising its quality. Because veterinarians aim to minimize toxic side effects, the intensity and duration of chemotherapy in dogs are far lower than in human oncology (Berg 1996).

Palliative radiation therapy provides an alternative treatment option for owners of animals with appendicular osteosarcoma where neither surgical amputation nor limb-sparing surgery is a feasible option. Radiation therapy involves administration of small fractions of ionizing radiation in a short time period for the purpose of pain relief, thus increasing the patient’s comfort level (Thrall and LaRue 1995). External beam radiation may be an option for an animal that is not responding to oral analgesics, often administered for the purpose of bone pain relief. It appears to have a role in treatment of primary bone tumors, metastatic bone lesions, and possibly non-skeletal tumors. In humans, methods of palliation include targeting the tumor, half-body irradiation that has produced significant dose-limiting side effects, and partial-body irradiation that has not been reported thus far in animals (Thrall and LaRue 1995). Lastly, bisphosphonates such as alendronate or pamidronate are being evaluated for their potential use as palliative therapeutic agents for primary and bone metastatic canine osteosarcoma. Although many clinicians are prescribing bisphosphonates for osteosarcoma, efficacy in dogs has yet to be established.
Bisphophonates

Bisphophonates are analogs of endogenous pyrophosphate, a naturally occurring inhibitor of bone metabolism, characterized by a carbon bond (P-C-P) with various side chains replacing the P-O-P bond of pyrophosphate (Coleman 2001, Diel et al. 2000, Porras et al. 1999, Rodan and Fleisch 1996, Tse et al. 1996). Their development occurred after the discovery that pyrophosphate inhibits the dissolution and formation of calcium phosphate crystals, therefore indicating a role for bisphosphonates in inhibition of bone resorption (Porras et al. 1999, Rodan and Fleisch 1996). The anti-resorptive activity of the bisphosphonates was first reported in 1968 (Fleisch et al. 1968) and was originally developed for osteoporosis. Recently this class of drugs has been gaining increasing popularity in the oncology field (Body and Abt 2001). The P-C-P group gives the bisphosphonates resistance to enzymatic hydrolysis, resulting in biochemical stability and insignificant metabolism, thus unaltered excretion (Rodan and Fleisch 1996). The variable side chains of the bisphosphonate result in large variations in potency and determine the pharmacological activity (Coleman 2001, Halasy-Nagy et al. 2001, Rodan and Fleisch 1996, Sonnemann et al. 2001, Tse et al. 1996). Based on the side chains, the bisphosphonates can be separated into two classes: nitrogen-containing BPs (alendronate and pamidronate) that contain one or more nitrogen atoms within R₂ or non-nitrogen BPs (etidronate or clodronate) that have CH₃, Cl⁻ or a nitrogen-free ring structure (tiludronate) at R₂ (Halasy-Nagy et al. 2001).

\[
\begin{align*}
R^2 & \\
\text{O}_3\text{P} - \text{C} - \text{PO}_3 & \\
R^1 & 
\end{align*}
\]

Figure 2-1. General chemical structure of a bisphosphonate
Bisphosphonates have a long half-life ranging from 1 to 10 years that is dependent upon the rate of bone turnover since bisphosphonates are liberated again only after the bone in which they are deposited is resorbed (Lin 1996). They are non-toxic to other organs and are clinically well tolerated (Fleisch 1998). The rapid clearance from plasma and low exposure of visceral tissues may explain why bisphosphonates are well tolerated while being so toxic to bone. Effects on bone growth, remodeling, mineralization, incidence of fracture and repair differ between bisphosphonates due to their chemical structure, pharmacological profile, dosage levels, and route of administration (Peter et al. 1996).

Bisphosphonates have been used successfully for many years in the treatment of diseases such as hypercalcemia, breast cancer, Paget’s disease, osteoporosis, multiple myeloma, and in the reduction of skeletal-related complications of metastases including pathologic fractures and bone pain. Currently, the anti-tumor effect of bisphosphonates is being investigated. Generally, bisphosphonates are used in treatment and/or prevention of diseases in which osteolysis occurs. Osteolysis, essentially caused by osteoclast stimulation by paracrine factors released from tumors, underlies most, if not all, bone metastasis, therefore providing a rationale for treatment with bisphosphonates. Eighty percent of patients with advanced cancer have skeletal metastases (Vinholes et al. 1996). Hypercalcemia, pathologic fractures and pain are the major sources of morbidity with metastases in humans with pain as the most common symptom occurring in 70% of patients with bone metastases (Vinholes et al. 1996). Bisphosphonates have been shown to reduce skeletal complications by approximately 30 to 40% (Diel et al. 2000). Hypercalcemia is a common metabolic complication associated with malignant diseases
(Tse et al. 1996) arising from increased bone breakdown caused by bone lesions or humoral factors (Coleman 2001). Hypercalcemia is expected to occur in 8 to 10% of all malignant diseases (Tse et al. 1996) and is best treated with intravenous bisphosphonates and hydration (Body and Abt 2001, Tse et al. 1996). A dramatic increase in bone resorption characterizes hypercalcemia, thereby increasing calcium levels (Body and Abt 2001). With bisphosphonate therapy, approximately 70% to 90% of human patients can achieve normal calcium concentrations, resulting in relief of symptoms and improved quality of life (Coleman 2001). Bisphosphonates cause a marked reduction in these skeletal events associated with metastatic cancer to bone, in addition to reducing the tumor burden in bone (Body et al. 1998, Coleman 2002). According to Coleman (2001), phase III studies in humans have shown that concomitant bisphosphonate therapy with radiation and/or other systemic therapy causes less of a deterioration in quality of life and less pain, than with systemic or radiation therapy alone.

Bisphosphonates may affect cells other than osteoclasts. Several studies (Fromigue et al. 2000, Garcia-Moreno et al. 1998, Shipman et al. 1997) have demonstrated the cytotoxic and proapoptotic effect bisphosphonates have on human tumor cells in vitro. However, conditions in vitro differ from osteolytic metastasis in vivo. Tumor cells secrete cytokines and growth factors stimulating tumor proliferation by increasing the formation and activity of osteoclasts. Bone resorption also stimulates the release of bone-bound growth factors (Goltzman 2001) while at the same time; bisphosphonate bound to bone is released and may exert a cytotoxic effect on both osteoclasts and tumor cells. The local concentration of bisphosphonate at the lytic tumor site is unknown, however values ranging from 0.1 to 1 mM have been calculated for
alendronate at an active osteoclastic resorption site (Sato et al. 1991). In human tumor
cell lines (myeloma, breast, prostate, pancreas), bisphosphonates have been shown to
exert cytotoxic and proapoptotic effects in a time-and dose-dependent manner at
concentrations from 5 to 2000 uM in vitro (Green 2003). These concentrations may
appear to be higher than expected as compared to levels obtained in the plasma.
However, these levels are obtainable within bone at active resorption sites (Sato et al.
1997) as bisphosphonate levels increase with repetitive dosing. A dose- and time-
dependent effect of bisphosphonates on human osteosarcoma cell lines has also been seen
in vitro (Sonneman et al. 2001). The dosing regimen for humans with established bone
metastases is 90 mg of intravenous pamidronate every 3 to 4 weeks, effectively
preventing or delaying the occurrence of skeletal complications. This dosing regimen
may also cause regression of bone lesions and improve overall survival. A frequent
dosing regimen with more potent bisphosphonates may be beneficial for achieving even
distribution of drug throughout the skeleton. Animal models have shown a
bisphosphonate-induced reduction in skeletal tumor burden compared with control
animals, as well as an inhibition of tumor-induced osteolysis (Coleman 2002).

In addition to tumor proliferation, the metastatic process includes other essential
steps such as tumor cell adhesion and invasion, secretion of growth factors and cytokines,
and angiogenesis (Boissier et al. 2000). Bisphosphonates alter the metastatic process and
create a less favorable microenvironment for tumor cell colonization, thus exerting an
anti-tumor effect. Bisphosphonates have been shown to directly inhibit adhesion of
tumor cells to the extracellular matrix of bone and to inhibit the process of invasion and
metastasis (Boissier et al. 1997, Boissier et al. 2000, Magnetto et al. 1999, van der Pluijm
et al. 1996). The binding of human breast and prostate cancer cells to mineralized and unmineralized matrices has been inhibited by bisphosphonate treatment (Boissier et al 1997, Magnetto et al. 1999, van der Pluijm et al. 1996). For tumor cell invasion to occur, the basement membrane must be digested by proteases. The activity of several matrix metalloproteinase enzymes (MMP-1, -2, -3, -7, -8, -9, -12, -13, -14) has been inhibited by bisphosphonates in vitro with concentrations in the range of 50-150 uM (Boissier et al. 2000, Teronen et al. 1997, Teronen et al. 1999). Growth regulatory substances are released during bone resorption, which stimulate tumor cell proliferation. Tumor cells secrete parathyroid horomone related protein (PTHrP), promoting osteoclastic bone resorption and causing additional release of more growth factors such as transforming growth factor β (TGF- β) and insulin-like growth factor-1 (IGF-1). TGF- β stimulates tumor cell production of PTH-rP, perpetuating a cycle of tumor osteolysis. This cycle is broken by bisphosphonate inhibition of osteoclast activity reducing the release of excess growth factors from bone resulting in reduction of tumor growth and the number of tumors in bone. Secretion of cytokines and growth factors from monocytes, osteoblasts, bone marrow stromal cells and macrophages are inhibited by bisphosphonates (Green 2003). Angiogenesis and neovascularization, which are essential for tumor growth and survival, are markedly inhibited by bisphosphonates (Boissier et al. 2000).

Bisphosphonates exert the greatest anti-tumor effect in bone, but may have a possible systemic anti-tumor effect. Findings from a study (Alvarez et al. 2002) in which mammary carcinoma cells were injected directly into the proximal tibia of rats showed a 95% reduction in lung nodules following treatment with 8 µg/kg of alendronate. Bisphosphonates may have an immunomodulatory effect that contributes to their anti-
tumor activity. In vitro and in vivo studies have shown bisphosphonate regulation on subpopulations of circulating lymphocytes and on the function of antigen-presenting cells (Cuenca et al. 2001, Pecherstorfer et al. 2000, Sansoni et al. 1995). N-BPs stimulate the proliferation of a specific gamma/delta T-cell subset in primates that is cytotoxic against lymphoma, myeloma, neuroblastoma, and carcinoma cell lines in vitro (Baker et al 2001, Das et al 2001, Kunzmann et al. 2000), and in vivo against MC-IXC tumors in SCID mice (Baker et al. 2001).

The main pharmacological action of bisphosphonates is inhibition of bone resorption without directly suppressing bone formation. However, the action mechanism is currently unknown. The targets of bisphosphonate action at the cellular level include osteoclasts, osteoblasts, macrophages, and possibly tumor cells (Diel et al. 2000). The final target is the osteoclast with bisphosphonate treatment first inhibiting osteoclast activity followed by osteoclast apoptosis. After administration, bisphosphonates travel to the site of exposed bone mineral and because of their strong affinity for calcium phosphate, strong binding to the hydroxyapatite occurs. The bone then absorbs the drug, leading to a high concentration of drug around the resorbing osteoclasts. Most of the tumor-induced skeletal destruction is due to osteoclast activity. All bisphosphonates can induce osteoclast apoptosis (Halasy-Nagy et al. 2001). Osteoclasts break down the calcium phosphate crystals, liberating the bound bisphosphonate, which is then internalized by the osteoclast, eventually leading to apoptosis (Coleman 2001). First generation bisphosphonates, such as clodronate, induce osteoclast apoptosis by becoming metabolized to cytotoxic analogs of ATP (Frith et al. 1997). Aminobisphosphonates, or second-generation bisphosphonates, appear to induce apoptosis by interfering with
mevalonate metabolism therefore inhibiting protein prenylation (Rogers et al. 2000). Prenylation is necessary for cell survival and function such as osteoclast signaling proteins, glutamyl transpeptidases (GTPases) that regulate osteoclast cytoskeleton organization, vesicle transport, membrane ruffling, and apoptosis. Thus, inhibition of prenylation yields a quiescent cell (Diel et al. 2000, Fisher et al. 1999, Luckman et al. 1998). Nitrogen-containing bisphosphonates (N-BPs) have demonstrated the molecular target in osteoclasts as farnesyl diphosphate (FPP) synthase (Bergstrom et al. 2000). Inhibition of FPP synthase reduces the downstream concentration of geranylgeranyl-diphosphate (GGPP) thereby disrupting the prenylation of small GTP-binding proteins, especially the proteins of around 20kDa, belonging to the rho, rac, Cdc42, and rab families, that are necessary for cell function and survival (Bergstrom et al. 2000, Dunford et al. 2001, Green 2003, Luckman et al. 1998, Rogers et al. 2000, van Beek et al. 1999). For instance, inactivation of rhoA was shown to cause osteoclast inactivation and rab proteins are important for vesicular fusion to membranes (Sogaard et al. 1994), which becomes impaired following administration of alendronate (Sato et al. 1991). Inhibition of FPP and the subsequent disruption of the function of these proteins can lead to osteoclast apoptosis and the consequent reduction in bone resorption (Fisher et al. 1999, Luckman et al. 1998, Reszka et al. 1999, van Beek et al. 1999). Halasy-Nagy et al. (2001) demonstrated that for alendronate and risedronate, osteoclast apoptosis is not the earliest or sole means by which bone resorption is inhibited. Their findings suggest that N-BPs inhibition of bone resorption occurs prior to and separately from osteoclast apoptosis since N-BPs have the ability to inhibit bone resorption in the presence of Z-VAD-FMK, an anti-apoptotic caspase inhibitor. The separation between inhibition of
bone resorption and the reduction in osteoclast number or apoptosis can be seen in time
and across a dose-response curve. The precise mechanism of their anti-resorptive action
at the cellular level is still unclear. Reduction of osteolytic bone resorption is thought to
occur through inhibition of osteoclast recruitment and activity on the bone surface, and a
possible cytotoxic effect on the osteoclast causing apoptosis (PDR 2002, Rodan and
Fleisch 1996). In addition, bisphosphonates decrease acid extrusion thus decreasing the
dissolution of bone mineral, stimulate changes in the cytoskeleton, and alter enzymatic
inhibition of bone resorption required lower doses of alendronate and risedronate as
compared to dosage requirements for osteoclast apoptosis. This suggests

geranylgeranylated GTPases controlling osteoclast function, such as cytoskeletal
organization, formation of ruffled border and vesicular traffic, might be more sensitive or
have a higher turnover rate than those responsible for controlling cell viability.

Another postulated mode of action is an indirect effect by production of an
inhibitor of osteoclast recruitment by the osteoblast (Fleisch 1994, Hu 2002, Rodan and
Fleisch 1996). A study conducted by Sahni et al. (1993) suggests that the inhibition of
the bisphosphonates on murine osteoclasts may occur through a mediated indirect action
on the osteoblasts. Mathov et al. (2001) reported bisphosphonates, olpadronate,
pamidronate, and etidronate, produced stimulatory effects on human osteoblastic cells by
through activation of extracellular signal-regulated kinases (ERKs). ERK-mediated
response occurred following short-term exposure of bisphosphonates with concentrations
ranging between $10^{-8}$ and $10^{-6}$M. Once higher concentrations were reached, the response
did not remain.
Pamidronate and Alendronate

Two second-generation bisphosphonates presently in use are pamidronate and alendronate. Both are nitrogen-containing aminobisphosphonates meaning the amino group is contained in the aliphatic carbon chain, yielding much higher potency and far greater selectivity for inhibiting bone resorption than etidronate, a non-nitrogen containing bisphosphonate and one of the first bisphosphonates used clinically. (Lin 1996, Rodan 1998). The length and contents of the aliphatic carbon chain appears to be an important factor in potency and pharmacological activity (Halasy-Nagy et al. 2001, Shinoda et al. 1983). Due to the differences in potencies, the clinical doses differ dramatically among the bisphosphonates.

Aredia, or pamidronate disodium (APD), is designated chemically as the disodium salt of 3-amino-1-hydroxypropylidene-1, 1 bisphosphonate, and its chemical structure is shown in Figure 2-2 (PDR 2002).

![Chemical structure of pamidronate disodium](image)

Figure 2-2. Chemical structure of pamidronate disodium

Its main pharmacological action is inhibition of bone resorption, although the mechanism of anti-resorptive action is not clearly understood. Pamidronate is administered intravenously at concentrations that have a minimal effect on bone mineralization while remaining a potent bone resorption inhibitor unlike earlier bisphosphonates (Boonekamp et al. 1987, Reitsma et al. 1980). It is available in 30 mg and 90 mg vials with standard administration of a 90 mg 1- to 2-hour infusion (Body 2001) given every 3 to 4 weeks continuously until patients present a clinical decline (Finley 2002). Oral administration
of pamidronate results in extremely poor absorption from the small intestine (<1%). Drug therapy at dosing regimens of 600 mg have proven effective in treatment of tumor osteolysis, but produced an unacceptable rate of side effects (Diel et al. 2000). Intravenous pamidronate will produce better prophylactic results. Pamidronate is not metabolized and is exclusively eliminated by renal excretion (PDR 2002). In rats given intravenous injections of radiolabeled pamidronate, approximately 50% to 60% of the compound was rapidly absorbed by bone and slowly eliminated from the body by the kidneys. The compound was cleared from circulation and mainly taken up by bones, teeth, spleen, liver, and tracheal cartilage. The majority of bone uptake occurred in areas of high bone turnover and the terminal phase of elimination half-life in bone was estimated to be approximately 300 days (PDR 2002). Concerning pharmacodynamics, decreases in serum phosphate and calcium levels, urinary calcium/creatinine and urinary hydroxyproline/creatinine ratios occur after administration of pamidronate, with the attainment of normal levels within 7 to 10 days (PDR 2002).

Side effects for use of pamidronate disodium include renal toxicity via renal tubular necrosis manifested as deterioration of renal function and potential renal failure. Primarily the kidney excretes unmetabolized pamidronate and those with impaired renal function have a greater risk for adverse reactions than does a person with a normal functioning kidney. Prior to, and concurrent with, administration of Aredia, serum creatinine levels should be assessed in patients to measure renal function. In humans, single doses should not exceed 90 mg and the duration of the intravenous infusion should be no less than two hours due to the risk of clinically significant deterioration in renal function, which may progress to renal failure (PDR 2002).
nephropathy has been associated with intravenous (bolus and infusion) administration of Aredia. A tolerance study was performed using dogs wherein Aredia was given 4 or 24 hours at doses of 1-20 mg/kg for up to 7 days. The compound was tolerated at 3 mg/kg, which is 1.7 times the highest recommended human dose for a single intravenous infusion when administered for 4 or 24 hours (PDR 2002). Adverse events of the liver and kidney occurred when Aredia was infused for 1 hour at doses of \( \geq 10 \) mg/kg. In another study, dogs received doses of 2, 4, 6 and 20 mg/kg of Aredia as a one-hour infusion, once a week, for 3 months followed by a one-month recovery period. At 2 mg/kg, which is 1.1 times the highest recommended human dose, no adverse renal effects were found. At 20mg/kg, moribundity/death and renal toxicity occurred. Elevated BUN and creatinine levels occurred at \( \geq 6 \) mg/kg and renal tubular degeneration occurred at \( \geq 4 \) mg/kg. The kidney changes were partially reversible at \( \geq 6 \) mg/kg (PDR 2002).

Aredia should also be used cautiously in pregnant women because of the harm that may come to the fetus. Pamidronate has been shown to cross the placenta in rats and has produced maternal toxicity and embryo/fetal effects when given during organogenesis at doses of 0.6 to 8.3 times the highest recommended human dose for a single intravenous infusion (PDR 2002). It is unknown whether pamidronate is excreted in human milk, and so caution should be exercised with nursing mothers. The safety and effectiveness in pediatric patients has not been established.

Adverse reactions that can occur with oral Aredia therapy tend to be specific to a disease. The reactions commonly due to intravenous therapy include asymptomatic hypocalcemia, fever, and redness, swelling, indurations, and pain upon palpation at the site of catheter insertion (Tse et al. 1996). Seizures can occur, although the majority
occurs in those with pre-existing seizure disorders. Other antagonistic reactions include nausea and back pain (PDR 2002).

Pamidronate inhibits bone resorption without affecting bone growth and mineralization (Fitton and McTavish 1991). An investigation administering pamidronate to dogs for one year revealed that trabecular thickness and trabecular bone volume had a dose-related increase following pamidronate treatment (Grynpas et al. 1992). The gains in trabecular bone volume and mechanical properties were maintained a year after completion of treatment. Pamidronate is the standard bisphosphonate used for treatment of bone metastases (Coleman 2001, Hilner et al. 2000) and is primarily used in the treatment of hypercalcemia, osteoporosis, Paget’s disease, breast cancer and skeletal-related events. Presently, pamidronate and clodronate are the two most widely used bisphosphonates in oncology (Body 2001). In a study performed by Hortobagyi et al. (1996), pamidronate was shown to prevent the emergence of skeletal-related events. Pamidronate 90 mg was administered intravenously to 382 breast cancer patients every 3-4 weeks. Compared to the placebo, the skeletal-related events, such as pathologic fracture, spinal cord compression, occurrence of bone pain, and hypercalcemia of malignancy, were reduced 45% versus 65%, and the time to the first skeletal-related event increased from 7.0 to 13.1 months (Coleman 2001). El Abdaimi et al. (2000) performed a study where the bisphosphonate pamidronate was administered subcutaneously to female nude mice inoculated with a human breast cancer cell line. Fewer than 10% of the mice injected with high doses of pamidronate developed bone metastases, whereas 85% of the mice receiving the vehicle alone developed metastases. Pamidronate reduced the total number of osteolytic bone lesions by more than 95% when
compared to the control. This indicates a potential anti-tumor effect of pamidronate on not only the primary bone tumor, but on metastases as well.

Hypercalcemia of malignancy is caused by osteoclastic hyperactivity resulting in excessive bone resorption. Polyuria and gastrointestinal disturbances occur with increasing dehydration and decreasing glomerular filtration rate, as a result of the excessive release of calcium into the blood as the bone is resorbed. A cycle of worsening systemic hypercalcemia results from the increased renal resorption of calcium. Hence, adequate fluid administration to correct volume deficits and correction of excessive bone resorption are essential to the management of hypercalcemia (PDR 2002). In a double blind clinical trial, 52 patients with hypercalcemia of malignancy received 30 mg, 60 mg, or 90 mg of Aredia as a single 24-hour intravenous infusion if their corrected serum calcium levels were $\geq 12.0$ mg/dL after 48 hours of saline hydration. Twenty-four hours after initiation of treatment, 64% of patients had decreases in serum calcium levels. By 7 days after initiation of treatment with pamidronate, normal-corrected serum calcium levels were achieved in 40% (30 mg), 61% (60 mg) and 100% (90 mg) of the patients (PDR 2002).

Paget’s disease (osteitis deformans) is an idiopathic disease characterized by excessive and disorganized resorption and formation of bone. These changes result in structurally defective bone that is thickened but weakened and prone to fracture or to bend under stress (Tse et al. 1996). Signs and symptoms may be bone pain, deformity, fractures, neurological disorders resulting from cranial and spinal nerve entrapment and from spinal cord and brain stem compression, increased cardiac output to the involved bone, increased serum alkaline phosphatase levels (reflecting increased bone formation)
and/or urine hydroxyproline excretion (reflecting increased bone resorption) (PDR 2002). Aredia is indicated in the treatment of patient’s with moderate to severe Paget’s disease of bone. Aredia therapy has been shown to decrease the risk of bone fractures (Peter et al. 1996) and significantly reduce serum alkaline phosphatase and urinary hydroxyproline levels (Gallacher et al. 1991, Ryan et al. 1992) by ≥50% in at least 50% of patients and by ≥30% in at least 80% of patients (PDR 2002). In one study involving 30 patients with symptomatic Paget’s disease, 15 mg, 30 mg or 45 mg pamidronate was given at 6 weekly intervals on three occasions (Stone et al. 1990). The patients were assessed 6 months after the beginning of the study for adequate control of bone turnover. If this was not achieved, the course of treatment was repeated. Sixteen (53%) patients developed a normal bone turnover within the first year of treatment. These findings were consistent with findings from another study in which 30 patients received weekly infusions of 30 mg pamidronate for 6 weeks (Ryan et al. 1992). Subjective improvement or resolution of pain in over 80% of the patients was reported in both studies. However, complete resolution of pagetic lesions was uncommon.

A current study produced by Sonnemann et al. (2001) examined the effect of pamidronate on human osteosarcoma cell growth in vitro. Pamidronate inhibited osteosarcoma cell growth in a time- and dose-dependent manner. Growth inhibition was found to be independent of apoptosis when examined using DNA fragmentation analysis. Previous studies have shown bisphosphonates to induce apoptosis on human breast cancer cells (Fromigue et al. 2000, Senaratne et al. 2000), human myeloma cells (Shipman et al. 1997), and murine osteoclasts (Hughes et al. 1995) and macrophages (Luckman et al. 1998). Sonnemann et al. (2001) found no caustic effect of the
bisphosphonate on cellular induction of apoptosis. Moreover, they demonstrated an inhibitory effect of pamidronate on the viability of human osteosarcoma cells, thus indicating an anti-apoptotic role of N-BPs.

Another second generation bisphosphonates is alendronate sodium (Fosamax). This particular bisphosphonate is a white, crystalline, nonhygroscopic powder that is chemically described as (4-amino-1-hydroxybutylidene) monosodium salt trihydrate. Alendronate’s structural representation is shown in Figure 2-3.

![Figure 2-3. Chemical structure of Alendronate](image)

Alendronate is a fairly safe drug, and is generally well tolerated pending the dosing recommendations are adequately followed and the patients do not have concurrent esophageal disease or symptomatic reflux (Body 2001, Baran 2001). It is not recommended for patients with severe renal insufficiency (creatinine clearance <35 mL/min) (Nace 1999). Patients are instructed to take Fosamax upon arising for the day with a full glass of plain water at least one-half hour before consuming any food; beverage or other medication to help ensure the tablet clears the esophagus quickly. Patients should not lie down for at least 30 minutes and until after their first food of the day to help reduce the risk of reflux. Absorption is poor with oral bioavailability of about 0.5 to 1.0% (Bone et al. 2000, Porras et al. 1999) and food, beverages (other than water), and waiting less than 30 minutes after drug administration substantially reduce
bioavailability, thus lessening the effect of the drug. A study performed in rats showed a 6- to 7-fold decrease in bioavailability when oral alendronate was administered in the presence of food (Lin et al. 1991). Products containing calcium, magnesium and other multivalent cations are likely to interfere with absorption of alendronate by forming insoluble complexes (Baran 2001, PDR 2002, Porras et al. 1999). Beverages such as orange juice or coffee can reduce bioavailability by about 60% as compared with water (Gertz et al. 1995). However, intravenous rantidine was shown to double the bioavailability of oral alendronate. The clinical significance of this increased bioavailability and whether similar increases will occur in patients given oral H₂ – antagonists is unknown (PDR 2002).

Failure to follow administration instructions may increase the risk of esophageal complications such as symptomatic reflux, epigastric pain, esophagitis, esophageal ulcers, and esophageal erosions, occasionally with bleeding and rarely followed by esophageal stricture or perforation (Baran 2001, Nace 1999, PDR 2002). Cases resulting in esophageal complications have been reported, but need to be put into perspective. In an earlier survey, >60% of the serious esophageal events were related to improper administration of alendronate such as swallowing the tablet without water or use in patients with such contraindications as swallowing disorders. Contraindications for use include abnormalities of the esophagus that delay esophageal emptying, inability to stand or sit upright for at least 30 minutes, hypersensitivity to any component of this product, and hypocalcemia which must be corrected before initiating therapy with Fosamax. Local irritation of the upper gastrointestinal mucosa may also occur. Caution should be used when Fosamax is administered to patients with active upper gastrointestinal
problems such as dysphagia, esophageal disease, gastritis, duodenitis or ulcers because of the possible irritant effects on the upper gastrointestinal mucosa (PDR 2002). In summary, the following adverse reactions have been reported with use of alendronate: hypersensitivity reactions including urticaria and rarely angioedema, occasional skin rash, rarely uveitis, esophagitis, esophageal erosions and ulcers, rarely esophageal stricture or perforation, and oropharyngeal ulceration. Gastric or duodenal ulcers have also been reported (PDR 2002).

A very long half-life lasting greater than 10 years is characteristic of this drug, as it will accumulate in bone with additional treatments. Alendronate inhibition of bone resorption and osteoclast activity (Peter et al. 1996, Porras et al. 1999) does not interfere with bone formation, thus leading to a positive bone balance. Hu et al. (2002) assessed the effects of 0.5 mg/kg of alendronate/day over 12 weeks on trabecular bone properties in dogs. They subjected 16 mature female hound dogs to bilateral femoral condyle implantation using the revision model technique of implant loosening (Bechtold et al. 2001). They reported a decrease in bone surface-to-volume ratio in all bone sites and significant increases in trabecular thickness and bone density thus improving mechanical properties. Like the other bisphosphonates, the mechanism of action for inhibition of resorption is unknown. Accepted thought is that alendronate bind to calcium salts, thus blocking the transformation of calcium phosphate into hydroxyapatite. This inhibits the formation, aggregation, and dissolution of hydroxyapatite crystals in bone that in turn leads to inhibition of bone mineralization. Alendronate does not interfere with the recruitment or attachment of osteoclasts; rather it preferentially binds to localized sites of greatest bone turnover and becomes solubilised by the acidic conditions generated by the
osteoclasts. Alendronate is then taken up by the osteoclasts rendering them inactive for bone resorption (Sahni et al. 1993). The osteoblasts subsequently commence forming new bone tissue at the former resorption site. The alendronate that is deposited at sites of resorption, if not taken up by the osteoclasts, is incorporated into the bone matrix and is no longer pharmacologically active until the drug is brought back to the surface by resorption; thus allowing it to interact with the osteoclasts (Porras et al. 1999). Alendronate reduces the number of sites at which bone is remodeled and produces progressive gains in bone mass at resorption sites by allowing bone formation to exceed bone resorption (PDR 2002). The exact molecular mechanisms of these actions are unknown, but it appears that alendronate causes disruption of osteoclast cytoskeleton and loss of the ruffled border, which are signs that the osteoclast is no longer active (Nace 1999, Porras et al. 1999). The anti-resorptive effects of bisphosphonates may be mediated by macrophages and osteoblasts (Nace 1999, Porras et al. 1999). Alendronate inhibits the production of interleukin (IL)-1β, IL-6 and tumor necrosis factor- α (TNF) by monocytes (Nace 1999), all which stimulate bone resorption. Inhibition of bone resorption diminishes after treatment completion, suggesting not all alendronate sequestered in bone is biologically active. Continuous administration is necessary to maintain osteoclast suppression on newly formed resorption surfaces. Not only is osteoclast activity reduced, but also the number of osteoclasts is also significantly reduced after long-term administration of alendronate. Whether this is secondary to the reduction in bone resorption and the dynamics of bone turnover, a separate effect to reduce osteoclast recruitment and/or differentiation, or the induction of osteoclast apoptosis, or all of the above, is not known (Hughes et al. 1995).
After systemic distribution, alendronate binds primarily to protein, more specifically the predominant protein albumin. Calcium concentration and pH are two parameters that affect the extent of the binding. In rats, dogs, and monkeys, with a drug concentration range of 0.1 to 0.5 mg/mL, approximately 80, 73, and 70% of alendronate is protein bound (Porras et al. 1999). In humans, approximately 78% of alendronate is bound to protein found in plasma. As stated previously, alendronate favors deposition at the most active sites of bone resorption. Since distribution within bone is determined by blood flow, trabecular bone uptake of the drug is greater than cortical bone, with greater uptake at the metaphysis as compared with the diaphysis of cortical bone (Lin et al. 1991). Trabecular and cortical bone is continuously remodeled at the microscopic level of its basic multicellular units (BMU). The numbers of BMU functioning at a given time determine the rate of bone remodeling to replace old or damaged bone. Generally the turnover rate is greater in trabecular than cortical bone because trabecular bone has a greater number of remodeling sites per unit of bone volume than does cortical bone (Baran 2001, Bone et al. 2000). A normal bone remodeling cycle is completed in 180 to 200 days and is comprised of four sequential steps: initiation, resorption, reversal, and formation. Initiation consists of alteration of lining cells that cover the bone surface, resulting in activation of the bone remodeling cycle (Baran 2001, Bone et al. 2000). Resorption is the next step in the remodeling cycle where osteoclasts resorb the exposed mineralized tissue left exposed by the removal of the lining cells. This occurs over a period of 2 to 3 weeks, creating microscopic pits (lacunae) on the bone surface (Kanis 1996). Over the course of the next 7 to 10 days, bone resorption is reversed, mediated partly by growth factors released by bone matrix. Lastly, the osteoblasts migrate to the
lacunae previously created by the osteoclasts and refill it with new osteoid, which
becomes well mineralized within 1 to 2 weeks, but continuously mineralizes over the
following 4 to 6 months (Bone et al. 2000, Kanis 1996).

Alendronate is cleared from the plasma by uptake in the skeleton or by the kidney
where it is excreted. The portions of the gastrointestinal tract with larger surface areas
appear to better absorb alendronate (jejunum>duodenum>ileum) (Lin et al. 1994). Forty
to sixty percent of an oral dose is retained for a long time in the body (Porras et al. 1999),
with approximately 50% being sequestered in bone over the short term (Bone et al.
2000). The drug is slowly released from bone accounting for the prolonged multiple-
phase elimination with the terminal half-life ($t_{1/2}$) dependent on the rate of bone turnover
in the specific species. In humans, the terminal half-life has been estimated at 12 years
(Khan et al. 1997). A half-life of at least 1,000 days has been estimated in dogs and
approximately 300 days in rats (Lin et al. 1991). About 1/3 of alendronate bound to
remodeling sites, slowly dissociates back into plasma over several weeks and is then
excreted by the kidneys (Porras et al. 1999). Within 24 hours of dosing, approximately
40% of a single IV dose is excreted in the urine while the other 60% is rapidly cleared
from the plasma (Khan et al. 1997). As with other bisphosphonates, there is no evidence

Alendronate is cleared from the body unmetabolized through renal excretion. A
negligible amount (<0.2%) is detected in feces after IV administration, suggesting little,
if any, is excreted in bile (Porras et al. 1999). In the rat, an active secretory system is
used for renal excretion of the drug with a maximum rate of about 25 mg/min/kg (Lin et
al. 1992). Certain compounds have shown an effect on the quantity of alendronate
excreted by the kidneys. Etidronate, a first generation bisphosphonate reduced the renal clearance in a dose-dependent manner, as did high concentrations of inorganic phosphate (Lin et al. 1992). In rats, the same effect was found using uranyl nitrate, which concurrently produced reductions in renal clearance in a dose-dependent manner and increased amounts of deposited alendronate in bone (Lin et al. 1992). The pharmacokinetics of alendronate are similar to other bisphosphonates and although these studies have not been undertaken in humans, the evidence is in strong support that the same pharmacokinetics of alendronate would apply. The majority of information has been collected from urinary excretion data since the concentrations in plasma following administration of therapeutic doses is not high enough for assay detection.

Alendronate is mainly used for the treatment and prevention of osteoporosis in postmenopausal women and is intended to inhibit bone resorption, reduce the rate of bone turnover and increase bone mass (Baran 2001). Osteoporosis is characterized by low bone mass that leads to an increased risk of fracture. This disease occurs in both males and females, but has a higher frequency of occurrence in females following menopause. Post-menopausal changes in the woman’s body cause an increase in bone turnover and the rate of bone resorption exceeds that of bone formation, thus leading to progressive bone loss. Common consequences of osteoporosis are fractures of the spine, hip and wrist; with hip fractures most commonly associating with substantial morbidity, disability and mortality (Baran 2001, PDR 2002). Clinically, alendronate is approved as an oral medication for the treatment (10 mg/d) and prevention (5 mg/d) of osteoporosis in postmenopausal women and corticosteriod-induced osteoporosis (5 or 10 mg/d), as well as for the treatment of Paget’s disease (40 mg/d for 6 months) (Bone et al. 2000, Porras et
al. 1999). Intravenous alendronate is being investigated for the management of hypercalcemia of malignancy (Porras et al. 1999). Alendronate has been shown to reduce the incidence of fractures and improve bone density in the hip and spine after 2 to 4 years of administration, with the majority of the increase occurring in the first year (Bone et al. 2000). In the Fracture Intervention Trial, 6,000 women enrolled: 2,000 women who already had vertebral compression fractures and established osteoporosis and 4,000 women who did not. The women with vertebral fractures were administered oral Fosamax for 3 years with a dosage of 5 mg/day for the first two years and 10 mg/day for the last year. The women without fractures were administered either oral Fosamax or a placebo. By the end of the trial, bone density increased in >90% of the women taking Fosamax whether or not they had vertebral fractures. (Cummings 1998). Quanot and Thompson (2000) showed alendronate consistently reduced the risk of new hip fractures in multiple trials with an overall risk reduction of greater than 50%. Data collected from another study on treatment of osteoporosis in postmenopausal women with Fosamax indicated the rate of bone turnover reached a new steady state, despite the progressive increase in the total amount of alendronate deposited within bone. A dose of 5 mg/day decreased osteocalcin and total serum alkaline phosphatase by approximately 40% and 15%, respectively. Similar reductions in the rate of bone turnover were observed in postmenopausal women during one-year studies with 70 mg once weekly Fosamax for the treatment of osteoporosis and 35 mg once weekly Fosamax for the prevention of osteoporosis. Patients given 10 mg/day for osteoporosis treatment, showed decreased markers of bone formation, osteocalcin, and bone specific alkaline phosphatase decreased by approximately 50%, and total serum alkaline phosphatase decreased by approximately
25% to 30%. These levels reached a plateau after 6 to 12 months (PDR 2002).

Generally, in studies of postmenopausal women with osteoporosis, BMD increases significantly following alendronate therapy within 3 months of initiation of treatment with a concurrent significant reduction in the incidence of nonvertebral fractures in as little as 12 months.

Studies in animal models of osteoporosis have been conducted to test the efficacy and safety of less than daily dosing of alendronate. Dosage amounts and mode of administration (parenteral or oral) were adjusted to provide relevance to human oral dosing for osteoporosis. Bone mineral density and bone strength both increased in the animal models receiving parenteral treatment at intervals up to 2 weeks (Bone et al. 2000). Balena et al. (1993) performed a study of the effects of a 2-year treatment with alendronate on bone metabolism, bone histomorphometry and bone strength in ovariectomized primates. Increases in bone turnover and decreases in BMD are common changes seen in women following menopause. Ovariectomized baboons also experience bone metabolism and bone strength changes. Ovariectomized baboons received intravenous alendronate once every two weeks for a period of two years (Balena et al. 1993). Alendronate produced reduced bone turnover in a dose-dependent manner when compared to control (nonovariectomized) levels. Dose-dependent increases in spine BMD and bone strength were produced, but indices of mineralization were not altered. Balena et al. (1993) concluded that bone strength was strongly related to BMD and that parenteral alendronate is effective given at a frequency of once every 2 weeks in animals. Parenteral dosing of alendronate less frequently than once daily has also prevented bone loss in preclinical studies of bone loss due to secondary hyperparathyroidism (dosed
twice weekly) (Rodan 1996), immobilization (dosed twice before immobilization) (Thompson et al. 1992), and hyperthyroidism in rats (dosed twice weekly) (Balena et al. 1993). These preclinical data proved a rationale for weekly dosing with alendronate for human osteoporotic bone loss, regardless of the primary etiology (Bone et al. 2000).

In 2001, the FDA approved two new strengths of alendronate sodium including 70 mg for treatment and 35 mg for prevention of osteoporosis for once a week dosing. These doses have been shown to be therapeutically equivalent to the standard 10 mg once daily regimen of the drug (Baran 2001). Inhibition of bone resorption diminishes after completion of treatment, suggesting that not all alendronate sequestered in bone is biologically active. Because the half-life of residence on bone surfaces is several weeks, weekly administration of alendronate should inhibit bone resorption to an overall extent similar to that of daily dosing, thereby producing similar increases in bone mass and strength (Bone et al. 2000, Nace 1999). The efficacy and safety of once weekly dosing versus daily oral alendronate therapy was compared in a randomized, placebo-controlled trial (Schnitzer et al. 2000). The trial comprised of 889 woman ages 42 to 95 years with postmenopausal osteoporosis defined by either decreased BMD of the lumbar spine or femoral neck at least 2.5 standard deviations less than the peak premenopausal mean or prior vertebral or hip fracture. For 12 months, 519 of the women received 70 mg/week and 370 of the women received 10 mg/day of Fosamax. Supplements of calcium and vitamin D were also administered. Similar reductions in bone turnover and increase in BMD occurred with both dosing regimens and the differences found between the daily and weekly doses were not statistically significant, thus the two dosing regimens were found to be therapeutically equivalent. With osteoporosis, bone loss is slow and
progressive, therefore effective management of this chronic disease requires long term adherence to drug treatment. A weekly dosing regimen offers a more convenient, therapeutically equivalent alternative to daily dosing regimens (Baran 2001, Bone et al. 2000).

Weekly or biweekly dosing regimens have been suggested to be less damaging to the esophagus. Keratinized tissue covers the esophagus and continuous exposure to bisphosphonates inhibits keratinocyte growth in culture (Halasy-Nagy et al. 1999). Normal proliferation resumes following removal of the bisphosphonate. Thus, tissue repair in response to insults such as regurgitated gastric acid may be inhibited by bisphosphonates. In 1994, Peter et al. performed studies on the effect the frequency of alendronate had on esophageal irritation. Their data suggested that repair of the esophageal keratized tissue due to gastric-acid induced injury may be inhibited by continuous exposure to oral bisphosphonates. Daily exposure of a low dose of alendronate caused esophageal irritation and ulceration in 25% to 75% of the dogs, while once weekly exposure to a relatively high does of alendronate, even under acidic conditions mimicking a worst-case scenario (acid reflux containing alendronate, with prolonged esophageal exposure at every dose), may not be sufficient to cause clinically significant injury to the esophagus. Exposure for only 1 day per week did not have as great of an affect, if any, on the esophageal mucosa is because it regenerates approximately every 5 days, allowing time for the tissue to heal before the next exposure, preventing cumulative injury. Thus, not only are weekly dosing regimens more convenient, but also the efficacy is equal to daily dosing regimens with a greater potential for less complications.
The development of osteoporosis and the associated fractures are commonly associated with sustained use of glucocorticoids (PDR 2002). Fosamax is indicated for the treatment of glucocorticoids-induced osteoporosis in men and women who have low bone mineral density and are receiving glucocorticoids in a daily dosage equivalent to 7.5 mg or greater of prednisone and (PDR 2002). These patients should receive adequate amounts of calcium and vitamin D. In clinical studies of up to two years duration, Fosamax 5 and 10 mg/day reduced cross-linked N-telopeptieds of type I collagen (a marker of bone resorption) by approximately 60%. Bone-specific alkaline phosphates and total serum alkaline phosphatase (markers of bone formation) were reduced by approximately 15 to 30% and 8 to 18%, respectively (PDR 2002). In a different study on the efficacy of Fosamax on glucocorticoids-induced osteoporosis, 5 mg and 10 mg of alendronate were administered once daily to men and women receiving glucocorticoids (at least 7.5 mg/day of prednisone or equivalent). This double blind, randomized, placebo-controlled study was performed twice over a one-year period, once in the United States and the other in 15 different countries. These studies enrolled 232 and 328 patients, respectively, between the ages of 17 and 83 years with a variety of glucocorticoids-requiring diseases. Patients were administered calcium and vitamin D supplements. Bone mineral density (BMD) was measured in the lumbar spine, femoral neck and trochanter. Significant increases relative to the placebo in BMD were seen at each of these sites in patients receiving either 5 mg/day or 10 mg/day; except in postmenopausal women not receiving estrogen therapy. Estrogen deficiency contributes to an increase in osteoclastic resorption and/or decrease in osteoblastic formation, leading to microscopic bone loss at the basic multicellular units (Baran 2001). In these women,
increases (relative to placebo) with Fosamax 10 mg/day were greater than those with 5 mg/day at the lumbar spine (4.1% vs. 1.6%) and trochanter (2.8% vs. 1.7%), but not at other sites. Fosamax was proven effective regardless of dose or duration of glucocorticoids use. Fosamax was similarly effective regardless of age (<65 vs. ≥65 years), race (Caucasian vs. other races), gender, underlying disease, baseline BMD, baseline bone turnover and use with a variety of common medications (PDR 2002).

The third disease alendronate has been approved for use in is prevention of Paget’s disease of bone. Paget’s disease of bone is a chronic, focal skeletal disorder characterized by greatly increased and disorderly bone remodeling. Excessive osteoclastic bone resorption is followed by osteoblastic new bone formation, leading to the replacement of the normal bone architecture by disorganized, enlarged and weakened bone structure (PDR 2002). Clinical indicators of this disease range from no symptoms to severe morbidity due to bone pain, bone deformity, pathological fractures, and neurological and other complications (PDR 2002). Candidates for treatment with Fosamax have alkaline phosphatase levels at least two times the upper limit of normal, those who are symptomatic or those at risk for future complications from this disease (PDR 2002). Clinically, Fosamax 40 mg once daily for six produced significant decreases in serum alkalkine phosphatase (the most frequently used biochemical index of disease activity) as well as in urinary markers of bone collagen degradation. Fosamax induced mild, transient and asymptomatic decreases in serum calcium and phosphate as a result of bone resorption inhibition (PDR 2002).

There has been only one case report published on the use of bisphosphonates in dogs with appendicular osteosarcoma. In a clinical trial conducted by Tomlin et al.
(2000) alendronate was used for the palliative management of osteosarcoma in two dogs. One dog, a 12-year-old male Belgian shepherd dog, suffering a pathologic fracture of the right tibia secondary to an osteosarcoma, underwent surgical reduction and stabilization with a six pin Type II transarticular external skeletal fixator. Forty days post-surgery, 10 mg of alendronate was administered daily. Five month after diagnosis, the tibial fracture had healed radiographically, although with a malunion. The degree of tibial osteolysis was unchanged. At seven months post diagnosis, the dog presented with lameness due to loosening of the external fixator. New osteolytic tracts had formed around the pin tracts and periosteal remodeling adjacent to the tumor site was evident. The external fixator was removed. By twelve months, the osteolytic tumor had grown progressively and the dog was euthanized due to limb ulceration and decreased activity level. Histopathy confirmed osteosarcoma with lung, liver and kidney metastases. The second dog, a 7-year-old male Labrador, had an osteosarcoma of the right zygomatic arch and caudal maxilla. A caudal maxillectomy was not performed and palliative management was initiated with 10 mg of alendronate every 24 hours starting 30 days after diagnosis. Two months post-diagnosis, the tumor had increased in size by approximately 25%, but the dog remained comfortable. The dose of alendronate was increased to 20 mg every 24 hours, but the mass continued to enlarge and after six months of alendronate treatment it was approximately 50% larger than originally. The alendronate dose was reduced to 10 mg every 24 hours due to expense and lack of remission exhibited by the tumor. Both dogs remained comfortable and survived for 12 and 10 months respectively after diagnosis. Both dogs had no clinically detectable side effects. Dog 1 survival time was equivalent to the survival time of a patient with limb-sparing tumor resection followed by
chemotherapy. Dog 2 survival time was approximately double the median survival time for axial osteosarcoma (Heyman et al. 1992, Hammer et al. 1995, Pirkey-Ehrhart et al. 1995) and showed no radiographic evidence of pulmonary metastasis after 6 months of treatment (Tomlin et al. 2000). This clinical trial indicates the clinical usefulness of bisphosphonates in the treatment of canine osteosarcoma. However, since this is the only published case report on the use of bisphosphonates in dogs with appendicular osteosarcoma, further research is needed to determine the value of this therapy on certain tumors, its side effects, and the most effective dosing regimens.
CHAPTER 3
METHODOLOGY

Cell Lines and Culture Maintenance

The canine osteosarcoma cell lines studied were POS and HMPOS. The POS (parent osteosarcoma) cell line was developed from a primary osteosarcoma of the left proximal femur of a 1.5-year-old male mongrel dog. Dr. Tsuyoshi Kadosawa at the University of Sapporo in Japan developed the HMPOS cell line (highly metastasizing primary osteosarcoma). The fibroblast cell line was derived from a canine trachea (ATCC). The osteosarcoma cell lines were cultured in RPMI 1640 medium supplemented with filtered additives of 200 mM L-glutamine, 100 mM sodium pyruvate, 10 mM non-essential amino acids solution, 10,000 µg/mL penicillin-streptomycin, and 10% fetal bovine serum in a humidified atmosphere of 5% CO$_2$ at 37°C. Fibroblasts were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with filtered additives of 200 mM L-glutamine, 100 mM sodium pyruvate, 10 mM non-essential amino acids solution, 10,000 µg/mL penicillin-streptomycin, and 10% fetal bovine serum in a humidified atmosphere of 5% CO$_2$ at 37°C. The cells were grown to approximately 80-90% confluency in a 75cm$^2$ flask, washed with 10 mL Hanks balanced salt solution, and detached using 5 mL of 0.25% trypsin. Flasks were placed into the incubator for approximately 10 minutes and were intermittently examined under a light microscope until cells detached from the flask. Ten milliliters of the respective medium was added to inhibit the trypsin. The cell-rich media was then removed with a pipette and placed into 15 mL tubes for centrifugation at 1300 rpm for 10 minutes. The supernatant was
decanted and the cells were resuspended in 5 mL of medium. Twenty microliters of the cells were stained with 20 µL trypan-blue dye and counted using a hemacytometer. HMPOS and POS cells were passaged once or twice weekly, and the fibroblast cells were passaged once every other week.

**Growth Curve**

The growth curves were performed in 6-well flat-bottomed microtiter plates. HMPOS cells were initially plated at 5,000 cells/well in RPMI and placed in the incubator. POS cells and canine trachea fibroblasts were initially plated at 10,000 cells/well in RPMI and DMEM, respectively, as their growth rates were slower than that of HMPOS. At 24-hour intervals, cells were washed with Hanks balanced salt solution and removed from the well using trypsin. Media was added to the detached cells to inhibit the trypsin. The cell-rich suspension was removed from the well and placed into a centrifuge tube and centrifuged at 1300 rpm for 10 minutes. The supernatant was decanted off and the cells were resuspended in 1 mL of respective media. Twenty microliters of the cells were stained with 20 µL trypan-blue dye and counted using a hemacytometer.

**MTT Assay for Cell Viability**

The assays were performed in 96-well flat-bottomed microtiter plates. Cells were seeded at 5,000 or 10,000 cells/well, depending upon their growth rates: HMPOS at 5,000, POS and fibroblasts at 10,000 cells/well. Plates were placed in the incubator for 24 hours. At 24 hours, the media was removed and replaced with 100 µL of media containing the respective bisphosphonate, alendronate or pamidronate. Mannitol was
also tested as pamidronate is composed of 470 mg mannitol and 30 mg pamidronate. For pamidronate and mannitol, dilutions of 1,000 µM, 500 µM, 100 µM, 50 µM, 10 µM, 5 µM and 1 µM with two control lanes containing no bisphosphonate were used (Figure 4A). The plate formation for alendronate included dilutions of 1,000 µM, 500 µM, 100 µM, 50 µM, 10 µM, 5 µM, 1 µM, 10⁻⁷ uM, 10⁻⁸ uM, 10⁻⁹ uM, and 10⁻¹⁰ uM with one control lane that contained no drug (Figure 4B). The 96-well flat-bottomed microtiter plate setup consisted of 1 row consisting of 8 wells per drug concentration with the pamidronate-treated plates having 2 rows or 16 wells of control. The alendronate-treated plates contained 1 row of control. Plates were incubated for 24, 48, and 72 hours, after which 10 µL of MTT reagent (thiazolyl blue; Sigma) in phosphate-buffered saline was added reaching a final concentration of 5.5 mg/mL in each well. A four-hour incubation period followed, after which 100 µL of 50% dimethylformamide in 10% SDS was added to dissolve the insoluble product. The absorbance of the wells was then measured at 540 nm using an Elisa microplate reader (EL 340 microplate Bio Kinetics Reader). The MTT assay was run in triplicate for each of the drugs at each different time interval.

**DNA Fragmentation Analysis for the Evaluation of Apoptosis**

Cells were grown to approximately 80-90% confluency in 75 cm² flasks, and prior to trypsinizing, the media containing non-attached cells was collected and centrifuged at 1,300 rpm for 10 minutes. The remaining attached cells were washed with 10 mL Hanks balanced salt solution, and then detached using 0.25% trypsin. Either DMEM or RPMI medium was added depending on the cell line to stop the trypsin reaction. The cell-containing media was then removed and centrifuged at 1,300 rpm for
10 minutes. The supernatant was decanted, the centrifuge tube vortexed and attached and non-attached cells were lysed in 5 mL of Tris buffer for 1 hour at 37°C. An additional 1 mL of Tris buffer was added along with 5 µL protease K, and the mixture was vortexed and incubated for 3 hours at 56°C. At the midpoint of the incubation time, an additional 5µL protease K was added. Equal amounts (1:1 ratio) of phenol and chloroform were mixed to form phenol-chloroform mixture from which samples were extracted. From the phenol-chloroform mixture, an equal amount matching the solution in which the cells were suspended was pipetted into the tube containing the cells. The tube was inverted until a cloudy solution formed and was centrifuged for 20 minutes at 2,200 rpm. The chloroform layer containing the DNA was extracted and placed into a new centrifuge tube. Additional chloroform-phenol mixture was added and extracted until a good separation of layers occurred. After the final chloroform layer was extracted, 100% ethanol was added in a 1:1 ratio with the remaining phenol along with 0.6 mL 3M sodium acetate which helped lower the pH, allowing the DNA to come out of solution. DNA was placed in the freezer at –20°C overnight.

After removal from the freezer, DNA was centrifuged at 2,200 rpm for 10 minutes. The supernatent (ethanol) was removed and the remaining DNA was vortexed and resuspended in 1 mL of 70% ethanol. DNA was vortexed again, transferred to a microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes. This process was repeated twice. Following the final centrifugation, ethanol was decanted and the microcentrifuge tube was inverted to dry. After the DNA was dry, 1 mL sterile water was added, vortexed and placed in 37°C water bath, with intermittent vortexing, for approximately 30 minutes or until the solution was not viscous. Two microliters of DNA
were removed and diluted with water purified with Barnstead Diamond Nanopure Water System (filtered and UV purified). Absorbance readings were taken at 260 nm for DNA content and 280 nm for protein content. DNA was placed in –20°C freezer until further use.

DNA was removed from the freezer and dissolved in TE buffer. The RNA was then digested with 50 µg/mL Rnase A at 37°C for 30 min prior to electrophoresis. Following digestion, aliquots of 4 µg of each sample were electrophoresed at 70 V with molecular weight markers of 123 bp multiples on a 1.0% agarose gel. Electrophoresis was completed once the dye front migrated 1cm from the bottom of the gel, which in this study took approximately 3 hours. Following the completion of electrophoresis, the gel was placed in 1 µg/mL ethidium bromide for 30 minutes causing the DNA to fluoresce. After 30 minutes, the gel was removed from ethidium bromide and placed in purified water for 30 minutes. At 30 minutes, the gel was removed from the purified water and gel images were captured on the computer.

**Cell Counts**

Cell counts were performed simultaneously with the final MTT assay for alendronate on one run at 24, 48, and 72 hours. Cell counts using trypan blue staining are a measurement of the number of viable versus dead cells. The same plate was used simultaneously for both the cell counts and the MTT cell viability assay. The cells in the last two wells of the 96-well flat-bottomed microtiter plates were stimulated and mixed thoroughly with a pipet prior to removal of 10 uL of cell-rich media. The last two wells in the plate used for cell counting, did not receive MTT reagent nor were they analyzed by the ELISA reader for cell viability data. The 10 uL of cell-rich media was placed in
another 96-well flat-bottomed microtiter plate and diluted with 2 uL trypan blue. The total volume of 12 uL was removed and placed on a hemacytometer for counting on a light microscope (40x).

**Data Analysis**

Statistical analysis comparing mean optical density between treated wells and control wells was performed on the MTT assay data using a Kruskal-Wallis one-way analysis of variance on ranks with a significant value of $p<0.01$ as compared to control. Kruskal-Wallis ANOVA on ranks was used because the data distribution was not adjusted to the normal distribution. More specifically, pairwise multiple comparison procedures, the Student-Newman-Kuels method was employed for non-parametric data with equal population size and the Dunn’s method was used for non-parametric data comparison of unequal sized populations. This analysis was performed separately for each time period.

The results of the DNA fragmentation analysis were assessed through visual observation. The lanes of the agarose gel were visually analyzed and compared to the control lane containing molecular weight markers of 123 bp multiples for signs of fragmentation thus indicating apoptosis. As the sample size was not large enough for a statistical analysis, only descriptive comments are provided.
Figure 3-4. Plate setup for MTT assay. A) A 96-well flat-bottomed microtiter plate used in the MTT assay for pamidronate and mannitol. Eight lanes have decreasing concentrations of pamidronate disodium or mannitol starting at 1000uM and decreasing to 1uM with 2 lanes as control, containing no drug (marked with 0). There were 8 wells for each concentration except for control with 16 wells. B) Eleven lanes have decreasing concentrations of alendronate starting at 1000uM and decreasing to $10^{-10}$ uM with 1 control lane containing no drug (marked with 0). There were 8 wells for each concentration and 8 wells for the control. The MTT assay was run in triplicate at each separate time interval of 24, 48, and 72 hours. There was a minimum of 24 wells or data points for each drug concentration at each specific time interval.
CHAPTER 4
RESULTS

Morphology and Growth Patterns in Cell Culture

The canine fibroblasts were large, very elongated spindle-shaped in appearance (Fig 4-5). They displayed a very slow growth rate and were the slowest growing cells with a doubling time of approximately 72 hours. Fibroblasts were grown in culture in 75 cm² flasks for 2.5 weeks before passaging. The POS cells contained several morphologic types of cells from small to large cells, spherical or polygonal in shape, and fibroblast-like cells (Fig 4-6B). The doubling time for the POS cells grown in culture for one week was between 24 to 48 hours. The reported growth rate ranges between 32±1.3 hr (Barroga et al. 1999) and 33hr (Kadosawa et al. 1994). The morphology of the HMPOS (highly metastasizing) cell line was comprised of medium sized, polygonal cells (Fig 4-6A). HMPOS cells had a rapid growth rate and a doubling time of less than 24 hours. These cells were seeded in 75cm² flasks at a low concentration of 2.5 x 10⁻⁵ cells/ml and were passaged every 4 to 5 days. At day 4, the HMPOS cell numbers were increased as compared to POS cells at day 4 (Fig 4-7).

Pamidronate

Effect on growth of POS cells by pamidronate

Mean optical density for 48 and 72- hour control samples was significantly higher than control mean optical density at 24 hours. Treatment with pamidronate demonstrated a significant inhibitory effect on cell growth as compared to the control following a 24-hour incubation at all drug concentrations 1 uM up to 1000 uM (Figure 4-8). Forty-eight
hours of incubation produced a significant decrease in mean optical density at 500 uM and 1000 uM. Following a 72-hour incubation period, pamidronate concentrations of 1, 10, 500, and 1000 uM produced significant growth inhibition on POS cells. At 72 hours, there was a significant increase in mean optical density at 50 uM and 100 uM. The lowest percent cell viability for POS cells was 47.16% and occurred with the 72-hour incubation at 1000 uM (Figure 4-8B).

Effect on growth of HMPOS cell line by pamidronate

Mean optical density for 72-hour control samples was significantly higher than control mean optical density at 48 hours, which in turn was significantly higher than control mean optical density at 24 hours (Figure 4-9). Pamidronate produced significant growth inhibition at concentrations of 1 uM and 1000 uM at 24 hours. A significant stimulatory effect at 100 uM occurred following a 24-hour incubation period. At 48 hours, cell growth was significantly inhibited at 500 uM and 1000 uM. A 72-hour incubation period at drug concentrations of 500 and 1000 uM produced significant decrease in cell viability. Pamidronate appeared to have a dose- and possibly a time-dependent inhibitory effect on HMPOS cells. In reference to Figure 4-9B, cell viability decreased from 100 uM to 1000 uM for drug incubations of 48 and 72 hours, and from 500 uM to 1000 uM for 24 hours. The 72-hour incubation period produced a decrease in cell viability from 98.38% at 100 uM to 27.43% at 1000 uM. The lowest percent cell viability for HMPOS cells was 27.43% and occurred with the 72-hour incubation at 1000 uM.

Effect on growth of fibroblast cells to pamidronate

No significant inhibition of cell growth as compared with the control (no drug) was produced at any drug concentrations (Figure 4-10). Drug incubation time had no
significant effect on fibroblast growth. The lowest cell viability was seen at 72 hours at a
drug concentration of 1000 μM was 77.3% or a 22.7% reduction in cell growth.
However, this reduction was not significant when compared to control.

Summary of pamidronate effects on POS, HMPOS, and fibroblast cell lines is
shown in Figure 4-11. At 72 hours and 1000 μM where growth inhibition was the
greatest, the cell viability was 47.16% for POS cells, 27.43% for HMPOS cells, and
75.60% for fibroblasts.

Mannitol

Effect of Mannitol on POS cell line

Pamidronate disodium is comprised of 470 mg mannitol and 30 mg pamidronate.
The effect of mannitol on cell growth was tested using a MTT cell viability assay.
Mannitol exhibited a significant stimulatory effect on POS cells at 24 hours with
concentrations ranging from 100 μM up to 1000 μM (Figure 4-12). Mannitol did not
significantly stimulate or inhibit POS cells at 48 or 72 hour time periods at any
concentration.

Effect of Mannitol on HMPOS cell line

A MTT cell viability assay was performed to determine the effect of mannitol on
the HMPOS cell line. A significant stimulatory effect was found at 24 hours with drug
concentrations 5, 10, 100, 500, and 1000 μM (Figure 4-13). A 72-hour incubation at 500
μM produced a significant stimulatory effect. No concentration of mannitol (1 μM –
1000 μM) exhibited a significant inhibitory effect on cell growth. Mannitol, if anything,
stimulated growth.
Alendronate

Effect of growth on POS cells by Alendronate

Mean optical density for 48 and 72-hour control samples was significantly higher than control mean optical density at 24 hours. A 24-hour incubation period produced significant growth stimulation at alendronate concentrations of 100 uM to 1000 uM (Figure 4-14). A 48-hour incubation showed growth inhibition at 50 uM and 500 uM. A 72-hour incubation produced significant growth inhibition at concentrations ranging from 50 uM to 1000 uM. Alendronate doses ≤10 uM do not appear to have an inhibitory effect on POS cells. A 72-hour incubation period resulted in the lowest cell viability of 45.08% at 50 uM. An incubation period of 24 hours produced the highest cell viability (148.7%) at 500 uM.

Effect on HMPOS cell growth by Alendronate

Mean optical density for 48 and 72-hour control samples was significantly higher than control mean optical density at 24 hours (Figure 15). No significant stimulatory or inhibitory effect on cell growth occurred after a 24-hour incubation period. At 48 and 72 hours, alendronate treatment induced a significant inhibitory effect on the HMPOS cell line at drug concentrations from 50 uM to 1000 uM. Concentrations ≤10 uM did not significantly affect cell growth. A stimulatory effect was seen at 500 uM after a 24-hour incubation, resulting in a cell viability of 127.5%. However, this was not a significant increase as compared to the control. Growth inhibition occurred in a time- and dose-dependent manner with 48 hours at 50 uM producing a cell viability of 59.73% and 72 hours at 100 uM producing the lowest cell viability (49.8%).
Effect on fibroblast cell growth by alendronate

Alendronate did not have a significant effect on fibroblast cells (Figure 4-16). The lowest cell viability (71.49%) was recorded after 24 hours at a drug concentration of 500 uM, however this was not a significant decrease from control.

Summary of alendronate effects on POS, HMPOS, and fibroblast cell lines is shown in Figure 4-17. At 72 hours where growth inhibition was the greatest, the cell viability at 100uM was 51.39% for POS cells, 49.83% for HMPOS cells, and 92.27% for fibroblasts. As 100 uM produced the lowest cell viabilities for HMPOS and fibroblast cells, 50 uM produced the lowest cell viability for POS cells at 45.08%.

Assessment of apoptosis in pamidronate and alendronate-treated cells

Apoptosis was evaluated by DNA fragmentation analysis. Drug treatments consisting of pamidronate concentrations of 500 uM and 1000 uM and 50 uM of alendronate were applied to the HMPOS and POS cell lines. These concentrations were determined by the MTT cell viability assay, as they appeared to have the greatest inhibition of cell growth (refer to Fig 4-8, -9, -14, -15). Lanes 2 (HMPOS) and 5 (POS) in the pamidronate gel contain the controls (no drug) and Lanes 3 (HMPOS) and 5 (POS) are the control lanes for alendronate. DNA cleavage was not observed in the pamidronate nor alendronate treated cells.

Assessment of cell viability using cell counts

Cell counts were an essential requirement for causal determination of cell growth inhibition as proven by the MTT cell viability assay. Cell counts using trypan blue staining are a measurement of the number of viable cells and dead cells. Additional cell counts are required reinforce the findings of the single cell count that was performed. Over time and increasing drug concentration, the cell viability percentage decreases as
shown in Figure 4-19. Hence, at 50 uM to 1000 uM alendronate concentrations, the percentage of dead cells increases with regards to concentration. Alendronate concentrations <10 uM did not appear to have an effect on POS cell viability.
Figure 4-5. Microscopic view of fibroblast cells. Canine trachea fibroblast cells (large, elongated spindle-shaped cells)

Figure 4-6. Microscopic view of HMPOS and POS cells. A) HMPOS cells (medium-sized, polygonal cells)  B) POS cells (small to large spherical or polygonal cells and spindle-like cells)

Figure 4-7. Fibroblast, POS, and HMPOS cell growth over time.
Figure 4-8. Pamidronate-treated POS cells. A) Results of the MTT cell viability assay for the effect of pamidronate on growth of the POS cell line. Mean optical density measured at 540 nm at 24, 48, and 72 hours for concentrations ranging from 0 (control) to 1000uM. * = Significant difference between the labeled data point and the control (p<0.01). B) Percent cell viability of the POS cell line after treatment with pamidronate. Cell viability was calculated using the following equation: (treated mean optical density/untreated mean optical density) x 100.
Table 4-1. Standard deviations for the effect of pamidronate on growth of the POS cell line for 24, 48, and 72 hours.

<table>
<thead>
<tr>
<th>Concentration (uM)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
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Figure 4-9. Pamidronate-treated HMPOS cells. A) Results of the MTT cell viability assay for the effect of pamidronate on growth of the HMPOS cell line. Mean optical density measured at 540 nm at 24, 48, and 72 hours for concentrations ranging from 0 (control) to 1000 uM. * = Significant difference between the labeled data point and the control (p<0.01). B) Percent cell viability of the HMPOS cell line after treatment with pamidronate. Cell viability was calculated using the following equation: (treated mean optical density/untreated mean optical density) x 100.
Table 4-2. Standard deviations for the effect of pamidronate on growth of the HMPOS cell line for 24, 48, and 72 hours.

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Figure 4-10. Pamidronate-treated fibroblast cells. A) Results of the MTT cell viability assay for the effect of pamidronate on fibroblast cell growth. Mean optical density measured at 540 nm at 24, 48, and 72 hours for concentrations ranging from 0 (control) to 1000 uM. * = Significant difference between the labeled data point and the control of the same time point (p<0.01). B) Percent cell viability of the fibroblast cell line after treatment with pamidronate. Cell viability was calculated using the following equation: (treated mean optical density/untreated mean optical density) x 100.
Table 4-3: Standard deviations for the effect of pamidronate on growth of the fibroblast cell line for 24, 48, and 72 hours.

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<td>0.105</td>
<td>0.035</td>
<td>0.042</td>
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<td>0.037</td>
<td>0.062</td>
<td>0.054</td>
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<tr>
<td>0</td>
<td>0.074</td>
<td>0.035</td>
<td>0.051</td>
</tr>
</tbody>
</table>

Figure 4-11. Mean optical density measured at 540nm at 72 hours represented by calculated cell viability of the POS, HMPOS, and fibroblast cell lines for pamidronate concentrations ranging from 0 (control) to 1000 uM.
Figure 4-12. Results of the MTT cell viability assay for the effect of mannitol on growth of the POS cell line. Mean optical density measured at 540 nm at 24, 48, and 72 hours for concentrations ranging from 0 (control) to 1000 uM. * = Significant difference between the labeled data point and the control (p<0.01).

Table 4-4. Standard deviations for the effect of mannitol on growth of the POS cell line for 24, 48, and 72 hours.

<table>
<thead>
<tr>
<th>Concentration (uM)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000</td>
<td>0.059</td>
<td>0.079</td>
<td>0.044</td>
</tr>
<tr>
<td>500</td>
<td>0.169</td>
<td>0.109</td>
<td>0.04</td>
</tr>
<tr>
<td>100</td>
<td>0.069</td>
<td>0.061</td>
<td>0.127</td>
</tr>
<tr>
<td>50</td>
<td>0.062</td>
<td>0.026</td>
<td>0.061</td>
</tr>
<tr>
<td>10</td>
<td>0.052</td>
<td>0.074</td>
<td>0.051</td>
</tr>
<tr>
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<td>0.049</td>
<td>0.059</td>
<td>0.073</td>
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</tr>
<tr>
<td>0</td>
<td>0.041</td>
<td>0.275</td>
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</table>
Figure 4-13. Results of the MTT cell viability assay for the effect of mannitol on HMPOS cell growth. Mean optical density measured at 540 nm at 24, 48, and 72 hours for concentrations ranging from 0 (control) to 1000 uM. * = Significant difference between the labeled data point and the control (p<0.01).

Table 4-5. Standard deviations for the effect of mannitol on growth of the HMPOS cell line for 24, 48, and 72 hours.

<table>
<thead>
<tr>
<th>Concentration (uM)</th>
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<th>72 hours</th>
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</thead>
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<td>0.033</td>
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<tr>
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<td>0.114</td>
<td>0.075</td>
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<tr>
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<td>0.094</td>
<td>0.048</td>
</tr>
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<td>0.27</td>
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<td>0.07</td>
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<tr>
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<td>0.14</td>
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<tr>
<td>0</td>
<td>0.041</td>
<td>0.155</td>
<td>0.071</td>
</tr>
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</table>
Figure 4-14. Alendronate-treated POS cells. A) Results of the MTT cell viability assay for the effect of alendronate on growth of the POS cell line. Mean optical density measured at 540 nm at 24, 48, and 72 hours for concentrations ranging from 0 (control) to 1000 uM. * = Significant difference between the labeled data point and the control (p<0.01). B) Percent cell viability of the POS cell line after treatment with alendronate. Cell viability was calculated using the following equation: (treated mean optical density/untreated mean optical density) x 100.
Table 4-6. Standard deviations for the effect of alendronate on growth of the POS cell line for 24, 48, and 72 hours.

<table>
<thead>
<tr>
<th>Concentration (uM)</th>
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<th>72 hours</th>
</tr>
</thead>
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<td>500</td>
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<td>0.037</td>
</tr>
<tr>
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<td>0.14</td>
<td>0.076</td>
<td>0.044</td>
</tr>
<tr>
<td>50</td>
<td>0.078</td>
<td>0.049</td>
<td>0.039</td>
</tr>
<tr>
<td>10</td>
<td>0.052</td>
<td>0.08</td>
<td>0.067</td>
</tr>
<tr>
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<td>0.09</td>
<td>0.073</td>
<td>0.067</td>
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<tr>
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<td>0.074</td>
<td>0.066</td>
</tr>
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</tr>
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<td>0.05</td>
<td>0.056</td>
</tr>
<tr>
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<tr>
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</table>
Figure 4-15. Alendronate-treated HMPOS cells. A) Results of the MTT cell viability assay for the effect of alendronate on growth of the HMPOS cell line. Mean optical density measured at 540 nm at 24, 48, and 72 hours for concentrations ranging from 0 (control) to 1000 uM. * = Significant difference between the labeled data point and the control (p<0.01). B) Percent cell viability of the HMPOS cell line after treatment with alendronate. Cell viability was calculated using the following equation: 
\[
\text{Percent viability} = \left( \frac{\text{mean optical density of treated}}{\text{mean optical density of untreated}} \right) \times 100.
\]
Table 4-7. Standard deviations for the effect of alendronate on growth of the HMPOS cell line for 24, 48, and 72 hours.

<table>
<thead>
<tr>
<th>Concentration (uM)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
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<td>0.067</td>
<td>0.107</td>
</tr>
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<td>0.097</td>
<td>0.079</td>
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</tr>
<tr>
<td>10^{-8}</td>
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<tr>
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<td>0.027</td>
<td>0.093</td>
<td>0.055</td>
</tr>
<tr>
<td>10^{-10}</td>
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<tr>
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<td>0.097</td>
<td>0.095</td>
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Figure 4-16. Alendronate-treated fibroblast cells. A) Results of the MTT cell viability assay for the effect of alendronate on fibroblast cell growth. Mean optical density measured at 540 nm at 24, 48, and 72 hours for concentrations ranging from 0 (control) to 1000 uM. * = Significant difference between the labeled data point and the control (p<0.01). B) Percent cell viability of the fibroblast cell line after treatment with alendronate. Cell viability was calculated using the following equation: (treated mean optical density/untreated mean optical density) x 100.
Table 4-8. Standard deviations for the effect of alendronate on growth of the fibroblast cell line for 24, 48, and 72 hours.

<table>
<thead>
<tr>
<th>Concentration (uM)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>72 hours</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.014</td>
</tr>
<tr>
<td>500</td>
<td>0.024</td>
<td>0.118</td>
<td>0.014</td>
</tr>
<tr>
<td>100</td>
<td>0.082</td>
<td>0.068</td>
<td>0.046</td>
</tr>
<tr>
<td>50</td>
<td>0.043</td>
<td>0.04</td>
<td>0.024</td>
</tr>
<tr>
<td>1</td>
<td>0.039</td>
<td>0.028</td>
<td>0.015</td>
</tr>
<tr>
<td>0</td>
<td>0.096</td>
<td>0.01</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Figure 4-17. Percent cell viability of the POS, HMPOS, and fibroblast cell line after treatment with alendronate concentrations ranging from $10^{-10}$ uM to 1000 uM. Cell viability was calculated using the following equation: 
(treated mean optical density/untreated mean optical density) x 100.
Figure 4-18: 1.0% agarose gel for DNA fragmentation analysis of the effect of pamidronate (Figure 4-18A) and alendronate (Figure 4-18B) on POS and HMPOS cell lines. Lane 2 contains HMPOS control, lane 3 contains HMPOS 1000 uM, lane 4 contains HMPOS 500 uM, lane 5 contains POS control, lane 6 contains POS 1000 uM, and lane 7 contains POS 500 uM (Fig A). In Figure 4-18B, lane 2 contains HMPOS 50 uM, lane 3 contains HMPOS control, lane 4 contains POS 50 uM, and lane 5 contains POS control.
Figure 4-19. Representation of cell viability (%) at 24, 48, and 72 hours produced from cell counts comparing the number of viable POS cells versus the number of dead POS cells upon exposure to alendronate. Cell viability = (viable cell number/total cell count) * 100.
Bisphosphonates were originally developed for use in osteoporosis and are used in the treatment and/or prevention of osteolytic diseases, thus they are gaining popularity in the oncology field. In the current study, the effects of two aminobisphosphonates, pamidronate disodium and alendronate, were evaluated on the growth of cultured canine osteosarcoma cells from POS and HMPOS cell lines. The effects of alendronate and pamidronate disodium on a normal canine fibroblast cell line were also evaluated.

In the current study, the range of drug concentration ($10^{-10}$ uM to 1000 uM) for alendronate treatment was established from previous reports showing stimulation of osteoblast precursors formation in human bone marrow cultures at concentrations below 100 nM (0.1 uM) (Giuliani et al. 1998). Conversely, concentrations equaling or higher than 100 uM alendronate decrease the viability of human osteoblasts (Garcia-Moreno et al. 1998) and alendronate concentrations ranging from 0.1-1 mM (100-1000 uM) have been reported at active osteoclast resorption sites (Green 2003, Sato et al. 1991). With pamidronate, sera concentrations have been reported to range from 0.5 uM to 10 uM depending on duration and dosage of intravenous infusion (Berenson et al. 1997, Lee et al. 2001, Leyvraz et al. 1992, Oiso et al. 1994). However, most *in vitro* studies, including the present one, report inhibitory effects of pamidronate at concentrations approximately 10 fold greater than sera concentrations reported in patients (Lee et al. 2001). Bisphosphonate concentrations have reached 1000 uM in the bone at sites of high resorption (Rogers 1997); therefore, a pamidronate concentration range of 1 uM up to
1000 uM was established. Bisphosphonate concentrations reached at sites of bone turnover are most likely much higher than sera and soft tissue levels due to the drug’s high affinity for bone. However, the clinical doses tumor cells are exposed too are unknown. Bisphosphonates are rapidly cleared from circulation in the body and are deposited in bone at sites of greatest turnover or are excreted unmetabolized by the kidneys. High levels of drug accumulate in bone at highest resorption sites, thus the concentrations of pamidronate and alendronate used in this study reasonably correspond to the in vivo situation. Measurements of bisphosphonate concentrations within tumors of treated dogs would determine whether osteosarcoma cells are in fact exposed to these drug concentrations in vivo.

Previous reports demonstrate bisphosphonates acting in a time- and dose-dependent manner (Green 2003, Sonnemann et al. 2001), thus time periods of 24, 48, and 72 hours were established. The current in vitro study found a time- and dose-dependent growth inhibitory response of alendronate concentrations on HMPOS and POS cell lines. Alendronate concentrations of 50 uM to 1000 uM after a 72-hour incubation period significantly inhibited growth of POS and HMPOS canine osteosarcoma cell lines while exerting no significant inhibitory effect on the canine fibroblast cell line. A biphasic effect of alendronate on colony forming units (CFUs) of fibroblasts was seen in a study searching for potential effects of etidronate and alendronate on formation of early and late osteoblastic cell precursors in human and murine bone marrow cultures (Giuliani et al. 1998). At concentrations 10^{-8} M to 10^{-13} M, stimulatory effects were seen on CFUs of fibroblasts while inhibition occurred at concentrations >10^{-7} M (0.1 uM).
In the present study, alendronate exerted no significant inhibitory or stimulatory effects on the growth of fibroblast cells, thus indicating normal fibroblast cells will most likely not be affected by this drug.

In the present study, the cell viability of the canine osteosarcoma cell lines POS and HMPOS were significantly inhibited at alendronate concentrations ≥50 uM at 72 hour incubation period, with concentrations ≤10 uM having no inhibitory effect on the cell lines. POS cell viability (45.08%) was lowest at 50uM after a 72-hour incubation period, with the lowest HMPOS cell viability (49.8%) occurring at 100 uM at 72-hours. Garcia-Moreno et al. (1998) reported the viability of normal human osteoblasts was inhibited at alendronate concentrations ≥100 uM. They found alendronate concentrations ≤10^{-5} M (10 uM) did not affect the viability of normal human osteoblasts. The effective dose (ED_{50} = inhibits resorptive capacity of osteoclasts by 50%) of alendronate as a bone resorption inhibitor is reported at 2x10^{-9} M (Sahni et al. 1993). The results from the present study and the study conducted by Garcia-Moreno et al. (1998) indicate that in vitro alendronate does not affect cell viability of osteoblasts at concentrations equal to its effective dose.

Stimulatory effects were also produced by alendronate on the POS and HMPOS cell lines generally at doses of 50 uM or greater. Alendronate (100 uM-1000 uM) produced significant growth stimulation on POS cells after a 24-hour incubation period with no significant growth stimulation seen on the HMPOS cells at the same time period. Giuliani et al. (1998) produced findings suggesting a direct bisphosphonate stimulatory action on human and murine osteoblasts. Their study showed an increase in osteoblastogenic potential of murine and human bone marrow cultures at alendronate
concentrations from $10^{-7}$ to $10^{-10}$ M with inhibition occurring at $10^{-5}$ M (10 uM). In another study (Garcia-Moreno et al. 1998), no stimulatory effect was reported for alendronate concentrations of $10^{-5}$ M (10 uM) to $10^{-12}$ M. The reason for the stimulatory effect is unknown. In the findings of the present study, the increase occurred at higher drug concentrations than previously reported which may be due in part to the response elicited by alendronate on neoplastic cell lines, whereas previous studies were using normal non-tumor cells. Variation between the cell lines of different species may also be a factor as the current study used canine-derived cell lines and previous studies have used human or murine cell lines. Studies (Tenenbaum et al. 1992, Tsuchimoto et al. 1994) have shown a bisphosphonate-induced increase in osteoblast function. At low concentrations, alendronate increased osteocalcin and collagen synthesis (Tsuchimoto et al. 1994). Another postulated mechanism for the proliferative effect may be due to production of basic fibroblast growth factor (bFGF). This local growth factor is involved in osteoblastogenesis and is a potent mitogen for mesenchymal cells (Giuliani et al. 1995). In human osteoblastic cells (MG-63) and in primary human bone marrow cells cultured for 48 hours, an increased production of bFGF by alendronate ($10^{-8}$-$10^{-12}$ M) was capable of stimulating osteoblastogenesis in vitro (Giuliani et al. 1995). Modulation of this growth factor may explain in part the bisphosphonate effect on osteoblastogenesis and indicate that the number of mesenchymal bone marrow cells committed to osteoblast phenotype may be increased by bisphosphonates.

Pamidronate disodium may demonstrate a time- and dose-dependent growth inhibitory effect on POS and HMPOS cell lines while exhibiting no significant inhibitory or stimulatory effect on canine fibroblast cells. Sonneman et al. (2001) reported a weak
sensitivity of human fibroblasts towards 50 uM pamidronate with a reduction in cell viability of 21% at 72 hours. In the current study, a non-significant 22.7% reduction in viability of canine fibroblast cells at 1000 uM pamidronate was found. This indicates bisphosphonates most likely will not exert a strong cytotoxic effect on normal, healthy cells. At 24 and 48 hours, Sonneman et al. (2001) did not find a pronounced growth inhibition by pamidronate on human osteosarcoma cell lines, however a time-dependency for efficacy of pamidronate was seen with the 72-hour incubation period producing the strongest growth inhibition. The present study showed some suggestion of a time-and dose-dependent effect. When comparing the HMPOS cell viability at 1000 uM for 72 hours of 27.43% to the HMPOS cell viability for 24 hours of 60.3%, a time-dependent effect is suggested. However, it is difficult to determine if a pamidronate did exert a time-dependent effect as POS cells exhibited inhibition after a 24-hour incubation at 100 uM with a cell viability of 65% as compared to the 72-hour cell viability of 117.9% at the same concentration. The results of the present study leave question as to whether a dose- and time-dependent effect was exhibited by pamidronate disodium on the canine osteosarcoma tumor cell lines studied. Studies (Crohns et al. 2001, Mackie et al. 2001) have well documented the time- and dose-dependent response of cell death to pamidronate at concentrations ≥10^{-4} M (100 uM). In the present study, the pamidronate concentration of 100 uM was not a significant inhibitory concentration on HMPOS cells. This may be due to the presence of fetal bovine serum (10%) in the media, which provides trophic factors to cells and decreases the effectiveness of pamidronate (Lee et al. 2001); and in part due to the effect of pamidronate on this particular cell line. In concurrence with the present study, Lee et al. (2001) found the lower doses <50 uM of
pamidronate appear to cause cell cytostasis. This is evident only on the pamidronate-treated HMPOS cells, as the HMPOS cell number did not significantly increase over time when compared to the control cells. Tumor cells have been indicated as potential targets of bisphosphonate action. Findings by van der Pluijm et al. (1996) showed a dose-dependent inhibition of human breast cancer cell adhesion and spreading on cortical and trabecular bone slices with varying concentrations (10^{-6} to 10^{-4} M) of pamidronate, olpandronate, alendronate, and ibandronate, which were used for an 18 hour cell treatment. Although the bisphosphonate response in this study is related to cellular function rather than cell death, it does show that bisphosphonates can elicit an inhibitory effect at an early time period as was seen in the present study with the growth inhibition of pamidronate-treated POS cells at 24 hours. At concentrations ranging from 1 to 500 uM, bisphosphonates have been shown to exert a dose- and time-dependent inhibition on proliferation and induction of apoptosis in a variety of human and murine tumor cell lines (Sato et al. 1991, Senaratne et al. 2000, Shipman et al. 1997, Sonneman et al. 2001). These studies support the results of the current study in which pamidronate-treated POS cells exhibited growth inhibition at concentrations ranging from 1 uM to 1000 uM for both 24 and 72-hour incubation periods. The most clinically relevant time period is 72 hours as drug therapies will be chronically administered. The 72-hour incubation period contained the majority of growth inhibition by pamidronate disodium at concentrations 500 uM to 1000 uM.

Pamidronate disodium produced stimulatory effects on the POS and HMPOS cell lines. Following a 72-hour incubation time with pamidronate, POS cells showed a significant stimulatory effect at 50 and 100 uM. A significant stimulatory effect was also
seen on 100 uM pamidronate-treated HMPOS cells at 24 hours. A possible cause of the growth stimulation may be the result of mannitol. The manufactured drug Aredia, containing phosphoric acid and 470 mg of mannitol, was used in the present study. The effects of phosphoric acid were not evaluated in this study. However, mannitol effects were evaluated on both the HMPOS and POS cell lines. The function of mannitol in pamidronate disodium is to aid in elimination of the drug, thus mannitol is a protective additive for the kidneys. Mannitol did not have a statistically significant inhibitory effect on either tumor cell line. However, following a 24-hour incubation at mannitol concentrations of 5, 10, 100, 500, and 1000 uM, and 72 hours at 500 uM, a statistically significant stimulatory effect on HMPOS cells was produced. When treated with pamidronate, the HMPOS cells exhibited a significant increase in optical density at 24 hours at 100 uM. POS growth stimulation was seen at 24 hours at mannitol concentrations of 100-1000 uM, thus mannitol, if anything, has a stimulatory effect on cell growth. Significant growth stimulation was seen with pamidronate disodium-treated POS cells at these same concentrations. Therefore, because the manufactured form of the drug was used in the current study, mannitol might have played a role in stimulating cell growth, making it difficult to accurately compare the results of the present study to previous studies where the pure form of the drug was used. It is possible that the inhibitory effect of pamidronate on canine tumor cell growth in the present study would have been even more pronounced had the pure form of pamidronate been used.

Studies have reported a stimulatory effect on cell growth when treated with the pure form of pamidronate. Mathov et al. (2001) reported a proliferative effect of bisphosphonates (olpadronate, pamidronate, etidronate) on human osteoblastic cells after
short-term drug exposure to concentrations between $10^{-8}$ M (0.01 uM) and $10^{-6}$ M (1 uM). In the current study, increases in cell viability with both drugs did occur, but generally at doses of 50 uM or greater. Giuliani et al. (1998) produced findings suggesting a direct bisphosphonate stimulatory action on osteoblasts. Their study showed an increase in osteoblastogenic potential of murine and human bone marrow cultures at alendronate concentrations from $10^{-7}$ to $10^{-10}$ M and inhibition occurring at $10^{-5}$ M. The reason for the stimulatory effect is unknown. The present study showed growth stimulation and inhibition occurring at higher drug concentrations than previously reported which may be due in part to the response elicited by pamidronate disodium on canine neoplastic cell lines, whereas previous studies were using normal human and murine non-tumor cells. Previous reports (Tenenbaum et al. 1992, Tsuchimoto et al. 1994) have shown a bisphosphonate-induced increase in osteoblast function with pamidronate stimulating both mineralization and alkaline phosphatase activity in chick periosteal osteoblasts (Tenenbaum et al.1992). As mentioned previously, the production of basic fibroblast growth factor (bFGF) may play a role in the proliferative effect of pamidronate (Giuliani et al. 1995). In previous animal studies of osteoclasts (Fisher et al. 1999, Rodan 1996) osteoclast number has initially increased upon treatment with bisphosphonates. The reason for the initial increase is unknown, although it could reflect an increase in osteoclast number to compensate for the decreased osteoclast activity (Bikle et al. 1994, Seedor et al. 1991). After long-term administration, the osteoclast number decreased (Seedor et al. 1991).

Inhibition of bone resorption by nitrogen-containing bisphosphonates seems to occur through disruption of the mevalonate pathway involving possibly FPP synthase
inhibition. FPP synthase inhibition leads to disruption of isoprenylation and its role in GTPase function thus indicating apoptosis as a separate and secondary event (Halasy-Nagy et al. 2001). Results of a cell count performed on alendronate-treated POS cells suggest that alendronate acts via non-apoptotic means. The cell counts provided evidence that the decrease in optical density at alendronate concentrations ≥50 uM seen in the MTT assay may be a result of cell death rather than decrease in cellular proliferation. However, this assumption is based on one cell count performed at 24, 48, and 72 hours with the final MTT cell viability assay. Because this was only performed on a limited number of samples at the end of the study, additional cell counts are required to reinforce the findings of the single cell count that was performed. Alendronate appears to act in a cytotoxic manner on POS cell line as cell viability decreased with dose and time. Findings by Garcia-Moreno et al. (1998) concluded alendronate concentrations ≥10^{-4} M (100 uM) caused a cell viability decrease in normal human osteoblast cells. Although the current study did not perform cell counts for pamidronate disodium, a cytotoxic effect may be the cause of the pamidronate-induced growth inhibition as the cytotoxic effect of pamidronate has been well documented (Crohns et al. 2001, Mackie et al. 2001). Higher pamidronate concentrations have been reported to produce a reported cytotoxic response. Cell counts performed in two studies (Crohns et al. 2001, Mackie et al. 2001) at 48 hours showed the decrease in cell viability on human breast cancer cells and murine osteosarcoma cell line is due to necrosis as opposed to induction of apoptosis. Necrosis was present at pamidronate concentrations 50 uM to 1000 uM. Lee et al. (2001) found that after a 48-hour pamidronate treatment at 100 uM, a significant decrease in human prostate cancer cell number occurred with the decrease due to cytotoxicity rather
than apoptosis. However the cell counts in the present study cannot distinguish between cell-death mediated by apoptosis and a cytotoxic effect. Fromigue et al. (2000) demonstrated at 100 μM, pamidronate produces a clearly cytotoxic effect on breast cancer cells. Based on the results of these studies, it can is likely that pamidronate and alendronate may act through non-apoptotic mechanisms.

The results of the DNA fragmentation analysis indicate that the inhibitory effect of pamidronate and alendronate on tumor cell growth appears to be independent of apoptosis. Fragmentation of the DNA of either cell line did not occur; indicating the method of cell death demonstrated in the MTT assay is non-apoptotic. For pamidronate, this is in agreement with the findings of Sonneman et al. (2001) where the inhibitory effect of pamidronate on human osteoblast cell growth was not via induction of apoptosis. Since the nitrogen-containing bisphosphonates induce a cytotoxic effect on the cells, rather than a direct apoptotic effect, alendronate and pamidronate must work through a different mechanism than seen in non-nitrogen containing bisphosphonates. Both drugs induce apoptosis in osteoclasts through inhibition of the mevalonate pathway that is critical for osteoclast cell function. It is possible that bisphosphonates act on bone through different mechanisms, some of which show a threshold effect and others a dose-dependent effect. Macrophages and osteoblasts may mediate anti-resorptive effects of the bisphosphonates. Presently, the specific molecular mechanisms for inhibition of bone resorption are still under investigation. Many theories have been formulated including inhibition of the activity of mature osteoclasts, inhibition of osteoclast formation/recruitment, inhibition of osteoclast activation, and induction of osteoclast apoptosis (Rodan 1998). Halasy-Nagy et al. (2001) reported that all bisphosphonates can
induce osteoclast apoptosis, but this action is not the sole or earliest means by which inhibition of bone resorption occurs. Intracellular targets of bisphosphonates are not well established and they may be involved in the different molecular mechanisms of the drugs. One target of both etidronate and alendronate is human protein tyrosine phosphatases (PTPs) involved in the transduction of signals controlling osteoblastic cell growth and differentiation (Giuliani et al. 1998). Both drugs inhibit the enzymatic activity of PTPs while at the same time stimulate osteoblast proliferation. Their study reported that nitrogen-containing bisphosphonates inhibit bone resorption prior to and separately from induction of apoptosis. Bone resorption was inhibited in the presence of the caspase inhibitor, Z-VAD-FMK that blocks osteoclast apoptosis induced by bisphosphonates. Also, inhibition of bone resorption and the reduction in osteoclast number or apoptosis occurred at separate times and doses. However, in previous studies, treatment with high doses of bisphosphonates induced apoptosis in murine osteoclasts (Hughes et al. 1995), murine macrophages (Luckman et al. 1998), human breast cancer cells (Fromigue et al. 2000, Senaratne et al. 2000) and human myeloma cells (Shipman et al. 1997).

Overall, alendronate appears to be more potent than pamidronate disodium. Alendronate caused the greatest decrease in cell viability at concentrations ≥50 uM, as compared to pamidronate disodium, which caused significant growth inhibition at concentrations ≥100 uM. Following a 72-hour incubation period with alendronate at 50 uM, POS and HMPOS cells treated with this drug had viabilities of 45.08% and 56.8%, respectively; compared to viabilities of POS and HMPOS cells treated with pamidronate of 121% and 112%, respectively. Alendronate appeared to have a greater effect on the HMPOS cell line with significant growth inhibition occurring at both 48 and 72 hour
incubation periods at concentrations \( \geq 50 \text{ uM} \), whereas the POS cell line exhibited inhibitory effects after 48 hours at 500 and 1000 \text{ uM} \) and after 72 hours at 100 \text{ uM} to 1000 \text{ uM} \). In addition, since alendronate is given orally, does not require hospitalization for dose administration, and is affordable to most pet owners (approximately $80.00 per month), it appears to be the drug of choice at this point.

In comparing the two canine osteosarcoma cell lines, HMPOS appears to be more resistant to the effects of bisphosphonates as compared to POS. Lower concentrations of bisphosphonates appear to have a greater affect on POS cells than HMPOS cells with the POS cells demonstrating a stimulatory effect at 24 hours with both alendronate and pamidronate disodium. The variability in the effect of bisphosphonates on the two different cell lines may be due to the different growth rates. The HMPOS cell line exhibited a doubling time of \(<24 \text{ hours} \) with the POS doubling approximately every 32 hours. Because the HMPOS growth rate is faster than the POS growth rate, it may take a higher drug concentration to inhibit its growth. Additionally, the HMPOS cell line represents a metastatic cancer whereas the POS represents the primary tumor. It may be that the lower bisphosphonate concentrations are potent enough to have an inhibitory affect on primary tumor growth whereas the metastatic tumors require a higher drug concentration to inhibit their growth.

In conclusion, alendronate demonstrated a time- and dose-dependent growth inhibitory effect on the POS and HMPOS canine osteosarcoma cell lines with a similar effect possibly seen with pamidronate disodium. Neither pamidronate disodium nor alendronate had a significant inhibitory or stimulatory effect on canine fibroblast cell growth. Although the mechanisms of action are unknown, both drugs have a stimulatory
effect on POS and HMPOS cells at 24 hours with the greatest growth inhibition occurring after a 72-hour incubation period at doses ≥50 uM alendronate and ≥100 uM pamidronate. In addition to limiting bone destruction in dogs affected with osteosarcoma, our results suggest that these drugs may also exert a direct cytotoxic effect on the tumor cells. These results reaffirm the documented usefulness of bisphosphonates in the treatment of canine osteosarcoma and suggest that they may also be useful in the treatment of the associated micrometastatic disease, perhaps even post-operatively. To our knowledge, this is the first study that has reported upon the effects of bisphosphonates on canine osteosarcoma cells in vitro. This study lays a foundation for future in vivo studies to confirm the same effects in tumor bearing animals. Measurements of bisphosphonate concentrations within the tumor also need to be accomplished. Further clinical investigation into bisphosphonate therapy as a potential treatment option for dogs affected with osteosarcoma needs to be undertaken, and determination of dosing regimens and physiological responses need to be performed.
LIST OF REFERENCES


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BIOGRAPHICAL SKETCH

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