EFFECTS OF AUROTHIOMALATE TREATMENT ON OSTEOSARCOMA

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To Cassandra Scott

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATM	Aurothiomalate
FBS	Fetal Calf Serum
HMPOS	Highly Metastatic Pulmonary Osteosarcoma
IACUC	International Animal Care and Use Committee
IP	Intraperitoneal
NSCLC	Non-Small Cell Lung Cancer
OSA	Osteosarcoma
PBS	Phosphate Buffered Saline
PC3U	Prostate Cancer Cells
PKC	Protein Kinase C
PrEC	Prostate Epithelial Cells
SRS	Stereotactic Radiosurgery
VEGF	Vascular Endothelial Growth Factor

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Spontaneously occurring tumors in the dog provide a useful model for evaluating treatments for dogs and humans affected by cancer. Similarities between canine and human osteosarcoma adds to the translational value of investigations of novel treatments for osteosarcoma in dogs. Despite advances in treatment protocols, osteosarcoma remains a highly fatal disease, with most affected dogs succumbing to metastatic disease within two years of diagnosis. The prognosis for humans with metastatic disease is similarly poor.

Aurothiomalate is a gold compound traditionally used in the treatment of immune-mediated diseases. Recent research has shown anti-neoplastic effects of these metallodrugs against several human tumor cell lines *in vitro*, and aurothiomalate was demonstrated to slow tumor growth in human lung cancer xenografts. Despite interest in the anti-cancer applications of chrysotherapy, there are no studies investigating the effects of gold compounds on sarcomas or on cancers of companion animals. Chapter 2 describes the effects of aurothiomalate treatment on human and canine osteosarcoma cell lines *in vitro*. We found that incubation with aurothiomalate significantly decreased

osteosarcoma cell survival. This study provided the basis for subsequent *in vivo* investigation of anti-tumor effects of aurothiomalate *in vivo*.

Chapter 3 describes the effect of aurothiomalate treatment on canine osteosarcoma in a mouse xenograft model. We demonstrated that canine osteosarcoma xenografts treated with aurothiomalate displayed slower tumor growth and lower incidences of tumor emboli and pulmonary metastasis compared to xenografts treated with a placebo. Aurothiomalate may therefore hold promise as an adjuvant drug in the treatment of canine osteosarcoma.

CHAPTER 1
INTRODUCTION

Clinical Significance of Canine Osteosarcoma

Osteosarcoma (OSA) is a devastating disease of dogs, and affected animals comprise a significant portion of dogs evaluated by veterinary oncologists for treatment. The incidence, currently reported to include approximately 8,000 new canine cases each year, may still underestimate the true prevalence of the disease since many dogs are euthanized based on a presumptive diagnosis without further reporting of the event.[1,2] Given the large impact of canine osteosarcoma on the companion animal population and its translational significance, a substantial amount of research into new treatment modalities has been generated in recent decades. Despite these advances in theoretical knowledge, median survival times for dogs with osteosarcoma remain largely unchanged with standard therapy, necessitating the continued need for research and development of new therapeutic strategies.[3-5]

Epidemiology

Osteosarcoma primarily affects middle-aged to older dogs, although there is a slightly bi-modal aspect to its distribution, with a small prominence of younger dogs diagnosed between the ages of 1.5 to 2 years. [6] The disease predominantly affects large to giant breed dogs, with the most commonly affected breeds including Great Danes, Saint Bernards, Doberman Pinschers, Rottweilers, German Shepherds, Golden Retrievers, Labrador Retrievers, Irish Setters, and Irish Wolfhounds.[6-8] More predictive than breed, however, are an individual dog's height and weight, with increasing values corresponding to an increased risk of osteosarcoma.[6] While the vast majority (95%) of osteosarcomas occurring in large breeds are appendicular, axial tumors account for the

majority (59%) of osteosarcomas occurring in dogs weighing less than 15kg.[9] Most literature also reports that males are slightly more at risk than females, with exceptions for certain breeds and primary axial osteosarcoma.[10-12] One study suggests that neutered dogs have twice the risk of developing osteosarcoma as intact dogs.[8]

Etiology

In both humans and dogs, the etiology of osteosarcoma remains largely unknown. Any cellular change that increases the rate of cell division theoretically increases the likelihood of the affected tissue becoming neoplastic, as increased mitotic activity increases the number of mutations that accumulate over time, potentially leading to an ultimately cancerous phenotype.[13] One simplistic theory is that micro-trauma to cells in the more rapidly-dividing physes of weight-bearing bones predisposes these cells to the subsequent development of osteosarcoma.[14] With their increased mitogenic potential, these cells theoretically have increased risk of accumulating mutations. This theory is consistent with the distribution of osteosarcoma in the major weight-bearing bones near late-closing physes in larger dogs.[14] A cadaver study, however, failed to show a significant difference between the incidence of micro-trauma in the radii of small- and large-breed dogs, calling into question whether micro-trauma actually comprises a significant predisposing factor for osteosarcoma development.[15]

Research has more definitively linked osteosarcoma development to previous fracture sites and the use of metallic implants.[16-18] This association has been attributed to chronic low-grade inflammation (potentially secondary to loosening of the implant), galvanic corrosion, and low-grade infection, all of which theoretically increase the opportunity for mutagenesis.[19-21] Furthermore, certain types of implants have been implicated due to their method of production and consequent predilection for

corrosion.[22,23] Although it is important to recognize this relationship, fracture-associated osteosarcoma represents the vast minority of cases compared to the incidence of spontaneously occurring osteosarcomas.[17,24] With growing awareness of the link between fracture repair and osteosarcoma and the consequent modifications of implant use, the incidence of implant-related osteosarcoma is likely to be further reduced in the future.

In both humans and dogs, osteosarcoma has been shown to develop secondary to exposure to ionizing radiation.[25] Dogs developed osteosarcoma secondary to plutonium, radium, and strontium exposure in experimental studies, although the distribution of these tumors differs markedly from that of spontaneously occurring osteosarcoma.[26-28] In a clinical setting, dogs have been reported to develop osteosarcoma as a late side effect of radiation therapy for other neoplasms, with the reported incidence ranging from 3% to 21% of those treated for a given condition.[29-31] Although this is a rare occurrence, it is an important consideration in planning radiation protocols to minimize exposure to surrounding tissues.

Osteosarcomas have also been observed in association with bone infarcts, although no causal relationship has been proven.[32] Of clinical significance is a report of osteosarcoma secondary to a bone infarct following a total hip arthroplasty.[33] Although this should heighten awareness of osteosarcoma as a potential sequella of total hip arthroplasty, the low incidence of this occurrence and the uncertainty of its etiologic link warrant further investigation before conclusive recommendations are made.

Numerous genetic and molecular factors have been investigated with regard to the pathogenesis of both human and canine osteosarcoma.[34-36] The relatively high

prevalence of osteosarcoma among certain breeds as well as within specific families is consistent with the theory that at least some aspect of the pathogenesis of osteosarcoma in dogs has a genetic basis.[14] The most well-established genetic abnormality associated with canine osteosarcoma is alteration of the tumor suppressor gene p53.[14] This gene has been shown to be both mutated and over-expressed in canine osteosarcoma and has been implicated in the development of human osteosarcoma.[34,37-39] Although many molecular and genetic factors have shown suggestive correlations with the development and/or progression of osteosarcoma in dogs, no single cause has been identified as a causative agent.[13] Nonetheless, various underlying molecular factors may prove to be significant in the development of therapeutic strategies and will be discussed in more detail with pathology and treatment options. Similarly, a better understanding of the genetic basis of osteosarcoma may provide new therapeutic options as well as the opportunity to reduce the incidence of osteosarcoma in dogs through changes in breeding practices.

Pathology

Osteosarcoma represents the malignant transformation of primitive bone (mesenchymal) cells, leading to the production of the extracellular osteoid matrix which is characteristic of osteosarcomas.[14] Further histological subclassification divides osteosarcomas into osteoblastic, chondroblastic, fibroblastic, poorly differentiated, and telangiectatic subtypes based on cellular characteristics and matrix amount and type.[14] Tumor subclassification in dogs has not been proven to correlate with biological behavior; histologic grade is more likely to be predictive of both tumor behavior and metastasis.[40]

Osteosarcoma in dogs occurs in the appendicular skeleton in approximately 75% of affected dogs, whereas approximately 25% of dogs present with axial lesions.[7] Documented cases of multicentric osteosarcoma at initial diagnosis comprise less than 10% of all cases, and extraskeletal primary sites have been reported but are rare.[41-43] The most commonly affected site for primary osteosarcomas is the metaphyseal region of long bones, with the thoracic limbs affected approximately twice as frequently as the pelvic limbs.[44] The most common sites are the distal radius and proximal humerus, and in the pelvic limbs, the proximal femur is slightly less frequently affected than the distal femur, distal tibia, and proximal tibia.[7,44] More distal appendicular lesions are rare in dogs.[45] Among cases of axial osteosarcoma, the majority are located in the mandible and maxilla, with the spine, cranium, ribs, nasal cavity or paranasal sinuses, and pelvis also reported.[10]

Locally, osteosarcoma is a very aggressive tumor, causing lysis, local bone production, or both, and is usually accompanied by soft tissue swelling.[14] Pain associated with the tumor is likely attributable to microfractures or periosteal disruption.[14] The lesions rarely cross a joint surface, which may be due to the activity of synovial collagenase inhibitors.[46,47] Pathological fracture through the tumor occurs with relative frequency and is an important consideration with bone biopsy or following radiation therapy.[48,49]

Metastasis of osteosarcoma primarily occurs through the hematogenous route, although tumor cells may rarely spread through regional lymphatics as well.[50] Metastasis of osteosarcoma in dogs is very common and tends to occur very early in the disease process; less than 15% of dogs have radiographically visible metastases at

initial diagnosis, but approximately 90% of dogs will have detectable metastases within one year when treated with amputation only.[7,51] The most common site of metastasis of osteosarcoma is to the lungs, although spread to other bones or soft tissues does occur.[52,53] It is suspected that the incidence of osseous metastasis in dogs may increase following systemic chemotherapy for osteosarcoma, as has been demonstrated in humans.[14,54] While there have been reported associations between metastatic behavior and certain primary tumor locations, there is not currently a widely accepted consensus regarding these trends.[55-57] Spontaneous regression of osteosarcoma in 4 dogs has also been reported but is exceedingly rare.[58]

The molecular pathology of osteosarcoma is the focus of much current research, as these underlying mechanisms can hold significance as potential therapeutic targets. Cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF) expression has been reported to be elevated and correlated with prognosis in canine osteosarcoma, although another study failed to confirm over-expression of COX-2 in this tumor type.[35,36,59,60] Both COX-2 inhibitors and VEGF-inhibitors are currently being investigated in the treatment of canine osteosarcoma.[61,62] Similarly, other molecules and oncogenes including growth hormone, *erb*B-2, PTEN, *sis*, c-kit, metalloproteinases (MMPs), and the telomerase reverse transcriptase gene have been shown to be altered in the pathogenesis of canine osteosarcoma.[34,37,38,63-66]

Diagnosis

History and Clinical Signs

The classic presentation for canine osteosarcoma is a middle-aged to older large- to giant-breed dog presenting for appendicular pain or lameness.[14] Given the multitude of orthopedic conditions afflicting large breed dogs and a potential history of

recent minor trauma frequently seen with these patients, a thorough orthopedic examination with an index of suspicion for osteosarcoma is warranted. Dogs with the typical signalment presenting for more severe pain may be more likely to have a pathological fracture.[14] Axial osteosarcoma presents with more variable clinical signs; pain is a less consistent presenting feature, whereas deformity or swelling due to the primary tumor may be more pronounced.[14] Systemic signs at initial diagnosis are uncommon; dogs with radiographically visible pulmonary metastasis usually develop decreased appetite and energy levels within one month and may develop hypertrophic osteopathy.[14]

Imaging

Orthogonal radiographs of the affected area are the first-line imaging for any suspected case of osteosarcoma. The radiographic appearance of a primary osteosarcoma may vary widely between osteolytic and osteoproliferative lesions, with many tumors showing both features.[67] Consistent features characteristic of osteosarcoma include cortical lysis, soft tissue extension and swelling, and new bone extending in either a palisading or radiating pattern from the cortex. "Codman's triangle" is the term for new periosteal bone from the cambium layer, elevating the periosteum to form a triangular appearance at the periphery of the lesion; this sign is suggestive of but not pathognomonic for osteosarcoma.[14] Other radiographic signs consistent with osteosarcoma include loss of the fine trabecular pattern in the metaphysis, an indistinct zone of transition from the affected medullary cavity to the cortex, and areas of fine punctuate lysis.[14] The most common differential diagnosis when evaluating a radiographic lesion consistent with osteosarcoma is osteomyelitis, particularly of fungal origin, which cannot be distinguished radiographically. Other radiographic differential

diagnoses for osteosarcoma include other primary bone tumors (chondrosarcoma, fibrosarcoma, hemangiosarcoma), metastatic bone tumors, multiple myeloma or lymphoma, and bone cysts.[14]

Additional imaging should be pursued to help stage dogs with osteosarcoma.[14] At minimum, three-view thoracic radiographs should be taken to evaluate for the presence of pulmonary metastases. Nodules 6 to 8 mm in diameter and larger are detectable radiographically.[14] Computed tomography (CT), magnetic resonance imaging (MRI), or proton emission tomography (PET) in combination with a CT afford additional sensitivity in detecting pulmonary or other bony metastases.[68,69] Prior to the advent of readily-available advanced imaging, bone survey radiography was employed to evaluate for metastasis and was found to be slightly more sensitive than thoracic radiographs in detecting second sites of osteosarcoma.[70] Nuclear scintigraphy is another imaging tool occasionally employed for the evaluation of osteosarcoma and subsequent metastases. Although a sensitive tool, it can only identify areas of osteoblastic activity and is thus not specific for bony neoplasia.[71-74] As a result, biopsy of potentially affected sites may be necessary to confirm whether metastasis is present.

Tissue Sampling

Histopathology is considered the basis for definitive diagnosis for osteosarcoma in dogs.[75] Cytology may be suggestive of osteosarcoma in dogs and is frequently used in diagnosing human osteosarcoma.[76] Alkaline phosphatase staining can help distinguish osteosarcoma from other tumor types, but currently no definitive cytological criteria exist for distinguishing canine osteosarcoma from inflammatory or proliferative bony lesions.[76,77] Thus, tissue biopsy becomes necessary to establish a definitive diagnosis. Risks of bone biopsy include infection, hemorrhage, and pathological

fracture, the latter of which is of particular concern when limb-sparing treatments are intended. Biopsy methods include open surgical, closed needle, or trephine biopsy.[14,78] Jamshidi needle biopsies are reported to be 91.9% accurate in distinguishing tumors from other disorders and 82.3% accurate for determining osteosarcoma subtype.[75] Biopsies should be taken from the center of the lesion to avoid peripheral reactive bone, and diagnosis should be confirmed with histopathological evaluation of the tumor following removal when possible.[14,75]

Staging

In addition to thoracic radiographs and additional imaging for metastases, a thorough orthopedic examination will occasionally detect sites of bone metastases.[14] Although lymphatic spread is rare, regional lymph nodes should be palpated and aspirated if enlarged or firm. A patient's systemic health should also be evaluated via a complete blood count including platelets, a chemistry profile, and a urinalysis, with particular attention paid to any cardiovascular or renal issues that could complicate anesthesia or chemotherapy during treatment.

Prognostic Indicators

Various factors have been identified as prognostic indicators in canine osteosarcoma. Large tumor size, location in the humerus, high tumor grade, microvessel density, and elevated serum alkaline phosphatase and vascular endothelial growth factor (VEGF) have all been demonstrated to be associated with a poorer outcome.[6,40,59,79-82] Similarly, dogs younger than 5 years of age or presenting with detectable metastasis or lymph node metastasis have also been shown to have a poorer prognosis.[50,51] For dogs with flat bone osteosarcoma, small body size, and completeness of excision are positive prognostic indicators.[83] Most osteosarcomas in

the axial skeleton are aggressive with a correspondingly poor prognosis, with exceptions including osteosarcomas of the mandible and potentially of the cranium.[55-57,84-87]

Treatment

Although an array of treatment modalities for canine osteosarcoma exist, employing a range of surgical, radiation, and chemotherapeutic options, there continues to be no repeatable way to achieve long-term survival in the face of osteosarcoma.[88] Despite new therapeutic developments over the last two decades, overall survival with osteosarcoma has not significantly improved.[3,49,80,89,90] The following is an overview of the different modalities currently available for osteosarcoma therapy with a description of the potential short-comings of each modality.

Treatment of the Primary Tumor

Surgery

Amputation, either at the scapulohumeral or coxofemoral joint, has long been the mainstay for treatment of appendicular osteosarcoma in dogs.[51] Similarly, surgical removal of axial osteosarcomas is pursued when feasible.[55,56,84,91,92] Given the fact that most dogs with appendicular lesions already have micrometastases elsewhere in the body at the time of initial diagnosis, surgical removal of the tumor alone is considered palliative therapy. Although most dogs, including older, large-breed dogs which are most commonly affected, do well following limb amputation, limb-sparing alternatives have become a viable option for a growing number of owners and clinicians.[14] The decision to pursue a limb-sparing procedure is sometimes based on owner reluctance or is opted for due to medical reasons such as concurrent orthopedic or neurologic disease elsewhere in the body.

While offering no prolongation of survival times, various limb-sparing surgical procedures may allow dogs to maintain good limb function in their affected leg without adversely affecting prognosis.[14] Dogs that have lesions which lack pathological fractures, affect less than 50% of the bone, have less than 360° involvement of the soft tissues, and have well-defined rather than edematous soft tissue involvement may be good candidates for limb-sparing procedures.[14] Tumor location also significantly influences the prognosis of a limb-sparing surgery, with tumors of the distal radius and ulna offering the most promising prognosis in terms of limb function following surgery.[14,79] Options for limb-spare surgery include the use of allograft, metal endoprosthesis, pasteurized tumoral autograft, intraoperative extracorporal radiation, medial ulnar bone transposition, and longitudinal bone transport osteogenesis.[14,93-99] Each of these has unique advantages and disadvantages, with all providing comparable long-term prognoses when used in combination with chemotherapy.[93,94,96-101] Major complications include recurrent local disease and allograft infection, although overall survival time was shown to be statistically prolonged in dogs with allograft infections compared to those without infected allografts.[102] Local polymer chemotherapy is frequently implanted at the time of surgery, although a significant reduction in the rate of tumor recurrence has yet to be definitively demonstrated.[103]

Radiation

Radiation therapy is primarily reserved for palliative effects in dogs with osteosarcoma, although radiation may also be employed with curative-intent stereotactic radiosurgery (SRS) as an adjunctive therapy with limb-sparing procedures. In one study, over 70% of dogs receiving palliative radiation divided into 2 or 3 treatments showed amelioration of pain and lameness for a median duration of 2

months.[104] Through necrosis of the primary tumor, fractionated high-dose radiation as a primary therapy with systemic chemotherapy was reported to achieve a median survival time of 209 days in dogs with appendicular osteosarcoma.[105] The most promising application of radiation therapy in canine osteosarcoma is the increasing use of SRS, which provided a median survival time of 363 days in a case series of 11 dogs, some of which received adjunctive chemotherapy.[49] Although expensive and complicated by the incidence of pathological fractures, SRS provides a potentially valuable means of sparing the affected limb while avoiding surgery, particularly in anatomic locations not amenable to limb-sparing surgery.

Chemotherapy

Although a critical component of adjuvant therapy to prolong survival, chemotherapy has failed to show demonstrable efficacy against primary osteosarcoma lesions.[14]

Treatment of Micrometastases

Adjuvant chemotherapy plays a critical role in the management of osteosarcoma, forming an integral part of the surgical protocols that achieve maximal survival.[4,80,89,106] The addition of chemotherapy to these protocols has led to the quadrupling of median survival times in dogs with osteosarcoma.[14] Nonetheless, the vast majority of dogs ultimately die of distant metastasis despite systemic therapy.[51,107] Thus the development of additional systemic therapies is the cornerstone of current osteosarcoma research. Below is an overview of the common chemotherapeutic agents used in the management of canine osteosarcoma, followed by a discussion of newly emerging treatment strategies.

Cisplatin and carboplatin are both platinum compounds that comprise the current standard of care for chemotherapeutic treatment of canine osteosarcoma.[4,80,89,106] Classic protocols involve the administration of the specified drug intravenously for 4 cycles approximately 3 weeks apart; studies have not yet clarified whether the timing of the initial dose (pre-operatively or post-operatively) has a significant effect on survival.[14] Median survival time reported for cisplatin and carboplatin in combination with amputation has been reported to be 325 days and 321 days, respectively.[80,106] The primary difference between the two drugs is the reduced nephrotoxicity of carboplatin, obviating the need for concurrent saline diuresis during administration.[14,80,108] Carboplatin, however, is almost exclusively renally excreted and should not be administered to patients with impaired renal function.[14] Another potential complication of either drug is myelosuppression; thus a complete blood count is checked prior to administration of each dose.[14]

Doxorubicin is another chemotherapeutic agent that has demonstrated efficacy against canine osteosarcoma. Although less effective as a single agent, doxorubicin is often given in conjunction with cisplatin or carboplatin to achieve median survival times ranging from 235 to 345 days.[3,5,89,90] The current literature does not support a significant advantage of these combined protocols over single-agent therapy.[3,5,90]

Newer areas of research in the chemotherapeutic treatment of canine osteosarcoma include the investigation of immunotherapy and the targeting of molecular factors. Immunotherapy, through the use of liposome-encapsulated muramyl tripeptide-phosphatidylehtanolamine (L-MTP-PE) to stimulate the immune system has shown modestly promising results.[109,110] In addition to enhancing the cytotoxicity of

macrophages to canine osteosarcoma cells *in vitro*, L-MTP-PE administered following cisplatin showed a longer median survival time (432 days) than dogs receiving placebo liposomes (291 days).[109,110] Palladia, a small molecule receptor tyrosine kinase inhibitor that has shown efficacy with several canine tumors, was evaluated in 3 dogs with osteosarcoma. None of the 3 dogs showed reduction in tumor size, although 2 of the 3 did not show any disease progression during the study period.[111] Further study is needed to determine whether a tyrosine kinase inhibitor could play a role in stabilization of canine osteosarcoma. The VEGF inhibitor bevacizumab has recently been shown to significantly slow tumor growth in a murine xenograft model of canine osteosarcoma, thus suggesting the potential role of VEGF inhibitors in slowing progression of canine osteosarcoma.[62] Bisphosphonates, which help alleviate pain associated with osteosarcoma through inhibition of osteoclast activity, are currently used by some oncologists as adjunctive therapy for canine osteosarcoma.[112,113] Although bisphosphonates have shown cytotoxic effects against osteosarcoma cells *in vitro*, neither an *in vivo* effect nor improvement in survival times has been demonstrated.[14,114-116]

Treatment of Macroscopic Metastases

In addition to treating the primary tumor, different modalities have been investigated for their efficacy against gross metastastic disease, particularly since distant metastasis is the ultimate cause of death in the vast majority of affected dogs.[14,51,107] Surgical removal of pulmonary metastasis was shown to extend survival time to a median of 487 days, with a median of 176 days survival following the pulmonary metastasectomy.[117] Given the potential morbidity of this procedure, however, certain guidelines are recommended in order to maximize the likelihood of

prolonged survival following metastasectomy. Those guidelines stipulate that: 1) the primary tumor be in complete remission, ideally with longer than 300 days without relapse; 2) one or two nodules visible on thoracic films; 3) there should be an absence of cancer detected outside of the lungs; and 4) a doubling time of longer than 30 days should be observed for the metastatic lesion, with no new sites noted within that time.[14]

Systemic chemotherapy for the treatment of macroscopic metastases has been largely disappointing, although aerosolized chemotherapeutic and immunomodulatory drugs administered directly to the lungs have elicited measurable responses among osteosarcoma metastases in several dogs.[118-121]

Canine Osteosarcoma as a Translational Model

In addition to being a costly and devastating disease of dogs, osteosarcoma holds additional significance as a translational model for studying human osteosarcoma and potential treatment modalities.[122,123] There are many parallels between the two forms of the disease; the primary differences between the two are a lower incidence overall of human osteosarcoma (approximately 1000 cases per year in the United States) and the younger age of human patients at initial diagnosis (generally in the first two decades of life).[14,122] Similar to dogs, approximately 80% of human osteosarcoma patients will have metastases at the time of diagnosis, with only 8 to 15% of those being radiographically detectable.[124,125] Five-year survival for humans without grossly evident metastasis at the time of diagnosis ranges between 65-75%, whereas humans presenting with evidence of metastasis have a 20% 5-year survival rate.[126,127] Although survival times have improved dramatically for humans with osteosarcoma over the last few decades, the overall survival rate remains low with distant metastatic disease comprising the primary cause of death.[14,122] Thus, despite advances in local tumor

treatment, more efficacious management of metastatic osteosarcoma is ultimately needed to achieve further improvement in the survival of canine and human osteosarcoma.

Sodium Aurothiomalate

History of Use

The medical use of gold compounds, termed chrysotherapy, is a practice originating with the ancient Egyptians and experiencing relative periods of popularity in both the Middle Ages and the Renaissance.[128] Known for its immune-modulating and anti-inflammatory effects, gold compounds have been evaluated in the treatment of many different afflictions over the years, but are currently limited primarily to the management of rheumatoid arthritis in people.[128,129] Traditionally, gold compounds were used in the treatment of pemphigus and other auto-immune disorders in veterinary medicine, although they have fallen out of favor with the advent of more effective drugs with fewer side effects.[130] In humans, medical use of gold compounds has been primarily limited by their potential for severe nephrotoxicity and by their relatively poor chemical stability in a clinical setting.[128] With the clinical success of the metal complex cisplatin in the treatment a wide array of cancers, however, there has been renewed interest in the use of gold compounds in anti-cancer therapy. Below is a description of the structure and action of these compounds with an overview of their applications to cancer therapy.

Molecular Structure

Medicinal gold compounds can be divided into gold(I) compounds which primarily form linear dicoordinate configurations, and gold(III) compounds, which exist as square planar complexes. Gold(III) complexes have the same electronic configuration (d^8) as

platinum(II) compounds (such as cisplatin), and it is relatively easy for the oxidation states +1 and +3 to interchange under physiological conditions. Gold(I) is a soft cation with a preference for soft ligands, making cyanide, thiolate, and soft halides good candidates to form stable anions, whereas phosphines, arsines, and other neutral ligands may easily form cationic complexes.[128] The most medicinally relevant complexes are those incorporating thiolate and phosphines, with thiolate ligands usually being more labile and thus more likely to undergo aquation and react readily with biomolecules.[128] Aurothiomalate (ATM) is an example of this type of compound and will be the focus of later discussion regarding anti-neoplastic effects of gold compounds.

Pharmacokinetics

Relatively little is known about the pharmacokinetics of gold(I) compounds.[128] Evaluation of sodium ATM in New Zealand white rabbits administered intravenously and intramuscularly has been reported.[131] Absorption was rapid (mean absorption half-life of 9.0 minutes) and a peak concentration of 6.0 ± 1.0 μg/mL was measured. Mean α half-lives were 0.738 hours and 1.78 hours for the IV and IM routes, respectively, and the terminal β half-lives were 54.1 hours and 63.0 hours, respectively. The mean (±SD) dose absorption following IM administration was 68.9 ± 12.4%. Gold(I) compounds are considered prodrugs which must undergo transformation within the body prior to rendering their pharmacological effects. Furthermore, the gold(I) center has a high affinity for sulfur and selenium ligands, which make proteins with accessible side chains the preferred targets for binding gold(I) compounds. Gold(I) thiolate drugs have been shown to react with glutathione, albumin, and metallothioneins (MTs).[132-134] Gold(I) compounds have been shown to bind extensively with MT, a heavy-metal binding protein which is found in large amounts in the mammalian kidney and liver and likely

plays a critical role in the storage and metabolism of gold compounds.[135] Gold(I) drugs may also be activated through conversion of the compound to aurocyanide ($Au(CN)_2^-$); the mechanism of this conversion is unknown but may be mediated through the enzyme myeloperoxidase.[136] Aurocyanide is often found in the blood and urine of patients receiving gold therapy and is a known inhibitor of neutrophils and monocytes and of lymphocyte proliferation.[137] Gold(III) may also form as a metabolite of gold(I) compounds; this phenomenon may account for the adverse immune reactions seen with ATM, as discussed later.[138] Of biological significance is the fact that gold(I) thiolates are water-soluble compounds that bind cell membrane surface thiols rather than crossing the membrane to enter cells. As a result, these drugs likely initiate their effects through preventing nutrient uptake by cells or by interfering with cell signaling pathways.[132,139,140] The transport process that mediates gold uptake across the cell membrane is not an energy-dependent active transport process, making it possible for intracellular and extracellular gold concentrations to establish equilibrium.[137,141]

Mechanism of Action

While the precise mechanism of gold compounds' anti-inflammatory and anti-neoplastic effects remain controversial, there is a growing body of knowledge regarding these compounds' biological pathways. The anti-inflammatory effects utilized in the treatment of rheumatoid arthritis are mediated through decreased production of pro-inflammatory cytokines and inhibition of proteolytic enzymes.[142] In the clinical setting, it is likely that gold's anti-inflammatory effects are much more varied.[142] In light of the emergence of the potential anti-neoplastic properties of gold compounds, research into their proposed molecular targets has been pursued with renewed interest. These potential targets have included DNA, thioredoxin reductase, apoptosis signaling

pathways, and protein kinases.[128] The following is a review of the current literature regarding the action of gold(I) compounds on each of these classes of targets.

DNA interference. Although DNA was originally considered to be an important target of the anti-arthritic gold(I) compounds, it has since been shown that these interactions are relatively weak.[143] The relative weakness of this interaction is particularly significant in comparison with the ability of these compounds to form strong interactions with the side chains of other potential protein targets.[144,145]

Thioredoxin redox system inhibition. Two such proteins with which gold(I) compounds have demonstrated strong binding capability are the enzymes thioredoxin reductase (TrxR) and thioredoxin (Trx).[146] These enzymes are found in both cytosolic (Trx1 and TrxR1) and mitochondrial (Trx2 and TrxR2) isoforms and have a wide variety of functions including reactive oxygen species scavenging, DNA synthesis, and activation of transcription factors for cell growth and survival.[128] Increased levels of cytosolic Trx1 have been shown to be correlated with the aggressive behavior and inhibition of apoptosis of certain human carcinomas, making this enzyme a target of great interest in the development of anticancer drugs.[147-150] Auranofin, a common gold(I) compound, has been shown to be both a specific and potent inhibitor of both cytosolic and mitochondrial forms of TrxR.[146,151,152] Gold(I) compounds have also demonstrated effects on mitochondria through alterations in mitochondrial membrane potential and permeability, leading to the release of cytochrome c into the cytoplasm, a process critical to the induction of apoptosis; thus by inhibiting Trx and TrxR, gold(I) drugs are able to induce apoptosis.[128,144,146,152,153]

Apoptotic signaling pathway activation. Caspase-3, along with other caspases, is a well-established mediator of apoptosis.[154] Gold(I) compounds have been shown to activate caspase-3 and lead to apoptosis in cancer cells through the generation of reactive oxygen species and selective accumulation within the mitochondria and ATP depletion.[155,156]

Protein kinase inhibition. Protein kinase C (PKC) is a family of serine/threonine kinases that perform a diverse set of functions involved in cellular proliferation, differentiation, polarity, and survival (Figure 1-1).[157] Alterations in PKC activity have been demonstrated in several cancers, and atypical protein kinase C iota (PKCι) is an established human oncogene necessary for the transformed growth of human cancer cells.[158,159]

The gold(I) drugs aurothiomalate (ATM) and aurothioglucose are potent *in vitro* inhibitors of PKCι interactions with Par6, a cysteine-containing polarity protein important in the PKCι signaling pathway.[159,160] ATM may also form gold-cysteine adducts on Cys69, the cysteine residue located within the active site of PKCι where Par6 normally binds (Figure 1-2).[159,160] This interaction, essentially blocking the binding of Par6 to PKCι at the conserved Phox Bem 1 (PB1) domain, effectively blocks the signaling pathway downstream of PKCι (Figure 1-3).

Toxicity

One of the limiting factors in the use of gold compounds in the treatment of rheumatoid arthritis is the occurrence of a variety of adverse effects.[142,161] These include glomerulonephritis, cytopenias, hepatitis, gastrointestinal effects, pneumonitis, and cutaneous reactions.[162] Thrombocytopenia is caused by anti-platelet antibodies, while the glomerulonephritis is thought to be immune-complex-mediated.[163,164] The

incidence of these reactions varies, but it is estimated that treatment is discontinued in up to one third of patients receiving gold compounds due to these side effects, and a genetic basis of the susceptibility to these reactions is suspected.[161,165,166] Reported side effects in dogs are similar and include bone marrow suppression, oral ulceration, glomerulonephropathy, and cutaneous reactions.[130,167] Systemic eosinophilia is believed to occasionally precede development of cutaneous reactions.[130]

Role in Anti-Neoplastic Therapy

Gold(I) compounds: A number of gold(I) and gold(III) compounds have been evaluated for their anti-neoplastic activity both *in vitro* and *in vivo*. Several gold(I) complexes were evaluated for their cytotoxicity against B16 melanoma cells and P388 leukemia cells as well as for their anti-tumor activity *in vivo* against P388 leukemia in mice.[168] Gold(I) thiosugar complexes with phosphine ligands were found to be the most potent. From these investigations, the following conclusions were made regarding the anti-neoplastic effects of gold(I) compounds: 1) lack of potency *in vitro* correlates with lack of anti-tumor activity *in vivo*, and 2) potent cytotoxicity *in vitro* is not necessarily predictive of activity *in vivo*. Although early studies of phosphine gold(I) thionucleobase compounds were promising, more extensive *in vitro* screening of their cytotoxicity against a panel of 60 tumor cell lines through the National Cancer Institute failed to demonstrate significant cytotoxic activity, although several human solid tumor cell lines have shown more response.[169,170]

Additional studies have demonstrated that the cytotoxicity of gold(I) compounds *in vitro* is not necessarily reflected by their *in vivo* activity.[171] The *in vivo* effects of most gold(I) compounds has thus far been relatively disappointing in light of their *in vitro* anti-neoplastic activity. In one of the more encouraging studies, auranofin, a gold(I) drug,

was found to prolong survival in a dose-dependent manner.[172] In a study evaluating the efficacy of auranofin against several human cancer types in a mouse xenograft model, intraperitoneally administered auranofin led to a 59% increased life span among mice with P388 leukemia, although none of the other cancers evaluated showed any sensitivity to this compound.[171]

Aurothiomalate

ATM is an example of a gold(I) compound and an established anti-rheumatoid-arthritis agent (Figure 1-4).[142] It has been investigated extensively for both its *in vitro* and increasingly its *in vivo* effects against certain cancers.[128] Given its acceptability as an established treatment for rheumatoid arthritis, its ease of availability, and its promise as an anti-neoplastic agent, ATM was selected as the gold(I) compound for evaluation in this study.

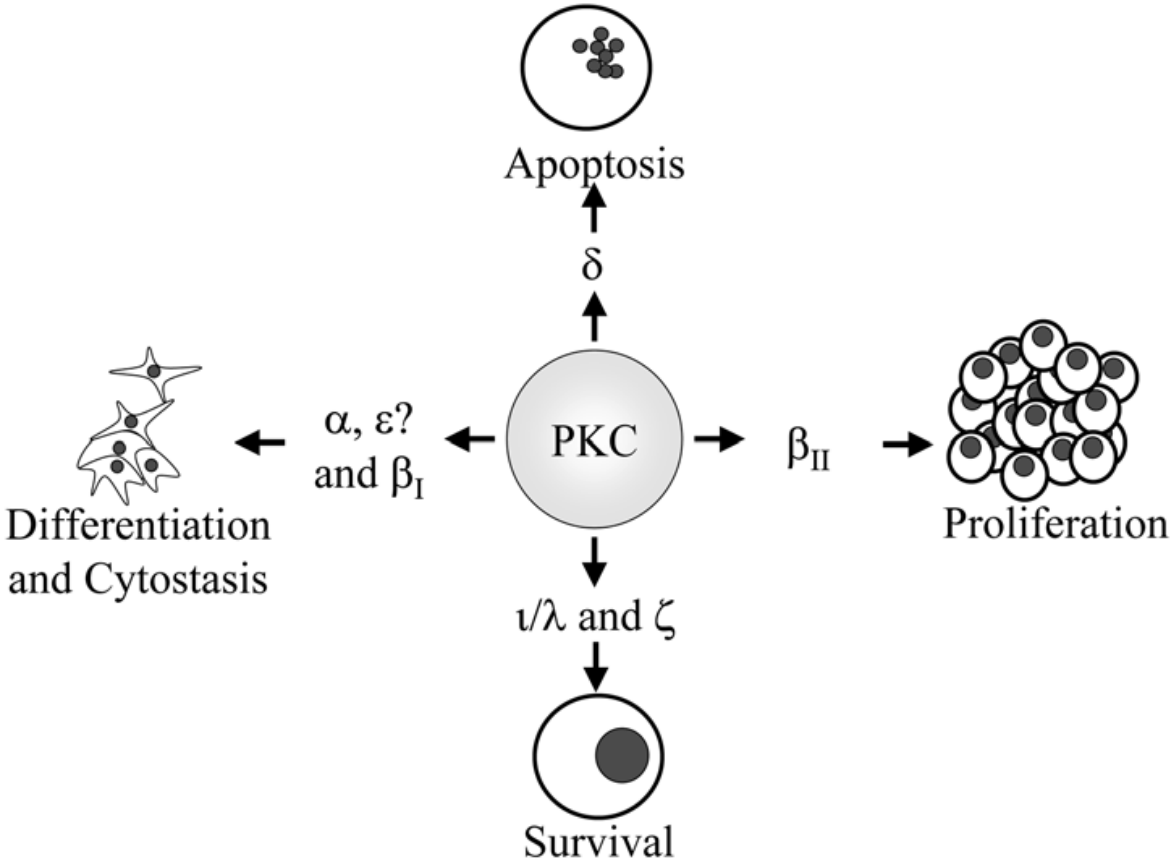


Figure 1-1. Established functions of PKC isozymes *in vivo*.[158]

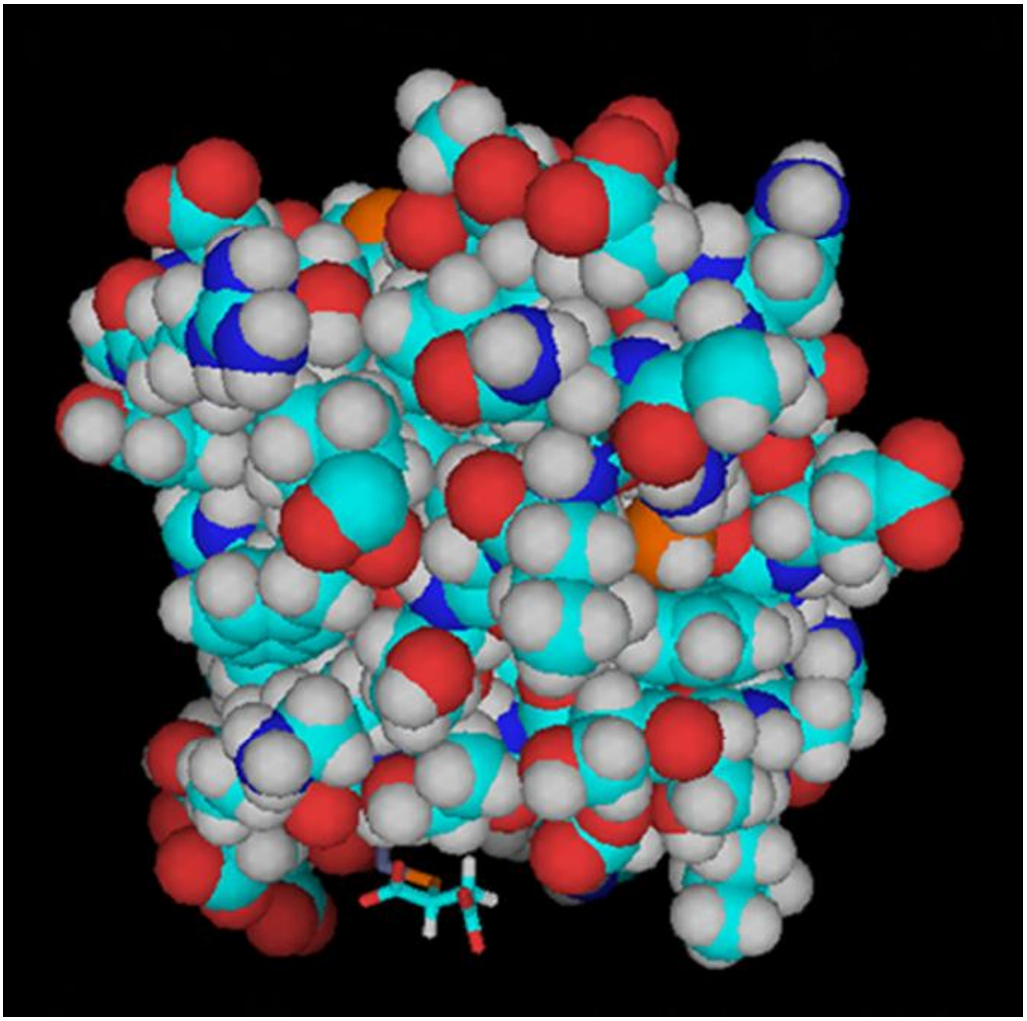


Figure 1-2. Illustration of PKCι-Cys69-ATM adduct. The Cys69-ATM adduct is depicted by the linear component of the model shown at the bottom of the diagram as it protrudes into the binding cleft normally occupied by Par6 in the PKCι-Par6 complex.[159]

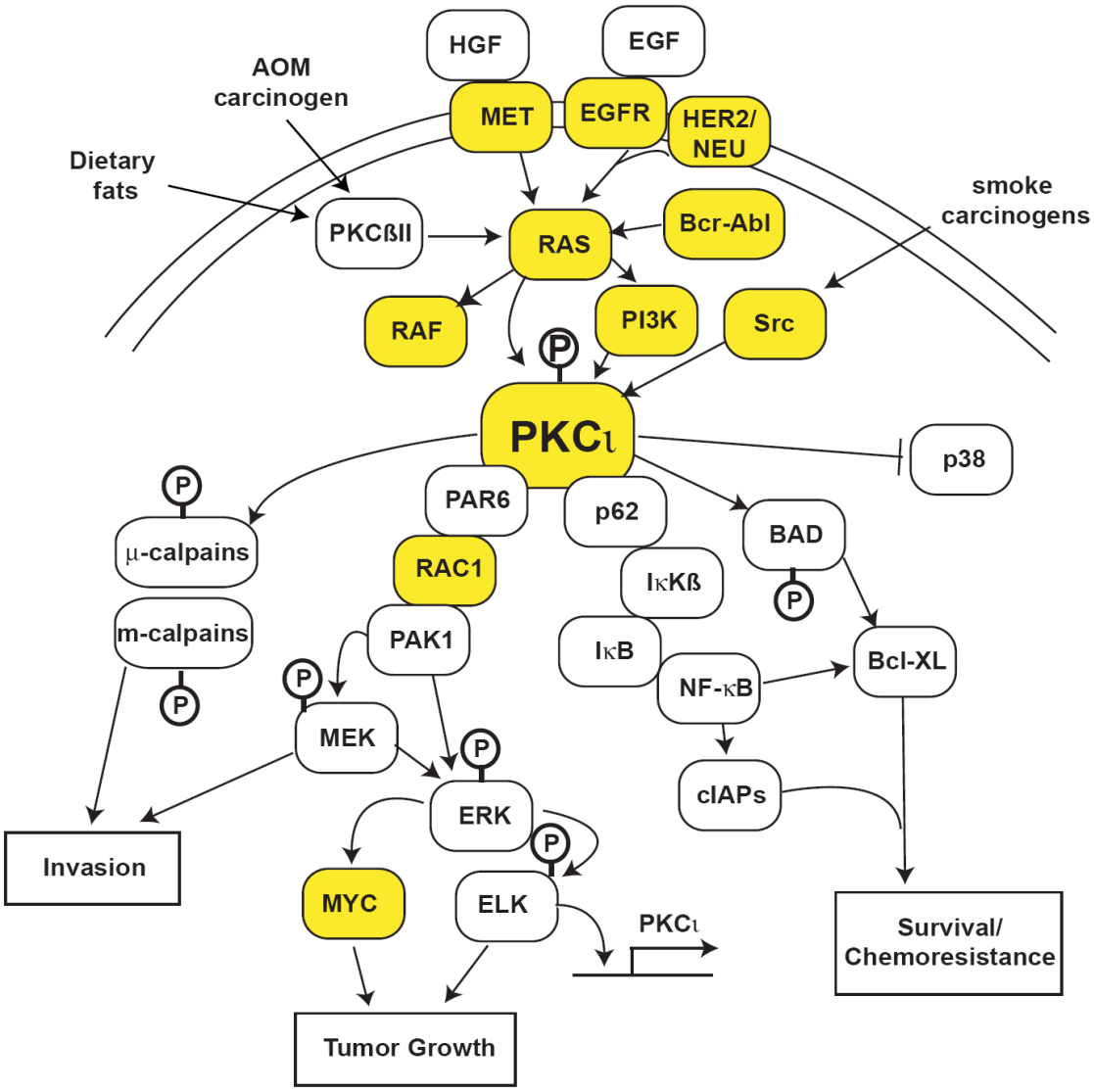


Figure 1-3. Schematic representation of the major oncogenic PKCι signaling pathways, including interactions with Par6, the protein to which binding by PKCι is blocked by ATM.[159]

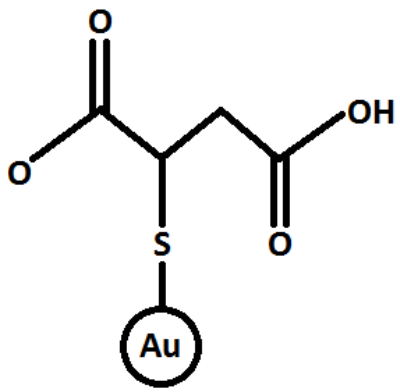


Figure 1-4. Molecular structure of aurothiomalate.

CHAPTER 2
ANTI-PROLIFERATIVE EFFECTS OF AUROTHIOMALATE ON HUMAN AND CANINE OSTEOSARCOMA CELLS IN VITRO

Background

Early investigation into the anti-neoplastic effects of ATM *in vitro* has been pursued using HCT-15 cells (human colorectal carcinoma), AGS cells (gastric epithelial cells derived from a human malignancy), and Meth/A cells (murine lymphosarcoma).[173] The results of this investigation revealed that all three cell lines showed fifty percent suppression at ATM concentrations ranging from 10 µg/ml to 125 µg/ml, depending on the cell line. Equivalent suppression of HCT-15 cells was seen at similar doses of cisplatinum. Concurrent flow cytometry on HCT-15 cells suggested that ATM blocked the S, G2 to M, and M phases of replication.

ATM has also been investigated in the treatment of human prostate cancer.[174] *In vitro* treatment of aggressive prostate cancer cells (PC3U) and normal prostate epithelial cells (PrEC) with ATM showed a significant apoptotic effect on the tumor cells without causing apoptosis of normal epithelial cells. The authors demonstrated that PKCι expression is elevated in PC3U cells compared to that of PrEC cells and that ATM treatment disrupted the binding of PKCι to the PB1 domain of Par6, a scaffold protein involved in cell division, migration, adhesion, and cytoskeletal reorganization.[159,174] ATM treatment led to caspase 3, p38, and JNK MAP kinase activation and to mitochondrial *cytochrome c* release in PC3U cells, all of which are important components of the apoptotic pathway.[174] This disruption led to increased activation of ERK, a kinase which when hyperactivated has been linked to apoptosis in some cancers, and to an increase in cleaved caspase 3 and release of mitochondrial *cytochrome c* into the cytoplasm, both of which are associated with apoptosis.[174]

Furthermore, *in vitro* treatment of the prostate cancer cells with ATM at 50 μM for 24 hours led to 40% of the PC3U cells displaying an apoptotic phenotype while no apoptotic changes were noted among normal prostate epithelial cells undergoing the same treatment, leading to the conclusion that ATM has a pro-apoptotic effect on prostate cancer cells *in vitro*.[174] Moreover, the cytotoxic effects of ATM were achieved at levels consistent with serum levels achieved in patients treated with ATM for rheumatoid arthritis.[174]

Additional findings by researchers at the Mayo Clinic evaluating the role of PKCι in cancer further supports the assertion that ATM inhibits binding of PKCι with the PB1 domain of Par6, and suggests that this interaction inhibits activation of the downstream signaling pathway normally initiated by PKCι in neoplastic cells.[160,181,182] To further support this theory, a significant correlation was demonstrated between Par6 mRNA and PKCι mRNA and between Par6 mRNA and ATM sensitivity *in vitro*.[177] The same study also showed that the sensitivity of several different types of lung cancer to ATM is not due to general sensitivity to cytotoxic therapeutic agents.[177]

Given the efficacy of ATM against several neoplastic cell lines *in vitro*, the objectives of this *in vitro* study were to: 1) evaluate the effects of sodium ATM treatment on cultured human and canine osteosarcoma cells, and 2) determine whether sodium ATM could achieve inhibition of tumor cell survival at doses comparable to serum concentrations achieved in human patients receiving therapeutic doses of ATM for other conditions. We hypothesized that: 1) ATM would inhibit survival of cultured canine and human osteosarcoma cells in a dose-dependent manner; and, that 2) cell survival

inhibition would be achieved at doses comparable to serum levels achieved in human patients receiving ATM for treatment of rheumatic diseases.

Materials and Methods

Aurothiomalate Preparation

Sodium aurothiomalate hydrate (ATM) was obtained as a stock powder with a molecular weight of 390.08 g/mol (Sigma-Aldrich®, St. Louis, Missouri). A stock solution of 128.2 mM was prepared by dissolving the sodium ATM hydrate in sterile phosphate buffered saline (PBS). The stock solution was protected from light throughout preparation and storage and was stored at 4°C. Fresh serial dilutions of ATM were prepared immediately prior to addition to the plates for each phase of the study by adding stock solution to the appropriate amount of media.

Cell Culture Preparation

Separate canine and human osteosarcoma cell lines were obtained for culture and *in vitro* testing. The canine osteosarcoma cell line, canine highly metastasizing parent osteosarcoma (HMPOS) cells (provided by Dr. Tsuyoshi Kadosawa, Laboratory of Veterinary Surgery, Hokkaido University, Sapporo, Japan), were used to evaluate the *in vitro* effects of ATM on canine osteosarcoma. HMPOS is a pulmonary metastatic subtype of parent osteosarcoma (POS), which originated from the proximal femoral osteosarcoma of a 1.5-year-old male dog.[31] HMPOS cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% heat-inactivated fetal calf serum (FBS), 1% Pen-Strep, 1% L-glutamine, vitamin solution, and non-essential amino acids. Cells were seeded (2×10^6) into 150-cm^2 flasks and maintained at 37°C under 5% CO_2 and 95% room air. The human osteosarcoma cell line, MG-63 (obtained from the American Type Culture Collection, Manassas, Virginia), was cultured

in minimum essential media (MEM) supplemented with heat-inactivated 10% FBS. MG-63 cells were seeded and maintained in an identical manner and environment as HMPOS cells. Both cell lines were passed using 0.25% trypsin.

Initial Determination of Cell Viability

Several pilot studies were performed to determine the appropriate range of ATM concentrations and incubation time to use for the evaluation of whether ATM has an inhibitory effect on osteosarcoma cells. These pilot studies were also used to determine the optimal number of cells per plate for the final phase of the *in vitro* study. The resazurin reduction assay (CellTiter-Blue® assay, Promega Corp., Madison, Wisconsin) was used to assess cell viability in these initial pilot studies. This assay works by using a fluorescent plate reader to quantify the fluorescent signal emitted by resorufin, a fluorescent molecule produced by conversion of the redox dye resazurin. Since only viable cells are able to convert resazurin to resorufin, the strength of the fluorescent signal reflects the quantity of viable cells present.[175]

Initially, HMPOS cells were seeded into 96-well plates in two groups of 5,000 cells per well and 10,000 cells per well. These cells were incubated at 37°C under 5% CO_2 and 95% room air for 24 hours, at which time ATM was added at concentrations of 0, 0.1, 1, 10, 100, and 1000 µM. Following an incubation time 24, 48, or 72 hours with the ATM, the resazurin assay was performed following the manufacturer's specifications. Fluorescence was quantified with a fluorescence plate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Five replicates of each group were analyzed in this initial phase.

A second pilot study evaluated a higher range of ATM doses (0, 1, 100, 1,000, 5,000, and 10,000 µM) on HMPOS (5,000 cells/plate) and MG-63 (5,000 and 10,000

cells/plate) using similar methodologies as those used in the first pilot study. Each group was tested in triplicate, and the resazurin assay was performed following 72 hours of incubation with ATM.

A third pilot study evaluated different dose ranges of ATM on HMPOS (0, 100, 1,000, 20,000, 50,000 μM) and MG-63 (0, 100, 250, 750, 1,000 μM), each with 5,000 cells per plate. These groups were tested in triplicate, and the resazurin assay was performed following 72 hours of ATM incubation with the media removed and replaced with new ATM-containing media every 24 hours. Thus, this pilot study also evaluated whether adding "fresh" ATM was a feasible option for evaluating the effects of ATM on cell viability.

Clonogenic Assays

Following the initial pilot studies evaluating cell viability using the resazurin assay, a final pilot study was performed using HMPOS (500, 1000, and 15000 cells per plate) and MG-63 (500 cells per plate) in 60-mm plates. ATM in concentrations of 0, 0.1, 1, 10, 100, and 1,000 μM were added to the designated plates after incubating 24 hours at 37°C under 5% CO_2 and 95% room air. Serial dilutions of ATM were created such that adding 2.5 mLs of the ATM-containing media created a plate containing 5 mLs of media with the appropriate total ATM concentration. All groups were tested in triplicate. The plates were then incubated at 37°C under 5% CO_2 and 95% room air and protected from light. The control plates were evaluated daily until the cell colonies were observed to reach confluence, which was noted after approximately 10 days of incubation. At this time, all plates were evaluated by manually counting the number of cell colonies per plate. This was performed by draining the media from each plate,

rinsing with 2 ml PBS, fixing the plates with 1 ml 70% ethanol for 3 minutes, and staining with 2 ml 0.1% crystal violet (Sigma-Aldrich®, St. Louis, Missouri) for 3 minutes.

Following evaluation of the plates from this pilot study, the final phase of the *in vitro* study was commenced following a similar procedure to that of the fourth pilot study. HMPOS and MG-63 were seeded into 60-mm plates (500 cells per plate in 2.5 mLs of media) and incubated at 37°C under 5% CO_2 and 95% room air for 24 hours, at which time ATM was added to the plates to create plates containing 5 mLs of media with 0, 0.1, 1.0, 2.5, 7.5, 10.0, and 50.0 µM concentrations of ATM. The plates were again incubated at 37°C under 5% CO_2 and 95% room air until cell colonies in control plates reached confluence (after approximately 10 days of incubation). At this time, all plates were drained of media, fixed, and stained with crystal violet as described above. Colonies on each plate were counted, and the percent cell survival was calculated by dividing the number of colonies in a given treatment group by the number of colonies in the control group. All groups were tested in triplicate, and each assay was repeated 3 times, thus providing a total of 9 data points for each ATM concentration for both cell lines.

Data Analysis

Data from the pilot studies was used to direct planning of the final phase of the *in vitro* study and was not analyzed statistically. Cell colony data from the clonogenic assays was analyzed using Sigma-Plot® software (SigmaPlot for Windows, version 11.00; Systat Software, Inc., Erkrath, Germany). Data was tested for normality using the Shapiro-Wilk test. Non-parametric data are reported as medians with an interquartile range (25-75%). The Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks was used to detect differences between treatment groups. Post-hoc

pairwise multiple comparisons were performed using the Tukey Test. For all statistical analyses, a P-value < 0.05 was considered statistically significant. The IC_{50} was defined as the ATM concentration at which 50% cell death was achieved compared to the control group. Cell survival data was fitted to a 4-parameter nonlinear regression model to determine the mean half maximal inhibitory concentration IC_{50} for both cell lines, and goodness of fit was reported as the R^2 value.

Results

Pilot Studies

The resazurin assays performed in the initial phases of the *in vitro* study demonstrated a clear dose-dependent effect of ATM on both HMPOS and MG-63 cell lines after 72 hours of incubation with the drug. This effect was repeated in the following pilot study using the clonogenic assay on both cell lines. The results of these preliminary studies were used to determine the cell numbers and ATM concentrations for the final clonogenic assays.

Effect of Aurothiomalate on HMPOS

In the final *in vitro* assays, ATM showed a clear dose-dependent inhibitory effect on HMPOS cell survival (P < 0.001) (Figure 2-1). No cells survived at a dose of 50 μM, and approximately 11% of cells survived at a dose of 10 μM. The calculated IC_{50} of ATM on HMPOS cells was 1.2 μM, with an R^2 value of 0.82 indicating the goodness of fit for the regression equation used to derive this value. The slope of the regression curve was -0.52.

Effect of Aurothiomalate on MG-63

MG-63 cells showed a similar dose-dependent decrease in survival fraction when treated with ATM (P < 0.001) (Figure 2-2). Approximately 3% of cells survived at an

ATM concentration of 10 μM, with no cells surviving at 50 μM. The derived IC_{50} of ATM on MG-63 cells *in vitro* was 3.0 μM, with an R^2 value of 0.98 indicating the goodness of fit for the regression equation used to derive this value. The slope of the regression curve was -3.26.

Discussion

The results of this *in vitro* study demonstrate a clear dose-dependent inhibitory effect of ATM on both canine and human osteosarcoma cells. Significant inhibition of HMPOS and MG-63 cell survival was achieved *in vitro* at ATM concentrations of 5 μM for both the HMPOS and MG-63 cell lines, and no cells from either osteosarcoma cell line survived at the 50-μM treatment level. Previous studies have shown that serum levels in human patients being treated with ATM for other disorders (primarily rheumatoid arthritis) range from 3-8 μg/mL.[176] These serum gold levels of 3-8 μg/mL correspond to ATM concentrations of 7.7 to 20.5 μM. Thus, consistent with our hypothesis, ATM achieved significant *in vitro* cytotoxicity at concentrations equivalent to those reached in patients receiving ATM at previously established therapeutic dosages with acceptably low incidences of adverse effects.[176] Given the potential toxicity associated with gold therapy, achieving targeted cytotoxicity within dose ranges currently accepted as “safe” is a valuable finding when considering the clinical application of ATM as an antineoplastic drug.

This study is the first to report an inhibitory effect of a gold compound on osteosarcoma and, to the author’s knowledge, the first to report cytotoxicity of a gold compound on a tumor of companion animals. Clonogenic assays are an accepted method for evaluating the inhibitory effect of a new potential drug and have been used to assess the *in vitro* efficacy of ATM against several types of cancer cells.[173] Previous

work with ATM used anchorage-independent growth assays to evaluate the inhibitory effect of ATM on non-small cell lung cancer.[177] The *in vitro* IC_{50} values reported here for HMPOS and MG-63 are 1.1 µmol/L and 3.0 µmol/L, respectively. These values are similar to the IC_{50} values of 0.3 and 1.3 µmol/L reported for two ATM-sensitive lung cancer cell lines (A427 and H1703).[177] In that study, several different lung cancers were classified as either ATM-sensitive (IC_{50} < 5 µmol/L) and ATM-insensitive (IC_{50} > 40 µmol/L). This categorization was correlated with *in vivo* experiments using murine xenografts in which A427 tumors demonstrated an *in vivo* IC_{50} of less than 2.5 µmol/L, and an insensitive cell line demonstrated a higher *in vivo* IC_{50} similar to its *in vitro* IC_{50}.[177]

According to this classification, the results presented here indicate that both HMPOS and MG-63 cell lines could be expected to be sensitive to ATM *in vivo*, with the lower HMPOS IC_{50} value potentially indicating increased ATM sensitivity compared to MG-63 cells. In contrast, the shape of the dose-response curves suggests that MG-63 cells may be more responsive than HMPOS cells to escalating ATM doses. The IC_{50} values reported for these cell lines may reflect a slightly decreased innate *in vitro* sensitivity to ATM compared to the most sensitive lung cancer cell lines. Alternatively, the slightly higher IC_{50} values may reflect differences related to the method of measurement of susceptibility (clonogenic assay versus anchorage-independent growth assay).

There are several limitations of this *in vitro* evaluation of ATM's efficacy against canine and human osteosarcoma. Although clonogenic assays are an accepted and well-established method of assessing a compound's cytotoxic effects on cultured cells,

an *in vitro* assessment does not always accurately reflect the relationship between the compound under evaluation and the cellular response *in vivo*. Tumors cells that are susceptible to a drug *in vitro* are often less susceptible to that drug *in vivo*, as other factors in the tumor microenvironment cannot be accounted for in an *in vitro* setting, and the *in vivo* response of osteosarcoma to ATM treatment has not been previously evaluated.[178,179] The *in vivo* IC_{50} for a relatively ATM-insensitive lung cancer cell line was actually less than the *in vitro* IC_{50} of the same cell line (25.6 μmol/L versus 46 μmol/L, respectively), suggesting that the *in vivo* response to ATM may actually be more profound than the *in vitro* response in some cancers.[177]

Although the aspects of gold metabolism remain under investigation, gold(I) compounds are commonly considered to be prodrugs due to the rapidity with which they undergo transformation in biological systems.[142,180] Gold(I) compounds have an extremely labile center and thus quickly undergo ligand exchange reactions in biofluids, cells, and proteins.[142] This high reactivity of gold(I) compounds with biological media may explain why the cytotoxic effects of ATM are observed in an *in vitro* setting despite the absence of biological systems typically involved in their metabolism and activation. Furthermore, gold(I) compounds and their metabolites are not readily taken up by cells *in vitro* or *in vivo*; instead these compounds bind to cell surface thiols to affect cell signaling pathways.[142] Although in vitro assays of ATM's cytotoxicity against cancer cells both in this and previous studies have not accounted for biotransformation of ATM, cytotoxic effects of this drug have been clearly demonstrated.[174] Given the multifaceted mechanisms of action of gold compounds and their propensity for additional

biotransformation, further activation of ATM and other gold(I) drugs *in vivo* may lead to increased *in vivo* efficacy compared to that seen in vitro.[142]

Another limitation is that the osteosarcoma cell lines in this study were not subjected to clonogenic assays performed using other cytotoxic agents to evaluate the specificity of the cell lines' sensitivity to ATM. Thus the ATM sensitivity demonstrated in this study could be associated with a general sensitivity to chemotherapeutic agents rather than to sensitivity specific to ATM. A previous study by researchers at the Mayo Clinic evaluated for such specificity in human lung cancer cell lines by simultaneously testing other chemotherapeutic agents in addition to testing ATM on ATM-sensitive and ATM-insensitive cell lines *in vitro*.[177] These researchers found that the cell lines displayed dramatically different sensitivities to ATM, whereas the sensitivity to other cytotoxic agents did not vary widely nor did it correlate with ATM sensitivity.[177] Additional clonogenic assays with multiple canine and human osteosarcoma cell lines subjected to ATM and additional cytotoxic agents are necessary to determine whether the ATM sensitivity seen in MG-63 and HMPOS cells is specific to ATM. Similarly, evaluating the effect of ATM treatment non-neoplastic canine and human osteoblasts would confirm that the cytotoxicity seen *in vitro* was the result of ATM's mechanism of action against neoplastic cells rather than an effect of general cytotoxicity from direct exposure to a compound in cell culture.

The current study did not evaluate the specific mechanism of cytotoxicity of ATM against HMPOS and MG-63 cells. Although clonogenic assays illustrate the effects of escalating drug doses on cell survival and thus reflect a drug's cytotoxicity, these assays do not delineate the mechanism(s) of action underlying this decreased cell

survival. Given the repeatable elucidation of PKCι's inhibition of PB1-PB1 binding demonstrated in previous studies, we suspect that this mechanism may also play a role in ATM's efficacy against osteosarcoma in vitro. Given the broad array of PKCι's cellular functions as well as the diversity of ATM's previously described mechanisms of action, ATM may have a multi-faceted role in inhibiting the survival of cancer cells, such as the induction of apoptosis noted in prostate cancer cells.[174] Although the disruption of PKCι binding at the PB1 domain appears to be a relatively ubiquitous mechanism of ATM's antineoplastic action *in vitro*, further studies are indicated to confirm the involvement of this pathway in ATM's effects on osteosarcoma.[159]

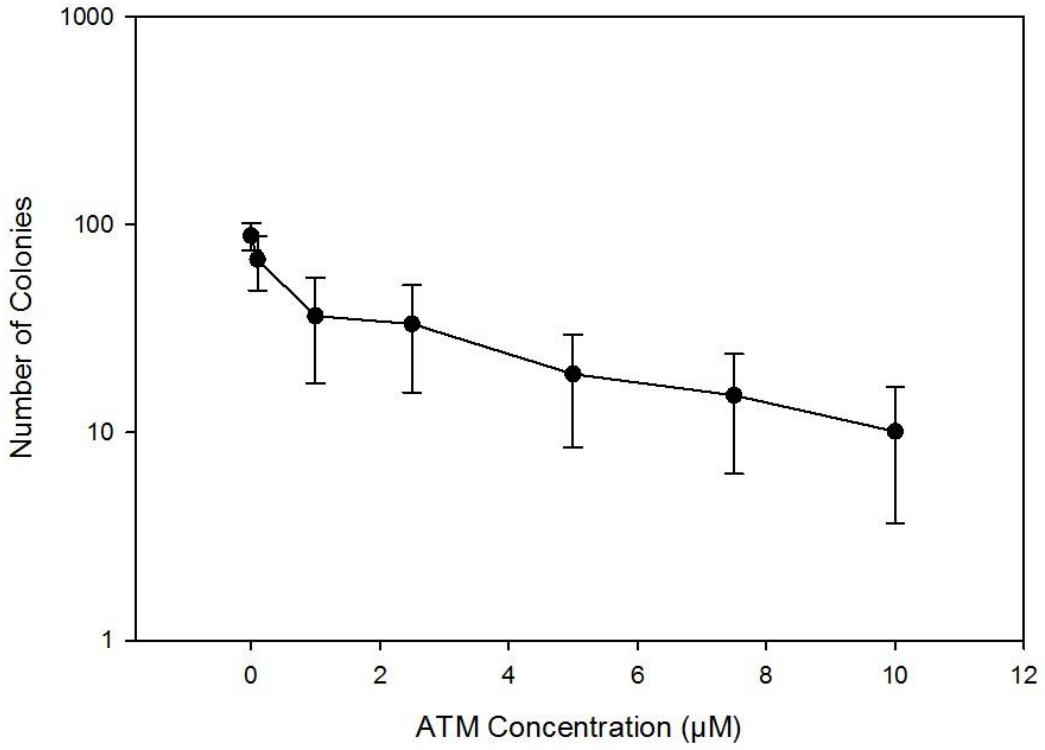


Figure 2-1. Dose-response curve for HMPOS cell colonies treated with ATM in vitro.

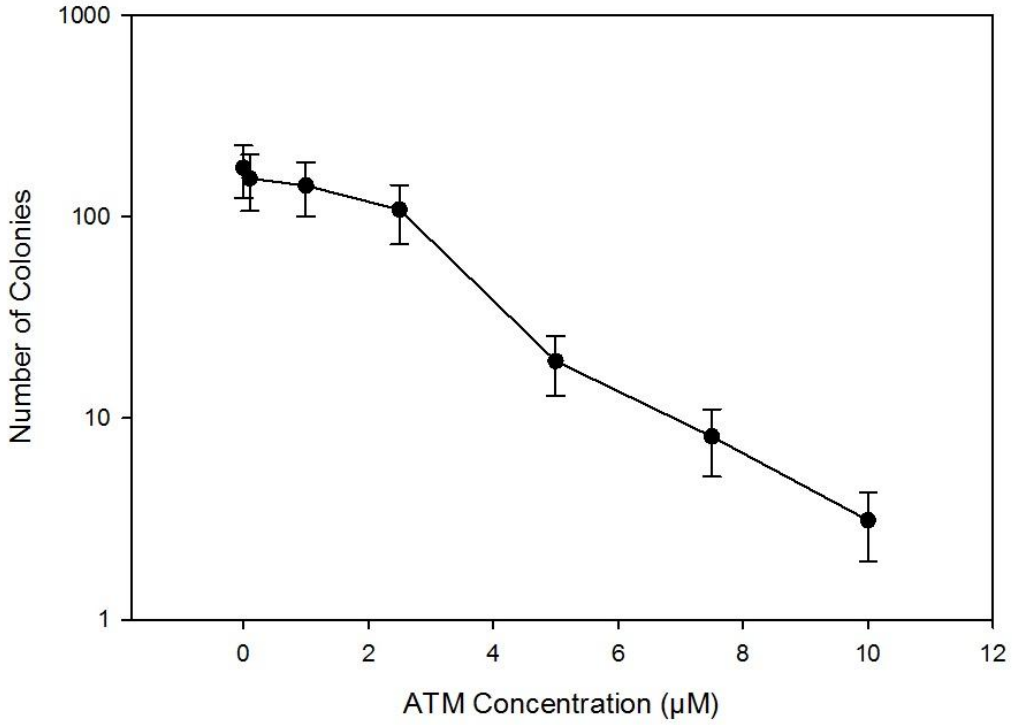


Figure 2-2. Dose-response curve for MG-63 cell colonies treated with ATM in vitro.

CHAPTER 3
EFFECTS OF AUROTHIOMALATE ON CANINE OSTEOSARCOMA IN A MURINE XENOGRAFT MODEL

Background

Early research evaluating the *in vivo* anti-neoplastic effects of ATM included the use of mice to evaluate the toxicity of ATM.[173,181] General tolerance of the drug was assessed by administering doses of 2 mg/kg and 10 mg/kg subcutaneously every other day for a total of 3 doses or by giving approximately 30 mg/kg/day in drinking water for 10 days. These mice were observed for twenty days with no adverse effects noted, whereas approximately 60% of mice receiving a single 10 mg/kg subcutaneous cisplatinum injection died within 10 days of receiving the injection.[173] In a second study, the authors demonstrated the effect of ATM on mice inoculated with Meth/A cells intraperitoneally.[181] ATM was injected subcutaneously every other day for 3 days or administered in drinking water daily for two weeks. Survival was significantly prolonged in mice receiving ATM at 30 mg/kg given subcutaneously every other day or 75 mg/kg given orally every day, whereas the survival time of mice treated subcutaneously with ATM at 125 mg/kg/day for 3 doses was not significantly prolonged. In this study, a group of mice received subcutaneous cisplatinum injections as a positive control. Of interest was the fact that cisplatinum had a narrower effective dose range and displayed marked toxicity at 125 mg/kg for 3 doses, whereas mice receiving ATM at 125 mg/kg showed no significant signs of toxicity.[181]

Much of the current research evaluating the potential applications of ATM in anti-cancer therapy stems from the work performed at the Mayo Clinic Comprehensive Cancer Center. This group's research focuses on human non-small cell lung cancers (NSCLC) and the role played by PKCι in these cancers' tumorigenicity.[182] Research

from the Mayo Clinic has demonstrated that PKCι is both highly expressed in human NSCLC and is required for transformed growth in soft agar in vitro and for tumorigenicity *in vivo*.[182,183] In subsequent studies, the authors demonstrated that ATM is a potent inhibitor of PKCι in vitro and inhibits NSCLC cellular transformation and tumorigenicity by inhibiting PKCι binding to Par6 in vitro and by disrupting the signaling pathway downstream of PKCι *in vivo*.[160,184] A panel of major human lung cancer subtypes was subsequently screened for responsiveness to ATM treatment, which revealed that ATM sensitivity correlated positively with PKCι and Par6 expression but not with that of thioredoxin reductase 1 or 2 (proposed target enzymes of ATM in rheumatoid arthritis treatment).[177] Sensitivity to ATM was also not associated with general sensitivity to other cytotoxic agents.[177] Furthermore, ATM inhibited tumorigenicity of both sensitive and insensitive lung tumors *in vivo* at plasma drug concentrations equivalent to those achieved in rheumatoid arthritis patients treated with ATM. This *in vivo* efficacy was mediated through inhibition of the Mek/Erk signaling pathway downstream of PKCι and through decreased cellular proliferation with no apparent effect on tumor apoptosis or vascularization.[177] The Mayo Clinic is currently sponsoring two Phase I Clinical Trials and planning for a Phase II Clinical Trial to evaluate the efficacy of sodium ATM as a treatment for human NSCLC.[185,186]

The purpose of this *in vivo* study was to evaluate the effects of sodium ATM on canine osteosarcoma in a murine xenograft model. We hypothesized that sodium ATM treatment would inhibit canine osteosarcoma tumor growth and prolong survival times. We further hypothesized that ATM treatment would achieve these effects by decreasing cellular proliferation without inducing tumor cell apoptosis. Lastly, we hypothesized that

sodium ATM, through its proposed anti-proliferative effects would decrease the incidence of micro- and macrometastasis.

Materials and Methods

Cell Culture Preparation

All experiments were performed at the University of Florida and were approved by the Institutional Animal Care and Use Committee (IACUC). Canine highly metastasizing parent osteosarcoma (HMPOS) cells were used to induce xenograft tumors. Cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FBS), 1% Pen-Strep, 1% L-glutamine, vitamin solution, and non-essential amino acids. Cells were seeded (2×10^6) into 150-cm^2 flasks and maintained at 37°C under 5% CO_2 and 95% room air.

Tumor Inoculation

Cells were grown to confluence (3 day passage time), washed with Hank's solution (pH 7.4), detached from their plates with 0.25% trypsin, resuspended in complete media, and counted with a hemocytometer. These cells were precipitated and re-suspended in phosphate buffered saline (PBS) to a concentration of 5×10^5 cells/0.01 mL (5×10^6 cells/mL) for subcutaneous inoculation in the final study. Different cell concentrations were used for the pilot studies as specified below; these pilot study preparations were composed in the same manner as were those for the final study. The HMPOS cell suspension was transported on ice to the animal housing facility. Tumor inoculation was performed by manually restraining the mice while using a 29-gauge needle to inject the tumor cells into the subcutaneous space between the scapulae (for the pilot studies) or along the right flank (for the final study).

ATM Preparation and Administration

Sodium ATM was prepared as a stock solution of 50 mg/mL by dissolving the ATM hydrate in PBS. The stock solution was protected from light throughout preparation and storage and was stored at 4°C. Each day, the weights of the mice from the previous day were used to determine the daily dose of ATM for each group. The highest mouse weight from each group was used to calculate the amount of ATM stock solution needed to add to PBS in order to create a solution that would provide the appropriate 60 mg/kg, 80 mg/kg, or 120 mg/kg dose in 100 µL PBS per mouse. This volume of the gold stock solution was then mixed under sterile conditions with the appropriate volume of sterile PBS to create approximately 2 mL of the desired ATM concentration for each group. The suspensions were protected from light and transported on ice to the animal housing facility. Each mouse in the treatment groups was manually restrained and received an intraperitoneal (IP) injection of 100 µL of the appropriate ATM-PBS solution through a 29-gauge needle. All mice in the control group were similarly restrained and received a 100 µL intraperitoneal injection of sterile PBS through a 29-gauge needle (Figure 3-1).

Pilot Study Design

Prior to beginning the final phase of the *in vivo* portion of this study, three pilot studies were performed to determine an effective number of HMPOS cells for inoculation to induce tumor development and to determine the most appropriate timing to initiate ATM administration. The first pilot study involved 10 mice, 8 of which were inoculated with 5×10^6 HMPOS cells. Two of these mice did not receive ATM, 3 received daily ATM injections beginning 24 hours after tumor inoculation, and 3 received daily ATM injections initiated once a palpable tumor was noted. Two

additional mice were inoculated with 2.5 x 10^6 HMPOS cells and received daily ATM injections beginning 24 hours after tumor inoculation. The ATM dosage in this pilot study was 60 mg/kg/day IP, administered with sterile PBS for a total injected volume of 100 µL. A second pilot study involved 4 mice, all of which were inoculated with 1 x 10^6 HMPOS cells and none of which received ATM. Mice in the first and second pilot studies were euthanized when their tumors reached 15 mm in diameter or when tumor ulceration was noted. A final pilot study was performed using 2 mice inoculated with 5 x 10^5 HMPOS cells and no treatment. The mice in the third pilot study were euthanized once tumor formation was observed.

Final Study Design

For the final phase of the *in vivo* study, a total of 58 mice were randomly assigned to one of three groups: a control group, a low-dose ATM treatment group (60 mg/kg/day IP) and a high-dose ATM treatment group (initially 120 mg/kg/day, then 80 mg/kg/day IP). All mice were inoculated with 5 x 10^5 cells subcutaneously along their right flank on Day 1 and daily treatments were initiated 24 hours after tumor inoculation (on Day 2). The control group received 100 µL of sterile PBS IP daily, whereas the two treatment groups received 60 mg/kg/day, 80 mg/kg/day, or 120 mg/kg/day ATM diluted in sterile PBS to a total injected volume of 100 µL.

Monitoring

All mice were approximately 5-week old athymic mice obtained from Charles River Laboratories International, Inc. (Wilmington, MA). The mice were housed in a specific pathogen-free (SPF) barrier facility with 12-hour light and dark cycles and were provided sterilized food and water ad libitum. Mice were weighed and monitored daily for tumor development and changes in body condition, attitude, and general

appearance (Figure 3-2). Once tumors were palpably detectable, calipers were used to measure the length, width, and height of the tumor, with the length defined as the longest diameter of the tumor in either a sagittal or transverse plane, width defined as the diameter of the tumor perpendicular to the length, and height defined as the distance from the base of the tumor to the tumor's farthest point from the body (Figure 3-3). Tumor size and volume were calculated according to the following equations:

$$Tumor\ Size = \sqrt{L \times W}$$

$$Tumor\ Volume = (L \times W \times H) \times 0.5236$$

where L = tumor length, W = tumor width, and H = tumor height.[182] Tumor weight was approximated from the tumor volume (using the estimation 100 $mm^3 \approx 0.1$ g) in order to monitor tumor percentage of body weight in accordance with institutional guidelines. The daily change in tumor volume was calculated by subtracting the previous day's tumor volume from a given day's tumor volume and was reported in mm^3/day. The mice were monitored daily for tumor ulceration, and the appearance of additional lesions was noted.

Daily net mouse weight (mouse weight minus estimated tumor weight), percent weight gain, and tumor growth rates were calculated. Mice were euthanized when tumor length reached 15 mm (Figure 3-4), when tumor ulceration was observed (Figure 3-5), if the mouse reached a body condition score of ≤ 2/5 or failed to attain a weight equal to at least 85% of the weight of untreated controls, or at the end of the 65-day study period. Survival was measured as the time from tumor inoculation until euthanasia due to a mouse's tumor length measuring 15 mm or greater. Mice that

failed to develop tumors or that died or were euthanized for reasons not related to tumor growth or ulceration were censored from survival data analysis.

Survival distributions were estimated by the Kaplan-Meier method with the Log-Rank test used to evaluate for differences in survival between the control group and the low-dose (60 mg/kg/day) and high-dose (80 mg/kg/day) ATM groups.[189] For all other gross data, the Shapiro-Wilk test was used to test for normality. Time to tumor development, daily tumor growth rates, incidence of tumor ulceration, and number of mice that did not develop a tumor or failed to develop a tumor length of 15 mm by the end of the study period were compared between groups using the Kruskal- Wallis one-way analysis of variance on ranks. These data were reported as medians with associated IQR, and pair-wise comparisons between groups were performed using Dunn's Method. One way analysis of variance was used to compare mouse net weight gain between the control group and ATM treatment groups. Mouse weight gain was reported as mean ± SD and pair-wise comparisons between groups were performed using the Tukey Test.

Necropsy and Histopathological Examination

Euthanasia was performed via CO_2 inhalation and confirmed by thoracotomy in accordance with institutional guidelines. Immediately following euthanasia of four mice in the high-dose group, blood was collected from the cranial vena cava for serum gold measurements. The blood was spun at 3,000 rpm for 10 minutes, transferred to an additive-free collection tube, and refrigerated at 4°C prior to submission. Following euthanasia, a complete necropsy was performed and the lower respiratory tract, including the larynx, trachea, and lungs, and the heart and mediastinal adipose tissue were removed. The lungs were inflated with 10% neutral-buffered formalin. The

primary tumor was dissected, bisected along its length, and the kidneys were similarly removed and bisected from the cranial to the caudal pole. All tissues were then fixed in 10% neutral-buffered formalin for 24 to 48 hours before being sectioned for histopathology. Representative samples of tumor, lungs, kidney, heart, and liver were embedded in paraffin, and 5-µm-thick sections were stained with hematoxylin and eosin (H&E) for microscopic examination. Histopathology was performed to assess tumor necrosis, the number of mitotic figures, microscopic tumor emboli, and micrometastasis. Analysis was performed individually by two board-certified pathologists and by one pathology resident who were blinded to individual identity and group assignment. When combining the histopathology data, the percent necrosis scores and number of mitotic figures were averaged between the three observers. For micrometastases and tumor emboli observations, any noted metastatic or embolic focus that was noted by any observer was included in the analysis, regardless of whether it was noted by all 3 observers, in order to increase the sensitivity of the analysis. Immunohistochemistry was performed by a single board-certified pathologist who was also blinded to individual identity and group assignment. The treatment effect of ATM on primary and metastatic tumors was evaluated using the parameters described below.

Tumor Necrosis: Tumor sections were examined under 400x magnification and the area of tumor necrosis was estimated and scored according to the following scale: 1 = 0-25% necrosis; 2 = 26-50% necrosis; 3 = 51-75% necrosis; and 4 = 76-100% necrosis. Ten high-powered fields were examined and median scores and interquartile ranges were calculated for each group

Mitotic Figures: The number of mitotic figures per ten high-power fields (400x magnification) were counted for representative tumor sections from each mouse in which a tumor developed.

Macrometastasis: Macrometastasis was noted and recorded during necropsy. Removal of the respiratory tract and inflation of the lungs with formalin allowed for a more sensitive evaluation for pulmonary metastases. The number, distribution, and approximate diameter of any suspected pulmonary metastases were recorded.

Micrometastasis: Representative sections of lung, liver, kidney, and heart were evaluated at 400x magnification for evidence of tumor foci. For each pulmonary section, the presence or absence of pulmonary micrometastasis was recorded. The location of any tumor foci in other organs (excluding those within vasculature) was noted.

Tumor Emboli: Sections of lung, liver, kidney, and heart were also evaluated for the presence of tumor cells within the vasculature. The presence or absence and location of any tumor emboli were recorded.

Tumor Immunohistochemistry: Tissue sections of the tumors were used for immunohistochemical evaluation of the expression of Ki67 and caspase-3.[187,188] Deparaffinization, antigen retrieval and immunostaining of formalin-fixed paraffin-embedded tissues were performed on an automated immunostainer (Bench Mark Automated Staining System, Ventana Medical Systems, Inc., Tucson, Arizona) using the Enhanced V-Red Detection (Alk. Phos. Red) Detection System (Ventana Medical Systems, Inc.) and a mouse monoclonal antibody against Ki67 (Dako Cytomation, Carpenteria, California) and rabbit polyclonal antibody against caspase-3 (Fitzgerald

Industries International, Acton, Massachusetts) at dilutions of 1:50 and 1:5,000, respectively. Antigen retrieval was achieved using the Ventana Medical Systems Retrieval Solution CC1 (Ventana Medical Systems, Inc.) for 60 min. Sections were counterstained with hematoxylin. Positive immunohistochemical controls included a canine reactive lymph node and canine skin to which the appropriate antisera were added. There was strong Ki67 expression in proliferating lymphocytes in the lymph node and in the basal cells of the skin and strong expression of caspase-3 in apoptotic cells in the germinal centers in the lymph node. To create negative controls, the primary antibodies were replaced with homologous non-immune sera. Only nuclear labeling was evaluated for Ki67 and cytoplasmic labeling for caspase-3. Caspase-3 and Ki67 labeling for the control group and low-dose group were compared using the Friedman repeated measures analysis of variance on ranks for caspase-3 labeling and a one way analysis of variance for Ki67 labeling. Pair-wise comparisons of Ki67 labeling were performed using the Holm Sidak method.

Data Analysis

Categorical histopathological data (tumor emboli, metastases) were converted to binary data with “1” indicating the presence and “0” indicating the absence of the specified variable. Normality was tested for using the Shapiro-Wilk test, and the Kruskal-Wallis one way analysis of variance on ranks was used to compare the following non-parametric variables between the treatment and control groups: tumor necrosis scores, tumor emboli, pulmonary macro- and micrometastases, and non-pulmonary metastases. Tumor mitotic figures were compared using a one way analysis of variance. Pair-wise comparisons of non-parametric data between groups were performed using Dunn’s Method, and the Mann-Whitney Rank Sum test was used to

compare pulmonary macrometastasis between groups. Non-parametric data were reported as median (IQR); parametric data were reported as mean ± SD.

Mouse and tumor parameters were also analyzed separately for comparison between the control and the low-dose (60 mg/kg) group excluding the high-dose (80 mg/kg) group using similar methods. A P-value of less than 0.05 was considered statistically significant for all statistical analyses. All data analysis was performed using SAS version 9.3 and SigmaPlot 11.0 software (SigmaStat® & SigmaPlot®, Systat Software Inc, Richmond, California).

Results

Tumor Development

One, 3, and 2 mice in the control, 60 mg/kg group, and 80 mg/kg group, respectively, did not develop tumors within the study period. Additionally, 2 mice in the control and 60 mg/kg groups and 4 mice in the 80 mg/kg group developed tumors that did not reach a tumor length of 15 mm by the end of the 65-day period. These mice were censored from the survival distribution analysis. There was no significant difference between groups in the number of mice that either did not develop a tumor or failed to develop a tumor length of 15 mm by the end of the study period (P = 0.613 and 0.067, respectively). The median time to tumor development (as detected by physical examination) in the control, 60 mg/kg , and 80 mg/kg groups was 8.0 days (7.0-14.8 days), 9.5 days (7.0-20.5 days), and 16.0 days (9.5-20.0 days), respectively (Table 3-1). The median time to sacrifice (due to development of a tumor length of 15 mm, tumor ulceration, or end of the study) was 36.0 days (23.0 – 49.5 days), 49.5 days (27.5 – 65.0 days), and 51.5 (32.8 – 65 days), respectively. Although there was a trend that the median time to tumor development and median time to sacrifice was shortest among

the control and longest among the 80 mg/kg treatment group, this difference was not statistically significant (P = 0.247 and P = 0.140, respectively). There was no statistically significant difference in survival among the control and ATM treatment groups with or without censorship of mice that developed tumor ulceration (P = 0.333 and 0.139, respectively) (Figure 3-6).

Tumor Ulceration

Tumor ulceration occurred in 7 mice in the control group, 8 mice in the 60 mg/kg group, and 4 mice in the 80 mg/kg group (Table 3-2). The mean tumor volume at the time of ulceration was 500.9 ± 448.6 mm^3 and the mean time to tumor ulceration was 25.9 ± 9.9 days. There was no significant difference in the incidence of tumor ulceration between groups (P = 0.493).

Tumor Growth Rate

The median daily tumor growth rates derived from daily estimated tumor volumes were 7.6 mm^3/day (0.0 – 50.6 mm^3/day), 2.7 mm^3/day (0.0 – 20.9 mm^3/day), and 1.6 mm^3/day (0.0 – 14.1 mm^3/day) in the control, 60 mg/kg, and 80 mg/kg groups, respectively (Table 3-1, Figure 3-7). The daily tumor growth rates were significantly different among the control and treatment groups (P < 0.001), with the daily tumor growth rates significantly slower in the 60 mg/kg group and 80 mg/kg group compared to the daily tumor growth rate in the control group (P < 0.05). Pair-wise comparisons did not show a statistically significant difference between the daily tumor growth rates of the 60 mg/kg and 80 mg/kg ATM treatment groups (P > 0.05).

Morbidity and Mortality

Net weight gain was significantly lower in the 80 mg/kg group compared to the control group (P < 0.001). Numerous mice, however, in the 80 mg/kg group became

dehydrated with marked weight loss after approximately 6 days of treatment with ATM at the initial dose of 120 mg/kg/day IP. At this time, 4 mice were euthanized due to failure to maintain body weights equal to at least 85% of those of the control group. An attempt was made to collect serum from these mice at euthanasia in order to measure serum gold levels; due to the size of the mice, however, a sufficient quantity of serum could not be obtained for analysis. As a result, ATM treatment of this group was discontinued; the remaining mice received 100 µL sterile PBS IP for 10 days. These mice regained adequate hydration status and comparable body weights during this 10-day period and were started at a dose of 80 mg/kg/day ATM IP 11 days after cessation of the 120 mg/kg/day treatment. This reduced dose was continued for the remainder of the study with no additional adverse effects noted; thus this high-dose group is referred to as the 80 mg/kg group.

One mouse in the control group was found dead in the cage on the day that its tumor reached the maximal allowable length. Extensive invasion of abdominal organs was noted on necropsy of this mouse. Another mouse in the control group that had not yet developed a palpable tumor was euthanized due to a hemoabdomen on Day 15 of the study; necropsy revealed approximately 2.5 mLs of serosanguineous fluid in the abdomen and diffusely reactive mesentery with no other gross changes. A mouse in the 80 mg/kg group was found deceased in its cage on Day 22 of the study. This mouse had a tumor that measured 6.5 mm in length; necropsy revealed some subcutaneous hemorrhage around the tumor and slight green discoloration in the left ventral abdomen but no other gross lesions. These mice were also censored from survival distribution analysis.

Pathology

Gross Pathology

Successful tumor inoculation was achieved in 46 of the 54 mice in the final study. Grossly tumors were commonly associated with subcutaneous hemorrhage around the tumor and frequently multi-lobulated in appearance. Most of the tumors were confined to the subcutaneous space and body wall of the flank, although 4 of the larger tumors in the control group were locally invasive, extending into the abdominal cavity and intra-abdominal organs. Pulmonary metastasis was the only gross distant metastasis observed.

Tumor Morphology

Tumors were histopathologically described as having an infiltrative growth pattern. All tumor cells were either round or polygonal, with a moderate amount of cytoplasm and round-to-oval, chromatin-stippled nuclei; all tumors contained both osteoid and bone. There were a large number of mitotic figures per high-power field in tumors of all groups with mean mitotic figures per 10 HPF of 152 ± 51, 167 ± 42, and 155 ± 57 in the control, 60 mg/kg, and 80 mg/kg ATM treatment groups, respectively (Figure 3-8). There was no significant difference between the number of mitotic figures between ATM treatment groups and the control group ($P = 0.294$). Percent tumor necrosis scores were also not significantly different between groups ($P = 0.439$), with mean scores of 1.7 ± 0.9, 1.5 ± 0.7, and 1.5 ± 0.8 for the control, 60 mg/kg group, and 80 mg/kg ATM treatment groups, respectively (Figure 3-9). These scores correspond to an average estimated percent tumor necrosis of less than 50% for the control and ATM treatment groups.

Metastasis

The incidence of gross metastasis to the lungs was significantly higher in the control group (Table 3-2, Figure 3-10). Five mice in the control group, no mice in the 60 mg/kg group, and 1 mouse in the 80 mg/kg group showed evidence of pulmonary macrometasasis on necropsy ($P = 0.033$). Pulmonary micrometastasis was present in 10 mice in the control group, 7 mice in the 60 mg/kg group, and 1 mouse in the 80 mg/kg group (Figure 3-11). This trend toward decreasing pulmonary micrometastasis with increasing ATM dose was statistically significant ($P = 0.011$); pair-wise comparisons revealed a significant difference in the incidence of pulmonary micrometastasis between the control group and the 80 mg/kg treatment group ($P < 0.05$). Pulmonary macrometastasis also occurred significantly more frequently in the control group compared with the 60 mg/kg treatment group ($P < 0.001$). Similarly, the control mice had a significantly higher incidence of tumor emboli compared with the 60 mg/kg and 80 mg/kg ATM groups ($P = 0.010$) (Figure 3-12). Non-pulmonary metastasis was uncommon but was noted in pulmonary lymph nodes, thoracic and pericardial fat, and subcutaneous tissue. There was no significant difference in the incidence of non-pulmonary micrometastasis between groups ($P = 0.089$) (Figure 3-10).

Tumor Immunohistochemistry

Mean Ki67 labeling in the tumors of the control group and 60 mg/kg group were 164 ± 20 per 5 grid areas and 95 ± 17 per 5 grid areas, respectively (Table 3-3). This elevation of Ki67 labeling in the control group was statistically significant ($P = 0.005$). Median caspase-3 labeling was 10% (5 – 10%) for the control group and 4% (4 – 4%) for the 60 mg/kg group; no statistically significant difference was detected between the two groups ($P = 0.063$).

Discussion

This study demonstrates that administration of ATM to mice with xenograft canine osteosarcoma significantly reduced tumor growth rate, tumor emboli, and pulmonary metastasis. Inhibition of tumor growth was achieved at an ATM dose of 60 mg/kg/day IP, consistent with serum gold levels (approximately 5 μg/mL) that are well within the safe therapeutic range reported in humans.[176,177] These findings are also compatible with previous work with non-small cell lung cancer, which showed that in vitro sensitivity to ATM corresponds with decreased xenograft tumor growth *in vivo* at serum gold levels achievable in human rheumatoid arthritis patients undergoing ATM therapy.[176,177] Consistent with our hypotheses, ATM treatment also significantly reduced the incidence of pulmonary micro- and macrometastases and the incidence of tumor emboli. Contrary to the effect on tumor growth rates, which was seen at both 60 and 80 mg/kg/day ATM dose levels, the effect on pulmonary micrometastases and tumor emboli was achieved only at the 80 mg/kg/day ATM dose. The slope of the curve elucidated by Regala, et al. to show the serum gold concentration achieved with varying doses of ATM in mice appears to begin to plateau as the ATM dose approaches 60 mg/kg/day.[177] Thus, it is reasonable to extrapolate that the slightly higher dose of 80 mg/kg/day would be unlikely to greatly increase the serum gold level beyond the level reached with the 60 mg/kg/day dose. It is also possible that ATM's anti-metastatic effect was achieved by the initial 120 mg/kg/day dose administered early during tumor formation; thus further elucidation of this effect of ATM on canine osteosarcoma is necessary to determine whether these results can be reproduced at serum gold levels safely achievable in human patients and dogs.[176,177] Non-pulmonary micrometastasis was not significantly different between the control and treatment groups. The overall

number of non-pulmonary micrometastases, however, was small (4 in the control group and 1 in the 60 mg/kg/day group); thus it is possible that a type II statistical error prevented detection of a decreased incidence of nonpulmonary micrometastis among the ATM treatment groups.

Ki67 labeling was significantly decreased among tumors of the 60 mg/kg/day treatment group compared with those of the control group, suggesting a possible mechanism for ATM's effects on canine osteosarcoma growth and metastasis. Ki67 is a nuclear protein expressed in all phases of the cell cycle but not in resting cells; thus measurement of Ki67 expression is considered a validated measure of tumor growth fraction.[187,190] The Ki67 index has been used as a prognostic indicator in both human and canine cancers, and has specifically been shown to correlate with increased mortality and pulmonary metastasis in humans affected with osteosarcoma.[191-196] The decreased Ki67 labeling in tumors of the 60 mg/kg/day group suggests that ATM decreases proliferation of HMPOS cells, thus providing an explanation for the decreased tumor growth rate and pulmonary metastasis among mice treated with ATM administration. This is consistent with the anti-proliferative effect measured using BrdUrd labeling in non-small cell lung cancer tumors treated with ATM.[177] In contrast, the absence of a significant difference in caspase-3 staining among tumors from both control and the 60 mg/kg treatment group in our study suggests that increased apoptosis is not a prominent mechanism underlying ATM's effects on canine osteosarcoma. TUNEL staining of lung cancer xenografts treated with ATM also failed to demonstrate a pro-apoptotic effect of the drug.[177] A previous study evaluating the in vitro effects of ATM on human advanced prostate cancer demonstrated increased

apoptosis among treated cells but did not test for effects on cellular proliferation.[174] These mechanistic differences may represent variation between ATM's in vitro and *in vivo* effects, or may reflect fundamental differences in the mechanism of action of ATM against different types of tumors.

The mitotic index is ratio of the number of cells in mitosis (those displaying mitotic figures) to the total number of cells present and is commonly used as a measure of cell proliferation. Interestingly, the number of mitotic figures was not significantly different between treatment and control groups in this study. The number of mitotic figures per high-powered field would be expected to be decreased in tumors of the ATM-treated mice, echoing the results of the Ki67 labeling. The mitotic index of tumor cells, however, is more reflective of cell proliferating speed, whereas the Ki67 index is more specifically representative of the tumor growth fraction.[197] Thus, it is possible that ATM treatment decreased the tumor growth fraction without decreasing the speed of cell proliferation. A lack of correlation between changes in Ki67 and mitotic indices has been reported for radiation therapy of humans affected with cervical cancer.[197,198] Since the number of mitotic figures per area reflects only the number of cells currently in mitosis whereas Ki67 labeling detects replicating cells throughout most of the cell cycle, a discrepancy between the two measures is not surprising. Central cross-sectional samples of tumors submitted for histopathology may also have been less sensitive than more peripheral samples for changes in cellular proliferation patterns since the majority of tumor cellular proliferation tends to occur near the margin of the tumor. Lastly, the standard deviations for the mitotic figures observations were high; as a result, variability within the measurements may have masked an underlying treatment effect.

Although there was a trend toward increased survival times in mice receiving higher doses of ATM, we were unable to substantiate a significant dose-related effect of ATM administration on survival times. The absence of a significant prolongation of survival with ATM treatment may be the result of a type II statistical error, as the tumor growth rate analysis involved a large amount of data points collected daily, whereas the number of mice analyzed for survival curves was substantially smaller. An additional factor complicating the evaluation of ATM's effect on survival times in this study was the high incidence of tumor ulceration. As tumor ulceration was a study endpoint mandated to mitigate morbidity of the mice, the high incidence of tumor ulceration artificially shortened the survival times reported in this study since the majority of tumors that ulcerated were markedly smaller than the 15 mm diameter endpoint. There was no significant difference in tumor ulceration between the control and treatment groups; thus it is unlikely that ulceration is affected by ATM treatment. Furthermore, no apparent association was observed between the incidence of tumor ulceration and tumor size. Survival analysis was performed both with and without censorship of tumor ulceration; both analyses showed a similar trend of increased survival with increasing ATM dose with no significant differences noted between groups.

The doses chosen for evaluation in this study were based on those used in previous research into the anti-neoplastic effects of ATM against various cancers.[173,181] In addition, the 60 mg/kg/day dose was specifically chosen due to evidence that this dose achieves serum levels in mice consistent with levels considered safely therapeutic in humans for other diseases.[176,177,181] An attempt was made to corroborate serum levels of gold reached during this study with those previously established in mice; the

volume of serum obtained from the mice in this study, however, was insufficient to perform that analysis. Mice receiving ATM at doses of 60 mg/kg/day and 80 mg/kg/day showed no signs of toxicity associated with ATM treatment, and none of the mice receiving these doses were euthanized for reasons other than tumor size, ulceration, or the end of the study period. One mouse in the control group was euthanized due to morbidity associated with a hemoabdomen suspected to be related to intraperitoneal injection since no evidence of neoplasia or other pathology was evident on necropsy. Mice receiving 120 mg/kg/day of ATM, however, showed evidence of toxicity manifested as marked dehydration and weight loss beginning approximately 4 days after starting ATM treatment. Four of these mice were euthanized due to probable toxicosis; the remaining mice all responded favorably to cessation of the 120 mg/kg/day treatment and later tolerated the 80 mg/kg/day dose with no observed adverse effects. Importantly, aside from the effects seen at the initial high-dose group, the mice treated with ATM seemed to tolerate the treatments well, supporting the assertion that therapeutic levels can be reached at levels that avoid systemic toxicity. A future study in larger animals would be useful to determine the gradations of serum gold corresponding to varied dose levels and to better determine at what serum gold level toxicity becomes apparent.

Sample size was also effectively reduced by the number of mice that did not develop tumors. The lack of tumor development in a substantial number of mice in the final phase of the *in vivo* study was an unexpected finding given that all mice in the pilot studies rapidly developed tumors following inoculation. It is possible that an inoculation number of 1,000,000 cells approximated a critical threshold for tumor development,

below which a significant number of mice do not develop detectable tumors or fail to do so within a practical time frame. Such a phenomenon would likely not have been detected by our pilot study using 500,000 cells since only two mice were used.

A similarly unexpected finding in the final phase of the *in vivo* study was the high incidence of tumor ulceration. During the pilot studies, tumor ulceration occurred more frequently than that subjectively observed with previous use of this model (James Farese, personal communication December 2010). Increased tumor ulceration might be due to local factors such as variations in local blood supply or tension in the overlying skin (Dietmar Siemann, personal communication, December 2010). The location of the inoculation site was changed from the interscapular region to the flank region in an attempt to mitigate these local factors. Tumor ulceration, however, continued to be observed at a relatively high rate in the final phase of the study. The increased incidence of tumor ulceration may instead have been a product of changes in the HMPOS cell line over time leading to a more aggressive phenotype (Shannon Roff, personal communication, March 2011), in which case establishing a murine xenograft model with a different canine osteosarcoma cell line with a less ulcerative phenotype would be highly beneficial in further evaluating the relatively slow-acting ATM.[128]

Table 3-1. HMPOS tumor development in mice treated with ATM

	Control	ATM (60 mg/kg)	ATM (80 mg/kg)	P-value
Median time to tumor development (days)	8.0 (7.0-14.8)	9.5 (7.0-20.5)	16.0 (9.5-20.0)	0.247
Median time to sacrifice (days)	36.0 (23.0-49.5)	49.5 (27.5-65.0)	51.5 (32.8-65.0)	0.140
Tumor growth rate (mm^3/day)	7.6 (0.0-50.7)	*2.7 (0.0-20.9)	*1.6 (0.0-14.1)	0.001

* indicates a significant difference from the control group (P < 0.05).

Table 3-2. HMPOS tumor characteristics in athymic mice treated with ATM

	Control	ATM (60 mg/kg)	ATM (80 mg/kg)	P-value
Tumor ulceration	7	8	4	0.493
Tumor emboli	9	5	*1	0.010
Pulmonary micrometastasis	10	7	*1	0.011
Pulmonary macrometastasis	5	0	1	0.033
Non-pulmonary metastasis	4	1	0	0.089

* indicates a significant difference from the control group (P < 0.05).

Table 3-3. Immunohistochemistry of HMPOS tumors with placebo and ATM treatment

	Control	ATM (60 mg/kg)	P-value
Ki67	164 ± 20	*95 ± 17	0.005
Caspase-3	10 (5-10)	4 (4-4)	0.063

* indicates results significantly different from values for the control group (P < 0.05).

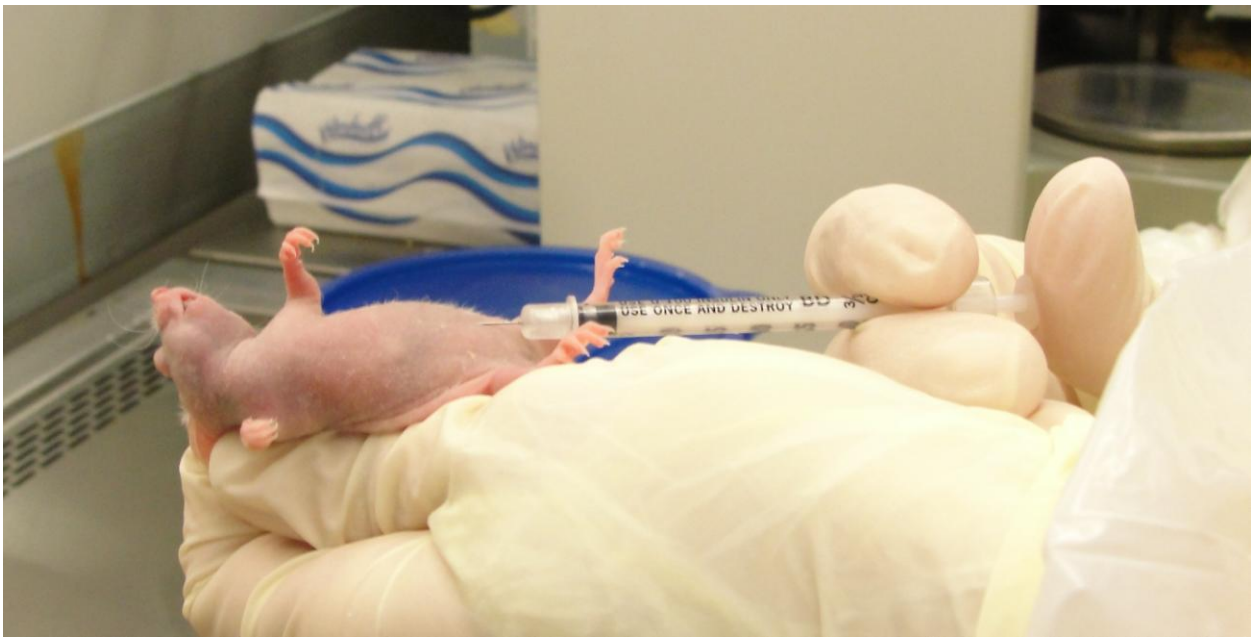


Figure 3-1. Intraperitoneal injection of ATM administered daily to athymic mice in the ATM treatment groups during the study period. Mice in the control group received a daily injection of sterile PBS using the same technique.

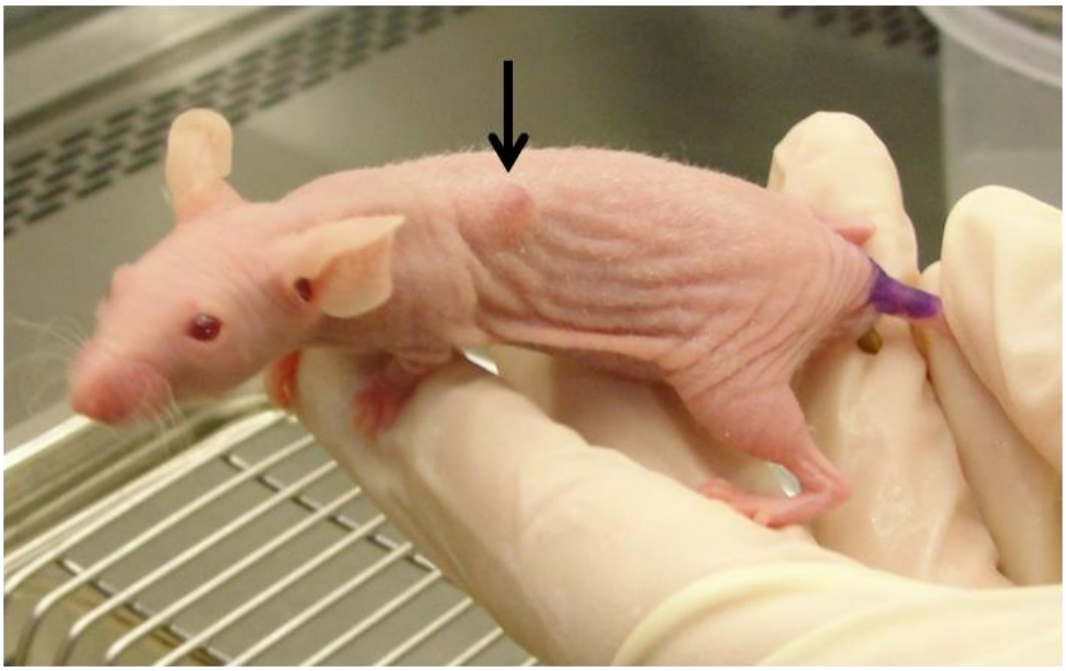


Figure 3-2. Early development of an HMPOS tumor (black arrow) following interscapular inoculation in a mouse during a pilot study. The inoculation site was changed to the right flank region for the final study.

Figure 3-3. Measurement of tumor parameters performed daily using calipers. Tumor length was defined as the longest tumor diameter, tumor width as the tumor diameter perpendicular to the tumor length, and tumor height as the distance from the base of the tumor to the tumor's farthest point from the body.

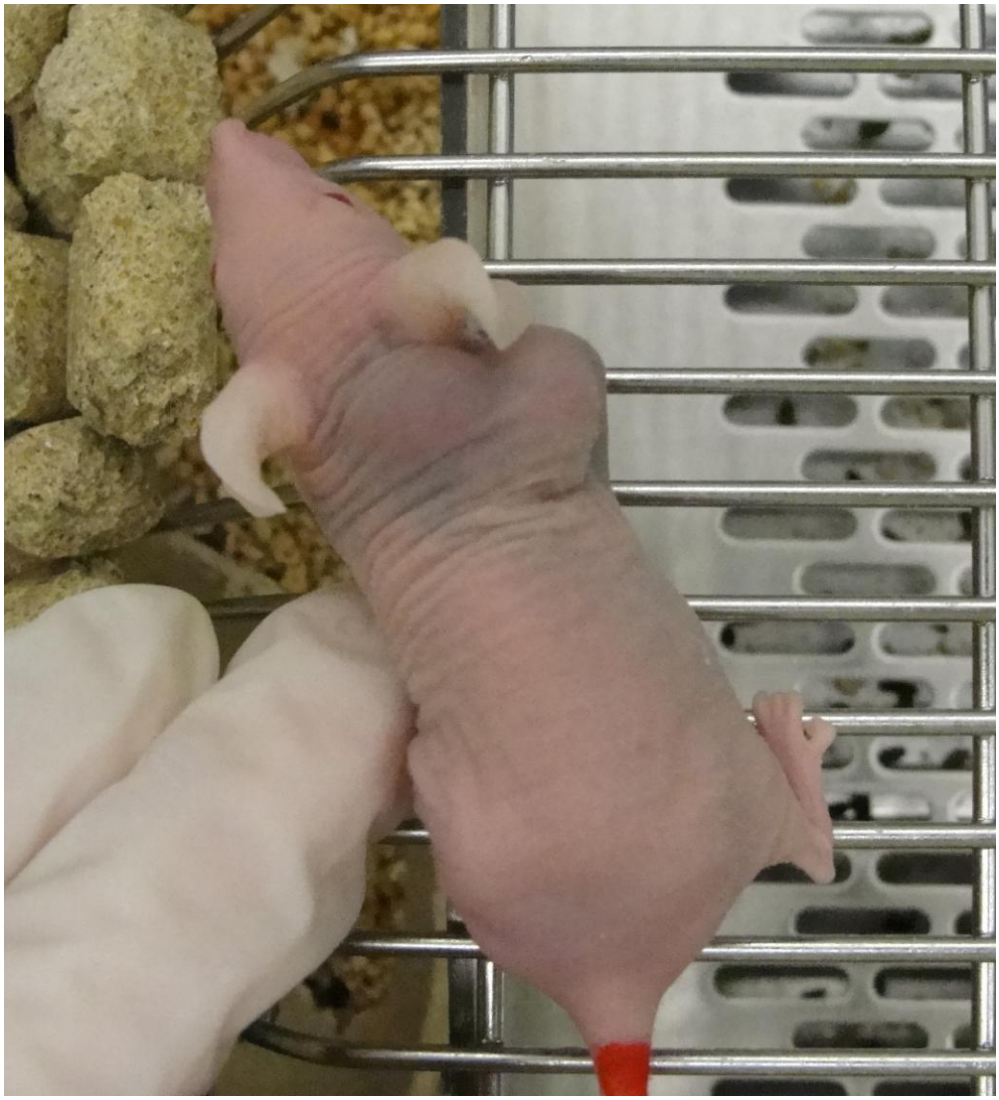
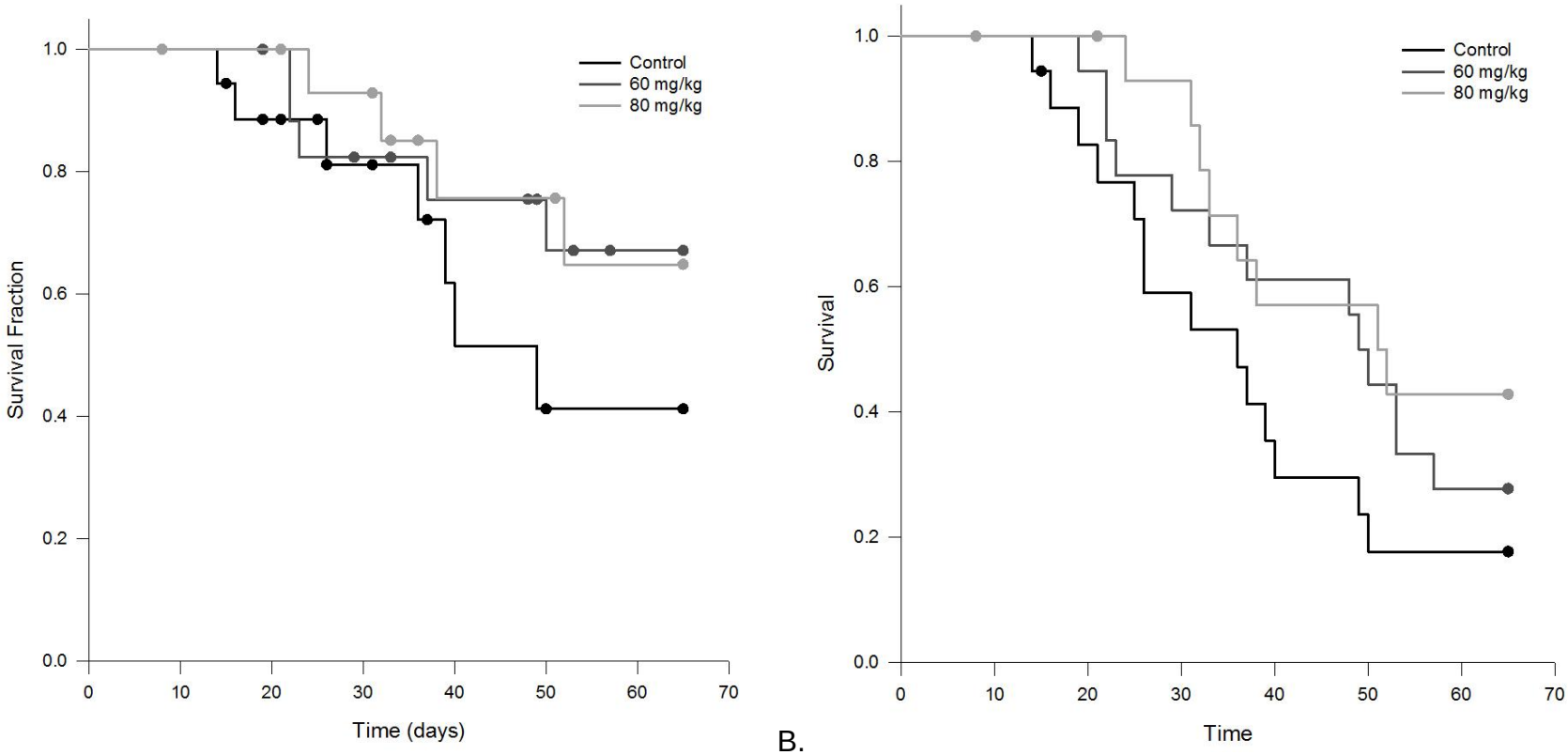


Figure 3-4. Development of interscapular HMPOS tumor in the pilot study. The grossly multi-lobulated tumor does not show evidence of bruising or ulceration as it approaches the 15-mm diameter endpoint.

Figure 3-5. This interscapular HMPOS tumor in the pilot study is approaching the 15-mm diameter endpoint and displays extensive discoloration along the superficial border of the tumor, consistent with tumor ulceration.



A. B.

Figure 3-6. Kaplan-Meier survival analysis of mice inoculated with HMPOS cells and treated daily with ATM or PBS injections. A) Survival curves for mice with censoring of mice euthanized for tumor ulceration. B) Survival curves for mice with no censorship for tumor ulceration. There was no significant difference in survival between ATM treatment groups and the control group with or without censor of mice that developed tumor ulceration (p-values of 0.333 and 0.139, respectively).

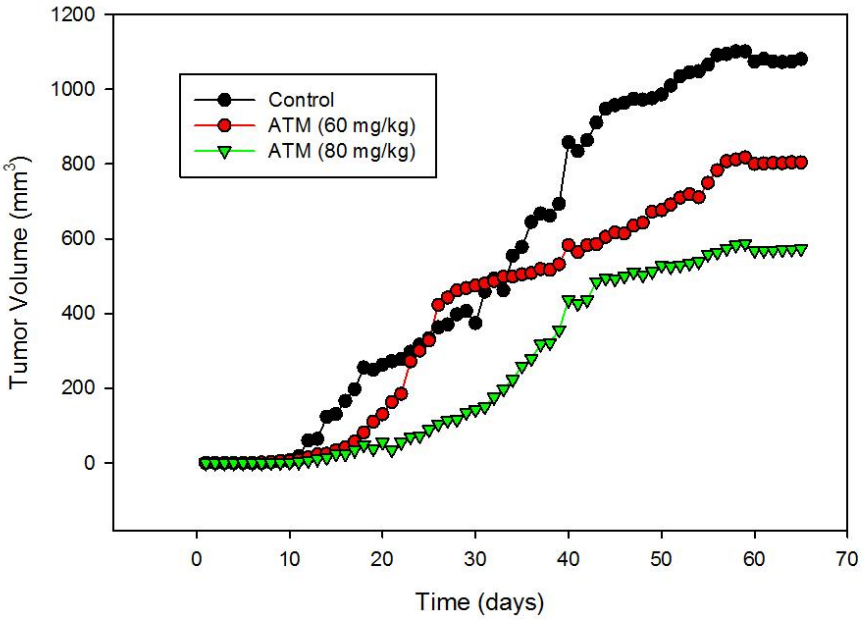


Figure 3-7. Daily HMPOS tumor volume among mice in the control and ATM treatment groups.

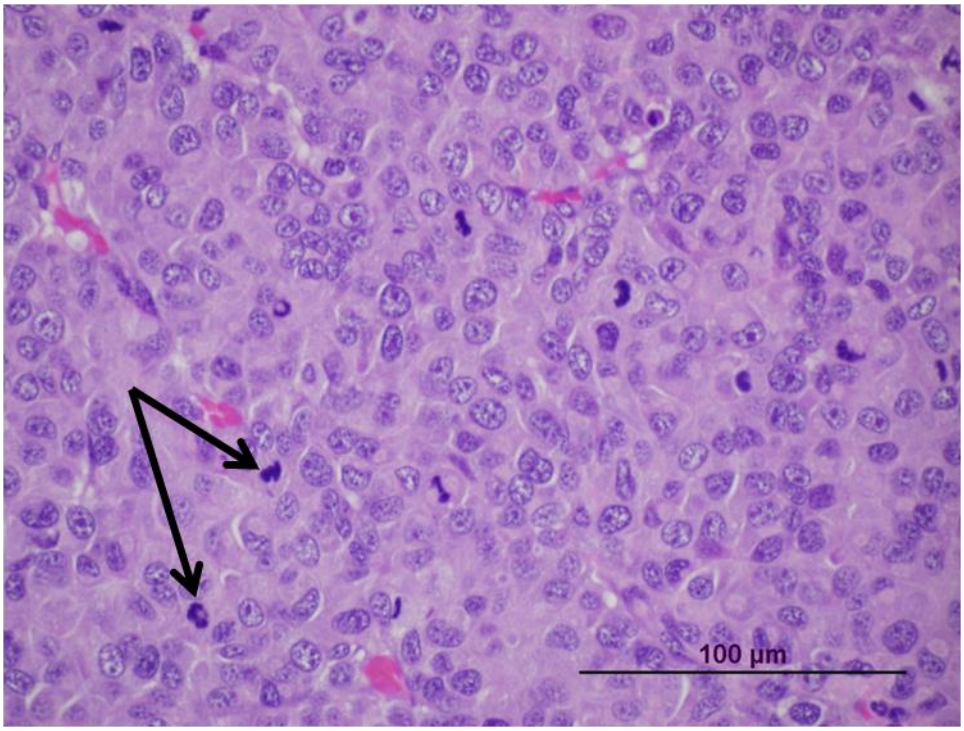


Figure 3-8. Cross-sectional image of HMPOS xenograft tumor showing increased numbers of mitotic figures (black arrows).

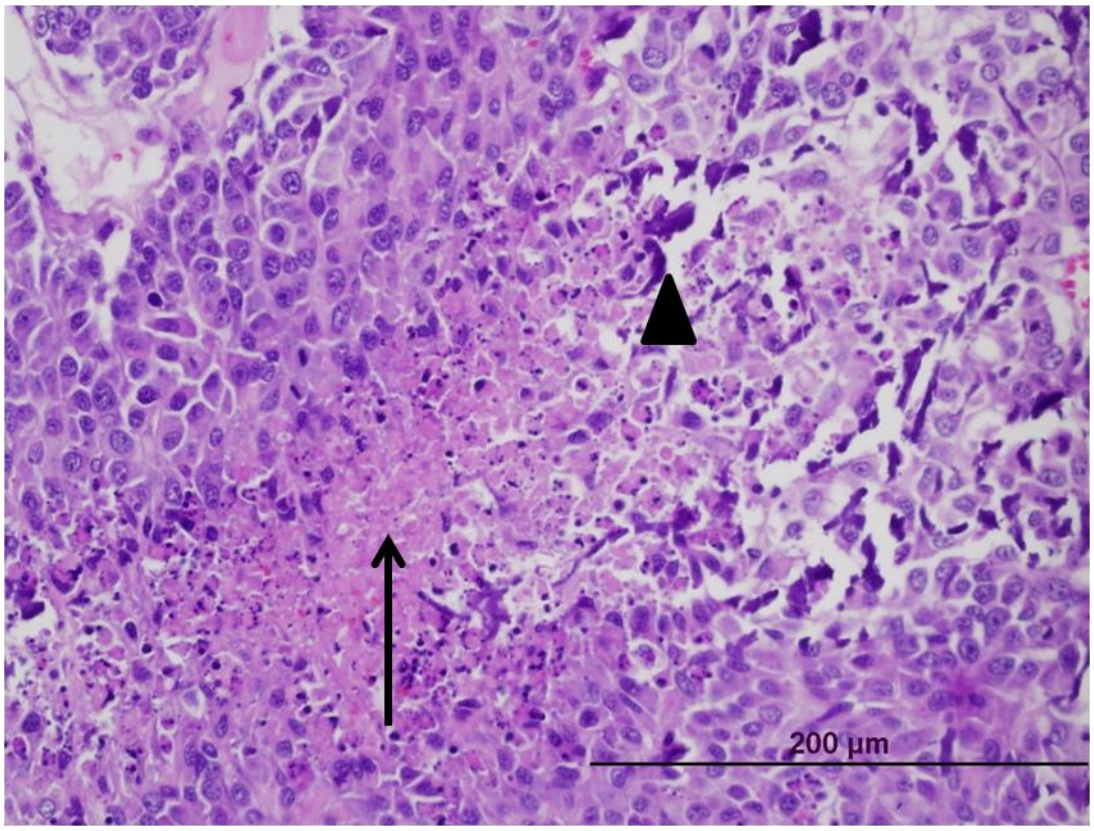


Figure 3-9. Cross-sectional image of HMPOS xenograft tumor showing necrosis (black arrow) and mineralization (black arrowhead) within the tumor.

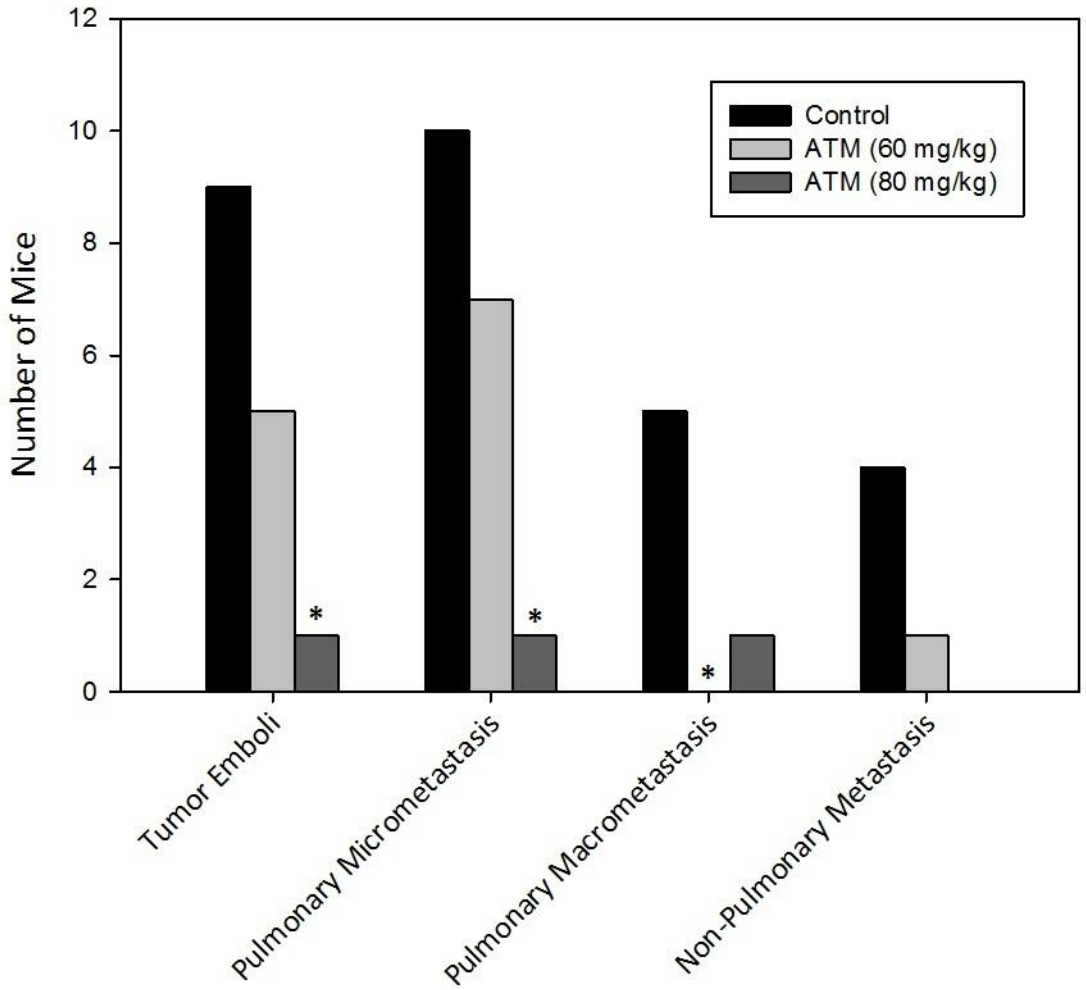


Figure 3-10. Incidence of tumor spread among mice in the control and ATM treatment groups.

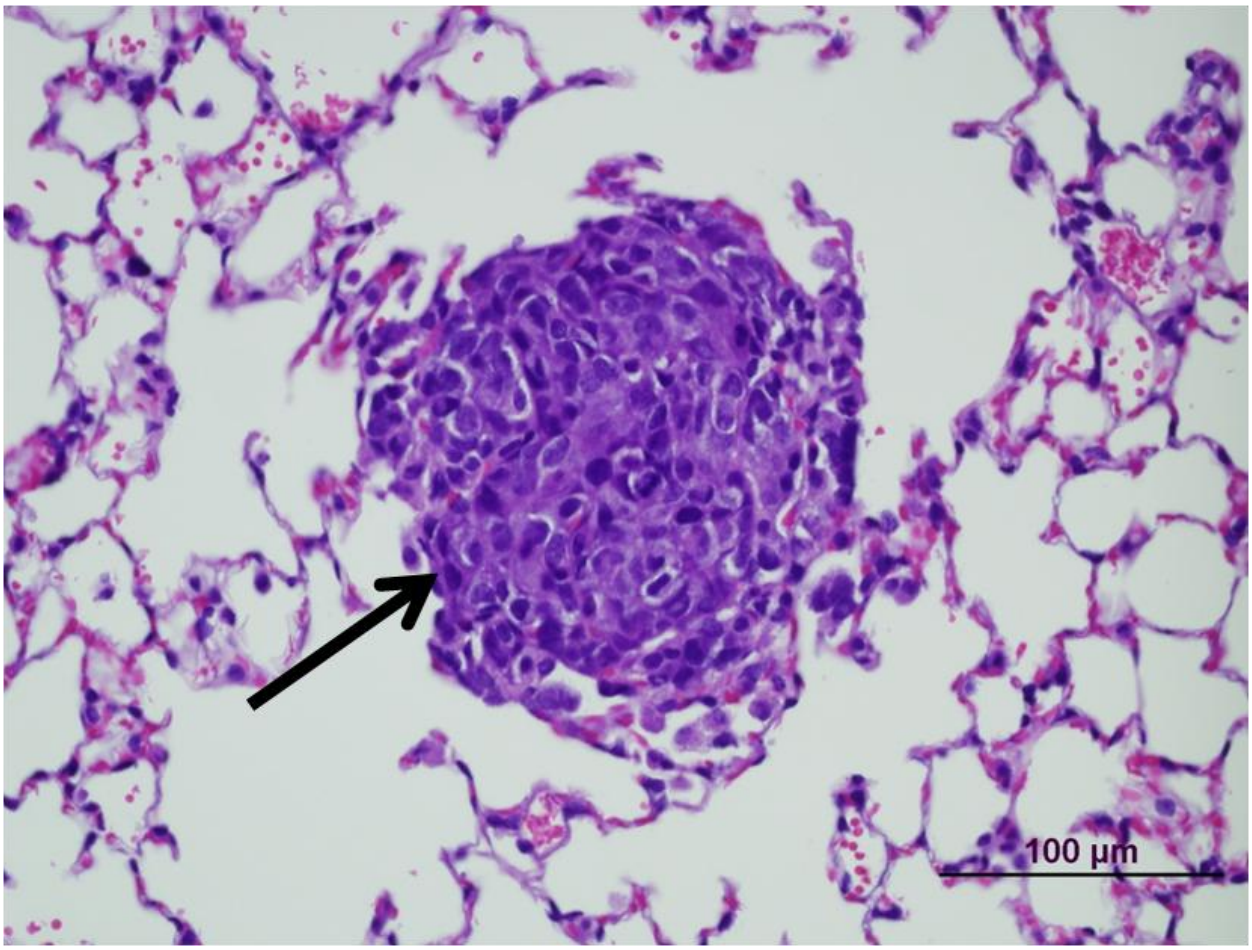


Figure 3-11. HMPOS micrometastasis (black arrow) within the pulmonary parenchyma.

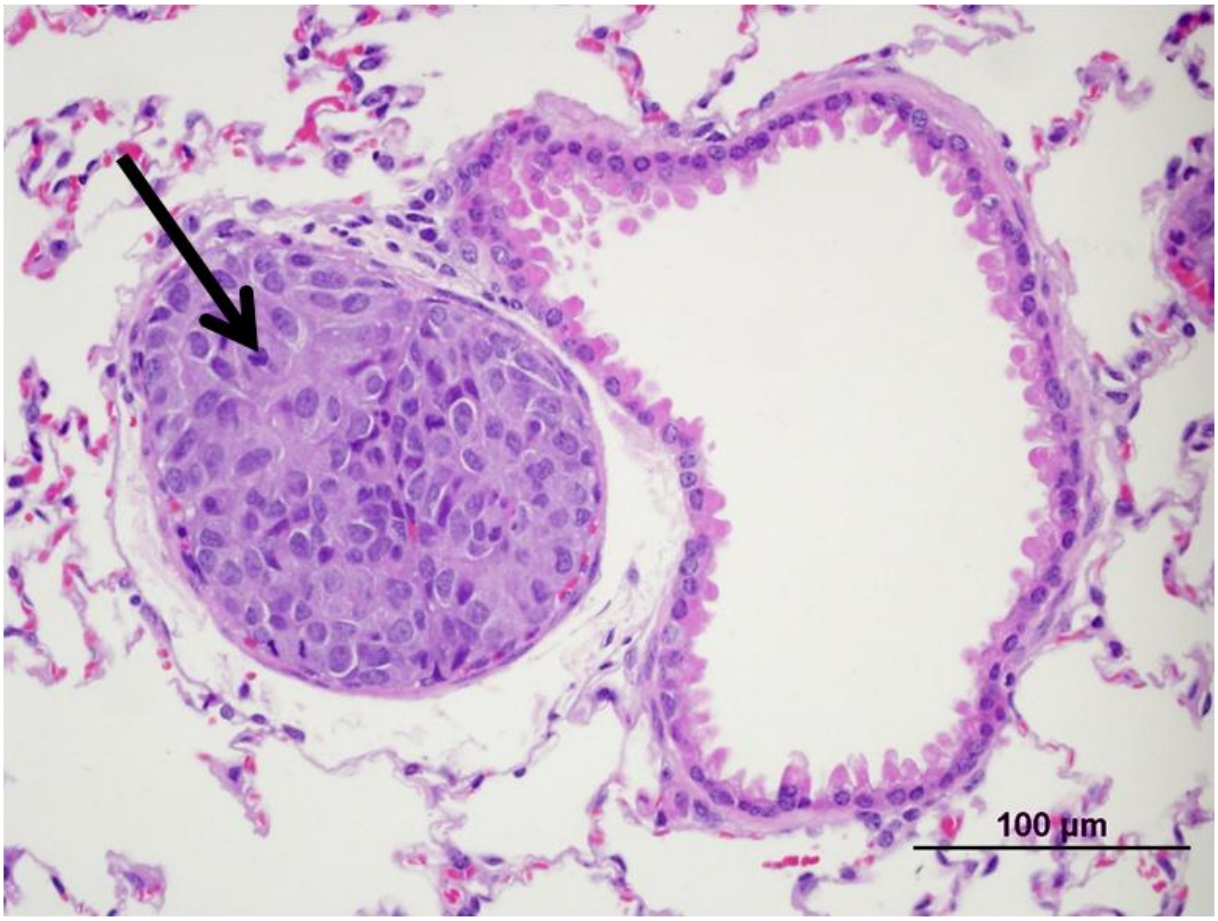


Figure 3-12. HMPOS tumor embolus (black arrow) within the pulmonary vasculature.

CHAPTER 4
CONCLUSION

The purpose of the *in vitro* study was to evaluate the effects of sodium ATM on canine and human osteosarcoma cells in order to establish whether ATM has potential therapeutic efficacy against this type of cancer. Previous studies have evaluated the *in vitro* effects of gold compounds on different cancer types but the effects of gold compounds on osteosarcoma have not been investigated. Similarly, although gold compounds have historically been used in veterinary medicine to treat a variety of diseases, the use of gold compounds as anti-neoplastic agents in companion animals has not been evaluated.[130] Results reported here indicate that both representative canine and human osteosarcoma cell lines showed marked inhibition *in vitro* with ATM incubation. Importantly, this inhibition was achieved at concentrations comparable to serum levels that are achievable in human patients receiving sodium ATM for the treatment of non-neoplastic diseases.[176,177] Previous studies have shown that *in vitro* sensitivity to sodium ATM correlates with *in vivo* sensitivity to this drug.[177] This study demonstrates that sodium ATM is a potent inhibitor of canine and human osteosarcoma *in vitro* and thus may have potential for treating dogs and humans affected with osteosarcoma.

The *in vivo* study was designed to determine whether the inhibitory effects of sodium ATM observed *in vitro* could be effective in a murine xenograft model of canine osteosarcoma. A secondary objective was to further elucidate the mechanism of action through which sodium ATM achieves anti-tumor effects against canine osteosarcoma. Previous studies have demonstrated both an anti-proliferative effect *in vivo* and an anti-apoptotic effect *in vitro* when tumor cells were treated with sodium ATM.[174,177] This

study showed that ATM treatment significantly delayed growth of canine osteosarcoma tumors *in vivo*. In addition, the incidence of pulmonary macrometastases and micrometastases and the incidence of tumor emboli were significantly reduced in mice treated with ATM. This study also demonstrated that ATM treatment decreases cellular proliferation within canine osteosarcoma tumors without a concurrent increase in apoptosis. This supports previous *in vivo* work demonstrating an anti-proliferative effect of ATM against lung tumor cells.[177] Furthermore, *in vivo* inhibition of canine osteosarcoma growth and metastasis was achieved at dosage levels comparable with those of humans safely receiving ATM for other disorders, further supporting the potential clinical applicability of these findings.

Investigating novel anti-neoplastic therapies poses several challenges to investigators. Ideally a new therapy is not evaluated in human patients until both efficacy and safety are demonstrated *in vitro* and *in vivo*. Thus the use of animal models becomes a critical tool in the development and screening of new anti-cancer drugs. While much of this research is performed using purpose-bred research animals, the use of anti-neoplastic therapies in client-owned veterinary patients can provide a wealth of information regarding the potential response of various cancers to novel anti-cancer therapies.[122,123] Canine osteosarcoma, although unique in several key aspects of its presentation among dogs, bears striking resemblance to its human counterpart and thus provides an excellent model for the study of therapies to treat both canine and human osteosarcoma.[122,123]

As with humans, the use of novel therapies in client-owned animals should not be employed without first establishing a basic understanding of a drug's potential efficacy

and safety. Cancers such as osteosarcoma, although devastating to the individual patient, pose an additional challenge to researchers due to their relatively low rate of spontaneous occurrence. Thus, much of the *in vivo* research into novel therapies relies on the use of animal models in which tumors are induced via inoculation of tumor cells into the research animal. A subcutaneous murine xenograft model of canine osteosarcoma was chosen for evaluation of ATM treatment due to several factors including feasibility, minimal morbidity, and previous successful experience with this model. The limitations of this subcutaneous xenograft model include the fact that the tumor environment does not mimic that seen in the clinical setting, unlike an orthotopic model in which tumor cells are inoculated directly into the bone of the research animal. Nonetheless, the use of a subcutaneous model provided adequate and easily measurable information regarding ATM treatment while avoiding the increased morbidity of an orthotopic model. Similarly, the highly aggressive nature of the canine osteosarcoma cell line used for tumor inoculation was beneficial in allowing evaluation of metastasis within the time frame of the study. In contrast, the highly aggressive nature of HMPOS made it difficult to assess the full potential effects of the relatively slow-acting sodium ATM prior to mice being euthanized for tumor size. This aggressive character of the HMPOS cell line may have also contributed to the increased incidence of tumor ulceration, essentially leading to a reduced study group size. It is possible that sodium ATM may show greater efficacy and prolonged survival in a less aggressive osteosarcoma model; such information, however, was beyond the scope of this study.

Another limitation of this study was the failure to demonstrate a clear dose-dependent response *in vivo*. The *in vivo* model was originally designed to test two dose levels in

addition to a placebo group. Due to toxicity experienced by the high-dose group, however, the protocol was adjusted, obviating any interpretation of a potential dose-dependent response. Further studies testing the *in vivo* response of canine osteosarcoma tumors at multiple dose levels is needed to better delineate a dose-dependent response and to elucidate the maximally effective dose achievable within a safe dosing range. Concurrent serum gold testing should be included with this research to more clearly define a safe and effective therapeutic range for osteosarcoma.

Although this study demonstrated anti-proliferative effects of sodium ATM on canine osteosarcoma *in vivo*, the exact mechanism of this effect has yet to be elucidated. Whether the inhibition of PKCι as a mechanism of action of ATM can be extrapolated from previous work in human lung cancer remains unknown. Future studies evaluating the levels of PKCι in canine and human osteosarcoma and osteoblast cell lines would be a valuable next step in addressing this question. Previous work has shown that PKCι expression in human lung cancer cell lines correlated positively with sensitivity to ATM.[177] Determination of PKCι expression and *in vitro* ATM-sensitivity of multiple human and canine osteosarcoma cell lines could determine whether the relationship between PKCι and ATM holds true for osteosarcoma. If this relationship is substantiated, PKCι expression screening could become a useful tool in predicting responsiveness to ATM therapy.

In conclusion, our study demonstrates that sodium ATM slows the growth of canine osteosarcoma and reduces the incidence of pulmonary metastasis in a murine xenograft model. Furthermore, it appears that this effect is mediated at least in part by reduced cellular proliferation within the tumor. This is consistent with previous work

showing an anti-proliferative effect of sodium ATM in the treatment of human lung cancer. This therapeutic effect was achieved at dose levels considered to be safe in human patients receiving ATM for treatment of other diseases. Given the role of canine osteosarcoma as a translational model for its human counterpart and the therapeutic challenges that both types of osteosarcoma present, further investigation of the anti-neoplastic effects of ATM on canine and human osteosarcoma is warranted.

LIST OF REFERENCES

1. Priester WA, McKay FW: The occurrence of tumors in domestic animals. Natl Cancer Inst Monogr:1-210, 1980.

2. Withrow SJ, Powers BE, Straw RC, et al: Comparative aspects of osteosarcoma. Dog versus man. Clin Orthop Relat Res:159-168, 1991.

3. Bailey D, Erb H, Williams L, et al: Carboplatin and doxorubicin combination chemotherapy for the treatment of appendicular osteosarcoma in the dog. J Vet Intern Med 17:199-205, 2003.

4. Thompson JP, Fugent MJ: Evaluation of survival times after limb amputation, with and without subsequent administration of cisplatin, for treatment of appendicular osteosarcoma in dogs: 30 cases (1979-1990). J Am Vet Med Assoc 200:531-533, 1992.

5. Mauldin GN, Matus RE, Withrow SJ, et al: Canine osteosarcoma. Treatment by amputation versus amputation and adjuvant chemotherapy using doxorubicin and cisplatin. J Vet Intern Med 2:177-180, 1988.

6. Misdorp W, Hart AA: Some prognostic and epidemiologic factors in canine osteosarcoma. J Natl Cancer Inst 62:537-545, 1979.

7. Brodey R, Riser W: Canine osteosarcoma: a clinicopathological study of 194 cases. Clin Orthop 62:54-64, 1969.

8. Ru G, Terracini B, Glickman LT: Host related risk factors for canine osteosarcoma. Vet J 156:31-39, 1998.

9. Kistler K: Canine osteosarcoma: 1462 cases reviewed to uncover patterns of height, weight, breed, sex, age and site involvement., Proceedings, Phi Zeta Awards, Philadelphia, Pennsylvania, 1981

10. Heyman SJ, Diefenderfer DL, Goldschmidt MH, et al: Canine axial skeletal osteosarcoma. A retrospective study of 116 cases (1986 to 1989). Vet Surg 21:304-310, 1992.

11. Straw RC: Tumors of the skeletal system, in Withrow SJ, MacEwen EG (eds): Clinical veterinary oncology, Vol. Philadelphia, Pennsylvania, WB Saunders, 1996.

12. Goldschmidt MH, Thrall DE: Malignant bone tumors in the dog, in Newton CD, Nunamaker DM (eds): Textbook of small animal orthopedics, Vol. Philadelphia, Pennsylvania, JB Lippincott, 1985.

13. Loeb LA: A Mutator Phenotype in Cancer. Cancer Research 61:3230-3239, 2001.

14. Dernell W, Ehrhart N, Straw R, et al: Tumors of the Skeletal System, in Withrow S, Vail D (eds): Withrow & MacEwen's Small Animal Clinical Oncology (ed Fourth), Vol. St. Louis, Missouri, Saunders Elsevier, 2007, pp 540-581.

15. Gellasch KL, Kalscheur VL, Clayton MK, et al: Fatigue microdamage in the radial predilection site for osteosarcoma in dogs. Am J Vet Res 63:896-899, 2002.

16. Sinibaldi K, Rosen H, Liu SK, et al: Tumors associated with metallic implants in animals. Clin Orthop Relat Res:257-266, 1976.

17. Stevenson S, Hohn RB, Pohler OE, et al: Fracture-associated sarcoma in the dog. J Am Vet Med Assoc 180:1189-1196, 1982.

18. Bennett D, Campbell JR, Brown P: Osteosarcoma associated with healed fractures. J Small Anim Pract 20:13-18, 1979.

19. Keel SB, Jaffe KA, Petur Nielsen G, et al: Orthopaedic implant-related sarcoma: a study of twelve cases. Mod Pathol 14:969-977, 2001.

20. Kumar K: Osteosarcoma associated with a metal implant. Int Orthop 20:335-336, 1996.

21. Li XQ, Hom DL, Black J, et al: Relationship between metallic implants and cancer: a case-control study in a canine population. Vet Comp Orthop Traumatol 6:70-74, 1993.

22. Boudrieau RJ, McCarthy RJ, Sprecher CM, et al: Material properties of and tissue reaction to the Slocum TPLO plate. Am J Vet Res 67:1258-1265, 2006.

23. Charles AE, Ness MG: Crevice corrosion of implants recovered after tibial plateau leveling osteotomy in dogs. Vet Surg 35:438-444, 2006.

24. Stevenson S: Fracture-associated sarcomas. Vet Clin North Am Small Anim Pract 21:859-872, 1991.

25. Miller SC, Lloyd RD, Bruenger FW, et al: Comparisons of the skeletal locations of putative plutonium-induced osteosarcomas in humans with those in beagle dogs and with naturally occurring tumors in both species. Radiat Res 160:517-523, 2003.

26. Lloyd RD, Taylor GN, Angus W, et al: Distribution of skeletal malignancies in beagles injected with 239Pu citrate. Health Phys 66:407-413, 1994.

27. White RG, Raabe OG, Culbertson MR, et al: Bone sarcoma characteristics and distribution in beagles fed strontium-90. Radiat Res 136:178-189, 1993.

28. White RG, Raabe OG, Culbertson MR, et al: Bone sarcoma characteristics and distribution in beagles injected with radium-226. Radiat Res 137:361-370, 1994.

29. Gillette SM, Gillette EL, Powers BE, et al: Radiation-induced osteosarcoma in dogs after external beam or intraoperative radiation therapy. Cancer Res 50:54-57, 1990.

30. Powers BE, Gillette EL, McChesney SL, et al: Bone necrosis and tumor induction following experimental intraoperative irradiation. Int J Radiat Oncol Biol Phys 17:559-567, 1989.

31. McEntee MC, Page RL, Théon A, et al: Malignant tumor formation in dogs previously irradiated for acanthomatous epulis. Vet Radiol Ultrasound 45:357-361, 2004.

32. Dubielzig RR, Biery DN, Brodey RS: Bone sarcomas associated with multifocal medullary bone infarction in dogs. J Am Vet Med Assoc 179:64-68, 1981.

33. Marcellin-Little DJ, DeYoung DJ, Thrall DE, et al: Osteosarcoma at the site of bone infarction associated with total hip arthroplasty in a dog. Vet Surg 28:54-60, 1999.

34. Sagartz JE, Bodley WL, Gamblin RM, et al: p53 tumor suppressor protein overexpression in osteogenic tumors of dogs. Vet Pathol 33:213-221, 1996.

35. Wergin MC, Kaser-Hotz B: Plasma vascular endothelial growth factor (VEGF) measured in seventy dogs with spontaneously occurring tumours. In Vivo 18:15-19, 2004.

36. Mullins MN, Lana SE, Dernell WS, et al: Cyclooxygenase-2 expression in canine appendicular osteosarcomas. J Vet Intern Med 18:859-865, 2004.

37. Mendoza S, Konishi T, Dernell WS, et al: Status of the p53, Rb and MDM2 genes in canine osteosarcoma. Anticancer Res 18:4449-4453, 1998.

38. Johnson AS, Couto CG, Weghorst CM: Mutation of the p53 tumor suppressor gene in spontaneously occurring osteosarcomas of the dog. Carcinogenesis 19:213-217, 1998.

39. Chandar N, Billig B, McMaster J, et al: Inactivation of p53 gene in human and murine osteosarcoma cells. Br J Cancer 65:208-214, 1992.

40. Kirpensteijn J, Kik M, Rutteman GR, et al: Prognostic significance of a new histologic grading system for canine osteosarcoma. Vet Pathol 39:240-246, 2002.

41. LaRue SM, Withrow SJ, Wrigley RH: Radiographic bone surveys in the evaluation of primary bone tumors in dogs. J Am Vet Med Assoc 188:514-516, 1986.

42. Kuntz CA, Dernell WS, Powers BE, et al: Extraskeletal osteosarcomas in dogs: 14 cases. J Am Anim Hosp Assoc 34:26-30, 1998.

43. Langenbach A, Anderson MA, Dambach DM, et al: Extraskeletal osteosarcomas in dogs: a retrospective study of 169 cases (1986-1996). J Am Anim Hosp Assoc 34:113-120, 1998.

44. Knecht CD, Priester WA: Musculoskeletal tumors in dogs. J Am Vet Med Assoc 172:72-74, 1978.

45. Gamblin RM, Straw RC, Powers BE, et al: Primary osteosarcoma distal to the antebrachiocarpal and tarsocrural joints in nine dogs (1980-1992). J Am Anim Hosp Assoc 31:86-91, 1995.

46. Brem H, Folkman J: Inhibition of tumor angiogenesis mediated by cartilage. J Exp Med 141:427-439, 1975.

47. Kuettner KE, Pauli BU, Soble L: Morphological studies on the resistance of cartilage to invasion by osteosarcoma cells *in vitro* and *in vivo*. Cancer Res 38:277-287, 1978.

48. Green EM, Adams WM, Forrest LJ: Four fraction palliative radiotherapy for osteosarcoma in 24 dogs. J Am Anim Hosp Assoc 38:445-451, 2002.

49. Farese JP, Milner R, Thompson MS, et al: Stereotactic radiosurgery for treatment of osteosarcomas involving the distal portions of the limbs in dogs. J Am Vet Med Assoc 225:1567-1572, 1548, 2004.

50. Hillers KR, Dernell WS, Lafferty MH, et al: Incidence and prognostic importance of lymph node metastases in dogs with appendicular osteosarcoma: 228 cases (1986-2003). J Am Vet Med Assoc 226:1364-1367, 2005.

51. Spodnick GJ, Berg J, Rand WM, et al: Prognosis for dogs with appendicular osteosarcoma treated by amputation alone: 162 cases (1978-1988). J Am Vet Med Assoc 200:995-999, 1992.

52. Bacci G, Avella M, Picci P, et al: Metastatic patterns in osteosarcoma. Tumori 74:421-427, 1988.

53. Giuliano AE, Feig S, Eilber FR: Changing metastatic patterns of osteosarcoma. Cancer 54:2160-2164, 1984.

54. Huth JF, Eilber FR: Patterns of recurrence after resection of osteosarcoma of the extremity. Strategies for treatment of metastases. Arch Surg 124:122-126, 1989.

55. Straw RC, Powers BE, Klausner J, et al: Canine mandibular osteosarcoma: 51 cases (1980-1992). J Am Anim Hosp Assoc 32:257-262, 1996.

56. Dernell WS, Van Vechten BJ, Straw RC, et al: Outcome following treatment of vertebral tumors in 20 dogs (1986-1995). J Am Anim Hosp Assoc 36:245-251, 2000.

57. Dickerson ME, Page RL, LaDue TA, et al: Retrospective analysis of axial skeleton osteosarcoma in 22 large-breed dogs. J Vet Intern Med 15:120-124, 2001.

58. Mehl ML, Withrow SJ, Seguin B, et al: Spontaneous regression of osteosarcoma in four dogs. J Am Vet Med Assoc 219:614-617, 2001.

59. Thamm DH, O'Brien MG, Vail DM: Serum vascular endothelial growth factor concentrations and postsurgical outcome in dogs with osteosarcoma. Vet Comp Oncol 6:126-132, 2008.

60. Mohammed SI, Khan KN, Sellers RS, et al: Expression of cyclooxygenase-1 and 2 in naturally-occurring canine cancer. Prostaglandins Leukot Essent Fatty Acids 70:479-483, 2004.

61. Sottnik JL, Hansen RJ, Gustafson DL, et al: Induction of VEGF by tepoxalin does not lead to increased tumour growth in a canine osteosarcoma xenograft. Vet Comp Oncol 9:118-130, 2011.

62. Scharf V, Coomer A, Farese J: Effect of bevacizumab on angiogenesis and growth of canine osteosarcoma cells xenografted in athymic mice. Accepted for publication. Am J Vet Res 2013.

63. Levine RA: Overexpression of the sis oncogene in a canine osteosarcoma cell line. Vet Pathol 39:411-412, 2002.

64. Levine RA, Forest T, Smith C: Tumor suppressor PTEN is mutated in canine osteosarcoma cell lines and tumors. Vet Pathol 39:372-378, 2002.

65. Flint AF, U'Ren L, Legare ME, et al: Overexpression of the erbB-2 proto-oncogene in canine osteosarcoma cell lines and tumors. Vet Pathol 41:291-296, 2004.

66. Yazawa M, Setoguchi A, Hong SH, et al: Effect of an adenoviral vector that expresses the canine p53 gene on cell growth of canine osteosarcoma and mammary adenocarcinoma cell lines. Am J Vet Res 64:880-888, 2003.

67. Thrall DE: Bone tumors versus bone infections, in Thrall DE (ed): Textbook of veterinary diagnostic radiology (ed Third), Vol. Philadelphia, Pennsylvania, W.B. Saunders, 1998.

68. Waters DJ, Coakley FV, Cohen MD, et al: The detection of pulmonary metastases by helical CT: a clinicopathologic study in dogs. J Comput Assist Tomogr 22:235-240, 1998.

69. Picci P, Vanel D, Briccoli A, et al: Computed tomography of pulmonary metastases from osteosarcoma: the less poor technique. A study of 51 patients with histological correlation. Ann Oncol 12:1601-1604, 2001.

70. Straw RC, Cook NL, LaRue SM, et al: Radiographic bone surveys. J Am Vet Med Assoc 195:1458, 1989.

71. Berg J, Lamb CR, O'Callaghan MW: Bone scintigraphy in the initial evaluation of dogs with primary bone tumors. J Am Vet Med Assoc 196:917-920, 1990.

72. Lamb CR: Bone scintigraphy in small animals. J Am Vet Med Assoc 191:1616-1622, 1987.

73. Hahn KA, Hurd C, Cantwell HD: Single-phase methylene diphosphate bone scintigraphy in the diagnostic evaluation of dogs with osteosarcoma. J Am Vet Med Assoc 196:1483-1486, 1990.

74. Parchman MB, Flanders JA, Erb HN, et al: Nuclear medical bone imaging and targeted radiography for evaluation of skeletal neoplasms in 23 dogs. Vet Surg 18:454-458, 1989.

75. Powers BE, LaRue SM, Withrow SJ, et al: Jamshidi needle biopsy for diagnosis of bone lesions in small animals. J Am Vet Med Assoc 193:205-210, 1988.

76. Reinhardt S, Stockhaus C, Teske E, et al: Assessment of cytological criteria for diagnosing osteosarcoma in dogs. J Small Anim Pract 46:65-70, 2005.

77. Barger A, Graca R, Bailey K, et al: Use of alkaline phosphatase staining to differentiate canine osteosarcoma from other vimentin-positive tumors. Vet Pathol 42:161-165, 2005.

78. deSantos LA, Murray JA, Ayala AG: The value of percutaneous needle biopsy in the management of primary bone tumors. Cancer 43:735-744, 1979.

79. Kuntz CA, Asselin TL, Dernell WS, et al: Limb salvage surgery for osteosarcoma of the proximal humerus: outcome in 17 dogs. Vet Surg 27:417-422, 1998.

80. Bergman PJ, MacEwen EG, Kurzman ID, et al: Amputation and carboplatin for treatment of dogs with osteosarcoma: 48 cases (1991 to 1993). J Vet Intern Med 10:76-81, 1996.

81. Coomber BL, Denton J, Sylvestre A, et al: Blood vessel density in canine osteosarcoma. Can J Vet Res 62:199-204, 1998.

82. Ehrhart N, Dernell WS, Hoffmann WE, et al: Prognostic importance of alkaline phosphatase activity in serum from dogs with appendicular osteosarcoma: 75 cases (1990-1996). J Am Vet Med Assoc 213:1002-1006, 1998.

83. Hammer AS, Weeren FR, Weisbrode SE, et al: Prognostic factors in dogs with osteosarcomas of the flat or irregular bones. J Am Anim Hosp Assoc 31:321-326, 1995.

84. Pirkey-Ehrhart N, Withrow SJ, Straw RC, et al: Primary rib tumors in 54 dogs. J Am Anim Hosp Assoc 31:65-69, 1995.

85. Hendrix DV, Gelatt KN: Diagnosis, treatment and outcome of orbital neoplasia in dogs: a retrospective study of 44 cases. J Small Anim Pract 41:105-108, 2000.

86. White R: Mandibulectomy and maxillectomy in the dog: long term survival in 100 cases. *J Small Anim Pract*:69-74, 1991.

87. Schwarz P, Withrow S, Curtis C, et al: Mandibular resection as a treatment for oral cancer in 81 dogs. *J Am Anim Hosp Assoc*:601-610, 1991.

88. Selvarajah GT, Kirpensteijn J, van Wolferen ME, et al: Gene expression profiling of canine osteosarcoma reveals genes associated with short and long survival times. Mol Cancer 8:72, 2009.

89. Berg J, Weinstein MJ, Springfield DS, et al: Results of surgery and doxorubicin chemotherapy in dogs with osteosarcoma. J Am Vet Med Assoc 206:1555-1560, 1995.

90. Kent MS, Strom A, London CA, et al: Alternating carboplatin and doxorubicin as adjunctive chemotherapy to amputation or limb-sparing surgery in the treatment of appendicular osteosarcoma in dogs. J Vet Intern Med 18:540-544, 2004.

91. O'Brien MG, Withrow SJ, Straw RC, et al: Total and partial orbitectomy for the treatment of periorbital tumors in 24 dogs and 6 cats: a retrospective study. Vet Surg 25:471-479, 1996.

92. Lascelles BD, Thomson MJ, Dernell WS, et al: Combined dorsolateral and intraoral approach for the resection of tumors of the maxilla in the dog. J Am Anim Hosp Assoc 39:294-305, 2003.

93. Vasseur P: Limb preservation in dogs with primary bone tumors. Vet Clin North Am Small Anim Pract 17:889-903, 1987.

94. LaRue SM, Withrow SJ, Powers BE, et al: Limb-sparing treatment for osteosarcoma in dogs. J Am Vet Med Assoc 195:1734-1744, 1989.

95. Liptak JM, Dernell WS, Lascelles BD, et al: Intraoperative extracorporeal irradiation for limb sparing in 13 dogs. Vet Surg 33:446-456, 2004.

96. Buracco P, Morello E, Martano M, et al: Pasteurized tumoral autograft as a novel procedure for limb sparing in the dog: A clinical report. Vet Surg 31:525-532, 2002.

97. Morello E, Vasconi E, Martano M, et al: Pasteurized tumoral autograft and adjuvant chemotherapy for the treatment of canine distal radial osteosarcoma: 13 cases. Vet Surg 32:539-544, 2003.

98. Ehrhart N: Longitudinal bone transport for treatment of primary bone tumors in dogs: technique description and outcome in 9 dogs. Vet Surg 34:24-34, 2005.

99. Kirpensteijn J, Steinheimer D, Park R, et al: Comparison of cemented and non-cemented allografts for limb sparing procedures in dogs with osteosarcoma of the distal radius. Vet Comp Orthop Traumatol:178-184, 1998.

100. Straw RC, Withrow SJ: Limb-sparing surgery versus amputation for dogs with bone tumors. Vet Clin North Am Small Anim Pract 26:135-143, 1996.

101. Liptak JM, Dernell WS, Straw RC, et al: Intercalary bone grafts for joint and limb preservation in 17 dogs with high-grade malignant tumors of the diaphysis. Vet Surg 33:457-467, 2004.

102. Lascelles BD, Dernell WS, Correa MT, et al: Improved survival associated with postoperative wound infection in dogs treated with limb-salvage surgery for osteosarcoma. Ann Surg Oncol 12:1073-1083, 2005.

103. Withrow SJ, Liptak JM, Straw RC, et al: Biodegradable cisplatin polymer in limb-sparing surgery for canine osteosarcoma. Ann Surg Oncol 11:705-713, 2004.

104. Ramirez O, Dodge RK, Page RL, et al: Palliative radiotherapy of appendicular osteosarcoma in 95 dogs. Vet Radiol Ultrasound 40:517-522, 1999.

105. Walter CU, Dernell WS, LaRue SM, et al: Curative-intent radiation therapy as a treatment modality for appendicular and axial osteosarcoma: a preliminary retrospective evaluation of 14 dogs with the disease. Vet Comp Oncol 3:1-7, 2005.

106. Berg J, Weinstein MJ, Schelling SH, et al: Treatment of dogs with osteosarcoma by administration of cisplatin after amputation or limb-sparing surgery: 22 cases (1987-1990). J Am Vet Med Assoc 200:2005-2008, 1992.

107. Farese JP, Fox LE, Detrisac CJ, et al: Effect of thalidomide on growth and metastasis of canine osteosarcoma cells after xenotransplantation in athymic mice. Am J Vet Res 65:659-664, 2004.

108. Canetta R, Franks C, Smaldone L, et al: Clinical status of carboplatin. Oncology (Williston Park) 1:61-70, 1987.

109. Kurzman ID, MacEwen EG, Rosenthal RC, et al: Adjuvant therapy for osteosarcoma in dogs: results of randomized clinical trials using combined liposome-encapsulated muramyl tripeptide and cisplatin. Clin Cancer Res 1:1595-1601, 1995.

110. Kurzman ID, Shi F, Vail DM, et al: *In vitro* and *in vivo* enhancement of canine pulmonary alveolar macrophage cytotoxic activity against canine osteosarcoma cells. Cancer Biother Radiopharm 14:121-128, 1999.

111. London CA, Hannah AL, Zadovoskaya R, et al: Phase I dose-escalating study of SU11654, a small molecule receptor tyrosine kinase inhibitor, in dogs with spontaneous malignancies. Clin Cancer Res 9:2755-2768, 2003.

112. Fan TM, de Lorimier LP, Charney SC, et al: Evaluation of intravenous pamidronate administration in 33 cancer-bearing dogs with primary or secondary bone involvement. J Vet Intern Med 19:74-80, 2005.

113. Tomlin JL, Sturgeon C, Pead MJ, et al: Use of the bisphosphonate drug alendronate for palliative management of osteosarcoma in two dogs. Vet Rec 147:129-132, 2000.

114. Ashton JA, Farese JP, Milner RJ, et al: Investigation of the effect of pamidronate disodium on the *in vitro* viability of osteosarcoma cells from dogs. Am J Vet Res 66:885-891, 2005.

115. Farese JP, Ashton J, Milner R, et al: The effect of the bisphosphonate alendronate on viability of canine osteosarcoma cells *in vitro*. *In Vitro* Cell Dev Biol Anim 40:113-117, 2004.

116. Poirier VJ, Huelsmeyer MK, Kurzman ID, et al: The bisphosphonates alendronate and zoledronate are inhibitors of canine and human osteosarcoma cell growth *in vitro*. Vet Comp Oncol 1:207-215, 2003.

117. O'Brien MG, Straw RC, Withrow SJ, et al: Resection of pulmonary metastases in canine osteosarcoma: 36 cases (1983-1992). Vet Surg 22:105-109, 1993.

118. Hershey AE, Kurzman ID, Forrest LJ, et al: Inhalation chemotherapy for macroscopic primary or metastatic lung tumors: proof of principle using dogs with spontaneously occurring tumors as a model. Clin Cancer Res 5:2653-2659, 1999.

119. Khanna C, Hasz DE, Klausner JS, et al: Aerosol delivery of interleukin 2 liposomes is nontoxic and biologically effective: canine studies. Clin Cancer Res 2:721-734, 1996.

120. Khanna C, Anderson PM, Hasz DE, et al: Interleukin-2 liposome inhalation therapy is safe and effective for dogs with spontaneous pulmonary metastases. Cancer 79:1409-1421, 1997.

121. Khanna C, Vail DM: Targeting the lung: preclinical and comparative evaluation of anticancer aerosols in dogs with naturally occurring cancers. Curr Cancer Drug Targets 3:265-273, 2003.

122. Mueller F, Fuchs B, Kaser-Hotz B: Comparative biology of human and canine osteosarcoma. Anticancer Res 27:155-164, 2007.

123. Brodey RS: The use of naturally occurring cancer in domestic animals for research into human cancer: general considerations and a review of canine skeletal osteosarcoma. Yale J Biol Med 52:345-361, 1979.

124. Link MP, Goorin AM, Miser AW, et al: The effect of adjuvant chemotherapy on relapse-free survival in patients with osteosarcoma of the extremity. N Engl J Med 314:1600-1606, 1986.

125. Ward WG, Hosseinian AA: Rehabilitation of Patients Following Resection of Extremity Soft Tissue Sarcomas. Cancer Control 1:606-612, 1994.

126. Bielack SS, Kempf-Bielack B, Delling G, et al: Prognostic factors in high-grade osteosarcoma of the extremities or trunk: an analysis of 1,702 patients treated on neoadjuvant cooperative osteosarcoma study group protocols. J Clin Oncol 20:776-790, 2002.

127. Harris MB, Gieser P, Goorin AM, et al: Treatment of metastatic osteosarcoma at diagnosis: a Pediatric Oncology Group Study. J Clin Oncol 16:3641-3648, 1998.

128. Nobili S, Mini E, Landini I, et al: Gold compounds as anticancer agents: chemistry, cellular pharmacology, and preclinical studies. Med Res Rev 30:550-580, 2010.

129. Bax DE, Amos RS: Sulphasalazine: a safe, effective agent for prolonged control of rheumatoid arthritis. A comparison with sodium ATM. Annals of the Rheumatic Diseases 44:194-198, 1985.

130. Rosenkrantz WS: Pemphigus: current therapy. Vet Dermatol 15:90-98, 2004.

131. Melethil S, Schoepp D: Pharmacokinetics of gold sodium thiomalate in rabbits. Pharm Res 4:332-336, 1987.

132. Ho SY, Tiekink ERT: ^{79}Au gold-based metallotherapeutics: use and potential, in Gielen M, Tiekink ERT (eds): Metallotherapeutic durgs and metal-based diagnostic agents: the use of metals in medicine (ed 507-527), Vol. New York, Wiley, 2005.

133. Stillman MJ, Shaw CF, Suzuki KT: Metallothionein : synthesis, structure, and properties of metallothioneins, phytochelatins, and metal-thiolate complexes. New York, VCH Publishers, 1992.

134. Klaassen CD: Metallothionein IV. Basel ; Boston, Birkhäuser Verlag, 1999.

135. Miles AT, Hawksworth GM, Beattie JH, et al: Induction, regulation, degradation, and biological significance of mammalian metallothioneins. Crit Rev Biochem Mol Biol 35:35-70, 2000.

136. Graham GG, Kettle AJ: The activation of gold complexes by cyanide produced by polymorphonuclear leukocytes. III. The formation of aurocyanide by myeloperoxidase. Biochem Pharmacol 56:307-312, 1998.

137. Shaw CF, Isab AA, Coffer MT, et al: Gold(I) efflux from auranofin-treated red blood cells. Evidence for a glutathione-gold-albumin metabolite. Biochem Pharmacol 40:1227-1234, 1990.

138. Goebel C, Kubicka-Muranyi M, Tonn T, et al: Phagocytes render chemicals immunogenic: oxidation of gold(I) to the T cell-sensitizing gold(III) metabolite generated by mononuclear phagocytes. Arch Toxicol 69:450-459, 1995.

139. Smith WE, Reglinski J, Hoey S, et al: Action of sodium gold(I) thiomalate on erythrocyte membrane. Inorganic Chemistry 29:5190-5196, 1990.

140. Smith W, Reglinski J: Gold drugs used in the treatment of rheumatoid arthritis. Perspect Bioinorg Chem:183-208, 1991.

141. Snyder RM, Mirabelli CK, Crooke ST: Cellular association, intracellular distribution, and efflux of auranofin via sequential ligand exchange reactions. Biochem Pharmacol 35:923-932, 1986.

142. Messori LM, Marcon G: Gold complexes in the treatment of rheumatoid arthritis, in Sigel A, Sigel H (eds): Metal ions in biological systems: metal ions and their complexes in medications, Vol 41. New York, NY, Marcel Dekker, Inc., 2004, pp 279-304.

143. Mirabelli CK, Sung CM, Zimmerman JP, et al: Interactions of gold coordination complexes with DNA. Biochem Pharmacol 35:1427-1433, 1986.

144. Rackham O, Nichols SJ, Leedman PJ, et al: A gold(I) phosphine complex selectively induces apoptosis in breast cancer cells: implications for anticancer therapeutics targeted to mitochondria. Biochem Pharmacol 74:992-1002, 2007.

145. Marzano C, Gandin V, Folda A, et al: Inhibition of thioredoxin reductase by auranofin induces apoptosis in cisplatin-resistant human ovarian cancer cells. Free Radic Biol Med 42:872-881, 2007.

146. Pia Rigobello M, Messori L, Marcon G, et al: Gold complexes inhibit mitochondrial thioredoxin reductase: consequences on mitochondrial functions. J Inorg Biochem 98:1634-1641, 2004.

147. Engman L, McNaughton M, Gajewska M, et al: Thioredoxin reductase and cancer cell growth inhibition by organogold(III) compounds. Anticancer Drugs 17:539-544, 2006.

148. Nguyen P, Awwad RT, Smart DD, et al: Thioredoxin reductase as a novel molecular target for cancer therapy. Cancer Lett 236:164-174, 2006.

149. Grogan TM, Fenoglio-Prieser C, Zeheb R, et al: Thioredoxin, a putative oncogene product, is overexpressed in gastric carcinoma and associated with increased proliferation and increased cell survival. Hum Pathol 31:475-481, 2000.

150. Raffel J, Bhattacharyya AK, Gallegos A, et al: Increased expression of thioredoxin-1 in human colorectal cancer is associated with decreased patient survival. J Lab Clin Med 142:46-51, 2003.

151. Gromer S, Arscott LD, Williams CH, et al: Human placenta thioredoxin reductase. Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. J Biol Chem 273:20096-20101, 1998.

152. Rigobello MP, Scutari G, Boscolo R, et al: Induction of mitochondrial permeability transition by auranofin, a gold(I)-phosphine derivative. Br J Pharmacol 136:1162-1168, 2002.

153. McKeage MJ, Maharaj L, Berners-Price SJ: Mechanisms of cytotoxicity and antitumor activity of gold(I) phosphine complexes: the possible role of mitochondria. Coordination Chemistry Reviews 232:127-135, 2002.

154. Porter AG, Jänicke RU: Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6:99-104, 1999.

155. Park SJ, Kim IS: The role of p38 MAPK activation in auranofin-induced apoptosis of human promyelocytic leukaemia HL-60 cells. Br J Pharmacol 146:506-513, 2005.

156. Jellicoe MM, Nichols SJ, Callus BA, et al: Bioenergetic differences selectively sensitize tumorigenic liver progenitor cells to a new gold(I) compound. Carcinogenesis 29:1124-1133, 2008.

157. Wodarz A, Näthke I: Cell polarity in development and cancer. Nat Cell Biol 9:1016-1024, 2007.

158. Fields AP, Gustafson WC: Protein kinase C in disease: cancer. Methods Mol Biol 233:519-537, 2003.

159. Fields AP, Regala RP: Protein kinase C iota: human oncogene, prognostic marker and therapeutic target. Pharmacol Res 55:487-497, 2007.

160. Erdogan E, Lamark T, Stallings-Mann M, et al: ATM inhibits transformed growth by targeting the PB1 domain of protein kinase Ciota. J Biol Chem 281:28450-28459, 2006.

161. Lockie LM, Smith DM: Forty-seven years experience with gold therapy in 1,019 rheumatoid arthritis patients. Semin Arthritis Rheum 14:238-246, 1985.

162. Havarinasab S, Johansson U, Pollard KM, et al: Gold causes genetically determined autoimmune and immunostimulatory responses in mice. Clin Exp Immunol 150:179-188, 2007.

163. Greinacher A, Eichler P, Lubenow N, et al: Drug-induced and drug-dependent immune thrombocytopenias. Rev Clin Exp Hematol 5:166-200; discussion 311-162, 2001.

164. Hall CL: Gold nephropathy. Nephron 50:265-272, 1988.

165. Rodriguez-Pérez M, González-Dominguez J, Matarán L, et al: Association of HLA-DR5 with mucocutaneous lesions in patients with rheumatoid arthritis receiving gold sodium thiomalate. J Rheumatol 21:41-43, 1994.

166. Sakkas LI, Chikanza IC, Vaughan RW, et al: Gold induced nephropathy in rheumatoid arthritis and HLA class II genes. Ann Rheum Dis 52:300-301, 1993.

167. Kummel BA: Medical treatment of canine pemphigus-pemphigoid, in Bonagura JB (ed): Kirk's Current Veterinary Therapy XII, Vol. Philadelphia, PE, W.B. Saunders, 1995, pp 363-368.

168. Mirabelli CK, Jensen BD, Mattern MR, et al: Cellular pharmacology of mu-[1,2-bis(diphenylphosphino)ethane]bis[(1-thio-beta-D-gluco pyranosato-S)gold(I)]: a novel antitumor agent. Anticancer Drug Des 1:223-234, 1986.

169. Crump D, Siasios G, Tiekink ER: A Study of the Antitumour Activity of Four Triorganophosphinegold(I) Thiolates: R(3) PAu(SR'), R = Ph, Cy, Et; SR'H = 6-Mercaptopurine and R = Et; SR'H = 6-Thioguanine. Met Based Drugs 6:361-368, 1999.

170. de Vos D, Clements P, Pyke SM, et al: Characterisation and *in vitro* cytotoxicity of triorganophosphinegold(i) 2-mercaptobenzoate complexes. Met Based Drugs 6:31-40, 1999.

171. Mirabelli CK, Johnson RK, Sung CM, et al: Evaluation of the *in vivo* antitumor activity and *in vitro* cytotoxic properties of auranofin, a coordinated gold compound, in murine tumor models. Cancer Res 45:32-39, 1985.

172. Simon TM, Kunishima DH, Vibert GJ, et al: Screening trial with the coordinated gold compound auranofin using mouse lymphocyte leukemia P388. Cancer Res 41:94-97, 1981.

173. Koide T, Kojima T, Kamei H: Antitumor effect of gold as revealed by growth suppression of cultured cancer cells. Cancer Biother Radiopharm 13:189-192, 1998.

174. Trani M, Sorrentino A, Busch C, et al: Pro-apoptotic effect of ATM in prostate cancer cells. Cell Cycle 8:306-313, 2009.

175. Cell Titer-Blue® Cell Viability Assay, in Corporation P (ed): www.promega.com, Vol. Madison, WI, Promega Corporation, 2009, pp 1-16.

176. Blocka KL, Paulus HE, Furst DE: Clinical pharmacokinetics of oral and injectable gold compounds. Clin Pharmacokinet 11:133-143, 1986.

177. Regala RP, Thompson EA, Fields AP: Atypical protein kinase C iota expression and ATM sensitivity in human lung cancer cells. Cancer Res 68:5888-5895, 2008.

178. Trédan O, Galmarini CM, Patel K, et al: Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst 99:1441-1454, 2007.

179. Teicher BA, Herman TS, Holden SA, et al: Tumor resistance to alkylating agents conferred by mechanisms operative only *in vivo*. Science 247:1457-1461, 1990.

180. Shaw III CF: Gold-based therapeutic agents. Chem Rev 99:2589-2600, 1999.

181. Kamei H, Koide T, Kojima T, et al: Effect of gold on survival of tumor-bearing mice. Cancer Biother Radiopharm 13:403-406, 1998.

182. Regala RP, Weems C, Jamieson L, et al: Atypical protein kinase Ciota plays a critical role in human lung cancer cell growth and tumorigenicity. J Biol Chem 280:31109-31115, 2005.

183. Regala RP, Weems C, Jamieson L, et al: Atypical protein kinase C iota is an oncogene in human non-small cell lung cancer. Cancer Res 65:8905-8911, 2005.

184. Stallings-Mann M, Jamieson L, Regala RP, et al: A novel small-molecule inhibitor of protein kinase Ciota blocks transformed growth of non-small-cell lung cancer cells. Cancer Res 66:1767-1774, 2006.

185. Menefee M: Sirolimus and gold sodium thiomalate in treating patients with advanced squamous non-small cell lung cancer, in Mayo Clinic Cancer Center NCI (ed). ClinicalTrials.gov, U.S. National Institutes of Heatlh, 2012.

186. Molina J: Gold sodium thiomalate in treating patients with advanced non-small cell lung cancer, in Mayo Clinic Cancer Center NCI (ed). ClinicalTrials.gov, U.S. National Institutes of Heatlh, 2009.

187. Bergin IL, Smedley RC, Esplin DG, et al: Prognostic evaluation of Ki67 threshold value in canine oral melanoma. Vet Pathol 48:41-53, 2011.

188. Kiupel M, Stevenson GW, Galbreath EJ, et al: Porcine circovirus type 2 (PCV2) causes apoptosis in experimentally inoculated BALB/c mice. BMC Vet Res 1:7, 2005.

189. Kaplan E, Meier P: Nonparametric estimation from incomplete observations. J Amer Statist Assn:457-481, 1958.

190. Brown DC, Gatter KC: Ki67 protein: the immaculate deception? Histopathology 40:2-11, 2002.

191. Hernández-Rodríguez NA, Correa E, Sotelo R, et al: Ki-67: a proliferative marker that may predict pulmonary metastases and mortality of primary osteosarcoma. Cancer Detect Prev 25:210-215, 2001.

192. Korabiowska M, Brinck U, Dengler H, et al: Analysis of the DNA mismatch repair proteins expression in malignant melanomas. Anticancer Res 20:4499-4505, 2000.

193. Korabiowska M, Brinck U, Brinkmann U, et al: Prognostic significance of newly defined ploidy related parameters in melanoma. Anticancer Res 20:1685-1690, 2000.

194. Korabiowska M, Brinck U, Middel P, et al: Proliferative activity in the progression of pigmented skin lesions, diagnostic and prognostic significance. Anticancer Res 20:1781-1785, 2000.

195. Peng TS, Qiu JS, Wu HX, et al: [Expressions of CD44s, MMP-9, and Ki-67: possible association with invasion, metastasis, and recurrence of osteosarcoma]. Ai Zheng 21:745-750, 2002.

196. Scotlandi K, Serra M, Manara MC, et al: Clinical relevance of Ki-67 expression in bone tumors. Cancer 75:806-814, 1995.

197. Nakano T, Oka K: Differential values of Ki-67 index and mitotic index of proliferating cell population. An assessment of cell cycle and prognosis in radiation therapy for cervical cancer. Cancer 72:2401-2408, 1993.

198. Nakano T, Oka K: Transition of Ki-67 index of uterine cervical tumors during radiation therapy. Immunohistochemical study. Cancer 68:517-523, 1991.

BIOGRAPHICAL SKETCH

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