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Evaluation of zoledronate for the treatment of canine stage III osteosarcoma: A phase II study

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Abstract

Background: Greater than 90% of dogs with appendicular osteosarcoma will develop pulmonary metastasis despite the standard of care. Available treatments have limited efficacy for stage III disease. Zoledronate, a bisphosphonate, induces apoptosis of canine osteosarcoma cells and appears to modulate the tumour microenvironment.

Objectives: This prospective, single institutional phase IIa trial investigated the use of single agent zoledronate in dogs with pulmonary metastases from osteosarcoma.

Methods: Zoledronate was administered once monthly, and thoracic radiographs were used to assess response.

Results: Eleven dogs were enrolled. Stable disease was achieved in two of eight dogs available for response assessment. The median progression-free survival was 28 days (range: 4–93 days). The median stage III-specific survival time was 92 days. Adverse events were reported in four dogs; two dogs developed grade III or higher toxicities. Notable adverse events included conjunctivitis, fever, hypocalcaemia, and hypophosphatemia.

Conclusions: Zoledronate appears to have limited efficacy as a single agent for stage III osteosarcoma and may be associated with unexpected toxicity in this population. This clinical trial was registered on the AVMA Animal Health Studies Database (AAHSD004396).

KEYWORDS
bisphosphonate, bone, lung, zoledronic acid

1 | INTRODUCTION

Osteosarcoma (OSA) is the most common bone tumour in dogs. Greater than 90% of dogs with appendicular OSA will develop pulmonary metastasis (Brodey & Riser, 1969). Reported median survival times are 10–12 months despite treatment with amputation and adjuvant chemotherapy (Bergman et al., 1996). Stage III disease (regional or distant metastasis) is generally considered refractory to treatment; dogs with pulmonary metastasis have a median survival time of 59 days (Boston et al., 2006). Chemotherapy appears minimally effective for stage III disease with response rates of 0%–12% for doxorubicin, cisplatin, mitoxantrone, or ifosfamide (Batschinski et al., 2014; Ogilvie et al., 1993). A prospective study demonstrated a 17.6% biologic response rate to toceranib phosphate, but notably 0% of dogs experienced a partial or complete response (Laver et al., 2018). The high frequency of metastasis despite the standard of care has

fuelled a search for effective therapies against macroscopic metastatic disease.

Bisphosphonates (BPs) are commonly used in humans and veterinary species to treat cancer-associated bone pain and hypercalcemia (Fan, 2007). They have also been investigated for anti-neoplastic properties. BPs concentrate within metabolically active bone and inhibit bone resorption. Zoledronate (ZOL), a third-generation BP, induces apoptosis in several cancer cell lines including canine OSA (Poirier et al., 2003). It inhibits farnesyl pyrophosphate synthase, an enzyme required for the prenylation of GTP-binding proteins (Fan, 2007). This results in disruption of intracellular metabolism, cell cycle arrest, and apoptosis. ZOL also modulates the tumour microenvironment by activating $\gamma\delta$ T cells (Das et al., 2001), inhibiting matrix metalloproteinase activity (Roelofs et al., 2010), promoting macrophage differentiation towards an anti-tumoral M1 phenotype (Roelofs et al., 2010), and decreasing vascular endothelial growth factor secretion (Ohba et al., 2014). ZOL reduced the development of pulmonary metastasis in some in vitro murine models (Koto et al., 2009; Ohba et al., 2014). Other studies have reported conflicting results but were unable to replicate the tumour–host microenvironment in an immunocompromised host (Wolfe et al., 2011).

The role of ZOL in human OSA treatment protocols has yet to be established. Although ZOL can be safely combined with chemotherapy, its addition in protocols for patients with non-metastatic disease is associated with an increased hazard of relapse and metastasis (Goldsby et al., 2013; Piperno-Neumann et al., 2016). ZOL may have greater utility in the macroscopic disease setting. A recent case series of patients with chemotherapy-resistant pulmonary metastases showed that ZOL induced long-lasting stable disease (Conry et al., 2016). Stabilization lasted for more than 19 months when historically, the median time to progression is 2 months for chemotherapy-resistant metastatic disease. While the results of Conry et al. are promising, the authors involved a limited number of patients, and a larger prospective study is necessary for validation.

The efficacy of BPs for canine metastatic OSA has yet to be determined. Most veterinary research has focused on cancer-associated bone pain. Pamidronate, a second-generation BP, provides pain control in 28% of dogs with OSA for at least 4 months' duration (Fan et al., 2007). It has been combined with carboplatin or doxorubicin and, although it was well tolerated, its addition did not influence prognosis (Fan et al., 2009; Kozicki et al., 2013). As a third-generation BP, ZOL exhibits a 100-fold higher anti-resorptive potency than pamidronate (Fan, 2007). The veterinary literature describing ZOL is limited to tolerability studies; it appears well tolerated with rare reports of renal azotaemia and osteonecrosis of the jaw (Brewer et al., 2021; Fan, 2007). ZOL has been shown to decrease plasma concentrations of CXCR4, a chemokine involved in metastasis, in dogs with OSA (Byrum et al., 2016). This preliminary information paired with the in vitro effects of ZOL on the tumour microenvironment warrants investigation of this drug for macroscopic OSA.

The purpose of this study is to obtain pilot data on the use of ZOL in dogs with pulmonary metastatic OSA. Outcomes of interest include adverse events (AEs), tumour response, and stage III survival.

2 | METHODS

2.1 | Patient Selection

This was a prospective, single institutional phase IIa study at Auburn University College of Veterinary Medicine Wilford and Kate Bailey Small Animal Teaching Hospital utilizing client-owned dogs with stage III OSA. The study design was approved by the institution's ethics committee and the animal care and use committee (#2017-3051). Written client consent was required prior to enrolment. Dogs were required to have at least one pulmonary nodule evaluable on thoracic radiographs and a histopathologic diagnosis of appendicular OSA. Given that pulmonary nodules could be due to diseases other than OSA, ultrasound-guided fine needle aspirates were offered if feasible. Prior treatment with surgery and/or chemotherapy was permitted, but cancer progression must be noted at the time of enrolment. Local disease control in the form of an amputation, surgical limb spare, radiation therapy, or analgesics was required. Exclusion criteria included the presence of a pathologic fracture, a life expectancy of less than 1 month, and/or the presence of azotaemia defined as a serum creatinine greater than 2.0 mg/dl. Patients were not allowed to receive concurrent treatment with chemotherapy, immunotherapy, or purported anti-tumour supplements. Dogs receiving non-steroidal anti-inflammatory drugs (NSAIDs) at the time of enrolment were permitted to continue administration for analgesia. NSAIDs were not initiated during the study due to reported anti-angiogenic effects (Mohammed et al., 2002).

2.2 | Study protocol

A baseline complete blood count (CBC), serum chemistry panel, and urinalysis was performed to confirm dogs were otherwise healthy. Three-view thoracic radiographs were obtained at enrolment and reviewed by a single board-certified radiologist. Dogs with known intra-abdominal metastatic disease also underwent an abdominal ultrasound. Intra-abdominal target lesions were measured by a board-certified radiologist. Images were obtained for comparison and subsequent assessments were performed within the same plane. Target lesions were defined and measured according to the Veterinary Cooperative Oncology Group Response Evaluation Criteria for Solid Tumors (VCOG-RECIST) to facilitate response assessment (Nguyen et al., 2015). Non-target lesions were defined as per VCOG-RECIST as present or absent.

ZOL (zoledronic acid, Mylan Institutional LLC, Canonsburg, PA, USA) was administered at a dosage of 0.1 mg/kg. Prior to July 2019, a maximum dose of 4.0 mg per dog was used based on the maximum dose established in human clinical trials (Goldsby et al., 2013). Patients enrolled after July 2019 were treated to a maximum dose of 8.0 mg per dog based on canine tolerability data (Byrum et al., 2016; Fan et al., 2008). ZOL was diluted in 0.9% sodium chloride to a total volume of 100 ml and delivered as a 15-min IV constant rate infusion.

Dogs were evaluated once every 4 weeks for the duration of the clinical trial. A physical examination, blood urea nitrogen, creatinine, urine

specific gravity, and three-view thoracic radiographs were performed at each visit. An abdominal ultrasound was also performed at each visit for patients with known intra-abdominal metastatic disease. ZOL was administered once per month for up to 6 months.

Owners completed a questionnaire documenting the patient's clinical status prior to enrolment (Supporting Information). Any adverse clinical events were expected to be reported via telephone at the time of the event and at each recheck exam on the institution's routine patient history form. The Veterinary Cooperative Oncology Group Common Terminology Criteria for Adverse Events (VCOG-CTCAE v1.1) was used to document AEs (Veterinary Cooperative Oncology Group, 2016). Since dogs were required to have local disease control, data pertaining to ZOL's pain control properties were not collected.

2.3 | Response assessment

Thoracic radiographs were reviewed by a single board-certified radiologist (Greg T. Almond). VCOG-RECIST definitions for complete response, partial response, stable disease, and progressive disease were used to describe response to treatment. Continued enrolment was offered if ZOL resulted in a biologic response (i.e., stable disease at minimum). Dogs were removed from the study at the time of OSA progression or based on the owner's request.

The primary end point was the development of progressive disease. Outcomes of interest included response to therapy, progression-free survival (PFS), stage III-specific survival time, and overall survival time (OST). PFS was measured from the date of enrolment (same day as ZOL administration) to the date of OSA progression. Patients that died prior to response assessment were included in PFS. Stage III-specific survival time was the time from detection of metastasis until the date of death. OST was defined from the time of OSA diagnosis to the time of death. Death from an undetermined cause was assumed to be due to osteosarcoma. Dogs were censored from survival analysis when they were lost to follow up or still alive at the time of data analysis.

2.4 | Statistics

Statistical analysis was performed using XLSTAT (Addinsoft, New York, NY, USA). The Kaplan–Meier product limit was used to calculate PFS, stage III-specific survival time, and OST with 95% confidence intervals (CI). Eleven dogs were recruited for enrolment i this phase II study. The null hypothesis was that single agent ZOL lacked efficacy for stage III osteosarcoma. A pre hoc power analysis was performed with the intent to pursue a prospective controlled clinical trial pending results from the current study. If at least a 20% response rate for ZOL was noted in the current study, then the authors intended to pursue a larger prospective study design of one control per three experimental subjects. If the true median OST of the control and experimental treatments were 60 and 180 days, respectively, then 31 experimental subjects and 10.23 control subjects would be required to reject the null hypothesis that the experimental and control survival curves are equal with a probability of 0.800. The type I error probability associated with the test of this null hypothesis is 0.05.

2.5 | Cell line validation statement

No cell lines were used in this study.

3 | RESULTS

3.1 | Patient population

A total of 11 dogs were prospectively enrolled at Auburn University College of Veterinary Medicine Wilford and Kate Bailey Small Animal Teaching Hospital between November 2017 and August 2020. Data for all dogs are shown in Table 1. The median age at diagnosis was 6 years (range: 1–12 years). Six were neutered males and five were spayed females. The median body weight was 34 kg (range: 17.8–52.6 kg). Three dogs had an elevated alkaline phosphatase at diagnosis.

The most common tumour location was the distal radius ($n = 4$). Other primary sites included the proximal humerus ($n = 2$), tibia (2), distal humerus (1), metacarpals (1), and metatarsals (1). Two dogs had metastatic disease at the time of diagnosis involving the regional lymph node ($n = 2$) and/or lungs (1). All 11 patients had achieved pain control of their bone tumour prior to enrolment via a limb amputation ($n = 9$), surgical limb spare (1), or analgesic medications (1; presenting with grade 1 lameness). A diagnosis of appendicular osteosarcoma was achieved via histopathology for all patients.

Nine dogs received prior chemotherapy and two were treatment naïve. Carboplatin was used in the microscopic disease setting for seven dogs. One dog was previously enrolled in a clinical trial and received sirolimus after completion of carboplatin. The dog exited the trial at the time of progression to stage III disease. Four dogs received chemotherapy for macroscopic metastasis prior to enrolment, consisting of carboplatin ($n = 3$) and doxorubicin (2). No responses were noted.

Pulmonary metastasis was detected radiographically in all patients at the time of enrolment. Two dogs had one pulmonary nodule, two dogs had two nodules, and the remaining seven dogs had three or more nodules. Two dogs also had metastasis to the regional lymph node ($n = 2$), liver (1), and skin (1). Both patients with nodal metastasis underwent lymphadenectomy at the time of primary tumour excision. The liver mass was considered a target lesion per VCOG-RECIST, and the skin nodules were non-target lesions. The median time from diagnosis to the development of metastasis was 75 days (range: 0–432 days).

Four of 11 dogs were receiving an NSAID at the time of enrolment. Other concomitant medications and supplements included gabapentin ($n = 2$), hydrocodone (1), tramadol (1), codeine (1), trazodone (1), phenylpropanolamine (1), alpha lipoic acid (1), and amikacin (1). The dog receiving amikacin (Case 6) had a multi-drug-resistant infection.

TABLE 1 Summary of 11 dogs enrolled in pilot study

Case number	Breed	Sex	Age at diagnosis (years)	Type of local therapy	Previous chemotherapy	Time to development of metastasis (days)	Location of metastasis
1	Labrador	FS	11	Amputation	Dox × 1, Carbo x 1	0	Pulm, liver, skin, LN
2	Newfoundland	MN	10	Amputation	Carbo × 6	203	Pulm
3	Mixed breed	FS	5	Amputation	N/A	111	Pulm
4	Airedale	MN	3	Amputation	Carbo × 4	211	Pulm
5	Rottweiler	MN	8	Amputation	Carbo × 2, Dox × 1	69	Pulm
6	Great Pyrenees	MN	6	Surgical limb spare	Carbo × 2	11	Pulm, LN
7	Great Pyrenees	FS	8	Amputation	Carbo × 4	432	Pulm
8	Golden retriever	MN	1	Amputation	Carbo × 4	147	Pulm
9	Standard poodle	FS	12	Analgesics	N/A	0	Pulm
10	Great dane	FS	6	Amputation	Carbo × 2	71	Pulm
11	Mixed breed	MN	3	Amputation	Carbo × 3	75	Pulm

Abbreviations: Carbo, carboplatin; Dox, doxorubicin; FS, female spayed; LN, lymph node; MN, male neutered; Pulm, pulmonary.

3.2 | Zoledronate administration

ZOL was administered the same day as enrolment at an intended dose of 0.1 mg/kg. The median total dose was 3.59 mg (range: 1.78–4.70 mg). Prior to July 2019, ZOL was administered at a maximum dose of 4.0 mg per dog. This resulted in four dogs receiving less than the target dose (range: 0.07–0.09 mg/kg). Patients enrolled after July 2019 were treated with a maximum dose of 8.0 mg. This affected one dog (Case 10) that received 4.70 mg. The median number of ZOL doses was 1 (range: 1–4).

3.3 | Toxicoses

Potential AEs were reported in four of 11 dogs (Table 2). Two dogs experienced a grade 3 or higher AE. Constitutional and gastrointestinal events consisted of lethargy (n = 3), anorexia (3), diarrhoea (2), and vomiting (1). Other AEs included fever (n = 2), tachypnoea (1), hypocalcaemia (1), and conjunctivitis (1).

Case 1 was reported to have grade 1 diarrhoea. This was documented a few weeks prior to enrolment and persisted after the first and second dose of ZOL. The dog received four doses of ZOL and did not have diarrhoea after subsequent doses. The owners reported prior to enrolment that the dog had a several years of history of intermittently loose stool.

Case 6 developed grade 3 anorexia and grade 2 lethargy. This dog had a mild fever, purulent discharge associated with the limb spare site, and newly documented pulmonary metastasis at the time of enrolment. The dog was reported to be doing well at home by the owner. A tissue culture from the limb spare site diagnosed a multi-drug-resistant *Enterobacter cloacae* with susceptibility to amikacin. Radiographs of the affected limb were not performed, but there was clinical concern for tumour recurrence. The dog was hospitalized for wound management and amikacin administration. ZOL was administered on the day of hospital admission. Anorexia and lethargy were noted the day of hospitalization and persisted for its duration (8 days). The dog was discharged from the hospital at the clients' request and was subsequently euthanized for lack of improvement.

Case 8 was euthanized due to a grade 5 fever. At the time of enrolment, the dog was hyporexic and mildly lethargic. Thoracic radiographs noted a 16 cm pulmonary mass and several smaller pulmonary nodules. A fine needle aspirate of the mass was performed and consistent with sarcoma. ZOL was administered the same day as the fine needle aspirate. The dog developed grade 3 lethargy, grade 2 tachypnoea, and grade 1 anorexia one day after administration. The dog was represented to the authors' institution 2 days post-ZOL and was febrile and tachypnoeic. Thoracic radiographs documented unchanged metastatic disease and no cause for the patient's clinical deterioration. A CBC, serum chemistry panel, and ionized calcium noted a mild mature inflammatory leukogram, mild hypophosphatemia, and grade 3 hypocalcaemia. The dog was hospitalized and treated with fluid therapy, a steroid, calcium supplementation, and antibiotics. The dog's fever progressed (105.0°F), and the owner elected for euthanasia after 2 days of hospitalization with no improvement.

Case 10 was reported to have grade 2 conjunctivitis, grade 2 anorexia, grade 2 lethargy, grade 1 fever, grade 1 diarrhoea, and grade 1 vomiting. The dog was clinically normal at the time of trial enrolment. The owner noted ocular abnormalities 1 day post-ZOL administration. The dog was evaluated by a veterinarian 12 days later after it developed gastrointestinal and constitutional AEs. A fever and bilateral conjunctivitis were recorded. The dog was treated with ophthalmic steroids and a systemic NSAID. The dog's clinical signs resolved by the time of presentation to the authors' institution 14 days later. Further testing for causes of conjunctivitis was not pursued.

TABLE 2 Adverse events (AEs), response, and outcome of 11 dogs treated with single agent zoledronate

Case number	Adverse events	Response to ZOL	No. of ZOL doses	PFS (days)	Outcome	Stage III survival (days)	OST (days)
1	Grade 1 diarrhoea	SD	4	93	Euthanized	226	226
2	None	SD	5	28†	Lost to follow up (361 days)	364‡	567‡
3	None	PD	1	28	Euthanized	182	293
4	None	PD	1	28	Euthanized	237	448
5	None	N/A	1	18	Euthanized	46	115
6	Grade 3 anorexia, grade 2 lethargy	N/A	1	12	Euthanized	71	82
7	None	PD	1	28	Euthanized	80	512
8	Grade 5 fever, grade 3 hypocalcaemia, grade 3 lethargy, grade 2 tachypnoea, grade 1 anorexia, hypophosphatemia	N/A	1	4	Euthanized	47	194
9	None	PD	1	28	Euthanized	92	93
10	Grade 2 conjunctivitis, grade 2 anorexia, grade 2 lethargy, grade 1 fever, grade 1 diarrhoea, grade 1 vomiting	PD	1	27	Lost to follow up (78 days)	78‡	149‡
11	None	PD	1	27	Euthanized	85	160

Abbreviations: N/A, not applicable; OST, overall survival time; PD, progressive disease; PFS, progression-free survival; SD, stable disease; ZOL, zoledronate.
†Patient had SD but exited trial and was censored from PFS, and went on to receive ZOL/toceranib for 139 days.
‡Censored from survival analysis.

Renal values were available for review 1-month post-ZOL for 8 dogs. There was no evidence of acute kidney injury based on VCOG-CTCAE. The dog that received concurrent amikacin (Case 6) had a urinalysis performed daily during treatment. Rare granular casts were noted after 3 days of amikacin. Amikacin was discontinued. A urinalysis was repeated 3 days later, and no casts were observed. Osteonecrosis of the mandible or maxilla was not observed in any patient.

3.4 | Response assessment

Three dogs (Cases 5, 6, and 8) were removed from the study at the owners' request prior to the 1-month assessment. Two of these dogs did not return for follow-up. Case 8 had thoracic radiographs rechecked 1 day after ZOL administration and documented stable disease.

Eight of 11 dogs returned 4 weeks later for response assessment (range: 27–28 days). Two had stable disease and six had progressive disease per VCOG-RECIST. Five dogs with progressive disease had developed new pulmonary nodules. The remaining dog's single target lesion had increased in size by 480%.

Case 1 experienced stable disease with single agent ZOL for a duration of 93 days. This dog had cytologically or histologically confirmed cutaneous, pulmonary, and hepatic metastasis. The dog was previously treated with doxorubicin and carboplatin in the macroscopic disease setting, and progressive disease was noted 21 days after each drug based on thoracic radiographs and abdominal ultrasound. This dog received a total of four ZOL doses before disease progression was ultimately noted.

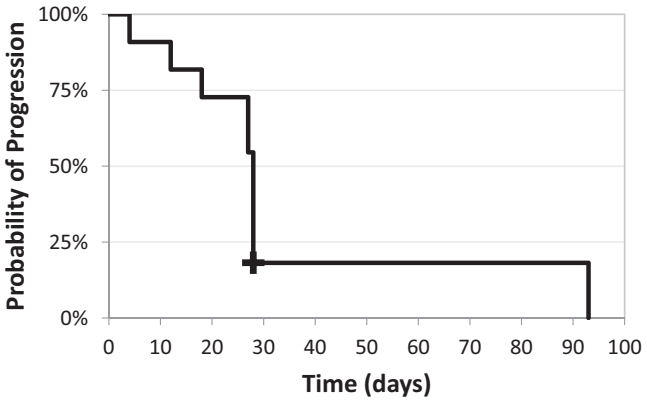

FIGURE 1 Kaplan–Meier curve showing progression-free survival for 11 dogs with osteosarcoma (OSA). Tick mark represents a censored dog. The median progression-free survival (PFS) was 28 days.

Case 2 experienced stable disease at the initial reassessment. This dog had two pulmonary nodules that were stable in size. The dog was removed from the trial at the owner's request with the intent to combine ZOL with toceranib phosphate. This patient received a total of five ZOL doses.

3.5 | Patient outcome

The median PFS for the 11 dogs was 28 days (range: 4–93 days; 95% CI: 18–28 days) (Figure 1). Three dogs received additional

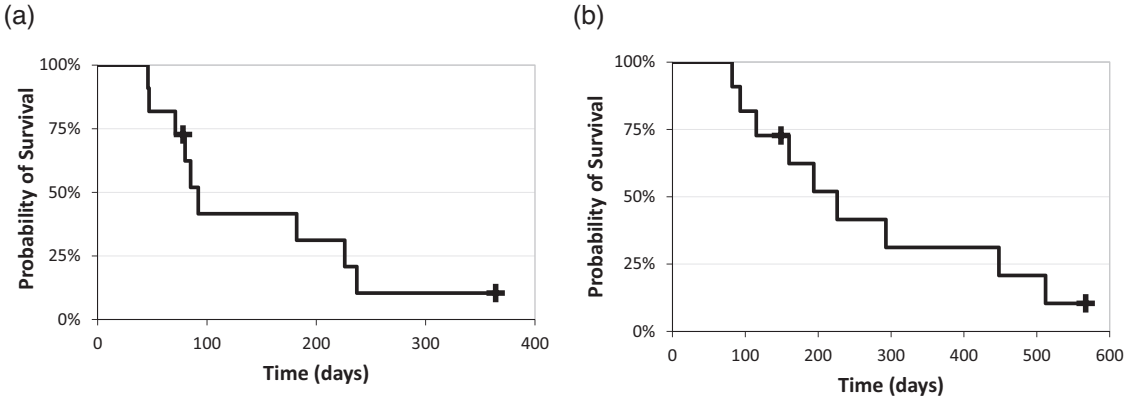

FIGURE 2 Kaplan–Meier curves showing stage III-specific survival time (a) and overall survival time (b) for 11 dogs with osteosarcoma (OSA). Tick marks represent censored dogs. The median survival time for stage III-specific disease was 92 days. The median overall survival time (OST) was 226 days.

chemotherapeutics after exiting the trial including sirolimus/piroxicam (n = 2), cyclophosphamide/sirolimus (1), toceranib/ZOL (1), and doxorubicin (1). Case 2 experienced stable disease with toceranib/ZOL (139 days) and doxorubicin (99 days). No other responses were noted.

Follow-up data and a date of death were available for 9 of 11 dogs. Seven dogs (Cases 1, 3, 4, 5, 7, 9, and 11) were euthanized due to progressive clinical signs attributed to OSA. Case 6 was euthanized 12 days post-ZOL for lethargy, anorexia, and a multi-drug-resistant infection. Case 8, the patient experiencing a fever, was euthanized 4 days post-treatment. Two dogs exited the study and were alive and lost to follow up 78 days (Case 10) and 361 days after enrolment (Case 2). Case 2 was no longer evaluated by the authors' institution 266 days post-ZOL, at which point the dog had progressive disease while receiving doxorubicin. The dog was lost to follow up but was noted to be alive as per referring veterinarian records 361 days post-ZOL. The median stage III-specific survival time was 92 days (range: 46–364 days; 95% CI: 71–226 days) (Figure 2). The median OST was 226 days (range: 82–567 days; 95% CI: 115–448 days).

Necropsy data were available for six dogs. Cases 7 and 8 had complete necropsy examinations performed at the authors' institution by a board-certified anatomic pathologist. Four dogs (Cases 1, 6, 9, 11) had limited necropsies performed by their primary care veterinarian. Incisional biopsies of gross abnormalities were submitted to the authors' institution. Histopathology confirmed a diagnosis of pulmonary metastatic OSA for all six dogs with available necropsy data. Additional sites of histologically confirmed metastasis at the time of death included the tracheobronchial lymph nodes (n = 1), liver (1), and skin (1). Sites of suspected metastasis included the bone (n = 2) and skin (1).

Case 8, the dog with a grade 5 fever, underwent a full necropsy examination. There was no evidence of hypertrophic osteopathy, central nervous system abnormalities, or intra-abdominal disease. The right middle lung lobe was almost entirely effaced by a solitary OSA with a central area of necrosis. The remaining portion of the right middle lung lobe contained severe chronic pyogranulomatous pneumonia and severe parenchymal necrosis. No infectious organisms were identified.

4 | DISCUSSION

Treatment with ZOL stabilized metastatic disease in two of eight dogs available for response assessment. No objective responses were noted. Case 1 experienced stable disease for a 3-month duration. This dog had rapidly failed carboplatin and doxorubicin in the gross disease setting, suggesting a clinically significant response to ZOL. Case 2 also achieved a biologic response, although stable disease was also noted with toceranib/ZOL (4 months) and doxorubicin (3 months), suggesting a more slowly progressive individual tumour behaviour.

AEs were reported in 36% of dogs (n = 4). Two dogs experienced AEs that have only been reported in humans, including fever, hypocalcaemia, hypophosphatemia, and conjunctivitis. One dog required outpatient medical management for fever and conjunctivitis. One dog was hospitalized and subsequently euthanized secondary to fever. The type and severity of AEs encountered in this study were unexpected based on the current literature in dogs.

Two dogs experienced AEs that were unlikely related to ZOL administration. Low grade constitutional signs may be attributed to ZOL or tumour burden. Gastrointestinal AEs such as diarrhoea (n = 2) and vomiting (1) may be associated with ZOL, dietary indiscretion, or administration of concomitant medications or supplements (carprofen, alpha lipoic acid). Diarrheal and vomiting have recently been described in a small proportion of ZOL-treated dogs (Conry et al., 2016). The AEs reported with Case 6 (lethargy, anorexia) may be related to the dog's surgical limb spare infection or ZOL administration. The wound likely played a role since the dog was febrile prior to enrolment.

Two dogs experienced AEs possibly related to ZOL. Case 10 developed a fever and conjunctivitis. Ocular inflammation is not a previously reported AE in dogs but occurs in 0.8% of humans (Kennedy et al., 2018). Uni- or bilateral conjunctivitis, episcleritis, and/or uveitis can occur at a median of 3 days post-treatment (range: 1–7 days). Most

cases are mild, and all have been steroid responsive (Kennedy et al., 2018). Alternative causes of conjunctivitis, such as trauma or exposure to inflammatory agents, could not be excluded. Hypertrophic osteopathy is considered unlikely as this patient had no evidence of lameness, limb swelling, or musculoskeletal pain on physical exam. Skeletal radiographs and/or necropsy data were not available.

Case 8 experienced a grade 5 fever. This patient also had sterile pneumonia detected upon necropsy. Aspiration of the pulmonary mass may have also precipitated a fever from secondary inflammation, though reported complications with this procedure are rare (DeBerry et al., 2002). The concurrent electrolyte derangements make ZOL toxicity more compelling. Acute-phase reactions occur in up to 43% of humans and are secondary to inflammatory cytokine production by $\gamma\delta$ T-cell lymphocytes (Fan, 2007; Izumi et al., 2018). Signs include fever, muscle and joint pain, nausea, vomiting, and oedema. Symptoms are typically self-limiting and resolve within 1–2 days. Acute-phase reactions appear independent of dose. Interestingly, they have been associated with an improved survival (Izumi et al., 2018). Acute-phase reactions have not been previously reported in ZOL-treated dogs.

Severe hypocalcaemia and moderate hypophosphatemia were also noted in Case 8. Hypocalcaemia could arise from ZOL toxicity or severe systemic inflammation. Hypophosphatemia could occur secondary to ZOL or respiratory acidosis from pneumonia. As this patient underwent a full necropsy, other causes are unlikely. Hypophosphatemia is encountered in 50% of humans and is the dose limiting toxicity (Goldsby et al., 2013). Electrolyte abnormalities are typically transient and reversible. These may be underreported in dogs as electrolytes are not routinely monitored. It is difficult to determine a timeline for monitoring as hypocalcaemia can occur days to months after ZOL administration in humans (Goldsby et al., 2013).

The OST encountered in the current study is 7.5 months. Historic median survival times are 4–6 months with local control and 10–12 months with the addition of chemotherapy (Bergman et al., 1996). This is likely influenced by enrolment of dogs with exclusively stage III disease, excluding the minority (<10%) of dogs with OSA that never develop metastasis. Two of 11 dogs did not receive chemotherapy, which likely impacted outcome. Additionally, five dogs (45%) had at least one of the following negative prognostic indicators: proximal humerus location, elevated alkaline phosphatase, and presence of metastasis at time of initial diagnosis. The median time to development of metastasis was 75 days in the present study, which is shorter than previous reports (183 days) (Boston et al., 2006). This may be due to the presence of negative prognostic indicators. As most dogs progressed despite ZOL, the median stage III survival time (92 days) was similar to previous studies (Boston et al., 2006).

The results of this study must be interpreted with caution due to the limited number of cases and risk of type II error. Inclusion criteria selected for cases with more biologically aggressive disease. Patients may have been understaged since only one had an abdominal ultrasound and no dog underwent computed tomography with or without positron emission tomography. Full necropsies were performed in only two of 11 patients, potentially failing to identify atypical metastases.

While ZOL appears well tolerated in healthy dogs, further research is needed in dogs with macroscopic cancer. Reported doses in healthy dogs range from 0.1 to 0.25 mg/kg every 3–4 weeks (Fan, 2007). The IC_{50} for ZOL has been established in two canine OSA cell lines, and it was variable similar to humans (Poirier et al., 2003). There are little published data regarding the pharmacokinetics of ZOL in dogs; this was established from a single dose of ZOL in healthy dogs at a dose higher than used in the present study (0.25 mg/kg) (Martín-Jiménez et al., 2007). To the authors' knowledge, the pharmacokinetics of ZOL in tumour-bearing dogs or at lower, clinically utilized doses (0.1 mg/kg) has not been published.

The present study initially utilized a maximum dose of 4.0 mg per dog before increasing to a maximum of 8.0 mg. One dog received a dose greater than 4.0 mg and was reported to have grade 2 conjunctivitis and several other AEs. A dosage greater than 0.1 mg/kg may be necessary as the anti-angiogenic effects of ZOL appear to be dose dependent (Koto et al., 2009). ZOL appears to have pleiotropic effects on OSA cell lines in that while higher doses inhibit cell growth, low doses may be pro-proliferative (Ouyang et al., 2018). Lower doses of ZOL (0.1 mg/kg) were found to downregulate CXCR4 expression and inhibit OSA cell migration in tumour-bearing dogs. This could lead to anti-tumour effects regardless of the impact on angiogenesis (Byrum et al., 2016).

Future research should involve establishment of pharmacokinetics in tumour-bearing dogs. Subsequently, a dose escalation in dogs with macroscopic OSA with monitoring of calcium and phosphorous could be pursued based on these results. Concurrent examination of CXCR4 and vascular endothelial growth factor plasma concentrations should be considered.

In conclusion, ZOL appears to have limited efficacy as a single agent for the treatment of canine stage III osteosarcoma and may be associated with unexpected toxicity. Further determination of a therapeutic dose and AEs are recommended before using either as a single agent or in combination therapies for stage III osteosarcoma in dogs.

AUTHOR CONTRIBUTIONS

Conceptualization, *data curation*, *formal analysis*, *funding acquisition*, *investigation*, *methodology*, *supervision*, *project administration*, *writing—original draft*, *and writing—review and editing* : Ashley A. Smith. Conceptualization, data curation, investigation, methodology, supervision, and writing - review and editing: Stephanie E.S. Lindley. *Conceptualization*, *data curation*, *formal analysis*, *investigation*, *supervision*, *and writing—review and editing*: Greg T. Almond. Data curation, investigation, and writing - review and editing: Noelle S. Bergman. Data curation, investigation, and writing - review and editing: Brad M. Matz. Conceptualization, data curation, funding acquisition, investigation, methodology, supervision, project administration, and writing - review and editing: Annette N. Smith.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting these findings are available from the corresponding author upon request.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The study design was approved by the institution's ethics committee and the animal care and use committee (#2017-3051).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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