

The Association of Endothelin-1 Signaling with Bone Alkaline Phosphatase Expression and Protumorigenic Activities in Canine Osteosarcoma

Z.L. Neumann, H.C. Pondenis, A. Masyr, M.L. Byrum, K.L. Wycislo, and T.M. Fan

Background: Canine osteosarcoma (OS) is an aggressive sarcoma characterized by pathologic skeletal resorption and pulmonary metastases. A number of negative prognostic factors, including bone alkaline phosphatase, have been identified in dogs with OS, but the underlying biologic factors responsible for such observations have not been thoroughly investigated. Endothelin-1-mediated signaling is active during bone repair, and is responsible for osteoblast migration, survival, proliferation, and bone alkaline phosphatase expression.

Hypothesis: The endothelin-1 signaling axis is active in canine OS cells, and this pathway is utilized by malignant osteoblasts for promoting cellular migration, survival, proliferation, and bone alkaline phosphatase activities.

Animals: 45 dogs with appendicular OS.

Methods: The expressions of endothelin-1 and endothelin A receptor were studied in OS cell lines and in samples from spontaneously occurring tumors. Activities mediated by endothelin-1 signaling were investigated by characterizing responses in 3 OS cell lines. In 45 dogs with OS, bone alkaline phosphatase concentrations were correlated with primary tumor osteoproliferativity.

Results: Canine OS cells express endothelin-1 and endothelin A receptor, and this signaling axis mediates OS migration, survival, proliferation, and bone alkaline phosphatase activities. In OS-bearing dogs, circulating bone alkaline phosphatase activities were positively correlated with primary tumor relative bone mineral densities.

Conclusions and Clinical Importance: Canine OS cells express endothelin-1 and functional endothelin A receptors, with the potential for a protumorigenic signaling loop. Increases in bone alkaline phosphatase activity are associated with osteoblastic OS lesions, and might be an epiphenomenon of active endothelin-1 signaling or excessive osteoproduction within the localized bone microenvironment.

Key words: Cell migration and survival; Osteoblastic response; Prognostic factor.

Bone alkaline phosphatase (bALP) is an enzyme anchored to the outer plasma membrane leaflet on both normal and malignant osteoblasts, and has been identified as a necessary factor for the initiation of skeletal mineralization.^{1,2} Given its essential role in bone homeostasis, serum bALP activity serves as a surrogate of osteoblastic bone formation and can be used as an adjuvant diagnostic test in cancer patients at risk for developing osteoblastic bone metastasis.^{3–5} In addition to its diagnostic role in skeletal metastases, increased serum bALP activity is a well-documented negative prognostic factor in dogs with appendicular osteosarcoma (OS) and is associated with shorter disease-free intervals and survival times.^{6–8} However, the exact reasons for it to serve as a negative prognostic

Abbreviations:

bALP	bone alkaline phosphatase
DEXA	dual energy x-ray absorptiometry
ET-1	endothelin-1
ET _A R	endothelin receptor type A
OS	osteosarcoma
rBMD	relative bone mineral density

factor in dogs diagnosed with OS remains incompletely defined. Elucidating the biologic mechanisms responsible for its association with a poor prognosis may help to identify novel therapies for the adjuvant treatment of OS in an effort to increase survival and quality of life in these patients.

In humans, and more recently in dogs, a correlation between serum bALP activity and tumor burden has been identified and mechanistically suggests that poor prognosis associated with increased serum bALP activity is simply a function of advanced disease stage, and consequently shorter metastasis-free survival and overall survival.^{9–11} However, this association between serum bALP activity and tumor burden is not always clinically apparent, and a subset of patients with substantial tumor burden does not uniformly have increases in serum bALP activity. Such observations suggest that other biologic mechanisms, in addition to tumor size, likely contribute to an increased serum bALP activity in dogs with OS.

Endothelin-1 (ET-1) is a potent peptide, originally discovered to be expressed by vascular endothelial cells, that plays a pivotal role in osteoblastic disease

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processes, mainly by the mediation of cellular differentiation and migration of bone cells.^{12,13} Endothelin-1 inhibits osteoclasts from resorbing bone, stimulates osteoblast survival and proliferation, and increases expression of osteopontin, osteocalcin, and bALP, resulting in new bone formation.^{14–16} These bone biologic effects are mediated by the endothelin A receptor (ET_AR) and are attenuated by antagonism of this receptor-mediator signaling pathway. As such, selective antagonists of ET_AR have been developed as an anti-cancer strategy to block the proliferative and survival effects induced by ET-1 on prostate cancer cells and associated reparative osteoblasts residing within the bone tumor microenvironment.^{17–19}

The involvement of ET_AR signaling in promoting malignant osteoblast migration, survival, and proliferation, as well as the regulation of bALP activity in OS of dogs has not been well characterized. As such, the purpose of this study was to investigate additional biologic mechanisms, specifically ET-1 mediated signaling, which might explain the prognostic relevance of serum bALP activity in appendicular OS-bearing dogs. The objectives of our study were (1) to demonstrate the expression and functionality of the endothelin axis in canine OS cells; (2) to evaluate the protumorigenic properties of ET-1-mediated signaling in vitro (3) to determine the effects of ET_AR antagonism on bALP expression and activity in vitro; and (4) to evaluate the relationship between serum bALP activity and primary tumor relative bone mineral density (rBMD) measured by means of dual energy x-ray absorptiometry (DEXA).

Materials and Methods

Cell Lines

Three canine OS cell lines including HMPOS (provided by Dr James Farese, University of Florida), D17 (purchased from American Tissue Culture Collection), and Abrams (provided by Dr Douglas Thamm, Colorado State University) were utilized in this study given their known cellular characteristics.^{20–22} The Jurkat (human T lymphocyte cells) and DU145 (human prostate carcinoma) cell lines were purchased from American Tissue Culture Collection and used as positive controls.^{19,23} Cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin (100 IU/mL), and 10% fetal bovine serum (FBS) in a humidified atmosphere supplemented with 5% CO₂. Cell cultures were maintained in subconfluent monolayers and passaged 2–3 times weekly as necessary.

Reagents and Antibodies

Human recombinant ET-1^a (E7764), anti-ET-1 antibody^a (mouse monoclonal; E166), and anti-ET_AR antibody^a (rabbit polyclonal; E3651) were purchased from Sigma-Aldrich. Atrasentan hydrochloride^b (ABT-627) was purchased from MedchemExpress. Phosphatidylinositol-specific phospholipase C^c (P-6466) was purchased from Life Technologies. Anti-phosphorylated c-jun antibody^d (rabbit monoclonal, 3270), anti-c-jun antibody^d (rabbit monoclonal, 9165), and anti-c-fos antibody^d (rabbit monoclonal; 2250) were purchased from Cell Signaling Technology. Horseradish peroxidase conjugated antimouse and antirabbit secondary

antibodies were purchased from GE Healthcare, UK. Anti-β actin antibody^c (mouse monoclonal; AC-15) and anti-ALP antibody^c (mouse monoclonal; ab108337) were purchased from Abcam.

Cell Protein Collection

Cells were grown to 80–100% confluence, the media was then removed and cells were washed twice with phosphate-buffered saline (PBS). Cells were trypsinized and centrifuged at 700 g at 4°C for 5 minutes. The resultant cell pellet was homogenized in 1 mL PBS and transferred to a 1.8 mL Eppendorf tube before centrifuging at 6,000 g at 4°C for 5 minutes. Cell pellets were homogenized with 100–150 μL of Mammalian Protein Extraction Reagent^c (M-PER) and mixed with fresh protease inhibitor cocktail solution^c for 15 minutes and then centrifuged at 10,000 rpm for 15 minutes at 4°C. The resultant supernatant was assessed for protein concentrations using a standard assay kit^c (Bicinchoninic Acid Protein Assay).

Western Blot Analysis

Endothelin-1 and ET_AR Expression. For each protein investigated, 50 μg samples were electrophoresed on 12% polyacrylamide gel and then transferred to a nitrocellulose membrane. Membranes were blocked with Tris-Buffered Saline with Tween 20 (TBST) with 5% milk for 1 hour at room temperature. Western blot analysis was performed using ET_AR and ET-1 antibodies at a concentration of 1 : 1,000 in TBST with 5% milk. The membrane then was washed three times with TBST and probed with the secondary antibody diluted 1 : 5,000 in TBST with 5% milk. Blots were developed using ChemiDoc XRS+ molecular imager system^f with Image Lab software^f. Band volume analysis was done using ImageJ software.^g Relative protein expressions were adjusted for β-actin or another reference protein used as a loading control. Results reported were derived from at least 2 independent experiments.

Expressions of c-fos, c-jun, and phospho-c-jun. The Abrams, D17, and HMPOS cell lines were grown in complete media to 85% confluence. Media was removed and the cells were serum starved for 24 hours. Cells then were exposed to experimental conditions including: (1) serum starved only or serum starved for 24 hours and then stimulated for 1 hour with (2) 10% FBS; (3) DMSO (vehicle); (4) 100 nM ET-1; (5) 40 μM ABT-627; or (6) 40 μM ABT-627 followed by 100 nM ET-1 for 1 hour (combo). After exposure to experimental conditions, cells were collected in 0% FBS DMEM to avoid changes in the phosphorylation of target proteins. Proteins were electrophoresed and transferred to membranes, and then incubated with either phosphorylated-c-jun antibody at 1 : 1,000 in TBST with 5% milk, c-jun antibody at 1 : 1,000 in TBST with 5% milk, or c-fos antibody at 1 : 1,000 in TBST with 5% milk. The membrane then was washed three times with TBST and probed with the secondary antibody diluted 1 : 5,000 in TBST with 5% milk and developed using a standard chemiluminescence detection kit.^h The enhanced chemiluminescence (ECL) blots were developed using ChemiDoc XRS+ molecular imager system^f with Image Lab software^f. Band volume analysis was done using ImageJ software^g. Results reported were derived from at least two independent experiments.

Immunohistochemistry

Ten canine appendicular OS tissue blocks were retrieved from the University of Illinois Veterinary Diagnostic Laboratory for immunohistochemical assessment. Slides were deparaffinized in xylene and rehydrated in ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for

15 minutes, and then slides were rinsed twice with wash buffer for 5 minutes. Slides were incubated with preheated 0.1% protease at 37°C for 20 minutes, and then rinsed in wash buffer for 2 minutes. Nonspecific staining was minimized with incubation for 10 minutes with Power Block,¹ and then blocking for 15 minutes with avidin and biotin block. Slides were incubated with antihuman primary antibodies against ET_AR (1 : 500) or ET-1 (1 : 2,000) for 1 hour at room temperature. Slides were incubated with a biotinylated secondary antibody for 20 minutes at room temperature, then washed in buffer before incubation for 20 minutes with a streptavidin-biotinylated horseradish peroxidase complex,³ and developed with 3,3'-diaminobenzidine (DAB) substrate for 5 minutes. Slides were counterstained with hematoxylin and evaluated by a single investigator (KLW). Negative controls for the samples were processed identically in the absence of the primary antibody.

Colony Forming Assay

The Abrams, D17, and HMPOS cell lines were seeded overnight in 6-well plates at a concentration of 200 cells per 3 mL of DMEM with 10% FBS. Upon adherence of individual cells, media was discarded and new media containing different experimental conditions were added including DMEM 5% FBS with vehicle (DMSO) or a range of ABT-627 concentrations (10–40 µM). Cells were allowed to grow undisturbed for 7 (Abrams) or 10 (HMPOS and D17) days. Subsequently, wells containing colonies were gently rinsed with chilled PBS, treated with 3 mL of solution containing 6.0% glutaraldehyde and 0.5% crystal violet for 30 minutes, and then rinsed with tap water. Each of the treatment conditions was performed in 3 separate experiments. The number of visible colonies per experimental condition was quantified 5 times by 3 different individuals blinded to treatment conditions.

Migration Scratch Assay

Qualitative analysis of cell migration was done using the “scratch assay” method.²⁴ The Abrams, HMPOS, and D17 cell lines were grown to 80% confluence in 6-well plates in DMEM supplemented with 10% FBS. Six experimental conditions for a duration of 24 hours were evaluated including complete media with vehicle (DMSO or H₂O), ET-1 (0.1–100 nM), or ABT-627 (10–40 µM). A standardized acellular gap was created through cell monolayers using a 200 µL pipette tip in the middle of each well. Images of the acellular gap were captured at time 0 (maximal gap) and 24 hours later for each experimental condition using an inverted microscope with a mounted digital camera. The average width of 5 representative acellular gaps per experiment conditions and cell line were used for quantitative comparisons, with percent residual gap $\left(\frac{\text{time 0 gap} - \text{time 24 gap}}{\text{time 0 gap}} \times 100\right)$ serving as the experimental readout. Each of the treatment conditions was performed in duplicate and the assay results are representative of 2 separate experiments. Data were analyzed with ImageJ software⁸.

Cytologic Detection of bALP Activity

The most robust bALP-expressing cell line, HMPOS, was plated into 0.5 mL chamber well slides at a concentration of 25,000 cells per well and allowed to adhere overnight in supplemented media. The media then was removed and the cells were exposed to serum-starved media for 48 hours with (1) media only, (2) DMSO (vehicle), (3) 40 µM ABT-627, or (4) 40 µM ABT-627 for 30 minutes followed by 100 nM ET-1 for 48 hours. The media then was removed and chambers were washed with PBS. The slides were allowed to dry and then were stained with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt

(NBT/BCIP), an ALP substrate, as previously reported.²⁵ Briefly, slides were incubated for precisely 8 minutes at room temperature with sufficient amounts of NBT/BCIP to coat the slide. Slides were rinsed with water, blotted dry, and examined microscopically. Positive staining was indicated by brown to black staining of the cell surface. Three representative images of each chamber were taken and quantitative analysis was performed on normalized pixel intensity measurements which accounted for two variables: (1) differences in cell densities and (2) staining intensities of positive cells among various treated and untreated conditions.

In Vitro bALP Protein Assessments

The most robust bALP-expressing cell line, HMPOS, was plated at a density of 5×10^5 cells/well in 6-well plates in 3 mL of 10% FBS DMEM. After cell adherence, media was removed and washed gently with PBS, then cells were exposed to serum-free media with varying conditions for 48 hours including (1) serum-starved DMEM only (control), (2) vehicle (DMSO), (3) 40 µM ABT-627, or (4) 40 µM ABT-627 for 30 minutes followed by 100 nM ET-1 (combo) for 48 hours. Media was removed, and cells were washed gently with 1 mL PBS, then scraped for collection. The cell pellet was resuspended in 0.5 mL PBS with 0.5 units of phosphatidylinositol-specific phospholipase C and incubated for 30 minutes at 37°C. After incubation, cells were pelleted and the supernatant was collected for quantitative analysis with a commercial test kit^k utilizing a murine monoclonal anti-bALP antibody. To normalize measured bALP activities, protein concentrations were determined in aliquots of each of the cell extracts using bicinchoninic acid (BCA) analysis. This cell layer protein was used as an index of cell number, which could have varied after exposure to the different conditions, and bALP was expressed as normalized activity units per gram of protein (U/L/g). Additionally, cleaved membranous bALP released into cell culture media was assessed by Western blot analysis using 20 µL aliquots of supernatant derived from the various conditions after PI-PLC incubation. A rabbit monoclonal antihuman/mouse bALP antibody was utilized at a concentration of 1 : 1,000 incubated overnight at 4°C followed by antirabbit secondary antibody at a concentration of 1 : 1,000 for 1 hour at room temperature.

Clinical Population, bALP, rBMD, and ET-1 Assessment

The study included a subset of dogs with OS that were evaluated at the University of Illinois Cancer Care Clinic between 2003 and 2012. All dogs had a diagnosis of appendicular OS confirmed by either histopathology or cytology with concurrent positive ALP staining. All dogs included in the study had orthogonal radiographs of the primary tumor, 3-view thoracic radiographs, DEXA scan, and serum collected at the time of diagnosis. Only dogs with untreated primary bone tumors and absence of macroscopic pulmonary metastases were used for associating serum bALP with primary tumor relative bone mineral density.

Serum bALP activity was evaluated with a commercial kit^k utilizing a murine monoclonal anti-bALP antibody, previously validated for use in dogs.²⁶ At initial presentation, DEXA scans were taken using a dedicated imaging unit^l of the primary tumor and an equivalent anatomic area of the unaffected contralateral limb. Bone mineral density (BMD) of the primary tumor was divided by BMD of the contralateral normal limb, and expressed as a ratio termed relative BMD (rBMD) as previously described.²⁷ For purposes of this study, a subset of 45 dogs was included for analysis and divided dichotomously into two categories (osteolytic or osteoblastic), which correlated with the lower (osteolytic: rBMD < 0.7) and upper (osteoblastic: rBMD > 1.3) rBMD

quartiles generated from a larger population of dogs ($n = 95$). To minimize primary tumor size as a confounding factor, a ratio of rBMD to the longest tumor measurement (RECIST) assessed by digital radiographs was used to identify any associations between normalized rBMD and serum bALP activity. Plasma endothelin concentrations were quantified using a commercially available kit[™] previously validated in dogs.²⁸

Statistical Analysis

The distribution of the continuous variable data was evaluated using the Shapiro–Wilk test, skewness, kurtosis, and q–q plots. Data were analyzed with either parametric or nonparametric methods depending upon the achievement of normality assumptions. For normally distributed data, 1-way ANOVA was used to evaluate for differences among groups with the use of Dunnett's posthoc test. Nonnormally distributed data were analyzed with the Kruskal–Wallis test and Dunn's comparison test. For the comparison of two datasets, student *t*-test or Mann–Whitney *U*-test was employed for normal or nonnormal distributed datasets, respectively. A Pearson product-moment correlation coefficient was used to assess the relationship between serum bALP activity and normalized rBMD/RECIST ratio. Statistical calculations were performed using commercial software programs,⁹ and $P < .05$ was considered statistically significant for all analyses.

Results

Protein Expression and Functionality of ET-1 and ET_AR in Canine OS

By Western blot analysis, ET-1 and ET_AR were expressed by the 3 immortalized canine OS cell lines utilized in this study (Fig 1A). In 10 spontaneously arising primary OS tumors, ET-1 demonstrated moderate to strong positive cytosolic expression in all (10/10) samples evaluated, but ET_AR expression was more variable

in both staining intensity and pattern. The majority of primary OS tumors were positive for ET_AR staining, either uniformly (4/10) or focally (5/10), but complete absence of ET_AR staining was identified in one of 10 samples examined (Fig S1).

To confirm the functionality of the endothelin intracellular signaling cascade in canine OS cell lines, components of the mitogen-activated protein kinase (MAPK) pathway, specifically c-fos and phospho-c-jun, were evaluated by Western blot analysis. In serum-starved HMPOS cells, exposure to either 10% FBS or exogenous ET-1 (100 nM) for 1 hour produced robust increases in c-fos and phospho-c-jun expression (Fig 1B–D). To demonstrate that c-fos and phospho-c-jun upregulation was mediated specifically by ET_AR stimulation, HMPOS cells were preincubated with a selective ET_AR antagonist (40 μM ABT-627) for 30 minutes before exogenous ET-1 (100 nM) incubation. Exposure to ABT-627 substantively inhibited protein expression of c-fos and phospho-c-jun, and persistent blockade of ET_AR signaling was achieved by ABT-627 in HMPOS cells continuously co-incubated with exogenous ET-1. Modulation of c-fos and phospho-c-jun expression also was identified in the D17 and Abrams cell lines, but some divergence in cell signaling responses was evident (Fig S2A,B). Despite comparable ET_AR expression by all 3 OS cell lines based on Western blot analysis (Fig 1A), response to ET_AR stimulation by either FBS or exogenous ET-1 was variable based upon changes in normalized c-fos protein expression (Fig S2C), which suggests that the molecular consequences of ET_AR stimulation or inhibition could produce heterogeneous biologic responses in OS cells of different origins.

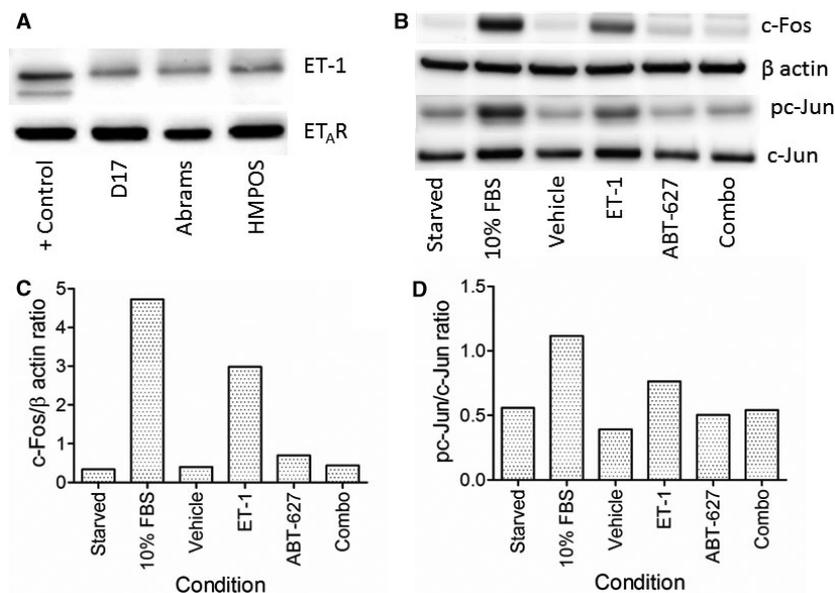


Fig 1. (A) Endothelin-1 (ET-1) and ET_AR expressed in canine OS cell lines by Western blot analysis. Positive control DU145 and Jurkat for ET-1 and ETAR, respectively. (B–D) ET_AR signaling pathway is functional in canine OS cells with upregulation of c-fos and phospho-c-jun following either 10% FBS or ET-1 (100 nM) stimulation. Similarly, blockade of ET_AR is achievable with ABT-627, a selective ET_AR antagonist.

ET_AR Antagonism Inhibits OS Cell Survival and Proliferation

To determine the contribution of ET_AR-mediated signaling in malignant osteoblast survival and proliferation, colony-forming assays were performed utilizing a range of ABT-627 concentrations (0–40 μM). Continuous exposure to ABT-627 for 7–10 days during colony formation inhibited cellular survival and proliferation in a dose-dependent manner across the 3 canine OS cell lines tested (Fig 2A,B; Table 1). For the D17 cell line (Fig 2A), exposure to ABT-627 at concentrations ≥30 μM decreased the number of colonies compared to vehicle (DMSO). Similarly, colony formation by the Abrams cell line was attenuated by ABT-627 at all con-

centrations ≥20 μM. In the HMPOS cell line, colony formation was impeded by ABT-627 at concentrations of 20 μM and 40 μM.

Endothelin-1 Signaling Mediates Canine OS Cell Migration

In a dose-dependent manner, the addition of exogenous ET-1 for 24 hours accelerated the closure of an acellular gap created in confluent cultures of all 3 canine OS cell lines (Fig 3A,B; Table 2). The migration of D17 (Fig 3A) and Abrams cell lines was uniformly promoted by ET-1 concentrations ranging between 1.0 and 100 nM. The promigratory effects of ET-1 were less pronounced in the HMPOS cell line, with only the highest ET-1 concentration (100 nM) accelerating acellular gap closure after 24 hours of exposure. Converse to the effects exerted by exogenous ET-1, blockade of ET_AR signaling for 24 hours by ABT-627 inhibited malignant OS cell migration in a dose-dependent manner (Fig 4A, B; Table 3). Based upon residual gap distances after 24 hours, the participation of ET_AR signaling in the promotion of cell migration was most pronounced in the Abrams cell line, with concentrations of ABT-627 ≥ 30 μM completely abrogating malignant OS cell migration (Fig 4A). The effect of ABT-627 to inhibit the migration of D17 and HMPOS cells also was achieved at concentrations ranging from 20–40 μM, however, the degree of inhibition (approximately 60%) was less pronounced compared to that achieved in the Abrams cell line (approximately 100%).

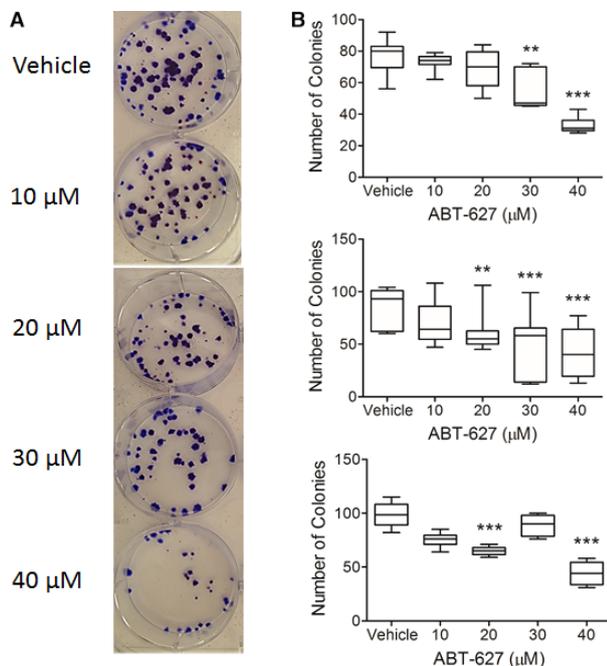


Fig 2. Visual reduction (A) in the number of colonies formed over 10 days in the D17 cell line following ET_AR signaling blockade by ABT-627. (B) General dose-dependent reduction in colony formation as a consequence of ET_AR signaling blockade by ABT-627 in three canine OS cells lines (top, D17; middle, Abrams; bottom, HMPOS). Data expressed as medians and significance defined as ***P* < .01 and ****P* < .001.

Table 1. ET_AR antagonism with ABT-627 reduces colony formation after continuous incubation for 7–10 days.

Condition	Abrams	D17	HMPOS
Vehicle	93 (60–104)	80 (56–92)	98.5 (82–115)
10 μM	64 (47–108)	74 (62–79)	76 (64–85)
20 μM	55 (45–106)**	70 (50–84)	65 (59–71)***
30 μM	58 (12–99)***	47 (45–72)**	90 (76–100)
40 μM	40 (13–77)***	31 (28–43)***	44 (31–58)***

Data expressed as median (range) with significance defined as ***P* < .01; ****P* < .001.

Endothelin A Receptor Antagonism Decreases bALP Expression and Activity

Modulation of bALP expression and activity as a consequence of ET_AR signal blockade was studied in the HMPOS cell line, given its robust expression of bALP (Fig S3A). Three complementary methods to study bALP modulation were employed including cytochemical analysis, enzymatic activity, and protein expression. Exposure of HMPOS cells to ABT-627 (40 μM) alone or pretreated with ABT-627 (40 μM) then followed by ET-1 (100 nM) stimulation resulted in decreased bALP activity as qualitatively appreciated by visual inspection of cytochemical staining intensity. ImageJ software was used to objectively quantify differences in staining intensity among treatment groups by expressing staining data as normalized pixel intensity scores, which account for cell density and positive cell staining intensity (Fig 5A,B). Normalized pixel intensity scores for ABT-627 alone or followed by ET-1 stimulation were 1201.4 ± 402.8 pixel intensity/cell or 1295.7 ± 294.1 pixel intensity/cell, respectively. In comparison, HMPOS cells exposed to control or vehicle conditions demonstrated significantly higher bALP-normalized pixel intensity scores of 4390.9 ± 808.3 pixel intensity/cell or 4460.5 ± 430.4 pixel intensity/cell (*P* < .05). Analogous to the cytochemical methodology, bALP activity quantified by ELISA and normalized for protein concentration yielded similar findings (Fig 5C).

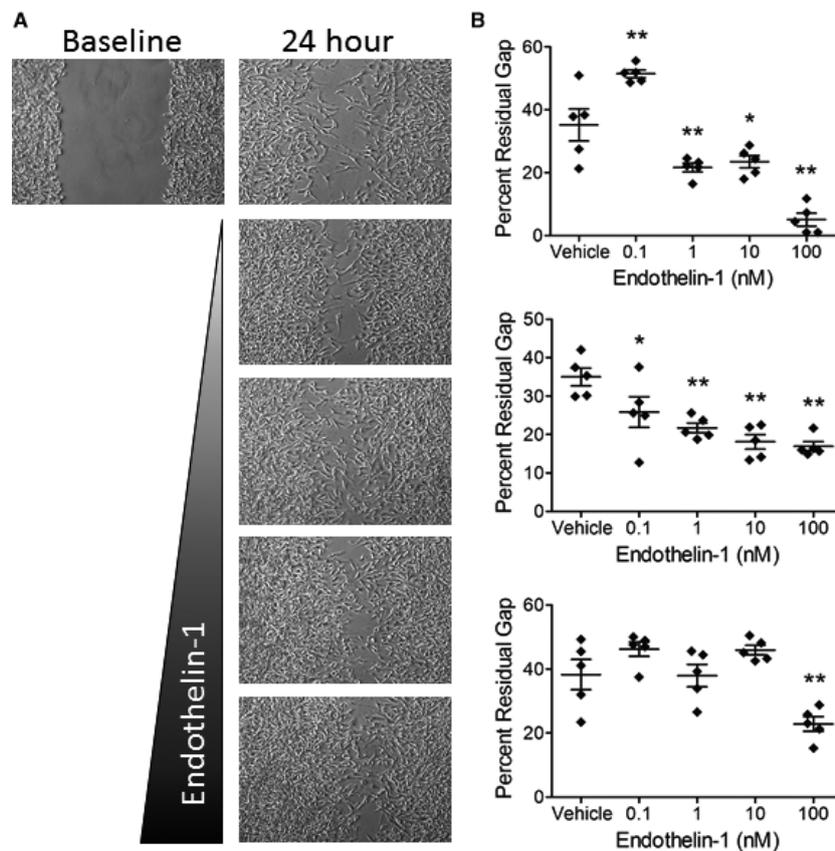


Fig 3. Visual enhancement (A) in cell migration with consequent reduction in residual gap after 24 hours of scratch induction in the D17 cell line following exogenous ET-1 supplementation. (B) General dose-dependent augmentation in cell migration induced by a wide dose range of exogenous ET-1 in 3 canine OS cell lines (top, D17; middle, Abrams; bottom, HMPOS). Data expressed as mean \pm SEM and significance defined as * P < .05 and ** P < .01.

Table 2. Endothelin-1 promotes cell migration and reduces the percent residual gap remaining after 24 hours of cell culture.

Condition	Abrams	D17	HMPOS
Vehicle	35.0 \pm 2.3	35.2 \pm 5.1	38.3 \pm 4.7
0.1 nM	25.9 \pm 4.0*	51.4 \pm 1.2**	46.2 \pm 2.2
1.0 nM	21.7 \pm 1.3**	21.6 \pm 1.4**	37.9 \pm 3.5
10.0 nM	18.1 \pm 1.9**	23.5 \pm 2.0*	45.9 \pm 1.5
100.0 nM	16.9 \pm 1.2**	5.1 \pm 2.0**	22.8 \pm 2.3**

Data expressed as mean \pm SEM with significance defined as * P < .05; ** P < .01.

Again, HMPOS cells exposed to ABT-627 alone or in conjunction with ET-1 demonstrated a marked reduction in normalized bALP activity at 386.4 \pm 46.4 U/L/g or 353.1 \pm 35.5 U/L/g, respectively, when compared to cells exposed to control or vehicle conditions (4303.7 \pm 300.2 U/L/g or 3967.6 \pm 172.1 U/L/g, respectively; P < .01). Lastly, cleavable membranous bALP was dramatically decreased in HMPOS cells after exposure to ABT-627 alone or in conjunction with ET-1 when compared to control- or vehicle-exposed conditions (Fig S3B,C).

Serum bALP Activity Correlates with Primary Tumor Bone Mineral Density

Primary tumor skeletal characteristics of 95 dogs diagnosed with appendicular OS were analyzed by digital radiography (Fig 6A) and DEXA scans. Concurrently, serum bALP activity was measured before to any interventional treatment. Primary tumor relative bone mineral density (rBMD) quantified by DEXA scans was used to arbitrarily categorize skeletal lesions as osteolytic (n = 25), mixed (n = 50), or osteoblastic (n = 20) by segregation of data into quartiles (Fig 6B). When evaluating primary tumors categorized as osteoblastic and osteolytic, circulating serum bALP activities were increased in dogs with osteoblastic primary tumor phenotype (median, 26.1 U/L; range, 11.2–86.3 U/L) as compared to dogs with osteolytic lesions (median, 15.7 U/L; range, 4.8–64.3 U/L; P = .005; Fig 6C). Circulating bALP activities associated with a mixed primary tumor phenotype (median, 21.5 U/L; range, 4.7–93.2 U/L) were not different than bALP activities of dogs with primary tumors categorized as either osteolytic or osteoblastic (Fig S4). Given the reported contribution of the primary tumor size to circulating bALP activity,¹¹ primary tumor rBMDs were normalized for tumor length (RECIST) and then

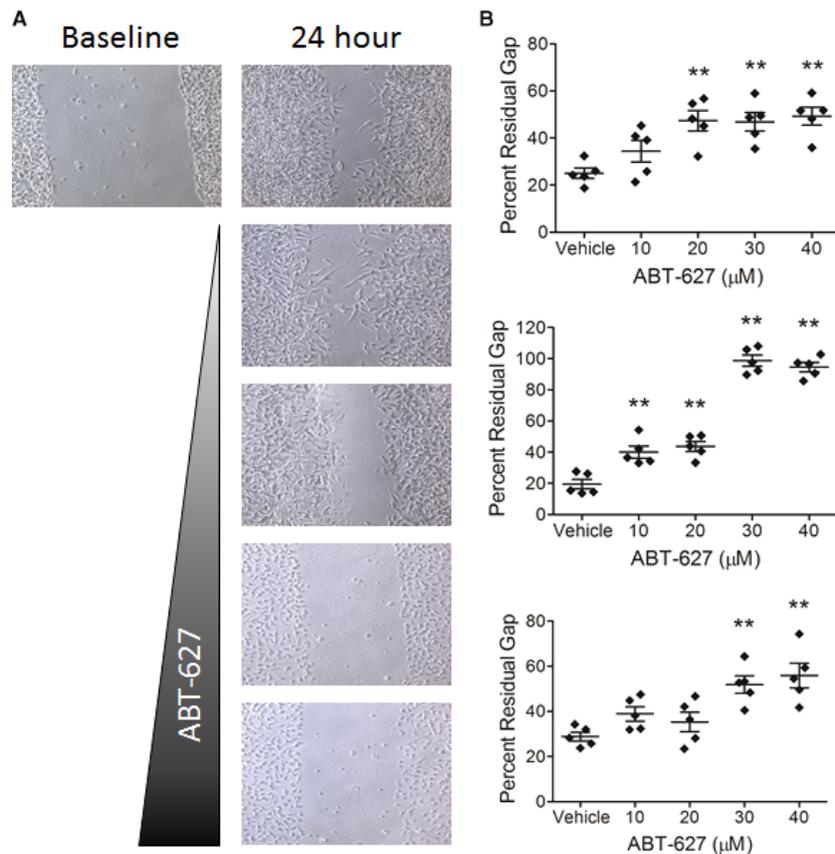


Fig 4. Visual inhibition (A) in cell migration following exposure to ABT-627, a selective antagonist of ET_A R, in the Abrams OS cell line. (B) Consistent dose-dependent inhibition of cell migration with ABT-627 in 3 canine OS cell lines (top, D17; middle, Abrams; bottom, HMPOS). Data expressed as mean \pm SEM and significance defined as $**P < .01$.

Table 3. ET_A R antagonism with ABT-627 inhibits cell migration and increases percent residual gap remaining after 24 hours of cell culture.

Condition	Abrams	D17	HMPOS
Vehicle	19.5 \pm 3.1	25.0 \pm 2.2	28.8 \pm 1.9
10 μ M	40.0 \pm 3.9**	34.5 \pm 4.6	38.9 \pm 3.2
20 μ M	43.7 \pm 3.2**	47.4 \pm 4.3**	35.3 \pm 4.3
30 μ M	98.7 \pm 3.6**	46.9 \pm 3.9**	51.9 \pm 3.9**
40 μ M	94.6 \pm 3.0**	49.3 \pm 3.8**	55.9 \pm 5.4**

Data expressed as mean \pm SEM with significance defined as $**P < .01$.

compared with circulating bALP activities (Fig 6D). A positive correlation between normalized rBMD and bALP activity was identified ($r = 0.38$; $P = .009$). No difference in circulating ET-1 concentrations was identified between dogs with osteoblastic (1.26 ± 0.1 fmol/mL) versus osteolytic (1.31 ± 0.1 fmol/mL) primary bone tumors ($P = .8$).

Discussion

With regard to malignant skeletal pathology, ET-1 serves as a key driver for the development and

progression of skeletal metastases, particularly for prostate carcinoma in humans.^{19,29} Within the bone microenvironment, carcinoma cells actively release ET-1 which promotes the successful development of osteoblastic metastases through the induction of reparative osteoblast proliferation and concurrent downregulation of osteoclast activities.^{18,19,29,30} The role of ET-1 in OS biology is less well characterized, but nonetheless indicates the capacity of ET_A R signaling to support several in vitro protumorigenic responses including invasion and chemoresistance.³¹⁻³⁴ Extending upon these in vitro observations, a limited number of in vivo studies have evaluated the participation of ET_A R signaling in the metastatic biology of OS.^{35,36} Collectively, ET-1 and ET_A R appear to be involved in critical cellular events that favor OS progression and metastases.

Our findings complement and extend existing knowledge regarding ET_A R signaling in OS, and in particular the relevance of ET-1 in the biology of canine OS.³⁷⁻³⁹ As indicated by the protein expression studies, canine OS cell lines and spontaneous tumor samples co-express both receptor and cognate ligand, findings that substantiate the potential existence of a paracrine or autocrine signaling pathway that could be subverted by malignant osteoblasts. The biologic consequences of ET-1 in canine OS cells specifically was evaluated in our study with emphasis on the MAPK pathway after stimulation

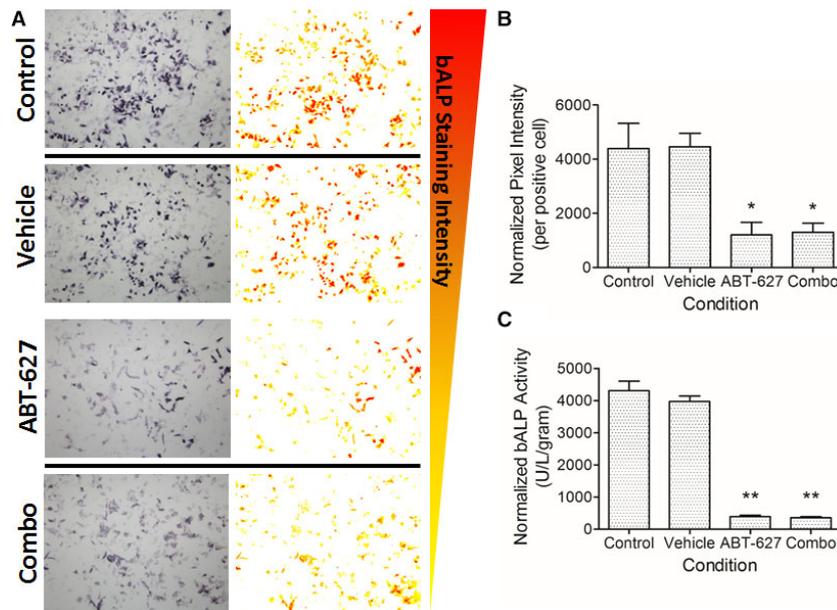


Fig 5. Cytochemical staining intensity representative of alkaline phosphatase activities (A) in the HMPOS cell line following exposure to various conditions including ABT-627. Marked reduction in cytochemical staining can be observed visually by inverted light microscopy (A; left panel) and digitally rendered pixel intensities (A; right panel), and (B) in the HMPOS cell line following exposure to ABT-627 alone or in combination with ET-1. Confirmation that ABT-627 reduces bone alkaline phosphatase activities in the HMPOS cell line by (C) enzyme-linked immunoassay. Significance defined as * $P < 0.05$ and ** $P < 0.01$.

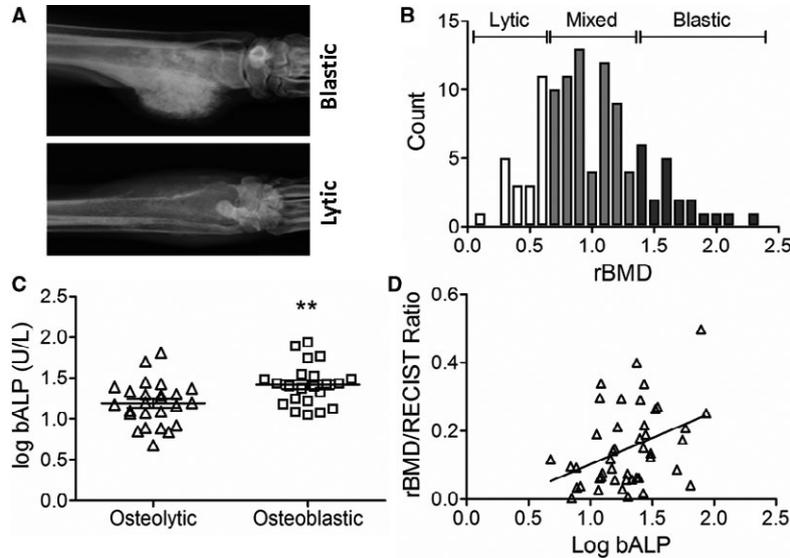


Fig 6. (A) Representative examples of primary OS bone lesions with either osteoblastic or osteolytic radiographic appearances. (B) Classification of 95 dogs with spontaneous appendicular OS with dual energy x-ray absorptiometry scans into quartiles representing osteolytic ($n = 25$), mixed ($n = 50$), and osteoblastic ($n = 20$) primary tumor relative bone mineral densities. (C) Dogs with osteoblastic primary tumor phenotypes have increased serum bone alkaline phosphatase in comparison with dogs possessing osteolytic bone tumors. (D) Positive association between serum bone alkaline phosphatase and normalized relative bone mineral densities adjusted for primary tumor size (RECIST). Significance defined as ** $P < 0.01$.

or inhibition of ET_A R signaling. Upon stimulation with ET-1, canine OS cells (specifically HMPOS and D17 cell lines) had the capacity to rapidly upregulate the expression of c-fos and phospho-c-jun, which can heterodimerize and form activator protein-1 (AP-1), a

transcription factor responsible for multiple cellular responses including differentiation, proliferation, migration, and survival.^{40,41} The inhibition of ET_A R signaling in canine OS cells was achievable with ABT-627, which dramatically blocked the expression of c-fos and

phospho-c-jun after ET-1 exposure in the majority of cell lines investigated. These findings support manipulation of ET_AR signaling as a potential therapeutic intervention in canine OS.

Recognition of ET_AR blockade as a potential therapeutic strategy for canine OS was supported by results derived from both colony forming and migration assays. Canine OS cells exposed to ABT-627 demonstrated a dose-dependent decrease in the number of colonies formed, indicative of altered cell survival and proliferative capacities as well as impeded cell migration. Although differences were observed among cell lines with regard to their sensitivity to ET-1 stimulation or to ABT-627-mediated inhibition, a consistent and directional effect elicited by ET_AR signaling blockade was conserved across all cell lines tested. However, the observed biologic effects exerted by ABT-627 might not be solely attributed to ETAR inhibition, because off-target effects of ABT-627 likely contributed to some of the cellular behavioral changes identified in our study. Nonetheless, the *in vitro* findings documented in our study are analogous with the findings derived from murine preclinical models of prostate skeletal metastases, in which ET_AR antagonism mitigated reparative osteoblast proliferation and survival induced by metastatic carcinoma cells within the bone microenvironment.^{42,43} Collectively, these *in vitro* and *in vivo* observations lend credence to the possibility that ET_AR blockade might have the potential to slow the growth and progression OS lesions arising within local and metastatic niches.

In addition to cell survival, proliferation, and migration, ET_AR blockade attenuated the expression and activity of membranous bALP. Different proteins are expressed along a continuum in association with osteoblast differentiation, with bALP serving as an early marker of pre-osteoblast maturation, which enzymatically contributes to extracellular matrix maturation.^{44,45} Based upon the *in vitro* findings of our study, canine OS cells responsive to ET-1-mediated signaling would have the potential for increased survival and proliferative capacity, cell mobility, and bALP activity. Given this triad of cellular characteristics, the negative prognosis associated with increased bALP activity in dogs with OS could simply be an epiphenomenon of active ET_AR signaling, in which OS cells under the influence of ET-1 would be endowed with “driver” protumorigenic properties and “passenger” bALP expression. The supposition of bALP serving in a “passenger” capacity is supported by recent studies in which the OS cell lines or primary tumor samples derived from dogs with either normal or increased bALP activity failed to show differences in biologic behaviors and gene expression profiles.^{46,47}

Given the role of ET-1 in osteoblast survival and proliferation, as well as the participation of bALP in extracellular matrix maturation, circulating bALP activity in dogs with OS could simply be a surrogate for the prevailing bone characteristics within the localized tumor microenvironment. Data derived from our study identified a wide range of rBMD in dogs with OS,

which could be phenotypically classified (osteolytic, mixed, osteoblastic) using an adapted methodology employed for the categorization of skeletal metastases in people.^{48,49} Dogs with osteoblastic tumor microenvironmental responses had higher bALP activity in comparison to dogs with predominant focal osteolytic characteristics, and a positive correlation between the degree of local tumor microenvironment osteoproduction and circulating bALP activity was identified. These findings suggest that in addition to primary OS size, which has been reported to correlate with serum bALP activity,¹¹ another host/tumor factor that might contribute to serum bALP activity is the net osteoproduction occurring within the localized tumor microenvironment.

Although our study provides new information pertaining to canine OS biology, several limitations should be acknowledged. First, the number of cell lines utilized in our investigation was limited, and therefore strong conclusions regarding the biologic consequences documented in this investigation associated with ET_AR blockade might not be conserved across all OS histologies. Given the heterogeneous nature of OS, responses to ET_AR blockade could be variable. This supposition would be supported by our findings because 60% of the spontaneously arising OS samples demonstrated either no (1/10) or variable expression (5/10) of ET_AR. Second, the concentrations of ABT-627 utilized *in vitro* to demonstrate the biologic consequences of inhibiting ET-1-mediated signaling were relatively high (10–40 μM) and used for prolonged exposure durations (1–24 hours), which are not likely achievable *in vivo* based upon existing pharmacokinetic data derived from human beings.^{50,51} Additionally, although ABT-627 is a potent and selective ETAR inhibitor, the micromolar concentrations evaluated in our study might have induced off-target effects, which could have contributed to the altered cellular behaviors documented. As such, the biologic consequences associated with ET_AR blockade observed *in vitro* might not be replicated in dogs with spontaneous OS receiving treatment with ET_AR inhibiting strategies. Third, our study did not provide any definitive evidence that increased serum bALP activity could be an epiphenomenon of ET-1-mediated signaling. Nonetheless, this speculation remains intriguing and warrants additional future studies. Last, although there proved to be a positive association between serum bALP activity and the osteoproduction tumor microenvironment, it was not possible to associate the contributions of either normal reparative osteoblasts or malignant OS cells with the observed increases in bALP activity. Despite being unable to define the exact cellular origin responsible for bALP liberation, our study identified a novel host/tumor factor that contributes to circulating bALP activity in dogs with OS.

In conclusion, our results showed that canine OS cells express both ET-1 and ET_AR, which are involved in protumorigenic processes including cell survival, proliferation, and migration. These processes were mechanistically associated with downstream changes in the MAPK pathway after ET-1 stimulation or blockade.

Expression of bALP is partially regulated by ET_AR-mediated signaling, and a plausible link between bALP-associated negative prognosis and the protumorigenic influences of ET-1 could be proposed. Finally, a new host/tumor factor, specifically osteoproduktivty associated with the local tumor microenvironment, contributes to circulating bALP activity in dogs with spontaneously arising OS. Collectively, these findings provide new information and in part elucidate the biologic mechanisms involved in bALP liberation in dogs with OS.

Footnotes

- ^a Sigma Aldrich, Saint Louis, MO
^b MedchemExpress, Monmouth Junction, NJ
^c Life Technologies, Grand Island, NY
^d Cell Signaling Technology, Danvers, MA
^e Abcam, Cambridge, MA
^f Bio-Rad, Hercules, CA
^g National Institutes of Health, Bethesda, MD
^h GE Healthcare Life Sciences, Pittsburgh, PA
ⁱ Thermo Scientific, Schaumburg, IL
^j ABC Vector Laboratories, Burlingame, CA
^k MicroVue BAP EIA kit, Quidel, San Diego, CA
^l QDR-4500W, Hologic, Bedford, MA
^m Endothelin ELISA (1–21), ALPCO, Salem, NH
ⁿ GraphPad InStat, Version 3.10, La Jolla, CA
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Conflict of Interest Declaration: Dr Fan is an associate editor for the Journal of Veterinary Internal Medicine.

Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Fig S1. Microscopic evaluation of canine OS primary tumors with hematoxylin and eosin (A-C), and immunohistochemistry for ET_AR (D, E) and endothelin-1 (F) expressions.

Fig S2. ET_AR signaling pathway is functional, but differ in response magnitude in (A) D17 and (B) Abrams canine OS cells. Variable upregulation of c-fos protein expression (C) in all 3 OS cell lines following either 10% FBS or ET-1 (100 nM) stimulation.

Fig S3. (A) Relative bone alkaline phosphatase expressions in 3 canine OS cell lines, with HMPOS demonstrating the most robust protein expression. (B-C) In the HMPOS cell line, membranous cleavage of bone alkaline phosphatase is reduced following exposure to ABT-627 alone or in combination with endothelin-1.

Fig S4. Comparison of serum bALP among 3 different osteophenotypes of canine appendicular osteosarcoma. Significance defined as **P* < 0.05.