

Pro-tumorigenic Effects of Transforming Growth Factor Beta 1 in Canine Osteosarcoma

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Background: Transforming growth factor beta 1 (TGF β 1) is a pleiotropic cytokine that contributes to reparative skeletal remodeling by inducing osteoblast proliferation, migration, and angiogenesis. Organic bone matrix is the largest bodily reservoir for latent TGF β 1, and active osteoblasts express cognate receptors for TGF β 1 (TGF β RI and TGF β RII). During malignant osteolysis, TGF β 1 is liberated from eroded bone matrix and promotes local progression of osteotropic solid tumors by its mitogenic and pro-survival activities.

Hypothesis: Canine osteosarcoma (OS) cells will possess TGF β 1 signaling machinery. Blockade of TGF β 1 signaling will attenuate pro-tumorigenic activities in OS cells. Naturally occurring primary OS samples will express cognate TGF β 1 receptors; and in dogs with OS, focal malignant osteolysis will contribute to circulating TGF β 1 concentrations.

Animals: Thirty-three dogs with appendicular OS.

Methods: Expression of TGF β 1 and its cognate receptors, as well as the biologic effects of TGF β 1 blockade, was characterized in OS cells. Ten spontaneous OS samples were characterized for TGF β RI/II expressions by immunohistochemistry. In 33 dogs with OS, plasma TGF β 1 concentrations were quantified and correlated with bone resorption.

Results: Canine OS cells secrete TGF β 1, express cognate receptors, and TGF β 1 signaling blockade decreases proliferation, migration, and vascular endothelial growth factor secretion. Naturally occurring OS samples abundantly and uniformly express TGF β RI/II, and in OS-bearing dogs, circulating TGF β 1 concentrations correlate with urine N-telopeptide excretion.

Conclusions and Clinical Importance: Canine OS cells possess TGF β 1 signaling machinery, potentially allowing for the establishment of an autocrine and paracrine pro-tumorigenic signaling loop. As such, TGF β 1 inhibitors might impede localized OS progression in dogs.

Key words: Bone resorption; Malignant osteolysis; Pleiotropic cytokine; Tumorigenesis.

Transforming growth factor beta (TGF β) is a pleiotropic cytokine expressed in a variety of tissues. Three distinct TGF β isoforms (TGF β 1, β 2, or β 3) have been identified, and exert their activities through receptor serine-threonine kinases.^{1,2} Secreted as latent homodimer precursors, TGF β isoforms require catalysis by proteases, such as plasmin and matrix metalloproteinases,^{3,4} to be fully activated.^{1,5} Of the 3 isoforms, TGF β 1 exists in highest concentrations in circulation, with platelets and bone matrix serving as the 2 largest biologic sources of TGF β 1 within the body.^{6,7}

Intracellular signaling mediated by TGF β requires the heterodimerization of its 2 cognate receptor serine-threonine kinases, TGF β RI and TGF β RII.^{1,2,8} Biologic effects exerted by TGF β are mediated by 2 divergent

Abbreviations:

NTx	N-telopeptide
OS	osteosarcoma
TGF β 1	transforming growth factor beta 1

signaling pathways, Smad-dependent (canonical) and Smad-independent (noncanonical). The canonical signaling pathway is well characterized and necessitates the phosphorylation and subsequent colocalization of Smad partnering complexes to the nucleus, which then serve as transcription factors for TGF β -mediated target genes.^{1,2,8–10} The noncanonical pathway is less well defined, but this signaling pathway contributes to cell proliferation, motility, and survival by activation of the mitogen-activated protein kinase, Rho GTPase, and PI3K/AKT pathways, respectively.^{9,10}

Physiologic activities of TGF β include regulation of skeletal health and remodeling. Normally, osteoblasts actively secrete TGF β 1, which becomes incorporated into the hydroxyapatite matrix of bone, and is subsequently released into the local microenvironment and systemic circulation after osteoclast-mediated bone resorption.^{11–13} During reparative or pathologic bone remodeling, TGF β 1 concentrations transiently increase as a result of excessive osteoclast activity and consequent release of bone-derived TGF β 1.^{14,15} Within regions of reparative skeletal healing, released TGF β 1 can promote osteoblast precursor recruitment and motility, osteoblast proliferation and differentiation, as well as osteoblast survival.^{16–19} Upon completion of bone healing and attenuated osteoclastic activities, the

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release and pro-osteoblastic activities of TGF β 1 become self-limiting.

Canine osteosarcoma (OS) is the most common primary bone tumor in dogs.²⁰ Clinically, affected dogs initially present for severe bone pain as a consequence of aggressive focal malignant osteolysis.^{21–23} If localized pain is adequately controlled with interventional therapies, the majority of affected dogs eventually will develop distant metastatic disease.^{24–27} Osteosarcoma progression within bone or distant organs can be rationally attributed to the unchecked growth of malignant osteoblasts, and although conventional therapies, including limb amputation and systemic chemotherapy, improve overall survival times of affected dogs, there remains a clinical need for biologically driven discovery of novel therapeutic targets.

In the context of canine OS, inhibiting cellular pathways involved in osteoblast proliferation, migration, and survival might lead to therapeutic improvements. Given the pivotal role of TGF β 1 in reparative bone biology, in conjunction with TGF β 1's participation in diverse tumorigenic processes including growth, migration, and angiogenesis,^{28–30} we sought to investigate the potential role of this signaling axis in canine OS. The specific aims of this study were (1) to characterize TGF β -associated ligand/receptor expressions in OS cell lines, spontaneous tumor samples, and OS-bearing dogs; (2) to study the *in vitro* effects of TGF β signaling blockade on malignant osteoblast proliferation, migration, survival, and vascular endothelial growth factor (VEGF) secretion; and (3) to evaluate the contribution of malignant osteolysis to circulating TGF β 1 concentrations in dogs diagnosed with OS.

Materials and Methods

Cell Lines

Five canine (POS, HMPOS, COS31, Abrams, and D17) OS cell lines, 1 human (143B) OS cell line, 1 normal canine osteoblast cell line (K9OB), and a human pulmonary carcinoma cell line (A549) were used in this study. The POS and HMPOS cell lines were provided by James Farese, University of Florida, and the COS31 line was provided by Ahmed Shoieb, University of Tennessee. The K9OB cell line was established from a fragment of normal canine trabecular bone at the University of Illinois, Comparative Oncology Research Laboratory. All other cell lines were purchased commercially from American Tissue Culture Collection (Manassas, VA). All cell lines were cultured in either RPMI1640 or DMEM, with 10% fetal bovine serum and 1% penicillin/streptomycin. Cell cultures were maintained at 37°C in 5% CO₂ and passaged twice weekly.

Reagents and Antibodies

Human recombinant TGF β 1^a was purchased and used for Smad phosphorylation experiments. A selective TGF β RI/II inhibitor, LY2109761,^b was purchased for *in vitro* inhibition studies. Antibodies purchased included mouse monoclonal antihuman Smad 2/3 antibody,^c rabbit monoclonal antihuman phosphorylated-Smad 2 antibody,^d rabbit polyclonal antihuman TGF β RI antibody,^e rabbit polyclonal antihuman TGF β RII antibody,^e rabbit polyclonal antimouse Phosphorylated-Akt antibody,^f rabbit

polyclonal antimouse Akt antibody,^f rabbit polyclonal antihuman β -actin antibody,^e and mouse monoclonal antihuman HIF-1 α antibody.^e Although the majority of antibodies utilized were validated for use in dogs based upon manufacturer specifications, specifically for TGF β RI and TGF β RII antibodies, canine cross-reactivity for western blot analysis and immunohistochemistry was supported by the detection of proteins of expected molecular weight and subcellular localization, respectively, in conjunction with the use of known human positive controls.

Western Blot Analysis

Protein Extraction and Loading Methodologies. For protein extraction, cell lines were grown to confluence in 100 mm petri dishes, and then scraped, harvested, and pelleted by centrifugation. Subsequently, proteins were extracted using a commercial reagent (M-PER Assay^g), mixed with a protease inhibitor cocktail,^h and concentrations quantified using a commercial kit (BCA Assay^g). For all western blots conducted, 50 μ g of protein was loaded and separated by SDS-PAGE using 10% polyacrylamide gels and transferred onto nitrocellulose membranes, with blocking at room temperature in 5% nonfat dry milk TBS-Tween immediately before incubation with target primary antibodies in 5% nonfat dry milk TBS-Tween overnight at 4°C; and after primary antibody incubation, membranes were washed and incubated for 1 hour with appropriate horseradish peroxidase-conjugated secondary antibodies (1 : 1,000) in 5% nonfat dry milk TBS-Tween and developed using a standard chemiluminescence detection kit.ⁱ

TGF β RI/II. Protein from 8 cell lines (5 canine OS, 1 normal canine osteoblast, 1 human OS, and 1 human lung carcinoma) was extracted and loaded as described. Membranes were incubated with either antihuman TGF β RI or TGF β RII antibodies (1:1000) in 5% nonfat dry milk TBS-Tween overnight at 4°C.

Smad 2/3 and Phosphorylated-Smad 2. The HMPOS cell line was grown to 80% confluence, and then serum starved with or without inclusion of LY2109761 for 24 hours. Protein was collected from 5 experimental conditions, including (1) serum starved for 24 hours only or serum starved for 24 hours and then stimulated for 1 hour with (2) 10% FBS media only; (3) 10% FBS media + human recombinant TGF β 1 (5 ng/mL); (4) 10% FBS media + LY2109761 (5 μ M); and (5) 10% FBS media + human recombinant TGF β 1 (5 ng/mL) + LY2109761 (5 μ M). Membranes were incubated with either antihuman Smad 2/3 antibody (1 : 500) or antihuman phosphorylated-Smad 2 antibody (1 : 1,000) in 5% nonfat dry milk TBS-Tween overnight at 4°C.

Akt and Phosphorylated Akt. The HMPOS cell line was grown in complete media and then serum starved with or without inclusion of LY2109761 for 24 hours. Protein was collected from four experimental conditions, including (1) serum starved for 24 hours only or serum starved for 24 hours and then stimulated for 1 hour with (2) human recombinant TGF β 1 (5 ng/mL); (3) LY2109761 (5 μ M) alone; (4) LY2109761 (5 μ M) + human recombinant TGF β 1 (5 ng/mL). Membranes were incubated with either a rabbit polyclonal antimouse phosphorylated-Akt antibody or a rabbit polyclonal antimouse Akt antibody, both at a 1 : 1,000 dilution.

HIF-1 α and β -actin. The Abrams cell line was grown in complete media to confluence and then exposed to LY2109761 (5 μ M) for varying exposure durations. Membranes were incubated with a mouse monoclonal antihuman HIF-1 α antibody (1 : 500). Subsequently, membranes were stripped and reprobed with a rabbit polyclonal antihuman β -actin antibody (1 : 5,000) and HRP-conjugated antirabbit secondary antibody (1 : 1,000). Quantitative dosimetry of HIF-1 α relative to β -actin was performed using Image J software and expressed as a ratio.

Immunohistochemistry

Antibody Validation using Cell Pellets. Five canine OS cell lines and a human positive control cell line, A549,³¹ were used to validate the application of TGF β RI/II antibodies for immunohistochemical staining of formalin-fixed paraffin-embedded samples. Adherent cell cultures were collected and washed in PBS, then pelleted by centrifugation. Each pellet was resuspended in 1 mL 10% formalin for 1 hour. Formalin was removed and cell pellets were resuspended uniformly into 1 mL 4% melted agarose gel by vortexing, and then immediately centrifuged to create an agarose-embedded cell pellet. Cell pellets then were trimmed and processed by an identical protocol routinely used for formalin-fixed paraffin-embedded tissue biopsy specimens. Briefly, paraffin-embedded cell pellets were sectioned every 3 μ m and placed on positively charged slides, and dried for 1 hour at 60°C. Slides were deparaffinized with 3 sequential xylene washes, and subsequently hydrated using 100, 95, and 70% ethanol for 2 minutes each; followed by a water rinse. Slides were placed in 3% hydrogen peroxide in methanol for 15 minutes, and then treated with citrate buffer (pH 6.0) at a temperature of 95°C for 10 minutes. SuperSensitive Wash Buffer and Peroxide block was applied for 10 minutes at room temperature to block nonspecific staining. The rabbit polyclonal antihuman TGF β RI and TGF β RII antibodies were used at a concentration of 1 : 100 for 1 hour at room temperature. Slides were rinsed with SuperSensitive Wash Buffer and treated with Super Enhancer for 20 minutes at room temperature, and subsequently treated with Polymer-HRP for 30 minutes at room temperature and incubated with 3,3'-diaminobenzidine (DAB) at room temperature for 5 minutes. Slides were washed with Super-Sensitive Wash Buffer and counterstained with Mayer's hematoxylin for 1 minute. Staining intensity for each canine OS cell line was qualitatively compared with the staining intensity of the human positive control using an arbitrary subjective integer scale: (–) no staining; (+) light uniform staining; (++) moderate uniform staining; and (+++) intense uniform staining.

Spontaneous OS Samples. Ten canine appendicular OS tissue blocks were provided by Dr. Luke Borst 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 by blocking for 10 minutes with Power Block, and then blocking for 15 minutes with avidin and biotin block. Blocked slides were incubated with rabbit polyclonal antihuman TGF β RI and TGF β RII antibodies at a concentration of 1 : 100 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, and developed with DAB substrate for 5 minutes and counterstained with hematoxylin. The negative control for the samples was processed identically in the absence of the primary antibody.

Colony-Forming Assay

The Abrams and HMPOS cell lines were seeded overnight into 6-well plates at a concentration of 50 cells per 3 mL of DMEM with 10% FBS. Upon adherence of individual cells, media was discarded and new media containing different experimental conditions was added, including DMEM 5% FBS only or DMEM 5% FBS with vehicle (DMSO) or a range of LY2109761 concentrations

(0.3–5 μ M). Cells were allowed to grow undisturbed for 7 (Abrams) or 10 (HMPOS) 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. The number of visible colonies and the average size of colonies per experimental condition were quantified using imaging software.¹ Two independent experiments were performed, with duplicates conducted for each experimental condition.

Scratch Assay

The Abrams, D17, and HMPOS cell lines were grown to 80% confluence in 6-well plates in DMEM supplemented with 10% FBS (complete media). Six experimental conditions for a duration of 48 hours were evaluated, including complete media only or complete media with vehicle (DMSO) or a range of LY2109761 concentrations (0.3–5 μ M). A standardized acellular gap was created through cell monolayers using a 200- μ L pipette tip in the middle of each well. Images of the acellular gap were captured at time 0 (maximal gap) and 48 hours for each experimental condition and analyzed with imaging software.¹ The average width of 5 representative acellular gaps per experimental condition and cell line was used for quantitative comparisons at 48 hours, and 3 independent experimental replicates were performed.

VEGF ELISA

The Abrams, D17, and HMPOS cell lines were plated in complete media at 37°C and 5% CO₂ with a seeding density of 5,000 cells per well in a 96-well plate. After adherence, complete media was removed and replaced with 1% FBS DMEM with either vehicle (DMSO) or various concentrations of LY2109761 (0.3–5 μ M) for 48 hours. Cell culture supernatants were collected and the concentration of secreted VEGF was quantified using a commercial kit (Canine VEGF Quantikine ELISA Kit^a) and normalized for cell proliferation by use of a colorimetric proliferation assay.^k Three independent experiments were performed, with hexaplicates conducted for each experimental condition.

TGF β 1 ELISA

The Abrams, D17, and HMPOS cell lines were incubated with 1% FBS DMEM at different cell densities ranging from 25 to 100 \times 10³ cells per well for 24 hours. Cell culture supernatants were collected and total TGF β 1 was determined with a commercially available ELISA kit (Quantikine[®] Mouse/Rat/Porcine/Canine TGF β 1 Assay^a). In 33 dogs with appendicular OS, heparinized plasma was collected pretreatment and 28 days after institution of standardized palliative radiation treatment and IV zoledronate. Plasma samples were stored at –80°C and batch analyzed using a commercially available ELISA kit (Quantikine[®] Mouse/Rat/Porcine/Canine TGF β 1 Assay^a).

Urine N-Telopeptide (NTx) assay

Pretreatment morning urine was collected from 33 dogs treated with standardized radiation therapy and IV zoledronate. Urine samples were immediately centrifuged at 4°C at 250 \times g for 10 minutes, and the supernatant was collected and stored at –20°C before analysis was performed. Urine creatinine and NTx concentrations were measured with commercial ELISA test kits (Parameter Creatinine ELISA^a).¹

Statistical Analysis

Data sets were assessed for normality using the Shapiro-Wilk test. For all data comparisons evaluating the effect of TGFβRI/II kinase blockade with LY2109761, a 1-ANOVA and posthoc Dunnett's test were used to identify significant differences among experimental conditions compared with DMSO vehicle control. In dogs with OS, Pearson correlation coefficient was used to characterize any relationship between pretreatment plasma TGFβ1 and urine N-telopeptide concentrations and a 2-sided, paired *t*-test was utilized to determine the significance of changes in plasma TGFβ1 concentrations before and after treatment with standardized radiation therapy and IV zoledronate. Significance was defined as *P* < .05.

Results

TGFβRI/II Expression and TGFβ1 Secretion by OS Cell Lines

By western blot analysis, OS cells derived from human and canine origin demonstrated the presence of both TGFβRI and TGFβRII (Fig 1A). Subjectively, TGFβRI was more robustly detectable in comparison with TGFβRII after normalizing for β-actin loading control. As expected based upon the participatory role of TGFβ in homeostatic skeletal biology, normal canine osteoblasts also expressed TGFβRI and TGFβRII. Expression of TGFβRI and TGFβRII also was identified in OS cell lines by immunohistochemistry (Fig 1B), demonstrating a membranous and cytosolic staining pattern consistent with the cellular localization of receptor serine-threonine kinases. Active secretion of the cognate ligand, TGFβ1, was demonstrated by titration studies in three canine OS cell lines (Fig 1C). At the highest cell densities evaluated (approximately 10⁵ cells), total TGFβ1 concentrations

achieved physiologically relevant and active concentrations in the ng/mL range. The HMPOS cell line demonstrated the greatest capacity to secrete TGFβ1, approximately doubling or quadrupling the concentrations liberated by Abrams and D17, respectively.

LY2109761 Blocks Canonical TGFβ Signaling in Canine OS Cells

To confirm the capacity of LY2109761 to block the canonical signaling pathway of TGFβ in malignant canine osteoblasts, HMPOS cells were exposed to 5 different in vitro conditions with subsequent characterization of phosphorylated-Smad 2 and Smad 2/3 by western blot analysis (Fig 2). Under serum starved conditions only, the HMPOS cell line demonstrated detectable, but low, concentrations of phosphorylated-Smad 2. Stimulation for 1 hour with 10% FBS increased phosphorylated-Smad 2 concentrations, whereas combined stimulation with 10% FBS and recombinant TGFβ1 markedly induced Smad 2 phosphorylation. The addition of 5 μM LY2109761 completely blocked 10% FBS-elicited Smad 2 phosphorylation, and substantially attenuated Smad 2 phosphorylation induced by concurrent stimulation with 10% FBS and recombinant TGFβ1.

LY2109761 Attenuates Pro-tumorigenic Activities in Canine OS Cells

Given the participatory role of TGFβ signaling in multiple facets of cancer progression, the potential anticancer effects of LY2109761, a highly potent and selective small molecule inhibitor of TGFβRI (K_i 0.04 μM) and TGFβRII (K_i 0.3 μM), on canine OS

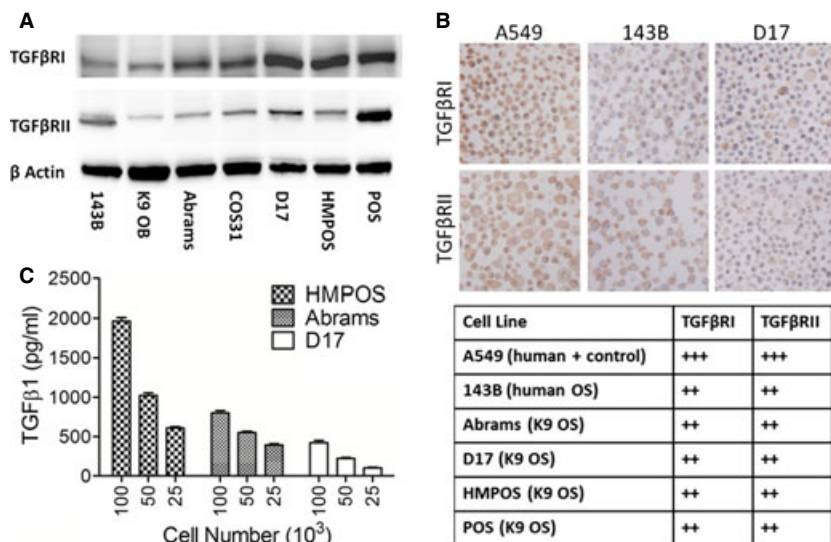


Fig 1. In a panel of immortalized cell lines, protein detection by (A) western blot and (B) immunohistochemistry identifies the expressions of TGFβRI and TGFβRII in OS cell lines. Tabulated summary represents qualitative positive staining relative to A549 positive control as detected by immunohistochemistry. By enzyme-linked immunosorbent assay (C), spontaneous secretion of TGFβ1 by canine OS cell lines is confirmed by titration studies that demonstrate a direct relationship between cell density and supernatant TGFβ1 concentrations. Data expressed as mean ± SD.

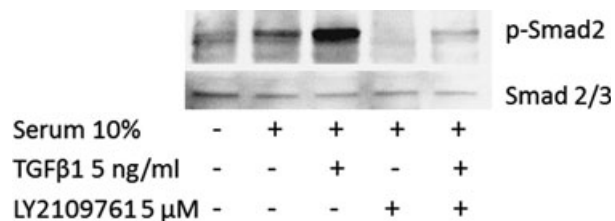


Fig 2. Western blot analysis demonstrating functional Smad-dependent signaling mediated through TGFβRI and TGFβRII in HMPOS canine OS cells. Increases in phosphorylated Smad 2 are induced by stimulation with either 10% fetal bovine serum alone or in combination with exogenous human recombinant TGFβ1. Pretreatment with LY2109761 (5 μM) completely prevents or attenuates Smad 2 phosphorylation after stimulation and substantiates the capacity for LY2109761 to block TGFβ signaling in canine cells.

cell proliferation, migration, survival, and angiogenesis were investigated. With the capacity to establish distinct, noncoalescing cell colonies in vitro, HMPOS and Abrams cell lines were selected to investigate the effect of LY2109761 on cell proliferation as evaluated by the number and size of colonies formed. Starting with an initial seed density of 50 cells per well, the antiproliferative effects of LY2109761 (0.3–5 μM) were characterized in HMPOS and Abrams cell lines by 7-day and 10-day colony-forming assays, respectively. For HMPOS cells, 10-day incubation with complete media with vehicle control (DMSO) resulted in an average generation of 265 ± 4 colonies. The addition of LY2109761 significantly decreased the number of colonies formed for all concentrations tested in comparison with vehicle control (Fig 3A,B), $P < .01$ for all comparisons. The average numbers of colonies

formed after incubation with LY2109761 at each respective concentration were 204 ± 2 (0.3 μM), 200 ± 3 (1 μM), 139 ± 2 (3 μM), and 65 ± 1 (5 μM). A similar, but less substantial, reduction in cell proliferation was elicited by LY2109761 in the Abrams cell line (Fig 3C). The average numbers of colonies formed for each experimental condition were 503 ± 7 (DMSO), 483 ± 5 (0.3 μM), 460 ± 42 (1 μM), 392 ± 4 (3 μM), and 344 ± 5 (5 μM). Paralleling the observed reduction in absolute number of colonies formed, higher concentrations of LY2109761 also modestly diminished the maximal diameter of cell colonies formed (Fig S1).

In addition to cell proliferation, the migration of malignant osteoblasts was attenuated by blocking TGFβ signaling. The extent of migratory inhibition, however, was highly cell lineage-dependent, represented in order of greatest to least inhibited being D17, HMPOS, and Abrams. The migration of the D17 cell line was most robustly inhibited by LY2109761 at all concentrations tested (Fig 4A,B), with percentage of maximal gap remaining after 48 hours of incubation being $50.2 \pm 9.9\%$ (DMSO), $72.6 \pm 11.3\%$ (0.3 μM), $73.8 \pm 10.4\%$ (1 μM), $79.1 \pm 8.9\%$ (3 μM), and $92.3 \pm 14.9\%$ (5 μM). Unlike the marked reductions in cell migration observed in D17 cells, only the highest concentration of LY2109761 was capable of slowing the rate and extent of migration relative to vehicle control in HMPOS cells, with percentage of maximal gap remaining after 48 hours of incubation being $62.8 \pm 7.4\%$ (DMSO), $70.6 \pm 8.3\%$ (0.3 μM), $65.6 \pm 7.1\%$ (1 μM), $61.3 \pm 8.5\%$ (3 μM), and $97.5 \pm 1.5\%$ (5 μM); (Fig 4C). The effect of LY2109761 on Abrams cell migration was marginal, and even the 2 highest concentrations of 3 μM and 5 μM

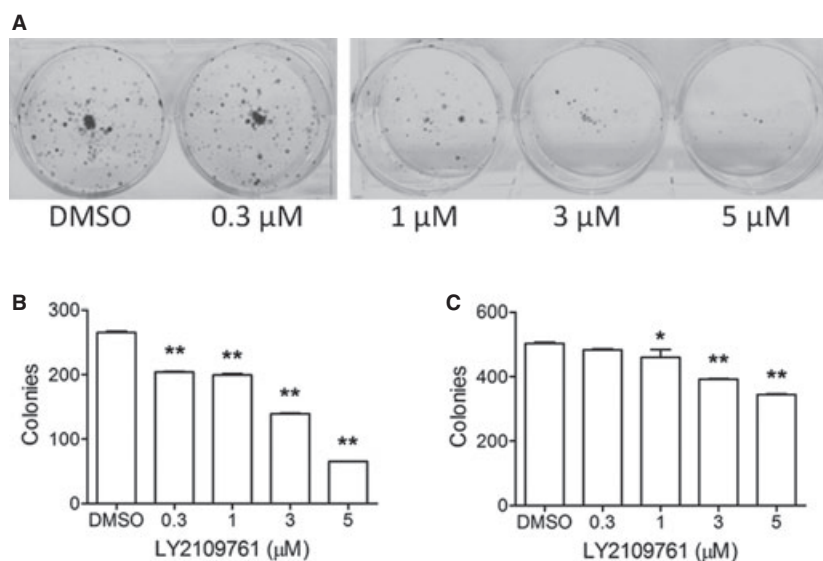


Fig 3. Visually evident (A) and quantifiable (B) dose-dependent antiproliferative effects exerted by LY2109761 in the HMPOS cell line demonstrated by colony-forming assay after 10 days of undisturbed growth. Similar quantitative (C) decreases in colony formation with LY2109761 exposure in the Abrams cell line after 7 days of undisturbed growth. Data expressed as mean \pm SD. * $P < .05$ and ** $P < .01$ in comparison with DMSO control.

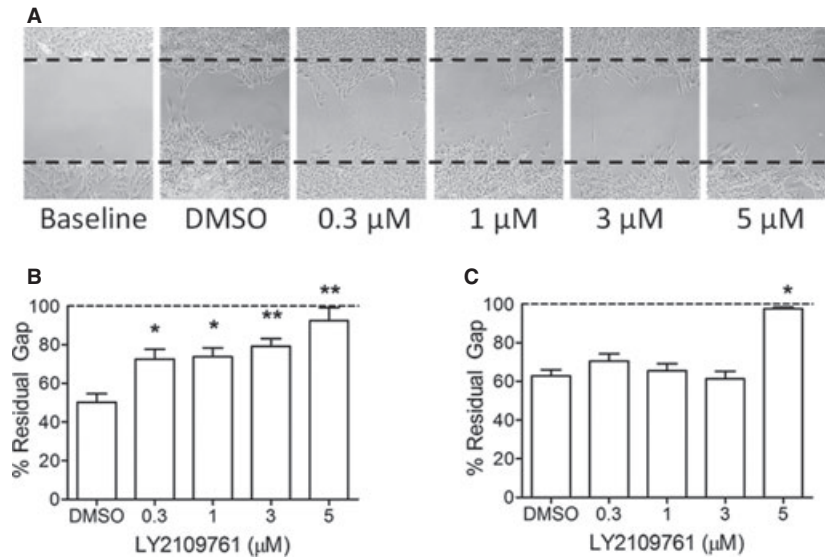


Fig 4. Visually evident (A) and quantifiable (B) dose-dependent inhibition of migration exerted by LY2109761 48 hours after scratch formation in D17 monolayer. Similar quantitative (C) attenuations in cell migration with LY2109761 exposure 48 hours after scratch formation in the HMPOS monolayer. Data expressed as mean \pm SD. * P < .05 and ** P < .01 in comparison with DMSO control.

only maintained a percentage of maximal gap of $11.0 \pm 1.9\%$ and $20.3 \pm 5.9\%$, respectively.

The ability of LY2109761 to modulate the pro-survival Akt pathway was evaluated in the Abrams cell line by western blot assessment of phosphorylated Akt and Akt. Even under nonstimulatory and serum starved conditions, phosphorylated Akt was robustly expressed, and stimulation with recombinant TGF β 1 (5 ng/mL) or inhibition with LY2109761 (5 μ M) did not alter the extent of phosphorylated Akt relative to total Akt (data not shown).

Given the pro-angiogenic properties of TGF β signaling, the effect of LY2109761 on VEGF secretion by D17, HMPOS, and Abrams cell lines was studied. Consistently, LY2109761 exerted a dose-dependent reduction in normalized secreted VEGF, generally at concentrations exceeding 1 μ M (Fig 5A). At the highest concentration of LY2109761, the secretion of VEGF was decreased by 40–45% in comparison with DMSO control for all cell lines tested. Canonical signaling through the TGF β pathway increases hypoxia-inducible factor 1- α (HIF-1 α) protein stability by downregulation of HIF-1 α -associated prolyl hydroxylase (PHD), a key enzyme involved in targeting HIF-1 α for proteasome degradation. To provide a plausible mechanism for the observed reductions in secreted VEGF after exposure to LY2109761, a relationship between TGF β signaling inhibition and decreased HIF-1 α protein stability was investigated. The Abrams OS cell line, which demonstrated the greatest reduction in secreted VEGF after LY2109761 exposure, was grown to confluence, serum starved overnight, and then incubated with 5 μ M LY2109761 for different exposure durations (0–60 minutes). In comparison with untreated cells (time 0 minutes), HIF-1 α concentrations decreased as a function of exposure time to

LY2109761 with maximal reductions in HIF-1 α approaching 35% after 60 minutes (Fig 5B,C). Relative reductions in HIF-1 α protein were mediated by LY2109761 and were not simply a by-product of DMSO exposure, because vehicle control experiments did not result in any change in HIF-1 α protein as a function of exposure time (data not shown).

TGF β Axis in Dogs with OS

To expand upon the potential clinical relevance of the TGF β signaling axis in dogs with OS, the expression of TGF β R1 and TGF β R2 was investigated in 10 primary canine osteoblastic OS samples. Similar to canine OS cell lines, malignant osteoblasts in all tumor samples consistently stained positive for TGF β R1 and TGF β R2 (Fig 6A). Given the focal malignant osteolytic nature of canine OS, in conjunction with bone matrix serving as the largest source of TGF β in the mammalian body, a correlation between pretreatment plasma TGF β 1 concentration and urine N-telopeptide excretion was investigated in 33 dogs with appendicular OS. The average concentrations for urine N-telopeptide excretion and plasma TGF β 1 were 191.1 ± 69.4 nM BCE/mM creatinine and 18.6 ± 8.7 ng/mL, respectively. Consistent with the notion that TGF β 1 might be released into circulation as a consequence of focal malignant osteolysis, a positive correlation was identified between pretreatment plasma TGF β 1 and urine N-telopeptide excretion (Fig 6B); ($r = 0.36$; $P = .04$). To further substantiate that focal malignant osteolysis associated with OS might contribute to circulating TGF β 1 concentrations, serial changes in TGF β 1 concentrations were characterized in 33 OS-bearing dogs before and after institution of effective palliative treatment comprised of

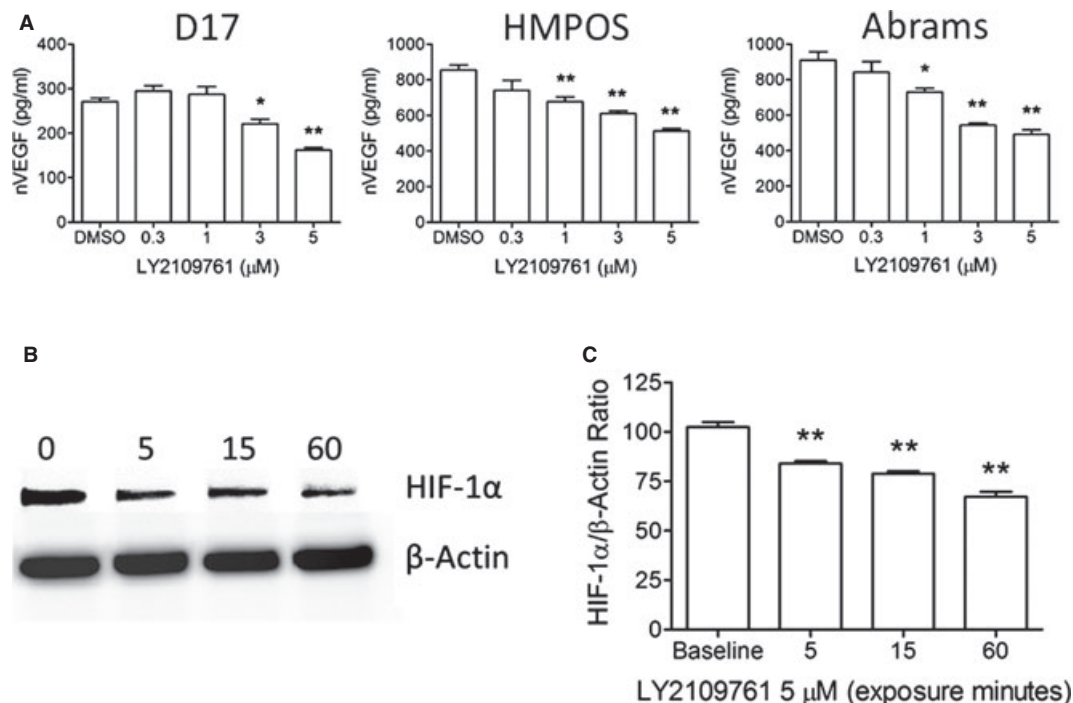


Fig 5. Quantification (A) of LY2109761 dose-dependent decrease in normalized secreted VEGF in 3 canine OS cell lines as measured by enzyme-linked immunosorbent assay. In Abrams OS cells, LY2109761 (5 μM) induces visually evident (B) and quantifiable (C) decreases in HIF-1α protein after brief exposure durations. Data expressed as mean ± SD. * $P < .05$ and ** $P < .01$ in comparison with DMSO control for VEGF secretion, or in comparison with time 0 minutes for HIF-1α expression.

ionizing radiation and IV zoledronate. The average pretreatment plasma TGFβ1 concentration of 18.6 ± 8.7 ng/mL was subsequently decreased to 13.8 ± 8.8 ng/mL after effective focal malignant osteolysis management (Fig 6C; $P = .001$).

Discussion

The biologic functions of TGFβ signaling actively participate in key cellular pathways, including apoptosis, cell cycle arrest, and immunity.^{32–35} In addition to normal physiologic processes, TGFβ's canonical and noncanonical signaling pathways probably orchestrate various tumorigenic properties, including cancer cell proliferation, migration, survival, and angiogenesis.^{1,36} With respect to malignant bone pathology, TGFβ has been identified as a key driver in perpetuating the successful development and progression of skeletal metastases, particularly for breast carcinoma.^{37–39} In the context of skeletal metastases, carcinoma cells successfully reaching the fertile bone microenvironment will subvert and dysregulate osteoclastic activities, thereby promoting excessive bone resorption, and consequently facilitate the release of TGFβ1 from the bone matrix.^{40,41} Serving as a potent mitogen for metastatic carcinoma cells, bone-derived TGFβ1 promotes carcinoma progression and accelerates additional osteoclastic bone resorption, with the establishment of a paracrine feedback loop termed the “vicious cycle”.³⁹

Although definitively a driver of skeletal metastases biology, the involvement of TGFβ signaling for the

establishment, maintenance, or progression of primary bone tumors, such as OS, remains incompletely investigated. Intuitively, given the role of TGFβ in reparative bone biology in which Smad-dependent and -independent signaling are responsible for normal osteoblast migration, proliferation, and survival,^{14,42–44} it would be rational to speculate that TGFβ signaling might also contribute to OS biology. Indeed, prior studies have investigated whether expression of different TGFβ ligand isoforms in tumor samples might influence OS biologic behavior in human patients,^{45–47} as well as if specific TGFβ receptor gene polymorphisms might serve as risk factors for OS development.^{48,49} Extending upon studies that only characterized either ligand or receptor, more comprehensive investigations suggest cooperation between TGFβ receptors and cognate ligands in the form of an autocrine feedback loop with the capacity to promote OS cell growth or drug resistance.^{50–52}

Complementing the results derived from studies of human and murine OS cells, the findings from the current investigation demonstrate that canine malignant osteoblasts similarly possess TGFβ receptor/ligand signaling machinery. All canine cells of osteoblast lineage, normal and malignant, expressed both TGFβRI and TGFβRII, suggesting that TGFβ signaling might not represent a malignant driver phenotype, but rather serve as a conserved pathway utilized by both reparative and malignant osteoblasts, alike. In addition, canine OS cells spontaneously secreted TGFβ1 at low ng/ml concentrations, a systemic concentration of

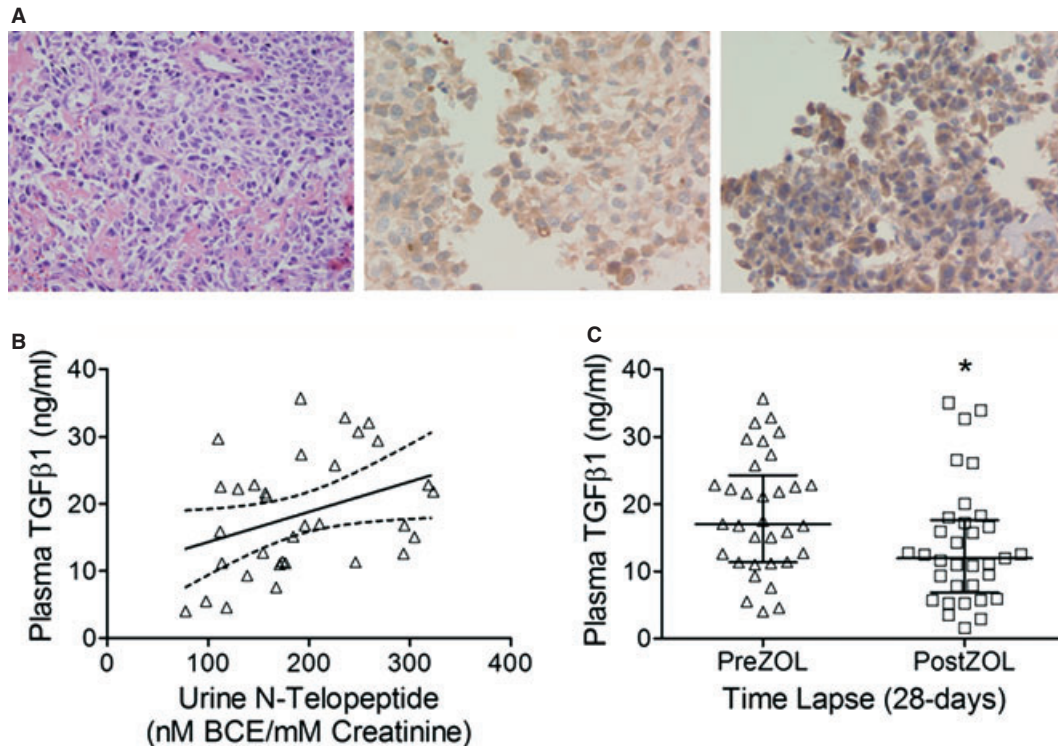


Fig 6. Microscopic appearance (A) of spontaneous canine OS sample by hematoxylin and eosin staining (left panel) and confirmed expressions of TGF β RI (middle panel) and TGF β RII (right panel) in malignant osteoblasts identified by immunohistochemical staining. In 33 treatment-naïve OS-bearing dogs, (B) positive correlation between baseline plasma TGF β 1 concentrations and urine *N*-telopeptide excretions. Decreases in TGF β 1 concentrations (C) in serially collected plasma samples derived from 33 dogs treated with standardized palliative treatment inclusive of ionizing radiation treatment and IV zoledronate administration. Data expressed as mean \pm SD. * $P < .05$.

cytokine that is capable of exerting active biologic effects *in vivo*.⁵³ Based upon the coexpression of both receptor and cognate ligand, it is plausible that canine OS cells have the potential to exploit either autocrine or paracrine TGF β signaling as a mechanism favoring protumorigenesis.

Given the potential for the existence of an auto-crine/paracrine signaling loop, in conjunction with the pivotal role that TGF β plays in cell cycle regulation and epithelial-mesenchymal transition (EMT), the current study explored the effects on canine OS cell proliferation and migration after exposure to LY2109761, a highly selective and potent small molecule inhibitor of TGF β RI and TGF β RII. The ability of LY2109761 to effectively block canonical TGF β -mediated signaling in canine OS cells was confirmed by the complete loss or substantial reduction in Smad 2 phosphorylation after stimulation with either 10% FBS alone or in conjunction with exogenous TGF β 1, respectively. With regard to OS cell proliferation, a dose-dependent antimetogenic effect of LY2109761 was demonstrated in both the HMPOS and Abrams OS cell lines. Although maximal decreases in colony formation and size were observed at the highest concentration of LY2109761 (5 μ M) and correlated well with attenuating Smad 2 phosphorylation, the antiproliferative activity of LY2109761 was operative at concentrations as low as 0.3 μ M, a finding consistent with the reported inhibition constant (K_i) of LY2109761 for TGF β RII.⁵⁴

In addition to cell proliferation, TGF β signaling blockade with LY2109761 decreased canine OS cell migration. The extent of impaired migratory ability appeared cell line dependent, with the motility of D17 and Abrams OS cell lines being markedly and minimally attenuated, respectively. Given that the highest concentration of LY2109761 (5 μ M) used in this study was expected to completely block canonical and non-canonical TGF β signaling, and yet the capacity of OS cells to migrate was not abolished completely, can be reconciled by alternate and redundant signaling pathways, such as c-Met,^{55,56} potentially exploited by canine OS for the preservation of cell motility.

Another pro-tumorigenic property investigated in this study was the influence of TGF β signaling on the secretion of VEGF by canine OS cells. As a proangiogenic cytokine, TGF β augments VEGF production by stabilizing HIF-1 α indirectly by selectively inhibiting the transcription and translation of HIF-1 α -associated prolyl hydroxylase (PHD), a negative regulator of HIF-1 α stability.⁵⁷ Exposure to LY2109761 resulted in a dose-dependent decrease in secreted VEGF in all OS lines evaluated, with maximal VEGF reductions approaching 50%. Importantly, the postulated mechanism for the observed decreases in VEGF was supported by the documented decreases in HIF-1 α , possibly through decreased stability, after exposure to LY2109761. Furthermore, the observed decreases in HIF-1 α in close temporal relationship with LY2109761

exposure are not unexpected, given the rapid post-translational regulation of HIF-1 α by proteasome degradation, and consequent short HIF-1 α half-life (<5 minutes).⁵⁸

The potential translational relevance of TGF β signaling in dogs diagnosed with OS initially was investigated by evaluation of TGF β RI and TGF β RII expression in 10 archived osteoblastic OS tissue samples. Consistent with the findings derived from immortalized canine OS cell lines, all 10 spontaneously arising OS samples demonstrated moderately strong and uniform positive staining for both receptors. Given that bone serves as the largest anatomic reservoir for TGF β 1 in the body, in conjunction with the focally osteolytic nature of OS, a correlative relationship between plasma TGF β 1 and urine N-telopeptide in 33 treatment-naïve OS-bearing dogs was explored and found to be significant. In the same 33 dogs, to indirectly support the notion that not only focal malignant osteolysis but also viable OS cells might contribute to circulating plasma TGF β 1 concentrations, serial plasma TGF β 1 concentrations were compared in each dog before and after the institution of effective palliative treatment comprised of standardized ionizing radiation and IV zoledronate. Not unexpectedly, in the majority of dogs (24/33), plasma TGF β 1 concentrations were substantially decreased after the institution of palliative treatment expected to decrease malignant osteolysis and induce OS cell apoptosis.

The collective findings of TGF β RI/II expression in OS samples and correlation between plasma TGF β 1 and focal osteolysis in OS-bearing dogs support the possibility that TGF β signaling might contribute to OS progression, and hence blockade of TGF β -mediated effects could be a rational therapeutic strategy. Although prolonged or complete TGF β blockade would carry the risk for development of severe adverse effects, including lymphoproliferative disorders and exacerbation of orthopedic pathology such as osteoarthritis, several potential blocking strategies and dosing regimens appear to be viable for the management of fibrotic diseases and cancer.⁵⁹ Based upon a large body of preclinical efficacy studies, coupled with early promising results in Phase 1, 2, and 3 studies in which TGF β blocking strategies are being evaluated for the management of diverse cancers in people, further investigations are clinically warranted to explore the suitability of inhibiting TGF β signaling as an adjuvant treatment in dogs with OS.

Although the findings from this study provide incremental advances in the potential role of TGF β signaling in OS, there are several limitations in the current investigation that should be recognized. First, no direct evidence, *in vitro* or *in vivo*, was provided to support the existence of a TGF β -mediated auto-crine/paracrine feedback loop in canine OS, and despite the demonstrated expression of cognate receptor/ligand pairs in OS cells, the biologic relevance of the identified cellular machinery cannot be ascertained. Second, although LY2109761 attenuated sev-

eral pro-tumorigenic characteristics *in vitro*, the concentrations (5 μ M) and exposure durations (24–48 hours) might not be biologically achievable or relevant in dogs with OS. However, preclinical murine models of skeletal metastases suggest that LY2109761 is tolerated and possesses favorable pharmacokinetics, as well as allowing for anticancer activities to be exerted within the bone microenvironment.^{60,61} Third, although LY2109761 decreased VEGF secretion in all canine OS cell lines tested, it cannot be concluded that this result is a direct consequence of decreased HIF-1 α protein stability after TGF β signaling blockade or simply an experimental design epiphenomenon. Fourth, the contributory origins of plasma TGF β 1 measured in OS-bearing dogs cannot be stated definitively. Nominal platelet aggregation, despite collection of blood in heparinized tubes, could have randomly influenced plasma TGF β 1 concentrations. Last, the decreases in plasma TGF β 1 after institution of ionizing radiation and IV zoledronate could not be strictly attributed to decreases in focal malignant osteolysis and OS cell viability, because whole skeletal antiresorptive effects of zoledronate would be anticipated to produce similar TGF β 1 reductive tracing.

In conclusion, findings from this investigation are novel from the perspective of canine OS biology, and corroborate existing studies conducted in human and murine OS cell lines. Future pharmacokinetic and tolerability studies in dogs with TGF β blocking molecules, such as LY2109761, will be necessary to guide the formulation of appropriate dosing regimens. The generation of preliminary and interesting data in this report provides impetus to further evaluate the feasibility and suitability of TGF β blocking strategies for the management of canine OS.

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Conflict of Interest Declaration: Timothy M. Fan serves as the Associate Editor for Oncology/Hematology in the Journal of Veterinary Internal Medicine.

Footnotes

^a R&D Systems, Minneapolis, MN

^b Selleckchem, Houston, TX

^c BD Bioscience, San Jose, CA (product no. 610842)

^d Millipore, Billerica, MA (product no. 04-953)

^e Abcam, Cambridge, MA (product nos. ab31013, ab61213, ab8227, ab463)

^f Cell Signalling, Danvers, MA (product nos. 9271 and 9272)

^g Pierce, Rockford, IL

^h Protease Inhibitor Cocktail kit, Thermo Scientific, Waltham, MA

ⁱ Amersham ECL kit, GE HealthCare Life Sciences, Piscataway, NJ

^j Image J Software, National Institutes of Health, Bethesda, MD
^k CellTiter 96[®] AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI
^l Osteomark N-telopeptide urine ELISA, Alere Inc, Waltham, MA

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Visually evident (A) and quantifiable (B) decrease in formed colony diameter exerted by LY2109761 in the HMPOS cell line after 10 days of undisturbed growth. Similar quantitative (C) attenuations in size of colonies formed with LY2109761 exposure in the Abrams cell line after 7 days of undisturbed growth. Data expressed as mean \pm SD. * $P < .05$ and ** $P < .01$ in comparison with DMSO control.