Cabozantinib inhibits prostate cancer growth and prevents tumor-induced bone lesions

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Translational Significance

Cabozantinib is a multi-kinase inhibitor that has the greatest impact on MET, VEGFR2 and RET compared to other kinases. In clinical trials, it has shown unprecedented resolution of bone metastatic lesions, based on bone scans, in men with prostate cancer. However, it is unclear in these studies if cabozantinib directly impacts the cancer or indirectly impacts the cancer through modulating the bone. In the current manuscript, a bedside-to-bench evaluation of cabozantinib's mechanism of action was performed. A combination of in vitro studies and in vivo murine models revealed that in addition to having direct impact on prostate cancer in both soft tissue and bone metastases, cabozantinib had a biphasic impact on bone. Cabozantinib targeted both MET and VEGFR2 in prostate cancer and osteoblast cells, respectively. These results reveal that targeting both the tumor and the bone microenvironment can have an important therapeutic impact on prostate cancer bone metastases.
Abstract

**Purpose:** Cabozantinib, an orally available multi-tyrosine kinase inhibitor with activity against MET and vascular endothelial growth factor receptor 2 (VEGFR2), induces resolution of bone scan lesions in men with castration-resistant prostate cancer bone metastases. The purpose of this study was to determine whether cabozantinib elicited a direct anti-tumor effect, an indirect effect through modulating bone, or both.

**Experimental Design:** Using human prostate cancer xenograft studies in mice we determined cabozantinib’s impact on tumor growth in soft tissue and bone. *In vitro* studies with cabozantinib were performed using (1) prostate cancer cell lines to evaluate its impact on cell growth, invasive ability and MET and (2) osteoblast cell lines to evaluate its impact on viability and differentiation and VEGFR2.

**Results:** Cabozantinib inhibited progression of multiple prostate cancer cell lines (Ace-1, C4-2B, and LuCaP 35) in bone metastatic and soft tissue murine models of prostate cancer, except for PC-3 prostate cancer cells in which it inhibited only subcutaneous growth. Cabozantinib directly inhibited prostate cancer cell viability and induced apoptosis *in vitro* and *in vivo* and inhibited cell invasion *in vitro*. Cabozantinib had a dose-dependent biphasic effect on osteoblast activity and inhibitory effect on osteoclast production *in vitro*, that was reflected *in vivo*. It
blocked MET and VEGFR2 phosphorylation in prostate cancer cells and osteoblast-like cells, respectively.

**Conclusion:** These data indicate that cabozantinib has direct anti-tumor activity; and that its ability to modulate osteoblast activity may contribute to its anti-tumor efficacy.
Introduction

Over 80% of men with advanced prostate cancer (PCa) develop bone metastases (1). The appearance of bone metastasis in men with advanced PCa is associated with compromised quality of life (QOL) and is a harbinger of death. Skeletal metastases result in skeletal-related events (SRE) (fracture, spine compression and instability, decreased mobility, pain, hypercalcemia), immunosuppression and anemia. Skeletal-metastatic pain is a problem in almost all patients and greatly impacts the patient’s QOL (2, 3). Both bisphosphonates and Denosumab (via RANKL inhibition) have been demonstrated to decrease SRE and improve QOL in patients with bone metastases but have not shown a significant survival benefit (4). Thus, it is critical that we continue to define mechanisms that promote bone metastasis to identify key targets to not only further enhance QOL but also improve survival.

Due to their importance in cell signaling and cancer progression protein kinases have been explored for their roles as anticancer targets. MET is a receptor tyrosine kinase, expressed in epithelial and endothelial cells (reviewed in 5). Under normal circumstances, MET is activated by hepatocyte growth factor (HGF) that is produced by stromal cells, such as fibroblasts, thus generating a paracrine activation loop. MET has been found to be highly expressed in PCa compared to benign prostate hyperplasia, and significantly correlated with higher tumor histology grade (6). Another kinase that contributes to cancer progression is vascular endothelial growth factor receptor (VEGF-R) which plays an important role in the progression of metastasis through its ability to promote angiogenesis.
upon activation by VEGF. Additionally, the VEGF pathway has been shown to promote the development of osteoblastic bone lesions in PCa (7, 8). Intriguingly, there is crosstalk between VEGF and MET pathways. VEGF induces phosphorylation of MET and thus activates the HGF/MET pathway in PCa (9). Taken together, these data demonstrate both the importance of MET and VEGF pathways in PCa and their ability to crosstalk indicating that targeting both of these pathways may have a greater benefit than targeting each pathway individually.

Cabozantinib (XL184) is an orally bioavailable tyrosine kinase inhibitor with activity primarily against MET and VEGFR2 as well as other tyrosine kinases (10). Cabozantinib was tested in a Phase 2 randomized discontinuation trial in men with metastatic castration resistant PCa (CRPC) (11). Cabozantinib treatment resulted in the regression of soft tissue lesions in 72% of patients evaluable for change in measurable disease. In addition, 68% of evaluable patients had improvement on bone scan, including 12% with complete resolution. Additionally, pain improved in 67% of evaluable patients with pain at baseline based on retrospective review. Among the patients with stable disease at week 12 that were randomly assigned to cabozantinib or placebo, median PFS was 23.9 weeks with cabozantinib and 5.9 weeks with placebo. While these results are promising, it remains to be better understood whether cabozantinib affects only the cancer or the bone cells, or both. This knowledge will help better understand the mechanism of cabozantinib’s activity in PCa skeletal metastases and to determine if tumor inhibition occurs in conjunction with the response.
observed in bone scan lesions. Accordingly, the purpose of the current study was to determine the efficacy of cabozantinib in models of PCa bone metastasis and determine if there was an anti-tumor effect, a bone effect, or both.
Materials and Methods

Animals. Six-week-old male severe combined immunodeficient (SCID) mice (Charles River, Wilmington, MA) were housed under pathogen-free conditions in accordance with the NIH guidelines using an animal protocol approved by the University of Michigan Animal Care and Use Committee.

Cell culture. Human PCa cell lines LNCaP and PC3 were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in RPMI 1640 (Invitrogen Co., Carlsbad, CA). C4-2B cells (UroCor, Oklahoma City, OK), which are LNCaP sublines selected to grow in bone (12), were grown in T medium. Human PCa cell line PC-3, a spontaneously immortalized cell line derived from a human vertebral PCa metastasis, was obtained from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in RPMI 1640 (Invitrogen Co., Carlsbad, CA). The canine Ace-1 luc PCa cell line (kindly provided by Dr. Tom Rosol, The Ohio State University, OH), was derived from a spontaneous dog prostate carcinoma (13) and was maintained at 37°C in Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DMEM/F12). The LuCaP35 human prostate cancer androgen-responsive xenograft (14) (kindly provided by Dr. Robert Vessella, University of Washington, Seattle, WA) was maintained as a xenograft in SCID mice. The MC3T3-E1 (clone MC-4) (kindly provided by Dr. Renny Franceschi, University of Michigan, Ann Arbor, MI), consists of pre-osteoblasts derived from murine calvariae that, when treated with ascorbate, express osteoblast-specific markers and are capable of producing a
mineralized matrix (15) was routinely maintained in α-MEM. ST-2 cells, mouse bone marrow stromal cell line, were obtained from RIKEN Cell Bank (Ibaraki, Japan) and maintained in α-MEM. C4-2B and PC3 cells infected with the pLazarus retroviral construct expressing luciferase were selected for stable transfectants in G418.

**Cell viability.** Cell viability was measured using WST-1 assays (Roche Applied Science) as directed by the manufacturer.

**Evaluation of osteoclastogenesis and osteoclast activity.** RAW 264.7 mouse macrophage/monocytes (ATCC) were seeded in 96-well plates (10⁳ cells/well) and allowed to attach to bovine bone slices in 96-well tissue culture plates. The culture medium was supplemented with 100 ng/ml recombinant murine RANKL. The cells were incubated 7 days at which time indicated levels of cabozantinib were added. The supernatants were collected at day 10 were stored at -80°C until analysis of TRACP 5b and CTX.

**TRACP 5b and CTX Measurements.** Secreted TRACP 5b was determined from the culture medium using MouseTRAP™ Assay (TRACP 5b) ELISA (IDS Inc., Fountain Hills, Arizona). Secreted TRACP 5b accurately reflects the number of osteoclasts formed in each well during the differentiation period. CTX, a measure of bone collagen degradation products was measured using Beta-Crossslaps (bCTx) ELISA (Uscn Life Science Inc.) A resorption index demonstrating mean
osteoclast activity was calculated by dividing the obtained resorption volume (CTX value) with the number of osteoclasts (TRACP 5b value) as described previously (16).

**Caspase-3/7 Assay.** Activity of Caspase-3/7 was determined using Apo-ONE Homogeneous Caspase-3/7 assay kits (Promega, Madison, WI) following the manufacturer’s instructions.

**Matrigel invasion assay.** Was performed using BD-Biocoat Invasion Chambers (BD Biosciences) as previously described (17).

**Prostate-specific antigen measurement.** Total PSA levels in serum or culture supernatants were determined using the Human PSA ELISA Kit (Abazym LLC, Needham, MA) as described by the manufacturer. The sensitivity of this assay is 1 ng/mL of PSA. For culture supernatants values were normalized to cell numbers as determined by the modified WST-1 assay.

**Bone Remodeling Assays.** ALP activity in cells was measured using SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec, Inc. Fremont, CA) as directed by the manufacturer. Osteocalcin was measured using mouse-specific ELISA as recommended by the manufacturer (Biomedical Technologies Inc., Stoughton, MA). ALP activity and osteocalcin were normalized to total protein content determined with bicinchoninic acid protein assay reagent.
(Thermo Scientific, Waltham, MA, USA). The deposition of calcium in cells was quantitated by Calcium assay kit (Cayman, Ann Arbor, MI) as directed by the manufacturer. Serum tartrate-resistant acid phosphatase (TRACP) 5b was measured using Mouse TRACP 5b Assay (IDS Inc., Arizona) and Serum Procollagen I N-Terminal Propeptide (PINP) was measured using Mouse PINP ELISA kit (IDS Inc., Arizona) as directed by the manufacturer.

Immunoblot analysis. For identification of MET, VEGFR2, AKT and ERK1/2 phosphorylation, whole cell lysates were prepared by incubating cells in ice-cold RIPA lysis buffer (Millipore, Billerica, MA). Lysates were precleared and the protein concentration was determined by the bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). For electrophoresis, lysates were supplemented with SDS loading buffer and separated on SDS-8% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes. The blots were incubated in TBS containing 0.1% Tween 20 and 5% BSA during the blocking and the antibody incubation steps, followed by Western blot analysis with rabbit anti-human p-MET monoclonal Ab (1:1000, Invitrogen), rabbit anti-human MET polyclonal Ab (1:1000, Santa Cruz), rabbit anti-human p-AKT monoclonal Ab, rabbit anti-human p-ERK1/2 monoclonal Ab (1:1000, Cell Signaling), rabbit anti-human AKT monoclonal Ab, rabbit anti-human ERK1/2 monoclonal Ab (1:1000, Cell Signaling), rabbit antimouse p-VEGFR2 polyclonal Ab (1:1000, Cell Application Inc), rabbit anti-mouse VEGFR2 polyclonal Ab (1:1000, Santa Cruz), mouse anti-human GAPDH monoclonal Ab (1:5000, Millipore). The Ab binding was revealed
using an HRP-conjugated anti rabbit IgG (1:3000, Cell Signaling), or anti-mouse IgG (1:3000, Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Antibody complexes were detected by SuperSignal West Pico Chemiluminescent Substrate, or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and exposure to X-Omat film (Kodak, Rochester, NY). Densitometric analyses for protein quantification were done using Image J 1.38x software.

**Immunohistochemistry (IHC).** Tibiae and subcutaneous tumors were fixed in 10% neutral buffered formalin for 24h. The tibiae were then decalcified for 48h in 10% EDTA and then both tibiae and subcutaneous tumors were processed for paraffin embedding. Five-micron (5µM) sections where used for H&E and IHC. Nonstained sections were deparaffinized and rehydrated then stained for the indicated antigens including Ki 67, Caspase 3/7, CD31, CD45, p-MET (pY1349, Novus Biologicals, Littleton, CO), MET (sc-161, Santa Cruz), VEGFR2 (Flk1, sc-504, Santa Cruz) and p-VEGFR2 (Abcam) .The percent of cells staining positive in each sample was determined in sections by counting the positive cells in 100 cells in 3 separate random sections of each slide at 40X.

**Bone Histomorphometric Analysis.** Was performed as we previously described (18). Briefly, 3-µm sections of the mouse tibiae that was processed for immunohistochemistry were stained with hematoxylin & eosin (H&E) or tartrate-resistant acid phosphatase (TRAP) and counterstained with hematoxylin. Osteoblast number [Ob.No./mm] and osteoclast number [Oc.No./mm] were
determined in trabecular bone, 0.25 mm from the growth plate. Nine discontinuous random regions of interest were examined within each bone to represent the bone fragment. Two sections were analyzed per bone, using BIOQUANT system (R&M Biometrics, Inc., Nashville, TN).

Animal Studies. All experimental animal procedures were approved by the University of Michigan Committee for the Use and Care of Animals. For PC-3 tumor subcutaneous studies single-cell suspensions (1 × 10⁶ cells) of PC-3 luc cells in RPMI media were injected in the flank at 100 μl/site using a 27-G 3/8-inch needle. Mice were randomized to receive either cabozantinib (n=12, 60mg/kg body weight/day, oral administration) or vehicle (n=12, distilled water) for 15 days once tumors were established at 35 days post tumor inoculation. The dose of 60 mg/kg was chosen because it was shown to be the maximum-tolerated dose for longer-term dosing (10). The cabozantinib gavage solution was made fresh daily. Subcutaneous tumor burden was determined using bioluminescence imaging (BLI), as described below, every five days. All animals were sacrificed at the end of day 15 of treatment. The tumor weights were measured. The subcutaneous tumors were harvested. Half of each tumor was kept for histological analysis and the other half flash frozen for molecular analysis.

For LuCaP 35 studies cells were maintained in SCID mice as xenografts, tumors were harvested and made into single cell suspensions as previously described. SCID mice were then injected subcutaneously with LuCaP-35 cells
(2x 10^6 in 100 μL RPMI 1640) and allowed to establish tumors over a period of 42 days. After establishment of tumor, treatment with either cabozantinib (60mg/kg/day, oral administration) (n = 12) or distilled water vehicle (n = 12) was initiated and continued for 10 weeks. Subcutaneous tumor growth was monitored weekly using calipers to measure two perpendicular axes. Tumor volume was calculated using the formula (volume = length × width^2/2). At 10 weeks, mice were euthanized; subcutaneous tumors were collected, weighed and saved in formalin for additional studies.

For intratibial studies, Ace1^{luc} cells, C4-2B^{luc} and PC-3^{luc} cells were inoculated intratibially to measure the effect of cabozantinib on tumor growth. For intratibial injection, mice were anesthetized with 2.5% isofluorane/air, and both legs were cleaned with betadine and 70% ethanol. The knee was flexed, and a 27-G3/8-inch needle was inserted into the proximal end of right tibia followed by injection of 20 μl single-cell suspensions of Ace1^{luc} and PC-3^{luc} cells (1 × 10^5 cells) and C4-2B^{luc} cells (3 × 10^5 cells). Bioluminescence imaging and radiograph were used to check tumor burden in bone as a primary outcome. Tumors were allowed to become established for 7 days for PC-3, 14 days for Ace1 and 30 days for C4-2B. Mice were randomized to receive either cabozantinib (n=12, 60mg/kg body weight/day, oral administration) or vehicle (n=12, distilled water) for 3 weeks (PC-3^{luc}), or 5 weeks (Ace1^{luc}) or 28 days (C4-2B^{luc}) once tumors had been established. The cabozantinib gavage solution was made fresh daily. The blood samples were taken by cardiac aspiration under anesthesia before treatment for mice with C4-2B^{luc} cells inoculation for
determination of serum PSA levels. Tumor development in bone was checked by bioluminescence imaging (BLI) and microradiography weekly or every 10 days. All animals were sacrificed at the end of week 3, or week 5 or week 7. Before sacrifice, blood samples and magnified flat radiographs were taken under anesthesia, and serum and tibiae were collected.

**Bioluminescence imaging (BLI).** BLI was performed as previously described (19).

**Bone Imaging:** Bone mineral content (BMC) of the excised tibiae were measured using dual-energy X-ray absorptiometry (DEXA) on an Eclipse peripheral DEXA Scanner using pDEXA Sabre software version 3.9.4 in research mode (Norland Medical Systems, Fort Atkinson, WI) as previously describe (18). Magnified flat radiographs of hind limbs were taken on a microradiography apparatus (Faxitron X-ray Corp., Wheeling, IL). The tibiae were scanned on a \(\mu\)CT system (GE Healthcare Systems, London, ON, Canada) and reconstructed with a voxel size of 25 \(\mu\)m for \(\mu\)CT analysis.

**Statistical analysis.** Statistical analysis was done using Statview Software (Abacus Concepts, Berkeley, CA). For comparison among two groups, Student's \(t\) test was used. For multiple comparisons, ANOVA was used for initial analyses followed by Fisher's protected least significant difference for post
hoc analyses. Differences with $P < 0.05$ were determined as statistically significant.

**Results**

Our initial studies were based on the observation that cabozantinib induced marked resolution of Tc$^{99}$ bone scan lesions in men with PCa skeletal metastases (11). Accordingly, we first sought to determine if cabozantinib inhibits PCa cell growth in vivo in bone in a murine model. We injected Ace$^{1\text{luc}}$ cells, which induce a strong osteoblastic reaction, into the tibiae of mice. Tumors were allowed to become established and then cabozantinib (60 mg/kg per os daily based on this being previously demonstrated as the maximum tolerated dose for long term administration (10) or vehicle administration was initiated and continued for 5 weeks. Cabozantinib inhibited tumor growth based on BLI (Fig 1A and B). As anticipated, the Ace$^{1\text{luc}}$ cells created osteoblastic lesions based on radiography and microCT, which was associated with an increase of BMC (Fig. 1C and D). Cabozantinib decreased the Ace$^{1\text{luc}}$-induced osteoblastic lesions based on both radiographs and micro CT and a decrease of BMC towards the normal baseline (Fig. 1C and D). While cabozantinib administration altered Ace$^{1\text{luc}}$-induced tumor bone remodeling, it did not alter serum markers of bone remodeling; although serum PINP showed a trend ($p=0.08$) towards increasing (Fig. 1E).

To determine the effect of cabozantinib on other PCa cell lines, we further evaluated C4-2B$^{luc}$, which create mixed osteoblastic/osteolytic lesions. In this...
model, cabozantinib administration was initiated after tumor had developed post-intratibial injection and was continued for a period of 7 weeks as C4-2B tumors grow slowly. Similar to the results with Ace1<sup>luc</sup>, cabozantinib inhibited tumor growth in bone based on BLI (Supplemental Fig 1A and B). Further evidence of an effect on tumor burden was provided by the observation that cabozantinib administration also decreased serum PSA levels in the C4-2B bearing mice (this cell line produces PSA, as opposed to PC-3 and Ace1) (Supplemental Fig. 1C). As expected, the C4-2B cells created mixed osteoblastic and osteolytic lesions based on radiography, microCT and decline of BMC (Supplemental Fig. 1D and E). Cabozantinib administration reversed the tumor-induced reduction in BMC to levels of non-tumor bearing bone (Supplemental Fig. 1E). This effect was opposite to that in the Ace1<sup>luc</sup> cell model where tumor growth was associated with excessive BMC which cabozantinib treatment reduced (Fig.1D). No impact on systemic bone remodeling markers was observed (Supplemental Fig. 1F).

To determine if the effects on BMC in Ace1 and C4-2B tumors could be due to direct effects on bone, we evaluated the non-tumor bearing tibiae in the mice. Under these treatment conditions, cabozantinib had no effect on BMC of the non-tumor-bearing bone (Figs. 1D, Supplemental Fig. 1E, Supplemental Fig. 2D; compare the BMC in the no tumor groups with and without cabozantinib). However, cabozantinib induced an increase in the osteoblast perimeter and a decrease of the osteoclast perimeter in the non-tumor bearing bone (Supplemental Fig. 3).
It is now recognized that some PCa lesions are highly heterogeneous and in some cases may have a strong osteolytic component. The cell line PC-3 is frequently used to model PCa both *in vitro* and *in vivo* and is highly osteolytic. To determine if the effects of cabozantinib extended to PCa of an osteolytic nature, we assessed the impact of cabozantinib on PC-3\(^{luc}\). After intratibial injection, tumors were allowed to become established and then cabozantinib or vehicle administration was initiated and continued for 3 weeks. In contrast to the ACE\(^{1\,luc}\) and C4-2B\(^{luc}\), cabozantinib had no impact on PC-3\(^{luc}\) tumor growth in bone (Supplemental Fig. 2A and B). Additionally, cabozantinib did not impact PC-3-induced bone loss (Supplemental Fig. 2C and D). Cabozantinib administration was associated with an increase in serum levels of the bone resorption marker TRACP 5b; whereas, it had no impact on bone production markers (Supplemental Fig.2E).

As cabozantinib inhibited both Ace-1 and C4-2B growth but not PC-3 growth in bone, we next determine if cabozantinib impacted soft tissue PC-3 lesions. We injected PC-3\(^{luc}\) in soft tissue on the flank of mice and cabozantinib administration was initiated after tumors were established over 5 weeks and continued for 15 days. Cabozantinib inhibited the development of PC-3\(^{luc}\) tumors in soft tissue (Fig. 2A-C) demonstrating that cabozantinib’s ability to inhibit tumor growth was not specific to tumors growing within bone. To ensure these results were not specific to PC-3\(^{luc}\) cells, we also evaluated the effect of cabozantinib on the androgen-dependent human PCa xenograft LuCaP 35. To model the clinically relevant transition to CRPC we implanted LuCaP 35 xenografts.
subcutaneously, then after tumors became established, mice were subjected to
orchiectomy at which time cabozantinib was initiated. We were able to measure
serum PSA levels in this model as LuCaP 35 produces PSA. Cabozantinib
prevented progression of CRPC tumor based on tumor burden (Fig. 2D and E)
and serum PSA levels (Fig. 2F). Taken together, these results demonstrate that
cabozantinib can inhibit PCa growth independent of the bone microenvironment.

As cabozantinib had a discordant impact on PC-3 growth in bone versus
soft tissue, we examined if a differential impact on tumor-associated
angiogenesis or infiltration of tumor by myeloid cell could account for this
difference as these activities are known to impact tumor growth (20).
Cabozantinib decreased tumor-associated vasculature in subcutaneous but not
intratibial PC-3 tumors (Supplemental Fig. 4A). In contrast, cabozantinib had no
impact on CD45+ myeloid cell numbers found within either subcutaneous or
intratibial PC-3 tumors (Supplemental Fig. 4B). These results suggest that a
diminished impact of cabozantinib-mediated inhibition of angiogenesis in bone
versus soft tissue could contribute to the decreased anti-tumor response
observed in PC-3 intratibial tumors.

Based on the overall inhibition of PCa growth, we next determined if
cabozantinib had an impact on cell proliferation and/or apoptosis in the cancer
cells. We therefore stained the tumor tissue from the cabozantinib-treated
animals for either Ki67 to evaluate for proliferation or activated caspase-3 to
evaluate for apoptosis. Ki67 expression was undetectable in the Ace1 luc tumors,
which is due to lack of cross reactivity of the antibody with the canine epitope in
these cells (Fig. 3A and B). However, Ki67 was decreased in the cabozantinib-treated C4-2B intratibial tumors and the PC-3\textsuperscript{luc} subcutaneous tumors, respectively, compared to vehicle-treated animals (Fig. 3A and B). In contrast, Ki67 expression was not altered by cabozantinib treatment in the PC-3\textsuperscript{luc} intratibial tumors (Fig. 3A and B). Caspase-3 expression was increased in the Ace1\textsuperscript{luc} and C4-2B\textsuperscript{luc} intratibial tumors and the PC-3\textsuperscript{luc} subcutaneous tumors but not the PC-3\textsuperscript{luc} intratibial tumors from the cabozantinib-treated mice compared to vehicle-treated mice (Fig. 3C and D). Taken together, these results suggest that cabozantinib inhibits overall tumor growth through inhibition of proliferation and promotion of apoptosis.

To determine if cabozantinib directly modulated the viability of PCa cells, we examined its effect on three representative cell lines \textit{in vitro}: LNCaP, C4-2B and PC-3. LNCaP cells were used to represent an androgen-responsive cell line, but they do not grow well in bone, thus were not used for the in vivo studies. Cabozantinib had no impact on cell viability at 24 hours (not shown); whereas, at 72 hours it inhibited cell viability of these cell lines in a dose-dependent fashion (Fig. 4A). We next sought to determine if cabozantinib achieved this effect, in part, through induction of apoptosis by measuring caspase 3/7 activity. Cabozantinib induced caspase in all three cell lines at 72 hours (Fig. 4B). To determine if cabozantinib’s ability to impact PCa cells extended into diminishing their metastatic phenotype, we assessed its impact on the invasive ability of the PCa cells. Cabozantinib inhibited the invasive ability of all three cell lines (Fig. 4C). Prostate specific antigen (PSA) is used to measure prostate tumor
response to therapies. To determine if cabozantinib impacted PSA expression, we measured intracellular PSA mRNA and PSA protein from cell culture supernatant in LNCaP and C4-2B cells exposed to cabozantinib. We found that cabozantinib induced a biphasic effect, first an increase then decrease in PSA mRNA and protein expression in the androgen-dependent LNCaP cells, but had no impact on PSA expression in the androgen-non-responsive C4-2B cells (Fig. 4D). In contrast to this in vitro result in the C4-2B model, in vivo a decline in PSA was observed which was associated with a reduction in tumor burden (Supplemental Fig. 1C). Taken together, these results indicate that cabozantinib can directly diminish PCa progression; and that measurement of PSA may not be the optimal assessment of tumor response.

Our earlier observation that cabozantinib had no impact on non-tumor bearing bone suggested that cabozantinib has no considerable direct effect on bone cells. However, impacts on BMC in healthy bone may take longer than the 7 week period of administration of cabozantinib and may be dose-dependent. Furthermore, the observations that cabozantinib induced a response on bone scan lesions in the clinical trial and that it impacted tumor-induced bone remodeling in the animal models suggests that there may also be a direct effect of cabozantinib on bone cells. We therefore examined if cabozantinib modulates osteoblastic activity and determined its impact on the ability of the pre-osteoblast cells MC3T3-E1 and ST-2 to differentiate and function as osteoblasts by measuring cell viability, alkaline phosphatase (an indicator of early osteoblast differentiation), osteocalcin (a measure of late osteoblast differentiation) and...
calcium (an indicator of mineralization). Cabozantinib decreased cell viability of both cell lines in a dose-dependent fashion with ST-2 cells being more sensitive to this activity (Fig. 5A). However, cabozantinib modulated alkaline phosphatase activity in both cell lines in a biphasic fashion (Fig. 5B). In contrast, cabozantinib diminished osteocalcin expression in both cell lines (Fig. 5C). Finally, cabozantinib had no impact on the ability of MC3T3-E1 cells to mineralize, except at the highest dose evaluated (Fig. 5D). Since ST-2 cells do not readily mineralize in vitro these cells were not evaluated. These results show that cabozantinib induces early osteoblast differentiation at low doses, but at higher doses can inhibit osteoblast differentiation, which may be due, in part, to the overall impact of cabozantinib on osteoblast viability. In addition to an impact on osteoblasts, we explored cabozantinib’s ability to modify osteoclast biology in vitro. Cabozantinib inhibited the differentiation of RAW pre-osteoclast cells into mature osteoclasts in a dose-dependent fashion (Fig. 5E). Similarly, cabozantinib inhibited the overall resorptive activity in these cultures (Fig. 5E). However, when resorptive activity was normalized for osteoclast numbers, no change in osteolytic activity was observed (Fig. 5E). These results indicate that cabozantinib’s effect on resorption in vivo was primarily due to a reduction in the numbers of osteoclasts, as opposed to inhibition of the activity of individual mature osteoclasts.

Cabozantinib is a multi-kinase inhibitor with high affinity for MET and VEGFR2 relative to other kinases. To ensure that cabozantinib was acting on these expected intracellular targets, we first confirmed cabozantinib’s ability to
block HGF-mediated activation of MET by measuring phospho-MET in PC-3 cells
(Fig. 6A). We next determined if cabozantinib targeted VEGFR2 in the pre-
osteooblast cell lines MC3T3-E1 and ST-2 (VEGFR2 is not expressed in the PCa
cell lines; data not shown). VEGF induced phospho-VEGFR2 expression in the
MC3T3-E1 cells, but not in the ST-2 cells which had high basal phospho-
VEGFR2 expression (Fig. 6B). Cabozantinib inhibited basal and VEGF-induced
phospho-VEGFR2 expression, respectively, in ST-2 and MC3T3 cell lines (Fig.
6B). Additionally, as the PI3K/Akt pathway is downstream of VEGFR2, we
evaluated if cabozantinib impacted VEGF-mediated activation of AKT. VEGF
induced phospho-AKT expression in the MC3T3-E1 cells, but not in the ST-2
cells which had high basal phospho-AKT expression, similar to the high basal
phospho-VEGFR2 expression observed (Fig. 6B). Cabozantinib inhibited basal
and VEGF-induced phospho-AKT expression, respectively, in ST-2 and MC3T3
cell lines (Fig. 6C).

To determine if these results extended to cabozantinib’s ability to target
these pathways in tumor cells in vivo we evaluated for phospho-Met and
phospho-VEGFR2 expression in the tumors. To accomplish this, we subjected
the intratibial and subcutaneous PC-3 tumors, the intratibial C4-2B tumors and
the subcutaneous LuCaP35 tumors to immunohistochemistry. Unfortunately, the
Ace-1 tumors are a canine cell line and although we attempted
immunohistochemistry on them, we were not successful. These results are
shown in Supplemental Figures 5 and 6. We found that cabozantinib inhibited
phospho-MET in all tumors in both subcutaneous and intratibial sites; however,
inhibition of phospho-MET in the intratibial PC-3 cells was about 25% of untreated level; whereas, inhibition of phospho-MET in the subcutaneous tumors was approximately 50%. Cabozantinib inhibited phospho-VEGFR2 in all tumors in both subcutaneous and intratibial sites; however, similar to what was observed for phospho-Met inhibition, cabozantinib-mediated inhibition of phospho-VEGFR2 was greater in the subcutaneous PC-3 versus intratibial PC-3 tumors. These results indicate that cabozantinib successfully targeted MET and VEGFR2; however, intratibial PC-3 cells were more resistant to cabozantinib. The diminished inhibition of both phospho-MET and phospho-VEGFR correspond to the decreased anti-tumor response and anti-angiogenic response in the intratibial PC-3 tumors compared to subcutaneous PC-3 tumors. Taken together, these results suggest that cabozantinib can effectively target MET and VEGFR2 signaling in tumor cells in both the soft tissue and the bone metastasis microenvironments; however, there may be instances in which tumors may have innate resistance to cabozantinib-mediated effects.
Discussion

Over 80% of men with advanced PCa develop bone metastases and most of those men will also have soft tissue metastases. Our results suggest that cabozantinib is effective against PCa in both soft tissue and bone sites. These results indicate that cabozantinib effectively induces a tumor response independent of the tumor microenvironment. Furthermore, our results suggest that the marked cabozantinib-induced responses observed on the bone scans of men with PCa bone metastases were due to an anti-tumor response and not just an impact on bone remodeling.

PCa bone metastases are characterized as primarily osteoblastic; however, it is now recognized that metastatic lesions are heterogeneous and contain areas of osteolytic activity (21). The models we evaluated included highly osteoblastic, mixed osteoblastic/osteolytic and highly osteolytic tumor types. Cabozantinib demonstrated effective anti-tumor activity for the osteoblastic and mixed lesions; but did not have an impact on the highly osteolytic PC-3 cells when grown in the bone. As pure osteolytic lesions, similar to those induced by PC-3, are rarely observed in PCa, these findings suggest that cabozantinib would be most active against the type of bone metastases that are present in the majority of men with PCa.

The observation that cabozantinib inhibits PC-3 tumor growth in soft tissue but not in bone demonstrates that PC-3 cells are sensitive to cabozantinib in vivo and also suggests that there may be a microenvironment effect that protects PC-3 cells from cabozantinib in the bone. Furthermore, the observation that
cabozantinib-mediated inhibition of both phospho-MET and phospho-VEGFR2 was diminished in intratibial versus subcutaneous PC-3 cells supports the hypothesis that the context of the tumor microenvironment impacts response to cabozantinib. However, this appeared specific to PC-3 cells as intratibial C4-2B cells responded well to cabozantinib and this was associated with reductions in phospho-MET and phospho-VEGFR2 in these cells. This *in vivo* observation is supported by the *in vitro* studies that demonstrated cabozantinib diminished viability and induced apoptosis in PC-3 cells. However, *in vitro*, PC-3 cells were less sensitive to cabozantinib inhibition than cells with more pronounced osteoblastic features such as C4-2B. Taken together, these results indicate that PC-3 cells are less susceptible to cabozantinib than the other PCa cell lines evaluated, and that an osteolytic bone microenvironment may potentially protect PC-3 from the anti-tumor activity of cabozantinib. The mechanisms through which osteolysis might protect PCa cells from cabozantinib were not determined at this time; however, it is well recognized that resorbing bone releases a variety of growth factors that could diminish the inhibition of proliferation or induction of apoptosis caused by cabozantinib.

PSA levels have generally been a good indicator of anti-tumor activity and thus are often used to measure treatment response and to monitor for recurrence. However, in the cabozantinib clinical trial that was previously performed in men with CRPC, PSA levels did not always correlate with the antitumor effects in bone and soft tissue (11). This suggests that measurements other than PSA should be used in monitoring tumor response and recurrence in
men undergoing therapy with cabozantinib. In our murine studies, PSA levels correlated with the C4-2B tumor response based on the decline in tumor volume. However, our in vitro studies demonstrated that cabozantinib induced a dose-dependent biphasic response in LNCaP cells; whereas, it had no impact on PSA response in C4-2B cells. In the context of LNCaP cells, the induction of PSA levels occurred at doses that decreased cell viability, thus suggesting that cabozantinib induced PSA expression (as opposed to just an increase in the number of cells producing PSA). Furthermore, the reduction of PSA expression in the LNCaP cells occurred at the dose of cabozantinib that had marked decrease in viability and increase of apoptosis. Taken together these data suggest that in the androgen-responsive LNCaP model, cabozantinib induces PSA expression that may be counteracted by cell death at higher concentrations resulting in overall reduction of PSA expression. This is consistent with the observation that cabozantinib had no impact on PSA expression in C4-2B cells in vitro, yet diminished PSA expression in vivo, suggesting that the PSA decline in vivo was reflective of the decline in tumor burden. In order to draw definitive conclusions regarding the impact of cabozantinib on PSA levels in vivo, correlation of cabozantinib serum levels with their impact on PSA expression must be performed. In our models, we did not measure cabozantinib serum levels, thus our results regarding PSA expression should be considered exploratory at this time.

A major goal of this study was to explore if the cabozantinib-induced bone scan effects were reflective of anti-tumor efficacy or a direct effect on bone since
bone scans only measure incorporation of radionuclide into the bone. Bone scans are thought to be a measure of osteoblastic activity and thus theoretically any effect on osteoblastic activity could impact the bone scan independent of a direct effect on tumor. Our results provide evidence that the impact on bone remodeling induced by cabozantinib in the context of tumor was, in part, due to an anti-tumor response. The evidence for this conclusion include (1) cabozantinib decreased tumor burden in bone; (2) in the osteoblastic tumor, the cabozantinib-induced tumor response was associated with a decrease of BMC towards normal levels; whereas, in the mixed osteolytic/osteoblastic tumor, the cabozantinib-induced tumor response was associated with an increase of BMC towards normal levels and in the highly osteolytic tumor, the lack of a cabozantinib-induced tumor response was associated with no change in BMC; and (3) cabozantinib had no impact on BMC in non-tumor bearing bone. However, the observation that cabozantinib altered both osteoblast and osteoclast perimeter in non-tumor bearing bone suggests that there is a primary bone effect that could impact tumor growth. These results are consistent with the in vitro observations that cabozantinib impacted osteoblastic differentiation and osteoclast production. Clearly distinguishing between direct versus indirect anti-tumor effects that are mediated through altering the bone will be challenging in these in vivo models. Several possibilities could account for the apparent contrast among in vitro and in vivo observations in the context of the tumor-induced bone phenotype including (1) that tumor-induced bone remodeling overshadows any cabozantinib bone-remodeling effect and (2) the time span these studies were performed in is
relatively short compared to modest effects induced by cabozantinib on
osteoblast differentiation.

Several lines of evidence suggest that the VEGF axis is important in PCa progression. Perhaps the most recognized impact of VEGF is on its ability to induce angiogenesis, which supports tumor growth. A role of the VEGF pathway in PCa is supported by studies that show (1) increased plasma levels of VEGF correlate with presence of bone metastasis in PCa (22) and (2) that VEGFR over-expression is associated with metastasis and poor outcome, while it appears to regulate epithelial to mesenchymal transition (EMT) of PCa cells (23, 24). In addition to pro-angiogenic effects, the VEGF axis has been shown to promote osteoblastic activity in PCa (7, 8). Due to its importance in tumor progression, targeting angiogenesis has received much attention. However, in general, clinical trial results with pure anti-angiogenesis inhibitors have been disappointing for multiple tumor types. One suggestion to account for this observation is that the hypoxia induced by angiogenesis inhibition leads to activation of the HGF/MET pathway which may promote tumor growth (25).

Multiple lines of evidence indicate that the HGF/MET pathway plays a critical role in PCa progression and appears to be an appropriate candidate for targeted therapy. HGF/MET expression has been associated with PCa aggressive behavior in tissue samples (26, 27). Over-expression of MET is an independent predictor of invasion and metastasis in PCa and its expression has been shown to be highly prevalent in bone metastases (28, 29). Moreover, increased serum levels of HGF are an independent prognostic marker in patients
with advanced disease stage (30, 31). Up-regulation of HGF and its receptor MET is associated with the transition to androgen-independent growth of PCa (32, 33). Pharmacologic inhibition of the MET signaling pathway by a variety of methods has been shown to reduce both the development and progression of PCa metastases in vitro and in vivo in animal models (34-36) including suppression of PCa growth in a mouse model (37). The observation that angiogenesis is important in tumor progression and that targeting angiogenesis promotes HGF/MET activation, which in turn may promote tumor growth, led to the concept that targeting both the VEGF and HGF/MET pathways may have greater anti-tumor efficacy than targeting either pathway alone.

Cabozantinib, targets both VEGFR2 and MET, and inhibited their activation in PCa and bone stromal cells, respectively. The ability to target MET in the cancer cells is consistent with cabozantinib’s ability to impact tumor growth. The ability of cabozantinib to target VEGFR2 in bone stromal cells is consistent with the observation that cabozantinib is active against bone marrow microenvironment cells and could account for the inhibition of stromal cell differentiation to osteoblasts we observed in vitro. These observations indicate that cabozantinib can target both the cancer and microenvironment components of the bone metastases through different pathways.

In conclusion, cabozantinib can effectively inhibit tumor growth and tumor-induced bone remodeling in murine models of PCa that have osteoblastic components. Cabozantinib-mediated inhibition of tumor-induced bone changes appeared to be primarily due to its ability to inhibit tumor growth as opposed to a
direct effect on bone. Cabozantinib’s ability to target both the tumor cells themselves in addition to microenvironment cells may result in effective anti-tumor therapy.
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References


Figure legends

Figure 1. Cabozantinib inhibits the progression of Ace-1\textsuperscript{luc} PCa cells in bone \textit{in vivo}. SCID mice were injected intratibially with Ace-1\textsuperscript{luc} cells (1x 10\textsuperscript{5} in 20\textmu L DMEM/F12) and allowed to become established over a period of 14 days. After establishment of tumor growth in bone, treatment with either cabozantinib (60mg/kg/once daily, per os) (n = 12) or distilled water vehicle (n = 12) was initiated and continued for 5 weeks. Mice were subjected to weekly bioluminescent imaging (BLI). At 5 weeks after initiation of cabozantinib, mice were euthanized, bones subjected to Faxitron X-ray analysis, microCT and dual-energy x-ray absorptiometry (DEXA), and blood was collected and separated into serum that was subjected to enzyme-linked immunoassay for bone markers (PINP, osteocalcin and TRAP 5b). A, Representative BLI imaging. Note the decreased signals in the tibiae of the cabozantinib-treated mice compared with vehicle-treated mice. B, Tumor burden as measured using BLI. Results are reported as relative light units (RLU). *P<0.05 versus vehicle-treated mice. C, Representative radiographic and microCT imaging. Note the decreased osteoblastic activity in the tibiae of the cabozantinib-treated mice compared with vehicle-treated mice. D, Bone mineral content (BMC) measured using DEXA. #P < 0.05 versus no tumor mice for each respective treatment group. *P<0.05 versus tumor-bearing vehicle-treated animals. E, serum PINP, osteocalcin and TRACP 5b levels.
Figure 2. Cabozantinib inhibits progression of PC-3\textsuperscript{luc} and LuCaP-35 PCa cells in soft tissue \textit{in vivo}. (A-C): PC-3\textsuperscript{luc} cells: SCID mice were injected subcutaneously with PC-3\textsuperscript{luc} cells (1x $10^6$ in 100 µL RPMI 1640) and allowed to grow for 35 days to become established. After establishment of tumor, treatment with either cabozantinib (60mg/kg/daily, per os) ($n = 12$) or distilled water vehicle ($n = 12$) was initiated and continued for 15 days. Mice were subjected to bioluminescent imaging (BLI) weekly. At 15 days post-initiation of cabozantinib, mice were euthanized, subcutaneous tumors were collected, weighed and saved in formalin for additional studies. A. Representative BLI of PC-3\textsuperscript{luc} tumors. Note the decreased signals in the tumors of the cabozantinib-treated mice compared with vehicle-treated mouse. B, Tumor burden of PC-3\textsuperscript{luc} tumors as measured using BLI. Results are reported as relative light units (RLU). *$P<0.05$ versus vehicle-treated mice. C, Tumor weight of PC-3\textsuperscript{luc} tumors. *$P<0.05$ versus vehicle-treated mice. (D-F): LuCaP-35 cells: LuCaP tumors maintained in SCID mice were made into single cell suspensions. SCID mice were then injected subcutaneously with LuCaP-35 cells (2x $10^6$ in 100 µL RPMI 1640) and allowed to develop into tumors over a period of 42 days. After establishment of tumor, treatment with either cabozantinib (60mg/kg/day, oral administration) ($n = 12$) or distilled water vehicle ($n = 12$) was initiated and continued for 10 weeks. The tumors were measured by caliper weekly. At 10 weeks, mice were euthanized; subcutaneous tumors were collected, weighed and saved in formalin for additional studies. D, Tumor volume of LuCaP-35 tumors. *$P<0.05$ versus control; **$P<0.05$ versus vehicle plus castration. E, Tumor weight of LuCaP-35
tumors. *P<0.05 versus control; **P<0.05 versus vehicle plus castration. F, PSA levels from mice with LuCaP-35 tumors. *P<0.05 versus control; **P<0.05 versus vehicle plus castration.

**Figure 3. Impact of cabozantinib on cellular proliferation and apoptosis of intratibial and soft tissue tumors in mice.** Tumors from the mice as described in Figures 1 through 4 were subjected to immunohistochemistry for (A) proliferation (using anti-Ki67) and (C) apoptosis (using anti-activated Caspase 3/7). *B: bone. *N: necrotic tissue. B and D The percent of positively stained cells was measured in three random 40x fields for each section. Results are shown as mean±SD for each section. *P<0.05 versus untreated for each cell line.

**Figure 4. Cabozantinib inhibits multiple parameters of PCa tumor progression in PCa cells.** A and B. LNCaP, C4-2B and PC-3 PCa cells were plated in 96-well plates (2 × 10^3 cells per well) in medium plus 10% FBS and incubated overnight, then media was replaced with 2% FBS-containing media and the indicated concentrations of cabozantinib. After 72 hours, (A) the cell viability was measured using WST-1 assays and (B) apoptosis activity was assayed by measuring caspase 3/7 activity based on cleavage of DEVD substrate using the Apo-ONE kit (Promega). Data are from five replicates and shown as mean±SD. *P<0.05 versus control (0 µM cabozantinib). The experiments were repeated three times. C. LNCaP, C4-2B and PC-3 cells (5
x10^4 cells) were added to the inserts of modified-Boyden chambers and treated with the indicated concentrations of cabozantinib (or saline). The plates were incubated for 22 h in a CO2 incubator at 37°C. The chamber inserts were then stained using the Diff-Quick staining kit (Dade-Behring) according to the manufacturer's instructions. The invasion was determined as the percent of cells that migrated through the membrane. Data are from triplicate samples and reported as mean±SD % of control. *P<0.05 versus control. The experiment was repeated three times. 

D. (1) PSA mRNA expression: LNCaP and C4-2B cells were plated at 5 x 10^5 cells/ml in 6-well plates and then treated with DHT (1nM) as positive control, DMSO as negative control and the indicated levels of cabozantinib. After 24 hours, total RNA was collected. Total RNA was subjected to PCR for PSA mRNA expression. (2) PSA protein expression: LNCaP and C4-2B cells were plated at 2 x 10^3 cells/ml in 96-well plates and after 24 hours treated with DHT (1nM) as positive control, DMSO as negative control and the indicated levels of cabozantinib. After 48 hours, the supernatants were collected and PSA level in the supernatants was measured by PSA ELISA and values were normalized to cell numbers as determined by the modified WST-1 assay. Data are from triplicate samples and reported as mean±SD. P<0.05 versus DMSO control.

**Figure 5. Cabozantinib impacts pre-osteoblast viability and differentiation.**

A. MC3T3-E1 and ST2 cells were plated in 96-well plates (2 x 10^3 cells per well) in α-MEM medium plus 10% FBS and incubated overnight, then media was
replaced with 2% FBS-containing media and the indicated concentrations of cabozantinib. After 72 hours, the cell viability was measured using WST-1 assays. Data are from triplicates and shown as mean±SD. *P<0.05 versus control (0 μM cabozantinib). The experiments were repeated three times. B and C. MC3T3-E1 and ST2 cells were plated in 12-well plates (5 × 10^4 per well) and grown in α-MEM containing 10% FBS. After the cells were confluent, the medium was replaced with osteoblast differentiating media (α-MEM with 10% FBS, 50 μg/mL ascorbic acid and 10 mmol/L β-glycerophosphate) the indicated concentrations of cabozantinib. Medium was refreshed every 3 days, at days 9, supernatants and cells were collected. (B) Alkaline phosphatase in cell lysates was measured using ALP assay Kit and (C) osteocalcin in media was measured using mouse osteocalcin EIA assay. Data are from triplicates and shown as mean±SD. *P<0.05 versus control (0 μM cabozantinib). The experiments were repeated three times. D. MC3T3-E1 cells were treated as in (B), but allowed to continue growth for 21 days after confluence at which time cells were collected and calcium in cell lysates was measured using a calcium assay that measure the reaction between o-cresolphthalein and calcium. Results were normalized to protein concentration in cells. Data are from three experiments and shown as mean±SD. *P<0.05 versus control (0 μM cabozantinib). (E) For measurement of osteoclast numbers and function RAW cells were plated in 96 well plates with bovine bone chips and RANKL to induce osteoclastogenesis. At day 7, cabozantinib was added to the indicated concentration, and at day 10 supernatant was collected for measurement of TRACP 5b and CTX. The
resorption index per osteoclast is calculated as CTX divided by TRACP 5b. Data are from three experiments and shown as mean±SD. *P<0.05 versus control (0 μM cabozantinib).

Figure 6. Cabozantinib inhibits c-MET, VEGFR2 and AKT phosphorylation in PCa cells and pre-osteoblasts. A. PC-3 cells were plated at 2 × 10^6 cells on 100mm plates. After 12 hours, the cells were pre-treated with cabozatinib (1μM) for three hours, then treated with either DMSO (as a negative control) or HGF (50ng/mL, as a positive control) for 20 minutes. Then total protein was extracted from the cells and subjected to immunoblot using anti-MET, anti-phosphorylated-MET (p-MET), anti-Akt, anti-phosphorylated-Akt (p-Akt), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies and appropriate secondary antibodies. GAPDH was used as an internal control. B and C. ST2 and MC3T3-E1 cells were plated at 2 × 10^6 cells on 100mm plates. After 12 hours, the cells were pre-treated with cabozantinib (1μM) for three hours, then treated with either DMSO (as a negative control), or VEGF (50ng/mL) for 20 minutes. Protein was then extracted from the cells and subjected to immunoblot analysis using (B) anti-VEGFR2, anti-phosphorylated-VEGFR2 (p-VEGFR2), anti-ERK1/2, anti-phosphorylated-ERK1/2 (p-ERK1/2) or (C) anti-AKT, anti-phosphorylated-AKT (p-AKT), and anti-GAPDH primary antibodies and appropriate secondary antibodies. GAPDH was used as an internal control. These results were obtained from at least three replicate experiments. Gel images were then subjected to densitometry using Image J 1.38x software and
densitometry values normalized to GAPDH band values for each lane are reported below each band.
Figure 4.
Figure 5.
Figure 6.
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Cabozantinib inhibits prostate cancer growth and prevents tumor-induced bone lesions

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