Cabozantinib Inhibits Prostate Cancer Growth and Prevents Tumor-Induced Bone Lesions

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Abstract

Purpose: Cabozantinib, an orally available multitryosine kinase inhibitor with activity against mesenchymal epithelial transition factor (MET) and VEGF 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 antitumor effect, an indirect effect through modulating bone, or both.

Experimental Design: Using human prostate cancer xenograft studies in mice, we determined the impact of cabozantinib on tumor growth in soft tissue and bone. In vitro studies with cabozantinib were performed using (i) prostate cancer cell lines to evaluate its impact on cell growth, invasive ability, and MET and (ii) 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 antitumor activity, and that its ability to modulate osteoblast activity may contribute to its antitumor efficacy.

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Introduction

More than 80% of men with advanced prostate cancer develop bone metastases (1). The appearance of bone metastasis in men with advanced prostate cancer 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, and hypercalcemia), immunosuppression, and anemia. Skeletal metastatic pain is a problem in almost all patients and greatly impacts the QOL of a patient (2, 3). Both bisphosphonates and denosumab [via receptor activator of NF-κB ligand (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.

Because 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 ref. 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 prostate cancer compared with benign prostate hyperplasia and significantly correlated with higher tumor histology grade (6). Another kinase that contributes to cancer progression is VEGF receptor (VEGFR), which plays an important role in the progression of metastasis through its ability to promote angiogenesis upon activation by VEGF. In addition, the VEGF pathway has been shown to promote the development of osteoblastic bone lesions in prostate cancer (7, 8). Intriguingly, there is crosstalk between VEGF and MET pathways. VEGF induces phosphorylation of MET and thus activates the HGF/MET pathway in prostate cancer (9). Taken together, these data demonstrate both the importance of MET and VEGF pathways in prostate cancer and...
Translational Significance

Cabozantinib is a multi-kinase inhibitor that has the greatest impact on MET, VEGFR2, and rearranged during transfection oncogene (RET) compared with 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 whether cabozantinib directly impacts the cancer or indirectly impacts the cancer through modulating the bone. In this study, 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.

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 II randomized discontinuation trial in men with metastatic castration–resistant prostate cancer (CRPC; ref. 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. In addition, 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. Although 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 to better understand the mechanism of the activity of cabozantinib in prostate cancer skeletal metastases and to determine whether tumor inhibition occurs in conjunction with the response observed in bone scan lesions. Accordingly, the purpose of this study was to determine the efficacy of cabozantinib in models of prostate cancer bone metastasis and determine whether there was an antitumor effect, a bone effect, or both.

Materials and Methods

Animals

Six-week-old male severe combined immunodeficient (SCID) mice (Charles River Laboratories) 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 (Ann Arbor, MI).

Cell culture

Human prostate cancer cell lines LNCaP and PC3 were obtained from the American Type Culture Collection (ATCC) and cultured in RPMI-1640 (Invitrogen Co.). C4-2B cells (UroCor), which are LNCaP sublines selected to grow in bone (12), were grown in T medium. Human prostate cancer cell line PC-3, a spontaneously immortalized cell line derived from a human vertebral prostate cancer metastasis, was obtained from the ATCC and cultured in RPMI-1640 (Invitrogen Co.). The canine Ace-1<sup>3</sup> prostate cancer cell line (kindly provided by Dr. Tom Rosol, The Ohio State University, Columbus, OH) was derived from a spontaneous dog prostate carcinoma (13) and was maintained at 37°C in Dulbecco’s Modified Eagle Medium/Ham’s nutrient mixture F12 (DMEM/F12). The LuCaP35 human prostate cancer androgen–responsive xenograft (ref. 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 preosteoblasts 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<sup>5</sup> 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 tartrate-resistant acid phosphatase (TRACP) 5b and carboxy-terminal collagen crosslinks (CTX).

TRACP 5b and CTX measurements

Secreted TRACP 5b was determined from the culture medium using MouseTRAP assay (TRACP 5b) ELISA (IDS Inc.). 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-Crosslaps (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) following the manufacturer’s instructions.

**Matrigel invasion assay**
The Matrigel invasion assay was perfomed using BD-Biocoat Invasion Chambers (BD Biosciences), as previously described (17).

**Prostate-specific antigen measurement**
Total prostate-specific antigen (PSA) levels in serum or culture supernatants were determined using the Human PSA ELISA Kit (Abayzme LLC) 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**
Alkaline phosphatase (ALP) activity in cells was measured using SensoLyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec Inc.), as directed by the manufacturer. Osteocalcin was measured using mouse-specific ELISA, as recommended by the manufacturer (Biomedical Technologies Inc.). ALP activity and osteocalcin were normalized to total protein content determined with bicinchoninic acid protein assay reagent (Thermo Scientific). The deposition of calcium in cells was quantitated by the Calcium Assay Kit (Cayman), as directed by the manufacturer. Serum TRACP 5b was measured using Mouse TRACP 5b assay (IDS Inc.) and serum procollagen I N-terminal propeptide (PINP) was measured using the Mouse PINP ELISA Kit (IDS Inc.) 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). Lysates were precleared and the protein concentration was determined by the bicinchoninic acid assay (Pierce Biochemicals). 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% bovine serum albumin (BSA) during the blocking and the antibody incubation steps, followed by Western blot analysis with rabbit anti-human p-MET monoclonal antibody (mAb; 1:1,000; Invitrogen), rabbit anti-human MET polyclonal antibody (1:1000; Santa Cruz Biotechnology), rabbit anti-human p-VEGFR2 polyclonal Ab (1:1,000, Cell Application Inc.), rabbit anti-mouse VEGFR2 polyclonal Ab (1:1,000, Santa Cruz Biotechnology), mouse anti-human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mAb (1:5,000; Millipore). The antibody binding was revealed using an HRP-conjugated anti-rabbit IgG (1:3,000, Cell Signaling Technology), or anti-mouse IgG (1:3,000; Amersham Pharmacia Biotech). Antbody 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). Densitometric analyses for protein quantification were done using ImageJ 1.38× software.

**Immunohistochemistry**
Tibiae and subcutaneous tumors were fixed in 10% neutral buffered formalin for 24 hours. The tibiae were then decalcified for 48 hours in 10% EDTA and then both tibiae and subcutaneous tumors were processed for paraffin embedding. Of note, 5-μm sections were used for hematoxylin and eosin (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), MET (sc-161; Santa Cruz Biotechnology), VEGFR2 (Flk1, sc-504; Santa Cruz Biotechnology), and p-VEGFR2 (Abcam). The percentage 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 ×40.

**Bone histomorphometric analysis**
The analysis was performed as we previously described (18). Briefly, 3-μm sections of the mouse tibiae that were processed for immunohistochemistry were stained with H&E or TRACP and counterstained with hematoxylin. Osteoblast number [Ob.No./mm²] and osteoclast number [Oc.No./mm²] 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.).

**Animal studies**
All experimental animal procedures were approved by the University of Michigan Committee for the Use and Care of Animals (Ann Arbor, MI). For PC-3 tumor subcutaneous studies, single-cell suspensions (1 × 10⁶ cells) of PC-3luc cells in RPMI media were injected in the flank at 100 µL per site using a 27-G 3/8-inch needle. Mice were randomized 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 5 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 histologic 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 (2 × 10⁶ in 100 μL RPMI-1640) and allowed to establish tumors over a period of 42 days. After establishment of tumor, treatment with either cabozantinib (60 mg/kg/d, oral administration; n = 12) or distilled water (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 × width²/2). At 10 weeks, mice were euthanized and 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 × 10⁶ cells) and C4-2B luc cells (3 × 10⁵ cells). BLI and radiography 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; 60 mg/kg body weight/d, 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 BLI (Fig. 1A and B). As anticipated, the Ace1 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 Ace1 luc-induced osteoblastic lesions based on both radiographs and microCT and a decrease of BMC toward the normal baseline (Fig. 1C and D). Although cabozantinib administration altered Ace1 luc-induced tumor bone remodeling, it did not alter serum markers of bone remodeling although serum PINP showed a trend (P = 0.08) toward increasing (Fig. 1E).

To determine the effect of cabozantinib on other prostate cancer 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 after intratibial injection and was continued for a period of 7 weeks as C4-2B tumors grow slowly. Similar to the results with Ace1 luc, cabozantinib inhibited tumor growth in bone based on BLI (Supplementary Fig. S1A and S1B). 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; Supplementary Fig. S1C). As expected, the C4-2B cells created mixed osteoblastic and osteolytic lesions based on radiography, microCT, and decline of BMC (Supplementary Fig. S1D and S1E). Cabozantinib administration reversed the tumor-induced reduction in BMC to levels of non–tumor-bearing bone (Supplementary Fig. S1E). This effect was opposite to that in the Ace1 luc cell model, in which tumor growth was associated with excessive BMC that cabozantinib treatment reduced (Fig. 1D). No impact on systemic bone remodeling markers was observed (Supplementary Fig. S1F).
To determine whether 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 (Fig. 1D and Supplementary Figs. S1E and S2D; 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 (Supplementary Fig. S3).

It is now recognized that some prostate cancer lesions are highly heterogeneous and in some cases may have a strong osteolytic component. The cell line PC-3 is frequently used to model prostate cancer both in vitro and in vivo and is highly osteolytic. To determine whether the effects of cabozantinib extended to prostate cancer of an osteolytic nature, we assessed the impact of cabozantinib on PC-3luc. After intratibial injection, tumors were allowed to become established and then cabozantinib or vehicle administration was initiated and continued for 3 weeks. In contrast with the ACE1luc and C4-2Bluc, cabozantinib had no impact on PC-3luc tumor growth in bone (Supplementary Fig. S2A and S2B). In addition, cabozantinib did not impact PC-3–induced bone loss (Supplementary Fig. S2C and S2D).
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 (Supplementary Fig. S2E). As cabozantinib inhibited both Ace-1 and C4-2B growth but not PC-3 growth in bone, we next determined whether cabozantinib impacted soft tissue PC-3 lesions. We injected PC-3luc 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-3luc tumors in soft tissue (Fig. 2A–C), demonstrating that the ability of cabozantinib to inhibit tumor growth was not specific to tumors growing within bone. To ensure these results were not specific to PC-3luc cells, we also evaluated the effect of cabozantinib on the androgen-dependent human prostate cancer 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 prostate cancer growth independent of the bone microenvironment.

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As cabozantinib had a discordant impact on PC-3 growth in bone versus soft tissue, we examined whether 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 (Supplementary Fig. S4A). In contrast, cabozantinib had no
impact on CD45+ myeloid cell numbers found within either subcutaneous or intratibial PC-3 tumors (Supplementary Fig. S4B). These results suggest that a diminished impact of cabozantinib-mediated inhibition of angiogenesis in bone versus soft tissue could contribute to the decreased antitumor response observed in PC-3 intratibial tumors.

On the basis of the overall inhibition of prostate cancer growth, we next determined whether 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 Ki67 to evaluate for proliferation or activated caspase-3 to evaluate for apoptosis. Ki67 expression was undetectable in the Ace1luc 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-3luc subcutaneous tumors, respectively, compared with vehicle-treated animals (Fig. 3A and B). In contrast, Ki67 expression was not altered by cabozantinib treatment in the PC-3luc intratibial tumors (Fig. 3A and B). Caspase-3 expression was increased in the Ace1luc and C4-2Bluc intratibial tumors and the PC-3luc subcutaneous tumors but not the PC-3luc intratibial tumors from the cabozantinib-treated mice compared with 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 whether cabozantinib directly modulated the viability of prostate cancer cells, we examined its effect on three representative cell lines 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 whether cabozantinib achieved this effect, in part, through induction of apoptosis by measuring caspase-3/7 activities. Cabozantinib induced caspase in all three cell lines (Fig. 4B). To determine whether the ability of cabozantinib to impact prostate cancer cells extended into diminishing their metastatic phenotype, we assessed its impact on the invasive ability of the prostate cancer cells. Cabozantinib inhibited the invasive ability of all three cell lines (Fig. 4C). PSA is used to measure prostate tumor response to therapies. To determine whether 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-nonresponsive C4-2B cells (Fig. 4D). In contrast with 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 (Supplementary Fig. S1C). Taken together, these results indicate that cabozantinib can directly diminish prostate cancer 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 whether cabozantinib modulates osteoblastic activity and determined its impact on the ability of the preosteoblast 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). Because 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 the ability of cabozantinib to modify osteoclast biology in vitro. Cabozantinib inhibited the differentiation of RAW preosteoclast 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 the effect of cabozantinib 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 multikinase 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 the ability of cabozantinib to block HGF-mediated activation of MET by measuring phospho-MET in PC-3 cells (Fig. 6A). We next determined whether cabozantinib targeted VEGFR2 in the preosteoblast cell lines MC3T3-E1 and ST-2 (VEGFR2 is not expressed in the prostate cancer 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). In addition, 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 whether these results extended to the ability of cabozantinib 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 IHC. Unfortunately, the Ace-1 tumors are a canine cell line and although we attempted IHC on them, we were not successful. These results are shown in Supplementary Figs. S5 and S6. 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.

Figure 4. Cabozantinib inhibits multiple parameters of prostate cancer tumor progression in prostate cancer cells. A and B, LNCaP, C4-2B, and PC-3 prostate cancer cells were plated in 96-well plates (2 \times 10^3 \text{ cells/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 \mu mol/L cabozantinib). The experiments were repeated three times. C, LNCaP, C4-2B, and PC-3 cells (5 \times 10^4 \text{ 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 hours 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 percentage 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, (i) PSA mRNA expression: LNCaP and C4-2B cells were plated at 5 \times 10^5 \text{ cells/mL} in 6-well plates and then treated with DHT (1 nmol/L) as positive control, dimethyl sulfoxide (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. (ii) PSA protein expression: LNCaP and C4-2B cells were plated at 2 \times 10^3 \text{ cells/mL} in 96-well plates and after 24 hours treated with DHT (1 nmol/L) 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.
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 antitumor response and antiangiogenic response in the intratibial PC-3 tumors compared with 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 micro-environments; however, there may be instances in which tumors may have innate resistance to cabozantinib-mediated effects.

Discussion

More than 80% of men with advanced prostate cancer develop bone metastases and most of those men will also have soft tissue metastases. Our results suggest that cabozantinib is effective against prostate cancer 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 prostate cancer bone metastases were due to an antitumor response and not just an impact on bone remodeling.

Prostate cancer 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...
Protein was then extracted from the cells and subjected to immunoblot analysis using (B) anti-VEGFR2, anti-phosphorylated-VEGFR2 (p-VEGFR2), anti-
below each band. then subjected to densitometry using ImageJ 1.38 secondary antibodies. GAPDH was used as an internal control. These results were obtained from at least three replicate experiments. Gel images were

**Figure 6.** Cabozantinib inhibits c-MET, VEGFR2, and AKT phosphorylation in prostate cancer cells and preosteoblasts. A, PC-3 cells were plated at 2 × 10^6 cells on 100 mm plates. After 12 hours, the cells were pretreated with cabozatinib (1 μmol/L) for 3 hours, then treated with either DMSO (as a negative control), or HGF (50 ng/mL), as a positive control) for 20 minutes. Then total protein was extracted from the cells and subjected to immunoblot analysis using anti-MET, anti-phosphorylated-MET (p-MET), anti-Akt, anti-phosphorylated-Akt (p-Akt), and anti-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 100 mm plates. After 12 hours, the cells were pretreated with cabozatinib (1 μmol/L) for 3 hours, then treated with either DMSO (as a negative control), or VEGF (50 ng/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 ImageJ 1.38 - software and densitometry values normalized to GAPDH band values for each lane are reported below each band.

highly osteoblastic, mixed osteoblastic/osteolytic, and highly osteolytic tumor types. Cabozantinib demonstrated effective antitumor 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 prostate cancer, these findings suggest that cabozantinib would be most active against the type of bone metastases that are present in the majority of men with prostate cancer.

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 seemed 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 prostate cancer cell lines evaluated, and that an osteolytic bone microenvironment may potentially protect PC-3 from the antitumor activity of cabozantinib. The mechanisms through which osteolysis might protect prostate cancer 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 antitumor activity and thus are often used to measure treatment response and to monitor for recurrence. However, in the cabozantinib clinical trial, which 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

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- 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. To draw definitive conclusions about 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 about PSA expression should be considered exploratory at this time.

A major goal of this study was to explore whether the cabozantinib-induced bone scan effects were reflective of antitumor efficacy or a direct effect on bone because 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 antitumor response. The evidence for this conclusion include (i) cabozantinib decreased tumor burden in bone; (ii) in the osteoblastic tumor, the cabozantinib-induced tumor response was associated with a decrease of BMC toward normal levels; whereas, in the mixed osteolytic/osteoblastic tumor, the cabozantinib-induced tumor response was associated with an increase of BMC toward normal levels and in the highly osteolytic tumor, the lack of a cabozantinib-induced tumor response was associated with no change in BMC; and (iii) cabozantinib had no impact on BMC in non–tumor-bearing bone. However, the observation that cabozantinib altered both osteoblast and osteoclast perimeters 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 antitumor effects that are mediated through altering the bone will be challenging in these in vitro 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 (i) that tumor-induced bone remodeling overshadows any cabozantinib bone-remodeling effect and (ii) the time span these studies were carried out in is relatively short compared with modest effects induced by cabozantinib on osteoblast differentiation.

Several lines of evidence suggest that the VEGF axis is important in prostate cancer 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 prostate cancer is supported by studies that show (i) increased plasma levels of VEGF correlate with the presence of bone metastasis in prostate cancer (22) and (ii) that VEGFR overexpression is associated with metastasis and poor outcome, whereas it seems to regulate epithelial-to-mesenchymal transition of prostate cancer cells (23, 24). In addition to proangiogenic effects, the VEGF axis has been shown to promote osteoblastic activity in prostate cancer (7, 8). Because of its importance in tumor progression, targeting angiogenesis has received much attention. However, in general, clinical trial results with pure antiangiogenesis 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 prostate cancer progression and seems to be an appropriate candidate for targeted therapy. HGF/MET expression has been associated with prostate cancer aggressive behavior in tissue samples (26, 27). Overexpression of MET is an independent predictor of invasion and metastasis in prostate cancer 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). Upregulation of HGF and its receptor MET is associated with the transition to androgen-independent growth of prostate cancer (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 prostate cancer metastases in vitro and in vivo in animal models (34–36) including suppression of prostate cancer 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 antitumor efficacy than targeting either pathway alone.

Cabozantinib targets both VEGFR2 and MET and inhibited their activation in prostate cancer and bone stromal cells, respectively. The ability to target MET in the cancer cells is consistent with the ability of cabozantinib 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 prostate cancer that have osteoblastic components. Cabozantinib-mediated inhibition of tumor-induced bone changes seemed to be primarily due to its ability to inhibit tumor growth as opposed to a direct effect on bone. The ability of cabozantinib to target both the tumor cells themselves in addition to microenvironment cells may result in effective antitumor therapy.

Disclosure of Potential Conflicts of Interest

D.T. Aftab is a senior Vice President and has ownership interest (including patents), F. Schimmoller has ownership interest (including patents) in Exelixis Inc. E.T. Keller received commercial research grant from Exelixis Inc. and Pfizer. No potential conflicts of interest were disclosed by the other authors.

References


Authors’ Contributions


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