Dual targeting c-met and VEGFR2 in osteoblasts suppresses growth and osteolysis of prostate cancer bone metastasis

Changki Lee, Young Mi Whang, Preston Campbell, Patrick L. Mulcrone, Florent Elefteriou, Sun Wook Cho, Serk In Park

PII: S0304-3835(17)30738-3
DOI: 10.1016/j.canlet.2017.11.016
Reference: CAN 13606

To appear in: Cancer Letters

Received Date: 17 October 2017
Revised Date: 14 November 2017
Accepted Date: 17 November 2017


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Dual Targeting c-Met and VEGFR2 in Osteoblasts Suppresses Growth and Osteolysis of Prostate Cancer Bone Metastasis

Changki Lee\textsuperscript{1,2,*}, Young Mi Whang\textsuperscript{3,*}, Preston Campbell\textsuperscript{1,2}, Patrick L. Mulcrone\textsuperscript{1,2}, Florent Elefteriou\textsuperscript{1,2,6}, Sun Wook Cho\textsuperscript{7}, and Serk In Park\textsuperscript{1,2,4,5}

Affiliations:
\textsuperscript{1} Department of Medicine and \textsuperscript{2} Vanderbilt Center for Bone Biology, Vanderbilt University School of Medicine, Nashville, TN, U.S.A.; \textsuperscript{3} Department of Urology, Chung-Ang University College of Medicine, Seoul, Korea; \textsuperscript{4} Department of Biochemistry and Molecular Biology; and \textsuperscript{5} The BK21 Plus Program, Korea University College of Medicine, Seoul, Korea; \textsuperscript{6} Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, TX, U.S.A.; and \textsuperscript{7} Department of Internal Medicine, Seoul National University Hospital, Seoul, Korea

* These authors contributed equally to this work.

Corresponding Author:
Serk In Park, D.D.S., Ph.D., Munsuk Medical Hall Room 319, 73 Inchon-Ro Seongbuk-Gu, Seoul 02841 Korea; Email: serkin@korea.edu; Phone: +82-2-2286-1305

Total Number of Words in the Main Body Text: 3497 (Abstract to Discussion)
Number of Figures: 6
ABSTRACT
Prostate cancer characteristically induces osteoblastic bone metastasis, for which no therapies are available. A dual kinase inhibitor of c-Met and VEGFR-2 (cabozantinib) was shown to reduce prostate cancer growth in bone, with evidence for suppressing osteoblastic activity. However, c-Met and VEGFR2 signaling in osteoblasts in the context of bone metastasis remain unclear. Here we show using cultured osteoblasts that hepatocyte growth factor (HGF) and VEGF-A increased receptor activator of NFκB ligand (RANKL) and M-CSF, two essential factors for osteoclastogenesis. Insulin-like growth factor-1 (IGF1) also increased RANKL and M-CSF via c-Met transactivation. The conditioned media from IGF1-, HGF-, or VEGFA-treated osteoblasts promoted osteoclastogenesis that was reversed by inhibiting c-Met and/or VEGFR2 in osteoblasts. *In vivo* experiments used cabozantinib-resistant prostate cancer cells (PC-3 and C4-2B) to test the effects of c-Met/VEGFR2 inhibition specifically in osteoblasts. Cabozantinib (60mg/kg, 3 weeks) suppressed tumor growth in bone and reduced expression of RANKL and M-CSF and subsequent tumor-induced osteolysis. Collectively, inhibition of c-Met and VEGFR2 in osteoblasts reduced RANKL and M-CSF expression, and associated with reduction of tumor-induced osteolysis, suggesting that c-Met and VEGFR2 are promising therapeutic targets in bone metastasis.

**Keywords:** Prostate Cancer, Bone Metastasis, Osteoblasts, c-Met, VEGFR2
1. INTRODUCTION

Bone metastases remain the major clinical problem of advanced-stage prostate cancer patients[1]. Improvement of surgical and chemotherapeutic modalities, as well as early detection methods, has increased the 5-year survival rate to almost 100% in early-stage patients (i.e. those only with the primary lesions). However, the patients with distant metastases, most frequently to bone, expect much lower survival rate (approximately 30%)[2]. Bone is a complex tissue comprised of diverse cell types including osteoblasts (bone-forming cells), osteoclasts (bone-resorbing cells), osteocytes, and hematopoietic bone marrow cells. Increasing evidence now clearly support the pro-tumorigenic functions of normal bone cells (i.e. stromal cells in the metastatic microenvironment) during the progression of bone metastasis[3]. In this regard, a few therapeutic approaches are designed to suppress the pro-tumorigenic function of the normal cells in the context of bone metastasis[4]. For example, bisphosphonates (e.g. Zometa) and anti-RANKL neutralizing antibody (e.g. Denosumab) suppress osteoclasts activity and differentiation, resulting in reduction of tumor-induced osteolysis, bone pain, and the incidence of pathologic fractures[5]. However, the osteoclast inhibitors are only palliative and do not cure bone metastasis. In addition, metastatic prostate cancer cells characteristically induce net osteoblastic reactions, in contrast to breast cancer that predominantly induce net osteolytic lesions. There are currently no targeted therapies available for the osteoblastic lesions. More importantly, increasing evidence supports that osteoblasts are master regulators of the bone microenvironment[6]. Therefore, osteoblast-targeted cancer therapy is a promising therapeutic strategy for metastatic prostate cancer.
Osteoblasts are derived from mesenchymal stem cells, and responsible for the synthesis and mineralization of the bone matrix[4]. Osteoblasts play a critical role in the process of osteoclast differentiation via expression of RANKL and M-CSF. This tight coupling between osteoblasts and osteoclasts provide the molecular mechanism of physiological bone remodeling which is an active dynamic balance between bone formation and resorption[7]. Interestingly, bone metastatic tumor cells hijack this physiological mechanism to induce osteolysis (i.e. tumor-induced osteolysis). For example, tumor cells produce numerous factors such as parathyroid hormone-related peptide (PTHRP), vascular endothelial growth factor-A (VEGF-A) and hepatocyte growth factor (HGF) which stimulate osteoblasts, leading to increased expression of RANKL and M-CSF, thereby stimulating osteoclastogenesis in the bone metastatic tumor microenvironment. Moreover, hematopoietic stem cell niche in osteoblasts (i.e. osteoblastic niche) plays critical roles in skeletal colonization and establishment of early metastatic lesions[8]. Accordingly, therapeutic approaches are under development for suppressing osteoblasts in bone metastasis. Most notably, tyrosine kinase inhibitors (TKI) targeting c-Met and VEGFR showed a partial or complete resolution of bone lesions measured by bone scans, as well as improvement of cancer-induced bone pain[9,10]. Bone scans using Tc-99m bisphosphonate directly measure bone turnover rate and osteoblastic activity in order to indirectly determine the bone tumor burden, and thus the clinical data strongly suggest that c-Met and VEGFR signaling axes are important in the stromal compartment of bone metastasis. However, the TKIs suppress both c-Met and VEGFR in tumor and bone compartments, and the clinical outcomes
from the c-Met- and/or VEGFR-targeted experimental therapeutics are mixed effects of suppressing tumor cells and the normal stromal cells. More precise analyses of the pharmacological efficacy and advanced knowledge on underlying mechanisms are essential to improve the forthcoming clinical trials of these novel therapies. We therefore aimed to dissect the net effects of suppressing c-Met and VEGFR specifically in the stromal compartment of the bone metastatic microenvironment using in vitro and in vivo models.

2. MATERIALS AND METHODS

2.1. Cells

Human prostate adenocarcinoma cells PC-3 and C4-2b were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin antibiotics. To establish cabozantinib-resistant PC-3 or C4-2b cells, cells were passaged in complete RPMI-1640 media containing 10µM cabozantinib for 8 weeks. Murine pre-osteoblasts, MC3T3-E1 subclone 4, were maintained in α-MEM supplemented with 10% FBS and antibiotics. Human fetal osteoblasts, hFOB1.19, carrying temperature-sensitive SV40T antigen were cultured at 33°C (permissive temperature; for expansion and maintenance) or at 37°C (non-permissive temperature; for experimental conditions) in DMEM/Ham’s F-12 media with 10% FBS and antibiotics[11]. Murine calvarial osteoblast cultures were established by collagen digestion of calvaria of 3- to 5-day old C57BL6 pups, and maintained in α-MEM supplemented with 10% FBS and antibiotics[12].
2.2. BrdU Cell Proliferation Assay

Cabozenzantinib sensitivity of the parental and cabozantinib-resistant prostate cancer cells (PC-3 and C4-2B) was determined by BrdU incorporation cell proliferation assays. Cells were plated on 96-well plates in 2% FBS-containing RPMI-1640 media followed by cabozantinib (10µM) or DMSO treatment. At 24-, 48-, 72- and 96-hour time points, cells were pulsed with bromodeoxyuridine (BrdU) for 4 hours (n=5/group). Wells were subsequently fixed, denatured, washed and detected with anti-BrdU antibody and HRP-conjugated secondary antibody (Cell Signaling), followed by 3, 3’, 5, 5’ tetramethylbenzidin (TMB)-based colorimetric quantification of the incorporated BrdU.

2.3. Determination of the IC50

The IC50 values of cabozantinib against the parental and cabozantinib-resistant prostate cancer cells (PC-3 and C4-2B) were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Cells were plated on 96-well plates in 2% FBS-containing RPMI-1640 media 24 hours before treatment with increasing doses of cabozantinib (0.1nM to 100mM) or DMSO (n=5/group). MTT assays were performed as previously described[13]. The IC50 values were calculated by nonlinear regression analyses of 4-parameter slopes of dose vs. response using GraphPad™ Prism version 5.

2.4. Cabozantinib
Cabozanitinib (also known as XL-184), an inhibitor of c-Met and VEGFR2 and additional receptor tyrosine kinases, was obtained from the Cancer Therapy Evaluation Program (CTEP) of the U.S. National Cancer Institute[14]. Cabozantinib was dissolved in DMSO (for *in vitro* assays) or suspended in distilled water using a sonicating water bath (for *in vivo* administration) according to the manufacturer’s instructions.

2.5. Small interfering RNA transfection

Human or mouse *MET*- and *KDR*-specific siRNA oligonucleotides were purchased from Santa Cruz Biotech. Scrambled sequence oligonucleotides were used as transfection controls. Lipofectamine 2000 was used to transfect oligonucleotides according to the manufacturer’s instructions[15].

2.6. Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent, followed by reverse transcription. Quantitative PCR was performed using the TaqMan gene expression assays. The primers included: murine *Rankl* (Mm00441908_m1), murine *Mcsf* (Mm00432686_m1), murine *Gapdh* (TaqMan 4308313), human *RANKL* (Hs00243522_m1), human *MCSF* (Hs00174163_m1) and human *GAPDH* (TaqMan 4310884E). Fold gene expression was calculated using $2^{\Delta\Delta Ct}$ method, and normalized to GAPDH.

2.7. Osteoclastogenesis

Bone marrow monocytes (BMM) were isolated via Ficoll-gradient centrifugation of femoral bone marrow cells and seeded onto 48-well plates ($1\times10^5$ cells/well). Cells
were cultured in α-MEM supplemented with 10% FBS and antibiotics for 5 days. For positive controls, murine M-CSF (30ng/mL, Peprotech) and RANKL (50ng/mL, Peprotech) were added. For experimental treatments, conditioned media were produced by treating MC3T3E1 subclone 4 cells with HGF (30ng/mL), VEGF-A (50ng/mL), IGF (100ng/mL) and cabozantinib (10nM). Tartrate-resistant acid phosphatase (TRAP) staining was performed to detect multi-nucleated TRAP⁺ cells.

2.8. Mice and in vivo tumors

All procedures were in accordance with guidelines and a protocol approved by the institutional animal care and use committee (IACUC) of the Vanderbilt University Medical Center. Four- to five-week old male athymic mice were purchased from Harlan Laboratories. Prostate cancer cells (5×10⁵ C4-2b cells; or 2×10⁴ PC-3 cells) were suspended in 20µL Hank’s balanced salt solution, and injected into the proximal tibia as previously described[16]. After 3 days, mice were randomized into two groups, and administered daily with cabozantinib (60mg/kg) or control diluent via oral gavage for 3 weeks. Tumor growth and bone destruction were determined by bioluminescence (Xenogen IVIS-200) or digital X-ray images (Faxitron).

2.9. Histology

At necropsy, tissues were harvested, fixed in 2% para-formaldehyde and decalcified in 20% EDTA solutions. Paraffin-embedded tissues were cut into 5µm sections. Modified H&E staining was performed as previously described[17].
2.10. Statistical analysis

Statistical analyses were performed by GraphPad™ Prism version 5. All data were tested for normality by the Shapiro-Wilk test. Student’s t-test (for normally distributed samples) or Mann-Whitney U-test (for non-parametric analyses) was performed to compare groups. All statistical analyses were two-sided.

3. RESULTS

3.1. HGF-induced M-CSF and RANKL expression in osteoblasts is suppressed by Cabozantinib

Cabozantinib (XL-184) is a multi-targeted kinase inhibitor that most potently inhibits c-Met and VEGFR2 receptors, whose ligands (HGF and VEGF-A, respectively) are enriched in the metastatic bone microenvironment. To investigate the effects of cabozantinib in the interactions between cancer cells and osteoblasts, three different types of osteoblastic cells, including mouse calvarial primary osteoblasts, MC3T3-E1 subclone 4 (murine pre-osteoblastic cell line) and hFOB1.19 (temperature-sensitive SV40T Ag-transformed human osteoblasts) were selected. HGF stimulation increased M-CSF and RANKL expression in osteoblasts, and this effect was suppressed by cabozantinib in a dose-dependent manner (Figure 1 and Supplemental Figure 1). We confirmed that both mRNA and protein levels of M-CSF and RANKL were changed in response to HGF with or without cabozantinib, indicating that HGF and cabozantinib affects secretory proteins as well as the membrane-bound forms of RANKL.
3.2. HGF, VEGF-A and IGF1, but not EGF, increased M-CSF and RANKL expression in osteoblasts

Recently, Varkaris et al. demonstrated that IGF1 trans-activates c-Met via Src[18], and Breindel et al. demonstrated that EGF trans-activates c-Met via MAPK[19]. Based on these findings and our results described above, we addressed whether VEGF-A, IGF1 and EGF can increase M-CSF and RANKL expression in osteoblasts, similar to HGF. We found that HGF, VEGF-A and IGF1, but not EGF, increased *M-csf* and *Rankl* expression in murine and human osteoblasts after 12- and 24-hour treatment (Figure 2). IGF1 treatment was stimulatory as well, but only after 24-hour treatment, in line with results from Varkaris et al. has shown that IGF1-mediated c-Met trans-activation required an incubation time longer than 16 hours (Figure 2C and D). These results support the hypothesis that the stimulatory effects of IGF1 on *M-csf* and *Rankl* expression in osteoblasts is mediated by trans-activation of c-Met. While EGF has been shown to activate c-Met in many types of cancer cells, we did not observe EGF-induced *Rankl* and/or *M-csf* expression in osteoblasts, although EGF increased *Mmp9* expression, thus confirming ligand potency (Figure 2E).

3.3. IGF1 increases M-CSF and RANKL via c-Met

In order to determine whether the induction of M-CSF and RANKL expression by IGF is indeed mediated by c-Met receptor activation, we knockdown down c-Met in MC3T3 E1 and hFOB1.19 osteoblasts and measured the expression of *M-csf* and *Rankl* following IGF1 treatment for 24 hours. Figure 3 shows that c-Met knockdown abolished the effects of IGF1 on M-CSF and RANKL expression, indicating that c-Met is required in
IGF1 effects on osteoblasts. **Supplemental Figures 3 and 4** confirmed that HGF- and VEGF-A-induced \( \text{Rankl} \) and \( M-csf \) expression are mediated by c-Met and VEGFR2, respectively. These results further support the notion that the stimulatory effect of IGF1 on \( M-csf \) and \( \text{Rankl} \) expression in osteoblasts is mediated by trans-activation of c-Met.

### 3.4. Cabozantinib suppresses *in vitro* osteoclastogenic activity of HGF-, VEGF-A- and/or IGF-conditioned media from osteoblasts

Data in **Figures 1, 2 and 3** collectively demonstrate that HGF, VEGF-A and IGF1 stimulate osteoblasts to express RANKL and M-CSF, two essential factors for osteoclastogenesis. To confirm the functional impact of HGF, VEGF-A and IGF1 effect on this process, we quantified the formation of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts following culture of murine bone marrow monocytes (BMM) with the cell-free conditioned media from MC3T3E1 osteoblasts treated with HGF, VEGF-A and/or IGF1 in combination with cabozantinib (10\( \mu \text{M} \)) or diluent vehicle (DMSO) for 24 hours. BMM formed TRAP-positive osteoclasts when treated with recombinant M-CSF and RANKL (**Figure 4A**, positive control). As shown in **Figure 4B** (upper panels), the conditioned medium from MC3T3E1 osteoblasts treated with HGF-, VEGF-A- or IGF- effectively induced osteoclastogenesis even without the addition of recombinant RANKL and M-CSF, suggesting that HGF-, VEGF-A- or IGF1-conditioned media from MC3T3E1 osteoblast culture contained sufficient level of functional RANKL and M-CSF. In addition, dual or triple combinations of HGF + VEGF-A or HGF + VEGF-A + IGF further increased osteoclastogenesis over single treatments alone. Most importantly, the addition of cabozantinib to the conditioned media from the
cells treated with HGF, VEGF-A or IGF significantly reduced osteoclastogenesis. We ruled out direct effects of HGF, VEGF-A or IGF on osteoclastogenesis by the observation that none of these factors induced osteoclast formation when added directly in the culture medium of BMM in the presence of saline CM (Figure 4C). In addition, we tested whether cabozantinib directly suppress osteoclastogenesis. Figure 4D shows that cabozantinib suppresses osteoclastogenesis compared with the BMM + M-CSF + RANKL treated control group in Figure 4A. However, cabozantinib-treated conditioned media effects were far more potent (lower panels in Figure 4B) which supports that the main mechanism of cabozantinib effects on osteoclasts is through the suppression of osteoblastic expression of RANKL and M-CSF.

3.5. Dual targeting c-Met and VEGFR2 in the stromal cells suppresses growth and osteolytic activity of prostate cancer bone metastases in vivo

The above in vitro data collectively demonstrate that inhibition of c-Met and VEGFR2 suppresses osteoblast-dependent and M-CSF/RANKL-dependent osteoclastogenesis, suggesting that anti-tumoral efficacy of cabozantinib observed in the recent clinical trials stems from mixed effects on both tumor and stromal compartments. We thus sought to analyze the net effects of cabozantinib on the tumor microenvironment in the context of prostate cancer cell growth and osteolytic activity, independently of its effect on cancer cells in vivo. To this aim, we created two cabozantinib-resistant prostate cancer cell lines by exposing PC-3 and C4-2b cells to high concentration of cabozantinib (10µM) for 8 weeks. As a result, cabozantinib-resistant PC-3 and C4-2b cells (termed CR-PC-3
and CR-C4-2b herein) showed a more than 600-fold increase in the IC50 of cabozantinib (Figures 5A and 6A). In addition, both cell lines showed cabozantinib-independent cellular proliferation in vitro (Figures 5B, 5C, 6B and 6C). We then inoculated CR-PC-3 cells in the proximal tibiae of male athymic nude mice, followed by cabozantinib treatment for 3 weeks. Cabozantinib-treated mice had significantly reduced CR-PC-3 tumor size measured by in vivo bioluminescence imaging (Figures 5D and 5E). Similarly, CR-C4-2b tumor growth was significantly suppressed by cabozantinib (Figure 6G). We subsequently isolated and cultured CR-PC-3 and C4-2B tumor cells from the mice, and confirmed that IC50 values of cabozantinib did not change after 3-week in vivo growth. These data indicate that the cabozantinib effects on the CR-PC-3 and CR-C4-2B tumors were more dependent on the alterations in the tumor microenvironment that in the tumor cells. Most importantly, tumor-induced osteolysis was significantly reduced in the cabozantinib-treated mice as determined by measuring cortical bone loss on the digital X-ray images tumor-bearing tibiae (Figures 5F, 5G, 6D, 6E, 6F). In agreement with our in vitro data, soluble RANKL and M-CSF protein levels in the flushed femoral bone marrow of cabozantinib-treated mice was significantly lower than in the control group (Figure 5H and 5I). Similarly, tumor-associated osteoclasts were decreased in CR-C4-2b tumor-bearing tibiae (Figure 6H).

4. DISCUSSION

The current study demonstrated that osteoblasts express RANKL and M-CSF in response to HGF, IGF1 and VEGF-A abundant in the tumor microenvironment, leading to increased osteoclastogenesis and osteolysis. Our data furthermore showed that
inhibition of c-Met and VEGFR2 specifically in osteoblasts effectively suppressed the pro-osteoclastogenic effect of HGF, IGF1 and VEGF-A \textit{in vitro} and the growth of prostate cancer bone metastases and osteolysis \textit{in vivo}, and that the positive effects of cabozantinib exhibited in the clinical trials in patients with bone metastatic prostate cancer are in part mediated by suppression of osteoblasts in the tumor microenvironment.

Cabozantinib, an inhibitor of receptor tyrosine kinases including c-Met and VEGFR2, indeed showed clinical efficacy in advanced prostate cancer patients, including reduction of tumor size, pain and narcotic use and increased progression-free survival in a Phase 2 randomized discontinuation trial[9,14]. More importantly, cabozantinib strikingly reduced Tc-99m bisphosphonate bone scans as well as bone turnover blood markers such as alkaline phosphatase (ALP, a bone formation marker) and c-telopeptide (CTx, a bone resorption marker) within 12 weeks implicating the effects of cabozantinib on the stromal compartment of the tumor microenvironment[10]. However, in the subsequent Phase 3 COMET-1 clinical trial encompassing 1028 metastatic castration-resistant prostate cancer patients, cabozantinib significantly improved bone biomarkers, bone scan responses (BSR), and radiographic progression-free survival (rPFS) but failed to meet the primary endpoint, i.e. improvement of overall survival (OS) compared with the control group (prednisone alone-treated group)[20]. These inconsistencies between Phase 2 and 3 trials, and also between BSR/rPFS and OS is not fully understood but may be in part be attributable to imbalances in subsequent anti-cancer therapy or differences in the patient population enrolled. Additionally, a
correlation of BSR/rPFS with OS has not been clearly established. We postulate that the clinical trials results may have been different if the patient had been selected more precisely on the basis of molecular mechanism, such as those with high osteoblastic activity measured either by bone scans or serum bone turnover markers, or if the primary endpoints had been reduction of the complications associated with skeletal-related events (e.g. pathologic fractures). Currently none of the bone-targeted agents such as Zometa® or other bisphosphonates do not have survival benefits, and thus cabozantinib, if the primary efficacy is on the stromal compartment, is reasoned not to have survival benefits. Therefore, a deeper understanding of cabozantinib’s and/or other c-Met inhibitors’ anti-tumoral efficacy warrant extensive further research.

The significance and novelty of our work include the use of drug-resistant cancer cells to dissect the effects of tumor microenvironment inhibitors in murine bone metastasis models. Numerous publications supported the role of HGF/c-Met axis, VEGF-A/VEGFR2 axis and IGF1 during the progression of bone metastasis, using various tumor models[21]. For example, Tsai et al. and Chen et al. demonstrated that HGF increases osteopontin and BMP-2 expression in osteoblasts, respectively[22,23]. Recently, Dai et al. showed that cabozantinib has direct anti-tumoral activity in their pre-clinical in vivo mouse models of metastatic prostate cancer, and more importantly, the data suggest that cabozantinib modulate osteoblast activity and contribute to anti-tumoral efficacy[24,25]. Watanabe et al. used another c-Met/VEGFR2 dual kinase inhibitor (TAS-115) and showed that the inhibitor directly suppressed FMS-dependent osteoclast differentiation and bone destruction induced by prostate cancer[26,27].
Fioramonti et al. provided additional evidence that cabozantinib decreases tumor-induced osteolysis via direct effects on osteoclasts as well as indirect effects on osteoblasts (reduction of RANKL and OPG expression) in agreement with our data[28]. More recently, Patnaik et al. demonstrated that cabozantinib induces CXCL12 and high mobility group box 1 (HMGB1) proteins, leading to increased neutrophil chemoattraction in the prostate tumor tissues of PTEN/p-53-deficient mice, suggesting that cabozantinib suppress prostate tumor by activating anti-tumoral innate immunity[29].

Collectively, the data from multiple groups consistently indicate that c-Met and VEGFR-2 play important roles in the complex interactions among tumor cells, osteoclasts and osteoblasts. Our data in this manuscript stand in line with all of the above clinical and pre-clinical data, and provide more direct evidence that cabozantinib efficacy is indeed in part mediated by directly suppressing osteoblastic activity in the tumor microenvironment. One potential limitation of our work is lack of an in vivo experiment comparing the effects of cabozantinib in parental (i.e. cabozantinib-sensitive) PC-3 and/or C4-2b intra-tibial or subcutaneous tumor models, with the data in Figure 5 and 6. These experiments could have been address the inhibition of stromal compartment is equally or more important as the direct effects on the tumor cells.

In conclusion, our data supports that c-Met and VEGFR2 in the osteoblasts are promising therapeutic targets of bone metastasis, and further studies on the molecular mechanisms of cabozantinib are beneficial to metastatic prostate and breast cancer patients.
ACKNOWLEDGEMENTS

The authors thank Exelixis, Inc. and the Cancer Therapy Evaluation Program (CTEP) of the National Cancer Institute, the U. S. National Institutes of Health for providing cabozantinib and also for reviewing this manuscript. The authors thank the Vanderbilt University Institute of Imaging Science for technical assistance with in vivo imaging (Faxitron X-ray and Xenogen IVIS bioluminescence imaging).

CONFLICT OF INTEREST

All authors declare no financial conflict of interest.

GRANTS SUPPORT

This work was in part supported by the National Research Foundation of Korea (Grants No. 2015R1C1A1A01051508 to S.I.P. and 2016RD1A1B03933826 to Y.M.W.) and the Ministry of Health and Welfare of the Republic of Korea (National R&D Program for Cancer, No. 1720140 to S.W.C. and S.I.P).
FIGURE LEGENDS

Figure 1. Effects of HGF and cabozantinib on RANKL and M-CSF expression in osteoblasts

Human and murine osteoblastic cells were stimulated with HGF (30ng/mL) in combination with cabozantinib (XL-184), followed by quantitation of M-CSF (A-C; measured by ELISA of culture supernatant) and RANKL (D-H; measured by quantitative RT-PCR of cell lysates (D-F) or ELISA of culture supernatant (G-H)). Data are mean ± SEM. *, P<0.05 and **, P<0.01, Student’s t-test vs. HGF alone treatment.

Figure 2. Effects of HGF, VEGF-A, IGF1 and EGF on M-CSF and RANKL expression in osteoblasts

(A, B) Murine (MC3T3E1 subclone 4) and human (hFOB1.19) osteoblastic cells were treated with HGF (30ng/mL), VEGF-A (50ng/mL), IGF1 (100ng/mL) or EGF (50ng/mL) for 24 hours, followed by quantitative RT-PCR analyses of MCSF and RANKL gene expression.

(C, D) The above experiments shown in the panels A and B were repeated but incubation time was reduced to 12 hours.

(E) EGF-treated MC-3T3E1 subclone 4 cells were analyzed for Mmp9 gene expression.

All data are mean ± SEM. *, P<0.05 and **, P<0.01, Student’s t-test vs. control.

Figure 3. Effects of c-Met knockdown on IGF-induced MCSF and RANKL expression

Murine (MC3T3E1 subclone 4; A, C) and human (hFOB1.19; B, D) osteoblastic cells
were transfected with \textit{MET}-(encoding c-Met) or \textit{KDR}-(encoding VEGFR1) specific siRNA oligonucleotides, followed by 24-hour IGF1 treatment. Non-transfected cells or scrambled sequence siRNA were used as controls. Quantitative RT-PCR was performed to measure \textit{MCSF} and \textit{RANKL} gene expression. Data are mean ± SEM. **, \(P<0.01\), Student’s t-test vs. respective saline treatment control.

**Figure 4. Osteoclastogenesis activities induced by HGF-, VEGF-A- or IGF1-conditioned media from osteoblastic cell cultures**

MC3T3E1 subclone 4 cells were treated with HGF, VEGF-A or IGF1 either alone or in combination, and with DMSO or cabozantinib (10\(\mu\)M) for 24 hours, followed by collection of cell-free supernatants (i.e. conditioned media). Subsequently, murine bone marrow monocytes (BMM) were treated with recombinant M-CSF and/or RANKL (\textbf{Panel A}; positive controls), or the conditioned media (\textbf{Panel B}). To rule out potential direct effects of HGF, VEGF-A, IGF1 or XL-184 on osteoclastogenesis, BMMs were treated with saline-conditioned media (Saline-CM), HGF, VEGF-A, IGF1, or XL-184 as indicated (\textbf{Panels C and D}). BMMs were cultured for five days, followed by TRAP staining. Experiments were performed in triplicates, and repeated more than 3 times. Representative bright field microscopic images are shown. Scale bar, 200\(\mu\)m.

**Figure 5. \textit{In vivo} efficacy of cabozantinib in a PC-3 prostate cancer bone metastasis model**

(A) Human prostate adenocarcinoma cells, PC-3, were exposed to high concentration of cabozantinib (10\(\mu\)M) for 8 weeks to produce cabozantinib (XL-184)-resistant PC-3
cells. IC50 concentration was determined by treating parental- or cabozantinib-resistant PC-3 cells in increasing concentrations of cabozantinib (10^{-1}~10^{8}\text{nM}), followed by MTT assay. IC50 and 90% confidence intervals (CI) are shown.

(B, C) Parental or cabozantinib-resistant PC-3 cells were treated with DMSO (dotted lines and open circles) or cabozantinib (10nM; solid lines and closed dots), and BrdU^{+} cells were quantitated at indicated time points. Data are mean ± SEM. P values are from linear regression analyses.

(D, E) Cabozantinib-resistant PC-3 cells were inoculated into the proximal tibiae of male athymic mice, followed by treatment with cabozantinib or diluent for 3 weeks. Tumor size was determined by in vivo bioluminescence imaging. Quantitation of photon emission (D) and images (E) are shown.

(F, G) Tumor-bearing hind limbs were analyzed by Faxitron digital X-ray imaging. Cortical bone loss was measured.

(H, I) RANKL and M-CSF protein levels were determined by ELISA of femoral bone marrow flush.

(D-I) Dots represent individual tumors, and whiskers represent median ± interquartile range. P values are from Mann-Whitney U tests.

**Figure 6. In vivo efficacy of cabozantinib in a C4-2B prostate cancer bone metastasis model**

(A) Human prostate adenocarcinoma cells, C4-2B, were exposed to high concentration of cabozantinib for 8 weeks to produce cabozantinib (XL-184)-resistant C4-2B cells. IC50 concentration was determined by treating parental- or cabozantinib-resistant C4-
2B cells in increasing concentrations of cabozantinib ($10^{-1}$~$10^8$ nM), followed by MTT assay. IC50 and 90% confidence intervals (CI) are shown.

(B, C) Parental or cabozantinib-resistant C4-2B cells were treated with DMSO (dotted lines and open circles) or cabozantinib (10nM; solid lines and closed dots), and BrdU+ cells were quantitated at indicated time points. Data are mean ± SEM. $P$ values are from linear regression analyses.

(D-F) Cabozantinib-resistant C4-2B cells were inoculated into the proximal tibiae of male athymic mice, followed by treatment with cabozantinib or diluent for 3 weeks. Tumor-bearing hind limbs were analyzed by Faxitron digital X-ray imaging. Osteolytic area and cortical bone loss were measured. Fractured fibula in the right panel of panel D is a histological artifact, not a biological effect.

(G, H) Tumors were H&E stained and photographed. Tumor areas were measured, and representative images were shown. White arrows indicate osteoclasts. Scale bar, 100µm.

(E-G) Dots represent individual tumors (area), and whiskers represent median ± interquartile range. $P$ values are from Mann-Whitney $U$ tests.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Effects of HGF and cabozantinib on RANKL and M-CSF expression in osteoblasts

Human (hFOB1.19) and murine (primary calvarial) osteoblastic cells were stimulated with HGF (30ng/mL) in combination with cabozantinib (XL-184), followed by quantitation of M-CSF (CSF1) measured by reverse transcription-quantitative PCR (RT-qPCR) (A, B) or by ELISA (C, D).

Supplemental Figure 2. Effects of HGF and cabozantinib on cellular proliferation of osteoblasts

Human (hFOB1.19) and murine (primary calvarial) osteoblastic cells were stimulated with HGF (30ng/mL) in combination with cabozantinib (XL-184), followed by quantitation of cell proliferation by BrdU uptake (A, B) or MTT (C, D) assays.

Supplemental Figure 3. Effects of c-Met or VEGFR2 knockdown on HGF-induced MCSF and RANKL expression

Murine (MC3T3E1 subclone 4; A, C) and human (hFOB1.19; B, D) osteoblastic cells were transfected with MET- (encoding c-Met) or KDR- (encoding VEGFR1) specific siRNA oligonucleotides, followed by 24-hour HGF treatment. Non-transfected cells or scrambled sequence siRNA were used as controls. Quantitative RT-PCR was performed to measure MCSF and RANKL gene expression. Data are mean ± SEM. **, P<0.01, Student’s t-test vs. respective saline treatment.
Supplemental Figure 4. Effects of c-Met or VEGFR2 knockdown on VEGFA-induced MCSF and RANKL expression

Murine (MC3T3E1 subclone 4; A, C) and human (hFOB1.19; B, D) osteoblastic cells were transfected with MET- (encoding c-Met) or KDR- (encoding VEGFR1) specific siRNA oligonucleotides, followed by 24-hour VEGFA treatment. Non-transfected cells or scrambled sequence siRNA were used as controls. Quantitative RT-PCR was performed to measure MCSF and RANKL gene expression. Data are mean ± SEM. **, P<0.01, Student’s t-test vs. respective saline treatment.
REFERENCES


Figure 1

A. Mouse Calvarial Osteoblasts

B. MC3T3E1

C. hFOB1.19

D. Mouse Calvarial Osteoblasts

E. MC3T3E1

F. hFOB1.19

G. Mouse Calvarial Osteoblasts

H. hFOB1.19

- Control
- HGF
- HGF+XL184 0.01µM
- HGF+XL184 10µM
Figure 2

A. 24-Hour Treatment

24-Hour Treatment

B. 12-Hour Treatment

C. 12-Hour Treatment

D. 12-Hour Treatment

E. EGF

MC3T3 E1

hFOB1.19

C: Saline Control
H: HGF
V: VEGF-A
I: IGF1
E: EGF

** P = 0.97
** P = 0.92
* P = 0.86
P = 0.95
P = 0.91
P = 0.43
P = 0.92
P = 0.90
P = 0.86
P = 0.59
P = 0.90
P = 0.65

P = 0.43
P = 0.92
P = 0.86
P = 0.59
P = 0.92
P = 0.95
P = 0.86
P = 0.65

P = 0.90
P = 0.92
P = 0.95
P = 0.86
P = 0.65
P = 0.59
P = 0.92
P = 0.95

P = 0.29
P = 0.21
P = 0.29
P = 0.29
P = 0.21
P = 0.21
P = 0.21
P = 0.21

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86

P = 0.90
P = 0.90
P = 0.90
P = 0.90
P = 0.90
P = 0.90
P = 0.90
P = 0.90

P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86

P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86
P = 0.86

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29
P = 0.29

E. EGF

12 hr. 24 hr.
Figure 3

A. MC3T3 E1

B. hFOB1.19

C. D.

**P = 0.84**

**P = 0.08**

**P = 0.83**

**P = 0.34**

Saline

IGF
Figure 4

A. 200µm

BMM  BMM +M-CSF  BMM +M-CSF +RANKL

B.

HGF  VEGF-A  IGF  HGF+VEGF  HGF+VEGF-A +IGF

HGF +XL184  VEGF-A +XL184  IGF +XL184  HGF+VEGF +XL184  HGF+VEGF-A +IGF+XL184

Conditioned Media from MC3T3E1 cells

C.

Saline-CM +HGF  Saline-CM +VEGF-A  Saline-CM +IGF

D.

BMM +M-CSF + RANKL +XL184
Figure 5

A. Parental PC-3
IC50=11.3µM
(95% CI=8.6~14.8)
XL184-Resistant PC-3
IC50=7.13mM
(95% CI=5.50~9.24)

B. Parental PC-3 + DMSO
Parental PC3 + XL184
BrdU (Abs 450)
P<0.01

C. Resistant PC-3 + DMSO
Resistant PC-3 + XL184
BrdU (Abs 450)
P=0.837

D. XL-184 Control

E. Control
XL-184

F. Cortical Bone Loss
P<0.05

G. Control
XL-184

H. RANKL (pg/ml)
P<0.01

I. M-CSF (pg/ml)
P<0.01
Figure 6

A.  

- Parental C4-2B  
  IC50=4.1µM  
  (95% CI=2.1~7.9)  
- XL184-Resistant C4-2B  
  IC50=2.7mM  
  (95% CI=1.9~3.7)

B.  

- Parental C4-2B + DMSO  
- Parental C4-2B + XL184

C.  

- Resistant C4-2B + DMSO  
- Resistant C4-2B + XL184

D.  

Control  
XL-184

E.  

Osteolytic Area (Pixels)

F.  

Cortical Bone Loss (x 1000 Pixels)

G.  

Tumor Area (Pixels)

H.  

Control  
XL-184

100µm
Supplemental Figure 1

A. MC3T3 E1

B. hFOB1.19

C. Mouse Calvarial Osteoblasts

D. hFOB1.19

- Control
- HGF
- HGF+XL184 0.01µM
- HGF+XL184 10µM
Supplemental Figure 2

A. Mouse Calvarial Osteoblasts

B. hFOB1.19

C. D.
Supplemental Figure 3

A. MC3T3 E1

B. hFOB1.19

C.

D.

**P = 0.06**

**P = 0.35**

**P = 0.52**

**P = 0.70**

Saline

HGF
Supplemental Figure 4

A. MC3T3 E1

B. hFOB1.19

C. 

D. 

**P = 0.42**

**P = 0.16**

**P = 0.09**

**P = 0.16**
HIGHLIGHTS

• HGF, VEGF-A and IGF1 increase M-CSF and RANKL in osteoblasts of the bone metastasis
• HGF, VEGF-A and IGF1 induce osteoclastogenesis via activation of osteoblasts
• A c-Met/VEGFR2 inhibitor suppresses osteoblasts and subsequent osteoclastogenesis
• Targeting c-Met and VEGFR2 in osteoblast suppresses prostate cancer bone metastasis
• Osteoblasts are promising stromal cell target for the treatment of bone metastasis