ARTICLE
Journal of Cellular Biochemistry 115:2033–2038 (2014)

Journal of Cellular Biochemistry

Antitumor Agent Cabozantinib Decreases RANKL Expression in Osteoblastic Cells and Inhibits Osteoclastogenesis and PTHrP-Stimulated Bone Resorption

Paula H. Stern[1]* and Keith Alvares[2]

[1]*Department of Pharmacology, Northwestern University Feinberg School of Medicine, Chicago, Illinois*

[2]*Department of Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, Illinois*

ABSTRACT

Cabozantinib, an inhibitor of vascular endothelial growth factor and hepatocyte growth factor signaling, decreases bone lesions in patients with prostate cancer. To determine direct effects of cabozantinib on bone, resorption in neonatal mouse bone organ culture and on gene expression, proliferation, and phenotypic markers in osteoblast and osteoclast cell lines were examined. Cabozantinib, 0.3 and 3 μM, prevented PTHrP-stimulated calcium release from neonatal mouse calvaria. Since the effect on resorption could reflect effects on osteoblasts to prevent osteoclast activation, or direct inhibition of osteoclasts, responses in osteoblastic and osteoclast precursor cell lines were examined. Twenty-four-hour treatment of osteoblastic MC3T3-E1 cells with 3 μM cabozantinib decreased expression of receptor activator of NFkB ligand (RANKL) and alkaline phosphatase. Forty-eight-hour treatment of MC3T3-E1 cells with 3 μM cabozantinib inhibited cell proliferation and decreased MTT activity. Effects on alkaline phosphatase activity were biphasic, with small stimulatory effects at concentrations below 3 μM. When RAW 264.7 osteoclast precursor cells differentiated with 20 ng/ml RANKL were co-treated for 24 h with 3 μM cabozantinib, expression of RANK, TRAP, cathepsin K, alpha v or beta 3 integrin, or NFATc1 were unaffected. Five-day treatment of RANKL-treated RAW 264.7 cells with 3 μM cabozantinib decreased TRAP and MTT activity. The results suggest that the osteoblast could be the initial target, with subsequent direct and indirect effects on osteoclastogenesis leading to decreased resorption. The multiple effects of cabozantinib on the cell microenvironment of bone are consistent with its effectiveness in reducing lesions from prostate cancer metastases. J. Cell. Biochem. 115: 2033–2038, 2014.

KEY WORDS: CABOZANTINIB; VEGF INHIBITOR; BONE; BONE METASTASES; BONE RESORPTION; OSTEOBLAST; OSTEOCLAST; RECEPTOR ACTIVATOR OF NFκB LIGAND (RANKL)

The skeleton is the primary site of prostate cancer metastases, with 70–80% of prostate cancer patients developing skeletal metastases [Chiarodo, 1991] that spread most commonly to the well vascularized areas of the skeleton such as the vertebral column, ribs, skull and the proximal ends of the long bones [Carlin and Andriole, 2000]. The skeletal changes resulting from skeletal metastases can cause severe bone pain, bone marrow suppression, leukopenia, hypercalcemia, and pathologic fractures [Chiarodo, 1991]. The skeletal metastases result in mixed osteoblastic and osteolytic lesions [Cook et al., 2006]. There is the appearance of woven bone [Charhon et al., 1983], a less organized bone that develops in response to injury and is associated with accelerated osteoblastic activity [Turner et al., 1992]. Studies have shown that osteoblastic metastases form at sites of previous osteoclastic resorption, indicating that bone resorption might be required to initiate subsequent osteoblastic bone formation [Carlin and Andriole, 2000; Keller et al., 2001; Zhang et al., 2001]. In addition to the metastatic lesion itself, androgen deprivation therapy and increasing age increase bone resorption and fracture risk, and these risk factors are also often present in the prostate cancer patients [Saylor et al., 2011].

There is a need for additional therapies to prevent and treat the bone metastases in patients with prostate cancer. Antiresorptive agents, specifically bisphosphonates such as zolendronate and the monoclonal antibody denosumab, initially developed for the treatment of osteoporosis, have been shown to have efficacy in the treatment of prostate cancer metastases [Saylor et al., 2011]. Bisphosphonates decrease the function and survival of osteoclasts through effects on protein prenylation and the promotion of apoptosis [Russell et al., 2008]. Denosumab, a monoclonal antibody, targets RANKL, a

*Correspondence to: Paula H. Stern, PhD, Department of Pharmacology, Northwestern University Feinberg School of Medicine, 320 E. Superior St, Chicago, IL 60611. Email: p-stern@northwestern.edu

Manuscript Received: 30 June 2014; Manuscript Accepted: 9 July 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 15 July 2014

DOI 10.1002/jcb.24879 • © 2014 Wiley Periodicals, Inc.

cytokine derived from osteoclasts and other cells that promotes osteoclast differentiation, activity and survival [Lacey et al., 2000]. Unfortunately, both of these agents have limitations at the doses required for treatment of bone metastases, one problematic side effect reported in a small but significant number of patients being the production of the condition osteonecrosis of the jaw (ONJ) characterized by impaired healing of the jaw after dental surgery [Almazrooa and Woo, 2009; Taylor et al., 2010]. Agents that could limit metastases by other mechanisms could be alternatives to these agents or could potentially be used in combination with lower doses of the antiresorptive drugs.

Cabozantinib (*N*-(4-((6,7-dimethoxyquinolin-4-yl) oxy) phenyl)-*N*-(4-fluorophenyl) cyclopropane-1,1-dicarboxamide), (XL184) an inhibitor of hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF) signaling, is a promising new agent for the treatment of prostate cancer. It has been shown to decrease tumor growth and angiogenesis and stabilize or decrease skeletal metastases, with marked decreases in radiologically detectable bone lesions [Saylor et al., 2011; Yakes et al., 2011; Lee et al., 2013; Smith et al., 2013]. Due to its effects on growth factor signaling, cabozantinib is an agent that would target bone metastases by quite different mechanisms from bisphosphonates and denosumab on bone cells and thus could substitute for or be used in conjunction with such other therapies.

In preclinical studies, cabozantinib inhibited the growth of intratibially injected prostate tumors and altered bone remodeling [Dai et al., 2013; Nguyen et al., 2013]. In those studies, osteoblast activity was affected in a dose-dependent biphasic manner, and osteoclast production was inhibited. The current experiments were carried out to further elucidate the effects of the drug on the bone microenvironment in the absence of tumor cells. Our current results demonstrate that cabozantinib inhibits resorption elicited by parathyroid hormone related protein (PTHrP). Both osteoblastic and osteoclastic cells were affected by cabozantinib treatment. The results are consistent with previously reported actions of cabozantinib on VEGF-dependent effects on bone and demonstrate that effects on the bone microenvironment could contribute to the marked effects on bone lesions observed in prostate cancer patients treated with cabozantinib.

MATERIALS AND METHODS

MATERIALS

Cabozantinib was obtained from SelleckBio; bPTHrP 1–34 was from BaChem; soluble hRANKL was from Peprotech.

ANIMALS

Mice were Harlan (Madison, WI) Sprague-Dawley, ICR strain. Six-day-old neonatal mice were used for the experiments.

CELLS

RAW 264.7 preosteoclast cells were purchased from American Type Culture Collection. They were used between passages 11 and 20. MC3T3-E1 osteoblastic cells were purchased from American Type Culture Collection. They were used between passages 15 and 25.

MEDIA

MC3T3-E1 cells and RAW 264.7 cells were maintained in α-MEM (Gibco/Life Technologies) supplemented with 10% fetal bovine serum (Cellgro), and 1% penicillin/streptomycin (Gibco/Life Technologies. Neonatal mouse calvaria were cultured in DMEM (Gibco/Life Technologies) supplemented with 10% fetal bovine serum (Cellgro) and 100 U/ml 1% potassium penicillin G (Sigma).

MTT ASSAY

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) activity was assessed in cells cultured on 96-well plates. Five hours before the end of the incubation, 20 μl of a sterile solution of 5 mg/ml MTT (Sigma) in phosphate-buffered saline (PBS) was added to each well of cells in culture medium in a 96-well plate. The plate was incubated for 5 h at 37°C in an atmosphere of 5% CO_2. At the end of the incubation, the medium was removed, and 200 μl demethylsulfoxide (DMSO) (Sigma) added to each well. The contents of the well were triturated to dissolve the crystals. After incubation at 37°C for 5 min, absorbance was read with a plate reader at 570 nm.

ALKALINE PHOSPHATASE ASSAY

Alkaline phosphatase activity was assessed in MC3T3-E1 cells cultured on 96-well plates, using a method published previously [Huang et al., 2000]. At the end of the culture period, medium was aspirated from each well, and the cells were washed with 0.2 ml ice-cold PBS. One hundred microliters of 50 mM diethanolamine pH 10.5, and 50 μl 5 mM Sigma phosphatase substrate (P 4744) in 100 mM glycine buffer, pH 10.5, containing 2 mM $MgCl_2$ were added to each well. The plate was then incubated at 37°C for 30 min, and then placed on ice. Fifty microliters of 0.1 N NaOH was added to each well and absorbance read at 410 nm.

TARTRATE-RESISTANT ACID PHOSPHATASE (TRAP) ASSAY

TRAP was assessed in RAW 264.7 cells cultured on 96-well plates. At the end of the culture period, medium was removed and 50 μl 10% formalin added to each well and incubated for 10 min. Formalin was removed and 100 μl 95% ethanol added to each well and incubated for 1–2 min. The ethanol was removed and the cells allowed to dry. The cells were then incubated for 30 min with 100 μl 5 mM Sigma phosphatase substrate (P 4744) in 50 mM Na citrate/10 mM Na tartrate buffer, pH 4.6 for 30 min. The substrate mix was added to 100 μl of 0.1 N NaOH in a 96-well plate and absorbance read at 410 nm.

DETERMINATION OF BONE RESORPTION BY NEONATAL MOUSE CALVARIA

The animal protocol [Stern and Krieger, 1983] was approved by the Northwestern University IACUC. Six-day-old ICR Harlan-Sprague Dawley mice were sacrificed by cervical dislocation and calvaria were dissected under aseptic conditions. The calvaria were placed in sterile tubes containing 2 ml of DMEM + 10%FBS + 1 mg/ml penicillin/streptomycin. Tubes were gassed with a mixture of 5% CO_2 in air, stoppered, and placed in a roller drum in an incubator maintained at 37°C. Media were changed and re-gassed at 24 and 48 h, and the final medium sample taken at 72 h. Calcium was determined with a Corning calcium titrator.

SEMIQUANTITATIVE ANALYSIS OF mRNA EXPRESSION BY REVERSE TRANSCRIPTION-RCR

Total RNA was isolated from the MC3T3 cells using the RNeasy mini-prep method (Qiagen) and first strand cDNA was synthesized using the Access RT-PCR system (Promega) following the manufacturers' protocols. The initial reverse transcription was done using AMV RT for 45 min at 45°C. The PCR reaction was as follows: 1 cycle at 94°C for 2 min, 55°C for 1 min, 68°C for 2 min; 30 cycles at 94°C for 30 s, 55°C for 1 min, 68°C for 2 min; 1 cycle at 94°C for 30 s, 55°C for 1 min, 68°C for 10 min.

The following mouse primer pairs were used:

Alkaline phosphatase (ALP) sense: 5′-GCC CTC TCC AAG ACA TAT A-3′;
antisense: 5′-CCA TGA TCA CGT CGA TAT CC-3′;
Receptor activator of NFκB ligand (RANKL) sense: 5′-ATC AGA AGA CAG CAC TCA CT-3′;
antisense: 5′-ATC TAG GAC ATC CAT GCT AAT GTT-3′;
Osteoprotegerin (OPG) sense: 5′-TGA GTG TGA GGA AGG GCG TTA C-3′;
antisense: 5′-TTT CTC GTT CTC TCA ATC TC-3′;
Receptor activator of NFκB (RANK) sense: ACC TCC AGT CAG CAA GAA GT;
antisense: TCA CAG CCC TCA GAA TCC AC;
Nuclear factor of activated T cells c1 (NFATc1) sense: CAA CGC CCT GAC CAC CGA TAG;
antisense: GGC TGC CTT CCG TCT CAT AGT;
Tartrate-resistant acid phosphatase (TRAP) sense: CAC GAT GCC AGC GAC AAG AG;
antisense: TGA CCC CGT ATG TGG CTA AC;
Integrin alphaV (alphaV) sense: GCC AGC CCA TTG AGT TTG ATT;
antisense: GCT ACC AGG ACC ACC GAG AAG;
Integrin beta3 (beta3) sense: TTA CCC CGT GGA CAT CTA CTA;
antisense: AGT CTT CCA TCC AGG GCA ATA;
Cathepsin K (CtsK) sense: GGA GAA GAC TCA CCA GAA GC:
antisense: GTC ATA TAG CCG CCT CCA CAG;
GAPDH sense: 5′-ACT TTG TCA AGC TCA TTT CC-3′;
antisense: 5′-TGC AGC GAA CTT TAT TGA TG -3′.

PCR products were analyzed by agarose gel chromatography with ethidium bromide staining. Results shown were replicated in a second experiment.

STATISTICAL ANALYSES

All studies were carried out at least in duplicate. Responses were analyzed for significance by ANOVA and Tukey's post test. Values $P \leq 0.05$ were considered significantly different.

RESULTS

EFFECT OF CABOZANTINIB ON BONE RESORPTION IN MOUSE CALVARIAL ORGAN CULTURES

Bone resorption elicted by PTHrP was assessed by release of calcium from cultured mouse calvaria. Cabozantinib, 3 μM, inhibited the evoked bone resorption elicited by PTHrP, 30 nM, and also inhibited calcium release from control bones (Fig. 1A). A lower cabozantinib concentration of 0.3 μM inhibited the effect of 3 nM PTHrP (Fig. 1B), but did not affect control bones (Fig. 1B). Since the effect of cabozantinib on resorption could reflect either direct inhibition of osteoclasts or effects on osteoblasts to prevent osteoclast activation, responses in osteoclastic and osteoblastic cell lines were examined.

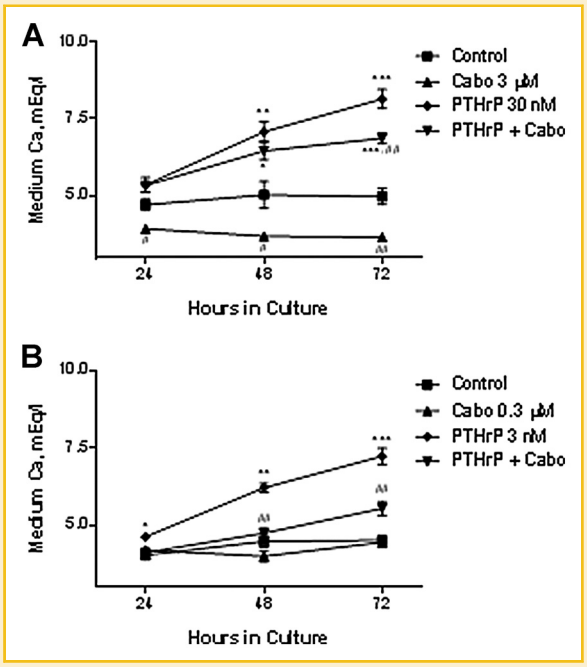

Fig. 1. Cabozantinib inhibits PTHrP-stimulated resorption of neonatal mouse calvaria. Calvaria were cultured for the indicated times and medium calcium determined. A: 3.0 μM cabozantinib, 30 nM PTHrP; (B) 0.3 μM cabozantinib, 3 nM PTHrP. Values are means ± SEM. n = 4 bones per data point. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. PTHrP or (part A, Cabo) $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. control.

EFFECT OF CABOZANTINIB ON MC3T3-E1 OSTEOBLASTIC CELLS

MC3T3-E1 osteoblastic cells were cultured for 17 days to promote their differentiation. Twenty-four-hour treatment of the differentiated cells with cabozantinib, 3 μM, decreased expression of RANKL and alkaline phosphatase, but not of osteoprotegerin (Fig. 2). In 48 h cultures, cabozantinib, 3 and 10 μM, but not 1 μM, decreased MTT activity, a metabolic assay used as a surrogate for cell proliferation and viability (Fig. 3). In other experiments, MC3T3-E1

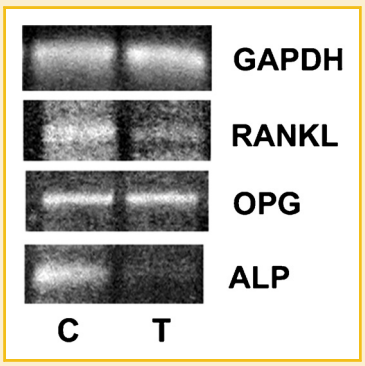

Fig. 2. Cabozantinib decreases RANKL and alkaline phosphatase gene expression in MC3T3-E1 cells. Cells were treated with cabozantinib, 3 μM, for 24 h.

cell number was decreased by 48 h treatment with 3 μM, but not 0.3 μM cabozantinib (not shown). Other investigators have reported that low doses of cabozantinib increased alkaline phosphatase activity in osteoblastic cells [Dai et al., 2013]. In our studies cabozantinib also had biphasic effects on alkaline phosphatase activity, eliciting small stimulatory effects at concentrations of 0.1–1 μM, and having no significant effect at 3 μM (Fig. 4).

EFFECT OF CABOZANTINIB ON RAW 264.7 OSTEOCLAST PRECURSOR CELLS

Twenty-four-hour treatment of preosteoclastic RAW 264.7 cells with 3 μM cabozantinib failed to affect the expression of RANK, cathepsin K, integrin alphaV, integrin beta3, TRAP or NFATc1 (Fig. 5). When RAW 264.7 cells were differentiated to promote osteoclastogenesis by 5-day treatment with 20 ng/ml RANKL, the resulting increase in TRAP activity was inhibited by co-treatment with 2 or 3 μM cabozantinib (Fig. 6A). In 5-day cultures incubated in parallel with those used to determine TRAP, cabozantinib inhibited MTT activity in both control and RANKL-treated cells (Fig. 6B), evidence that cabozantinib decreases the proliferation of osteoclastic cells and precursor cells in the longer term cultures.

DISCUSSION

The studies reveal effects of cabozantinib on bone cells and bone in the absence of tumor cells, indicating that cabozantinib can affect the bone microenvironment. As will be discussed, the effects that were observed on the bone environment are consistent with the effects of cabozantinib to affect the VEGF receptor, and could contribute to effects of cabozantinib to mitigate effects of prostate cancer bone metastases.

The current studies show that cabozantinib inhibits effects of PTHrP on resorption in a bone organ culture model. PTHrP acts through receptors on osteoblasts, eliciting the production of the membrane-bound cytokine RANKL, which then promotes the fusion, activity and survival of osteoclasts [Martin and Gillespie, 2001]. The effect of cabozantinib on PTHrP action is relevant to the effects of cabozantinib on prostate cancer bone metastases as PTHrP is produced by tumor cells and has been identified as a factor in the "vicious cycle" of stimulation of bone resorption by cancer metastases [Lacey et al., 2000]. The effect of cabozantinib to inhibit PTHrP-stimulated resorption is consistent with the action of cabozantinib to inhibit VEGFR2. In a mouse model of bone metastasis PTHrP stimulated VEGF expression, and the effect of PTHrP was inhibited by a VEGF-neutralizing antibody [Isowa et al., 2010]. In the current study, cabozantinib, 0.3 μM, inhibited PTHrP-stimulated resorption, but failed to affect bones that had not been treated with PTHrP. A higher concentration of cabozantinib,

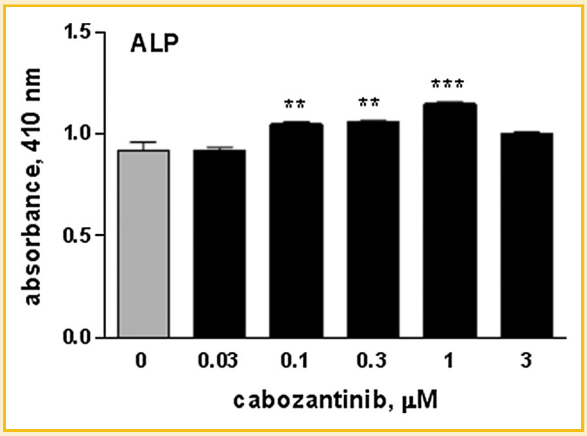

Fig. 4. Cabozantinib has biphasic effects on alkaline phosphatase activity in MC3T3-E1 cells. Cells were treated with the indicated concentrations of cabozantinib for 5 days. Values are means ± SEM. n = 6 wells per data point. $^{**}P < 0.05$, $^{**}P < 0.01$ vs. control.

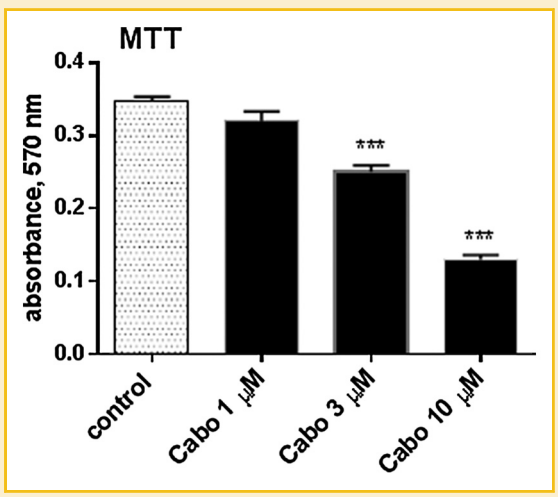

Fig. 3. Cabozantinib inhibits MTT activity in MC3T3-E1 osteoblastic cells. Cells were treated with the indicated concentrations of cabozantinib for 48 h. Values are means ± SEM. n = 6 wells per data point. $^{***}P < 0.001$ vs. control.

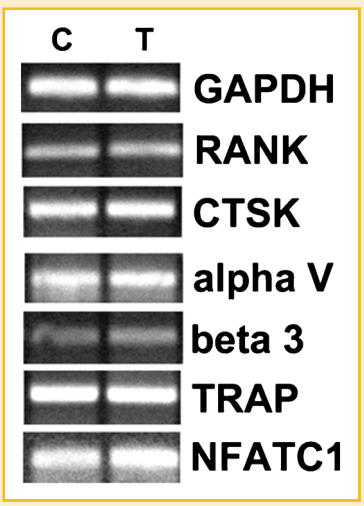

Fig. 5. Cabozantinib fails to affect expression of selected genes in short term cultures of RANKL treated-RAW 264.7 cells. Cells were treated with cabozantinib, 3 μM, for 24 h.

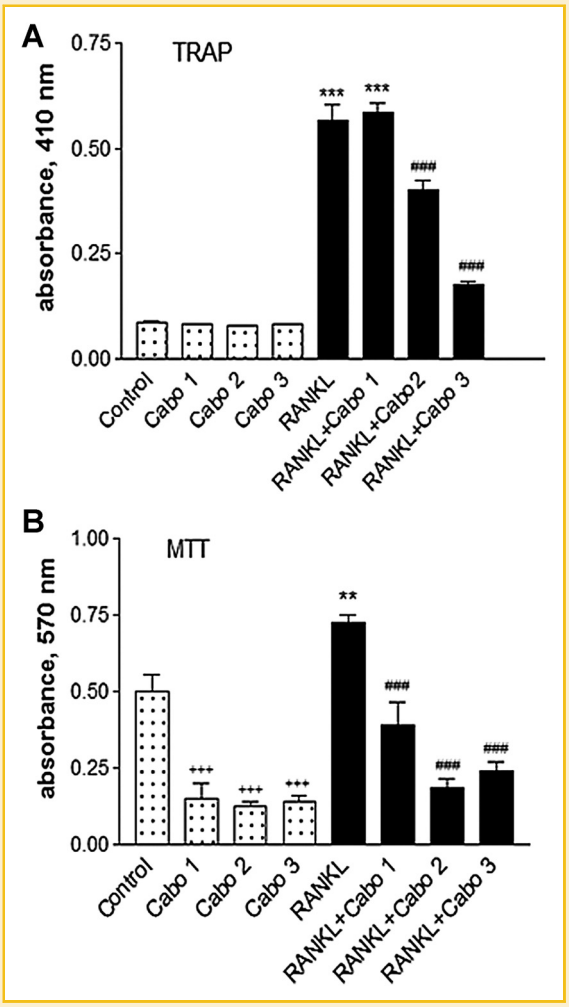

Fig. 6. Cabozantinib inhibits (A) TRAP and (B) MTT activity in RAW 264.7 cells. Cells were treated with or without cabozantinib, 1, 2, or 3 μM, for 5 days. Values are means ± SEM. n = 6 wells per data point. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; $^{###}P < 0.001$ vs. RANKL.

3 μM, inhibited calcium release both in the presence and in the absence of PTHrP. The different effects of the two concentrations could suggest that there may be more than one mechanism of cabozantinib action on the bones, with the higher concentration affecting cell viability in addition to eliciting other effects that attenuate resorption.

Two murine cell lines were used to further investigate effects of cabozantinib. Although neither was derived from the calvarial cultures, their responses to cabozantinib provide potential mechanisms for the effects of the drug on the intact calvarial bones. Cabozantinib treatment for 24 h decreased RANKL expression in osteoblastic MC3T3-E1 cells. The studies on osteoblastic cells suggest that the inhibitory effects of cabozantinib on resorption could be initiated through effects on osteoblasts. Additionally, cabozantinib at 3 and 10 μM, but not 1 μM inhibited MTT activity in the MC3T3-E1 cells. These effects of cabozantinib on osteoblasts are consistent with the inhibitory effects of the drug on VEGFR2, as VEGF promotes osteoblast survival [Street and Lenehan, 2009] and RANKL expression in MC3T3-E1 cells [Nakai et al., 2009].

Prostate cancer results in deranged bone formation, with excessive and abnormal bone being produced. In experiments designed to examine effects on osteoblast activity associated with bone formation, effects of cabozantinib on the osteoblast enzyme alkaline phosphatase were determined. Cabozantinib had mixed actions on alkaline phosphatase in the MC3T3-E1 cells. Twenty-four-hour treatment with 3 μM cabozantinib decreased alkaline phosphatase gene expression in the osteoblastic cells. Effects on alkaline phosphatase activity at a later time point were biphasic, with stimulatory effects elicited by lower concentrations of cabozantinib, consistent with previously published findings of other investigators [Dai et al., 2013]. The mechanism of the biphasic effect is unclear, as VEGF increases alkaline phosphatase in cultured osteoblasts [Midy and Plouet, 1994] and is a factor in the increased alkaline phosphate elicited by prostate cancer cell conditioned medium [Kitagawa et al., 2005]. However, overexpression of VEGF165 in bone marrow-derived mesenchymal cells inhibited BMP2-stimulated alkaline phosphatase activity [Lin et al., 2014]. Based on this finding, one could postulate that a small decrease in the VEGFR2 could promote the effect of an endogenous stimulator of alkaline phosphatase, whereas the inhibitory effect of higher concentrations of cabozantinib on VEGFR2 could override the stimulatory effect.

In cultures of osteoclast precursor RAW 264.7 cells treated with RANKL, 24 h treatment with cabozantinib failed to affect the expression of genes associated with resorptive activity, including NFATc1, cathepsin K, integrins alpha V and beta 3 and the RANKL receptor RANK, consistent with the initial effects of cabozantinib being mediated through effects on the osteoblasts.

In longer term cultures of the RAW 264.7 cells, effects were observed that could lead to inhibition of resorption. Five-day treatment with cabozantinib inhibited RANKL-stimulated TRAP activity, indicating an effect to decrease osteoclast differentiation. This finding is consistent with a mechanism mediated through inhibition of VEGF. VEGF-C is an expressed RANKL target gene in mouse osteoclasts, and the osteoclast-mediated resorption in that model is inhibited by a VEGF-C-specific inhibitor [Zhang et al., 2008]. Treatment of human monocytes by culture with RANKL and macrophage-colony stimulating factor (M-CSF) results in their differentiation into osteoclasts, and VEGF-A and VEGF-D were able to substitute for M-CSF in promoting RANKL-stimulated osteoclast formation [Taylor et al., 2012]. Similar effects were observed with hepatocyte growth factor (HGF) [Adamopoulos et al., 2006; Taylor et al., 2012]. In the current studies, cabozantinib also decreased MTT activity in the 5-day cultures, suggesting that decreased numbers of viable osteoclast precursors could contribute to the decrease in differentiated TRAP-secreting cells. Other studies have shown that VEGF promotes the survival of murine bone marrow osteoclast precursors [Yang et al., 2008].

In summary, this in vitro study provides evidence for potential effects on both osteoblasts and osteoclasts as components of the in vivo efficacy of cabozantinib in treating prostate cancer metastases. Cabozantinib inhibited resorption in an organ culture model. The drug was able to inhibit the expression of RANKL by osteoblastic cells, affected TRAP in osteoclastic cells, alkaline phosphatase in

osteoblastic cells and MTT activity in both cell types. The activity profile of cabozantinib on bone differs from that of the standard antiresorptive agents, which largely target osteoclasts. The difference in mechanism suggests that cabozantinib could be a useful alternative or adjunctive therapy for cancers that metastasize to bone.

ACKNOWLEDGMENTS

The authors thank Ms. Shirley Foster for expert technical assistance in carrying out the studies of bone resorption and the cell-based assays. We thank Drs. Eugene M. Silinsky, Douglas Laird, and Dana Aftab for reading a version of the manuscript. Funding for the research was provided to Dr. Stern by Exelixis.

REFERENCES

Adamopoulos IE, Xia Z, Lau YS, Athanasou NA. 2006. Hepatocyte growth factor can substitute for M-CSF to support osteoclastogenesis. Biochem Biophys Res Commun 350:478–483.

Almazrooa SA, Woo SB. 2009. Bisphosphonate and nonbisphosphonate-associated osteonecrosis of the jaw: A review. J Am Dent Assoc 140:864–875.

Carlin BI, Andriole GL. 2000. The natural history, skeletal complications, and management of bone metastases in patients with prostate carcinoma. Cancer 88:2989–2994.

Charhon SA, Chapuy MC, Delvin EE, Valentin-Opran A, Edouard CM, Meunier PJ. 1983. Histomorphometric analysis of sclerotic bone metastases from prostatic carcinoma special reference to osteomalacia. Cancer 51:918–924.

Chiarodo A. 1991. National Cancer Institute roundtable on prostate cancer: Future research directions. Cancer Res 51:2498–2505.

Cook RJ, Coleman R, Brown J, Lipton A, Major P, Hei YJ, Saad F, Smith MR. 2006. Markers of bone metabolism and survival in men with hormone-refractory metastatic prostate cancer. Clin Cancer Res 12:3361–3367.

Dai J, Zhang H, Karatsinides A, Keller JM, Kozloff KM, Aftab DT, Schimmoller F, Keller ET. 2013. Cabozantinib inhibits prostate cancer growth and prevents tumor-induced bone lesions. Clin Cancer Res 20:617–630.

Huang BK, Golden LA, Tarjan G, Madison LD, Stern PH. 2000. Insulin-like growth factor I production is essential for anabolic effects of thyroid hormone in osteoblasts. J Bone Miner Res 15:188–197.

Isowa S, Shimo T, Ibaragi S, Kurio N, Okui T, Matsubara K, Hassan NM, Kishimoto K, Sasaki A. 2010. PTHrP regulates angiogenesis and bone resorption via VEGF expression. Anticancer Res 30:2755–2767.

Keller ET, Zhang J, Cooper CR, Smith PC, McCauley LK, Pienta KJ, Taichman RS. 2001. Prostate carcinoma skeletal metastases: Cross-talk between tumor and bone. Cancer Metastasis Rev 20:333–349.

Kitagawa Y, Dai J, Zhang J, Keller JM, Nor J, Yao Z, Keller ET. 2005. Vascular endothelial growth factor contributes to prostate cancer-mediated osteoblastic activity. Cancer Res 65:10921–10929.

Lacey DL, Tan HL, Lu J, Kaufman S, Van G, Qiu W, Rattan A, Scully S, Fletcher F, Juan T, Kelley M, Burgess TL, Boyle WJ, Polverino AJ. 2000. Osteoprotegerin ligand modulates murine osteoclast survival in vitro and in vivo. Am J Pathol 157:435–448.

Lee RJ, Saylor PJ, Michaelson MD, Rothenberg SM, Smas ME, Miyamoto DT, Gurski CA, Xie W, Maheswaran S, Haber DA, Goldin JG, Smith MR. 2013. A dose-ranging study of cabozantinib in men with castration-resistant prostate cancer and bone metastases. Clin Cancer Res 19:3088–3094.

Lin Z, Wang JS, Lin L, Zhang J, Liu Y, Shuai M, Li Q. 2014. Effects of BMP2 and VEGF165 on the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. Exp Ther Med 7:625–629.

Martin TJ, Gillespie MT. 2001. Receptor activator of nuclear factor kappa B ligand (RANKL): Another link between breast and bone. Trends Endocrinol Metab 12:2–4.

Midy V, Plouet J. 1994. Vasculotropin/vascular endothelial growth factor induces differentiation in cultured osteoblasts. Biochem Biophys Res Commun 199:380–386.

Nakai T, Yoshimura Y, Deyama Y, Suzuki K, Iida J. 2009. Mechanical stress up-regulates RANKL expression via the VEGF autocrine pathway in osteoblastic MC3T3-E1 cells. Mol Med Rep 2:229–234.

Nguyen HM, Ruppender N, Zhang X, Brown LG, Gross TS, Morrissey C, Gulati R, Vessella RL, Schimmoller F, Aftab DT, Corey E. 2013. Cabozantinib inhibits growth of androgen-sensitive and castration-resistant prostate cancer and affects bone remodeling. PLoS ONE 8:e78881.

Russell RG, Watts NB, Ebetino FH, Rogers MJ. 2008. Mechanisms of action of bisphosphonates: Similarities and differences and their potential influence on clinical efficacy. Osteoporos Int 19:733–759.

Saylor PJ, Lee RJ, Smith MR. 2011. Emerging therapies to prevent skeletal morbidity in men with prostate cancer. J Clin Oncol 29:3705–3714.

Smith DC, Smith MR, Sweeney C, Elfiky AA, Logothetis C, Corn PG, Vogelzang NJ, Small EJ, Harzstark AL, Gordon MS, Vaishampayan UN, Haas NB, Spira AI, Lara PN, Jr., Lin CC, Srinivas S, Sella A, Schoffski P, Scheffold C, Weitzman AL, Hussain M. 2013. Cabozantinib in patients with advanced prostate cancer: Results of a phase II randomized discontinuation trial. J Clin Oncol 31:412–419.

Stern P, Krieger N. 1983. Comparison of fetal rat limb bones and neonatal mouse calvaria: Effects of parathyroid hormone and 1,25-dihydroxyvitamin D3. Calcif Tiss Intl 35:172–176.

Street J, Lenehan B. 2009. Vascular endothelial growth factor regulates osteoblast survival—Evidence for an autocrine feedback mechanism. J Orthop Surg Res 4:19.

Taylor RM, Kashima TG, Knowles HJ, Athanasou NA. 2012. VEGF, FLT3 ligand, PlGF and HGF can substitute for M-CSF to induce human osteoclast formation: Implications for giant cell tumour pathobiology. Lab Invest 92:1398–1406.

Taylor KH, Middlefell LS, Mizen KD. 2010. Osteonecrosis of the jaws induced by anti-RANK ligand therapy. Br J Oral Maxillofac Surg 48:221–223.

Turner RT, Backup P, Sherman PJ, Hill E, Evans GL, Spelsberg TC. 1992. Mechanism of action of estrogen on intramembranous bone formation—Regulation of osteoblast differentiation and activity. Endocrinology 131:883–889.

Yakes FM, Chen J, Tan J, Yamaguchi K, Shi Y, Yu P, Qian F, Chu F, Bentzien F, Cancilla B, Orf J, You A, Laird AD, Engst S, Lee L, Lesch J, Chou YC, Joly AH. 2011. Cabozantinib (XL184), a novel MET and VEGFR2 inhibitor, simultaneously suppresses metastasis, angiogenesis, and tumor growth. Mol Cancer Ther 10:2298–2308.

Yang Q, McHugh KP, Patntirapong S, Gu X, Wunderlich L, Hauschka PV. 2008. VEGF enhancement of osteoclast survival and bone resorption involves VEGF receptor-2 signaling and beta3-integrin. Matrix Biol 27:589–599.

Zhang J, Dai J, Qi Y, Lin DL, Smith P, Strayhorn C, Mizokami A, Fu Z, Westman J, Keller ET. 2001. Osteoprotegerin inhibits prostate cancer-induced osteoclastogenesis and prevents prostate tumor growth in the bone. J Clin Invest 107:1235–1244.

Zhang Q, Guo R, Lu Y, Zhao L, Zhou Q, Schwarz EM, Huang J, Chen D, Jin ZG, Boyce BF, Xing L. 2008. VEGF-C, a lymphatic growth factor, is a RANKL target gene in osteoclasts that enhances osteoclastic bone resorption through an autocrine mechanism. J Biol Chem 283:13491–13499.