RESEARCH ARTICLE

Effects of Cabozantinib on Human G292 Osteosarcoma Cells

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Abstract
Cabozantinib (CBZ) N-(4-((6,7-dimethoxyquinolin-4-yl) oxy) phenyl)-N-(4-fluorophenyl) cyclopropane-1,1-dicarboxamide) (XL184), an inhibitor of MET and vascular endothelial growth factor receptor (VEGFR-2), is an agent approved for the treatment of several types of carcinoma such as medullary thyroid and renal. Recent studies are encouraging for the effectiveness of CBZ in the treatment of osteosarcoma. Because of the complex nature of the microenvironment of osteosarcoma cell sites, in order to better understand the direct effects of CBZ on osteosarcoma cells, in vitro studies were conducted with the human osteosarcoma cell line, G292. Experiments were focused on the effects of CBZ on cell metabolic activity, differentiation, and apoptosis as well as the modulation of responses to growth factors such as platelet-derived growth factor (PDGF) and insulin like growth factor (IGF-I). The results indicate that CBZ can increase the activity of caspase 3/7 as an indicator of apoptosis in the G292 cells. CBZ also decreased G292 cellular activity, measured by MTT assay and differentiation assessed by alkaline phosphatase activity. The drug partially downregulated the effects of PDGF on cellular activity and significantly inhibited the effects of IGF-1 on cellular activity and production of VEGF by the osteosarcoma cells.
This study presents original information on responses of G292 human osteosarcoma cells to the chemotherapy agent, CBZ, and provides in vitro data consistent with the potential therapeutic effects of this agent for osteosarcoma.

Keywords
Cabozantinib, G292 cells, osteosarcoma

1. Introduction

Cabozantinib (CBZ) N-(4-((6,7-dimethoxyquinolin-4-yl) oxy) phenyl)-N-(4-fluorophenyl) cyclopropane-1,1-dicarboxamide) (XL184), a novel inhibitor of multiple tyrosine kinase receptors such as c-MET, vascular endothelial growth factor receptor (VEGFR-2), as well as Ret, Kit, Flt-1/3/4, Tie2, and AXL, has emerged as an effective agent in the treatment of several types of carcinomas and has been approved for the treatment of medullary thyroid cancer, hepatocellular carcinoma and renal cell carcinoma.[1-4] Although a phase III trial (COMET-1) in patients with heavily pretreated metastatic castrate-resistant prostate cancer (CRPC) did not demonstrate a statistically significant increase in overall survival with CBZ versus prednisone alone, a phase II randomized discontinuation trial in CRPC patients did show a significant reduction in progression-free survival (PFS) with reductions in bone turnover markers and bone pain for the CBZ-treated cohorts.[5-7] Additional clinical evidence of the potential benefits of CBZ on bone lesions associated with carcinomas was provided in the phase III clinical trial (METEOR study), in which treatment with CBZ was associated with a reduction of skeletal-related events and lower levels of osteolytic bone biomarkers in renal cell carcinoma patients with bone metastases (RCCBM).[8]

Preclinical animal models have provided important insight into the potential mechanisms by which CBZ might be producing inhibition of tumor growth as well as tumor related skeletal lesions. A murine bone study showed that CBZ can inhibit parathyroid hormone related hormone-inducted bone resorption with cell studies suggesting this response is initiated via decreases in *Receptor Activator of Nuclear Factor k B Ligand* (RANKL) production/secretion by osteoblastic cells.[9] This involvement of osteoblastic and osteoclastic cells in the action of CBZ's effects on bone was further substantiated in an in vitro study with human osteoblastic and osteoclastic cells at different stages of differentiation.[10] In this study, CBZ at non-cytotoxic doses, inhibited osteoclastic cell differentiation and bone resorption, down-regulating the expression of osteoclast markers genes as well as *Receptor Activator of Nuclear Factor k B* (RANK) with no significant effects on osteoblast viability or differentiation parameters such as alkaline phosphatase or mineralization but increases in osteoprotegerin (OPG) and decreases in RANKL expression and production.[10] A recent study using both in vitro and in vivo mouse models of RCCBM provided evidence that CBZ affects both osteoblasts and osteoclasts with significant enhancement of CBZ invoked increases in osteoblastic cell differentiation.[11]

The emerging studies with CBZ on the effects of this drug on the microenvironment of tumor sites have led to consideration of its effects on osteosarcoma (OS) cells. The clinical activity and safety of CBZ in osteosarcoma patients have been studied in a multicenter, single-arm phase II CABONE trial.[12] The results of this study, conducted in patients with advanced osteosarcoma with documented progression, showed significant results indicative of increased progression-free survival with the use of oral CBZ.[12]

Studies on the effects of CBZ in vitro have provided some insight into the potential mechanisms by which CBZ can influence the clinical progression of osteosarcoma. In a recent in vitro study, it was shown that CBZ, via its inhibition of the c-MET pathway, decreased proliferation of several human osteosarcoma (OS) cell lines such as HOS, MG-63, Saos-2, U-2OS cells with a stronger inhibition of RANK-positive OS versus RANK-negative cells.[13]

In realization of the complex nature of the microenvironment at osteosarcoma cell sites,

in this present study, the effects of CBZ were studied in another human OS line, G292, with focus on effects of the drug on cellular responses to platelet-derived growth factor – BB (PDGF-BB) and insulin like growth factor -I (IGF-I). These growth factors have been shown to be present in the microenvironment of bone, released during the resorptive process of bone remodeling, and to be involved in several aspects of bone remodeling either directly or indirectly via their regulation of the synthesis of other factors.[14,15] The *in vitro* results presented here suggest that CBZ can have direct effects on the G292 osteosarcoma cell line such that with appropriate concentrations of the drug, inhibitory effects on growth and differentiation of these cells along with decreased production of VEGF and increased apoptosis can be noted.

2. Materials and Methods

2.1. Materials and culturing conditions

G292 (clone A14181) cells were purchased from American Type Culture Collection (ATCC, VA). Cabozantinib (CBZ) was purchased from LC Laboratories (Woburn, MA).

Human recombinant insulin-like growth factor I (IGF-I) and human recombinant platelet-derived growth factor (PDGF-BB) and VEGF (human) ELISA Kit were purchased from Enzo Life Sciences, Inc (Farmingdale, NY). The Caspase-Glo 3/7Assay System for measurement of apoptotic activity was purchased from Promega (Madison, Wisconsin).

All cell cultures were obtained as frozen cultures and were suspended in alpha Minimum Essential Medium (MEM) culture medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin antibiotic (Gibco, Life Technologies, Grand Island, NY). All cells were seeded in a 75-ml culture flask with MEM-FBS and kept in an incubator at 37°C and 5% CO_2 until desired confluency was reached for use in experiments.

2.2. MTT assay

Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The detailed MTT assay protocol used here can be found in a previous study from our lab.[16] After incubation with appropriate concentrations of CBZ and growth factors for 48 hrs, culture medium was removed from each well. MTT reagent (Sigma-Aldrich, St. Louis, MO) was added to reduce the yellow tetrazolium by dehydrogenase enzymes from metabolically active cells. Cells were then incubated for 2 hours at 37℃ and 5% CO_2. MTT solution was removed and dimethylsulfoxide (DMSO) was added to solubilize the resulting purple formazan. After incubation for another 10 mins, absorbance readings were obtained with a Bio-Rad® micro plate spectrophotometer (Bio-Rad laboratories, NY, USA) at 540nm wavelength.

2.3. ALP assay

Osteoblastic cell differentiation was assessed by measurement of alkaline phosphatase (ALP). according to a previously published protocol.[16] After incubation of the cells with appropriate experimental conditions, culture medium was removed from each well and 200µL 1% Triton X-100 was added to lyse the cells. The cells were then incubated at 37℃ for 1 hour. After removing the culture solution, para-nitrophenol phosphate (pNPP) (Sigma-Aldrich) was added along with the alkaline buffer, 2-amino-2-methyl-1-propanol (Sigma-Aldrich), followed by incubation for 1 hour at 37°C. After NaOH was added to stop the reaction, absorbance readings with a Bio-Rad® micro plate spectrophotometer (Bio-Rad laboratories,

NY, USA) at 405nm wavelength were obtained.

2.4. VEGF production

The effect of CBZ on the production of VEGF by the G292 cells stimulated with IGF-1 was measured after 48 hrs in culture in 96 well tissue culture plates with the drug added simultaneously with the IGF-1. The levels of VEGF in the MEM media of the cells incubated under standard conditions were assayed with a quantitative ELISA according to the manufacturer's (Enzo) instructions. According to the product manual provided by the manufacturer (Enzo; Catalog # ENZ-KIT 156-0001), the sensitivity of the assay is 4.71 pg/ml with a 100% cross-reactivity for human VEGF 165.

2.4. Apoptosis Assay

The Caspase-Glo® 3/7 Assay (ProMega) that provides a caspase-3/7 DEVD-aminoluciferin substrate and luciferase in a single reagent was used as an indicator of cell apoptosis in G292 cells incubated with CBZ for 2 hrs under standard incubation conditions using MEM-FBS as described above for the other protocols. The assay was conducted according to the manufacturer's instruction provided in the Technical Bulletin available at www.promega.com/protocols/.

3. Results

3.1. Effects of CBZ on G292 cell activity

CBZ inhibited the cell activity of G292 cells in a concentration dependent manner. As shown in Figure 1, treatment of G292 cells for 48 hrs. with relatively high concentrations of CBZ significantly decreased the cell activity, as assessed by the MTT activity assay, compared to the control untreated group. The inhibitory effect was the highest when G292 cells were incubated with 600 and 900µM CBZ although 100 and 300 µM CBZ also produced significant decreases compared to controls.

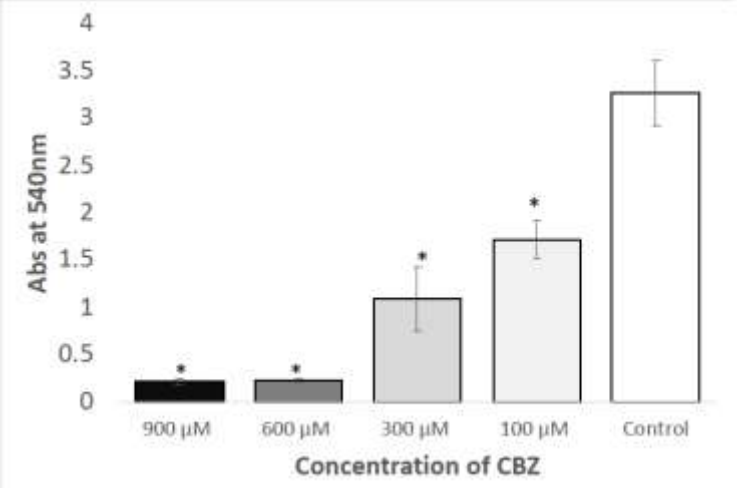

Figure 1. MTT assay: Effect of CBZ on G292 cells
48 hr. incubation of G292 cells with concentrations of CBZ from 100-900 µM produced significant decreases in cell activity. Values are the means +/- standard deviation; n= 4 samples/ group; * = significantly different from control; p< 0.001; one-way ANOVA followed by post hoc comparison using a Tukey HSD test.

3.2. Effects of CBZ on G292 cell ALP activity as a marker of differentiation

Figure 2 shows the result of the ALP assay testing the effects of CBZ on differentiation of G292 cells. The lowest level of cell differentiation occurred at 100µM CBZ and it gradually increased as the concentrations decreased to 1µM. However, the highest tested concentration of 300µM CBZ showed a higher level of cell differentiation than 100µM ($p<0.05$).

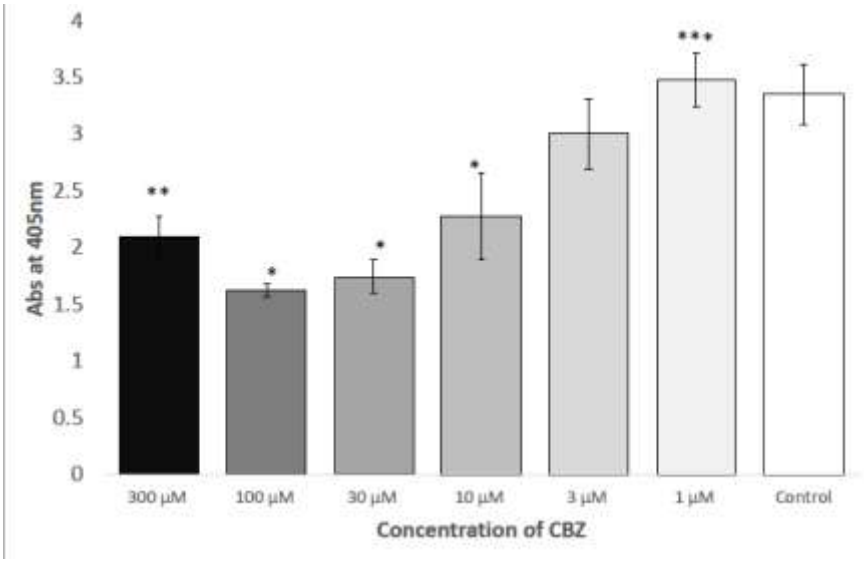

Figure 2. ALP assay: Effect of CBZ on G292 cells
72 hr. incubation of G292 cells with CBZ resulted in inhibitory effects on ALP activity. Values are the means +/- standard deviation; n= 4 samples/ group; * = significantly different from control; p< 0.001; **= a significant increase compared to the 100µM CBZ group; p<0.05; ***=significantly different from all CXZ treated groups; p<005; one-way ANOVA followed by post hoc comparison using a Tukey HSD test.

3.3 Effects of CBZ and PDGF on cell activity

The effects of CBZ on the response of G292 cells to PDGF are shown in Figure 3. Addition of 100 nM PDGF resulted in a small increase in MTT cell activity during a 48-hr. incubation and although CBZ alone (30µM) produced significant decreases in activity compared to controls, the simultaneous addition of PDGF (100nM) and CBZ (30µM) exhibited a significant increase compared to CBZ alone suggesting that the drug did not impair the ability of the cells to respond to the growth factor although the overall response of the cells to the combined treatment was significantly decreased compared to controls (incubated with appropriated amounts of the diluents of the drug and growth factor).

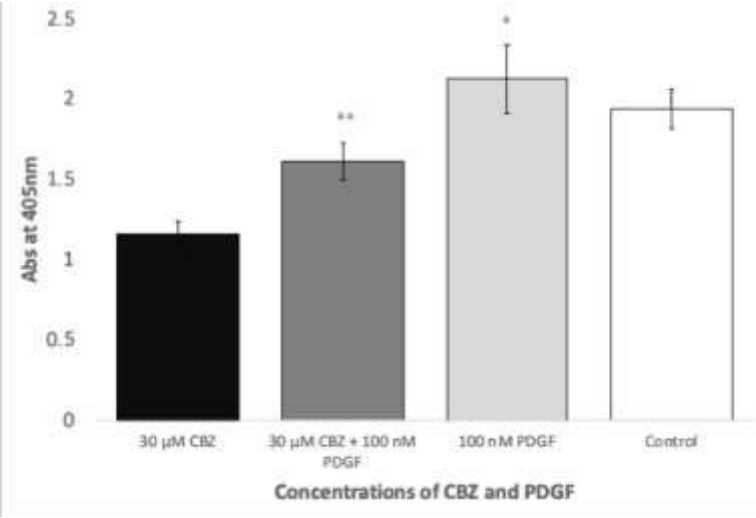

Figure 3. Effect of CBZ on G292 cells response to PDGF
With a 48-hour treatment with 30µM CBZ alone or a combination of 30µM CBZ and 100 nM PDGF a significant decrease in MTT cell activity compared to controls (no CBZ or PDGF) or PDGF alone was observed. The combination of CBZ and PDGF treatment group did exhibit a significant increase in cell activity compared to 30µM CBZ alone group. 100nM nM PDGF increased MTT activity compared to control; Values are the means +/- standard deviation; n= 4 samples/ group; **= p<0.001. *= p<0.05; one-way ANOVA followed by post hoc comparison using a Tukey HSD test.

3.4. Effects of CBZ and IGF-I on cell activity

With a 48-hour treatment with 10 nM IGF-I there was a significant effect on cell activity as assessed with the MTT assay compared to controls with no added IGF-I. This effect of IGF-I was not observed when cells were incubated with 30µM CBZ and 10nM IGF-I and this treatment group had no significant difference in activity from those treated with 30µM CBZ alone. IGF-I at 1 nM had no significant effect on cell activity in comparison to either control (no IGF-I added) cells or those treated with 30µM CBZ.

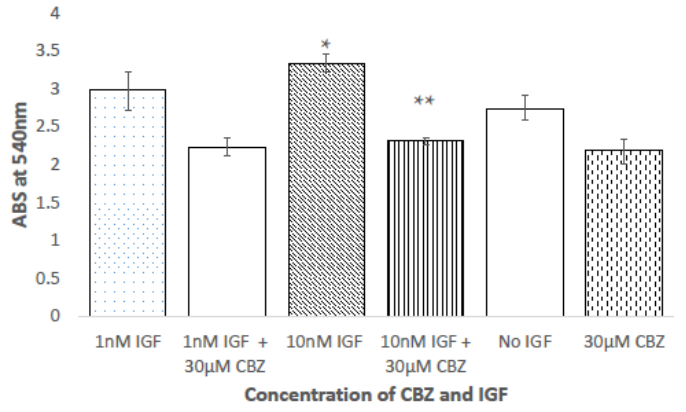

Figure 4. Effect of CBZ on G292 cells response to IGF-I
With a 48 -hour of incubation, 10nM nM IGF-I increased MTT activity compared to controls with no IGF added. The 30µM CBZ and 10 nM IGF-I treated cells exhibited no significant effects compared to the 30µM CBZ group that was significantly decreased compared to the control (no IGF) group. Values are the means +/- standard deviation; n= 4 samples/ group; **= p<0.001. *= p<0.05; one-way ANOVA followed by post hoc comparison using a Tukey HSD test.

3.5 Effects of CBZ on VEGF production in IGF-I treated cells

The results presented in Figure 5 indicate that CBZ inhibits the production of the VEGF in G292 cells incubated with IGF-I for a 48-hour period. Basal (control) levels of VEGF under these incubation conditions were not detectable with the ELISA assay used in this study (data not shown), but the levels of VEGF produced in the presence of either 10nM or 100 nM added IGF-I were significantly inhibited by either dose of CBZ (3µM, 30µM) tested.

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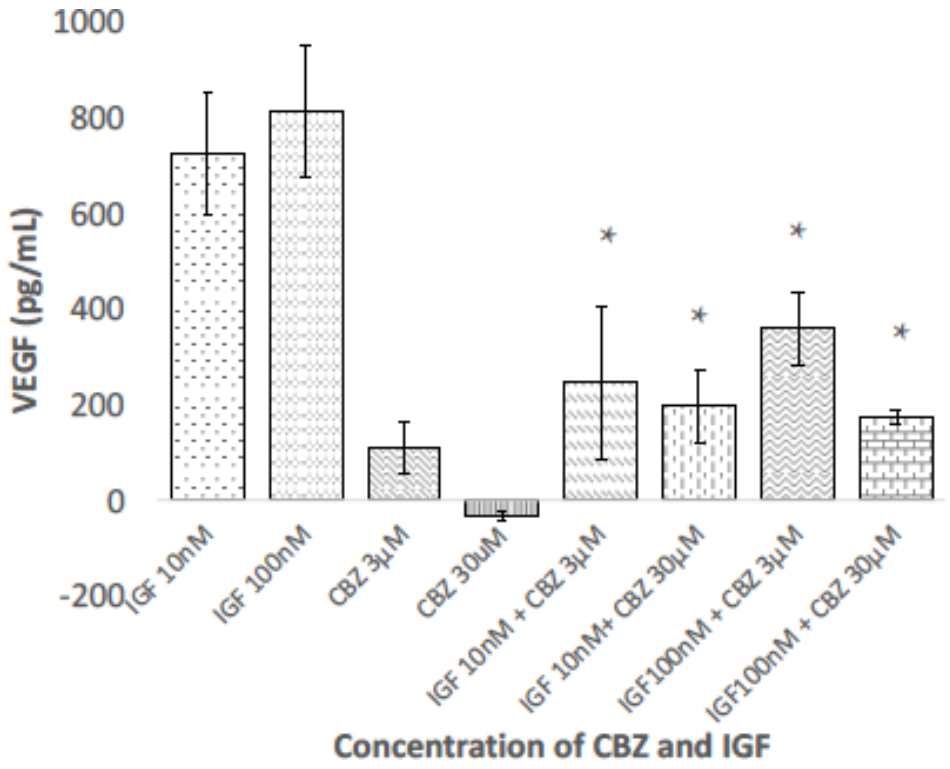

Figure 5. Effect of CBZ on IGF-I effects on VEGF production in G292 cells
With a 48-hour treatment with 3µM CBZ or 30µM CBZ, incubation with 10nM or 100nM IGF-I, resulted in lower concentrations of VEGF compared to 10nM or 100nM IGF-I only.
Values are the means +/- standard deviation; n= 4 samples/ group; *= $p<0.01$; one-way ANOVA followed by post hoc comparison using a Tukey HSD test.

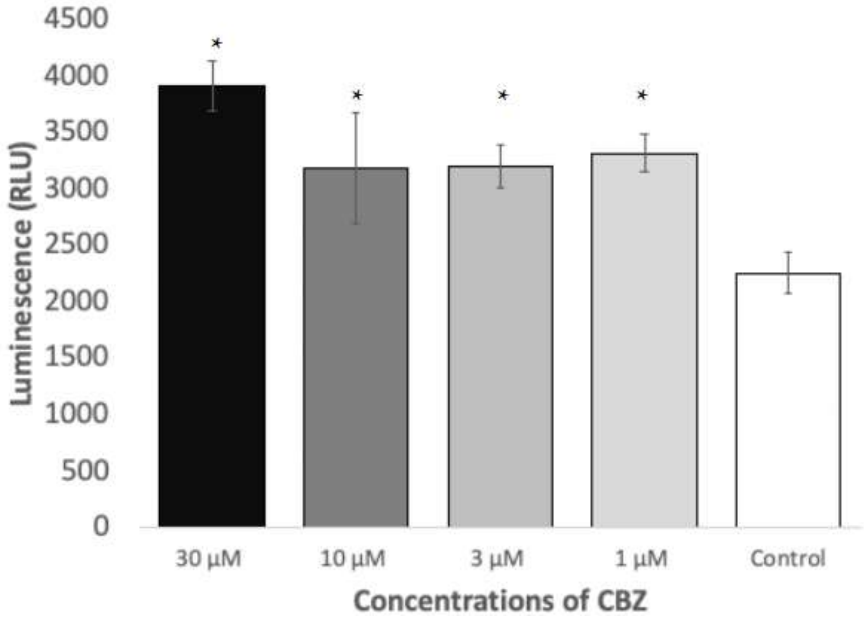

Figure 6. Effect of CBZ on apoptosis in G292 cells
A 2-hour treatment of CBZ at concentrations from 1- 30µM resulted in significant increases in luminescence generated by the Caspase-Glo® 3/7 assay as an indicator of apoptosis, compared to control. Values are means +/- standard deviation; 4 samples per group; *= $p<0.05$; one-way ANOVA followed by post hoc comparison using a Tukey HSD test.

Assessing apoptosis by measurement of caspase 3/7 activity using a luminescence assay, it was found that relatively low concentrations of CBZ (1-30 µM) significantly decreased apoptosis in G292 cells as shown in Figure 6.

4. Discussion

Osteosarcoma is a primary malignant bone tumor that has the basic histology of osteoid synthesis associated with malignant mesenchymal cells.[17] It is predominantly found in adolescents, and is ranked as the third most prevalent cancer in that age group, with the annual incidence of 5.6 cases per million patients under 15 years of age and peak incidence in individuals in their 20s.[18] The standard treatment for osteosarcoma includes neoadjuvant chemotherapy, surgery, followed with postoperative chemotherapy. Most recently there have been major advances in the use of tyrosine kinase inhibitors (TKI), targeted drugs that can specifically inhibit protein tyrosine kinases (PTKs). The use of TKIs in the treatment of osteosarcomas has recently been reviewed with an emphasis on those drugs that have been used in registered clinical trials. It appears based on emerging data that inhibition of several relevant receptor tyrosine kinases is needed for optimal osteosarcoma treatment.[19]

CBZ is a multiple target TKI and has activity not only for VEGFRs and MET, but also for KIT, RET, AXL, and PDGFRβ.[19] Consistent with this activity are the promising reported results of the recent multicenter, phase II trial (CABONE) with patients with advanced or metastatic osteosarcoma.[12] The longer median PFS results obtained with the CBZ treatment in this trial has been recognized as a major achievement in TKI treatment of osteosarcoma.[12,19] In light of the results of CABONE clinical trial, studies to further delineate the mechanism of action of CBZ can most likely aid in the establishment of

predictive factors for its use in patients who might be particularly responsive to the therapeutic and the best approaches for its administration.

The present in vitro study with G292 human osteosarcoma cells adds to the information obtained by others in different osteosarcoma cell lines to better understand how CBZ can produce direct effects on bone tumor cells. Although cell effects were evaluated here using different parameters, the inhibitory effects of CBZ in the G292 cells reflect similar overall growth inhibitory responses as those reported in HOS, MG-63, Saos-2 and U-2 OS cells.[13] Although our studies were initiated without awareness of the studies in these other OS cell lines and cannot be directly compared, it should be noted that the G292 cell line has been shown to have a substantially slower proliferation capacity than Saos-2 cells as well as 21 other osteosarcoma cell lines studied simultaneously.[20]

There is increasing evidence that development and progression of osteosarcoma are regulated not only by direct effects on the OS cells, but also by their interaction with normal, noncancerous osseous cells and the factors that these cells produce in the microenvironment of the OS cells.[13] These factors include several families of growth factors such as PDGF and IGF that mediate their effects via tyrosine kinase receptors. Because factors of this nature which have well characterized effects on the growth and migration of osseous cells can be released during the process of bone remodeling into the local microenvironment, the nature and regulation of their effects are important to be considered in the evaluation of the potential effectiveness of an agent such as CBZ.[21]

PDGF-BB has been shown to have effects on OS cells as well as normal osteoblasts to increase such parameters as growth and migration.[22] We have previously shown that PDGF-BB increases the activity of G292 cells.[16] In this present study, we report that CBZ (30μM) can decrease this response to the growth factor compared to cells without CBZ although there was still a significant increase in the CBZ+PDGF treated cells compared to those treated with CBZ alone. As discussed in detail in a recent review, there does not appear to be a direct correlation between the efficacy of drugs as inhibitors of PDGFRs and their efficacy for osteosarcoma suggesting that the PDGF/PRGFR signaling pathway may not be the principal mechanism for osteosarcoma progression and that other signaling pathways may be more critically involved.[19]

The studies conducted here with IGF-I are consistent with earlier studies that show that this growth factor is involved with the growth of human osteosarcoma cells including G292 cells and that production of VEGF is increased by the addition of IGF-I.[23] The data shown here indicate that CBZ is effective in inhibiting the effect of the growth factor on both MTT activity as well as VEGF production. These results suggest that downregulation of the action of IGF-I by the drug could be important in its mechanism of action on osteosarcomas. Inhibition of VEGF signaling has been shown to lead to suppression of tumor-induced angiogenesis and growth.[24] Moreover, poor prognosis in osteosarcoma has been associated with high levels of VEGF expression and its role in angiogenesis.[25]

Using Caspase 3/7 activity as an indicator of apoptosis, in this study CBZ at relatively low concentrations produced increases in this parameter in G292 cells, a response that would be desired for the drug's use as a therapeutic approach for osteosarcoma. The consistency of this response in other OS cells is not evident at this time since the literature appears to be scarce with respect to studies on direct effects of CBZ on apoptosis. Although

the data were not shown in the publication, it was reported that in the HOS cells treated with CBZ, Caspase-3 active protein levels, evaluated by Western Blots, did not indicate any activation of an apoptotic pathway.[13] Further studies are necessary to determine if the apoptotic effect observed in the G292 cells is specific to this particularly osteosarcoma cell line.

Results from both in vitro and in vivo preclinical studies as well as clinical trials such as the CABONE study are providing relevant information on biomarkers that can be critical in the use of targeted therapy for osteosarcoma.[12, 26] There is increasing evidence to suggest that there is a complex network of cross talk between the osseous tumor cells and the microenvironment of the bone site in which they are developing. Interactions with RANK/RANKL/OPG by both osteoclastic resorbing cells and osteoblastic bone forming cells appear to be involved in regulation of the net process involved in remodeling at the site of the tumor and OS cells expressing RANK are more responsive to CBZ. [9, 10, 13] Moreover, in the CABONE study, CBZ appeared to be more effective in patients with OS with highly soluble c-MET baseline values compared to those with low levels of c-MET.[12] Although we have not investigated the effects of CBZ on the RANK system or c-MET expression in G292 cells, the data presented here on MTT, ALP and Caspase 3/7 activities suggest that these OS cells would most likely be responsive to effects on these parameters as well.

Conclusions

The effects of CBZ on the G292 human osteosarcoma cell line reported here are consistent with the promising emerging data from other in vitro and in vivo studies as well as a multicenter phase II clinical trial on the effectiveness of this agent in the treatment of osteosarcoma. Previous studies with OS lines have not included G292 cells so the original results presented here should provide additional insight into mechanisms of action of CBZ and a possible basis for predictive factors for optimal responsivity to the drug.

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