TARGETED INHIBITION OF C-MET RECEPTOR BY A SELECTIVE C-MET INHIBITOR, TIVANTINIB, AND A SPECIFIC SHRNA REDUCES BREAST CANCER-DERIVED BONE METASTASES

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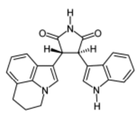
ABSTRACT

Breast cancer exhibits a propensity to metastasize to bone, resulting in debilitating skeletal-related complications associated with significant morbidity and mortality. Because of the clinical significance of this process, many research efforts are aimed at uncovering the molecular events in bone metastases to identify novel targets and to improve clinical management of metastatic bone disease. The interactions between metastatic cells and bone are critical to the development and progression of bone metastases, and their unravelling could lead to novel preventive or therapeutic approaches. We previously demonstrated the critical role of HGF/c-Met/β-catenin/TCF system in tumor-bone interaction leading to skeletal metastases of human breast cancer cells, suggesting the potential inhibition of this pathway *in vivo*. In this study, we evaluated the potential therapeutic efficacy of targeting the c-Met receptor by using both an oral, selective, small-molecule c-Met inhibitor, tivantinib, and a specific shRNA against c-Met in an experimental bone metastatic model of human breast cancer. Tivantinib exhibited dose-dependent anti-metastatic activity *in vivo*, and the 120 mg/kg dose, while being ineffective in reducing subcutaneous tumor growth, induced significant inhibition of bone metastatic growth and a noteworthy reduction of tumor-induced osteolysis. shRNA-mediated c-Met silencing did not affect *in vitro* proliferation of bone metastatic cells, but strongly reduced their migration, and this effect was further enhanced by tivantinib. These data were confirmed *in vivo*. Indeed, dual c-Met inhibition with both tivantinib and RNA interference strategy induces pronounced tumor growth suppression with concomitant marked decreases of lytic lesions and an improvement in survival. Overall, our findings highlighted the efficacy of c-Met inhibition in delaying the onset and progression of bone metastases and strongly suggested that targeting the c-Met receptor may have promising therapeutic value in the prevention and treatment of bone metastases from breast cancer. Most importantly, the finding that tivantinib is active as an anti-metastatic agent at low, non-cytotoxic doses suggests that its efficacy may be potentiated by combining it with other therapies that target cancer cell-bone interactions.

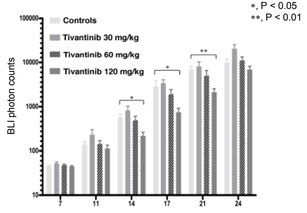
INTRODUCTION

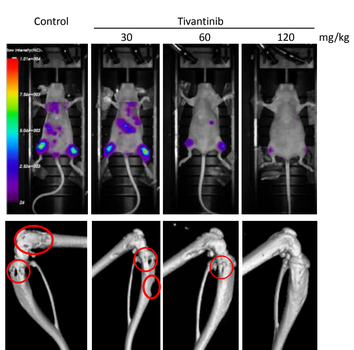
Breast cancer displays a remarkable predilection to metastasize to bone. Development of bone metastasis in breast cancer patients results in significant morbidity and mortality due to the skeletal-related complications like severe bone pain, pathologic fractures and spinal cord and nerve compression that diminish the patient's quality of life dramatically [1]. Currently, research on metastasis is focused on the complex bidirectional interplay between epithelial tumor cells and bone microenvironment establishing a "vicious cycle" that leads to a selective growth advantage for the breast cancer cells [2]. Therefore, understanding the cellular and molecular interactions between breast cancer cells and the surrounding bone cellular components may provide critical insights about the origin and maintenance of metastatic bone lesions and could lead to the identification of novel potential targets for the treatment of the skeletal metastases. Therapeutic targeting of tumor-bone interaction is under intensive investigation. A potential candidate is c-Met, the tyrosine kinase receptor for the hepatocyte growth factor (HGF). Primarily expressed on epithelial cells, c-Met drives different intracellular signaling pathways, ranging from proliferation, motility, and invasion to survival and angiogenesis, that are essential for the development and progression of many human cancers [3]. Aberrant signaling of the c-Met pathway, identified in a wide variety of human malignancies, has been associated with a poor prognosis, aggressive phenotype, increased metastasis, and shortened patient survival [4]. However, the role of c-Met signaling in human breast cancer bone metastasis has scarcely been investigated. Recently, we have reported that the c-Met receptor acts as an important mediator of the crosstalk between epithelial breast cancer cells and mesenchymal cells of the bone microenvironment, contributing to progression of osteolytic bone metastases *in vivo* [5]. In this work, we examined the potential therapeutic efficacy of targeting c-Met receptor using both a specific c-Met inhibitor (tivantinib) and RNAi technology in an *in vivo* murine model of breast cancer bone metastasis. Tivantinib is a novel, orally available, small-molecule, non-ATP-competitive c-Met inhibitor that is specific for the c-Met receptor [6]. Here we show that treatment with different concentrations of tivantinib affected bone metastasis progression in a dose-dependent manner. Moreover, treatment with tivantinib in combination with the silencing of c-Met protein expression by specific short hairpin RNA (shRNA) led to a much greater reduction in bone metastasis progression and cancer-induced bone destruction with an increase in overall survival.

Tivantinib:
the chemical structure



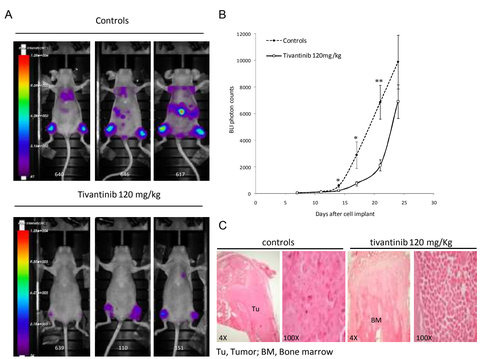
Treatment of 1833/TGL–injected mice with increasing doses of Tivantinib inhibits pathogenesis of breast cancer-derived bone /bone marrow metastasis and tumor-induced osteolytic lesions in a dose-dependent manner.



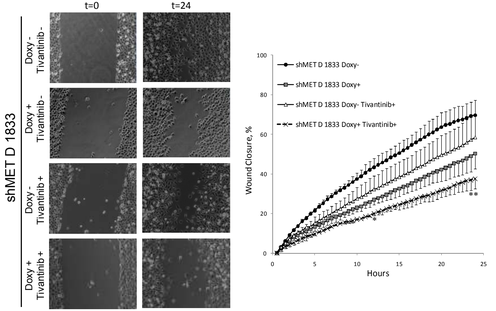


RESULTS

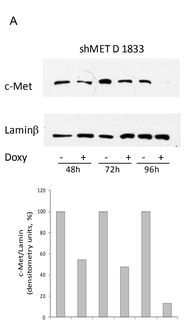
Chronic administration of tivantinib at the dose of 120 mg/Kg delays the development and progression of bone metastatic tumor growth (A-B) and reduced the formation of bone marrow metastatic tumor masses (C). The same dose was ineffective at inhibiting subcutaneously growing tumors.

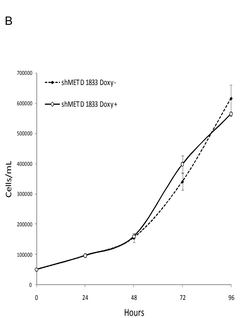


....reduced *in vitro* migration of shMET D 1833 cells. Interestingly, combination of doxycycline-induced c-Met silencing and tivantinib strongly decreased the migration potential of shMET D 1833.

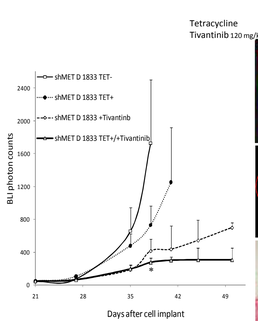


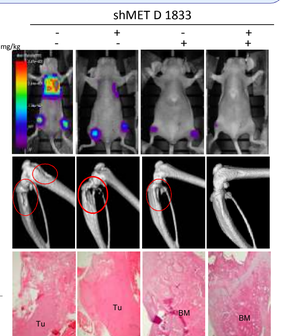
Specific doxycycline-induced shRNA-mediated c-Met silencing in shMET D 1833 cells was time-dependent (A). Inducible shRNA-mediated c-Met downregulation did not affect *in vitro* proliferation (B) but .....





shRNA-mediated c-Met silencing further increased Tivantinib efficacy: the combination of tivantinib (120 mg/Kg) plus c-Met downregulation induced a significant decrease in tumor burden in the bone, reduced lytic bone lesions and increased overall survival of injected mice.





MATERIALS AND METHODS

- **In vivo bone metastatic model:** 1833/TGL or shMET D 1833 cells [6] were i.c. injected into 4-week old athymic nude mice ($5X10^5$ cells/mouse). Implanted animals were randomized into groups of 10 mice each. Controls were treated with vehicle PEG 400: 20% vitamin E TPGS solution (60:40) or left untreated. Treated groups were administered with Tivantinib at different doses or with tetracycline (2mg/ml). Both vehicle and Tivantinib were administered daily *per os* starting 2 days after implant til the end of the experiment. Weekly bioluminescence imaging and micro-CT to monitor tumor burden and tumor-induced osteolysis were performed. During autopsy, hindlimbs were collected for histological analysis.
- **In vivo subcutaneous model:** Subcutaneously 1833/TGL-injected mice ($7.5x10^6$ cells/mouse) were randomized into 2 groups and chronically treated with vehicle or Tivantinib at the dose of 120 mg/Kg.
- **Inducible c-Met-silenced 1833 clone:** A tetracycline-inducible downregulation of c-Met was developed by using pSUPERIOR retro-based vector system to express inducible shRNA. Western Blot and densitometry analysis were performed to verify and quantify the doxycycline-inducible c-Met protein downregulation in shMET D 1833.
- **In vitro proliferation assay**: To test whether c-Met silencing could affect tumor cell proliferation, shMET D 1833 cells were cultured for 96 hours in absence or presence of doxycycline (2μg/mL), and cell growth at the indicated time points was determined.
- **In vitro migration assay**: To study directional cell migration *in vitro* the wound-healing assay was used. The extent of wound closure was quantified and expressed as the percentage of wound closure against the hours after wounding.

All the experiments were performed at "Mario Negri" Institute, Milan, Italy.

CONCLUSIONS

These results reveal that c-Met inhibition by tivantinib is effective in delaying the onset and progression of tumor growth in bone and bone marrow and in greatly diminishing osteolytic lesions, and these effects are not due to a direct cytotoxic effect of the compound. Moreover, the combination of the two modalities, tivantinib and shRNA against c-Met, with two different modes of action, could provide cooperative enhancement of c-Met inhibition with a consequent improvement in therapeutic efficacy. Therefore, these findings suggest that Tivantinib has significant therapeutic potential for the management of bone metastasis and that c-Met inhibition could be a potential clinical strategy for prevention of bone metastases from breast cancer. Most importantly, the finding that Tivantinib is active as an antimetastatic agent at low, non-cytotoxic doses provides the possibility to potentiate its efficacy by combinations with other therapies such as cytotoxic agents.

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