Bisphosphonates Inhibit Osteosarcoma-mediated Osteolysis Via Attenuation of Tumor Expression of MCP 1 and RANKL

Tetsuro oba¹, Hirotaka Haro².
¹Yamanahi university, Yamanashi, Japan, ²University of Wisconsin-Madison, Yamanashi, Japan.

Disclosures:  T. oba: None. H. Haro: None.

Introduction: Osteosarcoma (OGS) is the most common, primary bone tumor in children and young adults and accounts for over 50% of skeletal malignancies. Despite the success of chemotherapy, metastatic OGS has one of the lowest survival rates in pediatric cancer. As a consequence, there is a need for new therapies designed to treat OGS. Biologically, the aggressiveness of an OGS is proportional to the ability of the tumor to grow beyond the boundaries of the osseous compartment from which it originated. Hence, it is postulated that adjuvant therapies which reduce OGS proliferation and ability to induce bone destruction would slow tumor expansion and metastasis allowing for more time for chemotherapy to work. The purpose of this work is to explore the mechanisms responsible for, and means of inhibiting, OGS induced bone lysis. As evidence supports that cancer cells are not capable of direct bone resorption but rather from tumor cell activation of osteoclasts we tested the hypothesis that OGS induced bone loss is i) mediated by tumor induced osteoclastogenesis through the expression of RANKL and monocyte chemoattractant protein-1 (MCP-1) and ii) targeting this property will reduce OGS growth by attenuating the ability of the tumor to proliferate and ultimately escape the containment of its osseous compartment.

Here, we determined the expression of osteoclast activators (RANKL/MCP-1) and functional recruitment and subsequent formation of osteoclasts in vitro by OGS cell line of known differing aggressiveness. Finally, in both in vitro and in vivo models we determined the capacity of a bisphosphonate (Zolendronic Acid: ZOL) to reduce tumor expression of osteoclast activators and tumor growth.

Methods: In vitro: Murine osteosarcoma K-lines initially derived from a primary OGS in a Balb/C mouse were used for in vitro and in vivo. RT-PCR, Flow cytometry and ELISA were performed to measure expression and production of RANKL, OPG and MCP-1 from OGS cell line. Osteoclastogenesis and ability of bone resorption were measured using splenic monocytes with or without co-cultured osteosarcoma cell line. Cell migration was performed utilizing a modified Boyden Transwell chamber. K7M3 cells were cultured in the lower chamber with growth media after which cells were cultured in serum-free medium for an additional 24 hours with or without Zol treatment, and then RAW264.7 cells were added to the upper chamber.

In vivo: Single-cell suspensions (1 x 10⁵) of murine osteosarcoma (K7M3) cells were injected into the right tibias of a total of 33 (n = 15 saline; n = 18 Zol-treated) 6-week-old BALB/c mice as previously described. Zol or saline were injected subcutaneously (0.1 mg/kg) twice per week starting 1 day post tumor injection. Weekly radiographic evaluations were performed and assessment for enlargement of the soft tissues surrounding the injected tibia was conducted by analysis of radiographs as previously described. In addition, extent of osteolysis was determined with uCT of the trabecular bone volume within the tibial metaphysis. Long bones were fixed in 4% paraformaldehyde and sections were stained with hematoxylin and eosin (H&E) and for TRAP staining. Immunofluorescence for MCP-1, RANKL and
was performed using the manufacturer’s protocol and imaged with Nikon AZ100M fluorescent microscope.

**Results:** OGS cell line expression of RANKL was found to directly correlate with the malignant potential. Zol reduced RANKL expression and increased OPG production in aggressive K7M3 cells. Accordingly, we found that pretreatment (and removal) of K7M3 cells with Zol reduced K7M3-induced monocyte differentiation and ability of bone resorption. OGS cell line expression of MCP-1 was found to directly correlate with the aggressive potential and capacity to induce monocyte migration. Zol reduced expression and secretion of MCP-1 and inhibited K7M3 induced monocyte migration.

Zol significantly attenuated tumor growth compared to PBS-injected mice measured by radiographs and histology inoculated with osteosarcoma. In addition, these mice had reduced bone destruction as determined by bone fractional volume from μCT. Consistent with in vitro findings, immunofluorescent staining revealed that Zol treatment significantly reduced MCP-1 and RANKL expression by the tumor.

**Discussion:** We set out to explore the mechanisms by which OGS cells support osteoclastogenesis and if this activity enhances tumor growth. We determined that the OGS production of osteoclast stimulating molecular signals RANKL and MCP-1 and bone resorption correlates with the aggressiveness of OGS. We found that Zol attenuated OGS production of RANKL and MCP-1 reducing the capacity of the tumor to induce bone destruction. In vivo, these findings resulted in a significant reduction of osteosarcoma growth and bone destruction indicating that bisphosphonates may be a useful adjuvant therapy, not only by inhibiting osteoclast activation but, by directly attenuating the OGS ability to stimulate bone resorption.

It has been previously postulated that the RANKL/RANK/OPG axis offers a potential therapeutic target for the treatment of osteosarcoma. Rationale for this is supported by evidence that both local RANKL expression and increased osteoclast activity is associated with poor outcome in OGS patients. In addition, a recent study demonstrated that the rat OGS cell line UMR 106-01 expresses both RANKL and OPG. However, it had not been shown if RANKL directly correlates with OGS aggressiveness. Our data clearly demonstrates that RANKL expression correlates with OGS aggressiveness, and that the potential benefits of bisphosphonate treatment in an in vivo OGS model correlate with the local reduction of RANKL.

This is the first study to draw the correlation between OGS production of MCP-1 and its aggressiveness. MCP-1 is known to be expressed in osteoblasts and exhibits chemoattractant activity toward osteoclasts through its receptor, CCR2. In addition to inducing monocyte migration, MCP-1 was recently identified as an important factor acting directly on pre/osteoclasts to stimulate osteoclast formation and activity in the presence of RANKL. It has been shown to increases primary tumor growth and metastasis through tumor-associated macrophages (TAMs) and osteoclast recruitment in cancers. However the pathological role of MCP-1 in OGS progression had not previously been reported. We found that the aggressive K7M3 cells produce more than 35-fold more MCP-1 per million cells than osteoblastic MC3T3 cells.

Traditionally, Zol, a potent bisphosphonate, is thought to prevent bone destruction through inhibition of osteoclastogenesis. Here we provide evidence that, in addition to its effects on osteoclasts, Zol directly attenuates bone resorption by mitigating the molecular machinery utilized by the tumor cell to recruit and activate osteoclasts. As such we found that Zol reduces the ability of OGS to recruit monocytes (by decreasing MCP-1 production) and induce osteoclastogenesis (by altering the RANKL:OPG ratio).
Consistent with our overall hypothesis, we found that together, by targeting the ability of the tumor to induce bone destruction, in vivo Zol attenuates OGS-initiated osteolysis and proliferation in vivo.

**Significance:** We show that the high aggressive phenotype of OGS can be attributed to, in part, the expression of MCP-1 and RANKL and that bisphosphonates may provide key adjuvant therapy through its ability to directly down-regulate cancer cell expression and resultant bone destruction through MCP-1 and RANKL.