Proteasome Inhibitors Induce Differentiation, Cell Cycle Arrest And Apoptosis In Osteosarcoma.

Ali Zarezadeh MD. , Maria V. Guijarro PhD., Padraic P. Levings PhD., Elham Nasri MD., E.M. Viktoria Hyddmark, Margaret E. White, Glyn D. Palmer PhD., Steven C. Ghivizzani PhD., C. Parker Gibbs MD.

Musculoskeletal Oncology Laboratory, Department of Orthopaedics and Rehabilitation, University of Florida, Gainesville, Florida

Introduction: Osteosarcoma (OS) is the most common primary bone tumor in childhood and adolescence. Despite numerous treatment approaches, OS remains difficult to treat and often reoccurs after primary treatment. This failure may be explained by the intra-tumoral heterogeneity; an individual tumor is composed by cells with varying sensitivities to commonly used treatments. We have shown previously that we can identify and fractionate two distinct cell populations in OS, based on the cells' ability to activate an exogenous human Oct4/GFP reporter (hOct4/GFP). We found that tumorigenic potential is almost entirely restricted to the GFP+ population. Based on differential analysis of global gene expression, we identified the proteasomal degradation pathway as a potential target to be considered in treatment of OS. The aim of this study is to investigate the effects of Bortezomib (an FDA approved Proteasome Inhibitor) on osteosarcoma cells and to compare it to commonly used drugs such as Doxorubicin.

Methods: Human osteosarcoma cell lines were generated from patient biopsies and transfected with hOct4/GFP reporter. Xenograft tumors were generated by sub-cutaneous transplantation of GFP+ cells into NOD-SCID mice and fractionated into GFP- and GFP+ populations by FACS. GFP+, and GFP- cells from lung metastases were incubated with Doxorubicin and Bortezomib. Protein and mRNA were collected for western blots and gene expression analysis via real-time PCR, respectively. IC50s were calculated using Graphpad Prism®. Cell cycle analysis, apoptosis and cell viability was determined by Flow Cytometry. PicroSirius Staining was used to measure ECM collagen levels. Calcium deposition was measured by Alizarin Red staining. Cell migration was assessed using wound the healing method.

Results: Treatment of OS cells with Bortezomib, in doses ranging between 0.5 to 2 nM, induced differentiation towards an osteoblast-like phenotype, evidenced by production of collagen and extracellular calcium deposition.. At higher doses (from 2 to 10 nM), Bortezomib arrested OS cells in the G2/M phase of the cell cycle, and this was consistent with a reduction of CyclinB1/CDK1 protein levels, detectable by Western blot, and a corresponding increase in expression of the cyclin-dependent kinase inhibitor, P21. At a dose of 10 nM, Bortezomib induced apoptosis and this was found to correlate with induction of CHOP protein levels, which is associated with excessive endoplasmic reticulum (ER) stress. Cell migration assays, performed as a surrogate of metastasis, showed that Bortezomib inhibited migration more effectively than Doxorubicin Strikingly, IC50 data showed that non-tumorigenic GFP- cells in the tumor were almost three times more resistant to Doxorubicin (a conventional OS treatment) compared to the tumorigenic GFP+ cells.   In contrast, GFP- cells were more sensitive than GFP+ cells to Bortezomib. Our data also indicate that Bortezomib has a synergistic effect with Doxorubicin and the treatment of OS cells with Bortezomib can significantly sensitize OS cells to the effect of Doxorubicin.

Discussion: Although Proteasomes Inhibitors (PIs) have been used traditionally to treat blood malignancies, the application of PIs to treat OS is a novel idea. Our data show that Bortezomib exhibits a range of favorable dose dependent anti-cancer effects in OS cell lines. This includes a partial recovery of the differentiation machinery, which is defective in various OS tumors, and restoration of the G2/M and G1/S checkpoints. Considering that the prognosis of OS patients has not been improved for almost more than three decades, finding new strategies to fight OS is the utmost importance. Targeting Proteasomal Degradation Pathway in OS shows promising results *in vitro*. We are planning to test the drug in our xenograft model to generate *in vivo* data.