Proteasome Inhibition in Osteosarcoma Therapy
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Introduction
Osteosarcoma (OS) is a primary bone cancer that affects children and young adults. OS has a poor prognosis due to propensity for lung metastasis, resistance to radiation and inconsistent response to chemotherapy. We have previously determined that OS aggressiveness and resistance to apoptosis is associated with the increased NF-κB activity. In addition, we and others have shown that the loss of function of a major osteoblastic differentiation factor, Runx2, contributes to the aggressive and undifferentiated phenotype in OS and that gain of Runx2 function inhibits proliferation and induces apoptosis in OS cell lines. We also demonstrated that Runx2 transcriptionally activates a major pro-apoptotic gene, Bax, thereby sensitizing cells to apoptosis. Based on the above, we assumed that inhibition of NF-κB and induction of Runx2 may suppress OS growth and sensitize OS to apoptosis. NF-κB and Runx2 are inversely regulated by the proteasome and proteasome inhibitors have been shown to suppress NF-κB and, on the other hand, induce Runx2 function. We, therefore, hypothesized that the proteasome inhibitor, bortezomib (Bzm), via suppression of NF-κB and induction of Runx2, may inhibit tumor growth and induce apoptosis in OS.

Methods
Cell culture. We have used immortalized human osteoblasts, hFOB; primary rat osteoblasts (ROB); and osteosarcoma cell lines, HOS, 143B and OS187. Cell growth was assessed using an automated cell counter.
Real-time RT-PCR. Expression of genes of interest assayed using real-time RT-PCR approach was normalized to GAPDH.
Luciferase reporter assay. Cells were transfected with various luciferase reporters and with pRL-TK as a reference. The reporter signal was normalized to the renilla signal and expressed as RLU.
Western blotting. Cells were lysed and subjected to PAGE and immunoblotting using various primary antibodies. Blots were then stripped and re-probed for β-actin to verify equal loading.
Cellular and nuclear morphology assay. Cellular morphology was examined using an inverted microscope. A nuclear fluorescent stain, Hoechst 33342, was used to visualize nuclei.
Casparase-3 activity assay. Caspase-3 activity was measured using Ac-DEVD-AMC fluorogenic substrate cleavage assay.
In vivo OS xenograft model and treatment with Bzm. 143B cells expressing luciferase (143B-luc) were injected into tibiae of nude mice. Tumor growth was monitored longitudinally using bioluminescence imaging (BLI). Starting at day 8, mice were given PBS or Bzm I.P. at a dose of 1 mg/kg every 3 days for 3 weeks. At sacrifice tumors were measured using calipers.
Immunohistochemistry. Tumors were resected, fixed in formalin, decalcified and embedded in paraffin. Serial sections were prepared and processed for immunohistochemistry. The immunolabeling included Ki-67/active caspase-3, p65 NF-κB subunit, Runx2 and Bax. The number of Ki-67 and active caspase-3 positive cells was counted stereologically. Staining intensities of nuclear p65, nuclear Runx2 or cytosolic Bax were measured in 10 random fields per section and analyzed using the ImageJ software. The treated group was compared to the control group.

Results
Phenotype of OS cell lines. When compared to non-malignant proliferating hFOB osteoblasts, OS cell lines showed: 1) significantly higher proliferation rates; 2) similar Runx2 mRNA expression confirming their osteoblastic lineage; 3) decreased alkaline phosphatase (ALP) expression confirming their undifferentiated phenotype; 4) decreased Runx2 protein levels indicating increased degradation; and 5) 6-10 fold increase in NF-κB activity.

Discussion
Our study showed that the proteasome inhibitor, bortezomib, suppressed growth and induced apoptosis in osteosarcoma cells in vitro and in orthotopic osteosarcoma xenografts in vivo. Bortezomib showed selectivity towards osteosarcoma cells leaving non-malignant osteoblastic cells unaffected at clinically-relevant doses. The effect of bortezomib was associated with inhibition of NF-κB, and induction of Runx2 and Bax, although the direct relevance and significance of these molecular changes has to be further investigated. Our study is consistent with earlier reports that demonstrated a differentiating effect of bortezomib in osteoblasts related to stimulation of Runx2. Because bortezomib is already approved for treatment of multiple myeloma and lymphoma, it makes it an attractive candidate as a neo-adjuvant agent in osteosarcoma.

References
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![Fig. 1](image1.png)
**Fig. 1** A) Bzm suppresses growth of OS cells but not normal osteoblasts, hFOB or ROB in vitro; B) Bzm induces apoptotic markers in OS cells, such as nuclear fragmentation.

![Fig. 2](image2.png)
**Fig. 2** A) Bzm induces regression of xenografted OS tumors in mice; B) OS xenografts from Bzm-treated mice have increased number of apoptotic cells.