Title

# Suppression of Human Multiple Myeloma Cell Growth by TBL-12 in Combination with low doses of Velcade: Insight in to the modulation of IL-6/STAT-3 mechanisms

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## Abstract

Multiple myeloma (MM) is characterized by the latent accumulation of plasma cells in the bone marrow. As the side effects due to drugs that are currently in use are largely complex, targeting MM with natural agents in combination with FDA approved drugs seems to be a novel approach to overcome the side effects. **Methods**: In this study we tested the efficacy of proteasome inhibitor Velcade (Bortezomib) in combination with TBL-12, (an extract from Sea Cucumber, Unicorn Pacific Corporation) in human myeloma cells that are IL-6- dependent and-independent. To determine the effect of Velcade with TBL-12 on cell growth we performed dose and time dependent assays in cells stimulated with IL-6 (5ng/ml) and/or TNFα (5ng/ml). Effect on cell survival at different time points of 24, 48 and 72h were determined by MTT assays after treatment with TBL-12 (100ug/ml) alone and in combination with Velcade (1-10 ng/ml). Co-culturing of myeloma cells MM1 and U266 with human umbilical vein endothelial cells (HUVEC) followed by treatment with already established dose of Velcade with TBL-12 was conducted to determine cell adhesion and the effect on VEGF and VEGR2 levels to determine anti-angiogenic effects. **Results:** Using low doses of Velcade in combination with an already established dose for TBL-12 (100ugs/ml) we found a time and dose dependent inhibitory effect on cell survival determined by MTT assays. We observed cell survival rate reduced from 100 % to 30% at 48h and significantly reduced to 20% at 72h ($p<0.001$) in both MM1 and U266 cells. These findings suggest low dose effect of Velcade in combination with TBL-12 in a time dependent manner. Interestingly, co-culturing of myeloma cells MM1 and U266 with human umbilical vein endothelial cells (HUVEC) at the same time point followed by treatment with Velcade (5ng plus 100 ugs of TBL-12) showed a significant decrease (45%) in cells adhering to the surface of HUVEC determined by phase contrast and immunofluorescence microscopic observations. Findings from flow cytomtery analysis indicate a significant decrease in VEGFR2 level in TBL-12 treated cells. **Conclusion:** Overall findings from this study suggest the potential use of TBL-12 in combination with Velcade against MM. Ongoing trials with TBL-12 at NYUCI and this correlative data could support future clinical trials.

## Introduction

Multiple myeloma **(**MM) is a malignancy of terminally differentiated B cells accounting for approximately 10% of all hematological malignancies. The proteasome inhibitor bortezomib (Velcade) is widely used in the treatment of MM with remarkable response rates in both relapsed and newly diagnosed MM. However, the treatment response with Vecade is associated with drug resistance and toxicity issues. The immediate goal in treating MM is to get the disease under control and to keep the patient in remission with a good quality of life by supplementing with potential natural chemopreventive agents. Natural agents including resveratrol and curcumin were shown to exert antitumor activities in human cancers including myeloma and promyelocytic leukemia cells by reducing osteoclast formation and by sensitizing the cells that promote multiple mechanisms associated with apoptosis. Promising preclinical studies demonstrate the ability of Velcade in enhancing the sensitivity of myeloma cells to conventional anti-myeloma agents. Since the progression of multiple myeloma is correlated with angiogenesis, in this study we tested the effect of Velcade in combination with the natural agent TBL-12 and examined the effect on angiogenesis. We found that TBL-12 could inhibit myeloma cell growth, and prevent migration, cell adhesion and angiogenesis in human umbilical vein endothelial (HUVEC) and human pulmonary endothelial cells (HPEC). Our findings suggest that Velcade could sensitize and enhance the effect of TBL-12 by down regulating IL-6 and TNFα mediated signaling on VEGF and VEGFR2 expression. Overall, these findings suggest modified treatment regimens that could reduce toxicity with natural agents and prevent myeloma and the related disease progression.

## Methods

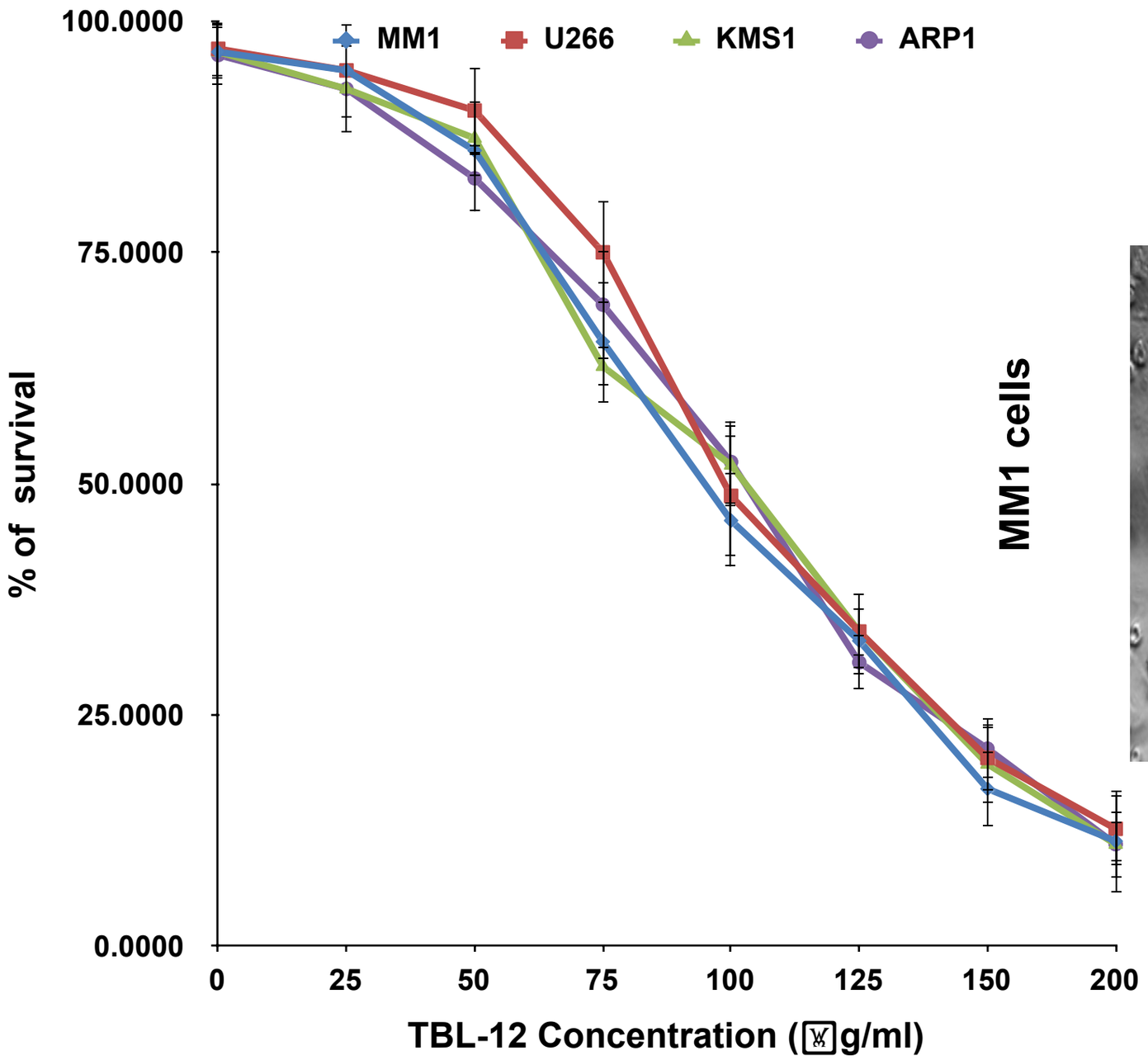
**Cell growth/viablity assay:** Exponentially growing human myeloma cells U266, MM1, KMS1 and ARP1 were cultured in 96 well plates and were treated with different concentrations of TBL-12 alone and in combination with Velcade. Cell viability was measured at different time points by conducting MTT assays as per the manufacturers directions. Results presented in this report are data gathered from three independent experiments. Cells not exposed to the agents served as the control. Calculation of 50% inhibitory concentration ($IC_{50}$) was done using SPSS 16.0 software.

**ELISA assays for VEGF:** Soluble vascular enodthelial growth factor (VEGF) and interlukin 6 (IL-6) levels in the myeloma cell culture medium collected at 48h after treatment with TBL-12 or Vecade was measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kit (Quantikine; R&D systems) according to the manufacturers instructions. Optical densities were measured at 450 nm using an ELISA reader and concentrations were determined based on the OD values of the standard curves.

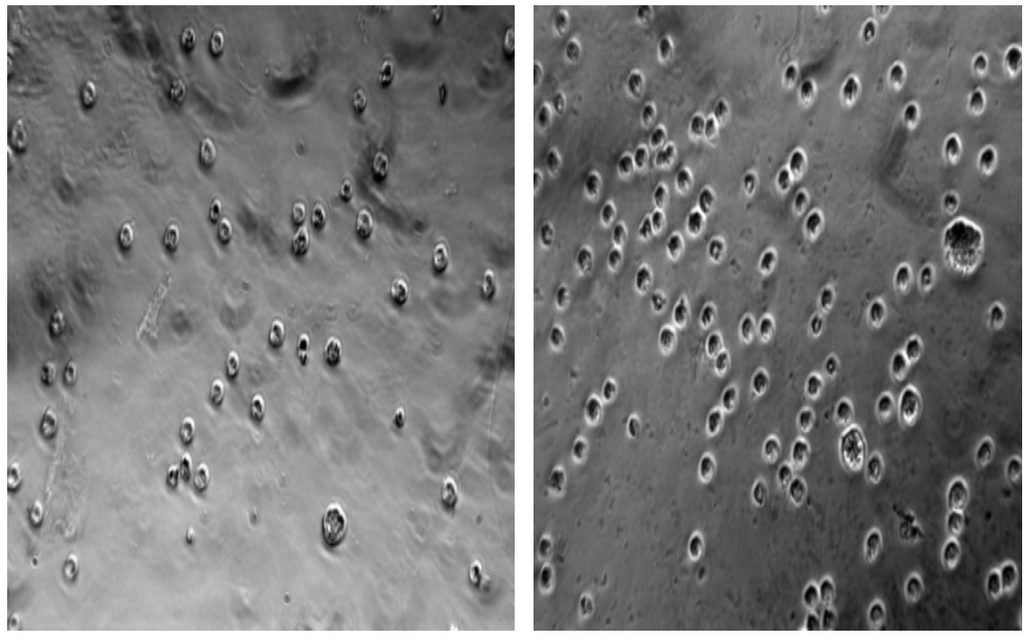
**Measuring CD309+ve (VEGFR-2/KDR) by flowcytometry:** Myeloma cells U266 were spiked with HUVEC cells followed by treatment with TBL-12 was stained with a high affinity receptor CD309 for VEGF. Briefly, cells labeled with antimouse CD309 (VEGFR-2/KDR)-Biotin (Miltenyi Biotec) were stained with antibiotin APC/PE as well as CD45-FITC. Flow cytomtery analysis was performed in cells labeled also stained FITC isotype as control.

**Endothelial tube formation assay**: Effect of TBL-12 on human endothelial cells HUVEC and HPEC were assessed by endothelial tube formation assay using CB4-200 (Cell Biolabs, San Diego, CA). Briefly, 96 well plates were coated with Matrigel provided by the manufacturer. Cells that are treated with TBL-12 or Valcade were added in the medium in the presence of 10 ng/ml VEGF and 10 ng/ml IL-6 with DMSO served as a control.. Cells seeded onto the matrigel coated plates at were incubated at 37°C. Tube formation images were captured at a higher magnification with a digital microscope camera system (Olympus, Tokyo, Japan) at different time points. The number of the tube formation was quantified by measuring the length of tubes in >5 randomly chosen fields from each well using an Image-Pro Plus software and was calculated against untreated groups.

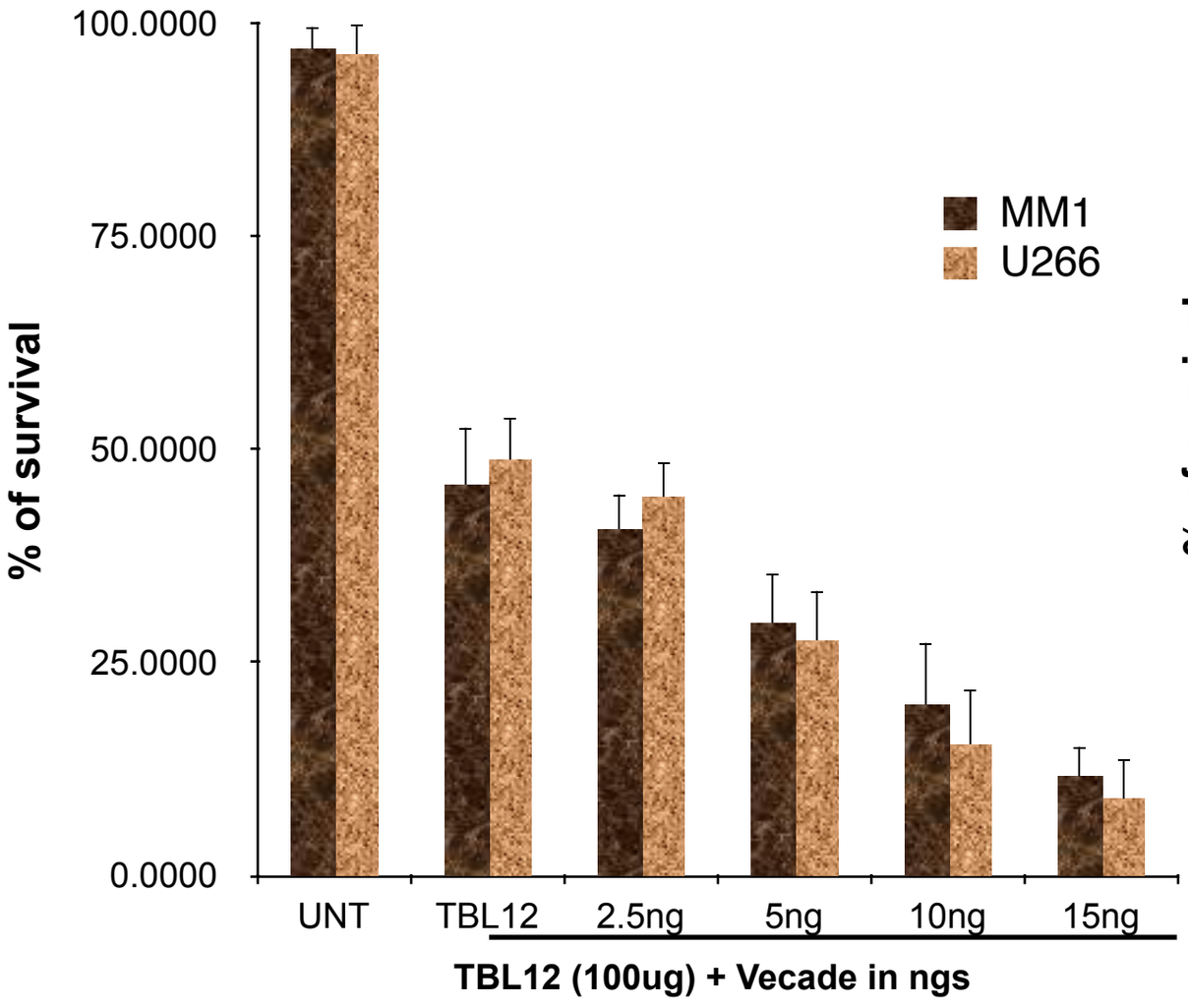
# Dose dependent effect of TBL-12 on myeloma cell survival



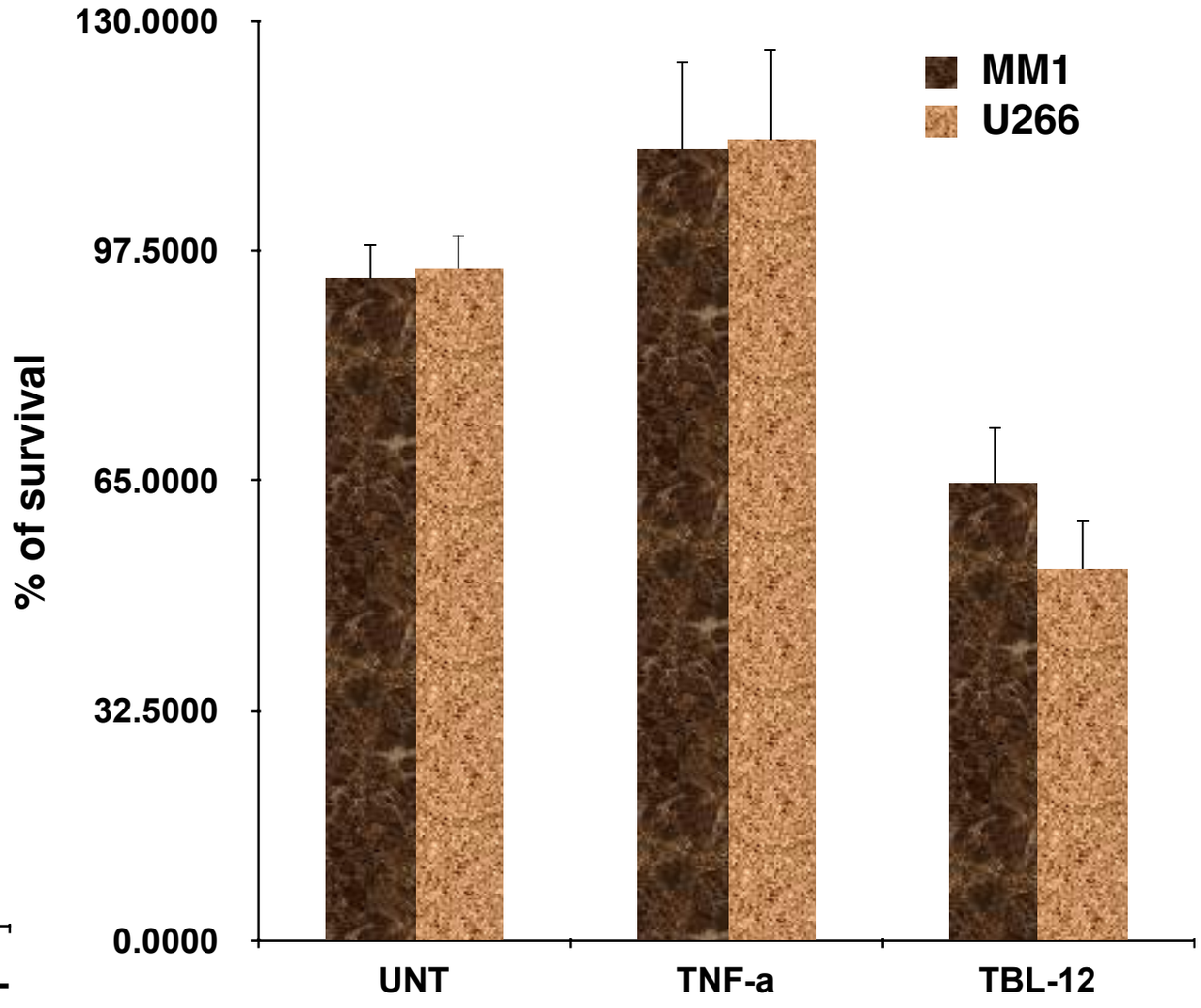

MM1 cells

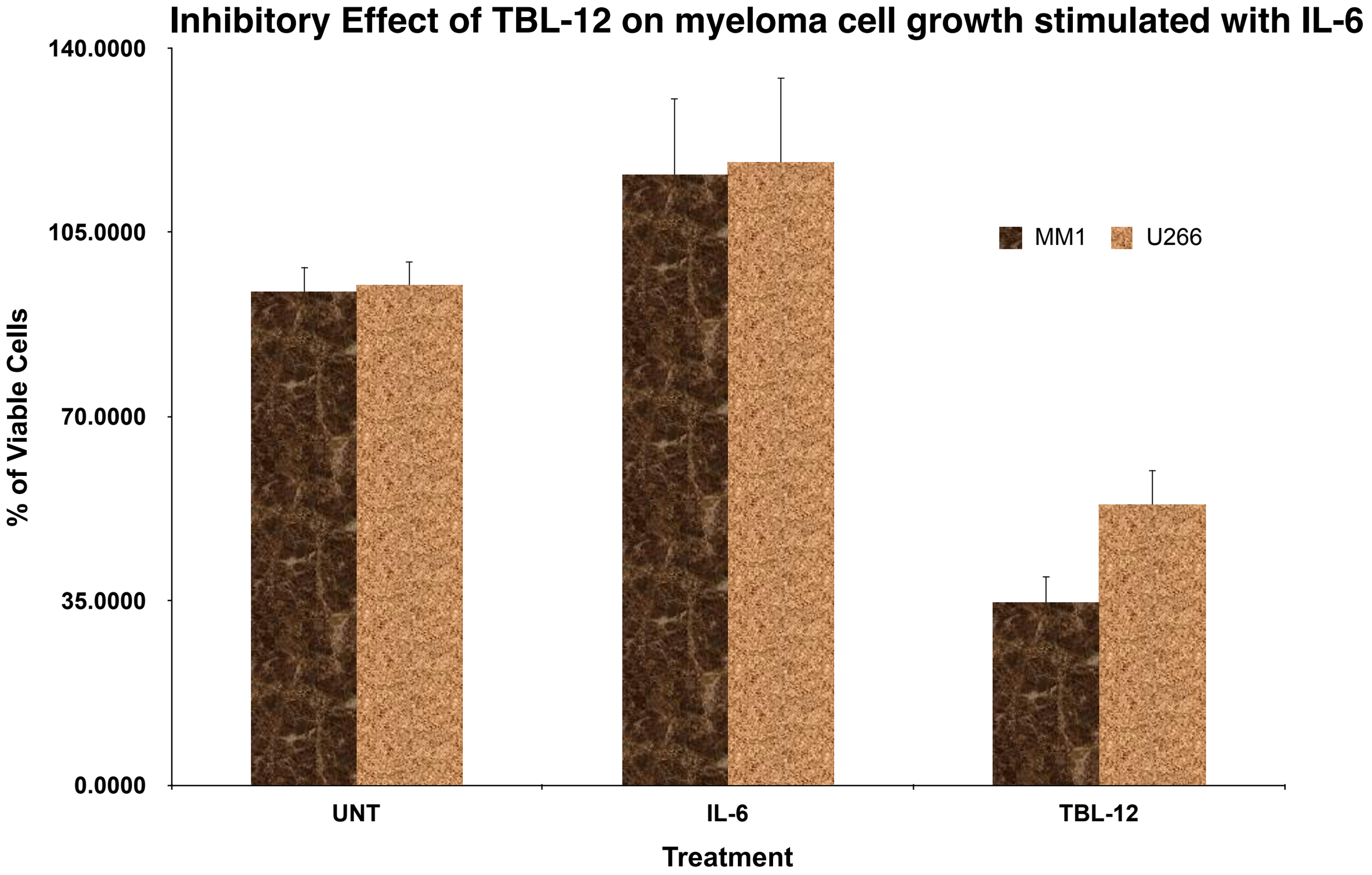


## Velcade enhance the inhibitory effect of TBL-12 at low doses in myeloma cells

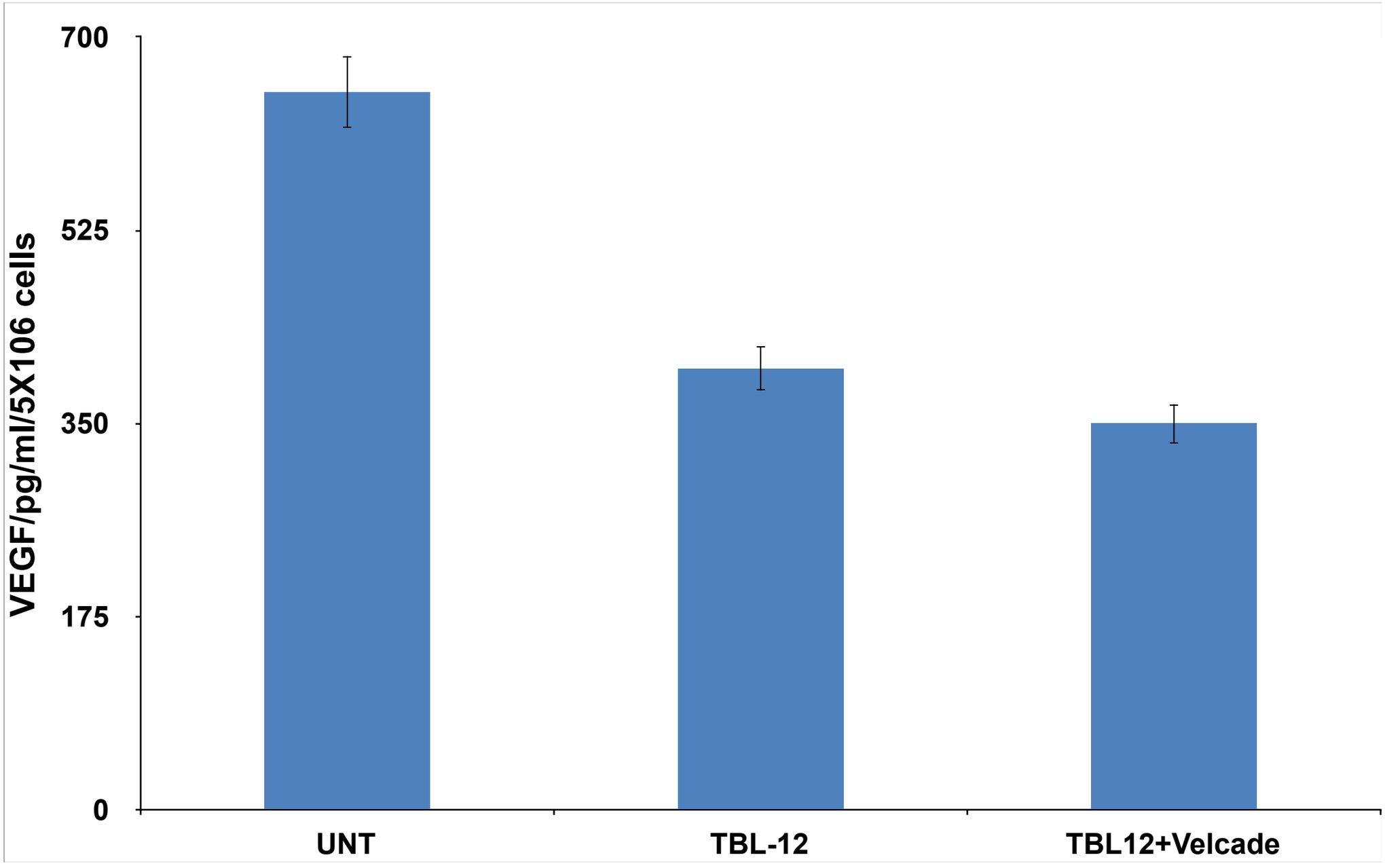



## Effect of TBL-12 on myeloma cells stimulated with TNF-α

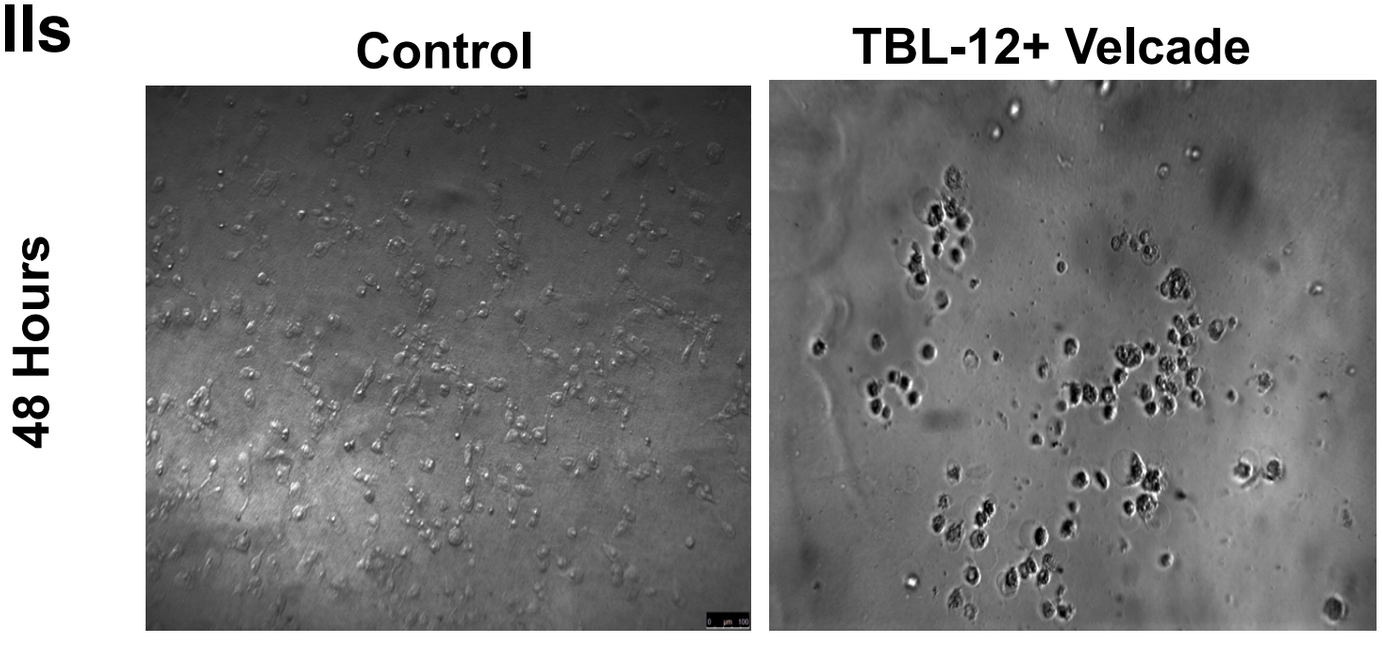



Inhibitory Effect of TBL-12 on myeloma cell growth stimulated with IL-6
140.0000
105.0000
70.0000
35.0000
0.0000
% of Viable Cells
MM1
U266
UNT
IL-6
TBL-12
Treatment

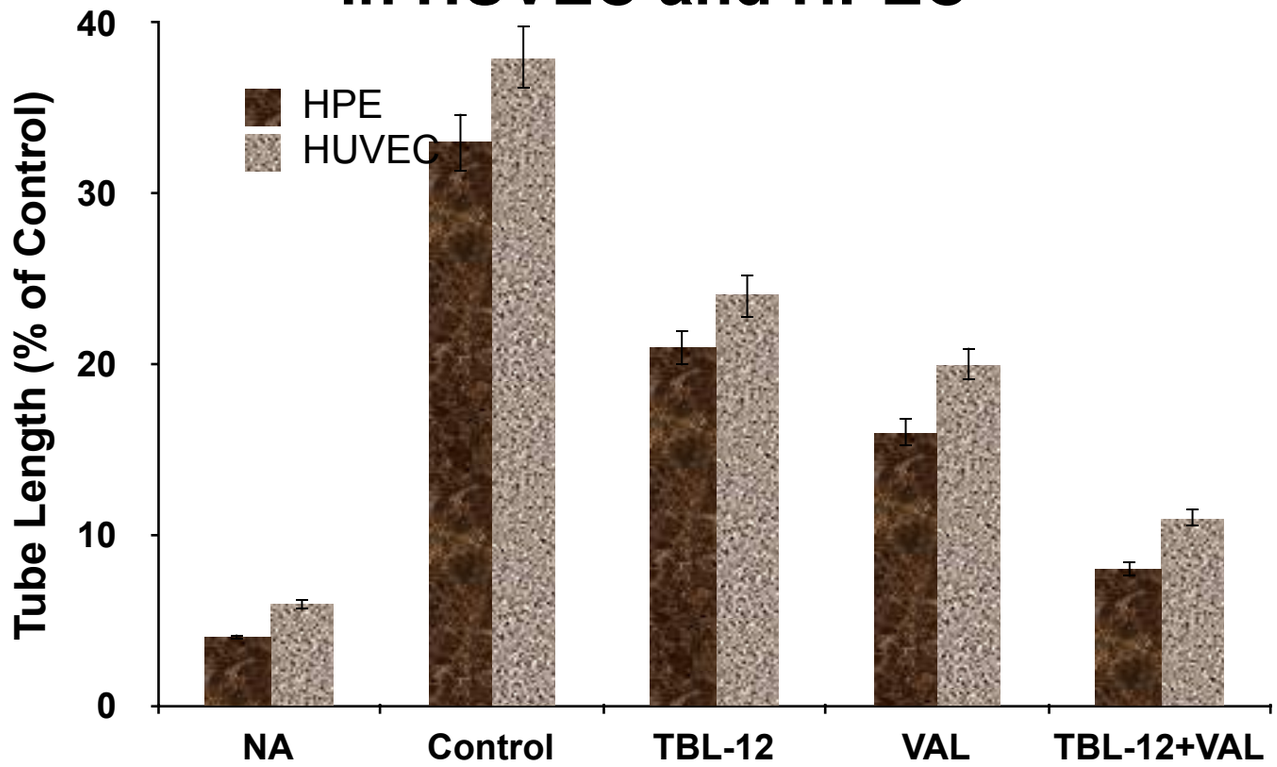
# Effect of TBL-12 plus Velcade on VEGF in HUVEC co-cultured with U266



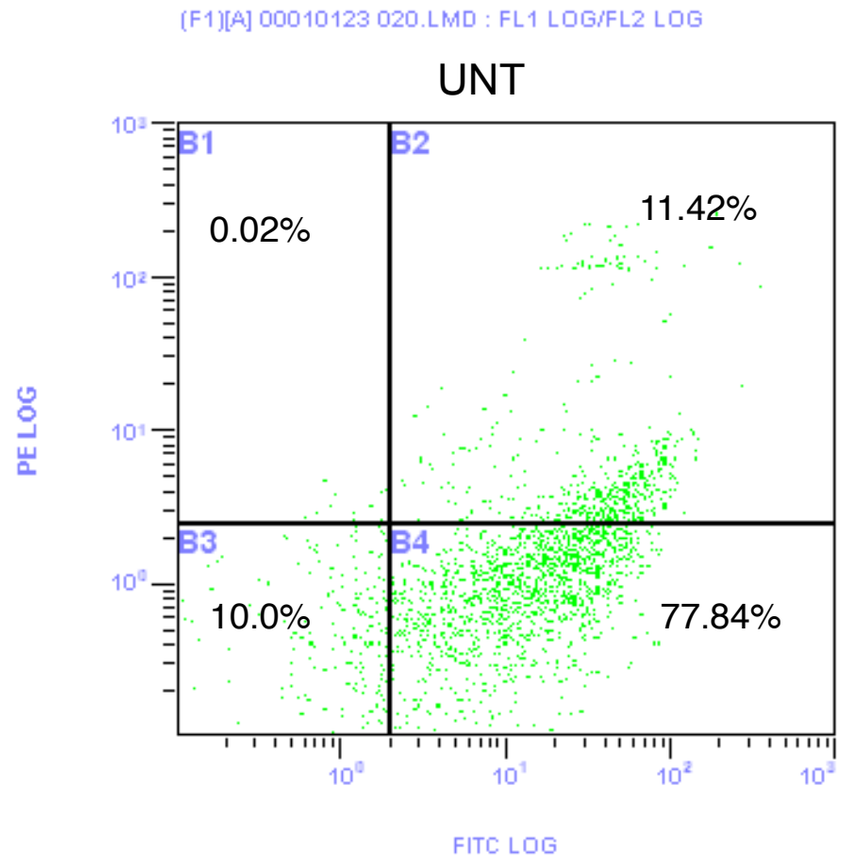
# Effect of TBL-12 on Tube formation in HUVEC cells

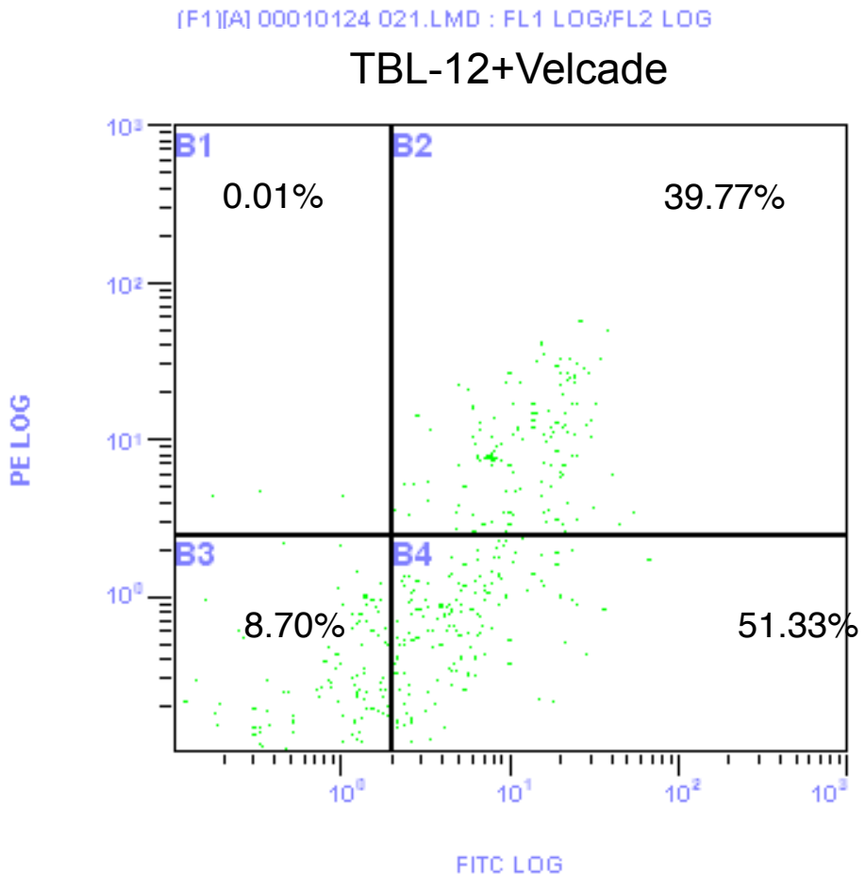



# Effect of TBL-12+Velcade on the tube formation in HUVEC and HPEC

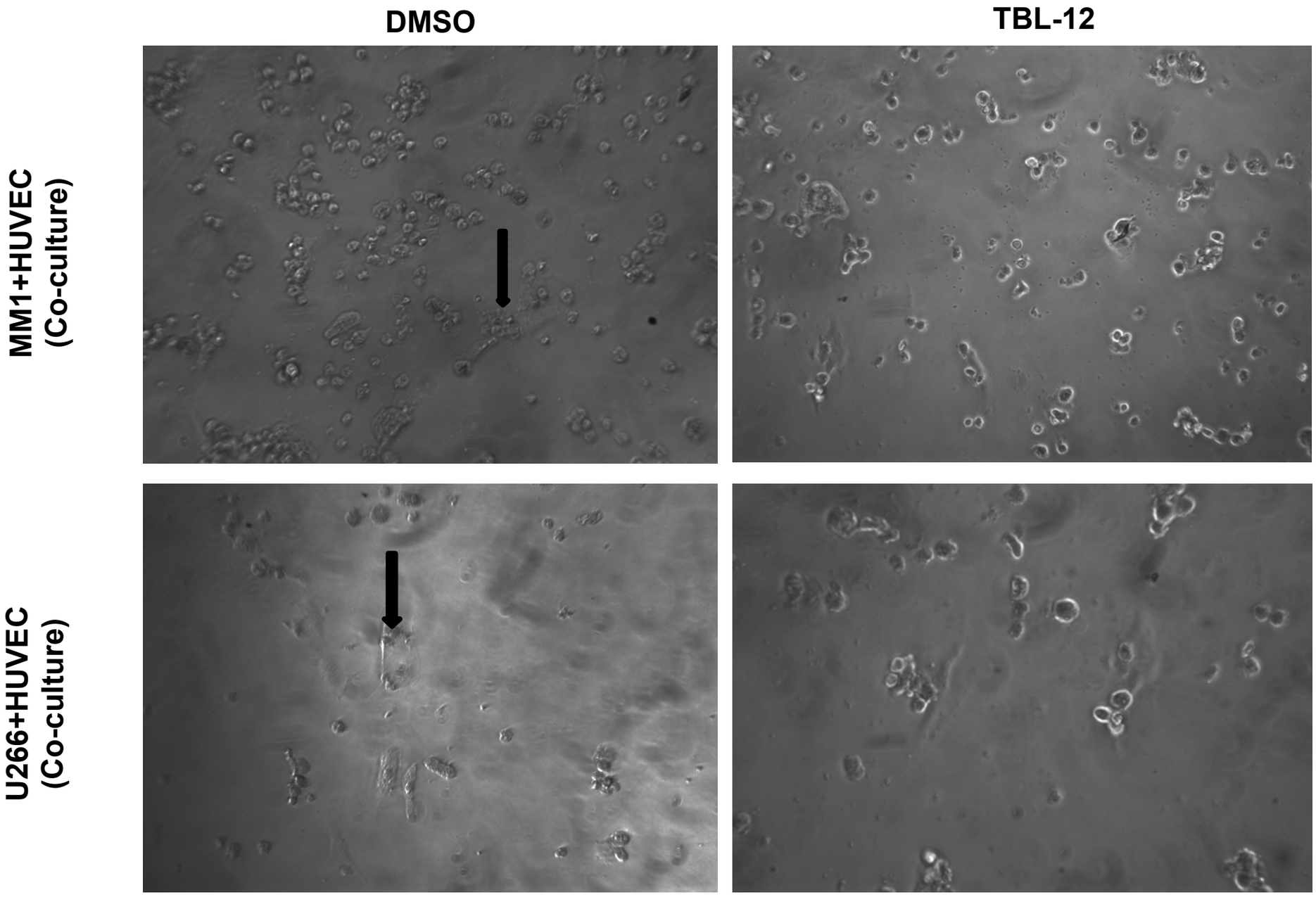


# Effect of TBL-12 on vasculoendothelial growth factor receptor (CD309+ve (VEGFR-2/KDR)



# Inhibition of cell adhesion by TBL-12 in human endothelial cells




Arrow indicate adhesion of myeloma cells on HUVEC

# Summary of Findings

**Effect of TBL-12 on myeloma cell growth:** Myeloma cells treated with TBL-12 showed a dose dependent effect on cell growth inhibition at an $IC_{50}$ dose of 100ug/ml in the four cell types tested.

- Velcade at concentrations ranging from 2.5ng to 10ngs with a single dose of TBL-12 at 100ug/ml showed inhibitory effect on cell survival with an IC-50 around 5ng/ml.
- TBL-12 inhibited IL-6 or TNFα induced cell growth; a significant inhibitory effect was observed in the presence of Velcade.
- TBL-12 blocked the proliferation of endothelial cells (HUVEC and HPEC) that are co-cultured with U266 cells for 48h at a dose 100ug/ml.

**Effect on angiogenesis:** Soluble vascular enodthelial growth factor (VEGF) levels measured in the myeloma cell (U266 and MM1-culture medium) collected at 48h after treatment with TBL-12 (100ug/ml) plus Velcade (5ng) showed a significant decrease in the soluble VEGF compared to that in the untreated cells. As myeloma cells could stimulate angiogenesis via VEGF, we investigated whether TBL-12 might affect VEGFR2 expression. Results from llow cytomtery analysis showed that when compared to the control, cells treated with TBL-12 reduced the level of VEGFR2 by >50%.

**Effect of Tube formation:** We tested the effect of TBL-12 in combination with Velcade on tube formation in an in vitro angiogenesis model using the matrigel tube formation assay. TBL-12 (100ug/ml) in combination with Velcade (5ng/ml) blocked VEGF mediated tube formation in HUVEC or HPEC with a significant effect on tube length and numbers. Effect of TBL-12 on IL-6/STAT-3 signaling is in progress.

# Conclusion and Future Directions

Overall findings from this study suggest the potential use of TBL-12, a natural agent that could modulate or block angiogenesis and prevent further progression of MM in combination with Velcade at low doses and reduce toxicity. In this context the ongoing trials with TBL-12 at NYUCI and this correlative data could support future clinical trials.

# Acknowledgement

We thank the Unicorn Pacific for providing TBL-12 and NIEHS Center Molecular Biology Core Facility for providing necessary services related to the molecular biological analyses.