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Effects of Heme Oxygenase 1 Inducer, t-BHQ, on Growth of Multiple Myeloma Cell Lines, and
on Osteoblast and Osteoclast Differentiation

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

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ABSTRACT

Expansion of plasma cells within the bone marrow constitutes the onset of multiple myeloma (MM). This disease manifests clinically primarily through the formation of osteolytic bone lesions that can lead to osteoporosis. The reason for the development of such lesions is the disruption of the equilibrium between bone resorption and bone formation as a result of proliferation of osteoclasts and reduction in the number of osteoblasts in the process of differentiation of mesenchymal stem cells (MSCs). The maintenance of bone architecture is critically dependent on osteoblasts and osteoclasts, the activity of which is underpinned by a range of soluble factors. The present study sought to reduce cellular genotoxicity by using t-BHQ to target the major antioxidant gene heme-oxygenase 1 (HMOX1).

This study provides a detailed investigation of the function of osteoclasts, osteoblasts MSCs and reactive oxygen species ROS in MM, especially with regards to disease progression. Since MM is associated with downregulation of HMOX1 expression, this study postulates that t-BHQ could be used to pharmacologically upregulate the expression of HMOX1. This pharmacological agent t-BHQ can trigger apoptosis in MM, confer cell protection against oxidative damage by upregulating HEME OXYGENASE 1, prevent osteoclasts from forming and ultimately avoid bone deterioration.

Student's two-tailed t-test was conducted to determine how the different types of cells (MM cell lines, MSCs, osteoblasts and osteoclasts) responded to t-BHQ. The *P*-value was less than 0.05, signifying that the results were of statistical significance.

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CHAPTER 1. BACKGROUND

1.1. Multiple Myeloma

The MM cells increase rapidly in terms of their proliferation rate because they contain cancer stem cells which downregulate many important genes (Yaccoby & Epstein, 1999). The microenvironments of MM patients are often heavily loaded with viruses that infiltrate the layer of endothelial cells, increasing the effects of angiogenesis and causing hole marks around myeloma cells (Yaccoby, Barlogie, & Epstein, 1998).

It is important to control the impact of the microenvironment in MM patients to prevent interaction with tumor cells that could progress the disease; this can be done by prohibiting osteoclast activity in the microenvironment (Yaccoby, Wezeman, Henderson et al., 2004). HMOX1 is downregulated in MM bones, and HMOX1 is required for osteoblasts and inhibiting osteoclast which will enhance the microenvironment of MM (Li, Ling, Khan, & Yaccoby, 2012).

In MM patients, where osteoblast activity increases, there is generally a decrease in cancerous myeloma cells (Yaccoby, Wezeman, Zangari, et al., 2006). Bone deterioration and lesion formation occur when monoclonal paraprotein is excessively expressed as a result of plasma cell hyperproliferation within the bone marrow. These phenomena characterize the B-cell malignancy known as multiple myeloma (MM). More specifically, bone deterioration is caused by osteoclasts developing and expanding abnormally coupled with suppression of osteogenesis (Gerecke et al., 2016). Evidence has been brought forth that the undetermined monoclonal gammopathy may be the cause of MM and therefore MM onset may be preceded by this pre-malignant event (Jewell et al., 2015). According to plasma cell cytogenic analysis, a range of MM types can be distinguished. Trisomies occur in around 40% of MM cases, whilst other cases exhibit translocations in the heavy chain of immunoglobulin situated on chromosome 14q23, and

there is also a small proportion of cases with both trisomies and translocations (Singhal & Mehta, 2006).

MM pathogenesis is believed to commence with trisomy and translocation of the immunoglobulin chain because these events are considered to occur at the start of the disease (Kumar et al., 2012). Additional molecular events accompany subsequent MM stages, including chromosome 1q gains, *RAS* mutations, chromosomes 1p, 13 and 17p deletions, and *MYC* oncogene translocations. Rajan and Rajkumar (2015) reported that a proportion of 15% of MM cases displayed T(11; 14) (q13; q32) in relation to the cyclin D1 (*CCND1*) gene, and Multiple myeloma SET domain (*MMSET*), whilst the MAF bZIP transcription factor B (*MAFB*) gene has also been associated with extra genomic aberrations. Prognosis and treatment response are particularly favorable in cases displaying only trisomies or t(11; 14) (q13) and t(6; 14). By contrast, MM is more likely to progress and life expectancy does not exceed two years in cases displaying t(6; 14), t(14; 16) and del(17p) (Binder et al., 2016).

Gaining insight into the abnormal molecular pathways that may critically underpin MM tumorigenesis is imperative, as this disease is mostly untreatable. Chng et al. (2007) suggested that impaired regulation of *CCND* and retinoblastoma (*Rb*) pathway may contribute to MM tumorigenesis. This observation is supported by the findings of Krämer et al. (2002), who reported that a minimum of 17% of MM cases displayed the *CCND1* gene, while around 28% of cases exhibited *Rb* deletions at chromosome 13q14; furthermore, MM unfavorable prognosis and recurrence were associated with p16^{INK4A} hypermethylation alongside *CCND1* translocations (Krämer et al., 2002).

It has been argued that mesenchymal stem cells (MSCs) in the bone marrow are stimulated to produce interleukin 6 (IL-6) when the histone H3 is present, being associated with

heightened MM tumorigenesis prevalence (McNee et al., 2017). Intracellular factors like basic fibroblast growth factor (bFGF) play an important role in the mediation in osteoclast stimulation by IL-6 in the context of MM tumorigenesis (Bisping et al., 2003). Furthermore, MM tumors are resistant to cytotoxic agents because IL-6 contributes to inhibit apoptosis and foster MM proliferation (van de Donk et al., 2005). IL-6 activates the phosphatidylinositol-3 kinase (PI-3K) and Janus Kinases/signal transducer and activator of transcription proteins3 JAK/STAT3 pathways, thus conferring apoptosis resistance, whilst IL-6 activation of the Ras-MAPK, JAK/STAT3 and PI-3K pathways is the basis for MM cell proliferation and expansion (Brocke-Heidrich et al., 2004).

MM cell lines and samples derived from patients have been found to contain the nuclear factor kappa-light-chain enhancer of activated B cells NF- κ B protein, suggesting that the NF- κ B signaling pathway plays a significant part in MM tumorigenesis. Keats et al. (2007) claimed that production and inactivation of cellular inhibitor of Apoptosis protein cIAP, cluster of differentiation CD40 and tumor necrosis factor receptor associated factor TRAF, which underpin NF- κ B signaling activation in MM, are enhanced by excessive activation of the NF- κ B signaling pathway. Meanwhile, Annunziata et al. (2007) reported that, in MM patient samples, the expression of genes regulated by NF- κ B was closely correlated with intensified nuclear p65 expression, alongside the occurrence of IL-6, a proliferation-inducing ligand APRIL or B- cell activating factor BAFF within the microenvironment of the bone marrow.

Currently, MM can be treated with a number of pharmacological agents. For instance, MM relapse can be managed and survival outcomes enhanced through separate or combined administration of bortezomib, lenalidomide, dexamethasone, cyclophosphamide, melphalan, carfilzomib, and daratumumab (Ludwig & Delforge, 2017). Thalidomide, lenalidomide and

pomalidomide contribute to the activation of cereblon E3 ubiquitin ligase and therefore play a role in the regulation of immunity. Cereblon E3 ubiquitin ligase serves as catalyst of ubiquitylation and breakdown of Ikaros zinc finger family IKZF1 and IKZF3, which are B cell proteins (Holstein & McCarthy, 2017). Besides having properties that can be damaging to DNA, these proteins have anti-angiogenic and immunomodulatory action, so they may exhibit cytotoxicity to MM cells, and they can also activate MM cell apoptosis by suppressing TNF (Bruno et al., 2005).

Relapsed MM can be managed with the proteasome-suppressing bortezomib, which can promote the activity of osteoblasts while downplaying the activity of osteoclasts. Furthermore, bortezomib makes MM cells more sensitive to other drugs, with cases administered combination therapy exhibiting a total response rate of over 50% (Field-Smith et al., 2006). MM cannot be completely cured at present, yet eradication might be possible in the future owing to the research progress that has been made with regard to targeting oncogenic signaling pathways and abnormal genomic events of critical importance.

1.2. MSC Microenvironment in Multiple Myeloma

Located in the bone marrow, MSCs are distinguished by the fact that they can differentiate into a range of types of cells, including osteoblasts (Uccelli et al., 2008), and they express numerous cell markers related to particular antigens (e.g. smooth muscle actin, cytokine and growth factor receptors and epidermal growth factor receptor EGFR, adhesion molecules, endoglin, L-selectin) (Lv et al., 2014). The molecular markers bone/liver/kidney alkaline phosphatase, osteopontin, osteocalcin, collagen type I and II and proteoglycans are associated with MSC terminal differentiation into osteoblasts (Minguell et al., 2001).

Owing to their ability to regenerate themselves, MSCs have received ample research attention in the field of regenerative medicine, as well as being considered in engraftment clinical applications. Their immunosuppressive properties have also legitimized the use of these cells as therapeutic agents in graft versus host disease and suggest that MSCs could be effective in preventing tumors from spreading (Granero-Molto et al., 2008). On the downside, as warned by Xu et al. (2018), by migrating to distal metastatic locations, MSCs could foster the formation of MM and other malignant neoplasms, while their differentiation into osteoclasts could be conducive to MM development.

Studies have addressed how MM-derived MSCs differ from normal MSCs in terms of molecular features. In this regard, Garderet et al. (2007) reported that MM-derived MSCs proliferated at a slower rate than normal MSCs, causing downregulation of the platelet-derived growth factor (PDGF) α and β , insulin-like growth factor-1 (IGF1), epidermal growth factor and basic fibroblast growth factor (bFGF). Furthermore, Zdzisińska et al. (2008) indicated that a correlation existed between the rate at which MM-derived MSCs proliferated and the MM stage; thus, MM-derived MSCs from patients at early disease stage developed and proliferated faster than those from patients at an advanced disease stage and displaying bone lesions. Moreover, MM-derived MSCs were found to have increased levels of interleukin6 IL-6 and a proliferation-inducing ligand (APRIL) protein, which were proven to have a damaging action and promoted MM progression (Matthes et al., 2016).

Increased levels of IL-3 and tumor necrosis factor TNF- α have been associated with MM-derived MSCs as well (Arnulf et al., 2007). The marker for poor MM prognosis, namely, growth differentiation factor 15 (GDF15), has also been found to occur in heightened levels in MM-derived MSCs (Corre et al., 2007). Growth/differentiation factor 15 GDF15 makes MM cells

more resistant to chemotherapy, therefore aiding MM to progress (Corre et al., 2012). Similarly, André et al. (2013) reported that β -galactosidase, a marker for senescence and relapse, was excessively expressed in MM-derived MSCs. Moreover, in a microarray analysis of MM-derived and normal MSCs, the former was observed to have higher levels of angiogenic factors and markers for bone marrow differentiation, as well as notable upregulation of peptidyl arginine deiminase 2 (PAD2). The latter presents the functionality of triggering histone H3 enzymatic deamination at arginine 26, increasing the protein levels of a major promoter of MM progression, namely, IL-6 (McNee et al., 2017).

A connection has been established between the IL-6 expression of MM-derived MSCs and diminished suppression of T cell dissemination and of modulation from T helper cells to T regulatory cell phenotype. MM-derived MSCs are involved in remote metastasis because they can upregulate the expression of factors regulating the immune response and angiogenesis, as well as because they can accelerate bone matrix disintegration (Giallongo et al., 2016). Furthermore, as suggested by Harmer et al. (2019), MM-derived MSCs can promote disease progression by interacting with the microenvironment of the tumor, synthesizing IL-6 and stimulating MM cells, thus helping the tumor to develop. MM cells encourage the production of Dickkopf-1 (DKK1), which prevents bone-derived MSCs from differentiating into osteoblasts, leading to a rise in the levels of IL-6 critical for supporting MM cells to proliferate (Zhou et al., 2013).

By interacting with MSCs from bone marrow, MM cells proliferate and become more resistant to the proteasome inhibitor bortezomib, which contributes to relapse in MM cases subjected to treatment with bortezomib due to chemokine receptor type 4 CXCR4 hyperexpression (Reagan & Ghobrial, 2012). Furthermore, MM growth and tumorigenesis have

been suggested to depend significantly on miRNA. In particular, MM pathogenesis seems to rely to a great extent on miR146a, which intensifies the MSC production of cytokines, thus helping MM to proliferate and metastasize (De Veirman et al., 2016).

The research cited above suggested that MM tumorigenesis was supported by MSCs. However, there is also research maintaining that MSCs inhibit MM tumorigenesis (Lee et al., 2019). There is some evidence that both placenta-derived and adipose tissue-derived MSCs prevent MM cells from growing. Li et al. (2011) postulated that the discrepancies related to the impact on the growth of MM cells were due to the fact that the MSCs from placenta and adipose tissue had dissimilar molecular and genomic profiles compared to MM-derived MSCs.

1.3. HMOX 1

The heme protein catabolic pathway is regulated by various heme-oxygenase protein isoforms that are encoded by the (HMOX1) (Dunn et al., 2014). The isoforms catabolize the breakdown of heme into iron, carbon monoxide and biliverdin in order to achieve regulation of the heme protein catabolic pathway (Maines, 1997). Normal function of HMOX1 inside cell (FIGURE1) (Otero Regino, Velasco & Sandoval, 2009).

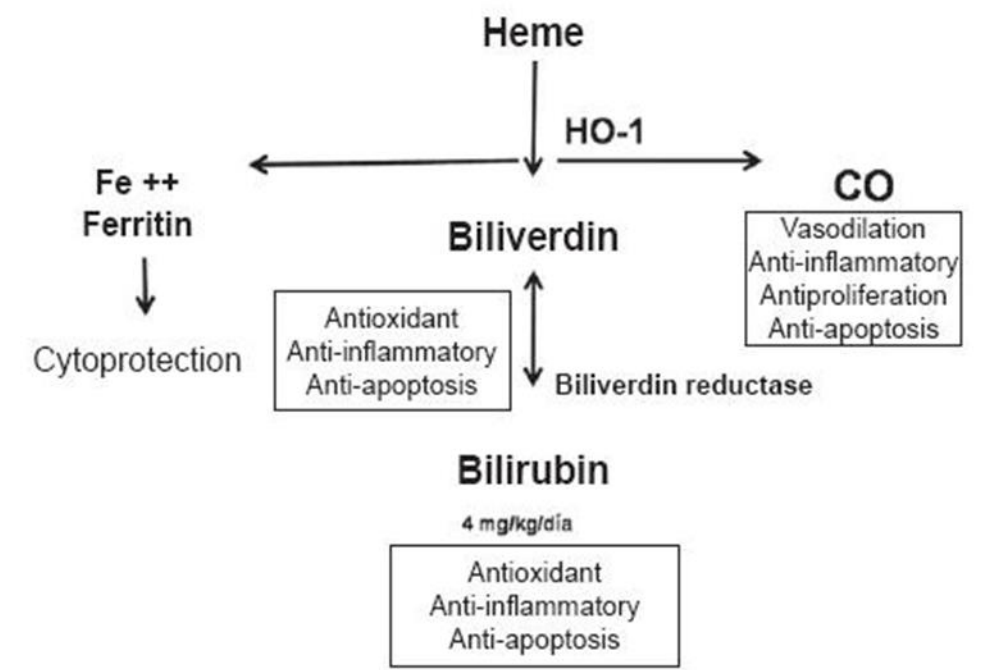


Figure 1. The function played by HMOX1 in the body.

The spleen, liver and bone marrow are among the tissues with expression of HMOX1 (Ryter et al., 2006). Within the bone marrow, HMOX1 is a key contributor to hemoglobin processing in macrophages. HMOX1 expression is modulated according to cellular response to stress, since it constitutes the sole isoform of the heme-oxygenase protein that is associated with a range of biological and cellular processes, including reactive oxygen species (ROS) and oxidative stress (Lin et al., 2007).

Evidence for HMOX1 displaying properties conducive to tumor development in a number of neoplasms may hint at the wide range of cellular functions fulfilled by this gene (Podkalicka et al., 2018). As reported by Chau (2015), the protein may be capable of inhibiting tumor growth, since the products of hemoglobin disintegration by HMOX1, namely, biliverdin and carbon monoxide, are known to have effects against inflammation and oxidants.

HMOX1 is abnormally activated by a number of genomic events, especially the occurrence of a short GT repeat polymorphism within the proximal area of the gene promoter (Kikuchi et al., 2005). Such repeat sequences have been associated with poor survival outcomes and heightened risk in gastric adenocarcinoma, as well as with greater probability of additional tumor growth in other cancer types (Sawa et al., 2008). Furthermore, hyperexpression of HMOX1 is related to poor prognosis in prostate, lung, thyroid and gastric cancers, and the protein can be found primarily in the cancer cell nuclear compartment, particularly in the macrophages within that compartment, and in the stromal microenvironment (Noh et al., 2013).

As cancer progresses, cancer cell turnover occurs at a high rate. This increases the levels of oxidative stress in the tumor microenvironment, which in turn intensifies the expression of HMOX1 through a number of cells signaling pathways, including nuclear factor kappa B NF- κ B and nuclear factor erythroid 2 Nrf2 signaling pathways. Additionally, HMOX1 expression is also actively modulated by the hypoxia pathway (Quail & Joyce, 2013). Since it occurs at a subcellular level, HMOX1/Nrf2 may help disease to progress, while a direct correlation has been established between protein presence in the nucleus and disease progression (Bekeschus et al., 2018). The signal peptide peptidase protein (SPP) can cleave and process HMOX1, generating a soluble HMOX1 that can permeate the nucleus and stimulate tumor development separately from its catabolic activities (Boname et al., 2014). Tibullo et al. (2013) reported that nuclear HMOX1 exhibited a cytoprotective effect in malignant neoplasms, since it reduced the susceptibility of chronic myeloid leukemia to imatinib. Meanwhile, downregulation of HMOX1 is significant for bone morphogenesis as it enhances the levels of the receptor activator of nuclear factor- κ B ligand (RANKL), which is capable of stimulating osteoclastogenesis and bone loss (Ke et al.,

2015). Therefore, HMOX1 is critical for bone morphogenesis as it may contribute to bone resorption.

Florczyk-Soluch et al. (2018) sought to shed more light on the involvement of HMOX1 in early-stage osteoclastogenesis in terms of the expression of RANKL by macrophages from bone marrow. Mouse models with HMOX1 knockout revealed that HMOX1 played a central role in osteoclast proliferation, thereby encouraging bone formation, as suggested by the heightened bone resorption of osteoclasts reflecting the terminal differentiation of osteoclast precursors. Meanwhile, Wu et al. (2016) noted that, in MM cases, cluster of differentiation CD138-positive bone marrow cells displayed marked hyperexpression of HMOX1. Moreover, the janus kinases2/signal transducer and activator of transcription proteins 3 JAK2/STAT3 pathway activation was found to contribute to reduced susceptibility to lenalidomide. The authors concluded that HMOX1 was a marker for the progression of MM and resistance to cytotoxic therapy (Wu et al., 2016).

There is also evidence that HMOX1 diminishes susceptibility to the proteasome inhibitor bortezomib, which is the main pharmaceutical agent used to treat MM. Tibullo et al. (2016) claimed that HMOX1 protein expression was intensified when MM cells were exposed to bortezomib, while MM cells became less resistant to bortezomib as a result of nuclear HMOX1, thus proving that it was the nuclear situation of HMOX1 rather than its catabolic activities that determined bortezomib resistance. Furthermore, MM was metabolically profiled by Maiso et al. (2015) in an effort to produce a drug resistance signature for this disease. According to the findings, by contrast to normoxic tumors, hypoxic MM tumors had a higher expression of hypoxia inducible factor 1 (HIF1), which was considered to be mediated by the occurrence of molecules and free radicals known to trigger HMOX1 expression, namely, ROS.

1.4. Osteoblasts, Bone Formation and Implications for Multiple Myeloma

Bone structure is maintained through the processes of renewal of old bone matrix and new bone formation, which are respectively performed by the osteoclasts and osteoblasts (Rosenberg et al., 2012). In vertebrate organisms, bone is formed either through intramembranous ossification, as is the case with the bones of the face and skull, and endochondral ossification, as is the case with most of the skeletal elements (Shahi et al., 2017). MSCs serve as mediators of endochondral ossification. They undergo differentiation into chondrocytes, which quickly proliferate, expand and die, and afterwards they are substituted by osteoblasts in the middle of the bone matrix, where ossification occurs (Ortega et al., 2004). In the case of intramembranous ossification, MSCs undergo differentiation directly into osteoblasts, which then initiate bone formation. In general, osteoblasts are found on the edges of the bone matrix and they regulate bone matrix mineralization, which usually takes place in matrix vesicles (Zhang et al., 2018).

Made up of osteoclasts, osteoblasts, blood vessels and connective tissue, the basic multicellular units (BMUs) are the structures where bone is resorbed and formed anew (Siddiqui & Partridge, 2016). Osteoblasts are the products of MSC terminal differentiation and their precursors are modulated primarily by hormones, cytokines and growth factors in the circulatory system (Cizkova et al., 2014). Soluble factors and growth factors produced by the bone matrix as bone matures underpin the development of the osteoblast precursors (Rathinavelu et al., 2018). Meanwhile, a range of signaling pathways modulating osteoblast apoptosis can facilitate the remodeling of osteoblast levels. For example, B-cell lymphoma 2 BCL2 is responsible for osteoblast maintenance and protects these cells against apoptosis, whilst the development of osteoblasts from corresponding precursors is suppressed by retinoblastoma protein Rb (Arias et

al., 2018). Among the determinants of osteoblast maturation from precursors in the BMU are precursor terminal differentiation into osteoblasts, the rate at which they proliferate and differentiate into osteocytes, hormones (e.g. parathyroid hormone), and apoptosis-related breakdown (Dempster et al., 1993).

Fibroblast growth factor FGF, insulin like growth factor IGF, osteotropic hormones and calcitonin are among the growth factors known to hinder osteoblast apoptosis (Gronowicz et al., 2004; Hill et al., 1997). On the other hand, osteoblast apoptosis is promoted by other factors, including tumor necrosis factor TNF, transforming growth factor beta TGF- β and IL-6 (Jilka et al., 1998). The wingless Wnt signaling pathway has been identified as the mediator of modulation of osteoblast maturation from MSCs (Houschyar et al., 2019). Proteins belonging to the Wnt family conduct their activities mainly by interacting with Frizzled and Lrp5/6, which constitutes the basis for the canonical Wnt pathway. The activation of this pathway occurs when the osteoblast structural integrity is modified by extrinsic factors, like events heightening the concentration of calcium ions in the cells (Day & Yang, 2008). Moreover, osteoblast modulation can be provided by the interaction between MSCs and osteocytes through excessive expression of the canonical Wnt pathway, which causes precursors to differentiate into osteoblasts to a greater extent and intensifies ossification via the sclerostin protein (Galli et al., 2010).

The maturation of osteoblasts is activated by the Hedgehog signaling pathway via the interplay between soluble factors Sonic hedgehog (Shh) in condensation MSCs and the membrane receptors T-box transcription factor 2 (Tbx2) and protein patched-like protein 1 (Ptc1) (Zhu et al., 2008). Furthermore, in the process of endochondral ossification, the number of osteoblasts is diminished by Ihh activity suppression, as this synergistically activates alkaline

phosphatase (ALP), which in turn encourages the osteoblasts to differentiate from MSCs (St-Jacques et al., 1999).

The number of osteoblasts is reduced in MM, with suppression of osteoblast differentiation from MSCs and promotion of osteoclast proliferation being the hallmarks of MM tumorigenesis (Drake, 2014). Disease progression is measured based on the extent of osteolysis, which occurs when osteoblasts have diminished activity, while osteoclasts have intensified activity (Kingsley et al., 2007). Kovacic et al. (2014) reported that early-onset MM was associated with exponential increase in bone formation and osteoclast proliferation, followed by reduction in the number of osteoblasts as the disease advanced.

According to a number of preclinical studies, MM and other hematopoietic malignancies were associated with diminished burden of osteoblasts and anti-neoplastic characteristics were exhibited by those cells (Taube et al., 1992). Meanwhile, Krevvata et al. (2014) observed that the tumor burden was alleviated and survival was improved when duodenal serotonin, a hormone decreasing the count of osteoblasts, was suppressed, leading to proliferation in osteoblasts within the bone marrow. In spite of such evidence, the role played by osteoblasts in the progression of MM has not been extensively empirically investigated. Ng et al. (2011) reported that osteoblasts were indeed involved in MM progression, as intensified osteolysis, diminished count of osteoblasts and high frequency of fractures were documented in cases of monoclonal gammopathy of undetermined significance (MGUS).

1.5. The Role Played by Osteoclasts in Bone Formation and Multiple Myeloma

With a composition dominated by proteins and minerals (e.g. calcium, potassium), bone tissue possesses the property of continual renewal, which is vital to maintain bone density and

thus minimize the rate of fracture occurrence (Granero-Molto et al., 2008). Bone renewal depends on the balance of the activities undertaken by osteoblasts and osteoclasts in the BMU structure (Rosenberg et al., 2012).

The sizable cells known as osteoclasts are the product of MSC terminal differentiation and comprise multiple nuclei (Ishii et al., 2010). Cytokine macrophage (MCSF) and RANKL mediate the transformation of MSCs into osteoclasts. MSCs generate the osteoprotegerin receptor RANKL to suppress the differentiation of osteoclasts and thus preserve the equilibrium between osteoblasts and osteoclasts during bone remodeling (Lacey et al., 2012). The osteoclasts fulfill a functional part in bone regeneration as they produce proteolytic enzymes (e.g. cathepsin K) that disintegrate the soluble organic and inorganic constituents of bone tissue (Ikeda & Takeshita, 2015).

Consisting of numerous osteoclasts developed at the edge of the osteoclast apical membrane, resorption lacunae are the usual site of bone regeneration (Boyce, 2013). After bone is resorbed, MSCs are mobilized at the resorption lacunae to differentiate into osteoblasts, which synthesize osteoid, the organic element of bone, which becomes mineralized when hydroxyapatite is added (Raggatt & Partridge, 2010). The osteoblast-osteoclast cross-talk is achieved by a number of signaling pathways, including the Wnt and Hedgehog pathways, as well as cellular factors during bone resorption. Such cross-talk facilitates the mobilization of osteoblast and osteoclast precursors in the resorption lacunae to initiate bone regeneration (Kim et al., 2013).

There is evidence that a range of osteoclast subtypes exist, since osteoclasts located in the trabecular, cortical and intramembranous areas of the BMU differ slightly in the genes that they express (Boyce et al., 2009). MSCs are mobilized to bone surfaces during bone resorption under

the control of the osteoclast-produced transforming growth factor beta1 (TGF- β 1). At the bone surfaces, MSCs undergo terminal differentiation into precursor cells (Tang et al., 2009).

Bone regeneration occurs with the involvement of the cytokines collagen triple helix repeat containing1 (CTHCR1), sphingosine-1-phosphate 1 (SIP1) and C3a. When bone resorption begins, the MSCs and osteoclasts start producing CTHCR1 under stimulation from bone morphogenic protein 2(BMP2). Later stages of bone resorption are associated with heightened levels of Ca, while the transformation of osteogenic precursors into osteoclasts is underpinned by SIP1 (Ishii et al., 2010; Kimura et al., 2008).

Several cell surface receptors (e.g. ephrin 1 and 2 and associated cognate ligands) serve as mediators for the osteoclast-osteoblast cross-talk (Matsuo & Otaki, 2012). Osteogenic precursors differentiate into osteoclasts but development of osteoblasts is suppressed when the osteoclast ephrin A2 ligand attaches to the osteoblast EphA2 receptor. On the other hand, the development of osteoblasts is promoted when the osteoclast ephrin B2 attaches to the osteoblast EphB4 receptor, which prevents osteogenic precursors from terminally differentiating into osteoclasts (Zhao et al., 2006).

There are a number of subtypes of bone metastasis, including osteolytic, osteosclerotic and combination bone phenotypes (Vinayachandran & Sankarapandian, 2013). Osteolytic bone metastasis leads to full ablation of the bone structure and its substitution with tumorigenic cells. When osteoclasts are activated to excess and osteogenic precursors differentiate into osteoclasts to an overwhelming degree, extreme bone resorption can occur, which results in osteolytic lesions. A particularly noteworthy characteristic of MM is that the formation of osteolytic lesions can be stimulated by the interaction between osteoclasts, stromal cells, osteoblasts and MM-related fibroblasts (Terpos et al., 2018).

The mechanisms of osteolytic lesion formation in MM have not been studied extensively. In spite of this, however, evidence has been provided regarding osteoclast participation in the formation of such lesions because osteoblast resorption in the BMU is diminished (Hameed et al., 2014). Furthermore, the effectors of osteogenic precursor differentiation into osteoclasts, namely, RANKL and Dickkopf-related protein 1 DKK1, have been observed to occur in lower levels in the serum of MM patients with favorable response to bortezomib therapy, whereas the levels of bone formation markers (e.g. osteocalcin, alkaline phosphatase) were higher. This confirmed that osteogenic bone disease in MM could be effectively managed with bortezomib (Terpos et al., 2006).

CHAPTER 2. MOTIVATIONS

2.1. Issues on MM

To gain insight into the implications that mesenchymal stem cells MSCs can have for the management of Multiple Myeloma MM and other chronic illnesses, it is necessary to have good comprehension of their basic functions. MM is a pernicious condition that currently has no cure. In the US, after non-Hodgkin lymphoma, MM is the hematological tumor with the second highest prevalence, accounting for 2% of malignant tumor diagnoses and associated with a rising rate of mortality as it is developed mainly by older individuals (Bianchi, 2014). A B cell malignancy, MM presents as its main clinical manifestations the aggregation of neoplastic plasma cells in the bone marrow and the formation of lytic bone lesions (Lin et al., 2014). The condition was first identified by Macintyre in 1850, while the first case demonstration was provided by Kahler in 1889. Bone lesions as a diagnostic characteristic of MM were first distinguished by radiological investigations at the beginning of the 1900s. Subsequently, during the 1940s, bone marrow biopsy helped to shed more light on MM morphology. The detection of odd electrophoresis patterns in MM sample analysis determined Kekwick to establish the basic principles for the use of electrophoresis (Souter, 1998).

There are great lacunae in knowledge about MM etiopathology. Environmental factors, genetic factors and family history have all been proposed as possible risk factors or causes for this disease. According to Bianchi (2014), nuclear radiation and petroleum products constitute the only risk factors related to environmental and occupational exposure that are acknowledged; however, pesticides have also been highlighted by epidemiologic studies as potential environmental risk factors, since MM seems to disproportionately affect individuals working in agriculture and wood and leather manufacture. As regards genetic risk factors, genetic mutation,

especially oncogene mutation, causes the translocation of plasma cells from the bone marrow into tumor cells. The tumor cells developed from plasma cells invade the bone marrow and replace the hematopoietic stem cells, leading to the formation of lytic bone lesions, the main characteristic of MM (Mehta et al., 2014).

MM-related morbidity is related primarily to the development of myeloma bone disease. By contrast to normal, healthy bone, growth occurs abnormally in myeloma bone disease, with reduced bone formation (osteogenesis) and increased bone resorption (osteoclasts). This imbalance gives rise to bone pain and leads to a higher rate of bone fractures. The gravity of MM stems from the fact that it is not restricted to bone, but it affects other parts of the body as well and is usually accompanied by high levels of calcium in blood, anemia, kidney failure, infection, and disorders of metabolism and coagulation. Moreover, because it shares some characteristics with other diseases (e.g. metastasis carcinoma, rheumatic arthritis), diagnosis of MM is not always straightforward.

2.2. ROS

HMOX1 is downregulated in MM bones (Li, Ling, Khan, & Yaccoby, 2012). The products of synthesis by electron transport chain pathway within mitochondria, whereby oxygen molecules are converted into a superoxide anion via univalent oxygen reduction reactions (Yang et al., 2018), reactive oxygen species (ROS) represent susceptible molecules. Additional pathways of ROS synthesis include DNA damage due to ionizing radiation, anaerobic respiration, and catabolic reactions performed by nicotinamide adenine dinucleotide phosphate oxidase NADPH and redox biology (Schieber & Chandel, 2014). Tejero et al. (2019) argued that ROS may be directly involved in modulating normal physiological mechanisms within the

human body, including vasculature maintenance and oxygen sensing functions on which cells depend to survive.

When ROS are synthesized with hypoxia mediation, the expression of hypoxia-inducible factor1 HIF1 is activated, which in turn activates angiogenesis and enhances the level of cellular oxygen, thus contributing to the maintenance of cellular homeostasis (Movafagh et al., 2015).

The modulation of the innate immune system also relies on ROS. The activity of T cells is triggered by hyperproduction of ROS by phagocytes and detects antigenic peptides on pathogen surfaces, and consequently, viral and bacterial pathogens can be eliminated from circulation (Chen et al., 2018). With regard to the modulation of cellular physiology, Powers et al. (2011) indicated that ROS contributed to the physiological state of skeletal muscles by controlling the amount of glucose that is assimilated as muscles contract and by preserving redox reaction homeostasis during physical activity, which makes cells less susceptible to the harmful effects of oxidative stress reactions.

Evidence has been provided that cellular signaling pathways are regulated with ROS participation. This participation takes the form of post-translational covalent alteration of histone acetylation and deacetylation states, which modulate the transcription of numerous proteins controlling cellular processes like DNA damage and repair and cell apoptosis, autophagy and senescence (Zhang et al., 2016). Particular research attention has been focused on the joint function of ROS and transcription factor E2F1 in the modulation of the apoptosis-promoting function of Sirtuin 1 proteins (Sirt1).

The Sirt1 protein undergoes deacetylation as a result of oxidative stress, leading to deactivation of the deacetylating into an acetylation capable of activating an apoptosis-promoting form of the Sirt1 protein, which facilitates cellular apoptosis alongside p53 (Rajendran et al.,

2011). To deal with the impact of oxidative stress, a range of appropriate mechanisms have been evolved by cells, such as the activation of a sequence of cell signaling cascades that ensure that cells can survive in media with high levels of oxidation.

In the context of infection, phagocyte stimulation maximizes ROS production, which in turn triggers lymphocytes to mobilize at the infection sites (Brieger et al., 2012). A sequence of signaling cascades are subsequently activated and regulated by the lymphocytes, resulting in transcriptional activation of redox-responsive factors (e.g. activating protein1 AP-1, nuclear factor kappa-light-chain enhancer of activated B cells NF- κ B). The latter attach to the promoter area of various genes, like thioredoxin (Trx), which work alongside the glutathione system to prevent fluctuations in the intracellular redox state, thus conferring cellular protection from the oxidizing effects of ROS. HMOX1 and cystine transporter xc2 are also genes with redox protective function (Alfadda & Sallam, 2012).

Investigations have been conducted on ROS regarding their promotion of cancer, diabetes and other diseases (Dröge, 2006). The DNA promotion capacity of ROS has attracted attention to their function in carcinogenesis, as this capacity can make it more likely for cells to attain genomic mutations conducive to cancer development. ROS can trigger genomic mutations in a nuclear factor erythroid 2-related factor 2 Nrf2 transcription factor, which experiences stimulation within cells during oxidative stress and its activity is based on gene detoxification (Aitio, 2006). Nrf2 constitutive activation or suppression of kelch-like ECH-associated protein1 KEAP-1 attachment to Nrf2 can be the outcome of Nrf2 gene somatic mutations, leading to the transcriptional activation of Nrf2-modulated genes involved in the stimulation of tumor growth (Tonelli et al., 2017). Pancreatic adenocarcinoma is associated with high Nrf2 levels, while excessive Nrf2 expression has been observed to reduce susceptibility to some tumor-targeting

therapies. Furthermore, Nrf2 transcriptional activation seems to be enhanced by oncogenic mutations like protooncogene KRAS, serine/threonine-protein kinase BRAF and proto-oncogene MYC, which suggests that ROS may have an oncogenic function in Nrf2 activation, therefore promoting carcinogenesis (DeNicola et al., 2011).

The serum of MM cases has been shown to contain high levels of ROS and reduced levels of antioxidants. For instance, Cieslar et al. (2002) reported that, compared to individuals without MM, those with MM had lower levels of antioxidants, vitamin C and vitamin E in the serum, but significantly higher levels of oxidative stress markers. Furthermore, Mulligan et al. (2006) indicated that oxidative stress had a negative effect on the efficiency of proteasome inhibitors (e.g. bortezomib) that are employed as the primary strategy for managing MM. ROS are synthesized prior to the activation of the apoptotic signal cascade by bortezomib. Additionally, Obeng et al. (2006) claimed that bortezomib may be ineffective in MM patients who have antioxidant by-products.

The Kruppel like factor9 KLF9 transcription factor has been proposed as a new mechanism for oxidative stress activation by bortezomib in MM. It has been found that patients who responded favorably to bortezomib therapy had higher levels of KLF9 and additionally, KLF9 reduced the expression of the Thioredoxin Redutase2 TXNRD2 protein, which fulfills the function of decreasing oxidative stress in cells (Zucker et al., 2014). When MM cells were exposed to bortezomib, TXNRD2 expression diminished whilst at the same time ROS levels increased, reflecting the elevation in oxidative stress. Fink et al. (2016) observed that bortezomib was less effective against MM cells when TXNRD2 was expressed in excess, and those cells no longer underwent apoptosis. Meanwhile, Raninga et al. (2015) discovered that ROS-dependent apoptosis intensified when MM cells were exposed to inhibitors that repressed TXNRD1

activity. Hence, MM could potentially be treated through a strategy that integrates ROS-triggering agents and bortezomib.

2.3. Issues on MSCs

A.J. Friedensatein was the first to identify MSCs during the 1960s and he also observed that these cells were produced mainly by the bone marrow (Rebeca, 2011). MSCs are classified as non-hematopoietic stem cells that can be subdivided into mesenchymal and non-mesenchymal stem cells. The latter are capable of differentiation into neurons and astrocytes both *in vitro* and *in vivo*. MSCs present various applications and can contribute to the treatment of a range of illnesses, which is why they have attracted ample research attention. These cells are particularly important in regenerative medicine, which is concerned with tissue damage rejuvenation, and in numerous disease processes that are challenging to treat (Rebeca, 2011). BMC injection was originally clinically trialed in 1995, and since then, allogeneic or autologous MSCs have been used in over 2000 cases to treat different illnesses, refractory wounds, and bone/cartilage defects, as well as for the purposes of organ transplants. In July 2013, the website of the United States National Institutes of Health specified that over 200 clinical trials of MSC-based treatment had either been finalized or were in the process of being conducted (Ikebe & Suzuki, 2014). Indeed, the treatment of bone and skeletal diseases, in particular those associated with undetermined genetic defects, relies greatly on MSCs (Bernardo et al., 2012).

As a new therapeutic strategy within the field of medicine, MSCs have received approval for use in the management of a wide array of chronic conditions and for tissue regeneration, including myocardial infarction and corneal damage. Owing to ethical considerations, MSCs are extracted from adult human tissue and they have the property of synthesizing anti-inflammatory

proteins such as tumor necrosis factor that is capable of tissue damage repair (Wei et al., 2013). There is thus clear evidence that MSCs have the potential to transform regenerative medicine. However, as outlined by the International Society of Cellular Therapy, to be used in regenerative medicine applications, MSCs of *in vitro* derivation must satisfy a number of requirements, such as adherence to plastic in standard conditions of tissue culture, demonstration of expression of particular cell surface markers (e.g. CD37, CD90, CD105) and lack of expression of other markers (e.g. CD45, CD34, CD14, CD11b, CD79 α , and HLA-DR surface molecules), as well as capability for differentiation into osteoblasts, adipocytes and chondroblasts under particular conditions (Wang, 2011).

2.4. Issues on Treatments of Multiple Myeloma Using MSCs

Precipitously the Animal Model for MM have been tested to evaluate using MSCs as a cytotherapy for MM. It has been reported that MSCs cytotherapy enhanced recovery of osteolytic lesion in MM during active and remission stage (Li et al,2011). This recovery was initiate after HMOX1 was upregulated (Li et al, 2012) (Grochot-przeczek, Kozakowska, Starowicz, & Jagodzinska, 2013). The latest research advances have led to the introduction of stem cell therapy as a feasible treatment for MM that can significantly improve disease prospects. The usefulness of MSCs for the treatment of MM has been widely espoused. For instance, Yu (2013) reported that lytic bone lesions worsened the prevalence of morbidity in MM cases and that the Fas/Fas ligand (FasL) pathway underpinned the action of MSCs on MM cells.

MSCs play a vital role in preventing MM cells from growing and in stimulating tumor cell apoptosis. In addition to their tumor inhibiting effect, MSCs also promote bone development when the disease is in remission. Consequently, bone formation is initiated anew by osteoblasts,

while bone resorption by osteoclasts is slowed down. However, as emphasized by the studies under review, despite the great potential displayed by MSCs for use in treating MM, MSC-based therapy is not successful in all cases, and therefore additional investigation is necessary. The empirical work conducted by Yu (2013) did not provide details regarding the manner in which MSCs were injected into bone or regarding the mechanism of suppression of tumor cell growth by MSCs. Furthermore, despite the therapeutic benefits afforded by MSCs, it has been proven that these cells can also stimulate MM expansion in the absence of FasL.

Atsuta et al. (2013) also addressed the issue of whether tumor growth was mediated or inhibited by MSCs. The authors demonstrated that bone formation was aided by MSCs with elevated Fas-L expression, but they did not recognize that the FAS-L mechanism contributed to the process of MM repair. Consequently, there remains a lack of clarity about this mechanism. Furthermore, the study failed to provide a comprehensive explanation regarding the function of MSCs in protecting against MM. The study did not elucidate how normal MSCs differed from MM-derived MSCs and neither did it indicate which of these two variants had high FAS-L expression *in vivo*. Since the empirical work was conducted *in vitro*, additional investigations must be conducted on MM patients to determine the type of MSCs most suitable for therapy purposes. The results derived from such investigations could be applied to preclinical and clinical practice.

Andre et al. (2013) claimed that, during disease relapse, the renewed growth of tumor cells was occasionally stimulated by MSCs. They indicated that MSC aberrations were enhanced by the occurrence of senescence-related B-galactosidase in MM-derived MSCs. Nevertheless, those aberrations were minimized through treatment integrating different types of pharmaceutical agents, although it was not specified whether such treatment eradicated or decreased the

dimensions of MSCs. Information about this aspect is critical because it is indicative of the likelihood of post-treatment recurrence or relapse of MM. Moreover, the study also failed to state whether treatment helped cells to recover their normal functionality, whether cells suffered degeneration or whether they diminished in size.

As an approach for treating MM bone disease, MSC cryotherapy has attracted a significant amount of research attention, particularly in terms of the efficiency of MSC sources for improving MM therapy. Given the noted limitations of bone marrow MSCs (e.g. aberrations, increased likelihood of disease relapse), other MSC sources were considered, including adipose tissue, which seems to be especially promising for the development of a new strategy for treating MM bone disease. In the empirical work conducted by Lin et al. (2014), the process of osteogenesis was explored to comparatively analyze the effectiveness of MSCs from the adipose tissue or bone marrow of MM patients against osteoblasts. According to the findings obtained, osteogenesis was significantly influenced by MSCs from adipose tissue owing to broader availability of calcium by comparison with MSCs from bone marrow. This work has paved the way for future investigation of MSCs from adipose tissue as a potential therapy for MM bone disease. On the downside, the work was limited in the diversity of samples gathered and did not specify whether lytic bone lesions were healed by calcium-rich MSCs from adipose tissue or whether such cells merely enhanced bone strength.

Considering the results of the previously cited studies, it is clear that a wide range of issues must be addressed before MSCs can be employed as a viable treatment for MM. Development of a standard MM treatment requires performance of clinical trials on human subjects. Within this context, the purpose of the present study is to expose MM cells, MSCs, osteoblasts and osteoclasts to the pharmaceutical agent t-BHQ because it triggers the expression

of HMOX1 (Yamaguchi et al,2014) and assess the impact of this drug on those different types of cells. MM has been associated with reduced expression of HMOX1, and therefore administration of t-BHQ to pharmacologically increase HMOX1 expression is deemed to be an antioxidant target. The overall aims are to trigger MM cell apoptosis, confer cell protection against oxidative damage, prevent the development of osteoclasts, and ultimately hinder bone deterioration.

CHAPTER. 3. MATERIALS AND METHODS

Tert-butylhydroquinone (t-BHQ) was applied in different doses to various MM cell lines (i.e. U266, JJN3, H929, 8266, OPM2 and ARP) as well as to MSCs, osteoblasts and osteoclasts.

3.1. Viability, Culturing and Analysis of Cells

3.1.1. Multiple Myeloma Cell Culture

U266, JJN3, H929, 826, OPM2 and ARP1 were the six MM cell lines that were employed. Cells were numbered under a microscope to determine their viability, which should be 90-100%. The culturing of MM cells was performed in RPMI media with a content of L-glutamine (5 ml), fetal bovine serum (50 ml), and antibiotics (5 ml). The plating of the various MM cell lines was performed on plates with 96 wells and t-BHQ in different doses. Following the addition of t-BHQ, proliferation of cells was assessed via an MTT assay, while HMOX1 expression was identified via the luciferase assay. MM transfected cells were prepared as previously described (Qiang, Shaughnessy, & Yaccoby, 2008).

3.1.2. MTT Assay (Tetrazolium dye Assays)

An MTT assay was conducted to evaluate cell proliferation after the six MM cell lines were incubated in 96-well plates for 14 days. The procedure involved addition of MTT solution consisting of 25 μ l of 5-mg MTT in 1-ml PBS, followed by two-hour incubation of the MM cells at 37°C. Cell lysis was subsequently carried out through addition of 100- μ l MTT lysis buffer. Measurements at 570 nm were performed a day later.

3.1.3. Luciferase Assay

Following the incubation of the six MM cell lines on 96-well plates, the luciferase assay was conducted to determine HMOX1 expression and therefore gain insight into how the proliferation of cells was affected by t-BHQ. The procedure involved addition of 200 μ l of 50 μ l 5 \times luciferin solution to every one of the 96 wells with MM cell lines. The measurement of luciferase photons was undertaken five minutes later.

3.1.4. Mesenchymal Stem Cells

DMEM enriched with fetal bovine serum (25 ml) and penicillin (2.5 ml) was used for MSC growth. To collect the MSCs from the walls of the culture flasks, the old media was poured out and PBS was used to wash the cells before it was disposed of. Removal from the flasks was facilitated through addition of trypsin and two-minute incubation at 37°C. A microscope was subsequently used to assess the MSCs in terms of their shape, dimensions and mobility. The MSCs that were cultured and awaiting use were supplemented with DMEM. In line with the recommendation that fresh DMEM or AlphaMEM should be used for twice weekly feeding of MSCs, preparation of AlphaMEM was undertaken through addition of fetal bovine serum (10 ml) and antibiotics (5 ml) in the MSC growth media. The next step was seeding the MSCs on a plate with six wells and various drug doses. For RNA extraction and evaluation of HMOX1 expression, collection was performed at different intervals.

3.1.5. Mesenchymal Stem Cells Differentiation into Osteoblasts

The counting and seeding of MSCs onto a plate with 48 wells were performed. A sufficient amount of time was permitted for cell growth to a minimum of 60% confluence prior

to triggering differentiation into osteoblasts. To extract RNA and identify osteoblast markers, it is essential to obtain a suitable number of osteoblasts. Therefore, the MSCs were subjected to two-day incubation, followed by addition of osteoblast reagents to every one of the 48 wells. Ascorbic acid, beta-glycerophosphate and dexamethasone were the growth factors applied to the osteoblasts differentiated from MSCs. The osteoblasts were exposed to various doses of drug and fed nutrients once weekly. Satisfactory osteoblasts were obtained in two weeks.

3.1.6. Mesenchymal Stem Cells Differentiation into Osteoclasts

Osteoclast growth was achieved with peripheral blood stem cells (PBSCs). Culturing was performed in AlphaMEM media containing fetal bovine serum (50 ml) and antibiotics (5 ml), with addition of the growth factors RANK-L and MCSF at ratios of 1:2000 and 1:4000, respectively, to activate osteoclast development. Satisfactory osteoclasts were obtained in 14 days.

3.2. Examination of Gene Expression

3.2.1. Extraction and Measurement of RNA

For assessment of HMOX1 gene expression, RNA was extracted from every cell type, namely, the six MM cell lines (U266, JJN3, H929, 8266, OPM2 and ARP1), MSCs, osteoblasts and osteoclasts. After removal of the growth media, PBS was used for gentle washing of the wells containing the various cell types. To extract RNA, RTL buffer (350 µl) with mercaptoethanol:10 µl in 1-ml RLT was added to every well and the guidelines pertaining to the RNeasy MinElute Cleanup Kit (QIAGEN) were followed. A Fluorometer Nanodrop device set for RNA selection was employed for quantification of the RNA extraction. For every sample, the

RNA quantity was no less than 10 ng, as this is the quantity necessary for HMOX1 gene expression. For the purpose of fluorometry, water without RNA was used as a blank and measurement of the extracted RNA involved cleaning and placing 2- μ l sample on the spot.

3.2.2. Synthesis of cDNA

cDNA synthesis was performed on every RNA sample from every type of cell. A reverse transcription kit was employed for amplification of the sequences of cDNA from the isolated mRNA for the purposes of HMOX1 gene expression. RNase inhibitor was not used to prepare the PCR master mix, which contained buffer, dNTP mix, RT random primers, reverse transcriptase and water without nuclease. The next step was pipetting 10- μ l PCR master mix on 10- μ l RNA derived from every sample. The total reaction was performed in a DNA ENGINE device and lasted for one hour and five minutes, with temperature and time adjustments.

3.2.3. Real-Time PCR

DNA binding dyes were employed for amplification of the different types of cells through real-time PCR. The fluorescent signal issued by the dyes helped to detect HMOX1 gene expression. A B-actin primer served as sample endogenous control for the purposes of comparison of amplification fold-changes in HMOX1 expression. The entire procedure was conducted with the 2Xsyber green qPCR kit. For amplification of HMOX1 gene expression for quantitative real-time PCR, a mixture was created by combining Bio tool 10 μ l SYPER Green Master Mix, 2- μ l template, 10- μ l primer, and 7- μ l water. Furthermore, in line with the manufacturer's guidelines, a total volume of 20 μ l was obtained by adding 18- μ l PCR master mix to 2- μ l cDNA from every sample. The plate with 96 wells was then subjected to five-minute

centrifugation at 200 rpm. Temperature adjustment was performed on amplification reverse transcribed RNA by real-time PCR according to the cycles of denaturation, annealing and extension. The longest length of time was 120 minutes. The resulting Cq values reflected how much nucleic acid the samples contained. A high level of HMOX1 gene expression was reflected by cQ values that were (<29 cycles) lowered 29, while a low level of HMOX1 expression was reflected by cQ values that were (>38 cycles) higher 38.

3.3. Histochemistry

3.3.1. Use of Alkaline Phosphatase for Osteoblast Staining using florescence microscope

To determine whether the different drug doses induced an increase in HMOX1 expression, staining of the osteoblasts was performed, with image acquisition to visualize how ALP activity was deposited at various drug doses. Elevated drug doses determined significant ALP deposition, at which point real-time PCR was conducted to identify the osteoblast marker genes runt-related transcription factor 2 (RUNX2), collagen alpha 1 (COL1A1), and bone gamma carboxy glutamic acid containing protein (BGLAP). A kit from Sigma-Aldrich was employed to conduct the ALP protocol and images were captured to observe how ALP synthesis promoted osteogenic differentiation.

3.4. Use of Titrate-Resistant Acid Phosphate for Osteoclast Staining using florescence microscope

To determine whether the different drug doses induced an increase in HMOX1 expression, staining of the osteoclasts was performed and a microscope was used to count the osteoclasts. Identification of osteoclasts with multiple nuclei was based on staining with titrate-

resistant acid phosphate (TRAP), which was performed using a kit from Sigma-Aldrich and following the manufacturer's guidelines. It was observed that formation of osteoclasts was associated with significant ALP production after hemin treatment. During excessive HMOX1 expression, images were captured at 20× and 40× magnification.

3.5. Statistical Analysis

Student's two-tailed t-test was conducted to determine how the different types of cells (MM cell lines, MSCs, osteoblasts and osteoclasts) responded to t-BHQ. The *P-value* was less than 0.05, signifying that the results were of statistical significance.

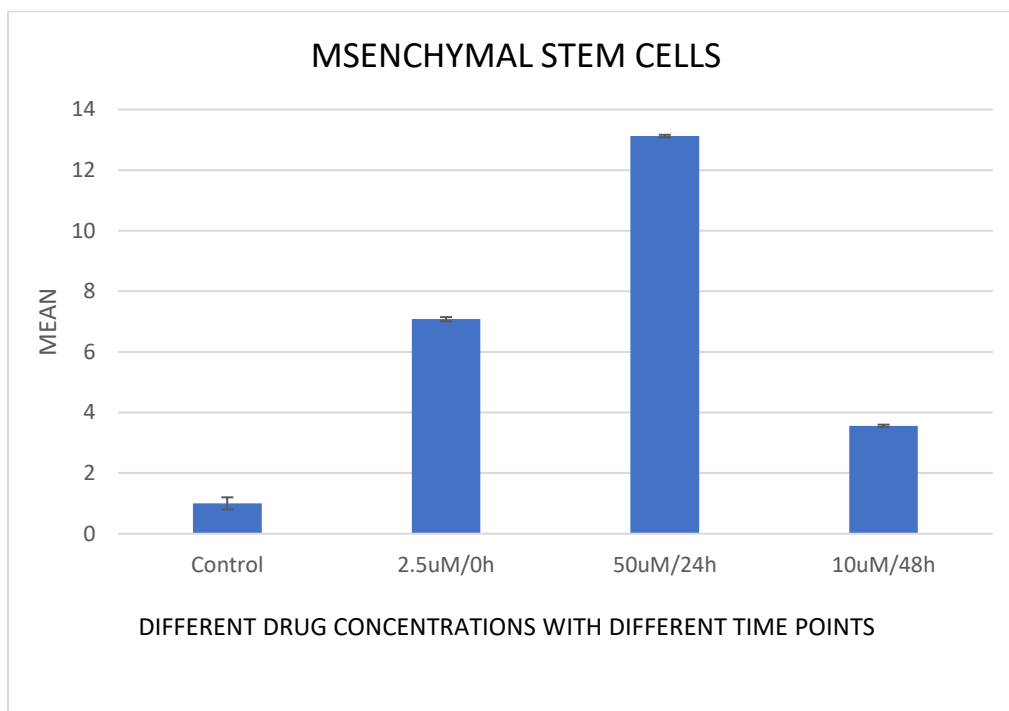
CHAPTER 4. RESULTS AND DISCUSSION

4.1. Mesenchymal stem cells Cytotherapy: Consolidation of MM microenvironment and prevention of damage related to tumor expansion through use of t-BHQ to increase HMOX1 expression in MSCs

Various t-BHQ doses were employed for upregulation of HMOX1 expression within MSCs for intervals of varying lengths (0, 24 and 48 hours). The aim pursued in upregulating HMOX1 with t-BHQ was augmentation of MSC stemness to boost formation of osteoblasts and diminish formation of osteoclasts. As previously reported by Funes et al. (2014), upregulation of HMOX1 within MSCs hindered those cells from acquiring an oxidant-promoting state induced by tumor oncogenes. Figure 2 illustrates the administration of t-BHQ in different doses to increase the expression of HMOX1 within MSCs at intervals of 0, 24, and 48 hours. Besides its contribution to MSC self-regeneration, t-BHQ also promotes osteogenic development by modulating the NRF2/P35-SIRT1 pathway (Yoon, Choi & Lee, 2016).

Funes et al. (2014) discovered that tumor oncogene increase was promoted by the absence of NRF2 from MSCs. As a result, osteoclasts may proliferate and bone damage may be intensified. Conversely, osteoblasts proliferate and bone is renewed due to upregulation of HMOX1 and NRF2. The results obtained in this study indicated that t-BHQ promoted differentiation of osteoblasts and suppressed formation of osteoclasts by enhancing HMOX1 expression in MSCs and therefore has strong potential for use in MM treatment.

Exposure of MSCs to various t-BHQ doses led to upregulation of HMOX1 expression at different time points



Drug Concentrations	<i>P</i> -value	Mean	Standard Errors
Control	-	1.00	0.2
2.5uM/0hours	9.9965E-08	7.08	0.069
50uM/24hours	2.29E-09	13.12	0.041
10uM/48hours	4E-07	3.56	0.04

Figure 2. Use of t-BHQ in various doses in the context of MSC cytottherapy led to HMOX1 upregulation across different time points. HMOX1 plays a central role in bone remodeling with MSCs in MM patients and improves outcomes regarding the control of tumor development microenvironment. It recorded the highest level in 50Um/24hours. Also, their p-value showed more highly statistical significance than concentration 2.5Um/24hours.

4.2. Suppression of osteoclast formation through upregulation of HMOX1 in MSC by t-BHQ

The impact of the antioxidant pathway on osteoclasts was assessed by culturing MSCs in osteoclast media with t-BHQ in doses of 5, 10 and 50 μ m, while the impact of t-BHQ on the count of multinucleated osteoclasts was assessed by TRAP staining immunohistochemistry. TRAP staining revealed that use of t-BHQ in growing doses suppressed the differentiation of multinucleated osteoclasts (Figure 3). An earlier study by Suda et al. (1993) reported that the formation of ROS, which are key contributors to osteoclast development, was diminished by HMOX1 activation. The present study also found that formation of osteoclasts owing to oxidative stress, was inhibited when t-BHQ was used to activate HMOX1 (Figure 4).

Yamaguchi et al. (2014) reported that modulation of the pathway based on heme-oxygenase (HO1)/high mobility group box1 (HMGB1) helped to distinguish the molecular mechanism through which t-BHQ acted on osteoclasts. RANK-L, which activates ROS production, was reduced by HMOX1 upregulation. This consolidates supports for the use of t-BHQ to improve bone density and hinder bone deterioration through modulation of the microenvironment and tumor development in MM.

t-BHQ upregulation of HMOX1 expression in MSC cytotherapy contributed significantly to the suppression of osteoclast formation per culture dish

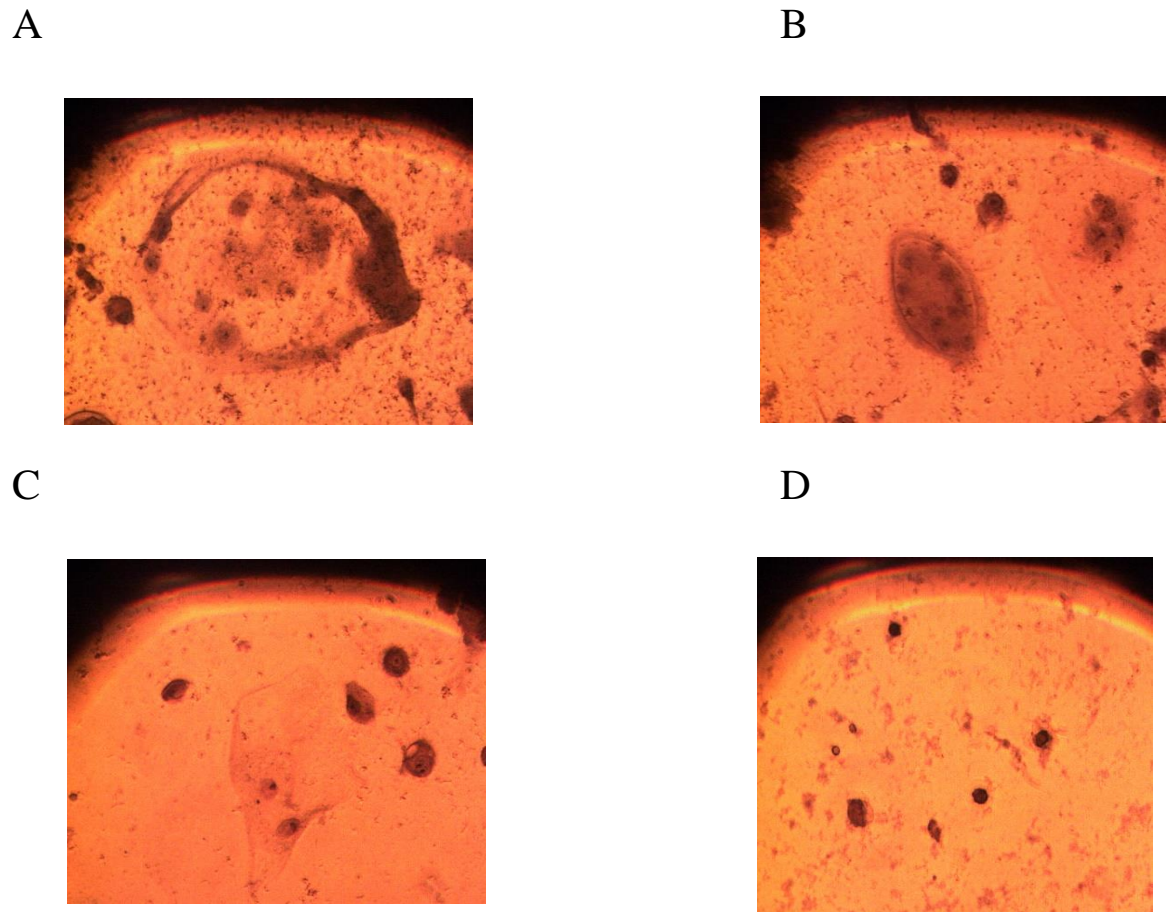
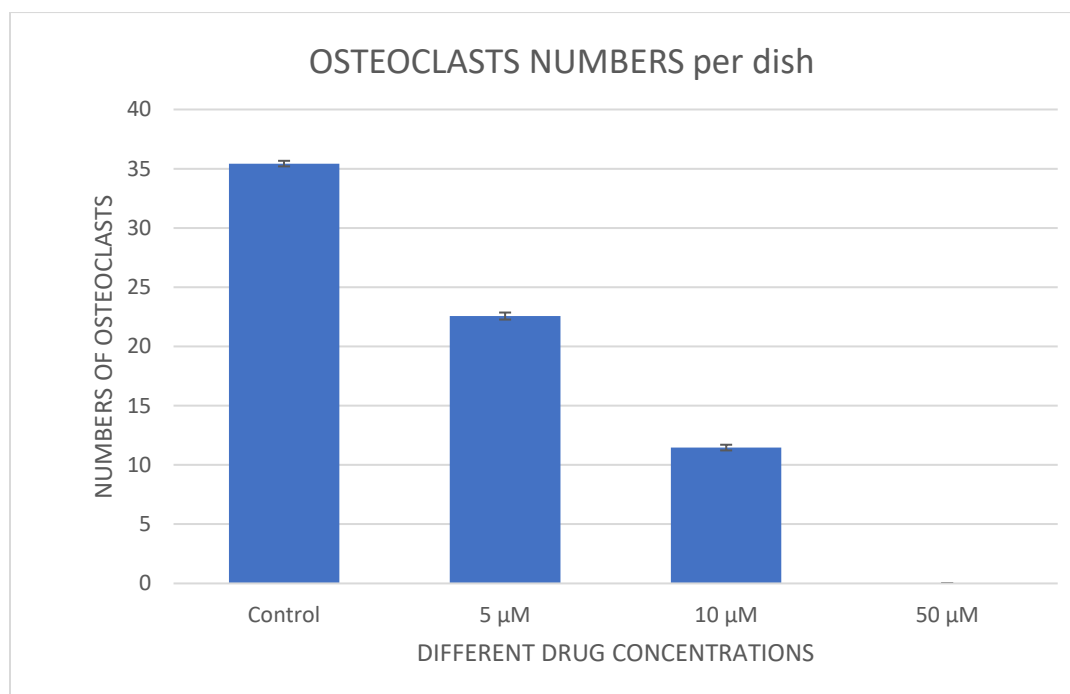


Figure 3. Development of osteoclasts was suppressed when t-BHQ was used to upregulate HMOX1 during differentiation of osteoclasts. Osteoclasts were observed in tartrate-resistant acid phosphatase (TRAP). There was a reduction in the number of multinucleated osteoclasts stained with TRAP. (A) The cell count associated with the control was 35. (B) Treatment with 5- μ M t-BHQ reduced the number of osteoclasts to 23. (C) Treatment with 10- μ M t-BHQ reduced the number of osteoclasts to 11. (D) Treatment with 50- μ M t-BHQ reduced the number of osteoclasts.



Drug Concentrations with Osteoclasts	Osteoclasts Number per Dish	P-value	Mean	Standard Errors
Control	35	-	-	-
5uM	23	4.4029E-06	22.56	0.296
10uM	11	2.29E-07	11.46	0.240
50uM	Disappear	1.13E-08	0	0

Figure 4. Increase in t-BHQ concentration led to a reduction in the cell count of osteoclasts that was of highly statistical significance (P -value = $1.13\text{E-}08$). Suppression of osteoclast development necessitates high expression of HMOX1.

4.3. Use of t-BHQ to increase HMOX1 expression in MSCs promotes osteoblast formation and related markers

The present empirical work involved using t-BHQ in various doses and at various time intervals to stimulate osteoblast formation. To determine whether the process of osteoblast formation was accompanied by the production of ALP, immunohistochemistry staining was carried out and osteoblast markers were detected during HMOX1 activity via real-time PCR. Furthermore, as confirmed by the analysis of global gene expression profile, HMOX1 was not expressed in MM, but the formation of osteoblasts was promoted as a result of increase in HMOX1 expression by t-BHQ. The initialization of ALP deposition during the differentiation of osteoblasts was indicated by the results obtained (Figure 5). A previous study confirmed that t-BHQ triggered NRF2 phosphorylation so its use could promote formation of osteoblasts and MSC self-regeneration (Yoon, Choi & Lee, 2016).

As anticipated, t-BHQ robustly promoted an increase in the expression of HMOX1, which in turn stimulated osteoblast markers to develop. The osteoblast precursor markers RUNX2, COLLAGEN and bone gamma-carboxyglutamate protein BGLAP were intensely stimulated to confirm the increase in HMOX1 expression in osteoblasts. There was a significant increase in Runt- related transcription factor 2RUNX2 expression in the initial seven days, with administration of t-BHQ in doses of 1 and 2.5 μM resulting in four-fold modifications, whereas administration of t-BHQ in doses of 5 and 10 μM yielded only two-fold modifications. However, as the t-BHQ dose was elevated from 10 to 50 μM , four-fold modifications were achieved in a three-week interval (Figure 6). It was thus confirmed that upregulation of additional osteoblast differentiation markers was promoted by the emergence of RUNX2. For instance, at 1- μM t-BHQ, the levels of osteocalcin BGLAP markers increased in three weeks, with five-fold modifications. This corroborated earlier

findings by Zou et al. (2015) about the significance of RUNX2 upregulation for the activation of osteoblast marker formation (e.g. collagen1 alpha, ALP, osteocalcin BGLAP) in the extracellular matrix. Based on such results, it can be concluded that, in MM, osteoblast formation can be promoted while osteoclast formation can be diminished by using t-BHQ to increase HMOX1 expression.

Osteoblast formation was promoted when t-BHQ upregulated HMOX1 expression
in MSC cytottherapy

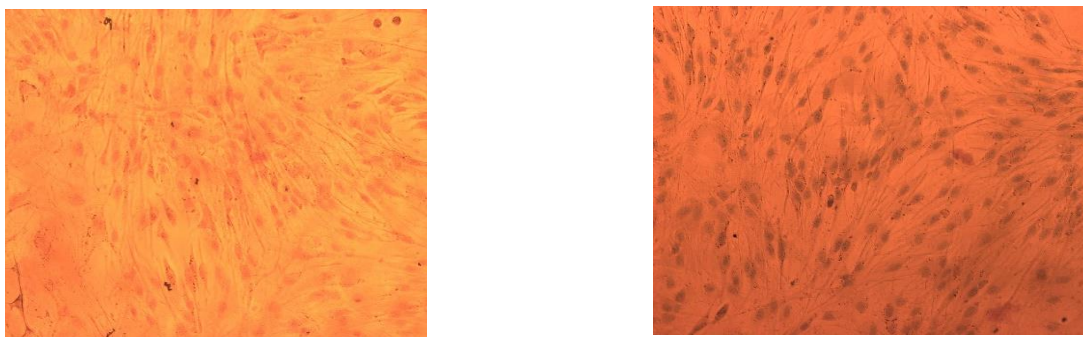
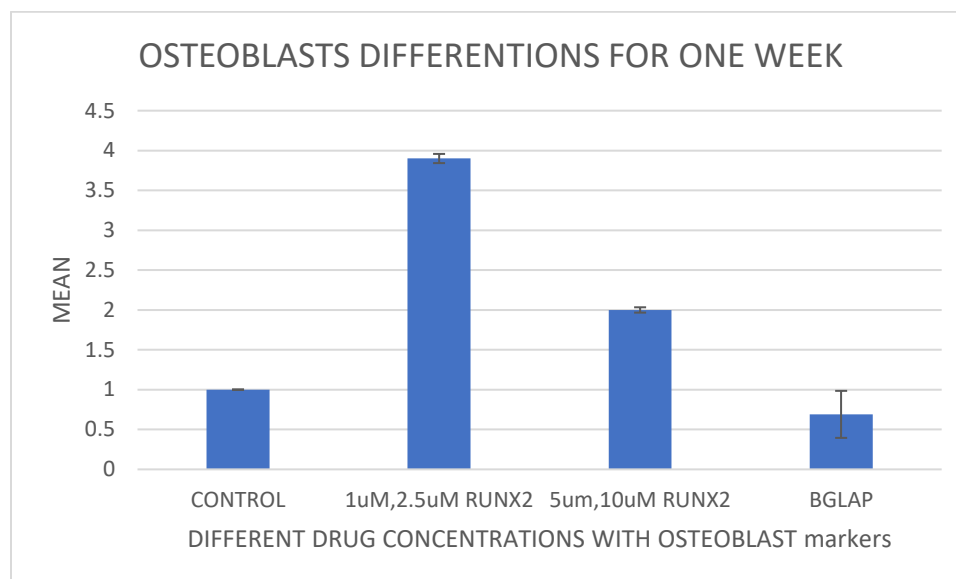


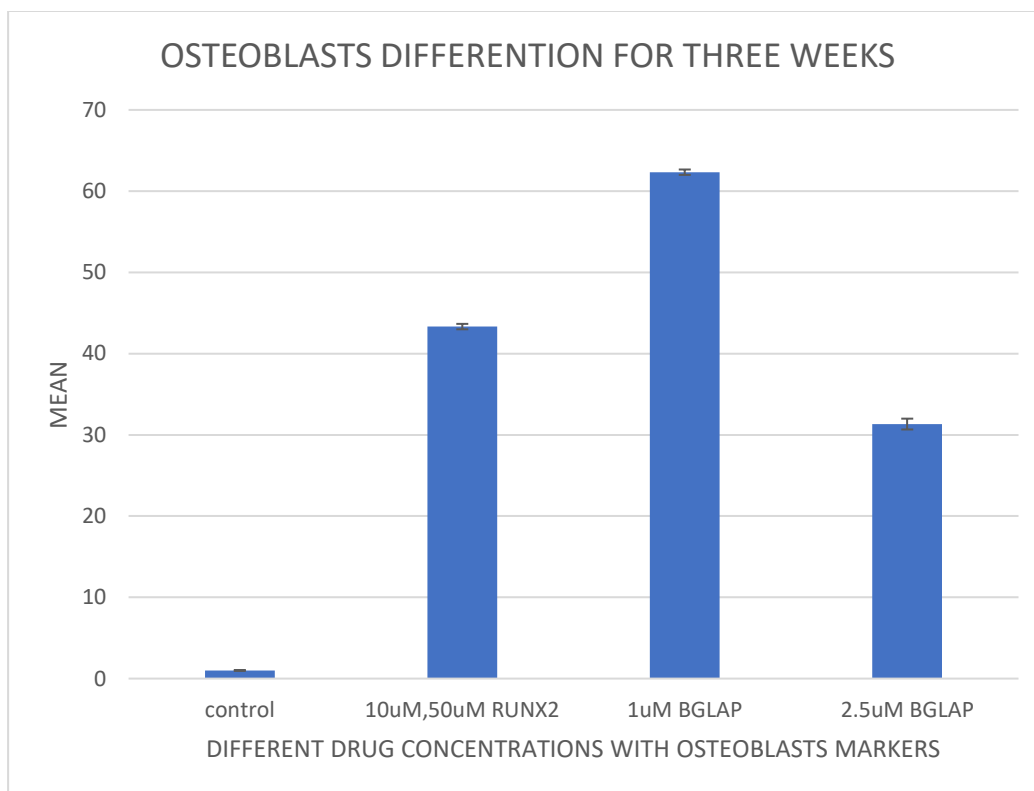
Figure 5. Differentiation of osteoblasts from MSCs subjected to t-BHQ treatment and ALP staining. (A) Control MSCs with osteoblast media. (B) MSCs with osteoblast media with t-BHQ treatment. Upregulation of HMOX1 led to deposition of ALP, which represents a key marker for differentiation of osteoblasts.

The number of osteoblasts differentiated from MSCs was increased by HMOX1 expression upregulation by t-BHQ at different time point expression upregulation by t-BHQ at different time points



Drug Concentrations with Osteoblast Markers / One Week	<i>P</i> -value	Mean	Standard Errors
Control	1	1	-
1uM, 2.5uM RUNX2	3.81E-07	3.9	0.057
5uM,10uM RUNX2	9.85E-07	2	0.033
BGLAP	NONE	0.69	0.295

Figure 6-A. Use of t-BHQ for upregulation of HMOX1 in MSCs contributes to bone regeneration by promoting differentiation of osteoblasts and associated markers (e.g. RUNX2, BGLAP). Normalization was achieved with B-actin.



Drug Concentrations with Osteoblast Markers/ Three Weeks	<i>P</i> -value	Mean	<i>Standard Errors</i>
Control	1	1	-
10uM,50uM RUNX2	2.87E-07	43.33	0.333
1uM BGLAP	2.64E-06	62.33	0.333
2.5uM BGLAP	1.08E-06	31.33	0.666

Figure 6-B. Use of t-BHQ for upregulation of HMOX1 in MSCs contributes to bone regeneration by promoting differentiation of osteoblasts and associated markers (e.g. RUNX2, BGLAP). Normalization was achieved with B-actin.

4.4. HMOX1 Upregulation via t-BHQ to Diminish Cell Proliferation and Suppress Tumor Growth in MM

The empirical work conducted in the present study involved exposure of four transfected MM cell lines (i.e. JJN3/EL, U266/EL, 8266/EL and H929/EL) to various doses of t-BHQ. Following drug administration, cell proliferation was assessed via a luciferase assay. Furthermore, MM heterogeneity was validated by conducting real-time PCR to measure increase in MM-related HMOX1 expression for U266 and H929. Given that the HMOX1 gene is absent in MM, the antioxidant drug t-BHQ, which is capable of strongly activating the HMOX1 gene, was employed to observe detoxification in MM.

A large number of key genes and factors (e.g. antioxidant HMOX1, NRF2) may be inhibited in MM as a result of progression of ROS and increase in acidity caused by accumulation of lactate. As highlighted by Kann et al. (2005), cells experience oxidative stress when the levels of glutathione antioxidant genes are low. The present study sought to reduce cellular genotoxicity by using t-BHQ to target the major antioxidant gene HMOX1. Owing to the heterogeneity exhibited by MM, the different MM cell lines that were investigated were impacted by t-BHQ in two distinct ways, as illustrated in Figure 7 (A-D). In MM, numerous key CD markers are absent from the surface of plasma cells, and therefore these cells generate a wide range of mutated antigens that are challenging to manage (Hajek, Okubote & Svachova, 2013). The results obtained in the present study suggested that t-BHQ in concentration of 10 μ M had a significant impact on the JJN3/EL cell line (P -value = 4.23455E-05) as well as on the U266/EL cell line (P -value = 3.28732E-22) (Figure 7 A and B). On the other hand, t-BHQ had no impact on the 8266/EL and H929/EL cell lines (P -value = 0.02 and 0.7, respectively) (Figure 7-C). The findings indicate that t-BHQ has enormous potential to be used in the treatment of MM. The

MTT assay validated the heterogeneity (Figure 8 A-F). Evidence was produced regarding the fact that hypoxia and oxidant burden caused the t-BHQ repair gene to miss up on the level of the cell via modulation of key cellular mechanisms such as cell growth and maintenance, modulation of cell cycles and control of the genes underpinning DNA repair (Kann et al., 2005).

The molecular mechanism of the gene targeted by t-BHQ was investigated as well. The MM cell lines U266 (Figure 9-A) and H929 (Figure 9-B) were exposed to various doses of t-BHQ to assess HMOX1 expression at a range of time points. The findings obtained exhibited good statistical significance, indicating that the HMOX1 expression was increased in U266 and H929 after 12 and 24 hours. In H929, the rate of cell proliferation was unaffected by HMOX1 expression, but the increase in HMOX1 expression in this MM cell line could nevertheless reduce ROS levels owing to oxidative stress associated with aberrant plasma cells.

The increase in the expression of HMOX1 led to a reduction in MM cell proliferation. In this regard, the study is consistent with an earlier work by Shih et al. (2005), which reported that, by increasing the levels of NRF2 antioxidant transcription factors, t-BHQ could modulate a suppressor of the citric acid cycle in mitochondria, namely, the toxic nitropropionic acid (3-NP). Such findings suggest the possibility of HMOX1 reforming within various MM cell lines, enhancing the survival chances of individuals with MM (Otero Regino, Velasco & Sandoval, 2009). The results obtained are positive and support the use of t-BHQ to treat MM, as it can target the HMOX1 molecular mechanism and thereby reduce cell proliferation. Overall, we used two methods for growth luciferase assay and MTT assay. The results from two methods are inconsistent in all cell lines but to U266. Thus, the only U266 responded to the drug based on the results while the results with JJN3 Cell need further validation.

The outcomes of the luciferase assay confirmed that t-BHQ had an impact on the MM cell lines with control DMSO

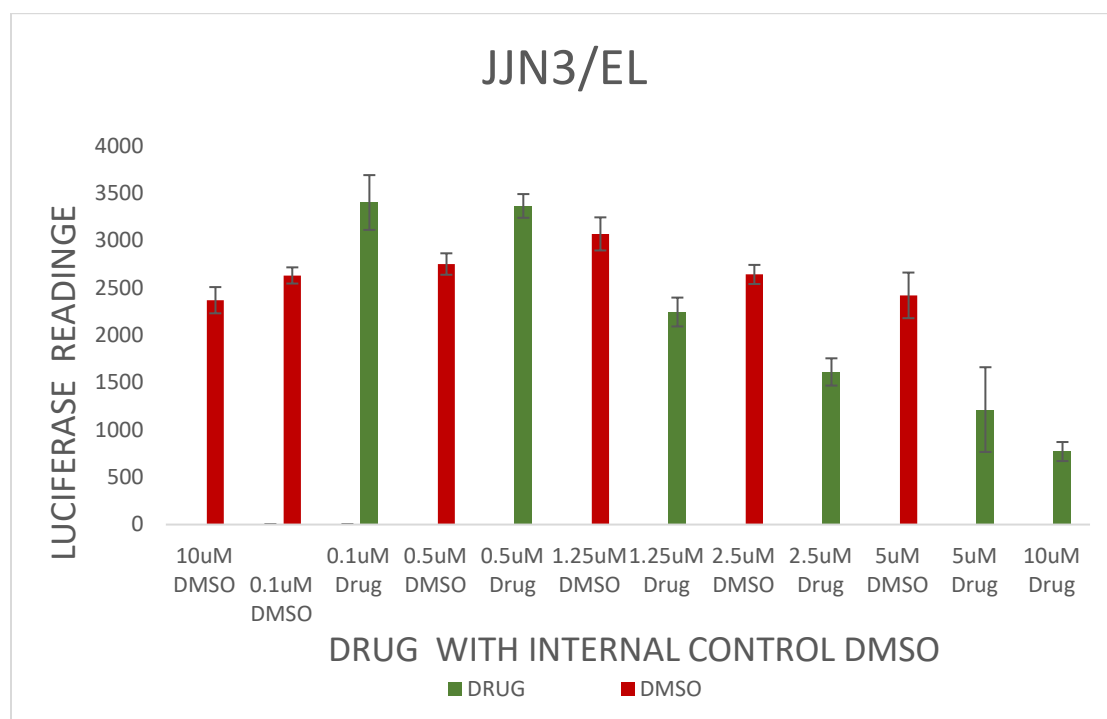


Figure 7-A. By comparison to the internal control, DMSO, MM cell proliferation was suppressed by systemic upregulation of HMOX1 by t-BHQ. The proliferation of JJN3/EL cells diminished as the drug levels were increased. Results were of statistical significance, with drug concentrations of 10, 5 and 2.5 μ M being associated with *P*-value of 4.23455E-05, 0.00016 and 0.001198, respectively.

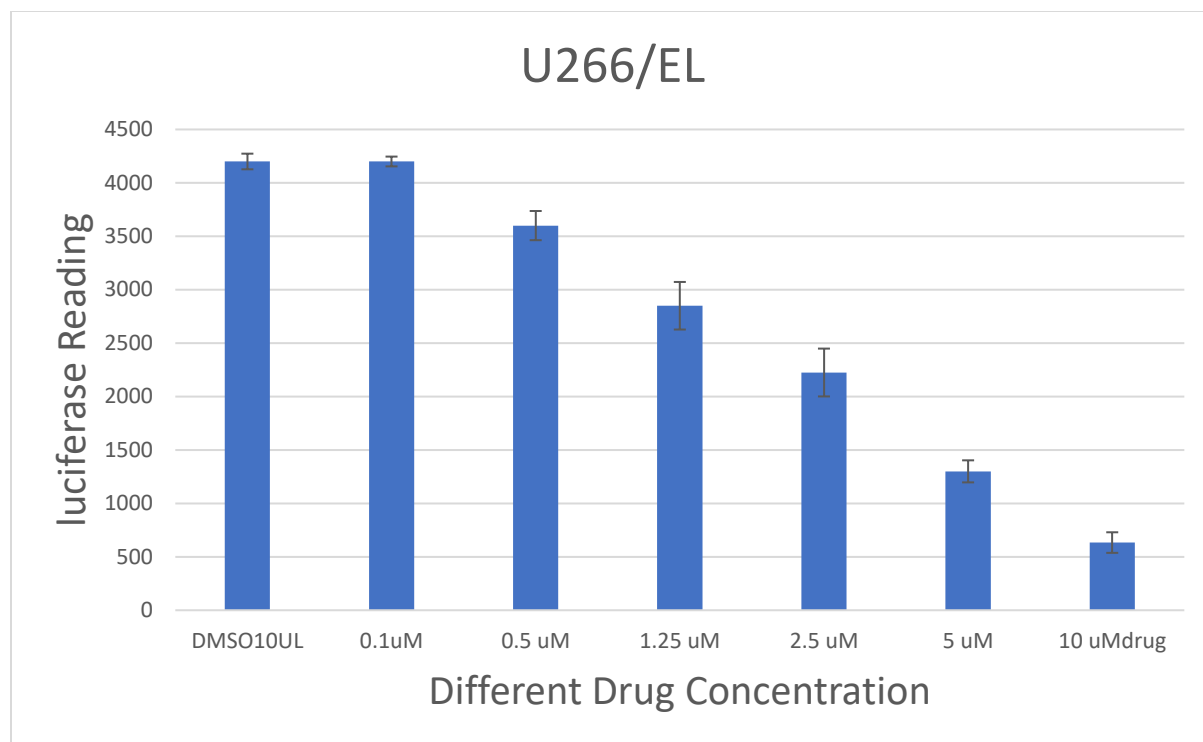


Figure 7-B. By comparison to the internal control, DMSO, MM cell proliferation was suppressed by systemic upregulation of HMOX1 by t-BHQ. The proliferation of U266/EL cells diminished as the drug levels were increased. Results were of statistical significance, with drug concentrations of 10, 5, 2.5, 1 and 0.5 μ M being associated with *P*-value of 3.28732E-22, 5.99E-15, 5.1E-08, 2.07E-05 and 0.000103, respectively.

The outcomes of luciferase assay indicated that MM cell lines exhibited resistance to t-BHQ

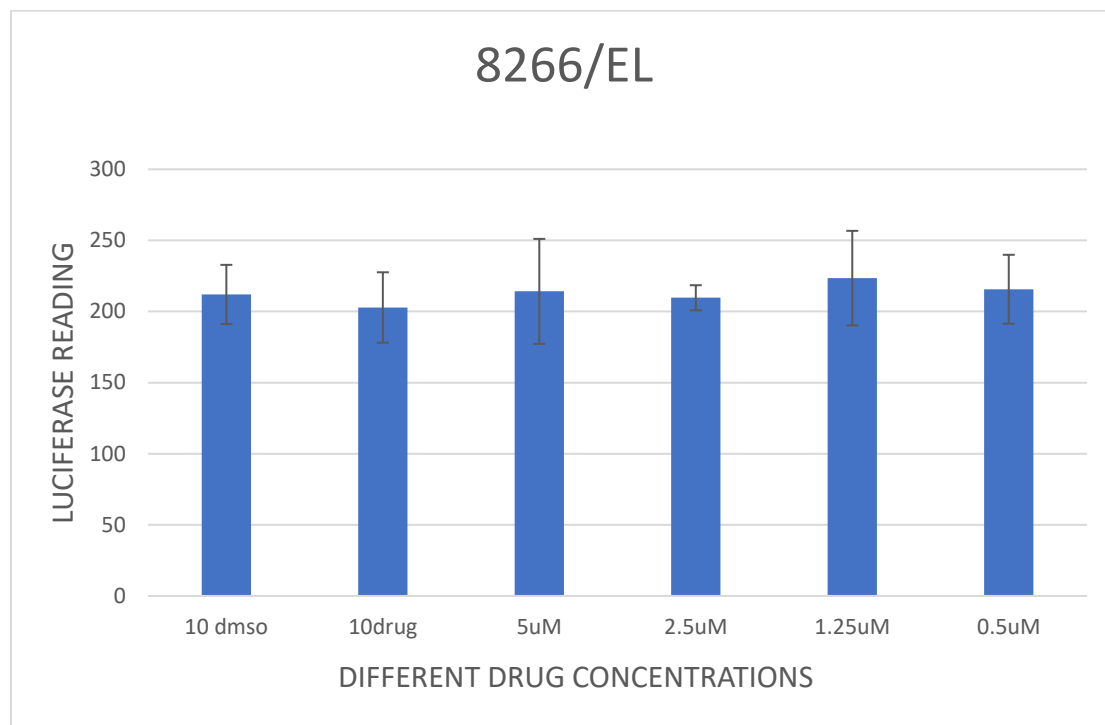


Figure 7-C. t-BHQ had no impact on MM 8266/EL cell lines and, by comparison to DMSO, heightening of drug levels and H929/EL proliferation showed no correlation of statistical significance (P -value = 0.02). Such results corroborate the notion that MM represents a heterogeneous disease.

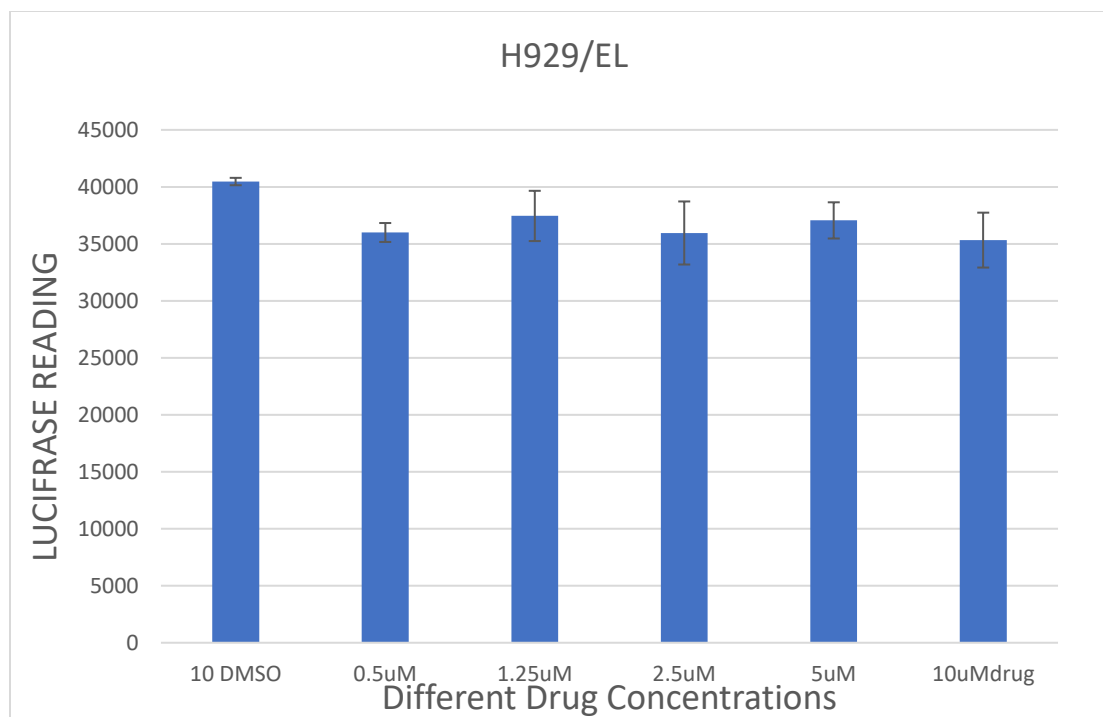


Figure 7-D. t-BHQ had no impact on MM H929/EL cell lines and, by comparison to DMSO, heightening of drug levels and H929/EL proliferation showed no correlation of statistical significance (P -value = 0.7). Such results corroborate the notion that MM represents a heterogeneous disease.

The outcomes of MTT assay indicated that t-BHQ had an impact on MM cell lines with control DMSO

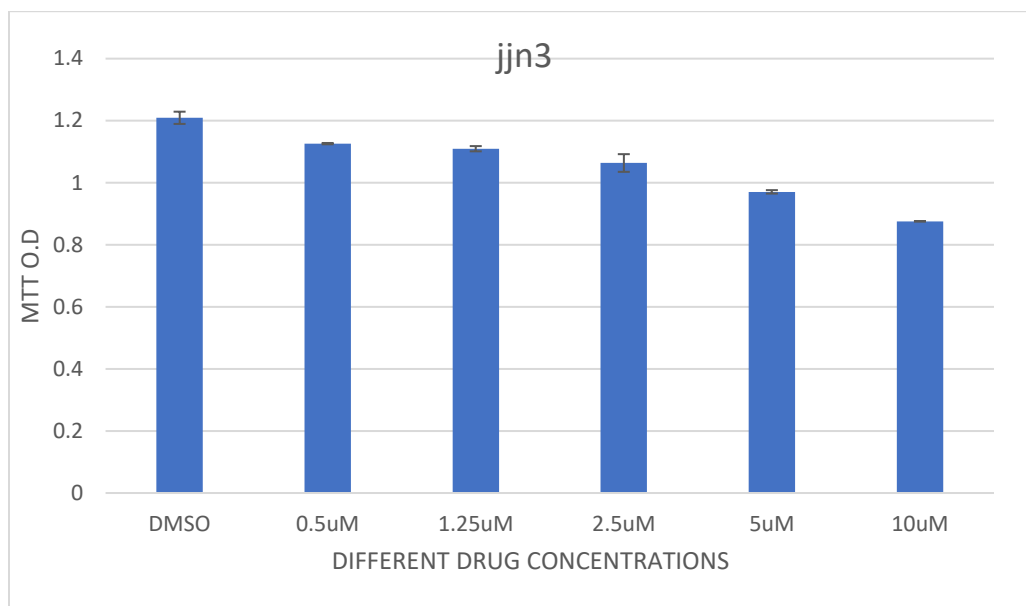


Figure 8-1 (A-B-C). t-BHQ had an impact on the JJN3, MM144 and U266 MM cell lines. By comparison to the internal control, DMSO, MM cell proliferation was suppressed by systemic upregulation of HMOX1 by t-BHQ. The proliferation of JJN3 cells diminished as the drug levels were increased. Results were of statistical significance, with drug concentration of μM being associated with *P*-value of 4.45E-05, 0.00065 and 0.02 for JJN3, MM144 and U266 cell lines, respectively.

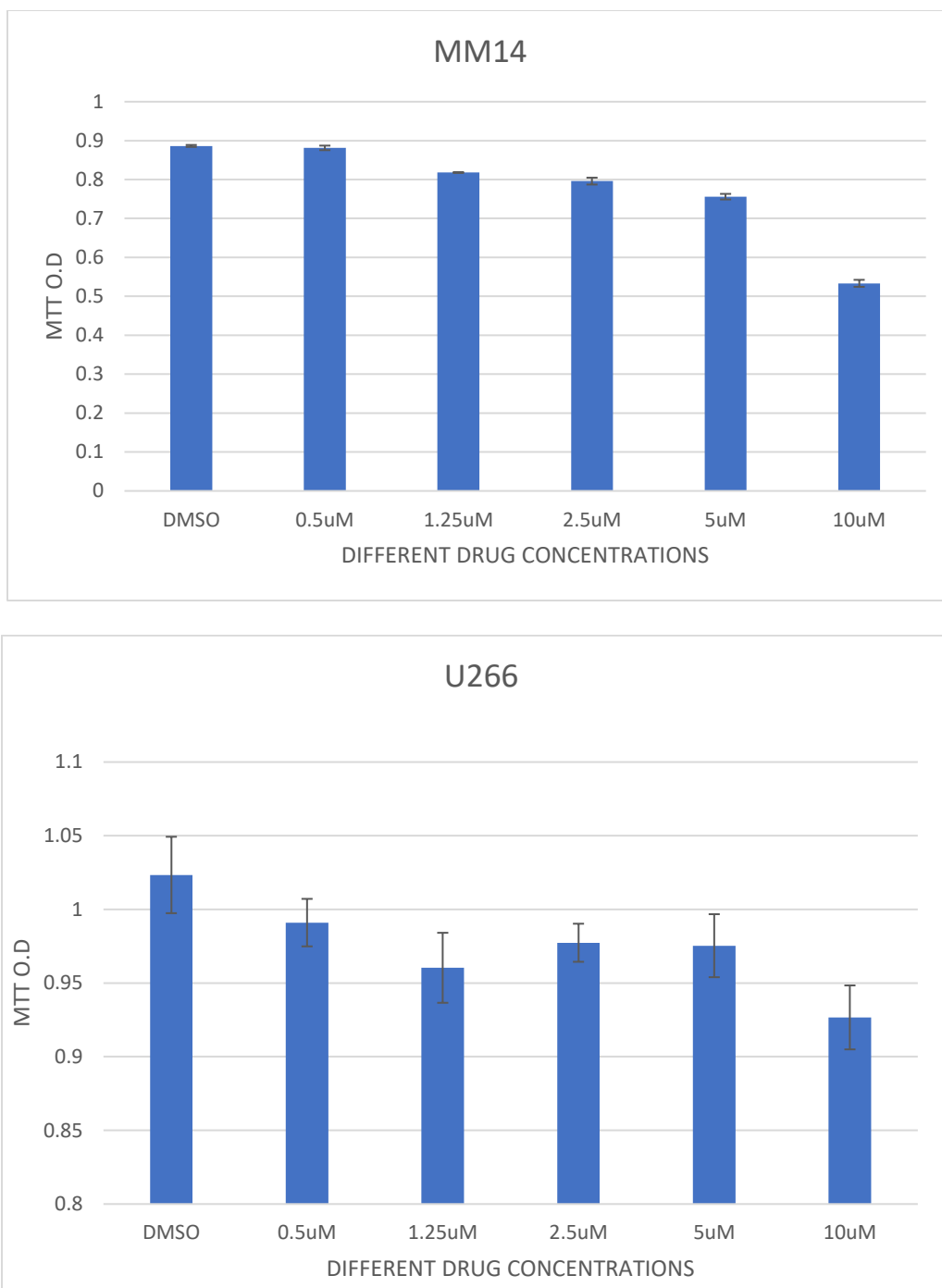


Figure 8-2 (A-B-C). t-BHQ had an impact on the JJN3, MM144 and U266 MM cell lines. By comparison to the internal control, DMSO, MM cell proliferation was suppressed by systemic upregulation of HMOX1 by t-BHQ. The proliferation of JJN3 cells diminished as the drug levels were increased. Results were of statistical significance, with drug concentration of μM being associated with P -value of $4.45\text{E-}05$, 0.00065 and 0.02 for JJN3, MM144 and U266 cell lines, respectively (Cont.).

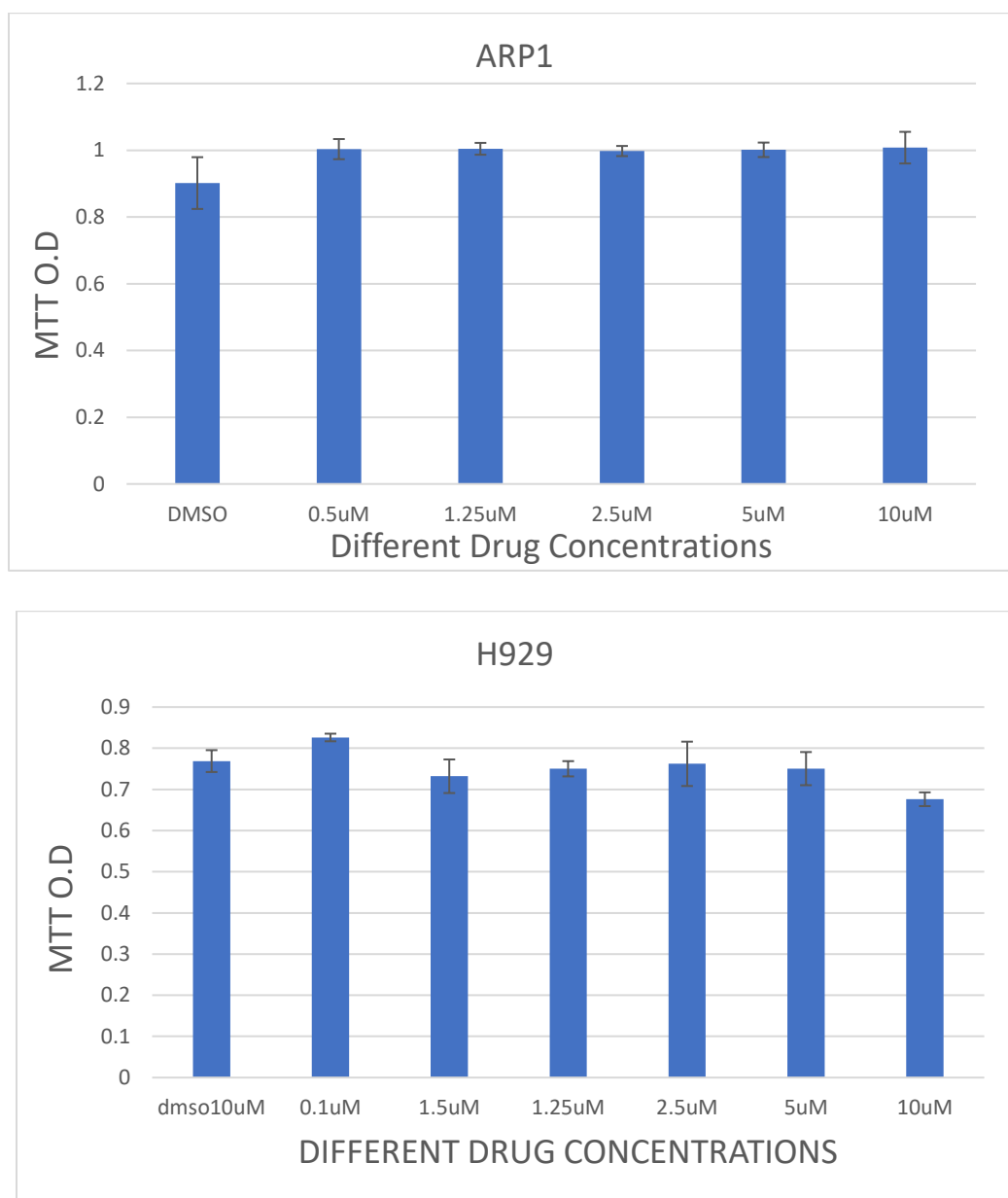
MTT assay results showing that MM cell lines displayed resistance to t-BHQ with control**DMSO**

Figure 8-3 (D-E-F). t-BHQ had no impact on MM ARP1, H929 and 8266 cell lines and, by comparison to DMSO, heightening of drug levels and cell proliferation showed no correlation of statistical significance. Such results corroborate the notion that MM represents a heterogeneous disease.

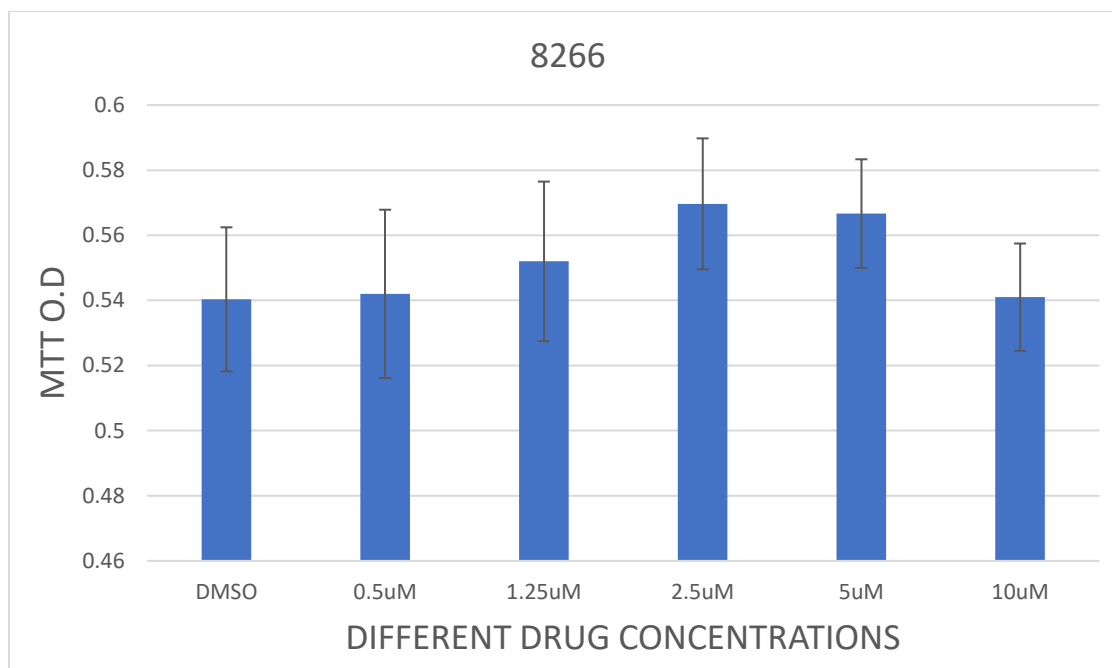
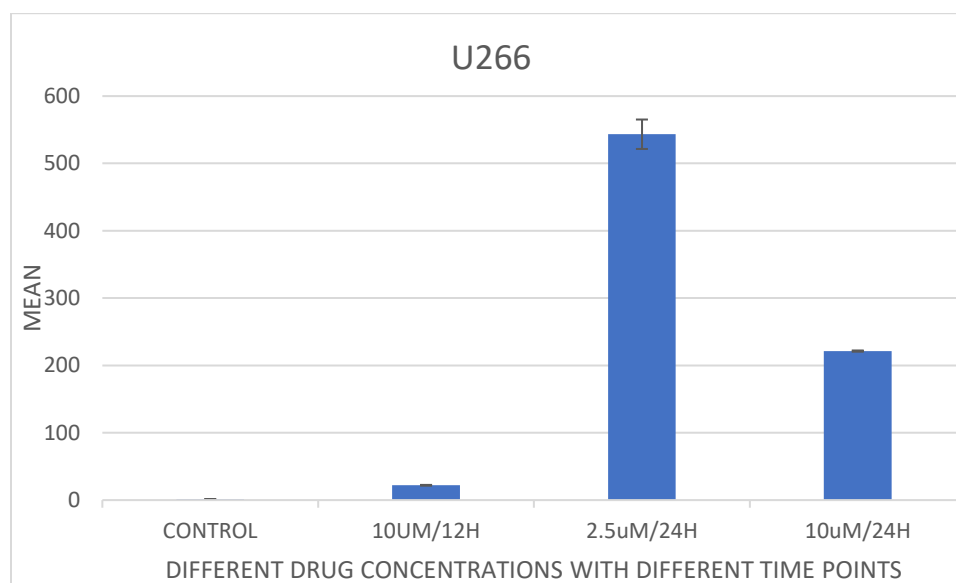


Figure 8-4 (D-E-F). t-BHQ had no impact on MM ARP1, H929 and 8266 cell lines and, by comparison to DMSO, heightening of drug levels and cell proliferation showed no correlation of statistical significance. Such results corroborate the notion that MM represents a heterogeneous disease (Cont.).

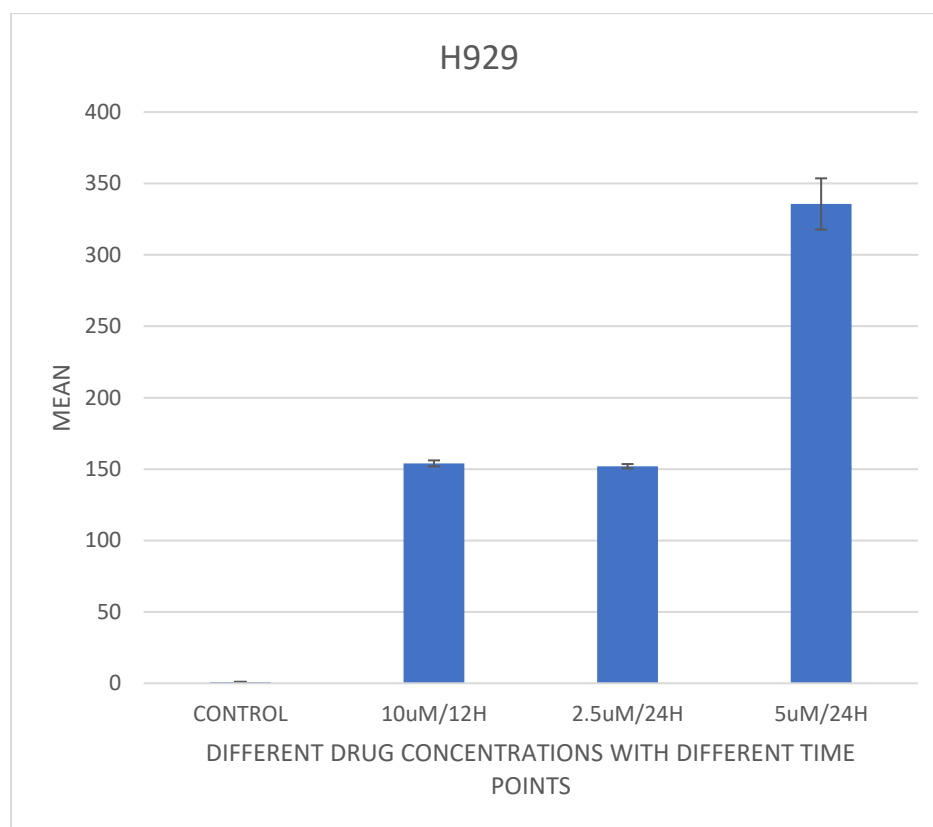
Real-time PCR indicated that t-BHQ had an impact on HMOX1 upregulation in U266 at various time points



U266 Drug Concentrations with Different Time Points	P-VALUE	Mean	Standard Errors
B-ACTIN Control	1	1	-
10uM /12hours	2.8E-09	22	0.577
2.5uM /24hours	6.27E-12	543.33	21.85
10uM /24hours	2.77E-16	221.33	0.881

Figure 9-A. In MM U266 cell line, t-BHQ exhibited an antioxidant protective effect by promoting HMOX1 upregulation.

Real-time PCR indicated that t-BHQ did not have an impact on HMOX1 upregulation in H929 at various time points



U266 Drug Concentrations with Different Time Points	P-VALUE	Mean	Standard Errors
B-ACTIN CONTROL	1	1	-
10uM /12hours	4.75E-10	154	2.081
2.5uM /24hours	5.03E-09	152	1.527
5uM /24hours	3.37E-12	335.66	17.94

Figure 9-B. In MM H929 cell line, t-BHQ exhibited an antioxidant protective effect by promoting HMOX1 upregulation.

CONCLUSION

The aim pursued in upregulating HMOX1 with t-BHQ was augmentation of MSC stemness to boost formation of osteoblasts and diminish formation of osteoclasts. upregulation of HMOX1 within MSCs hindered those cells from acquiring an oxidant-promoting state induced by tumor oncogenes. The results obtained in this study indicated that t-BHQ promoted differentiation of osteoblasts and suppressed formation of osteoclasts by enhancing HMOX1 expression in MSCs and therefore has strong potential for use in MM treatment.

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