IDENTIFICATION OF SMALL MOLECULES THAT ENHANCE MURINE BONE MARROW DERIVED MESENCHYMAL STEM CELL EXPANSION

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ABSTRACT

IDENTIFICATION OF SMALL MOLECULES THAT ENHANCE MURINE BONE MARROW DERIVED MESENCHYMAL STEM CELL EXPANSION

Mesenchymal stem cells became a great candidate for transplantation-based therapies with their immunomodulatory traits (ability to regulate immune response), homing capability (migration to injured sites), differentiation ability to all three embryonic lineages; only after perfecting isolation protocols and expension tecniques. They are present in the adult body, they can self-renew themselves and exhibit multipotency. They can be obtained from a variety of tissue types including; bone marrow, adipose tissue, umbilical cord, dental tissue. On the other hand, there are major challenges on mobilization, expansion and understanding the differentiation mechanism. If these challenges can be overcome, MSCs show great potential for experimental and clinical applications. In our study we focused on expansion of mBM-MSCs with small molecule treatment. With that, we hope to achive increased mobilization results as well. We selected four effective molecules primarily by WST-1 cell viability assay, the assay was supported by hoechst staining for cell counting; SKF96265, SB203580 (P38-MAPK Inhibitor), GSK-3 Inhibitor and StemReginin 1 (AhR Antagonist). To see the effects on cell mechanism; cell cycle analysis with DAPI and Hoechst staining, apoptosis analysis with Annexin V-FITC and PI staining were conducted. The results did not suggest any misconduct on our MSC culture by the expanding effects of selected small molecules except SFK96265, a CCE inhibitor, which caused G1-phase arrest and the cell population treated by had increased cell death rates. Next, we performed RT-PCR to check if there were any negative changes on HDR, CDKI, S-Phase-related gene expressions. SFK96265 also showed highly significant upregulation of RAD51 and PCNA HDR-related genes expressions. Our first molecule is a GSK-3 inhibitor that stabilizes free cytosolic β- catenin and inhibits differentiation. Next candidate is a p38-MAPK inhibitor, which is the most effective small molecule. In conclusion, we layed a foundation for a safe and reliable way of *in vitro* expansion of murine BM-MSCs. Our next step is treatment of the mixture of compatible small molecules, *in vivo* trials by small molecule treated stem cell transplanation for GvHD and immunomodulation therapy. With all this, our goal is to carry this knowledge to therapeutic field.

ÖZET

FARE KEMİK İLİĞİ KÖKENLİ MEZENKİMAL KÖK HÜCRELERİNDE ÇOĞALMAYI İNDÜKLEYEN KÜÇÜK MOLEKÜLLERİN İDENTİFİKASYONU

Mezenkimal kök hücreler (MKH), immüno modülatör özellikleri (immün sistemi kontrolü), homing yetenekleri (hasarlı bölgelere göç edebilme) ve farklılaşma kapasiteleri ile transplantasyon-terapileri için çok önemli bir aday haline geldiler. MKH'ler yetişkinlerde bulunan, multipotensi özelliği gösteren ve kendini yenileme kabiliyetine sahip hücrelerdir. Kemik iliği, adipoz dokusu, kordon, dental doku dahil olmak üzere bir çok dokudan izole edilebilirler. Ancak, mobilizasyon, ekpansiyon ve farklılaşma kapasitelerinin anlaşılması konularında bir çok soruna rastlanmaktadır. Eğer bu sorunların üstesinden gelinebilirse, deneysel ve klinik çalışmalar için çok önemli bir aday haline gelebilirler. Çalışmamızda, fare kemik iliğinden izole edilen MKH'leri küçük moleküller ile çoğaltmaya odaklandık. Ayrıca küçük molekül muamelesi sonrası mobilizasyonda da artış görmeyi umduk. Öncelikli olarak, WST-1 denemeleri ve Hoechst boyamaları sonucunda dört etkin molekül seçildi; SKF96265, SB203580 (p38-MAPK İnhibitörü), GSK-3 İnhibitörü ve StemReginin I (AhR Antagonist). Hücre mekanizmalarında etkilerini görmek için, hücre döngüsü (DAPI ve Hoechst boyamaları) ve apoptoz analizleri (Annexin V-FITC ve PI boyamaları) yapıldı. Kontrolle (DMSO) karşılaştırıldığında, SKF96265 ile muamele edilmiş MKH populasyonu haricinde sonuçlarda herhangibir olumsuz etki görülmedi. SKF96265 (CCE İnhibitörü) hücrelerin G1 fazında duraklamaya neden oldu ve yüksek ölüm oranları gözlendi. Sonrasında, HDR, CDKI ve S-Fazı genlerindeki değişimleri görmek için RT-PZR yapıldı. SKF96265'in RAD51 ve PCNA HDR gen ekspresyonlarını anlamlı bir şekilde arttırdığı görüldü. İlk seçilen molekül olan GSK-3 inhibitörü serbest sitozolik β-katenini stabilize ederek farklılaşmayı engellemektedir. SB203580 ise p38-MAPK yolağına özgü bir inhibitördür ve seçilenler arasından en etkili molekül olduğu gözlemlenmiştir. Sonuç olarak, bu çalışma ile murin kemik iliği kökenli MKH'lerin *in vitro*'da çoğaltılması için sağlam ve güvenilir bir yöntemin temelleri atılmıştır. İleride, etkili küçük moleküllerden yapılan kokteyllerin denenmesi, immüno modülasyon ve GvHD için küçük moleküllerle muamele edilmiş MKH'ler ile *in vivo* çalışmalar yapılabilir. Temel amaç, bu bilgileri klinik alanda kullanabilir hale getirmektir.

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LIST OF SYMBOLS/ABBREVIATIONS

μL	Microliter
μM	Micromolar
mL	Mililiter
BDNF	Brain derived neurotrophic factor
BM	Bone marrow
CDK	Cyclin-depended kinases
CDKI	Cyclin-dependend kinase inhibitors
CFU-F	Colony forming unit - fibroblasts
DMEM	Dulbecco’s modified Eagle’s media
DMSO	Dimethyl sulfoxit
DNA	Deoxiribo nucleic acid
DPBS	Dulbecco’s phosphate-buffered saline
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FCS	Fatal calf serume
FGF	Fibroblast growth factor
GvHD	Graft vs host disease
HDR	Homology directed repair
HLA	Human leukocye antigen
HSC	Hematopoietic stem cells
IDO	Indoleamie
IFN	Interferone
IL	Interleukine
MHC	Major histocompability complex
MSCs	Mesenchymal stem cells
NBCS	New born calf serum
NGF	Nerve growth factor
NHEJ	Non-homology end joining

NK	Natural killer cells
NT-3	Neurotrophin-3
PD	Population doublings
PGE2	Prostaglandin E2
RNA	Ribonucleicacid
RPMI	Roswell park memorial institute medium
SC	Stem cells
SM	Small molecules
TGF	Transforming growth factor

1. INTRODUCTION

1.1. MESENCHYMAL STEM CELLS

The discovery of the regenerative potential of adult stem cells in developed organisms raised the interest in the field of genetics and regenerative biology [1]. First achievement on this subject was hematopoietic stem cells [2]. It was thought that the human bone marrow (BM) mainly consisted of hematopoietic stem cell (HSC) niches which are the small hypoxic environments on the bone cavity that allows stem cells (SCs) to grow and maintain themselves in a steady state [3]. But then in 1968, Friedenstein and his colleagues did a heterotrophic transplantation of bone marrow to identify the potential of bone marrow (BM) cells. The results were, instead of hematopoiesis, reticular tissue development (a considerable amount of bone evolvement) in the graft. These postnatally existing stem cells are having the ability to regenerate to all types of bone tissues. Later discovered, these progenitors', which were named mesenchymal stem cells (MSCs), ability to differentiate was not limited to only bone tissue, but they also could generate tendon, muscle, fat and ligament and many more different cell types [4].

Other than tissue repair abilities; in bone marrow cavity, HSC niches are undergrided by MSCs consistently secreting cytokines and this support system is vital for hematopoiesis and engraftment of HSC [5]. Which also benefit transplantations if the population is co-cultured with MSCs. BM-MSCs immunomodulatory traits (ability to regulate immune response) [6], homing capability (migration to injured sites) [7], differtiation ability to all three embryonic lineages makes them a great candidate for cell based therapy, only after perfecting isolation protocols and expension tecniques.

1.1.1. Sources and Differentiation Potential of Mesenchymal Stem Cells

The initial encounter of MSCs was including but not limited to bone marrow. It has been shown that MSCs can be found in various tissue types such as adipose [8], dental tissues [9] amniofluid and membrane [10]**,** limb bud [11], peripheral blood [12], salivary gland

[13], skin and foreskin [14], Wharton's jelly [15], and umbilical cord blood [8].

MSCs from these different tissue types show various epigenetic features. There are significant changes on gene expressions, immunomodulatory potential, proteome that depend only to source, and are not heterogenitiy in the population [8,16–18].

Table 1.1. Source tissues for mesenchymal stem cells and *in vitro* differentiation potency

	MSC Source Tissue	***in vitro* Differentiation Potency**	**References**
1.	**Bone Marrow**	Osteocytes Chondrocytes Adipocytes Hepatocytes Cardiomyocytes Neuronal Cells Pancreatic Cells	[8,19–23]
2.	**Adipose Tissue**	Osteocytes Chondrocytes Adipocytes Hepatocytes Cardiomyocytes Neuronal Cells Pancreatic Cells	[8,23–25]
3.	**Dental Tissue**	Osteocytes Chondrocytes Adipocytes Melanocytes Pancreatic Cells Neuronal Cells	[26,27]
4.	**Amniotic Fluid/Membrane**	Adipocytes Osteocytes Chondrocytes Neuronal Cells	[14,28]

5.	**Skin and Foreskin**	Osteocytes Chondrocytes Adipocytes Myogenic Lineage	[16,29]
6.	**Peripheral Blood**	Adipocytes Osteocytes Chondrocytes	[30]
7.	**Limb Bud**	Adipocytes Osteocytes	[11,31,32]
8.	**Wharton's Jelly**	Osteocytes Chondrocytes Adipocytes	[15,33–35]
9.	**Umbilical Cord Blood**	Osteocytes Chondrocytes Adipocytes Cardiomyocytes Neuronal Cells Pancreatic Cells	[15,33,36,37]
10.	**Endometrium**	Osteocytes Chondrocytes Adipocytes	[38,39]

1.1.2. Charasteristics and Morphology

In 1999, Pittenger et. al. experimented and revealed new features of MSCs which later become the main principles in the field of mesenchymal stem cells [40].

i. Adherent bone marrow stem cell cultures are consisting mesenchymal stem cells
ii. Stem cells can be expanded in cell culture
iii. Expended stem cells can differentiate when induced
iv. *in vitro* induced-differentiated cells can be used for *in vivo* assays
v. Markers used to characterize cultured putative stem cells, preferably to uncultured stem cells.

These features have become the main rules and the guideline in the field of MSC expansion [40].

After subsequent discoveries, the main definition of mesenchymal stem cells (MSCs) came to be: multipotent stem cells that can be obtained from adult human and animal sources [41], they show adherence properties and form spindle-shaped colonies [42,43]. These adhesion molecules expressed by MSCs is listed as: vimentin, laminin, fibronectin and osteopontin [44].

To address a population as MSCs, the population is obliged to express CD73, CD90 (Thy-1) and CD105 cell surface markers; and they must be negative for hematopoietic antigens (leukocyte and B-cell markers, antigens expressed by monocytes and macrophages) CD14, CD34, CD45 or HLA class II and CD19 or CD79a and CD11b. The minimal guideline for surface marker rate is >95% for positive markers; <2% for negative ones. Unfortunatelly, murine MSC surface marker characterisation is not well established yet and surface markers listed above may not apply to murine systems [45], but only to humans. For this reason, experiments should be conducted for all measures to support characterization; morphological tests, CFU-F assays for colony formation.

MSCs have the ability to differentiate into mesodermal lineage (ergo, named "mesenchymal", as they are from the same embryonic origin) including; osteocytes, chondrocytes, adipocytes [46], ectodermal and endodermal lineages (exp. cell types; neurocytes and hepatocytes) [15]**,** in spesific *in vitro* differentiating conditions. Also it is shown that MSCs can generate bone tissue following transplantation to immunodeficient SCID mice, which shows MSCs *in vivo* capability. [47,48].

Table 1.2. *in vitro* MSC differentiation factors.

	Lineage	**Target Tissue**	**Factors**	**References**
1.	**Mesoderm**	**Osteogenic**	Dexamethasone β-glycerophospate Ascorbic Acid	[49]
2.	**Mesoderm**	**Condrogenic**	TGF-β3 TGF-β2	

			TGF-β1 Insulin Transferrin Selenium Linoleic Acid	[50]
3.	**Mesoderm**	**Adipose**	Dexamethasone Isobutyl-methylxantine Indomethacin	[50,51]
4.	**Mesoderm**	**Cardiomyocytes**	5-azacytidine TGF-β	[52]
5.	**Ectoderm**	**Neuronal**	DMSO BHA KCl Forskolin Hydrocortisone bFGF β-mercaptoethanol NT-3 NGF BDNF Neurogenin	[53,54]
6.	**Endoderm**	**Pancreatic**	Nicotinamide β-mercaptoethanol Acitvin A	[55,56]
7.	**Endoderm**	**Liver**	Hepatocyte Growth Factor Oncostatin EGF bFGF Nicotinamide	[57]

1.1.3. Immunomodulation

Human MSCs are also known in clinical field for their immunomodulatory properties; meaning they can modulate adaptive and innate immune system responses. The inhibition of immune system is a complicated mechanism that includes skills to regulate maturation and activation of antigen presenting cell types (including T and B cells) in addition to cytokine secrating cells like NK (natural killer cells), dendritic cells, and decreased immunogenicity [58–61].

Their suppressive immunoregulatory activities arise with the presence of soluble factors which are substantively expressed and secreted by MSCs or released after the presence of a stimulatory factor on an inflammatory environment [62]. For instance, IDO and PGE2 expressions have been seen to increase in MSCs [63]. IDO's presence in the environment causes quinurenin and tryptophan to decrease. Which results in inhibition of growth of the immune system cells due to toxicity effects to their catabolism or simply, nutrient insufficiency. Moreover, immunomodulatory effects of PGE2 are associated with releases of cytokines, particularly IFN-γ and IL-2. PGE2 simply alters an inflammatory environment to an anti-inflammatory state [64]. Recent studies also indicate that when IFNγ is present, it stimulates HGF (hepatocyte growth factor) and TGFβ1 expressions and with that suppresses allo-responsiveness [65].

In addition, decreased MHC-I and lack of MHC-II expressions together with co-stimulatory molecules (e.g. CD40, CD80, CD86) ensures mesenchymal stem cells' protection from NK cells (natural killer cells, innate immune system effector cells) lysis [66]. Escaping recognition by NK cells is also provided by HLA-DR expression. Quite similar effects are seen but different pathways are involved in cytotoxic T-cell suppression by MSCs. IFN-γ and LIF (leukemia inhibitory factor) are too involved on these pathways [67]. And recent studies have shown that MSCs have effect on three important pathways of dendritic cell maturation; i. Up regulation of antigen presenting molecules' gene expressions ii. Ability to present antigens and iii. Migration capability [7,68].

In a study conducted by Polchert, D. et. al., they have shown that when murine BM-MSC population gets exposed to IFN-c (interferon-c), they become activated and repress graft versus host disease (GvHD) *in vivo*. In this manner, MSCs's immunomodulatory effects

raise an opportunity on tissue damage repair induced by immune system/ auto immune diseases such as rheumatoid arthritis, Crohn's disease, type-1 diabetes, GvHD of the liver, skin, gut after allogenic cell transplantations, and also MSCs can be used to prohibit organ transplant rejections [65]. To summarize, these findings opens door for not only MSC based cell therapy options but also co-cell/tissue transplantation with mesenchymal stem cells treatments.

Both *in vivo* and *in vitro* studies have shown that MSCs having the ability to suppress T-cell proliferation, B-cell activation, macrophage, natural killer cells and dentritic cells [69,70]. And these results indicate that MSCs have a great potential for treating diverse immune disorders in animal models and also humans with this feature.

1.1.4. Homing of Mesenchymal Stem Cells

Homing term is used when a cell/cell population is migrated to a site of inflammation and injury. And to be precise for MSC; homing is the flow in vasculars of a tissue, in pursuit of transmigration over the endothelium. There are variety of factors such as culture conditions (oxygen levels, cytokine existence), passage number, cell's senescence that affect mesenchymal stem cell's homing efficiency [71,72].

This procedure is regulated by homing receptors on the migrating cells to intertwine with interested endothelial co-receptors. Thereafter, chemokine induced activation of integrin viscidity, adhesion and extra-vasasion [68,73,74].

Integrins are a major component for homing. They have been known to play an important part on migrating, chemo taxis and cell-to-cell adhesion. mBM-MSCs express a variety of integrins on their membranes [75]. The list in volves integrin α4 and β1; whose binding peers (VCAM-1) are upregulated in the ischemic mycardium [74,76,77]. These integrins modulate the capture-rolling-attachement proceses of MSCs [78]. The inflammatory cytokines (TNFα, TGFβ1, ILβ1) increase the migrating MSCs by up-regulating matrix metalloproteinases (MMPs) [72].

1.1.5. Growth Conditions and Aging of Mesenchymal Stem Cells

Isolated MSCs from different sources can be cultured using conditioned media such as: Dulbecco's Modified Eagle's Media (DMEM, ideally low-glucose) [45,70], αMEM [80–82] DMEM-F12 [56,83,84], RPMI (Roswell Park Memorial Institute Medium) [85], DMEM-HG [86,87]. These primary culture mediums can be supplemented with 10-15% FBS [79,81], NBCS (new born calf serum) [81] and/or FCS (fatal calf serum) [88]. Oxygen concentration also affects the proliferation and expansion of MSCs**,** hypoxic culture conditions are preffered.

Mesencymal stem cells show charasteristic losses on their potency during sub-culturing and also at high passage numbers. MSCs' becoming senecent at long-termed cultures was seen after passage 5. Meaning, until the aimed quantitiy of cells obtained, they might have already lost their potency. The decrease in telomerase activity and differentiation potential, telomere shortening and morphological changes are some of the indications of the loss of potency [89].

The age of MSCs in culture is decided by PDs (population doublings) time. In 10 weeks, colonies from a single cell has seen around 50 PDs [42], whilst long-term cultured MSCs have shown 30 PDs in 18-20 weeks [89]. In a similar experiment setup, it has been also shown that MSC count in stem cell cultures drops after the 4^{th} passage and the differentiation potential significantly declines after the 6^{th} passage along with other present morphological abnormalities and telomere shortening. In conclusion, MSCs undergo a phase of ageing very early on *in vitro* cultures compared to *in vivo* studies [89].

High passage number in MSC cultures when transplanted also leads to tumor growth and metastasis [90]**.** Moreover, aging features and morphological defects can be seen in the later development. Complete understanding of MSC modulation and regulation are important for the fast and safe expansion methods with the optimized assessment of growth conditions. The quality of growth conditions directly affects the therapeutic efficiency of *in vitro* expansion [46,91]

1.2. POTENTIAL THERAPEUTIC OPTIONS AND NEED FOR MESENCHYMAL STEM CELL EXPANSION

Safe and easily accessible stem cell sources have become one of the hot topics in regenerative biology studies to provide reliable and consistent research material for the researchers. The advances thriven in stem cell technologies, have let the stem cell therapy to grow over the past decade. Various isolated sources of stem cells have become commercially available. The development of commercially available stem cell sources has let the researchers to modulate the immune system and provide valuable assets for regenerative medicine and cell-based tissue repairing systems [89,92].

The interest around MSCs and their therapeutic potential increased significantly over the last decade. Ease of accessibility, *ex vivo* expansion, ease of isolation techniques, immunomodulatory abilities, tissue repair potential made MSCs an important source for cell-based therapy applications. While our understanding of MSCs improved, new therapeutic applications were developed not only in tissue engineering area, but also on MSCs immunomodulatory and reperative traits on wound healing and recitification of defective immune systems.

In spite of these traits, the researchers seem to have one big main problem as described by Ahmad H., Thambiratnam K. et. al. The study showed that there is a significant decline in the expansion ability of stem cell cultures through time in three different human MSCs isolated from dental tissue, umbilical cord and Wharton's Jelly. The expansion of MSCs was observed to be successful until the fifth passage and to be irregular/decreased following the fifth passage [93].

A series of experiments have been conducted by Laube, M. , Stolzing, A. et. al. (2016) [94] [94] to understand the potential of MSCs in the treatment of pulmonary complications. Although the overall research had promising results, the researchers have reported that there is an urgent need for optimization of MSC culture conditions before clinical trials [94]. It is clear that regulations, and innovations of new techniques are required to optimize MSC culture conditions in terms of passage number dependency, elimination of aging factors, improving of cryopreservation and preconditioning, and the determination of optimal tissue origin suitable for each specific context.

In another study investigating MSC-based treatment possibilities on autoimmune diseases based on immunomodulatory effects of MSCs by Erin Collins, Gary Gilkeson et. al. have also reported that further research is mandatory to optimize MSC microenvironment in cell cultures [95]. The optimum environment (stem cell niche) is essential for MSC activation and sustainability in cell culture conditions.

Recently, the scientists show that the importance of MSCs in the treatment of muscle dystrophy (MS). Treatments for muscle dystrophy are mainly depend on activating the host satellite cells, forming new myofibers at the injury zone. These applications reveal the rapid loss in the grafted MSC population. Therefore, the therapeutical requirement of MSC expansion increases [96].

To conclude, it has been clearly seen that the importance of MSC expansion tecniques on the field of use of MSCs increased rapidly due to the clinical trials that demands large population of MSCs. Although they are dividing cells, their expansion capability does not match with the requirement of clinical trials. While a minimal dosage requirement for transplantation is $1\text{-}2x10^6$ cells per kilogram, bone marrow cell population consists of only 0,3% of MSCs. In the cord blood and peripheral blood samples, the ratio is even lower [89,92]. To reach the sufficient amount for transplantation either long-term culture of MSC is requiered ,which is unsafe due to senecense, loss of potency, tumor growth potential, or developing new and safe expansion techniques.

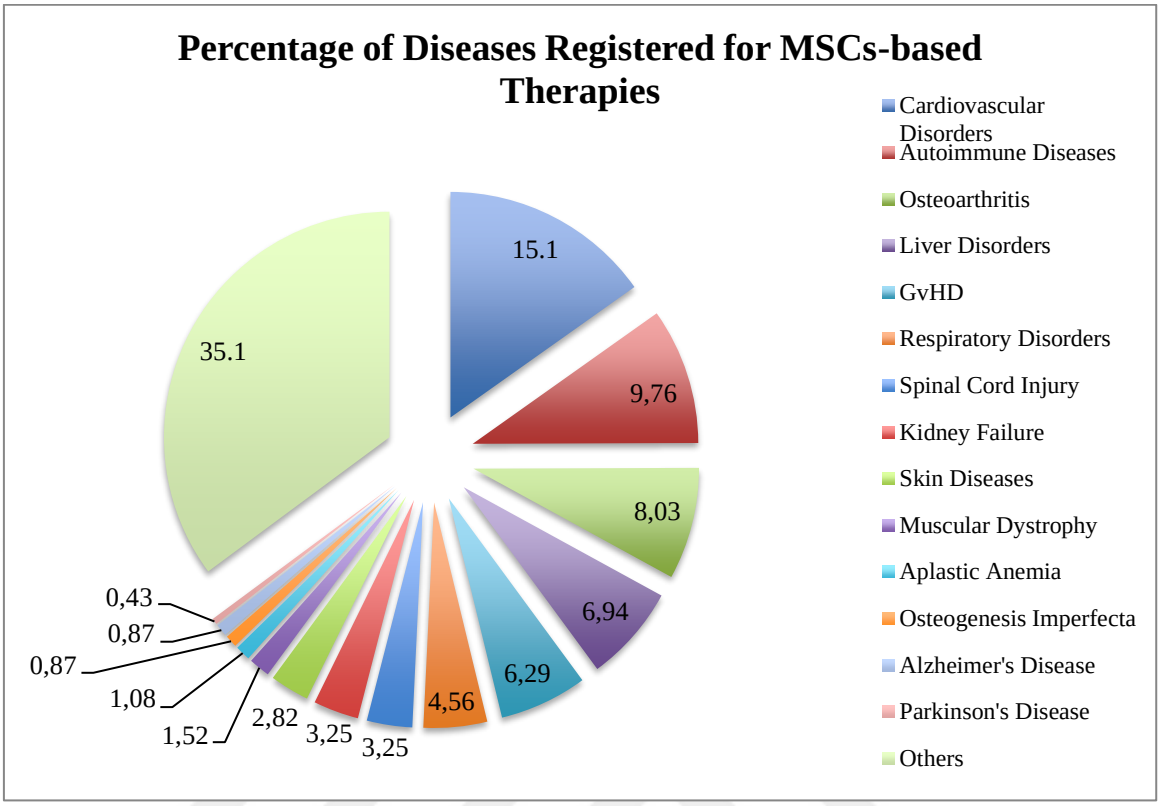


Figure 1.1. Pie chart of the percentile of diseases registired for MSCs-based therapies [4]

1.3. HOMOLOGY DIRECTOR REPAIR, S-PHASE AND CYCLIN-DEPENDENT KINASE INHIBITOR RELATED GENES

1.3.1. HDR (Homology Directed Repair) and S-phase Related Genes

DNA stability is provided by efficient and accurate repair of double stranded breaks (DSB). There are two major pathways for DNA repair, first being HDR (Homology-Directed Repair) and second, NHEJ (Non Homologous End Joining). NHEJ pathway is formed on the basis of ligation of two-ends when there are only a few complemantary nucleotids, whilist HDR happens when there are longer forms of homolog sequences, accuracy is advaced. Homologous recombination is the most prevalent form of homology-directed repair [97,98].

S-phase, synthesis phase, is a step on cell cycle where DNA replication occurs. Cells enter this phase as a diploid cell (2n); exits with 2 set of genome content (4n). This phase occurs between G1 and G2 phases. Efficient and correct DNA-replication is crutial in this step to

prohibit genomic abnormalities which usually pioneer diseases or cell death. Regulatory pathways and related genes are highly conservative on this subject because of its importance on survival [99,100].

Table 1.3. Homology directed repair and S-Phase related genes and functions.

GENE	FUNCTION	REFERENCE
RAD51	Recombinase. Repair and homologous recombination. S-Phase and HDR.	[101]
PCNA	Proliferating Double-stranded break repair nuclease. S-Phase and HDR.	[102]
RAD17	Cell cycle check point gene. S-Phase	[103]
MCM2	Minichromosome maintenance protein. Genome stability. S-Phase.	[99]

1.3.2. CDKI (Cyclin Dependent Kinase Inhibitor) Related Genes

CDKs are an evalutionarily conserved cell cycle regulator protein kinase family. They regulate gene transcription [104,105]. Cyclin dependent kinase inhibitors are small proteins that play a crucial role in cell cyle. As the name gives: they have inhibitory effects on cyclin kinase complexes which are functionary on cell cycle. The genes coding CDKIs are often deleted (mutated) in cancer cell lines, and they are a newly considered canditates of tumor suppressor genes (e.g. P16, P21). CDKIs' act as cell cycle regulators. They generally suppress CDK activity in G1 phase in response to damaged DNA in order to stop cell cycle until DNA repair occurs [106].

Table 1.4. CDKI related genes and their functions

GENES	FUNCTIONS	REFERENCES
P15	CDK4 and CDK6 inhibitor. Passage trough G1 check point	[107]
P16	Tumor suppressor. Blocks MDM2 induced p53 degradation. Induces apoptosis.	[108]
P18	Cell growth inhibition. Connected functions with RB (retinoblastoma protein)	[109]
P19	Cell cycle regulator.	[110]
P19(Arf)	Inhibits ribosome biogenesis.	[111]
P21	P53-activated fragment 1. Suppressor of malignant tumors.	[112]
P27	CDK2 inhibitor.	[113]
P57	Negative regulator for cell proliferation.	[114]

1.4. SMALL MOLECULES

Small molecules (SM) are low molecular weighted substances. They are well defined, easily characterised substances. SMs are chemically synthesized and their identical copies can be made inexpensively**.** Once they enter the cell, they can affect other molecules. To be more spesific, they are synthesized in a way to target biological pathways incorporated with growth, expansion, signal transduction, apoptosis, cell cycle phases, differentiation and many more [115,116].

Their stability and non immunogenic properties are wanted and needed traids in thereupathic fields, especially for cancer drug discovery [117]. The most known small molecule drug example is aspirin, with its prevalent clinical use. Aspirin only has 21 atoms

and measures at 180 daltons. Its stability and low immune response stimuli makes it the optimum small molecule [118].

In this study, small molecule treatment is selected and thought to be a safe and reliable way of solving the expansion problem of murine bone marrow derived mesenchymal stem cells. And with that, increase the potential use of MSC in cell based therapy. A total of four molecules were selected for investigating their effects on mBM-MSC expansion. These small molecules are; CHIR99021 (a GSK-3 inhibitor), StemReginin I (Aryl hydrocabon receptor antagonist), SB203580 (a P38/MAPK inhibitor) and SKF96395 (a Capacitative Ca^{2+} entry inhibitor).

CHIR99021 is an an amino pyrimidine derivative. This small molecule inhibits GSK-3 and this results in Wnt signalling pathway (migration, neuronal patterning, cell survival establishing, organogenesis) activation [119].

StemReginin I is an aryl hydrocarbon receptor antagonist SR1 exhibits HSC expansion capabilities in numerous researches [120,121].

SB203580 is a P38/MAPK inhibitor. MAPK is connected to differentiation, apoptosis, autophagy and it is persistantly active and ageing-inducing. Its inhibition showed activity against fibrosis and muscle regeneration was induced with SB203580 treatment [122–124].

SKF96395 is an appealing new anti-cancer drug candidate. It showes an anti neoplastic activity by inducing cell cycle arrest and apoptosis by inhibitin capacitative Ca^{2+} entry [125,126].

2. MATERIALS AND METHODS

2.1. ANIMAL INFORMATION

Balb-C mice have been used throughout this study. Animal studies were used under decision number 651 which was approved by the Institutional Clinical Studies Ethical and the Institutional Animal Care and Use Committee of Yeditepe University (YUDHEK).

2.2. MESENCHYMAL STEM CELL ISOLATION FROM MOUSE BONE MARROW AND CELL CULTURE CONDITIONS

BalbC mice were dissected; femur and tibia bones were separated from flesh. Cold DPBS (Dulbecco's Phosphate-Buffered Saline, Gibco cat. no. 14190250) were flushed through the bone marrow cavity using a 26-G needle. The marrow cell suspension was filtered through a 70 μm cell strainer (Falcon cat. no. 0877102). The cell suspension was centrifuged at 1500 rpm for 5 minutes. Supernatant was removed. Pellet was resuspended in low-glucose DMEM media (Gibco, cat. no. 10567014), supplemented with 15% FBS (Gibco, cat. no. 10082147), 1% PSA (Gibco, cat. no. 15240062). Then, cells were counted on hemocytometer. The cells were cultured in T75 flasks (20 million cells per flask) with the media volume of 8 mL, or T25 flasks (8 million cells per flask) with the media volume of 3 mL. Flasks were placed in incubator set on 37°C and 5% CO_2 overnight. 24h later media with non-adherent cells was discarded, fresh media was added to flasks. Media was replaced with fresh media every 3-4 days. After two weeks of initial culture, adherent cells (MSCs) were collected.

2.3. SMALL MOLECULE TREATMENT TO MOUSE BM-MSC CULTURE

Small molecules were dissolved in DMSO for stock. SM stocks were at 20 mM concentration. The next dilution was done with DPBS at concentrations 0,01 mM, 0,1 mM and 1 mM and treatment was done by using these stock solutions. mBM-MSCs were seeded at the density of 5000 cells per well to 96-well plates. 24h later, the media was

replaced with fresh media and cells were treated with before-mentioned doses of small molecules to the final concentrations of 0,1 μM, 1 μM and 10 μM, three replica for a dose. Thereafter, the cells were incubated at 37°C and 5% CO_2 conditions for five days.

2.4. WST-1 CELL VIABILITY ASSAY

WST-1 Reagent (Cell Proliferation Reagent WST-1, Roche, cat. No. 11644807001) were diluted in 1:10 ratio with cell culture media and added as 100 μL to each well. Samples were incubated in humidified incubator at 37°C and 5% CO_2 in dark. After 4 hours, the absorbance was measured using a spectrophotometer microplate reader (Thermo Scientific, Varioskan Lux) at 420-480 nm.

2.5. HOECHST STAINING

mBM-MSCs were seeded at the density of 5000 cells per well to 96-well plates. 24h later, the media was replaced with fresh media and cells were treated with before-mentioned doses of small molecules, three replica for a dose. The cells were incubated at 37°C and 5% CO_2 conditions for five days. Media was chanced with a fresh media with a volume of 200 μL for each well. 1,5 μL from 200X Hoecsht stain (Thermo Scientific, cat. no. 62249) was added per well. Plate was placed in incubator for 30 minutes (in dark). Cells were washed with DPBS. Cell imaging device (GE, Cytell) was used for analysis.

2.6. CELL CYCLE ANALYSIS

P1 murine BM-MSC cells were seeded at 20000 per well density at a 24-well plate. 24h later, cells were treated with small molecules. Five days later, cells were trypsinized, centrifuged at 1500 rpm for 5 minutes at room temprature. Supernatant was discarted. Pellet was resuspended with 200 μL of fresh media. Suspension was placed in 37°C and 5% CO_2 incubator for 15 minutes. 1,5 μL from 200X Hoecsht stain (Thermo Scientific, cat. no. 62249) was added per well. Plate was placed in incubator for 30 minutes (in dark). After that step 2 μL from 100X Pyronin Y (Sigma-Aldrich, cat. no. 213519) was added per

well. The cells were incubated at 37°C and 5% CO_2 for 15 minutes. Flow cytometry device was used for analysis (Beckman Coulter, Cytoflex S).

2.7. APOPTOSIS ANALYSIS

P1 murine BM-MSC cells were seeded at 20000 per well density at a 96-well plate. 24h later, cells were treated with small molecules. Five days later, cells were trypsinized, centrifuged at 1500 rpm for 5 minutes at room temprature. Supernatant was discarted. For this analysis Invitrogen Annexin V-FITC Apoptosis Detection Kit, cat. no. BMS500FI-100 was used. Pellet was resuspended with 50 µL of 1X Binding Buffer. 2,5 µL Annexin V and 2,5 uL of PI was added to each well. The cells were incubated at room temprature, in dark, for 15 minutes. 200 µL 1X Binding Buffer was added to each well. Flow cytometry device (Beckman Coulter Cytoflex S) was used for analysis.

2.8. RNA ISOLATION

Total RNA Isolation was done by using NucleoZOL kit (Macerey-Nagel, cat. no. 740404200). Cell culture media was removed and 1 mL of NucleoZOL was added to the wells. Complete lysis was ensured by vigorous pippetting. 200 µL of RNase free water added per 500 µL of NucleoZOL/lysed cell suspension. The suspencion was incubated at room temprature for 5 minutes then centrifuged at 12000 xg for 15 minutes. 500 µL of supernatant was transferred to a fresh tube, 500 µL isopropanole was added. The suspension was incubated at room temprature for 10 minutes and centrifuge at 12000 xg for 10 minutes. Supernatant was discarted and 500 µL of 75% ethanol was added. Sample was centrifuged for 3 minutes at 8000 xg. The ethanol-washing step was repeated two times. The RNA pellet was dissolved in RNase free water. Concentration and purity (A230/A280) results were obtained by Nanodrop.

2.9. CDNA SYNTHESIS

This experiment was done using ProtoScript II, First Strand cDNA Syhnthesis Kit (cat. no. E6560S). Up to 1 µg of template RNA, 2 µL d(T)23VN, 10 µ L of ProtoScript II Reaction

Mix (2X), 2 µL ProtoScript Enzyme Mix (10X) was mixed in a PCR tube. Nuclease free water was added as necessary to complete the mixture to a total volume of 20 µL. The mixture was incubated first at 42°C for 1 hour, then at 80°C for 5 minutes.

2.10. REAL TIME PCR

Promega, GoTaq qPCR Master Mix, cat. no. A6001 was used. 4 µL of nuclease free water, 0,75 µL of Forward Primer (100 mM), 0,75 µL of Reverse Primer (100 mM), 7,5 µL of Master Mix (Syber Green) and 2 µL of the sample's cDNA was mixed. 1 cycle of Hot Start Activation at 95°C for 2 minutes, 50 cycles of Denaturation (95°C for 15 seconds) and Annealing (60°C for 1 minute) protocol was set on Roche, Light Cycler 96. GAPDH and β-Actin was used as internal control. Data was analyzed by using 2-ΔΔCt Method.

2.10.1. Primer List

Table 2.1. Complete list of primers used in this study.

CONTROL	S-PHASE and HDR	CDKI
mGAPDH	mRAD51	mP15
mβ-ACTIN	mRAD17	mP16
	mPCNA	mP18
	mMCM2	mP19
		mP19 Arf
		mP21
		mP27
		mP57

Table 2.2. Oligo sequences of primers used in this study. 5’ to 3’ for forward primers; 3’ to 5’ for reverse primers.

	Gene	Forward Primer	Reverse Primer
1	mGAPDH	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT
2	mβ-ACTIN	ATGGAGGGGAATACAGCCC	TTCTTTGCAGCTCCTTCGTT
3	mRAD51	GGTGGTCTGTGTTGAACCCT	ACACCGAGGGCACCTTTAG
5	mPCNA	GGAGACAGTGGAGTGGCTTT	GGAGACAGTGGAGTGGCTTT
6	mMCM2	TCAGCTCCTCCACATCTTCA	TCAGCTCCTCCACATCTTCA
7	mRAD17	CACATCCTGGAGGACCTGTTA	CACATCCTGGAGGACCTGTTA
8	mP15	CAGTTGGGTTCTGCTCCGT	AGATCCCAACGCCCTGAAC
9	mP16	GGGTTTCGCCCAACGCCCCGA	TGCAGCACCACCAGCGTGTCC
10	mP18	CTCCGGATTTCCAAGTTTCA	GGGGGACCTAGAGCAACTTAC
11	mP19	TCAGGAGCTCCAAAGCAACT	TTCTTCATCGGGAGCTGGT
12	mP19 Arf	GTTTTCTTGGTGAAGTTCGTGC	TCATCACCTGGTCCAGGATTC
13	mP21	ATCACCAGGATTGGACATGG	CGGTGTCAGAGTCTAGGGGA
14	mP27	GGGGAACCGTCTGAAACATT	AGTGTCCAGGGATGAGGAAG
15	mP57	TTCTCCTGCGCAGTTCTCTT	CTGAAGGACCAGCCTCTCTC

2.11. CFU-F ASSAY

mBM-MSCs were seeded on 6-well plates at 25000 cell per well density. The cells were incubated at 37°C and 5% CO_2 conditions for twelve days. Media was chanced with a fresh media every 3-4 days. After twelve days, cells were washed gently with DPBS twice. Cells were incubated and fixed at room temperature fo 20 minutes with 2 mL of 1% folmaldehid. Formaldehid was discarted. Giemsa (Thermo Scientific, cat. no.10434969) was d,luted with dH_2O at 1:20 ratio and added to the wells and incubated for 30 minutes. Giemsa was rinsed thoroughly with destilled H_2O. Later on, colonies were observed and counted on confocal microscope. Colony condition was 100 cells or higher.

2.12. CELL SURFACE MARKER CHARACTERIZATION

P2 murine BM-MSC cells were seeded at 20000 per well density at a 96-well plate. Each well were stained seperately with Anti mouse CD90 (Thy-1) - PE, Anti mouse CD105 - PECy7 and Anti mouse CD11b – PE (Invitrogen cat. no. in order; 4280969, 12105742, 11011842) deluted in DPBS at 1:2000 ratio. 50 µL of deluted antibodies was added to each well containing 200 µL media. The cells were incubated in ice, in a dark place for 15 minutes. Later on, flow cytometry analysis was conducted

2.13. STATISTICAL ANALYSIS

Two tailed t-test was used to determine the significance of the data. The results were labeled as significant if the t-test values is less than 0.05, and high significant if the value is less than 0.025.

3. RESULTS

3.1. SMALL MOLECULE LIST TESTED FOR THIS STUDY

Small molecules were selected via *in silico* drug screening based on their hematopoietic stem cell expansion capabilities acquired from previous research. Small molecules are labed with numbers (#) to ease data analysis.

Table 3.1. Small molecules tested for this study.

NO	Gene Symbol	Drug Selected
1	PTPMT	Alexadine Dihdrochloride
2	HAT (p300&CBP Inhibitor IV)	HAT Inhibitor-505298/p300&CBP Inhıbıtor VI
3	Inppp5d	AS1949490
4	Dnmt3a	RG 108
5	HAT Inhıbıtor	HAT Inhibitor-Garcinol and derivatives
6	GSK-3	CHIR-99021
7	Puma (Bbc3)	ZINC 19882186-C#7
8	c-Myc	c-Myc inhibitor II
9	Tet2	SC1 (pluripotin)
10	Trp53 or p53	Pifithrin-α
11	II2	Ro 26-4550 trifluroacetate
12	Gli1	GANT 61
13	Hif1a	400083 I HIF-1 Inhibitor

14	AhR	Stem Reginin 1, 182706 I AhR Antagonist II, SR1
15	Cdc42	CASIN
16	Cxcr4	CXCR4 Antagonist 1, AMD3100
17	Id1	Cannabidiol (CBD)
18	Runx1	219506 I CFB-β-Runx1 Inhibitor II, Ro5-3335
19	Skp2	SKP2-C25, 506305 I SKP2 E3 Ligase Inhibitor III
20	Pten	bpV (Hopic)
21	Gjb1 (Cx32)	2-aminoethoxydipenyl-borade (2APB)
22	Sirt1	EX 527&Nicotinamide
23	Unidentified	Tauroursodeoxycholic acid (TUDCA)
24	p38	SB203580&SB239063, a p28 MAPK inhibitor
25	Unidentified	α-Tacopherol
26	iNOS	L-N&-(1-iminoethyl)-lysine hdrochloride (L-NIL)
27	-	BIO (6-bromoindirubin-30-oxime)
28	-	SKF 96395 hydrochlorine
29	-	Mdivi-1
30	-	(5Z)-7-Oxozeaenol
31	-	SB203580 and p38 MAPK inhibitor
32	-	trans-2-phenylcyclopropyşamine hdrochloride
33	-	Tetraethylammonium chloride
34	-	K252c

35	-	BML-260
36	-	N-4-Tosyl-L-arginine methyl ester hydrochloride
CM1	-	Meis1 inhibitor
CM2	-	Meis1 inhibitor
NAE	-	
ST	-	
CA5	-	MMV688852, Radezolid
DG8	-	MMV688372, Kinetoplastids

3.2. MBM-MSC CULTURE IMAGE

After seven days of initial culture following isolation, adherent MSCs and spindle shaped colony formation was seen. Cells were observed on brightfield microscopy to validate the morphology of the isolated cells.

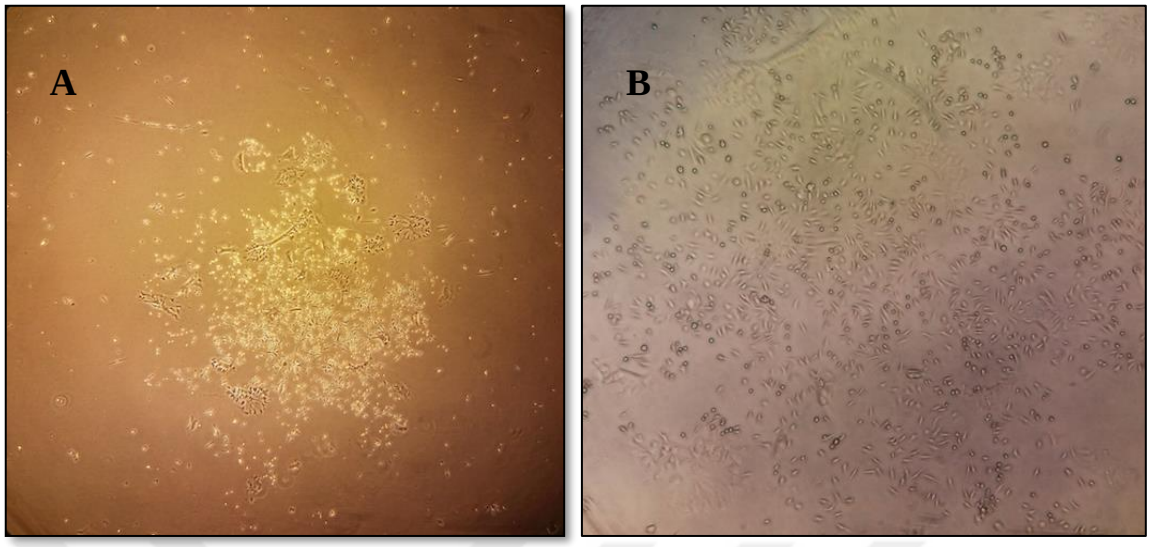


Figure 3.1. Passage 1, day 7 image of mBM-MSC on bright field microscopy. A; 10X magnification, B; 20X mignification.

3.3. CFU-F ASSAY

After 12 days of culture of passage number 3 mBM-MSC on T25 flask, 27 colonies ,with >100 cells per colony, were counted on brightfield microscopy.

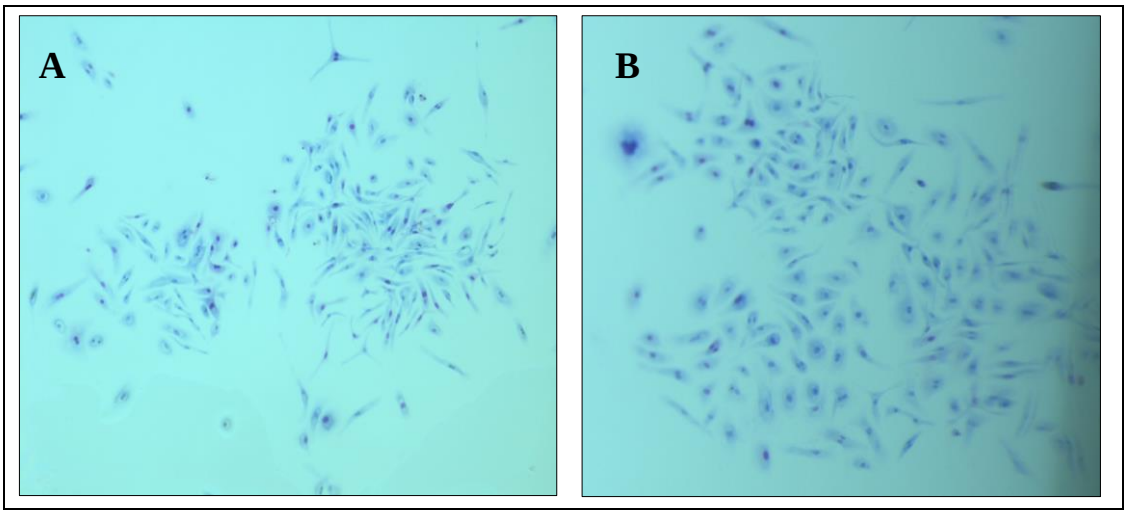


Figure 3.2. CFU-F assay images taken on brightfield microscopy on day 12 of culture. A; 10X magnification, B; 20X mignification.

Table 3.2. CFU-F count of P3 mBM-MSC after formaldehid fixation followed by giemsa staining.

Number of CFU-F per avg. of 100 cells	27

3.4. CELL SURFACE MARKER CHARACTERIZATION FLOW CYTOMETRY RESULTS

mBM-MSCs were isolated and cultured for 14 days on 37°C and 5% CO_2 incubator then immediately after tripsinizing, they were stained with CD90-PE and CD105-PECy7 for positive selection. CD90-PE yielded <98% of positivity; while CD105-PECy7 showed <83% of positivity. Negative control was CD11b-PE and the results were <94% negative.

A **B**

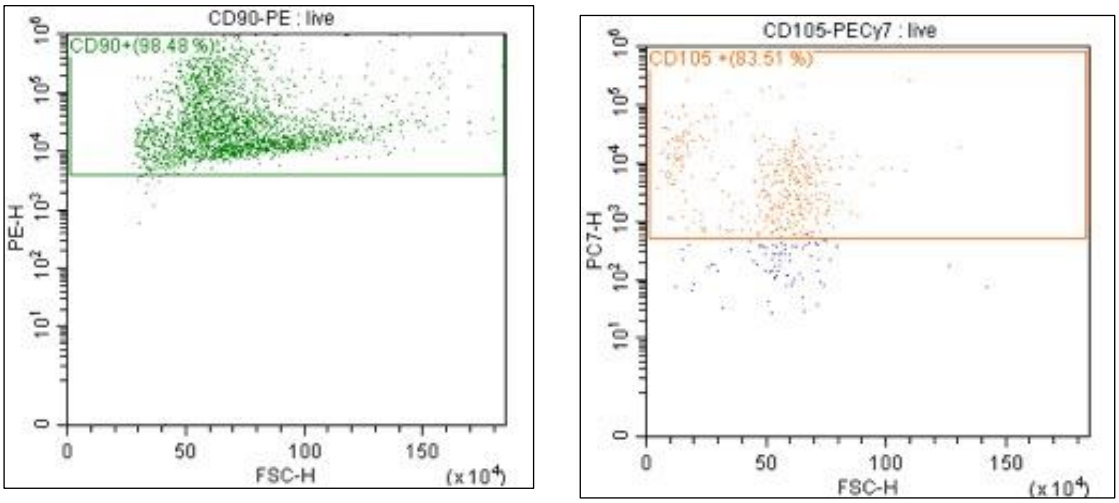


Figure 3.3. mBM-MSC characterization with anti mouse antibodies. A; CD90 for positive selection. B; CD105 for positive selection.

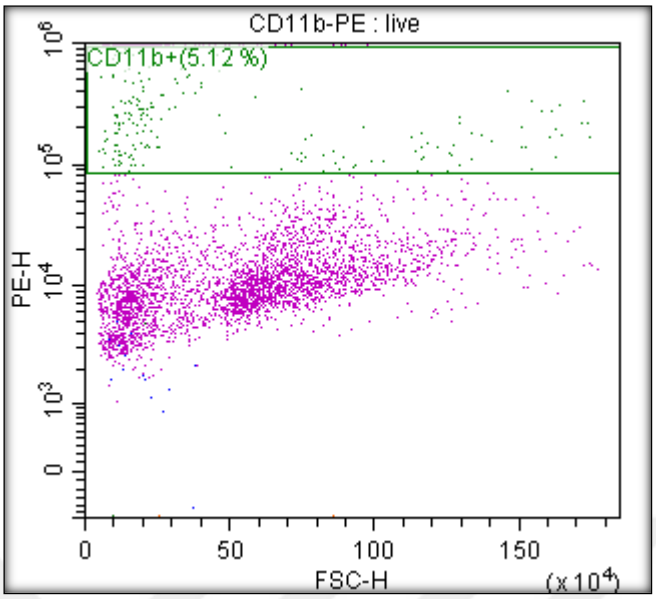


Figure 3.4. mBM-MSC characterization with anti mouse antibody CD11b for negative selection.

3.5. WST-1 CELL VIABILITY ASSAY RESULTS

WST-1 Cell Viability assay was performed at the first stage of the experiments to evaluate and select the effective small molecules (Figures 3.5 and 3.6). First, cells were treated for five days with a single dose (1 µM, middle dosage of the following experiments) of small molecules to see the overall effects of the molecules on mBM-MSC (Figure 3.5) . Second set of experiments was done with 0,1 µM, 1 µM and 10 µM doses of small molecules numbered 12, 14, 22, 24, 28, 31, 33 (Table 3.1. and Figure 3.6).

Treatment named "Mix" includes small molecules numbered #3, #5, #23, #28 because of its positive effects on mouse bone marrow derive hematopoietic stem cell expansion. This cocktail of small molecules did not show any synergic effect.

Significant results were seen for #6 at 1 µM, #14 at 1 µM, #24 and #28 at 10 µM concentration. Small molecules numbered #6 (GSK-3 inhibitor), #14 (StemReginin I), #24 P38/MAPK Inhibitor), #28 (CCE Inhibitor) affected mesenchymal stem cells towards inducing expansion. #14 was first seen to inhibit mBM-MSC proliferation, but on following experiments the error was noticed. No conflicting data was obtained on

following experiments, the expanding effects of #14 (StemReginin I) was clearly seen via dose and time dependent WST-1 cell viabiliy assays.

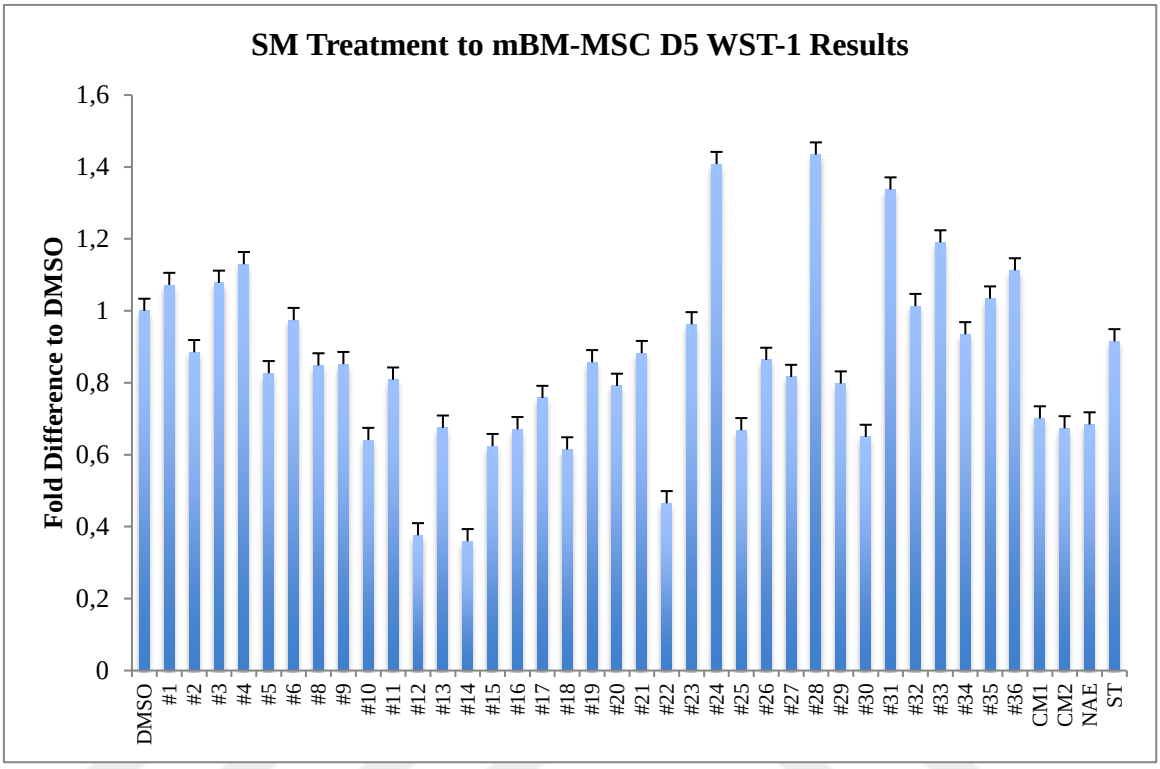


Figure 3.5. WST-1 cell viability assay results following 5 days of treatment with all of the listed small molecules at 1 μM final concentration (Table 3.1.).

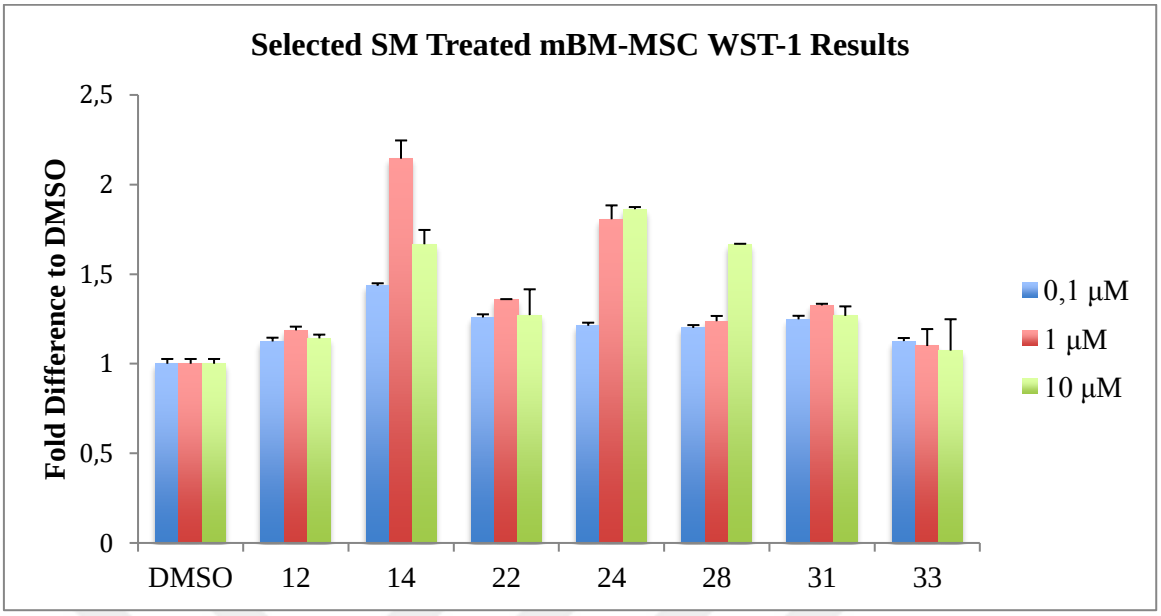


Figure 3.6. WST-1 cell viability assay results following 5 days of dose dependent (0,1 µM, 1 µM, 10 µM) treatment with selected small molecules.

After selection of four small molecules, time and dose (0,1 µM, 1 µM, 10 µM) dependent treatment experiment was conducted. WST-1 assay results were obtained on days 1, 3, 5 and 7 (Figures 3.7, 3.8, 3.9 and 3.10). Optimum expanding effects were seen after five days of treatment. After day 5, viability of the population started to drop (Figure 3.10.). Following experiments are decided to be performed after five days of treatment throughout this study.

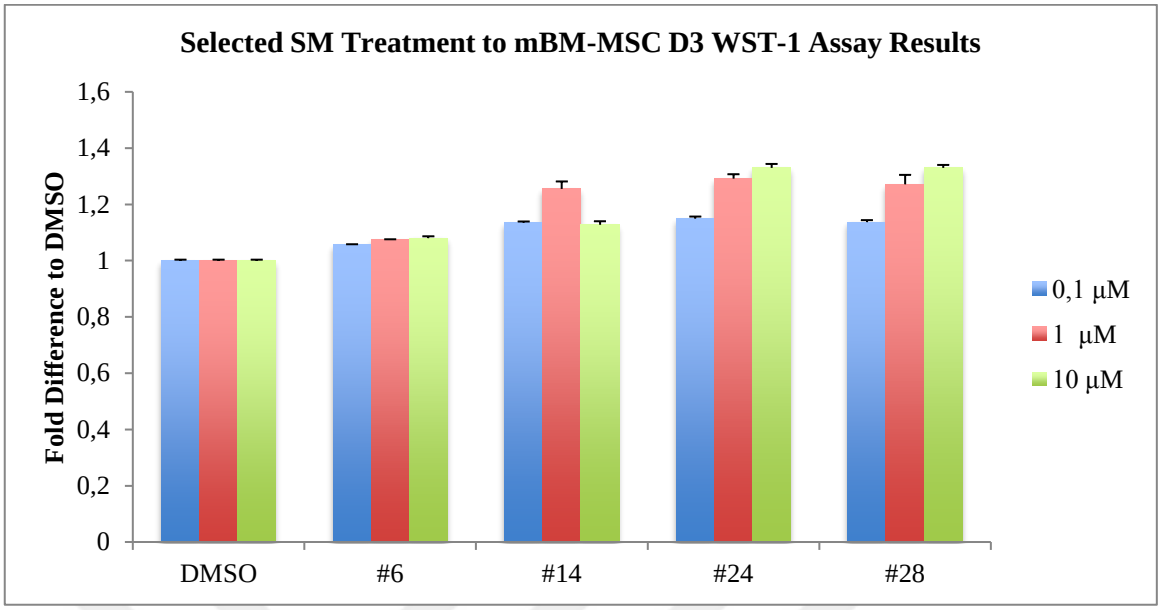


Figure 3.7. WST-1 cell viability assay results following 3 days of dose dependent (0,1 µM, 1 µM, 10 µM) treatment with selected small molecules.

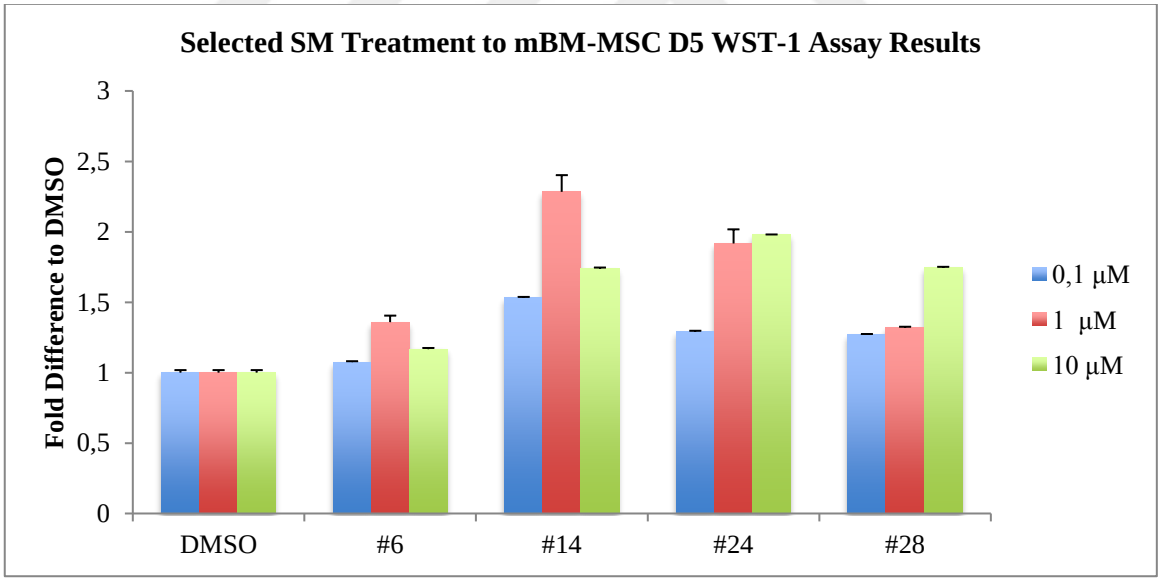


Figure 3.8. WST-1 cell viability assay results following 5 days of dose dependent (0,1 µM, 1 µM, 10 µM) treatment with selected small molecules.

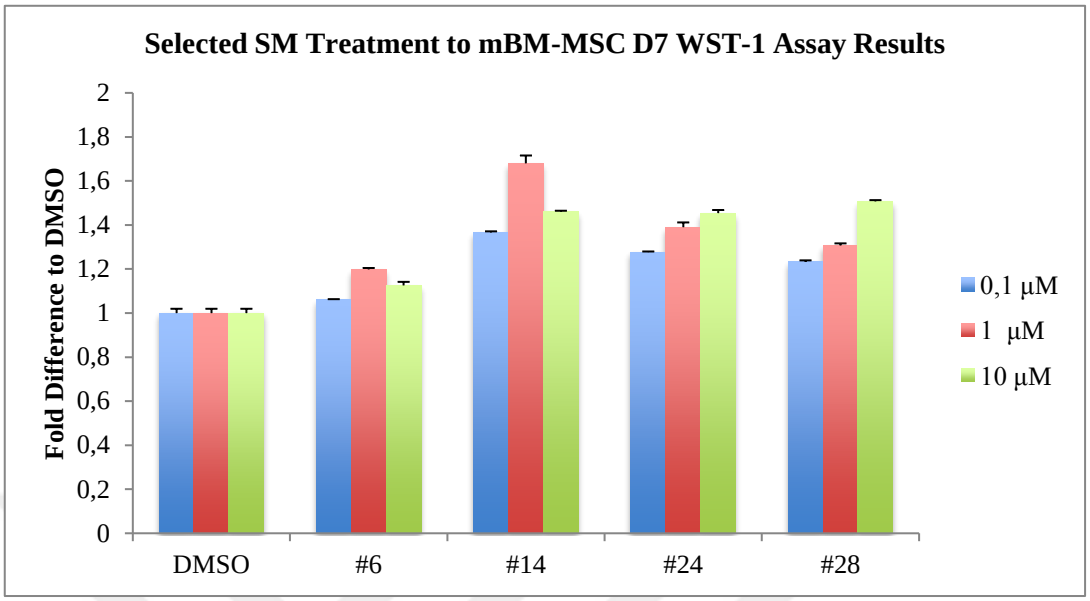


Figure 3.9. WST-1 cell viability assay results following 7 days of dose dependent (0,1 µM, 1 µM, 10 µM) treatment with selected small molecules.

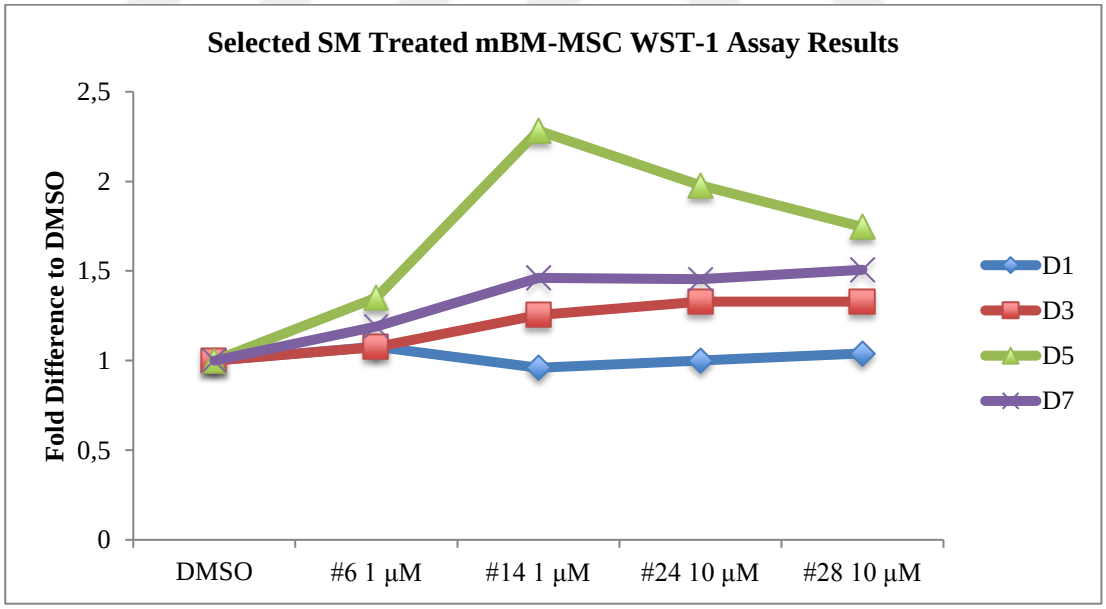


Figure 3.10. WST-1 cell viability assay results of time (D1-3-5-7) and dose dependent (0,1 µM, 1 µM, 10 µM) treatment with selected small molecules.

To also see the slightly long-term effects of small molecules labeled #57, CA5, DB8 and Mix (#3-5-23-28) on mBM-MSC a WST-1 cell viability assay was conducted after 7-days of treatment. No positive change was seen between 5-days of treatment (Figure 3.11.).

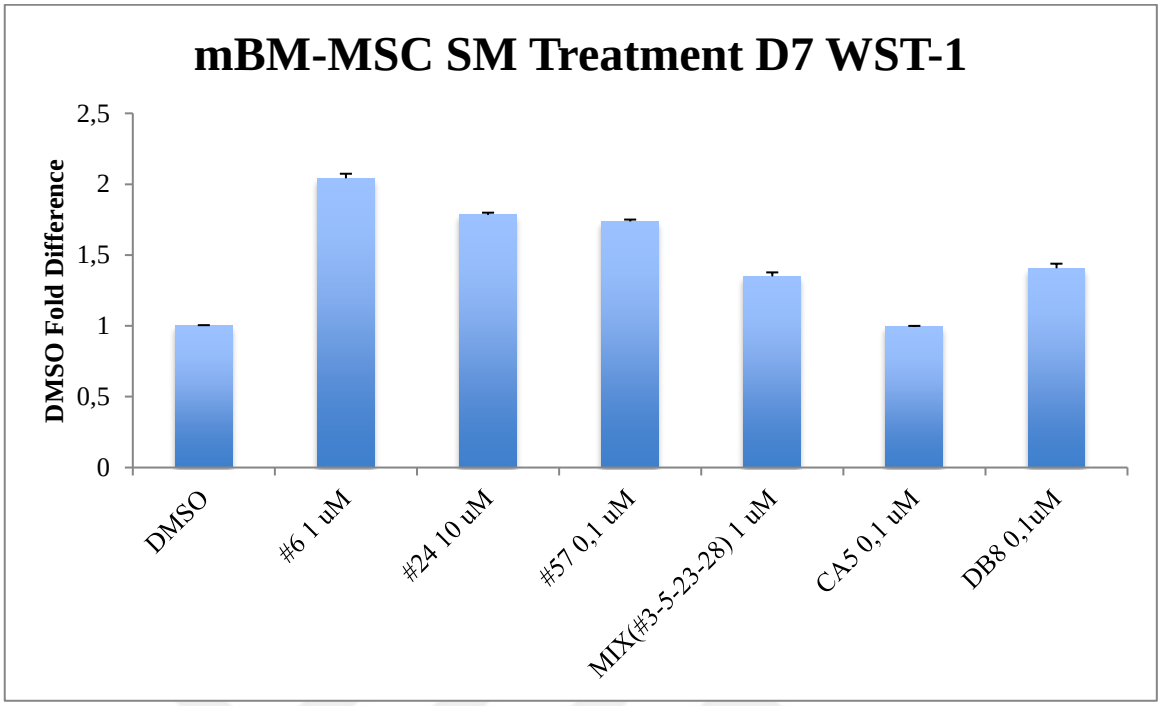


Figure 3.11. WST-1 cell viability assay results for mBM-MSC treated for 7 days with selected small molecules on selected doses.

3.6. HOECHST STAINING CELL COUNTING

To see the time-dependent effects of small molecules nuclei were counted after hoechst staining on days 1, 3, 5 and 7. It was seen that five days of treatment yielded the best results for small molecules #6, #24, Mix (#3-4-23-28), CA5 and DB8 (Figure 3.12.). Reperesentative images of mBM-MSC stained with Hoechst stain at 20X magnification can be seen in Figure 3.13.

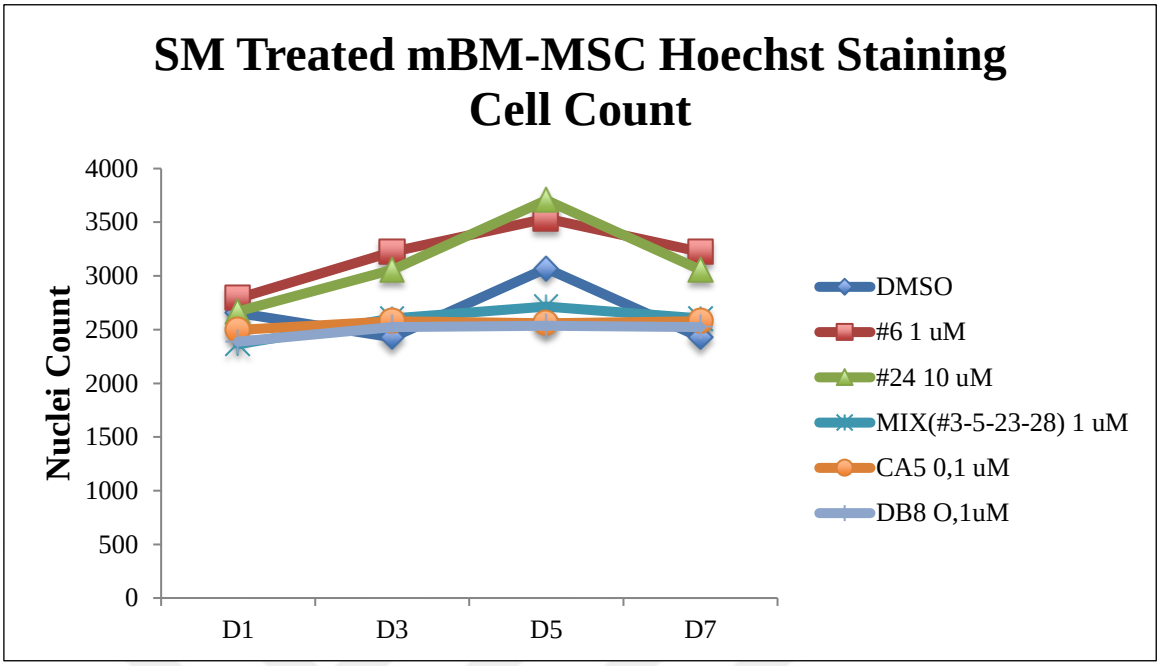


Figure 3.12. Cell count data after Hoechst staining. The data was acquired on days 1, 3, 5 and 7. Cell counting was done by Cytell Imaging Systems.

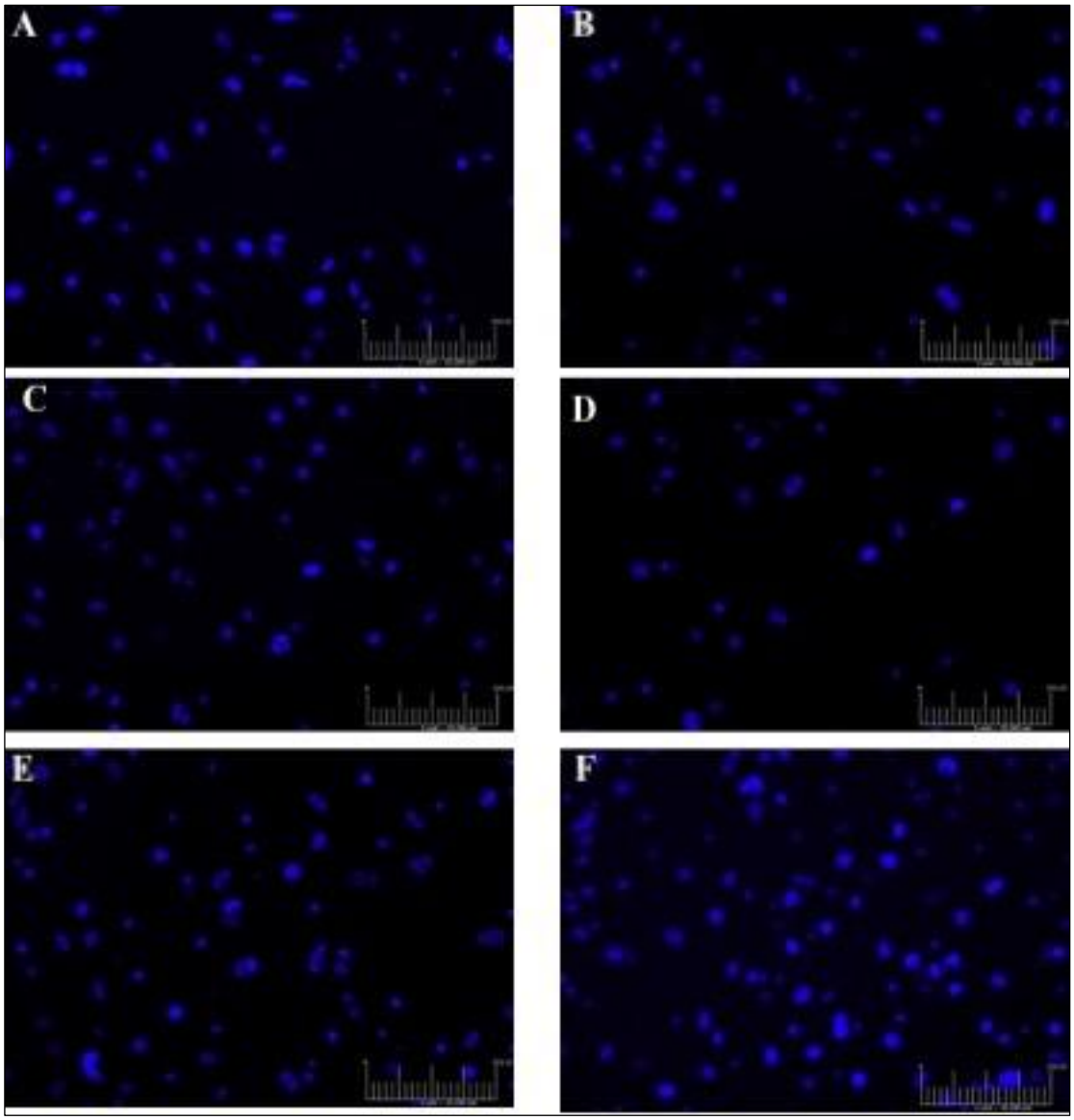


Figure 3.13. Representative images of m-BM-MSCs stained with Hoechst stain at 20X magnification. The image was taken with Cytell Imaging Systems (GE). A, B, C, D, E and F represent cultures after 5 days of treatment with DMSO and SM. A DMSO, B #6 at 1 μM conc., C #24 at 10 μM conc. , D Mix (3-5-23-28) at 1 μM conc., E CA5 at 0,1 μM conc. and F DG8 at 0,1 μM conc.

3.7. CELL CYCLE ANALYSIS

After 5 days of staining cell cycyle analysis was done with Hoechst and Pyronin-Y staining. The results were obtained by flow cytometry. Hoechst is labeled as DAPI-H; and Pyronin-Y is labeled as PE in flow cytometry dot-blots (Figures 3.14, 3.15, 3.16, 3.17, 3.18).

Significant changes were seen on numbers #24 and #28. Treatment with #28 (Capacitative Ca^{2+} Entry Inhibitor) caused G1 arrest while #24 (P38/MAPK Inhibitor) tripled the S/G2/M phase population rates indicating that MSCs were indeed expanding rapidly. #6 (GSK-3 Inhibitor) and #14 (StemReginin I) showed no significant alterations on cell cycle phases (Figure 3.19.).

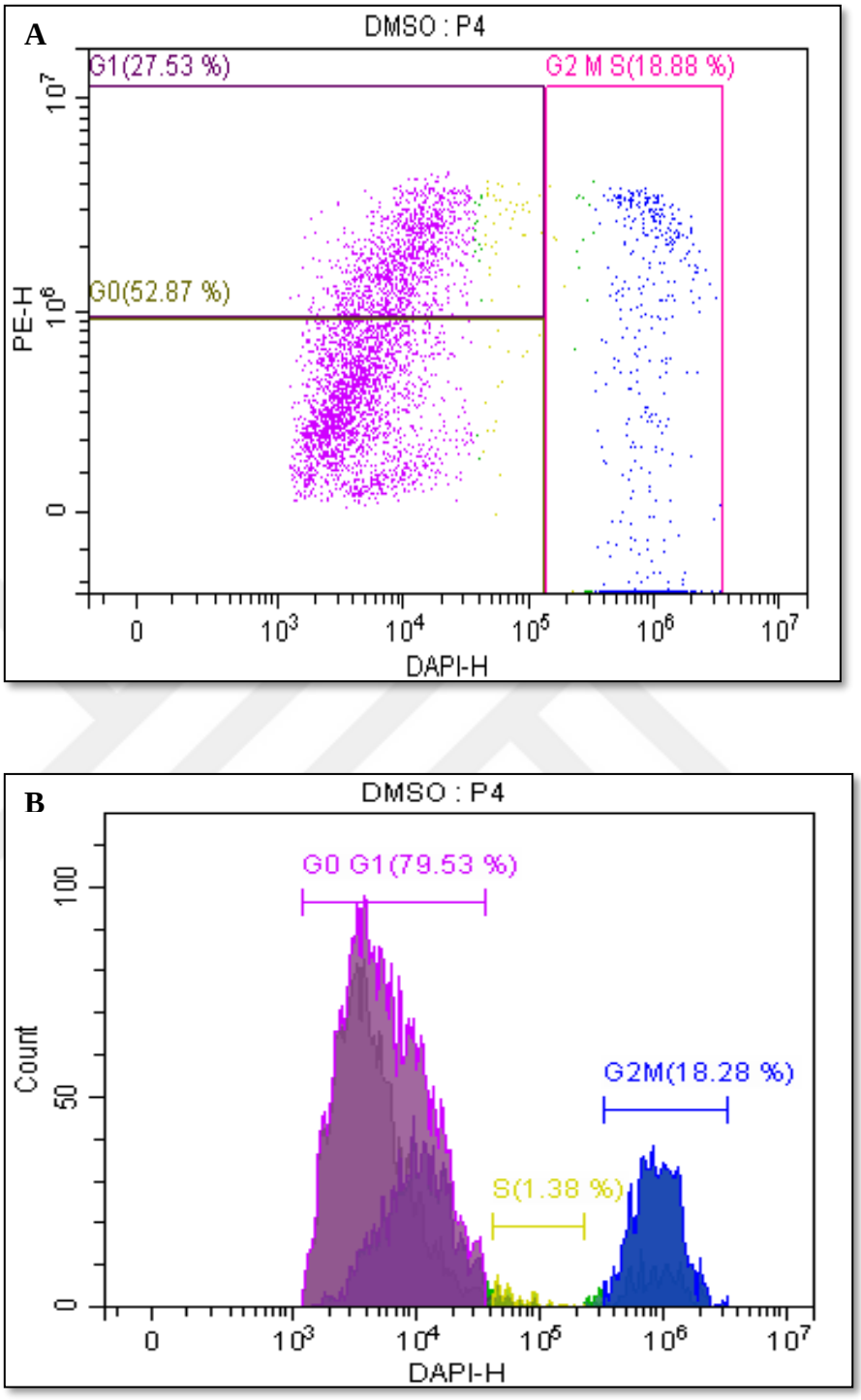


Figure 3.14. Cell Cycle Analysis on mBM-MSCs treated with DMSO as control. Hoechst and Pyronin Y were used for DNA and RNA content staining. A; G0, G1 and G2-M-S phases, B; shows the distinguish between S and G2-M phases.

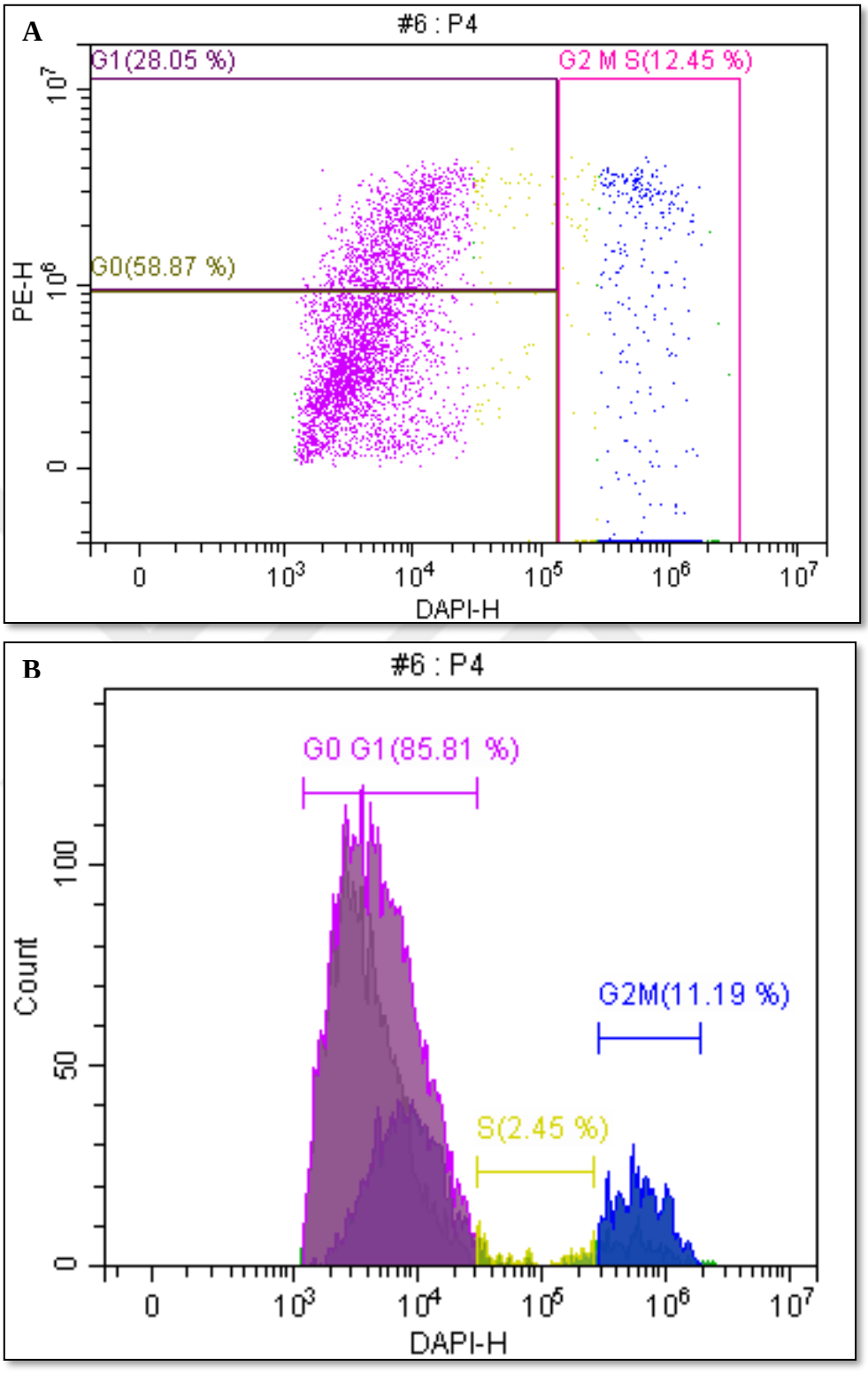


Figure 3.15. Cell Cycle Analysis on mBM-MSCs treated with #6. Hoechst and Pyronin Y were used for DNA and RNA content staining. A; G0, G1 and G2-M-S phases, B; shows the distinguish between S and G2-M phases.

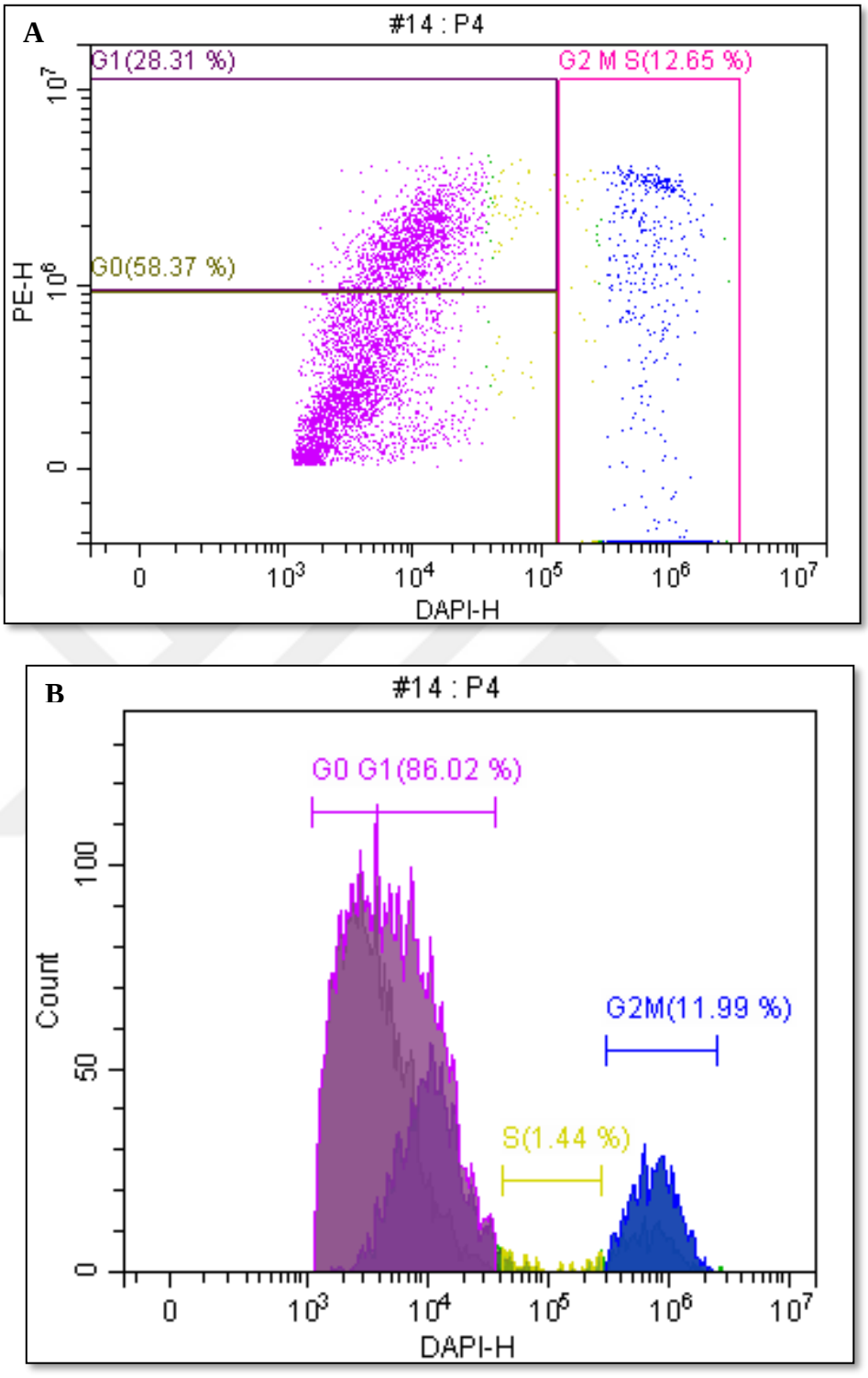


Figure 3.16. Cell Cycle Analysis on MSCs treated with #14. Hoechst and Pyronin Y were used for DNA and RNA contents' staining. A; G0, G1 and G2-M-S phases, B; shows the distinguish between S and G2-M phases.

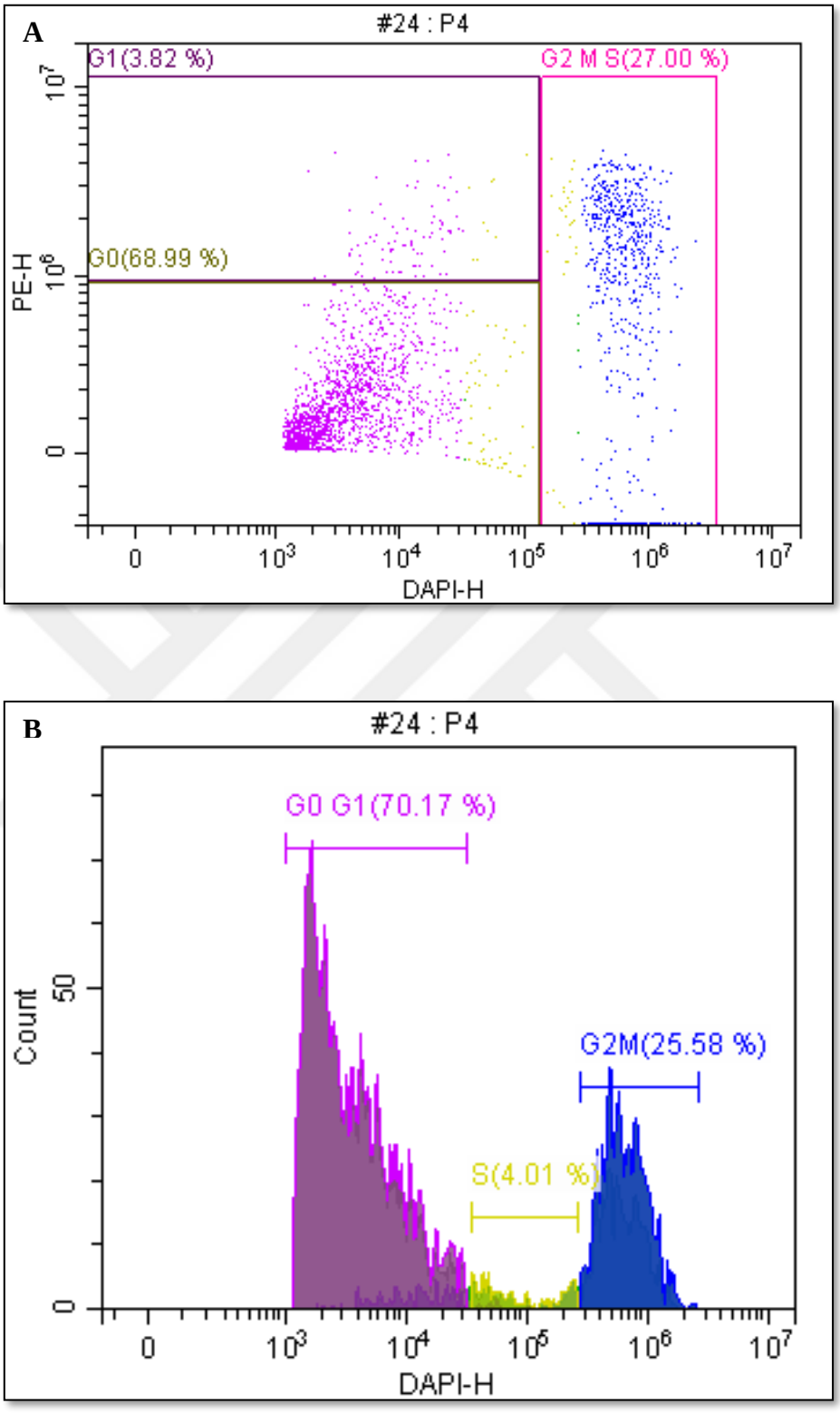


Figure 3.17. Cell Cycle Analysis on mBM-MSCs treated with #24. Hoechst and Pyronin Y were used for DNA and RNA content staining. A; G0, G1 and G2-M-S phases, B; shows the distinguish between S and G2-M phases.

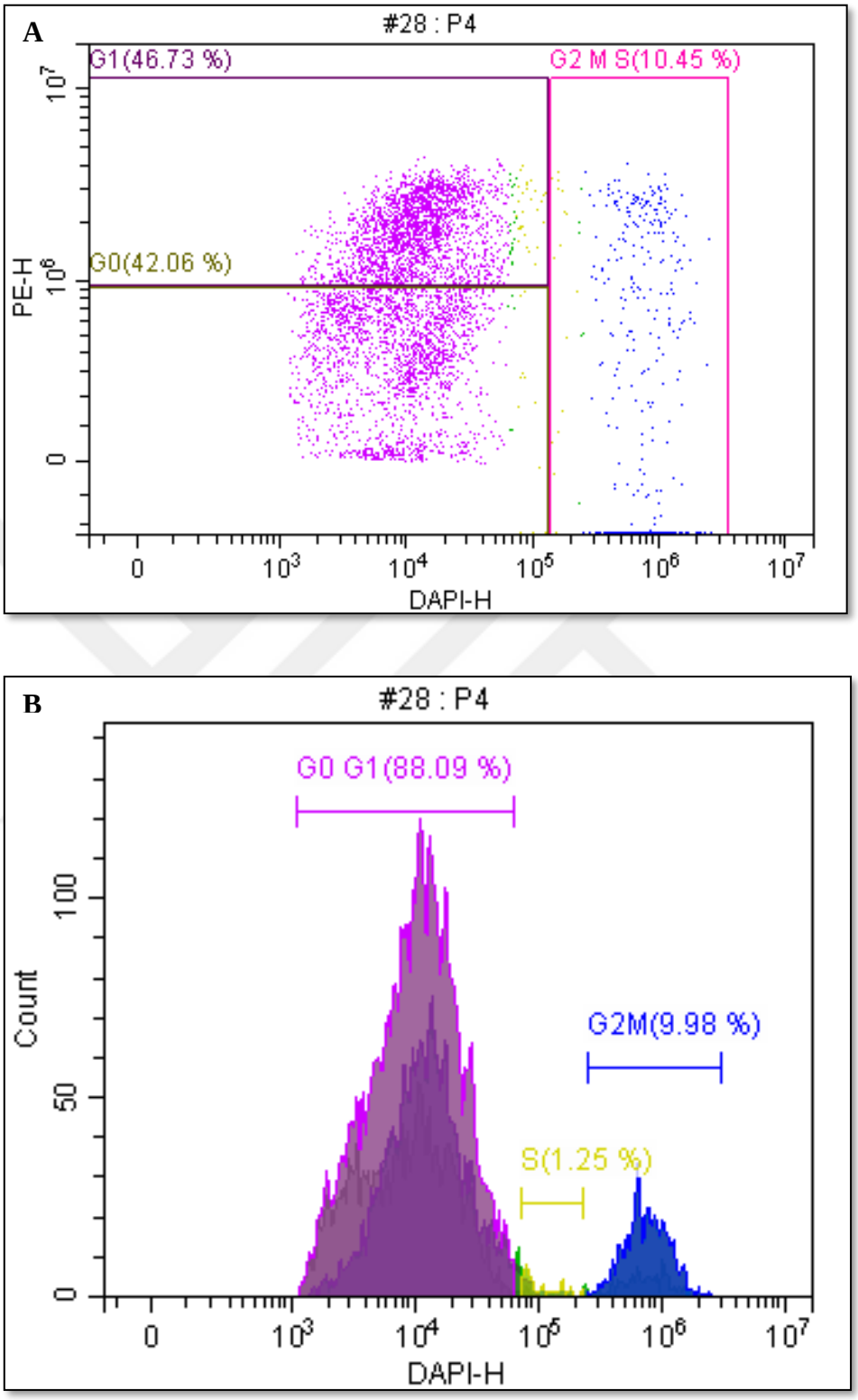


Figure 3.18. Cell Cycle Analysis on mBM-MSCs treated with #28. Hoechst and Pyronin Y were used for DNA and RNA content staining. A; G0, G1 and G2-M-S phases, B; shows the distinguish between S and G2-M phases.

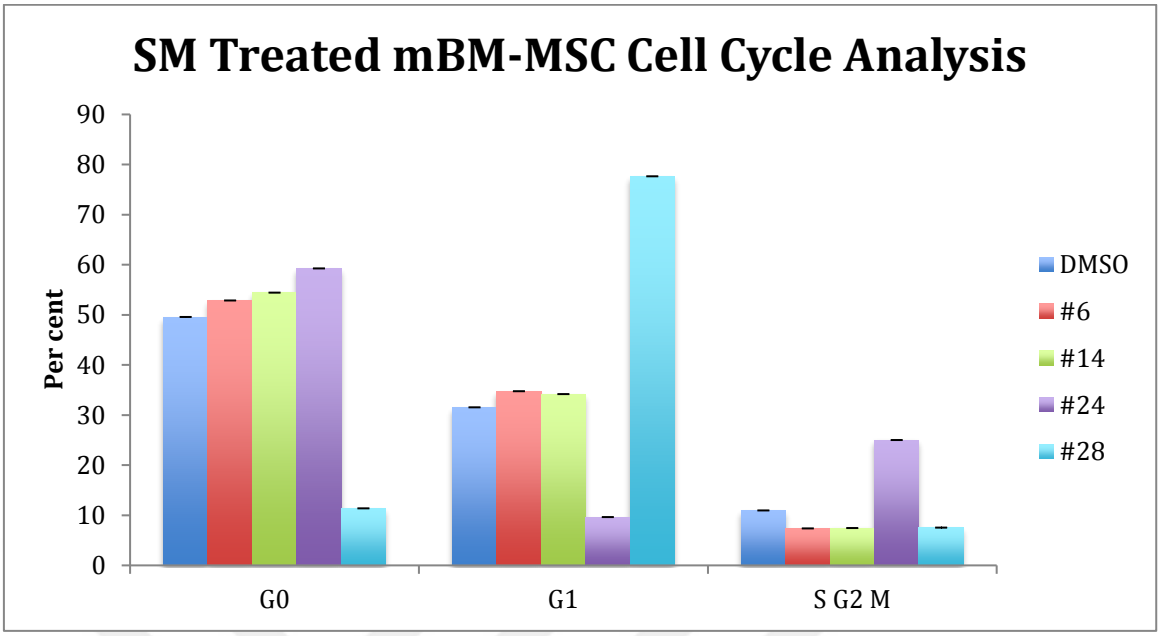
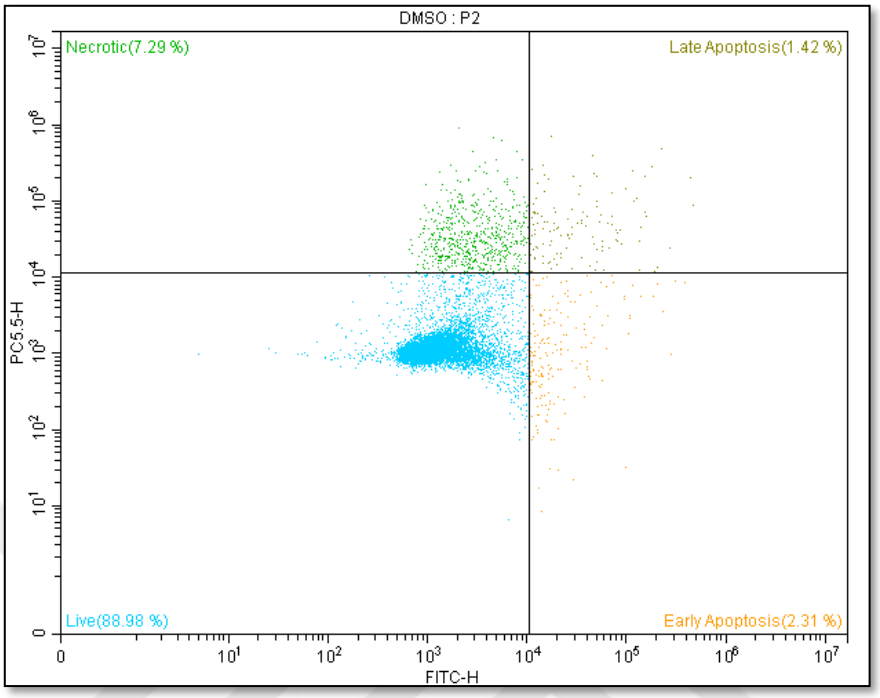


Figure 3.19. Cell Cycle Analysis on mBM-MSCs treated with selected small molecules. Hoechst and Pyronin Y were used for DNA and RNA contents' staining.

3.8. APOPTOSIS ANALYSIS

Rate of apoptosis was determined by Annexin V-FITC and PI apoptosis assay following flow cytometry. Early apoptosis, late apoptosis and necrotic cell rate was coherent with the control for #6 (GSK-3 Inhibitor), #14 (StemReginin I) and #24 (P38/MAPK Inhibitor); while #28 (CCE Inhibitor) treated cells showed decreased dead/necrotic cell percentage and slightly higher early apoptotic cell rate (Figures 3.20, 3.21, 3.22, 3.23.)

A



B

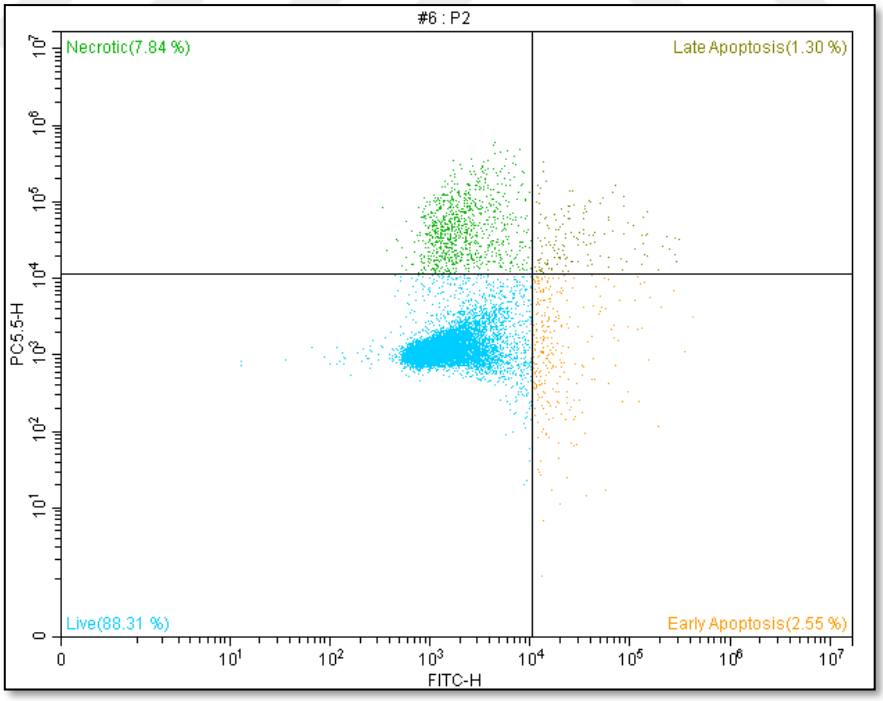
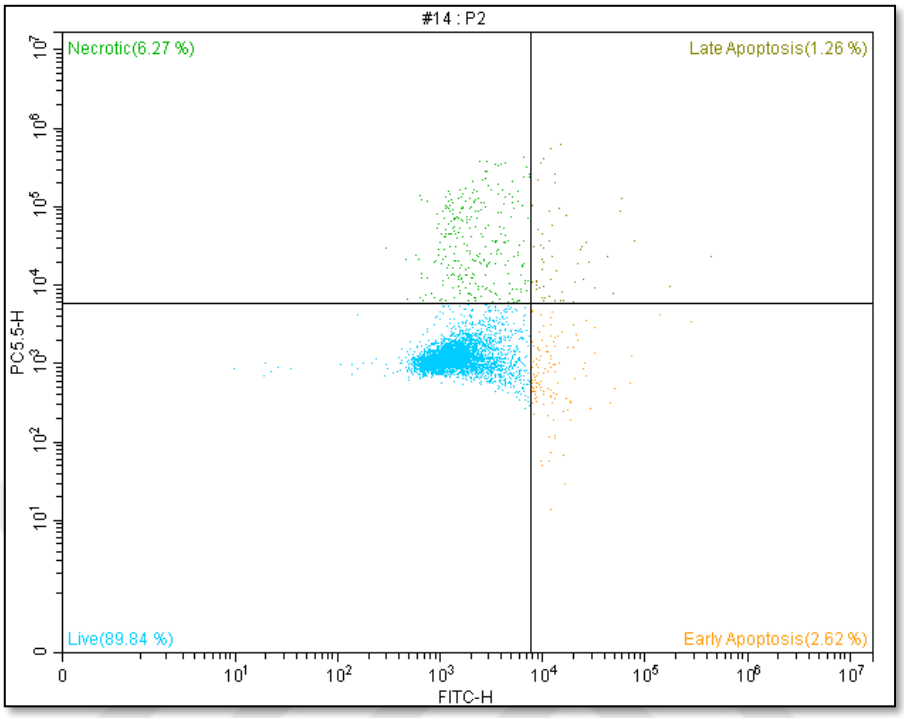


Figure 3.20. Annexin V-FITC and PI stained apoptosis analysis results on mBM-MSCs treated with A; DMSO and B; 1 µM final dose of #6.

A



B

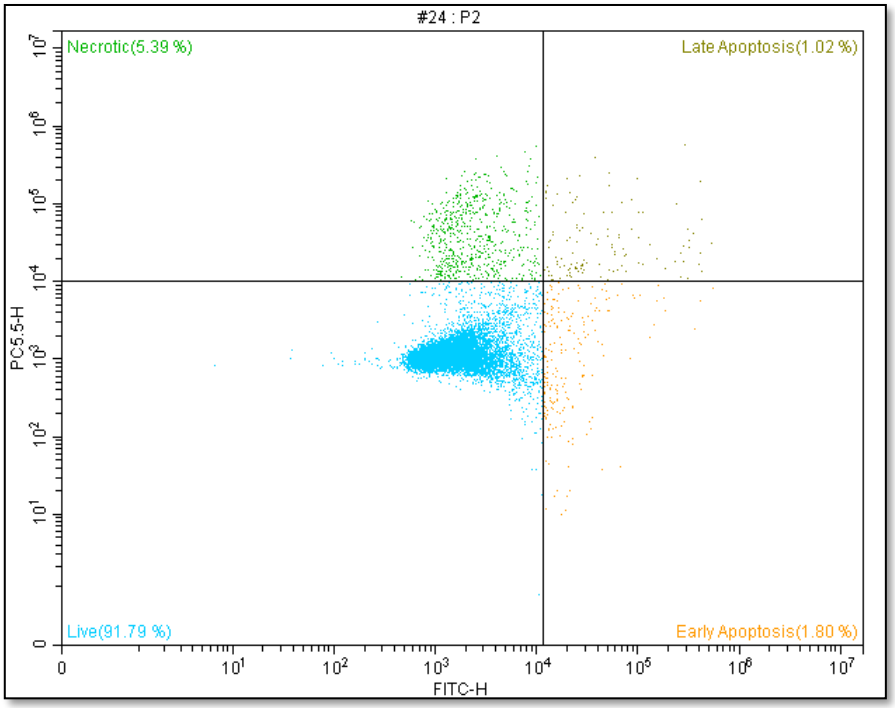


Figure 3.21. Annexin V-FITC and PI stained apoptosis analysis results on mBM-MSCs treated with A; 1 µM final dose of #14 and B; 10 µM final dose of #24.

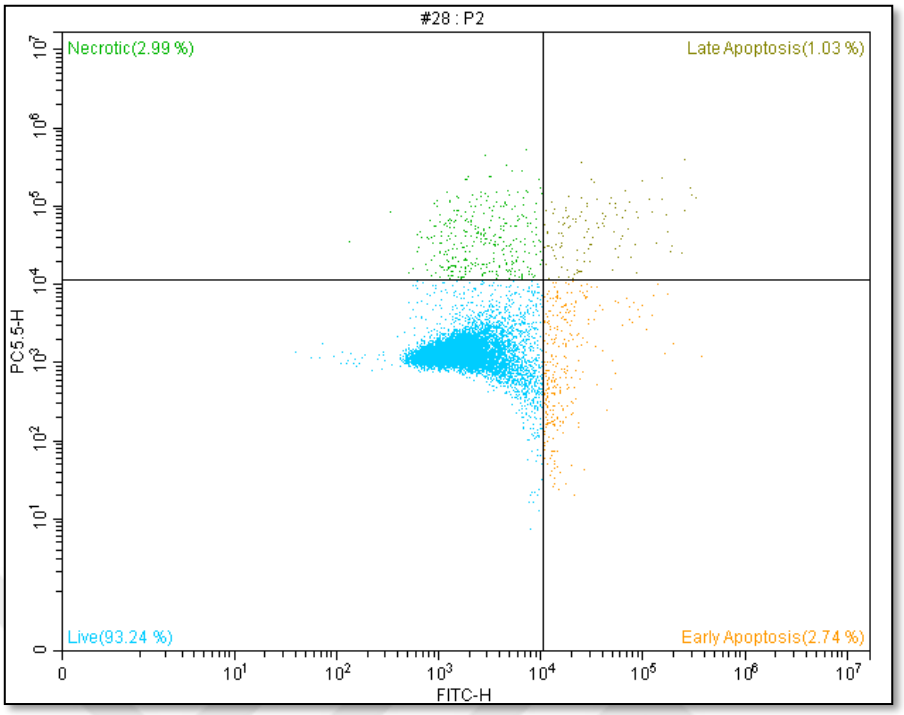


Figure 3.22. Annexin V-FITC and PI stained apoptosis analysis results on mBM-MSCs treated with DMSO and 10 µM final dose of #28.

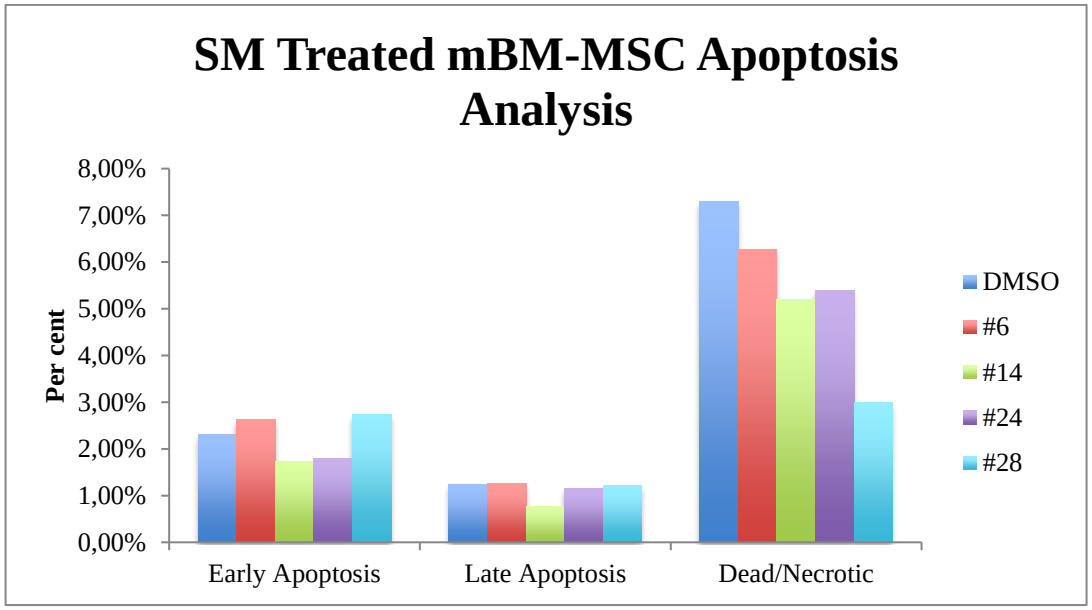


Figure 3.23. Collective results of Annexin V-FITC and PI stained mBM-MSCs treated with DMSO and selected molecules. Early apoptosis, Late apoptosis and dead (necrotic) cell population percentages.

3.9. REAL TIME PCR RESULTS

RNA isolation was done following 5 days of selected small molecule treatment to mBM-MSCs. Thereafter cDNA was synthesized, Real Time-PCR was conducted to estimate CDKI, HDR and S-Phase related gene expression alterations (Table 3.2.) (Figures 3.24 and 3.25).

For CDKI related gene expression results; P21 was significantly down regulated by #24 and #28. #6 up regulated P16 and also down regulated P57 genes' expressions. P15 and P18 was up regulated by #14 and #28 (affectted the ladder).

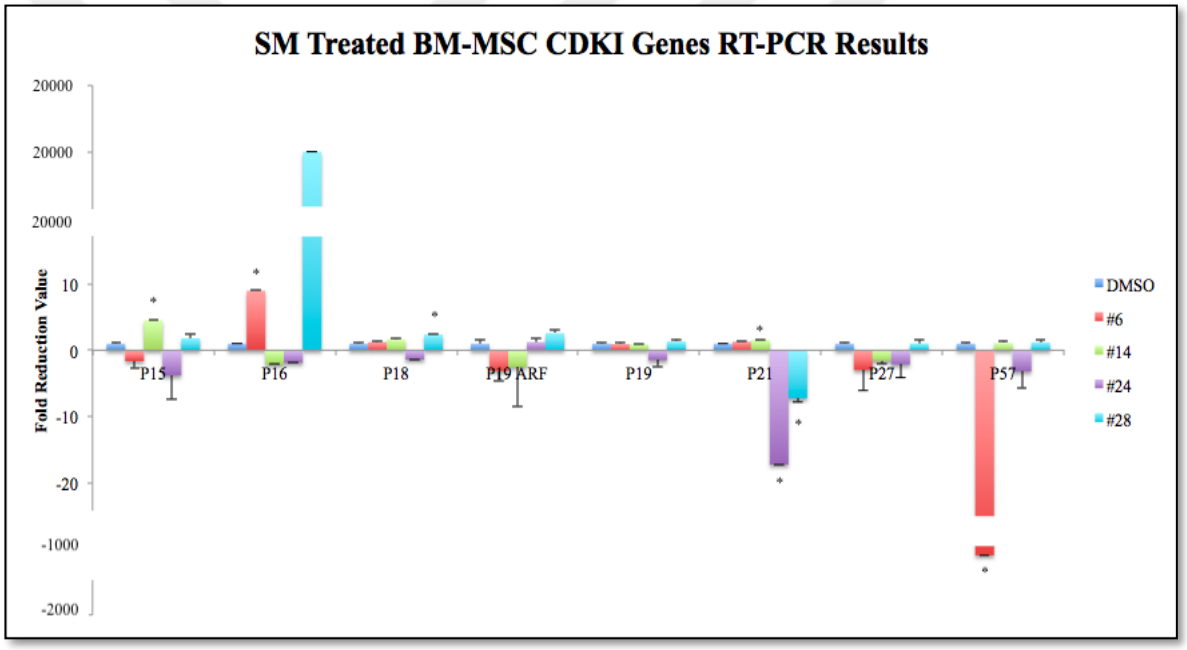


Figure 3.24. RT-PCR results of mBM-MSCs treated with selected doses of small molecules.

MCM2 gene expression was significantly upregulated by #6. #28 RAD51 and PCNA gene expressions. #14 and #24 showed no significant efffect on any HDR/S-Phase gene expressions.

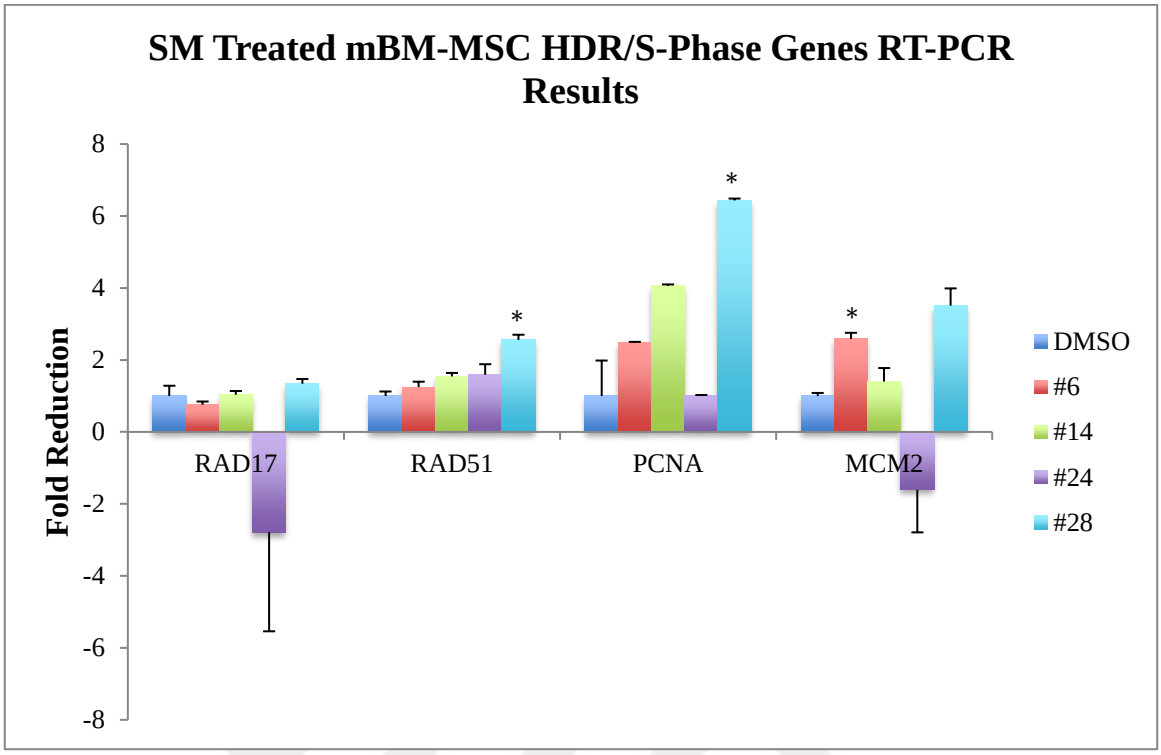


Figure 3.25. RT-PCR results of selected doses of #6, #14, #24 and #28 small molecules treated mBM-MSCs

4. DISCUSSION

Stem cell sources have become one of the hot topics in tissue engineering and regenerative biology. The progression on stem cell technologies have let stem cell therapy to grow over the past decade [67]. The ease of access, low immunogenic effects, reliable ways of *in vitro* and *in vivo* transplantaiton abilities are set to be the desired requirements for application. With more research being done, it was concurred that mesenchymal stem cells were one of the most suitable stem cell sources. MSCs immunomodulatory traits present a great potential for treating various diseases including immune disorders and it enables them to act as a co-transplatation population to reduce the immune response; their homing capability is found to be advantageous, hence their interaction with the host-tissues [59]. The differentiation ability of mesenchymal stem cells to all three embryonic lineages also makes them a great canditate for cell based therapy [25,56,57,79,127]. On the other hand, there is a significant decline in the expansion ability of mesenchymal stem cell cultures through time. MSCs show charasteristic losses on their potency during sub-culturing and also at high passage numbers. MSCs' becoming senecent at long-termed cultures is seen after passage 5 [93]. Although they are dividing cells, their expansion capability does not match with the requirement of clinical trials. Meaning, until the aimed quantitiy of cells obtained for therapeutic options, they might lose their potency. To conclude, it has been seen clearly that the need for MSC culture enhancement and expansion tecniques on the field of use of MSCs increased rapidly due to the clinical efficacy; which demands large population of MSC for broad implementation of MSC terapies.

Developmental studies for MSC expansion started with a simple adjustment on the oxygen levels of cell culture conditions [128]. The standard O_2 levels in bone marrow cavitiy is known to be around 5%. Yet, O_2 gradient has been seen throughout the bone marrow. Hematopoietic stem cell niche is extremely hypoxic and it has been shown that culturing HSC at low O_2 %, helps to protect the the potency of the cells [129]. Based on this information, MSCs also have been cultured on hypoxic conditions and the results indicated that, hypoxia does not only promote MSC expansion, but it also allows the cells to preserve their potency [130].

Platelet lysate was proposed as FBS subrogate for MSC expansion. The hypothesis was that platelet lysate growth factors can support MSC expansion. A substantial number of researches have been completed and they propose that the expansion of MSC is quicker and faster set against to FBS-supplemented media. However, platelet lysate also shows expression alterations on BM-MSC resulting in decrease on immunomodulatory effects [131]. Similarly, it is clearly seen that further studies are necessary for the quality of MSCs expanded in platelet lysate protocols to decrease the potential patient's exposure to the hazards of serum due to possible immunogenic reactions and contaminations [132,133].

In our study, a number of small molecules were analyzed to identify a novel mBM-MSC expanding small molecule. For this purpose, murine bone marrow mesenchymal stem cells were isolated, cultured and treated with small molecules in a dose-dependent manner. Following cell viability assays and hoechst cell counting data, we decided to continue the study with four molecules selected for MSC expansion; and one for inhibition. The selected expanding molecules are; #6- CHIR99021 (GSK-3 Inhibitor), #14-StemReginin I, #24-p38 MAPK Inhibitor and #28 SKF96365.

GSK3 (Glycogensynthase kinase-3) is an evermore active kinase that is connected to cell proliferation and apoptosis pathways [134,135]. It is tied to both promotion and inhibition of apoptosis. GSK3 is an regularly-active enzyme and its inhibition, not activation, effects these pathways including insulin pathway and Wnt signaling (migration, neuronal patterning, cell survival establishing, organogenesis). The GSK-3 Inhibitor (#6) is an amino pyrimidine derivative [136]. While it inhibits GSK-3, it also functions as Wnt signaling activator by stablizing beta catenin. In a study conducted by Yongyan Wu, Zhiying Ayi et. al. in 2013 they have shown that CHIR99021 stimulates self-renewal, and it increases the derivative efficiency of murine embryonic stem cells. It also regulates pluripotency signaling pathways via alterations on epigenetic regulation genes [119].

In our study, our results on expansion capability of CHIR99021 was compatible with the literature. #6 on the optimum dose of 0,1 μM expanded mBM-MSC safely. More spesifically, 5 days was the most convenient timespan for treatment rather than a longer time period because at day 7, cell count was observed to drop. Cell cycle and apoptosis analysis demonstrates that #6 treated mBM-MSCs did not show any irregular patterns on either pathways. Alterations on gene expressions of CDKI genes were seen. P16 (tumor suppressor) expression was upregulated while P57 (Cell proliferation negative regulation)

was downregulated. In the hope of application of #6 on gene editing technologies on further studies (especially CRISPR/Cas9) the effects on HDR and S-phase gene expressions were also examined via RT-PCR. Promising results were achieved; with the result of upregulation of PCNA, MCM2. However, downregulation of XRCC2 and ERCC1 was also seen.

AhR (aryl hydrocarbon receptor) regulates the toxiferous effects of many molecules including TCDD, polycyclis aromatic hydrocarbons. It is a member of ligand activated transcription factors partinent to CNS development, physiology, hypoxia-response, day and night adaptations [121]. StemRegenin 1 (SR1) is an AhR Antagonist. SR1 exibits CD34+ cell (hematopoietic stem cells) expansion capabilities in numerous researches. A 50 fold expansion in vitro; and a 17 fold increase in cells which preserved their engraftment properties was seen [120,137].

In our study, the results on expansion capability of StemReginin 1 was compatible with the literature. #14 on the optimum dose of 1 μM expanded mBM-MSC safely. Cell cycle and apoptosis analysis demonstrates that #14 treated mBM-MSCs did not show any irregular patterns on either pathways. Alterations on gene expressions of CDKI genes were seen via RT-PCR. P15 (G1 check point) and P21 (tumor suppressor) expression was upregulated. In the hope of application of #14 on gene editing technologies on further studies (especially CRISPR/Cas9) the effects on HDR and S-phase gene expressions were also examined. But there was no significant changes on gene expressions. Even still, these results indicates that #14 expand MSCs without altering their crutial cell mechanisms.

P38, a mitogen activated protein kinase (MAPK), takes part on response to stress factors. For instance; UV, inflammatory cytokine presence, heat and osmotic-shocks [123,138]. To be exact, they are connected to differentiation, autophagy and apoptosis. SB203580 is a P18 inhibitor and its primary effect is to block the catalysis activity of P38-MAPK [124,139]. Similarly, it also inactivates the enzyme by binding to it in an inactive state. More spesifically, by binding to its substrate MAPKAP K2. Research studies are focused on the effects of using SB203580 on; cardiac-stress relations, auto immune diseases and inflammatory pathways. It has been shown that SB203580 exibits activity against fibrosis and COPD pathways which evokes a therapeutic response in chronic airway disease [140]. In addition, SB203580 show inhibition potential on cardiac stress activated protein kinases (SAPKS) and C Jun N Terminal Kinases (JNKS). These effects could be the results of

elimination of the ageing-inducing persistantly active p38/MAPK pathway; which impairs muscle regeneration [122].

In our study, our results on expansion capability of SB203580 was one of the highest among the tested small molecules. #24 on the optimum dose of 10 μM expanded mBM-MSC safely. More spesifically, 5 days was the most convenient timespan for treatment rather than a longer time period because at day 7, cell count was observed to drop. Cell cycle analysis results showed a significance increase on G2/M phases. Apoptosis analysis demonstrates that #24 treated mBM-MSCs did not show any irregular patterns on apoptosis analysis. CDKI gene expressions were compatible with control (DMSO-treated culture) on mRNA level. There was a decrease in only P21 (tumor suppressor) gene expression. In the hope of application of #24 (a P38 inhibitor) on gene editing technologies on further studies (especially CRISPR/Cas9) the effects on HDR and S-phase gene expressions were also examined. Significant data was acquired only on ERCC1 (endonuclease subunit), gene expression was downregulated. Meaning #24 has no effect on homology directed repairement, therefore is not a suggested small molecule for CRISPR/Cas9 applications.

SKF96365 a CCE (Capacitative Ca^{2+} Entry) inhibitor blocks Ca^{2+} entry by a receptor mediated manner [125]. In diagnostics, SKF is used as a TRPC (transient-receptor potential canonical type) channel inhibitor. SKF is an appealing new anti cancer drug candidate. It shows a anti neoplastic activity by inducing cell cycle arrest and apoptosis [126].

In our study, our results on expansion capability of SKF96365 was one of the highest among the tested small molecules. #28 on the optimum dose of 10 μM expanded mBM-MSC but also araised many concernes. Cell cycle analysis results showed a G1 phase arrest on the cell population, which concurred with the literature. Apoptosis analysis demonstrates that #28 treated mBM-MSCs decreased necrotic cell death. CDKI gene expressions were compatible with control (DMSO-treated culture) on mRNA level. In the hope of application of #28 on gene editing technologies on further studies (especially CRISPR/Cas9) the effects on HDR and S-phase gene expressions were also examined. Significant increase was seen on HDR genes RAD51 (homology directed repair) and PCNA (proliferating double stranded break repair endonuclease). Even with the G1 arrest

results, #28 demonstrated the best results on mBM-MSC expansion and also on HDR gene expressions' upregulation.

5. CONCLUSION

Overall, this study assesed the premise of finding a novel small molecule for mBM-MSC expansion. Further studies should be conducted to achieve a final result. Characterization assays should be conducted after the treatment of SM also too see the effects of the treatment on MSC's self-traits. Validating experiments should be done on immunomodulation aspects of mBM-MSC after treatment of small molecules. Making a cocktail of the complementing molecules #6, #14 and #24 might result in higher expansion potential. Subsequent to these results, if a safely expanding small molecule can be found, *in vivo* trials on mice should be started. In addition to these, with the effects of #6 and #28's significant increase on HDR and S-Phase gene expressions, this study can be carried to a new topic of optimizing gene editing on mouse bone marrow derived mesenchymal stem cells.

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APPENDIX A: ETHICAL COMMITTEE APPROVAL

Ethical committee approval for project titled " Development of Hematopoietic Stem Cell Expansion Technologies" is dated 22.02.2018 and the decision number is 651.

YEDİTEPE ÜNİVERSİTESİ

T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU (YÜDHEK)

ETİK KURUL KARARI

Toplantı Tarihi	Karar No	İlgi	Proje Yürütücüsü
22.02.2018	651	19.02.2018	Yrd.Doç.Dr. Fatih KOCABAŞ

'Hematopoietik Kök Hücre Çoğaltma Teknolojilerinin Geliştirilmesi (Development of Hematopoietic Stem Cell Expansion Technologies)' adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

Etik Onay Geçerlilik Süresi: 3 Yıl	**Hayvan Türü ve cinsiyeti:** Fare ♀	**Hayvan Sayısı:** 30

GÖREVİ	ADI SOYADI	
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Yardımcısı	Prof. Dr. Erdem YEŞİLADA	
Raportör	Vet. Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Doç. Dr. Soner DOĞAN	
Üye	Doç. Dr. Ediz DENİZ	**KATILMADI**
Üye	Prof. Dr. Gamze TORUN KÖSE	**KATILMADI**
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	