

SMALL MOLECULE-MEDIATED HUMAN HSC EXPANSION AND
DEVELOPMENT OF HSC EXPANSION MEDIUM



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Esra Albayrak

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DEVELOPMENT OF HSC EXPANSION MEDIUM

APPROVED BY:

Assoc. Prof. Dr. Fatih Kocabaş
(Thesis Supervisor)
(Yeditepe University)

Prof. Dr. Fikrettin Şahin
(Yeditepe University)

Prof. Dr. Gamze Torun Köse
(Yeditepe University)

Prof. Dr. Tunç Akkoç
(Marmara University)

Assist. Prof. Dr. Zeynep Tokcaer Keskin
(Acıbadem University)

DATE OF APPROVAL:/....../2020



*This thesis is dedicated to my beloved
parents Fatma and Hasan Albayrak...*

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ABSTRACT

SMALL MOLECULE-MEDIATED HUMAN HSC EXPANSION AND DEVELOPMENT OF HSC EXPANSION MEDIUM

Hematopoietic stem cells (HSC), known by their ability of multipotency and self-renewal are mostly utilized in HSC transplantation for treatment of hematological disorders and malignancies. Umbilical cord blood (UCB) and mobilized peripheral blood (mPB) which are alternatives to bone marrow (BM) for HSC transplantation, are limited by lack of efficient HSC number. To overcome this limitation, *ex-vivo* expansion of HSCs is a highly promising therapeutic applications rather than usage of double cord blood unit, which causes delay of hematopoietic recovery. We have previously shown that knockout of HSC quiescence genes could increase HSC pool *in vivo*. Thus, we hypothesized that targeting the HSC quiescence regulators using small molecules may be utilized for *ex vivo* mPB and UCB-HSC expansion. We aimed to identify the most effective hematopoietic small molecules (HSMs)/HSM combinations and develop good manufacturing practises (GMP)-grade human HSC expansion medium including the identified HSMs for successful HSC transplantation. We used 35 HSMs targeting HSC quiescence genes for HSC expansion. We found that BML-260 and TAME molecules increased the mPB and UCB-HSC content and triggered the re-entry to cell cycle. Colony forming-unit (CFU) assay confirmed their enhanced multilineage potential effects. BML-260 was safer rather than TAME on viability of the expanded cells. Gene expression analysis showed that BML-260 and TAME molecules contribute to HSC expansion through modulation of cell cycle kinetics including p27, Skp2 and Cdh1. On the other hand, the preliminary data about identification of the effective chemically-defined HSC expansion medium showed that RPMI 1640 basal medium supplemented with 10 percent FBS, cytokines (SCF, TPO, FLT-3), insulin-transferrin-selenium (ITS) and BML-260 molecule could be promising for development of GMP-grade human HSC expansion medium. These findings reveal that these small molecules and this medium composition could be improved for human HSC expansion.

ÖZET

KÜÇÜK MOLEKÜL-ARACILI İNSAN HKH ÇOĞALTIMI VE HKH ÇOĞALTIMI İÇİN BESİYERİ GELİŞTİRİLMESİ

Multipotent ve kendini yenileme yetenekleri ile bilinen hematopoietik kök hücreler (HKH) bilhassa hematolojik kanser ve hastalıklarının tedavisi için HKH transplantasyonu için kullanılır. HKH transplantasyonu için alternative olan göbek kordon kanı (GGK) ve mobilize periferik kan (mPK), HKH sayısının azlığı ile sınırlıdır. Gecikmiş hematopoietik iyileşmeye sebep olan çift GGK unitesi kullanımına kıyasla HKH'lerin *ex vivo* çoğaltılması yaklaşımı, bu sınırlandırmanın üstesinden gelebilmek için daha umut verici bir terapötik yaklaşımdır. Biz daha önce HKH dormansi genlerinin susturulmasının *in vivo* HKH havuzunu arttırdığını gösterdik. Bu yüzden, HKH dormansi düzenleyicilerinin küçük moleküller kullanılarak hedeflenmesinin, *ex vivo* insan mPK ve GKK kökenli HKH çoğaltılması için kullanılabileceğini hipotez olarak ileri sürdük. En etkili hematopoeitik küçük molekülleri ve kombinasyonunu tanımlamayı ve bu küçük molekül veya kombinasyonu içeren iyi üretim uygulamaları (İÜU)-kalitesinde bir insan HKH çoğaltma besiyeri geliştirmeyi amaçladık. Bu çalışmada, HKH dormansi genlerini hedefleyen 35 hematopoietik küçük molekül kullandık. BML-20 ve TAME moleküllerinin hem mPK'da hemde GKK'nda HKH içeriğini arttırdığını ve hücrelerin tekrar hücre döngüsüne girişini tetiklediğini bulduk. Koloni oluşturu birim analizi de moleküllerin çok-soylu olma potansiyelini arttırıcı etkisini teyit etmiştir. Ayrıca, BML-260 molekülünün TAME molekülüne kıyasla çoğalan hücrelerin canlılığı konusunda daha güvenilir olduğu bulunmuştur. Gen ekspresyon analizleri, BML-260 ve TAME moleküllerinin hücre döngüsü elemanlarının modülasyonunu sağlayarak insan HKH'lerini çoğalttığını göstermiştir. Diğer yandan, etkili olan kimyasal olarak tanımlanan HKH çoğaltma besiyerinin tanımlanması ile ilgili öncü sonuçlar göstermektedir ki; 10 percent fetal bovin serum, sitokinler (SCF, TPO and FLT-3), insülin-transferrin-selenyum (ITS) ve BML-260 molekülü eklenmiş RPMI 1640 besiyeri kimyasal olarak tanımlanmış insan HKh çoğaltma besiyeri için umut vaadedici olabilir. Bu bulgular, bu moleküllerin ve bu besiyeri içeriğinin, insan HKH çoğaltılması için geliştirilebileceğini ortaya koymaktadır.

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LIST OF SYMBOLS/ABBREVIATIONS

2-APB	2-aminoethoxydiphenyl borate
2-PCPA	Tranlycypromine hydrochloride
5-aza-D	5-aza-2'-deoxycytidine
5-HT	5-hydroxytryptamine
7AAD	7-aminoactinomycin
°C	Celcius degree
μM	Micro molar
Min	Minutes
ml	Mililiter
rpm	Revolutions per minutes
v/v	Volume per volume
AD-MSC	Adipose derived mesenchymal stem cell
AGM	Aorta-gonad-mesonephros
AhR	Aryl hydrocarbon receptor
ALDH	Aldehyde dehydrogenase
ANG1	Angiopoietin-1
ANGPTLs	Angiopoietin like proteins
APC	Allophycocyanin
ATP	Adenosine-triPhosphate
BFU-E	Burst forming unit-erythrocyte
BIO	6-bromoindirubin-3'-oxime
BM	Bone marrow
BM-MNC	Bone marrow mononuclear cell
BM-MSC	Bone marrow mesenchymal stem cell
BMP4	Bone morphogenic protein 4
CAFC	Cobblestone area forming cells
CAKs	CDK activating kinases
CAR	CXCL12 abundant reticular
CBP	Cyclic AMP response element-binding protein

CDKIs	CDK inhibitors
CDKs	Cyclin-dependent kinases
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming unit
CFU-E	Colony-forming unit-erythroid
CFU-GEMM	Colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte
CFU-GM	Colony forming unit-granulocyte and macrophage
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
C-MPL	Myeloproliferative leukemia protein
CO ₂	Carbondioxide
CRISPR	Clustered regularly interspaced short palindromic repeats
Cu	Copper
DAPI	4',6-diamidino-2-phenylindole
DCFDA	2',7' –dichlorofluorescein diacetate
THBP	Tert-butyl hydroperoxide
DEAB	Diethylaminobenzaldehyde
Dhh	Desert hedgehog
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DNMT	DNA metyltransferase
DPBS	Dulbecco's phosphate buffer saline
ECs	Embyronic cells
EPO	Erythropoietin
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FL	Fetal liver
FLT-3L	Fsm like trosine kinase 3 ligand
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte/macrophage colony stimulating factor
GMP SCGM	Good manufacturing practise-stem cell growth medium
GMP	Good manufacturing practise

GMPs	Granulocyte & macrophage progenitors
GSK3 β	Glycogen synthase kinase 3 beta
GVHD	Graft versus host disease
HDAC	Histone deacetyltransferase
HDR	Homology directed repair
Hh	Hedgehog
HIFs	Hypoxia inducible factor
Hif-1 α	Hypoxia inducible factor 1 alpha subunit
Hif-1 β	Hypoxia inducible factor 1 beta subunit
HLA	Human leukocyte antigen
HOX	Homeobox
Hoxa9	Homeobox a9
HREs	Hypoxia-responsive elements
HSCs	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
HSM	Hematopoietic small molecule
HSPCs	Hematopoietic stem and progenitor cells
HUVECs	Human umbilical vein endothelial cells
HTS	Highthroughput screening
ICH	International conference on harmonisation
IGF-2	Insuline growth factor 2
Ihh	Indian hedgehog
IL	Interleukin
IL3	Interleukin 3
IL6	Interleukin 6
IL11	Interleukin 11
Ink4	Inhibitors of CDK4
ISO	International organization for standardization
LepR	Leptin receptor
Lin-	Lineage negative cells
LSC	Leukemia stem cell
LSK	Lin-Sca1+Ckit+ cell population
LSKCD34 ^{low}	Lin-Sca1+Ckit+CD34 ^{low} native HSCs

LT-HSC	Long-term hematopoietic stem cell
LTC-IC	Long-term culture-initiating cell
LT-IC	Long-term-initiating cell
LT-RC	Long-term-repopulating cell
MCM3	Minichromosome maintenance complex component 3
MEIS1	Myeloid ecotrophic insertion site 1
MEP	Myeloid erythroid progenitor
MGDF	Megakaryocyte growth and development factor
MPPs	Multipotent progenitors
MSC	Mesenchymal stem cells
N2N1	1-naphthoquinone-2-monoxime
NAM	Nicotinamide
Nes	Nestin
NH4Cl	Ammonium chloride
NK	Natural killer
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
NSG	NOD scid gamma
OB	Osteoblast
OC	Osteoclast
ODD	Oxygen-dependent degradation
OM	Osteomac
OPN	Osteopontin
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PES	Polyether sulfone
PGE2	Prostaglandin e2
PHD	Prolyl hydroxylases domain
PI	Propidium iodide
PLA	Polylactic acid
PMBCs	Peripheral mononuclear blood cells
PSA	Penicillin streptomycin amphotericin
PTCH	Protein patched homolog
PU	Polyurethane

Rb	Retinoblastoma family proteins
RBC	Red blood cell
ROS	Reactive oxygen species
RPMI	RPMI Roswell Park memorial institute
RT-PCR	Real-time PCR
SCF	Stem cell factor
SDF-1	Stromal-cell derived factor-1
Shh	Sonic hedgehog
SM	Small molecule
SMO	Smoothened
SIRT-1	Sirtuin 1
SR-1	Stemregenin-1
SRC	SCID repopulating cell
ST-HSC	Short-term hematopoietic stem cell
TAME	N-4-tosyl-l-arginine methyl ester hydrochloride
TCPS	Tissue culture polystyrene
TEPA	Tetraethylenepentamine
TGF β	Transforming growth factor beta
TNC	Total nuclear cell
TSA	Trichostatin a
TPO	Thrombopoietin
TUDCA	Tauroursodeoxycholic acid
UCB	Umbilical cord blood
UCB-MNC	Umbilical cord blood mononuclear cell
VA	Violuric acid
VEGF	Vascular endothelial growth factor
VPA	Valproic acid
WMDA	World marrow donor association

1. INTRODUCTION

1.1. HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSCs) are known to be mother of the all blood cell types and play a critical role on hematopoiesis process defined as production of blood cells. HSCs are characterized by high capacity of self-renewal and differentiation to all blood cell types. They differentiate to the blood cells by loss of self-renewal capacity during hematopoietic development. HSCs generate the multipotent progenitors (MPPs) by reduction of self-renewal ability. Then, hematopoietic lineages primarily consist of two oligopotent progenitors, which differentiates into several but not all lineages of system; common myeloid progenitor (CMP) and common lymphoid progenitor (CLP). Afterwards, CMPs give rise to megakaryocyte/erythroid progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). MEPs generate red blood cells and megakaryocytes while GMPs generate mast cells, eosinophil, neutrophil and monocytes/macrophages. On the other hand, B, T and natural killer (NK) cells are derived from CLPs.

Characterization of hematopoietic stem and progenitor cells (HSPCs) is main issue for HSC studies. Surface markers identified for characterization of HSPCs differ between mouse and human species. HSCs which have long-term self-renewal capacity are identified as Lin-Ckit+Sca1+Flk2-CD34-Slamf1+ in mouse, Lin-CD34+CD38-CD90+CD45RA- in human. During differentiation to MPPs in mouse, Flk2 and CD34 marker expression are upregulated while Slamf1 expression is downregulated. CLPs are identified as Lin-Ckit^{low}Sca1^{low}IL7R α + in mouse, Lin-CD34+CD38+CD10+ in human. CMPs are characterized as Lin-Ckit+Sca1-CD34+FcyR^{low} in mouse, and Lin-CD34+CD38+IL7R α ^{low}CD45RA+ in human. MEPs are identified as Lin-Ckit+Sca1-CD34-FcyR- in mouse and Lin-CD34+CD38+ IL7R α -CD45RA- in human (Figure 1.1)[1].

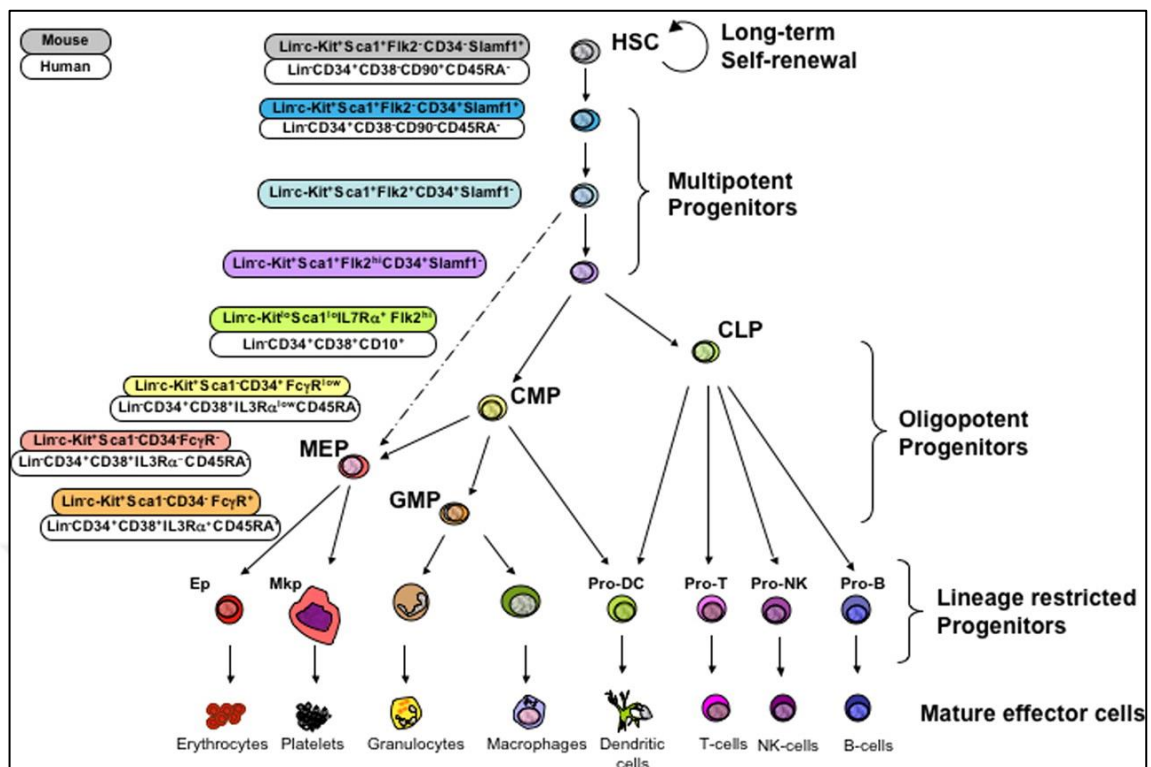


Figure 1.1. Hematopoietic hierarchy during hematopoiesis[1]

HSCs are involved in two different stage as long-term HSC (LT-HSC) and short-term (ST-HSCs) in the development. Actually, LT-HSCs give rise to ST-HSCs. LT-HSCs have self-renewal capacity whereas ST-HSCs do not. Although HSCs are stem cell, they do not always proliferate and regenerate themselves without any inducer such as extrinsic or intrinsic factors. They are arrested at the cell cycle and entry to the G0 phase. This situation is called as ‘‘quiescence’’. However their limited self-renewal capacity, HSCs are pupil of the regenerative medicine because of their stem cell properties for the treatment of hematological malignancies and blood disorders.

1.1.1. Origin of Hematopoietic Stem Cells During Embryogenesis

Hematopoiesis is processed at different regions during embryogenesis. The studies on mice have shown that hematopoiesis begins at embryonic yolk sac post fate specification from hemangioblast and then, transfers to the aorta-gonad-mesonephros region (AGM), the fetal liver (FL) and bone marrow (BM) during embryogenesis, respectively (Figure 1.2)[2]. BM

is the state that HSCs acquire quiescent form post loss of fetal liver characteristics. The initial blood production is occurred in mammalian yolk sac and this hematopoiesis called as primitive hematopoiesis is responsible for production of red blood cells to provide oxygenation for tissue against rapidity of development. Then, the primitive hematopoiesis is converted to definitive hematopoiesis. While definitive hematopoiesis is responsible for the colonization of fetal liver and bone marrow, primitive hematopoiesis carries out de novo HSC generation[3,4].

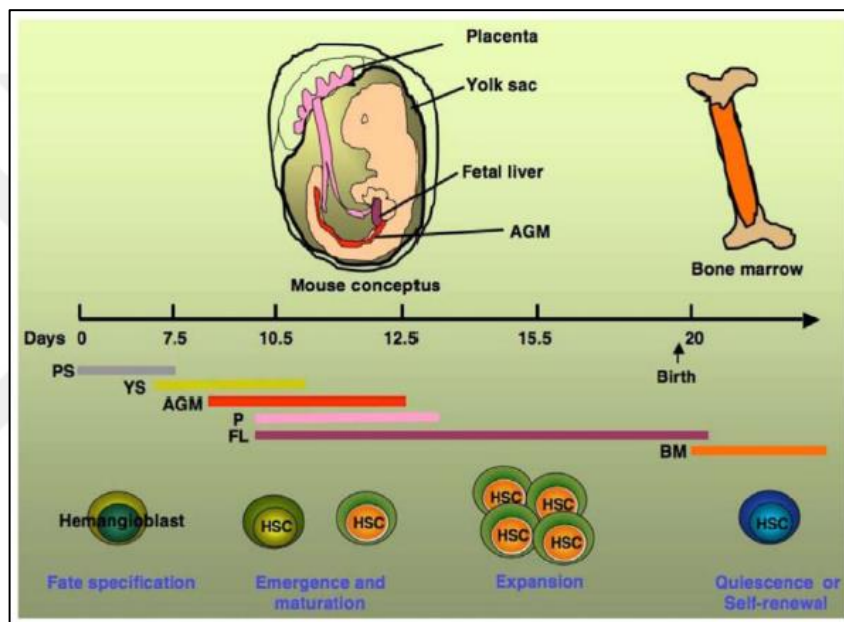


Figure 1.2. Development of hematopoietic stem cells during mouse embryogenesis[5]

Fetal and adult HSCs have different capacity of proliferation and regulation processes. For instance; proliferating fetal HSCs are replaced by quiescent adult HSCs in BM after 4 weeks of birth. Fetal HSCs are highly active on cell cycle profile compared to adult and aged HSCs. The fetal cells are more cycling cells and the adult HSCs are characterized by quiescent cells. As HSCs aged, their cycling level and functions are reduced. Immune senescence and oncogenic property depending on accumulation of many mutations in their lifelong progress[6]. Besides, red blood cells produced in yolk sac are nucleated and have embryonic hemoglobin, but erythrocytes produced by HSCs in FL and adult BM are non-nucleated and have adult hemoglobin. In human, the niche of HSCs in fetal liver is regulated by Wnt signaling while the niche of HSCs in BM is regulated by Notch signaling pathway[5]. The

mechanism of transition between proliferative fetal HSCs to quiescent adult HSCs remains unclear.

1.1.2. The Niche of Hematopoietic Stem Cells

Adult HSCs are localized in a special microenvironment in BM, also called as ‘‘niche’’. The niche consists of two region; endosteal and perivascular niche including heterogeneous populations. Specifically, quiescent HSCs are involved in endosteal region of BM where is near to arteriolar niche while cycling HSCs reside in sinusoidal niche[7]. The niche regulate self-renewal, differentiation, stemness and maintenance of HSCs through direct interaction or secretion of soluble factors. The endosteal niche includes osteoblastic cells such as osteoblasts (OB), osteomacs (OM) and osteoclasts (OC). The arteriolar niche in perivascular niche includes endothelial cells (ECs), sympathetic nerves, Schwann cells, Nes+GFP^{dim}Lepr⁺ and Nes+GFP^{bright}NG2+Lepr⁻ mesenchymal stem cells (MSC), and the sinusoidal niche in perivascular niche include leptin receptor (LepR)⁺ MSCs, CXCL12-abundant reticular (CAR) cells and megakaryocytes. Besides that, macrophages are interacted with Nestin (Nes)⁺ cells for regulation of HSC retention[8].

Osteoblasts in endosteal niche are responsible for regulation of homing, mobilization and quiescence of HSCs by production of soluble factors and cytokines including CXC-chemokine ligand 12 (CXCL12), Osteopontin, Angiopoietin-1 and Ca⁺² secretion. CXCL12, also known as stromal-derived factor-1 (SDF-1) is produced by immature osteoblasts and endothelial cells in perivascular niche and it is known to regulate HSC homing, retention and repopulation. Besides that, osteoblasts have been indicated to have a role in the regulation of B lymphopoiesis which require CXCL12 for differentiation of pre-pro-B cells to B cell. Osteopontin (OPN) is a phosphorylated matrix glycoprotein secreted by osteoblasts and known to be involved in the retention, migration and downregulation of HSC proliferation and differentiation. Angiopoietin-1 (Ang-1) is expressed by osteomacs and, its interaction with receptor tyrosine kinase Tie2 regulates adhesion and quiescence of HSCs. Genetically deletion of Tie1 and Tie2 receptors in mice showed the role of Ang-1 in the regulation of hematopoiesis by resulting loss of HSC maintenance in BM. Another regulator is calcium releasing by osteoclasts and was demonstrated to regulate HSC maintenance while removal of mineralized bone following bone formation[9,10].

Secretion of CXCL12 from perivascular niche cells; endothelial cells and CXCL12-abundant reticular (CAR) cells is main regulator for maintenance and homing of HSCs. Besides, endothelial cells and CAR cells provide the stemness and proliferation of HSCs by secretion of stem cell factor (SCF). Endothelial cells also induce HSC self-renewal and prevent HSC exhaustion by Notch ligand Jagged-1. Sympathetic nerves and non-myelinating Schwann cells regulate maintenance of quiescent HSC by regulating transforming growth factor- β 1 (TGF- β 1) activation[7]. In addition to them, MSCs have a critical role in HSC regulation. Nes+GFP^{bright} NG2+ Lepr⁻ mesenchymal cells reside in arterioles while Nes+GFP^{dim}Lepr⁺ MSCs reside in sinusoid in the bone marrow. Both two MSC types support the maintenance of quiescent and cycling HSCs by secretion of CXCL12 and SCF via interaction with macrophages. More proliferative HSCs interact with Nes+GFP^{dim} Lepr⁺ MSCs in sinusoidal region while quiescent HSCs interact with Nes+GFP^{bright} NG2+ Lepr⁻ MSCs in arterioles[11]. Megakaryocytes provide the support for HSC regulation by secretion of the chemokine CXCL4, TGF- β 1 and TPO. Deletion of CXCL4 in megakaryocytes increased numbers of murine HSCs. Besides, TGF- β 1 deletion studies in megakaryocytes was found to increase HSC proliferation. Taken together, TGF- β 1 and CXCL12 maintain HSC quiescence. TPO is known to be regulator of HSC maintenance (Figure 1.3)[12].

The niche cells have a critical role in regulation of HSC and hematopoiesis. The secreted components determine the HSC cell fate. Therefore, *ex vivo* culture of HSCs requires the providing of the niche factors for efficient culture.

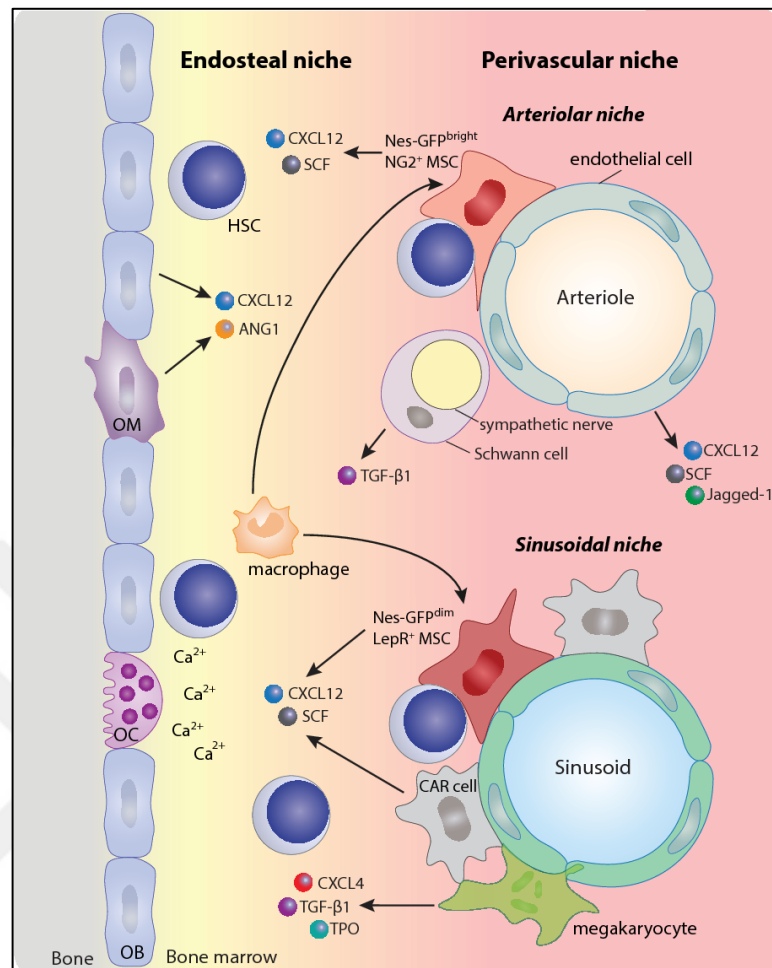


Figure 1.3. Endosteal and perivascular niches of the bone marrow which regulate HSC maintenance through direct interaction or secretion of soluble factors[8]

1.1.3. Metabolism of Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are localized in endosteal region of bone marrow, which is characterized by low oxygen tension, as mentioned previous section. The specialized hypoxic microenvironment of HSCs is regulates self-renewal and differentiation of HSCs. Because of that, HSCs prefer anaerobic glycolysis to sustain their life. The preference of anaerobic glycolysis against to mitochondrial oxidative phosphorylation provides the advantages for survival in this hypoxic niche. The main handicap of mitochondrial oxidative phosphorylation on cells is reactive oxygen species (ROS) production. ROS is known to induce cellular damage on the cells. It has been demonstrated that the high levels of ROS resulted in loss of HSC function and HSC exhaustion. Regulation of ROS is main issue for

HSC function. To adapt this condition, HSCs have to minimize their metabolic activity. Because, in regard of mitochondrial oxidative phosphorylation, anaerobic glycolysis results in less adenosine triphosphate (ATP) production. Therefore, HSCs are generally involved in quiescent state, which resulted by cell cycle arrest at G0 phase of cell cycle and divide only in response to stimulus to replenish blood system, for economical utilization of limited energy[13].

Hypoxia-inducible factor-1 α (HIF-1 α) is a major transcriptional regulator which promotes the metabolic switch from aerobic mitochondrial oxidative phosphorylation to anaerobic glycolysis in response to hypoxic condition. It has been demonstrated that HIF1 α is highly expressed in HCs and loss of its function resulted in HSC dysfunction[14]. HIF1 α interacts with MEIS1 protein which is required for transcriptional activation of HIF-1 α in HSC[15]. MEIS1 is modulators of oxidant defense mechanisms in HSCs by regulation of HIF-1 α and thus, ROS production. Higher differentiation capacity of HSCs, the metabolic profile is changed. ROS production is increased in differentiated cell along with increase in mitochondrial oxidative phosphorylation level. The transcriptional network regulates metabolic balance, fate and function of HSC.

1.1.4. Cell Cycle Kinetics of Hematopoietic Stem Cells

Cell cycle is a vital process for every cell types, which is necessary to maintain essential cellular activity for tissue regeneration. Cell cycle is a complex process regulated by intrinsic and extrinsic factors. Cell intrinsic factors which regulate the quiescence of HSCs, are cyclins, cyclin-dependent kinases (CDKs), cyclin-dependent kinase inhibitors (CDKIs), cyclin-dependent kinase activating kinases (CAKs) and retinoblastoma (Rb) protein family.

Cyclins are regulatory proteins and work together with CDKs cell cycle regulators. There are four cyclins in the family; Cyclin-A/B/D/E. Especially, cyclin-D is expressed in HSCs. It was reported that the lack of cyclin-D family members reduced HSPC number. A similar pattern was observed for Cyclin-A about HSC cell cycle regulation. Cyclins are activated by formation of Cyclin/CDK complex. CDKs consists of CDK4&CDK6 which bind to cyclin-D and positively regulate G1 phase, CDK2, which regulate G1-S transition with cyclin-E, CDK1, which plays the regulation of the cell cycle through G2 and M phases with cyclin-A and cyclin-B[6]. The critical point on HSC quiescence is the control of whether HSCs enter

the G1 phase of the cell cycle or arrested in G0 by cyclin D–Cdk4/6 complex[16]. Deficiency of cyclin D members showed the decrease in HSC number and frequency of HSCs in S-G2-M phases in mice[17]. This complex works activation by CAKs following phosphorylation process and also, controlled by CDKIs including Cip/Kip family (p21Cip1, p27Kip1, p57Kip2) and Ink4 family (p15Ink4b, p16Ink4a, p18Ink4c, p19Ink4d). To control the cell cycle, the members of the Cip/Kip and Ink4 families bind to Cyclin-D/E/A-dependent kinases and CyclinD-CDK4/6 complex to inhibit their protein activity. Ink4 family members are related to transcriptional repression by Bmi1, a polycomb group family member. It was known that deficiency of Bmi1 resulted in loss of HSCs[6,18]. CDKIs are critical for progression of the cell cycle. For example; researchers have revealed that while p18 has an inhibitory effect on HSC self-renewal, p16, p57 remained HSC function and quiescence, respectively. All CDKIs have not same regulatory effect such that contrary to p16, p18 and p57, p21 reduces exhaustion of HSCs and p27 does not effect on HSC self-renewal[6,19]. The retinoblastoma (Rb) family consists of pRb, p107, and p130 transcriptional repressor proteins which limit cell cycle entry by repression of positively cell cycle regulator E2F transcription factors. Inactivation of Rb family members, phosphorylated by cyclin D–Cdk4/6 complex leads to cell cycle progression through G1. Transition to S phase is performed by inactivation of Rb-mediated inhibition of E2F post phosphorylation by cyclin E–Cdk2 complex. Deletion of Rb family members in adult mice has been resulted in an increase in HSC proliferation and absolute cell numbers. Rb family members have a critical role in quiescence and self-renewal of HSC[6].

The regulation of HSC self-renewal and function mainly depends on the cell cycle process. Hence, the critical checkpoints components involved in progression of cell cycle are main regulator factors for HSC expansion and regulation.

1.1.5. Molecular Pathways Involved in Hematopoietic Stem Cell Regulation

There is a balance between differentiation and self-renewal capacities of HSCs during their lifelong. Several molecular pathways play a critical role to be provided this balance. Cell extrinsic factors such as Notch, Wnt, Hedgehog, Angiopoietin-mediated and hypoxia signaling pathways and Hox (Homeobox) gene family are involved in regulation of HSC function.

1.1.5.1. Notch Signaling Pathway on HSCs

Notch signaling, which is evolutionarily conserved pathway, primarily promotes the cell fate determination such as differentiation, proliferation, apoptosis and cell adhesion. Five ligands; Jagged 1-2, Delta 1-3-4 play a role in Notch signaling pathway. HSC studies showed that Notch ligands and receptors have been detected in the bone marrow niche of mouse and human HSCs. Manipulations on the Notch signaling promote the regulation of the HSC function[20]. Barbara Varnum Finney et al. determined the expression of the Notch in murine whole bone marrow cells and LSK (Lin- Sca1+ C-kit+) cells. Then Notch ligand, Jagged-1 was transfected to the cells and thus, it was observed that binding of Notch to its ligand, Jagged-1, increased the self-renewal and expansion of primitive precursor cell population by inhibition of differentiation[21]. Frances N. Karanu et al. demonstrated that Notch ligand, Jagged-1, expanded the human HSCs. They transplanted the cells expanded on presence of Jagged-1 to NOD/SCID (nonobese diabetic/severe combined immunodeficient) mice and found that Jagged-1 provided enhancement of survival and reconstitution capacity[22]. On the other hand, it was demonstrated that while another Notch ligand, Delta-1, inhibits the differentiation of human CD34+ cells to B cells. Notch ligands exhibit different effect on the cells. Besides, it was demonstrated that Notch2 regulates self-renewal of LSK cells and repopulation following transplantation and inhibition of myeloid differentiation[23]. These findings revealed that activation of Notch members and ligands have shown to regulate the self-renewal and expansion of human and mouse HSCs.

1.1.5.2. Wnt Signaling Pathway on HSCs

Another evolutionary conserved pathway involved in HSC regulation is Wnt signaling pathway. The pathway includes Dishevelled, TCF, and β -catenin components, commonly expressed in the AGM region in mouse. The components involved in this pathway are secreted as glycoprotein growth factors and induce the canonical pathway, also known as Wnt/ β -catenin dependent pathway and the non-canonical pathway, also known as β -catenin-independent pathway. Wnt proteins mainly regulate cell fate determination as well as Notch pathway and stem cell renewal by canonical Wnt/ β -catenin pathway. In this pathway, β -Catenin has a major membrane-bound form and this form interacts with E-cadherin. After this interaction, membrane-bound β -catenin is released into the cytosol by connection to actin filaments. This cytosolic β -catenin plays a role as a downstream protein of the canonical Wnt signaling pathway. In the nucleus, β -catenin activates cyclin D1 and c-myc

target genes and thus, the Wnt signaling pathway is important for the development of hematopoiesis and HSC proliferation[20,24,25]. Embryonic hematopoiesis studies showed that Wnt signaling is involved in development of primitive and erythromyeloid progenitors in the mouse. Activation of Wnt pathway by glycogen synthase kinase 3 (GSK3 β) inhibition that caused upregulation of β -catenin was resulted in increased HSC function post hematopoietic reconstitution. Besides, inhibition of Wnt with a small molecule resulted in decrease in HSCs [26]. Wnt3a and Wnt5a are the common Wnt signaling proteins that are involved in the regulation of HSC fate. David J. Van Den Berg et al. have determined that effect of the Wnt proteins on human HSC *in vitro*. They co-cultured the human HSCs and fibroblasts as a feeder layer along with Wnt-5A, Wnt-2B, and Wnt-10B activation. They indicated that the Wnt genes increased the cell number of Lin-CD34⁺ according to colony forming unit assay [27]. Timothy W. Austin and et al. used the conditioning media obtained from cells transfected with Wnt-1, Wnt-5a, or Wnt-10b proteins on the murine fetal liver and human bone marrow HSC and found that 7-11 fold increase in HSC number[28]. The studies based on β -catenin component showed prostaglandin E2 (PGE2) provides stabilization of β -catenin and thus, induces expansion of UCB HSC by enhancement of canonical Wnt signaling. Moreover, activation of Wnt/ β -catenin pathway using GSK3- β inhibitor with Rapamycin (inhibitor of mTOR pathway) resulted in increase in the number of murine LT-HSCs *in vivo*[2]. These findings shows that Wnt signaling pathway display a critical role from primitive hematopoiesis to definitive hematopoiesis during development.

1.1.5.3. Hedgehog Signaling Pathway on HSCs

Hedgehog (Hh) is involved in a secreted protein family and consists of three members in mammals such as sonic hedgehog (Shh), Indian Hedgehog (Ihh), Desert Hedgehog (Dhh). Hh pathway is a major regulator of cell-fate decision on stem cells. Hh proteins bind to the Patched (Ptch) receptor. In the absence of Hh, this receptor play a role as a negative regulator of 7 membrane spanning receptor, also called as Smoothened (Smo). When Hh protein binds and inhibits Ptch function, Smo signaling is induced. Induction of the signaling cascade activates activation of the Gli transcription factor family. The effect mechanism of Hh signaling pathway differs depending on the primitive and definitive hematopoiesis during development and cell types such as HSCs or mature blood cells in hematopoiesis. In primitive hematopoiesis, the secretion of Ihh and Hh target genes including Ptch1 and Gli1 have a role in hematopoiesis and vasculogenesis. Deficiency of Ihh in mice resulted in death

of mice by yolk sac abnormalities showed the critical role in primitive hematopoiesis. The studies about definitive hematopoiesis showed that the Hh pathway genes were highly expressed on the human cord blood derived CD34⁺CD38⁻ primitive cells[29]. Besides, addition or blockage of the components of Hh pathway showed the role on the proliferation and differentiation of HSCs. In addition, addition of these proteins enhanced the hematopoietic recovery and reconstitution in NOD/SCID mice. They suggested that the expansion mechanism is dependent on BMP4 (Bone Morphogenic Proteins 4) by inhibition of the sonic Hh-induced proliferation following using of BMP4 inhibitor Noggin. Therefore, the finding suggested that sonic Hh regulates the HSC proliferation depending on BMP4 signaling pathway. Recent studies have shown that BMPs, which belongs to TGF- β superfamily, regulate the HSC function. They are critical components for stem cell fate determination. Zhang J et al. revealed that BMP4 reduced the differentiation of UCB CD34⁺CD38⁻ cells. However these findings, more information about the effect mechanisms of Hh signaling pathway is need to be clear[25,30].

1.1.5.4. Angiopoietin-mediated Signaling Pathway on HSCs

Tie-2 is a receptor encoded by receptor tyrosine kinase family. Its ligand, Angiopoietin-1, and Tie-2 are highly expressed in endothelial cells and quiescent HSCs. Ang-1 protein is produced by OBs in adult BM niche and Tie2/Ang-1 signaling regulates adhesion capacity of HSCs to OBs. It was indicated that the interaction of Tie-2 and Angiopoietin-1 enhanced the *in vivo* long-term repopulation ability of HSCs. They have reported that angiopoietin-1 induces the adhesion of the HSCs to BM niche by promoting quiescence of HSCs for protection of HSCs from stress factors. The other effect mechanism of Tie2/Ang-1 signaling is based on N-cadherin expressed by HSCs and OBs. Moreover, upregulation of the expression of N-cadherin in Tie2⁺LSK cells by Ang1 provided maintenance of long-term culture-initiating cells (LTC-IC). These findings suggested that N-cadherin-mediated adhesion between HSCs and OBs contributes to HSC maintenance in HSC BM niche[31].

1.1.5.5. Hypoxia Signaling Pathway on HSC Regulation

HSCs are localized in endosteal regions of the bone marrow that is the zone responsible for vascular organization to constitute the arterioles as previously described above. They have a microenvironment characterized by low oxygen tension, also known as hypoxia. The hypoxic niche are involved in HSC regulation by induction of the expression of various genes which regulate the modulation of stem cell metabolism, proliferation and survival[32].

In the niche, HSCs display low proliferative activity and their quiescent state is the result of adaption to the hypoxic niche. The mitochondrial activity is reduced in the cells because of that their anaerobic glycolysis preference, and thus they are protected from DNA damage resulted by production of ROS.

HIFs is a transcription factor family to be related to hypoxic response in HSCs. This protein consists of two subunits; HIF-1 α , which regulates quiescence state of HSCs and HIF-1 β . HIF-1 α and HIF-2 α have the oxygen-dependent degradation (ODD) domain which includes prolyl residues. These residues are hydroxylated by prolyl hydroxylases (PHDs) which are activated with molecular oxygen under normoxic condition. The hydroxylation initiates the ubiquitin-mediated proteolytic degradation of HIF1- α by E3 ubiquitin ligase complex. Contrarily, hydroxylation of the ODD domain is reduced by inactivation of PHDs by lack of oxygen under hypoxic condition and thus, inhibited ubiquitin-mediated protein degradation of HIF- α . Therefore, HIF1- α translocates into the nucleus and generates a complex by dimerization with HIF-1 β /ARNT which results in interaction with the transcriptional co-activators p300/CREB-binding protein (CBP). This interaction initiates the adaptive response against to hypoxic stress by recognition of the hypoxia-responsive elements (HREs) involved in downstream of hypoxia-responsive genes[33,34]. The other transcription factor involved in HSC glycolytic metabolism is MEIS1 (Myeloid ecotropic viral integration site 1). MEIS1 is associated with HIF-1 α and regulates transcriptional activation of HIF-1 α with their cofactors; Pbx1 (Pre-B-Cell Leukemia Transcription Factor 1) and Hoxa9[14,35]. It has been found that culture of HSCs under hypoxic conditions increased the number of HSCs by arresting of the cells in the G0 phase and thus, reduction of proliferation via upregulation of p27Kip1 and p57Kip2 CDKI genes[36,37]. Several studies carried out in mice and human have demonstrated that deletion of MEIS1 was resulted in downregulation of HIF-1 α and HIF-2 α and therefore, it was observed the loss of HSC quiescence and re-entry to cell cycle[38]. Pernilla Eliasson et al. revealed that when they performed hypoxic condition to mouse LSK cells, they found the reduction of HSC proliferation by accumulation of the cells to G0 phase[39]. Another mechanism of HIF-1 α is related to Cripto, a member of the EGF-CFC protein family and highly expressed on HSCs and BM-derived osteoblastic cells. HIF-1 α upregulates Cripto/GRP78 and induces its transcriptional activation in HSCs at BM niche by directly binding to the promoter region of Cripto. These findings indicate that Cripto/GRP78 signaling contributes to maintenance of

HSC quiescence by enhancement of glycolysis in the BM niche[40]. In addition to its regulator role in HSCs quiescence, HIF-1 α is also involved in the homing and mobilization of HSCs. It has been reported that CXCR4 chemokine receptor contributed to homing of HSCs by stabilization of HIF-1 α [32]. Therefore, treatment of PHD inhibitors for stabilization of HIF-1 α in HSPCs was carried out and found the enhancement of homing capacity of HSPCs to the BM[41]. Besides, the PHD inhibitor induced mobilization of HSCs synergistically with identified mobilization agents such as Granulocyte-colony stimulating factor (G-CSF) and AMD 3100. These findings suggest that the stabilization of HIF-1 α functionally provides the contribution to the homing and mobilization capacity of HSCs[42]. Briefly, hypoxic condition in HSC niche is clearly effect on proliferation, homing and mobilization capacity of HSCs by regulation of their metabolism.

1.1.5.6. The Role of Hox Transcription Factors in HSCs

The clustered Hox family belongs to evolutionarily conserved Homeobox transcription family. This family consists of four main sub-family (A-D) in mammals and 39 Hox genes. The hematopoiesis studies showed that Hox genes involved in A, B and C clusters are involved in mouse and human bone marrow and highly expressed in hematopoietic cells. Hox genes display a key role in regulation of HSCs, and dysregulation of Hox genes is related to leukemic transformation[43]. Hox genes are clearly found in progression of leukemia. For instance, overexpression of Hox genes; Hoxb3, Hoxa9 and Hoxa10 led to myeloid differentiation resulting to myeloablative diseases or leukemia[44]. Knockout and knock-in studies have shown the role of Hox family members on HSC regulation. It was demonstrated that overexpression of Hoxa10, Hoxb3 and Hoxb6 in mouse BM cells triggered the blockage of B and T cell differentiation which facilitates the progression of myeloproliferative disorders and leukemia. Moreover, overexpression of Hox genes in mouse BM and fetal liver-derived HSCs and human cord blood derived progenitors showed the effects of Hox genes on differentiation and proliferation of HSCs[45,46]. The strongest member about HSC expansion is HOXB4. Overexpression studies performed by transduction of HOXB4 gene to bone marrow cells on mice showed that the HOXB4-transduced cells regenerated primitive HSCs *in vivo*[47]. Sophie Amsellem et al. have shown the effect of the HOXB4 protein on human HSCs. They used genetically engineered-stromal cells (expressing of HOXB4 gene) and found that transplantation of HOXB4-transfected cells increased the human Long-term Initiating Cells (LT-ICs) and repopulating cells in

NOD/SCID mice[48]. Knockout of Hoxb1-Hoxb9 involved in Hoxb cluster indicated that the Hoxb cluster is dispensable for normal hematopoiesis[49]. Hoxa9 is also expressed in primitive hematopoietic cells likewise Hoxb4[49]. Its expression is reduced during differentiation. Deficiency of Hoxa9 generated the defects in hematopoietic lineages and repopulation of HSCs post BM transplantation. These findings suggest that overexpression of Hoxa9 is related to progression of leukemia by induction of *in vivo* HSC regeneration as well differentiation[50]. HOX proteins are known with its interaction with Pbx1 and MEIS1[51]. Any changes of Hox genes affect HOX-dependent pathways. Therefore, Pbx1 and MEIS1 mutant animals had been the HSC defects in Hox knockout mouse model. These findings showed the effective role of these genes in self-renewal and proliferation of HSCs[52,53].

1.1.6. Epigenetic Control of Hematopoietic Stem Cells

Epigenetic modifications allow changes in every cell phenotype without generation of any changes in genetic during development. The two most commonly noticed epigenetic modifications on HSCs are DNA methylation and histone modifications that affect the gene expression level. Dnmt1, Dnmt3a and Dnmt3b are DNA methyltransferases (DNMT). Dnmt1 has a role in maintenance of DNA methylation while Dnmt3a and Dnmt3b have critical role in *de novo* methyltransferases. In the studies focused on the role of methyltransferases in HSCs, it was found that loss of Dnmt1 in HSCs resulted in defects in self-renewal while knockout of Dnmt3a caused a loss of differentiation potential after transplantation of the genetically modified cells. Deficiency of both Dnmt3a and Dnmt3b in HSCs reduced the HSC differentiation. It has been reported that hypomethylation regulates the proliferation of HSCs. Knockout studies of DNA methyltransferase in mouse models showed that Dnmt1 functions in HSC self-renewal, niche retention and differentiation of the myeloid lineage step by step differentiation of HSC to ST-HSC/MPPs and ST-HSC/MPPs to myeloid progenitor[54,55].

Histones are proteins that allow the DNA package in order to generate more compact chromatin structures. Post-translational histone modifications including acetylation, methylation, phosphorylation, sumoylation, and ubiquitination tightly regulate gene silencing and activation. DNA methylation interacts with H3K27me3 and this interaction

showed loss of DNA methylation resulted in deficiencies of H3K27me3. The genes that highly expressed in committed progenitor populations have high level of the H3K4me2 observed in HSCs. Besides, genome-wide analyses showed that young HSCs express high levels of the H4K16ac activation mark compared to old HSCs. Methylation of H3K4 (H3K4me3), a mark of active chromatin; methylation of H3K27 (H3K27me3), a mark of repression; and methylation of H3K36 (H3K36me3) are found in young and old HSCs with their different expression levels. The alterations of histone modification levels in young and old HSCs have shown epigenetic modifications contribute to the functional changes on HSC aging[54,56]. Epigenetic mechanisms are important for promotion of critical processes of HSCs as well as every cell types. Hence, modulation of epigenetic is one of the most popular approaches for HSC regulation.

1.2. HEMATOPOIETIC STEM CELL TRANSPLANTATION:CLINICAL ASPECTS AND LIMITATIONS

The series of statistical analysis have shown that millions of people are exposed to lymphoma, leukemia, immune-deficiency and autoimmune diseases, congenital metabolic defects, hemoglobinopathies, myelodysplastic and myeloproliferative syndromes and about 50,000 people are treated by HSC transplantation (HSCT) in every year according to the data obtained from World Marrow Donor Association (WMDA). There are variety of the HSC sources for allogenic which is the transplantation of another person's stem cells or autologous transplantation which is the transplantation of the person's own stem cells. Bone marrow and peripheral blood are main sources of HSCs but, the lack of the (human leukocyte antigen) HLA-matched donor dispatched to find the alternative source. Thus, umbilical cord blood (UCB) have been used for alternative source because of the advantages about usage of mismatched donors without HLA-matched which provide the less stringent criteria for selection of HLA donor recipient, easily obtaining from donor and the reduced incidence of graft versus host disease (GVHD) post transplantation. WMDA reported that more than 730,00 cord blood units in worldwide are waiting for any patient who needs transplantation, and around 35,000 cord blood transplants have been performed until today. The main limitation of single unit UCB transplantation is that HSC number is not enough for effective transplantation. The clinical studies showed that hematopoietic recovery is delayed after UCB engraftment, but this handicap is tolerated by using higher number of nucleated and

CD34+ cells. The clinical studies showed that minimum cell dose of 3×10^7 nucleated cell/kg is required for successful engraftment. To overcome this, two different UCB unit have been used but, this approach is not certain solution. Because, usage of double CB unit results in some problems for engraftment capacity and recovery rate. [20,57–59].

To obtain sufficient HSC, many different studies have been carried out. They are based on stimulate to HSC proliferation by using cytokines related to HSC regulation, effective secreted molecules found in molecular pathways which play role in stem cell regulation, stromal cells known that effective for HSC expansion or large scale production by bioreactors with the best culture systems, 3D culture systems which allows the mimicking of the HSC niche and small molecule-based culture. These expansion methods are going to be particularly explained in the upcoming sections.

1.3. HSC EXPANSION TECHNOLOGIES

The most important limitation in clinical area of HSC transplantation is not only about insufficient HSC number, but also the lack of homing and reconstitution capacities which are important to enhance hematopoietic recovery. The usage of double UCB units have precluded the insufficient HSC number, but the delay of neutrophil and platelet recovery that is necessary for the successful transplantation remains still insolubleness. To prevent this handicap, researchers have developed new strategies to expand the cells and enhanced the recovery efficiency post HSC transplantation[60]. In this regard, several strategies have been developed such as cytokines, manipulations on molecular regulation pathways, co-culture with stromal cells, chemical molecules, and bioreactors that allows large-scale production. Many cell-extrinsic and cell-intrinsic factors have a key role in regulation of HSC self-renewal and expansion. The studies about cell-intrinsic factors have been identified as transcription factors, epigenetic and cell-cycle regulators while cytokines and growth factors have been identified as cell-extrinsic factors.

1.3.1. Cytokine-mediated HSC Expansion

Cytokines are the secreted molecules which are released by different cell types localized in the HSC niche, and regulate quiescence, self-renewal, differentiation, apoptosis,

mobilization and homing of HSCs. Neighboring cells of HSCs in their special niche in BM and synthesize many HSC cytokines adhesion proteins, and other cellular signals produced by these stromal cells[61]. Cytokines and other proteins have been research topics for maintenance of HSCs in cell culture and *ex vivo* expansion to develop new strategies for HSC-based cell therapy.

Stem cell factor (SCF) is a ligand of c-kit that is highly expressed in mouse HSCs, and involved in stem cell regulation via binding to c-kit receptor. SCF was found to increase in repopulating HSCs and prevents HSC apoptosis[62,63]. It has been reported that presence of SCF regulates in survival, mobilization, and self-renewal of HSCs in culture and HSC niche. Therefore, SCF is essential component for cytokine combinations that provide HSC expansion[61].

Thrombopoietin (TPO) is the main cytokine involved in the development of megakaryocyte and platelet, and TPO and its receptor Mpl regulates the HSC expansion post transplantation and maintenance of HSC quiescence *in vivo*[61]. Deficiency of Mpl/TPO resulted in reduction of repopulating HSC number[64]. Besides, TPO has synergistically effect with SCF and IL-3. Their effect induced the formation of colony-forming unit granulocytes/macrophages (CFU-GM), colony-forming unit erythrocytes (CFU-E), and colony-forming unit granulocytes/erythrocytes/monocytes/megakaryocytes (CFU-GEMM) colonies derived from CD34⁺Ckit^{low} CD38^{low} cell population in human BM cells[65]. Moreover, it was found that TPO positively promotes promoting the survival of repopulating HSCs. Its role in early hematopoiesis during embryogenesis contributes to definitive hematopoiesis by HSCs generation[66].

FMS-like tyrosine kinase 3 (FLT3) receptor and its ligand (FL) are involved in HSC maintenance and expansion. Especially, combination of FLT3 with SCF, IL3, and granulocyte/macrophage colony stimulating factor (GM-CSF) is more effective on HSC regulation[67,68]. Moreover, FL was found to increase the number of LTC-ICs after 10 days culture of CD34⁺CD38⁻ cells in human BM cells[69].

Interleukins (ILs) is a group of cytokines that regulate the immune system by promoting of cell communication between the immune cells. ILs are highly expressed in hematopoietic cells and regulates the interaction between them[70]. An example for that, IL-10 receptor is expressed on mouse LSK cells and induced the frequency of HSCs in culture. IL-6 is

essential with certain cytokine combinations such that IL-6/IL-6R induced *ex vivo* expansion of human HSCs for transplantation[69]. They are not effective on HSC expansion alone, they play a role together as positive regulator on HSC expansion with different combinations.

From other signaling factors, Notch ligands, Delta and Jagged, regulate the generation, differentiation, and expansion of HSCs. Wnt signaling is involved in regulation of HSC generation and expansion. TGF- β induces the inhibition of HSC function while BMPs from members of the TGF- β superfamily, have a critical role in HSC specification during embryogenesis. The cooperation between BMP and Hedgehog signaling promotes the human HSC expansion. Fibroblast growth factors (FGF) which is expressed on repopulating HSCs derived from BM, FGF-1 and FGF-2 regulate the *in vitro* expansion of ST- HSCs and survival of LT-HSC. Tie-2/Ang1 signaling pathway is associated the maintenance of HSCs quiescent[61]. Angiopoietin-like proteins (Angptls) which is involved in the family of seven secreted glycoproteins. Angptl2 and Angptl3 from these proteins are known to be inducer on *ex vivo* expansion of BM-HSCs[71]. Moreover, Angptl5 was also found to induce *ex vivo* expansion of human UCB-SCID repopulating cells post transplantation[72]. Besides, insulin growth factor-2 (IGF-2) is specifically expressed in HSC and display an important role in promoting of *ex vivo* expansion of HSCs derived from fetal liver and bone marrow[73]. Pleiotrophin is also identified as a growth factor for HSC expansion. Himburg et al. demonstrated that pleiotrophin increased the mouse long-term repopulating HSC and NOD/SCID repopulating cells post transplantation of human cord blood HSCs[74]. Therefore, they identified pleiotrophin as a growth factor for HSC expansion. HSC function including the quiescence, self-renewal, differentiation, apoptosis, and mobilization is regulated by combined effect of these cellular secreted proteins. The combinatorial usage of cytokines and other signaling proteins is main strategy for *ex vivo* HSC culture. In respect to this, Shpall et al. have demonstrated in 37 patients suffered from hematological malignancies or breast cancer that there was 56 fold increase in total nucleated cell (TNC) number, 4 fold increase in CD34+ cells following transplantation of manipulated by *ex vivo* expansion with SCF, TPO, G-CSF for 10 days and unmanipulated units, but any differences was not observed for neutrophil and platelet engraftment[75]. McNiece et al. used two step culture technic for expansion. For that they cultured the CD34+ cells selected from UCB unit in large volume following culture in small volume with SCF, G-CSF and megakaryocyte

growth and development factor (MGDF). They found that 438 fold increase in TNC number and 29 fold increase in CD34⁺ cell number post transplantation of the expanded cells to the patients[76]. Marcos De Lima et al. from MD Anderson used two step culture technic, too. They carried out the study in 71 patients with *ex vivo* expansion and without *ex vivo* expansion. CD133⁺ cells selected from UCB unit were expanded in presence of SCF, G-CSF and TPO. Transplantation of CD133⁺ cells resulted in 23 fold increase in TNC number and 2.3 fold increase in CD34⁺ cells[77]. Another example of these studies have been performed by Himburg et al., They used a cytokine cocktail including SCF, Flt-3L and TPO cocktail with Pleiotrophin molecule and they observed 4-fold increase in Lin⁻ CD34⁺ CD38⁻ cells and increased frequency of repopulating cells (RCs) in NOD/SCID mice[74]. In addition, the combination of IGF-2 with SCF, TPO, and FGF-1 found to promote increase in frequency of HSCs in culture[73]. The combinatorial effect of Notch with identified cytokines showed that human CB cells cultured with Delta 1, IL-3, IL-6/sIL-6R, SCF, TPO and Flt3L in serum-free medium provided the increase in SRC number[78].

The positively regulation of cytokines and signaling proteins have clearly shown that they are essential for maintenance and survival of HSCs in their niche and culture. However, there are many studies carried out about this topic, identification of ideal HSC cytokines and combinations still remain to be investigated.

1.3.2. Stromal Cell-mediated HSC Expansion

HSCs are involved in a microenvironment including hematopoietic or non-hematopoietic components. These components affect the hematopoietic regulation by secreting many molecules that control cellular response, activity. The most commonly appeared cell types are mesenchymal stem cells. MSCs are multipotent stem cells that can differentiate into a variety of cell types, including mainly adipocytes, osteoblasts, chondrocytes; and especially neuronal cells, myocytes and β -pancreatic islets cells. MSCs support hematopoiesis[60,79]. It is known that MSCs have immunomodulatory effect and thus, they are important cells to prevent GVHD post HSC transplantation. Recent studies have shown that MSCs affect T- and B-cell response by suppression of T-cell proliferation, cytokine secretion and cytotoxicity. Besides, MSCs may inhibit the proliferation and arrest the cell cycle. In addition, MSCs also inhibit the maturation, activation of dendritic cells and IL-2-induced

NK cell activation[59]. MSCs are used as a feeder layer for HSC expansion. Researchers have performed co-culture of UCB MNCs with MSCs during 14 days. They indicated that 10-20 fold increase in expansion of TNCs, 7-18-fold increase in expansion of committed progenitor cells, 2-5 fold increase in expansion of primitive progenitor cells and 16-37-fold increase in expansion of CD34⁺ cells[80]. Marcos De Lima et al. have used MSC co-culture to expand the HSCs and they found that 12 fold increase in expansion of TNC and CD34⁺ cells[77]. Na Li et al. have determined the mechanism of MSC effect on HSC expansion. They revealed the secreted protein profile from MSC comparing usage of MSC co-culture and MSC conditioning medium and found that MSCs play an inducer role in expansion of peripheral blood stem cells by IL-6 secretion[81]. Besides MSCs, osteoblastic cells are localized on the microenvironment of HSCs in bone marrow. Osteoblastic cells induce HSC number by activation of Notch1 *in vivo*[82]. Another component of the HSC niche is endothelial cells. N Li et al. demonstrated that human umbilical vein endothelial cells (HUVEC) promote the PBPC expansion by co-culture of CD34⁺ cells with HUVEC via secretion of IL-6 growth factor[83].

Another strategy for utilization of HSC niche to expand HSCs *ex vivo* is 3D culture technic that allows mimicking of the niche components by using biomaterials as extracellular matrix (ECM)[7]. The 3D culture based on scaffold has some advantages including controlled biodegradation rate of the material (main criteria), no cytotoxicity, promotion of cell-cell interactions, no immune response, adaptation to large-scale production, compatibility with physiological conditions, easy transplantation capacity to the human[84]. The materials used as scaffold can be produced as nanofibers such as natural and synthetic polymers, ceramics and semiconductors. Natural polymers consist of collagen, gelatin fibrin, silk fibroin, keratin, cellulose, hyaluronic acid, chitosan and alginate while synthetic polymers consist of polylactic acid (PLA), polyether sulfone (PES), tissue culture polystyrene (TCPS), polyurethane (PU), polycaprolactone (PCL) and polyethylene terephthalate (PET)[85]. Many studies showed the efficiency of this strategy to enhance the ability of HSC expansion. Modified fibronectin-coated PET has been reported to increase in the number of human HSCs compared to unmodified biomaterials[86,87]. Besides, aminated-PES biomaterial and cytokines including SCF, Flt3, THPO, and IL-3 increased the expansion of CD34⁺ HSCs derived from UCB compared to tissue culture polystyrene. 3D culture systems have been more efficient to increase in human HSC self-renewal compared to 2D cultures[88]. 3D

culture system is in demand according to 2D culture. This system has been efficiently used as well co-culture with stromal cells because of their advantages about mimicking of HSC niche.

1.3.3. Bioreactor-mediated HSC Expansion

The clinical stem cell therapy needs high number of stem cells such as that $1-9 \times 10^6$ cells/kg are required for stromal cell therapy while around 2×10^5 cells/kg are required for hematopoietic cell therapy for efficient neutrophil recovery post transplantation[89]. The limited HSC number handicap for successful HSC transplantation is achieved by Good Manufacturing Practices (GMP)-compliant culture systems including production and quality control procedures in clinic. The major GMP-compliant systems consists of GMP-grade expansion products and GMP-grade bioreactors that allows the large-scale production. GMP-grade HSC expansion products including special media, efficient supplements and culture flasks/bags are generally used for traditional static culture system. Recent studies showed that dynamic culture system was more efficient to expand HSCs than static culture because of limited cell productivity, difficulties on monitoring and control of culture parameters, low oxygen and lack of nutrient occurred in static culture for HSCs that has ability of clump at the bottom of T-flasks, multiwell plates and culture bags[89,90]. The mentioned handicap limits the efficiency of *ex vivo* HSC expansion. Therefore, the usage of bioreactors become an alternative to static culture systems. Dynamic culture in bioreactors provides the flexibility for manipulations on requirements, large-scale production and incorporation of monitoring and control tools[89].

The bioreactors are designed according to requirements of stem cell properties. For example; rotating wall vessels in addition to stirred environment are developed to reduce shear stress for shear-sensitive cell types such as HSPCs[91]. The wave bioreactor is used for stem cell expansion such as T lymphocytes[89]. Bioreactors can integrate with other expansion technologies such as usage of cytokines, small molecules and growth factors as supplements or biomaterials for 3D culture systems[92]. Especially, the integration of bioreactor with biomaterials was found to induce stem cell expansion and differentiation. There are many clinical studies on usage of bioreactor. Timmins and collaborations designed a two-phase system including firstly expansion of CD34+ cells in static culture and then, a 10 L-scale

production in a wave bioreactor and found production of up to 30 doses (2×10^{10} cells/dose)[93]. The main aim in clinical usage of stem cells is prevention of contamination and variability risks. They also designed an automated closed system for the isolation and expansion of human placental MSC in a wave bioreactor system following isolation of MSC from placental tissue[94]. This technic is important for reduced operator manipulation by integration of isolation and expansion protocols in designed system. Another application about integration of different steps for expansion of stem cells in bioreactors is CliniMACS® Prodigy developed by Miltenyi Biotec. This newly designed device provide the culture of required cell populations in a closed and automated system following the enrichment or depletion of specific cell populations from heterogeneous hematopoietic cell population using CliniMACS® cell separation system[89]. Another designed bioreactor for MSC expansion is developed as Quantum Cell Expansion System which is an automated and closed hollow-fiber bioreactor and allows obtaining of 1×10^8 MSCs[95]. Besides, Aastrom Bioscience has developed an automated continuous perfusion culture system device designed for large-scale expansion by nutrient delivery and gas exchange. In one phase I trial, UCB unit was expanded in the device with growth factor cocktail; PIXY321, FLT-3L, Erythropoietin (EPO). The expanded cells were determined post 12 days of the transplantation and any differences in myeloid, erythroid and platelet engraftment was not observed[96]. In a second study with new bioreactor which uses Dideco ‘‘pluricell system’’ medium, expanded UCB cells increased 230 fold in MNC expansion and 21 fold in CD34+ cells. These system can be used to perform large-scale augmentation of UCB cells[79,97].

The utilization of bioreactor systems enabling of integration with other expansion technologies under GMP conditions has been in demand to overcome the identified limitations for stem cell therapies.

1.3.4. Chemical Molecule-mediated HSC Expansion and Clinical Aspects

Small molecules which have low molecular weight (<900 Dalton) and easily diffuse from cell membrane are chemical molecules. They are also undetected as a foreign determinant by the immune system. They are very valuable tools to improve therapeutic approaches because of their features including lower cost, facility of manipulation, and enabling of control[98]. It is known that small molecules are able to regulate stem cell fate by affecting

to stem cell regulation pathways, cell cycle regulation or preventing from apoptosis[99]. Therefore, small molecules could be therapeutically used in clinics instead of usage of double cord blood unit for transplantation that results in increased risk of GVHD and thus, reduced efficiency of neutrophil and platelet recovery. Recent studies have shown that small molecules become very important stage for clinically HSC expansion along with improvement of computational methods that allows virtual design with high-throughput screening.

Stemregenin-I is a firstly identified small molecule for *ex vivo* expansion of HSPC. It was identified as a purine derivative post screening from a library of 100,000 heterocycles by Boitano et.al. The function of Stemregenin-I (SR-I) was firstly determined on mobilized peripheral blood-CD34⁺ cells. Under previously identified culture condition including SCF, TPO, FLT3 and IL-6 cytokines in serum-free media, SR-I increased the TNC number more than 2,5 fold after 7 days of culture and resulted in 11 fold increase in TNC number and 73 fold increase in CD34⁺ cell number post 3 weeks of culture. Besides, SR-I did not show proliferative effect on the cells in the absence of cytokines. Moreover, the result determined that the absence of SR-I caused the differentiation of the cells showed that SR-I had reversible effect. The study carried on UCB cells showed that after 5 weeks of culture, SR-I increased the proliferation of TNCs and CD34⁺ cells around 10- and 47-fold, respectively. The increase in the CFU-G, CFU-E, CFU-GM and CFU-GEMM colony number indicated that SR-I induced the multilineage potential of expanded cells. Transplantation studies of SR-I expanded UCB CD34⁺ cells into NSG mice demonstrated that SR-I enhanced the repopulation capacity of the expanded cells. Second transplantation provided the determination of adjuvant effect of SR-I on SCID repopulating cell (SRC) frequency (17 fold). Besides, secondary transplantation of the cells obtained from BM of firstly transplanted NSG mice proved the remarkable effect of SR-I on long-term engraftment. The gene expression analysis showed that SR-I treatment reduced the AhR gene expression. Luciferase assay including human AhR-dependent luciferase reporter gene have proved that SR-I is antagonist of AhR protein. The shRNA-mediated knockdown of AhR was resulted in the similar effect with SR-I. It was reported that the effect mechanism of SR-I was based on AhR inhibition which is associated with HES-1, C/EBP, PU.1, β -Catenin, CXCR4, STAT5, C-Myc hematopoietic regulators[100]. Clinical Phase I/II trials carried on 19 patients suffered from high risk hematological malignancies reported that SR-I expanded

cells (yielded 330 fold increase in CD34⁺ and 854 fold increase in TNCs) were transplanted to the patients post myeloablation. The HSC835-named product including the SR-I expanded cells under GMP conditions increased the engraftment capacity (100 percent) compared to 111 historical control (transplanted with unmanipulated double cord blood unit) (87 percent). Neutrophil and platelet recovery time that is one of the main limitations for double cord blood unit transplantation was faster (median 15 days for neutrophil, 49 days for platelets) in HSC835-transplanted patients compared to historical patients (median 24 days for neutrophils, 89 days for platelets). The efficiency and safety of SR-1 proved in this phase I/II study by showing enhancement of immune recovery and engraftment capacity, no significant induction of GVHD, mortality and telomeres shortening compared to traditional transplantation[101,102]. Moreover, SR-I was used with gelatin-based hydrogel cross-linked by microbial transglutaminase for 3D culture system by utilization of the adjuvant effect of SR-I on stemness and expansion[103]. SR-I was also found to increase the CRISPR/Cas9 Gene-Edited NSG-repopulating cells and the study is promising for efficient gene edited HSC transplantation[104].

E478 compound is another antagonist of AhR gene and developed for gene-edited HSC technology. This compound enhanced the engraftment post transplantation of expanded mPB cells into NSG mice more than 10 fold. E478 also increased the engraftment and homology directed repair (HDR) rate of the expanded cells which were transduced by lentiviral vector and by CRISPR/Cas9 knockout more than 10 fold. mPB CD34⁺ cells cultured with E478 for 4 days before and after stimulation for HDR showed the 6 fold increase in the rate of HDR and 134 fold increase in CD34⁺CD90⁺ cell number. Transplantation of these cells into NSG mice showed the 4 fold increase in engraftment and 12 fold increase in HDR rate. These findings revealed that E478 is a promising for gene-edited HSC technology[105].

UM729 is a pyrimidoindole derivative and identified among a library of 5280 compounds by computational screening according to ability of mPB-CD34⁺CD45RA⁻ cell expansion. UM729 was seen as a potent among 7 best molecules from library because of independency of its effect on inhibition of AhR gene. Then, 300 newly synthesized analogs of UM729 were screened under the same criteria. **UM171** was found to have 10-20 fold more potential than UM729 for mPB-CD34⁺CD45RA⁻ cell expansion. The study of determination of UM171 efficiency was carried out on UCB CD34⁺ cells in comparison with SR-I small

molecule in the fed-batch culture system. Alone SR-I or UM171 had similar effect on CD34⁺ cell expansion while combined usage of them increased the CD34⁺ cell number. UM171 was found to enhance the CFU-GEMM multilineage potential, CD34⁺CD45RA⁻ cell number compared to SR-I alone. Besides, LT-HSC frequency was increased 13 fold for UM171 treatment after 20 weeks of transplantation. RNA sequencing revealed that UM171 induces HSC expansion independently from Ahr inhibition[106,107]. Clinically phase I/II studies were carried out on 27 patients suffered from hematological malignancies. Four patients of them were transplanted with UM171-expanded cells and unmanipulated unit. Twenty-two patients were transplanted with UM171-expanded cells. One of them was excluded because of expansion failure of the cord blood unit. Post transplantation, it was reported the reduced transplant-related mortality (around 5 percent), enhanced survival rate (around 95 percent), low incidence of severe acute GVHD, no incidence of severe chronic GVHD[108,109]. These findings highlighted that UM171 is feasible and safe. Phase II trial for niche indications and then, randomized phase III trial will required for active usage in clinic.

P18IN003 and P18IN011 are the small molecules and were identified by in silico 3D database docking screening according to inhibition capacities of p18 CDKI which blocks transition to G1 phase by inhibition of CDK4/6 complex. Deficiency of p18 was resulted in the increased self-renewing division of HSC without enhancement of proliferation rate. Transplantation of daughter cells from the first division of HSCs showed the increase in self-renewing cell division of p18^{-/-} mice compared to p18^{+/+} mice. These findings proved the inhibitory effect of p18 in self-renewing division of HSC. Targeting to the p18 CDKI is promising for the HSC *ex vivo* expansion because of its inhibitory effect on HSC self-renewal. For that, Yingdai Gao et al. identified these two novel small molecules based on p18 protein structure and predicted binding surface cavity by using 3D database docking screening method. They tested the effect of P18IN011 and P18IN003 and found that they significantly increased the frequency of mouse primitive hematopoietic cells (LSKCD34⁻) in culture. Besides, they enhanced the long-term repopulation capacity post 16 weeks of transplantation of expanded LSK cells in CD45.1 cells[110].

Another identified small molecule **XIE18-6** was found to be highly interacted with p18 and responsible for disruption of the interaction between p18 and CDK6. It was reported that XIE18-6 promoted the mouse HSC expansion by more than 2 fold increase in the number

of Lin-Sca-1⁺ HSPC. Post determination of the effect of XIE18–6, the researchers synthesized the analogs of it. Among them, representatively named as compound 40 was found to be most efficient and increase the number of LT-HSCs around 2.61 fold. Besides, compound 40 enhanced the number of cycling cells in LT-HSCs, but not HSPCs and progenitor cells. In addition to mouse HSCs, it was reported the adjuvant effect of compound 40 on human HSCs expansion[111]. These findings highlighted that compound 40 promotes HSCs expansion by inhibition of p18 and thus, activation of CDK4/6 and may be therapeutically used as a drug in clinic.

dmPGE2 (16, 16 dimethyl prostaglandin E2) is a derivative of prostaglandin E2, which is an eicosanoid and modulates many physiological systems. It is known that PGE2 stimulate proliferation, cycling and differentiation processes in quiescent cells. PGE2 has been reported to enhance murine HSC expansion by the 4 fold increase in HSC frequency and engraftment post serial transplantation and 2 fold increase in LSK cell cycle activity. In addition to expansion, the molecule contributed to HSC homing by enhancement of CXCR4[112]. Besides, PGE2 caused the reduction of apoptosis *in vitro*. The anti-apoptotic effect of PGE2 was proved *in vivo* study[113]. Treatment of mice *in vivo* with PGE2 showed significantly decrease in apoptosis of HSPCs compared with control mice. Besides, dmPGE2 was found to enhance the survival and hematopoietic recovery after radiation injury in mice[114]. In addition to mouse study, transplantation of human UCB hematopoietic cells into the NSG mice suggested that PGE2 increased the engraftment capacity of human UCB HSC. In parallel with mouse results, PGE2 is related to enhance homing capacity of UCB HSC[112]. Moreover, Phase I trial was carried out with 21 patients transplanted with dmPGE2-manipulated UCB units in optimized conditions. It was reported the increase in neutrophil recovery (median 17,5 day vs median 21 day) and long-term engraftment capacity by enhancing homing compared to historical controls[115]. Phase II trial has being carried out.

Epigenetic modifications which allow heritable changes in gene function without generation of any genetically changes during development, are critical for cell fate programming or regulation of pluripotency genes. The two most commonly noticed epigenetic modifications are DNA methylation and histone acetylation modifications that synergistically affect the gene expression level[116]. The small molecules that are associated with reversion of epigenetic modifications could be effective to expand HSCs. Araki et al. identified two

chromatin modifying agents; **5-aza-2-deoxycytidine (5azaD) and Trichostatin A (TSA)**. These modifying agents were used together with cytokine combination (SCF, TPO, FLT3, IL3 or IL6) on UCB-CD34⁺ cells. It was reported the increase in CD34⁺CD90⁺ cell number (12,5 fold), CFU-GEMM (9,8 fold), cobblestone area forming cell (CAFC) (11,5 fold), frequency of SRC (9,6 fold) post 9 days of treatment with 5AZA/TSA[117]. In addition to UCB source, these agents were tried on mPB-CD34⁺ cells. In similar with the results obtained from UCB cells, it was found the increase in CD34⁺CD90⁺ cell number (9,6 fold), CFU-GEMM (10,1 fold), cobblestone area forming cell (CAFC) (2,2 fold). Besides, hypomethylation occurred by using 5AZA/TSA agents caused upregulation of self-renewal-related genes (HOXB4, GATA2, EZH2) and downregulation of differentiation-related gene (PU.1)[118]. The changes in regulation of identified genes have been mainstay to determine the effect mechanism of these agents on human HSC expansion.

Another identified compound against to histone acetylation, **Garcinol** is a derivative of α -benzophenone and selected from 92 natural products[119]. To evaluate the effect of Garcinol, derivatives of it were produced such as Isogarcinol, O-monomethylisogarcinol, O-dimethylisogarcinol. Post 7days of treatment of UCB-CD34⁺ cells with Garcinol and its derivatives in SCF and TPO cytokines containing culture condition, the significant increase in CD34⁺CD38⁻ number was found for Garcinol and Isogarcinol (4,5 fold, 7,4 fold, respectively). Besides, Garcinol and Isogarcinol induced the enhancement of CFU-GEMM colony count. Moreover, limiting dilution assay showed the 2,5 fold increase in SRC frequency post transplantation to NSG mice[120]. Another study about Garcinol proved the adjuvant effect of Garcinol on human UCB HSC. It was found that treatment of UCB-CD133⁺ cells with Garcinol induced CD133⁺ cell expansion more than 2 fold. Besides, it was reported that Garcinol increased the homing capacity of the cells by upregulation of CXCR4 gene[119].

Valproic acid (VPA) from epigenetic modifiers is an inhibitor of histone deacetylase (HDAC) and used as a neurological agent. VPA has been known to modulate the HSC expansion by providing of cell cycle progression via reduction of p21 CDKI[121]. In the study about effect of valproic acid on human HSC, researchers used VPA in expansion medium with cytokine cocktails (Flt3L, TPO, SCF, IL-3). VPA treatment increased the CD34⁺ cell number derived from CB, mPB and BM treated[122]. Moreover, the other human HSC study showed that CD34⁺CD45⁺ and CD34⁺CD133⁺ cell populations were

induced by using VPA in two phase culture system including the enrichment and culture[123]. Besides, *In vivo* study showed that VPA enhanced the engraftment capacity of expanded UCB cells by upregulation of CXCR4[123,124]. The adjuvant effect of VPA compound dependent on induction of cell cycle progression on human HSC expansion is promising for usage of VPA in clinic.

Nicotinamide (NAM) which is a precursor of NAD is an inhibitor of HDAC and sirtuin family. Deficiency of Sirt1 gene showed that Proliferation rate of HSCs increased in Sirt-1-deficient mice[125]. Peled et al. investigated the effect of NAM on UCB-HSC expansion and found that NAM increased in absolute number of CD34+CD38- cell post 1 week of culture and also, CD34+ and CFU colon count at 8 weeks of culture. Besides, NAM-treatment increased 7,6 fold the frequency of SRC in NOD/SCID mice post transplantation. Moreover, NAM was involved in delay of differentiation and enhancement of homing capacity of expanded cells[126]. Phase I/II trials were performed for the NiCord product including *ex vivo* expanded cells with Nicotinamide. The trial carried out on 43 patients suffered from high risk of hematologic malignancies resulted in faster neutrophil engraftment, high incidence of platelet at 100 days of transplantation, lower severe acute GVHD and mortality compared to historical controls (n=1037)[127]. These findings revealed that the feasibility and safety of NiCord stem cell product manufactured under GMP conditions and Phase III trial will be needed.

CHIR99021 is an inhibitor of GSK3 β and increases the β -galactosidase in HSCs and **Rapamycin** is a mTOR suppressor. It has been known that the inhibition of GSK3 β not only activates self-renewal of HSCs by activation of Wnt/ β -catenin signaling pathway[128], but also, activates mTOR pathway that regulates lineage commitment, HSC depletion and reduction of self-renewal of HSCs. Therefore, *Huang et al.* cultured mouse LSK cells and human UCB CD34+ cells in serum-free, cytokine-free medium supplemented with GSK3 β inhibitor CHIR99021 and mTOR inhibitor Rapamycin for 7 days. When they transplanted the cells to immunodeficient mice, they found that CHIR99021 and Rapamycin combination increased LT-HSC maintenance and the cell population in S/G2/M phase[129].

6-bromoindirubin 3'-oxime (BIO) is a pharmacological inhibitor of GSK3 β . It was reported that the BIO synergistically effects the human HSCs with C-kit ligand. The optimal concentration (0,2 μ M) of BIO increased the expansion of UCB primitive hematopoietic cells by using with C-kit ligand. Higher concentration of BIO (2 μ M) reduced the proliferation of

total cells, CD34⁺ cells[130]. Ko et al. showed that BIO treatment caused the enrichment of human primitive cells by delay of cell cycle progression through up-regulation CDK inhibitor p57 and thus, down-regulation cyclin D1. Besides, BIO was found to increase the frequency of SRC post transplantation of BIO-expanded cells into NSG mice *ex vivo*-expanded HSCs[131].

NR-101 is a small molecule and antagonist of myeloproliferative leukemia virus proto-oncogene (c-MPL), which is receptor of TPO. TPO is known to be necessary for megakaryocyte differentiation and platelet production by interaction of TPO and c-MPL which are associated with three pathways; Janus Kinase (JAK)/ signal transducer and activator of transcription (STAT), Ras/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT pathways[132,133]. It was reported that Deficiency of TPO or c-MPL in mice caused the defects in maintenance of HSC quiescence and also, knockout of TPO reduced engraftment capacity of HSCs[134,135]. Researchers identified the NR-101 molecule to inhibit c-MPL and found that NR-101 increased human CD34⁺CD38⁻ cells *ex vivo* and repopulating cells following transplantation to NOD/SCID mice. They also indicated that NR-101 increased the cell population in G0/G1 phase in cell cycle. Gene expression analysis showed that NR-101 was responsible for upregulation of transcription factor STAT5 and accumulation of HIF1 α protein and thus, upregulation of its target genes[136].

Recent reports have showed that metal ions; iron, calcium, zinc, magnesium and copper modulate hematopoietic differentiation. It has been demonstrated that Cu ions are important for hematopoietic differentiation[137]. Studies about effect of Cu chelator on human HSC expansion have showed that treatment of ***Tetraethylenepentamine (TEPA)*** along with SCF, TPO, FLT3 and IL-6 cytokines increased the CD34⁺, CD34⁺CD38⁻ population percentage in short-term[138]. Large-scale production of CD133⁺ derived from UCB isolated by CliniMacs in the clinical-grade closed culture bags containing the identified cytokines resulted in the 89 fold increase in CD34⁺ cell number, 30 fold increase in CD34⁺CD38⁻ cell number and 171 fold increase in CFU colony count. Besides, TEPA reduced differentiation of expanded cells and induced engraftment and self-renewal capacities in NOD/SCID mice by reduction of Cu level in CD34⁺ cells[139]. These findings showed the utilization efficiency of TEPA on clinical manufacturing. In the phase I/II studies carried out at MD Anderson, they transplanted the 1.8×10^7 TNC per kilogram and 1.5×10^5 CD34⁺ cells per

kilogram UCB unit expanded with TEPA, also known as StemEx product, into ten patients and then, they analyzed the rate of cell expansion, capacity of engraftment, GVHD and mortality ratio. They found that 2,9 fold increase in TNC number and 6 fold increase in CD34+ cell number. They did not observe any cases of grades 3–4 GVHD. Besides, 100-day survival ratio was 90 percent because 9 out of 10 patients were alive after 100 days following the transplantation[140]. These findings have revealed that TEPA usage is feasible. Moreover, Farah Zaker et al. used co-culture of UCB-CD34+ with BM-MSC combined with TEPA and recombinant cytokines and compared to culture with recombinant cytokines/ BM-MSC+recombinant cytokines/ recombinant cytokines+TEPA. They found that approximately 118 fold increase in TNCs, 110 fold increase in CD34+ cells and 172 fold increase in colony numbers in BM-MSC co-culture+recombinant cytokines+TEPA combination compared to other culture conditions[141].

Aldehyde dehydrogenase (ALDH), is an enzyme which catalyze the conversion of retinol (vitamin A) to retinoic acids. ALDH is also known to be highly expressed in HSCs. Therefore, it is metabolic marker for human HSCs. *Chute et al.* showed that ALDH plays a critical role in HSC differentiation. Inhibition of ALDH by **diethylaminobenzaldehyde (DEAB)** reduced the human HSC differentiation and lineage commitment. The treatment of DEAB along with SCF, TPO and FLT3 cytokines showed the 3.4-fold increase in expansion in the UCB primitive cells and enhancement of UCB-CD34+CD38- expansion. In addition, *in vivo* studies revealed that DEAB increased engraftment and frequency of SRC in short-term culture post transplantation to NOD/SCID mice. Moreover, inhibition of ALDH directly reduced the Retinoic acid production in cells. Reduction of Retinoic acid led to inhibition of HSC differentiation. The adjuvant effect of DEAB on self-renewal capacity of human HSCs was dependent on upregulation of HOXB4. These findings showed that inhibition of ALDH and retinoid activity by DEAB is promising for the expansion of human HSCs[142].

zVADfmk and zLLYfmk are inhibitors of caspase and calpain which are cysteine proteases and regulate apoptosis process, respectively[143]. zVADfmk and zLLYfmk was reported for protection of HSCs from apoptosis and increase in LT-RCs and engraftment capacity by three mechanisms; upregulation of bcl-2 gene anti-apoptotic gene, decreased activity of caspase 3, activation of Notch pathway. Besides, these inhibitors enhanced 2 fold CXCR4 and thus, migration dependent on enhanced SDF-1[144,145].

5-hydroxytryptamine (5-HT), also known as serotonin is a neurotransmitter. Yang et al. used 5-HT to expand the human UCB HSCs and found that the treatment of UCB CD34+ cells with 5-HT in addition to SCF, TPO and FLT3 cytokines induced the TNCs and CD34+CD38- cell expansion and CFU colony formation post 8 days of treatment. *In vivo studies* also showed the enhanced engraftment capacity of 5-HT-treated cells. Moreover, serotonin was associated with reduction of apoptosis by downregulation of Caspase 3[146].

SB203580 is an inhibitor of p38 protein which belongs to the MAPK family of signal transduction kinases. It has been reported that enhanced p38 function results in HSPC proliferation and differentiation. Deficiency of p38 in mice showed the increased reconstitution capacity of hematopoietic cells. Activation of p38 also induces the HSC exhaustion and senescence by upregulation of p16Ink4a in HSCs. Pharmacologically inhibition of p38 is promising for HSC self-renewal and expansion. To evaluate that, Wang et al. cultured the mouse LSK cells in serum-free medium supplemented with SCF, TPO, FLT3 and SB203580 inhibitor. It was found that inhibition of p38 by SB203580 resulted in the around 8 fold increase in the LSK number post 7 days of treatment and 7,6 fold increase in CAFCs post 35 of treatment. *In vivo* transplantation study indicated the 5,2 and 9,8 fold increase in engraftment capacity at 8 and 12 weeks, respectively. Besides, secondary transplantation of BM cells obtained from primary recipients showed the 11,2 fold increase in engraftment. Moreover, the adjuvant effect of SB203580 on self-renewal and engraftment depends on upregulation of HOXB4 and CXCR4 genes[147]. Human HSC study carried out on UCB cells showed that treatment of UCB-CD133+ cells with SB203580 increased the TNCs around 50 fold post 7 days of treatment and induced the CD133+ (more than 10 fold) and CD133+CD38- (more than 85 fold) cell expansion. CFU assay confirmed these findings by increase in CFU-GEMMs colony count post treatment with SB203580. Besides, transplantation of UCB-CD133+ expanded with SB203580 showed the 3 fold increase in engraftment capacity of cells[148]. These findings obtained from mouse and human studies reveals that inhibition of p38 promotes *ex vivo* expansion of human UCB and mouse HSC and SB203580 is promising for therapeutically approach.

4-[2-(1-fluoronaphthalen-2-yl)-4(5)-(4-fluorophenyl)-1H-imidazol-5(4)-yl]pyridine (C7) is a novelazole-based small molecules and an analog of SB203580 compound (p38 MAPK inhibitor). C7 is identified among 50 small molecules by structure-activity-relationship studies. C7 was reported to increase 1,554.1 fold UCB-MNCs compared to SB203580 and

DMSO controls post 11 days of treatment. Besides, C7 also increased 600 fold expansion of CD34+CD38-CD90+CD49f+ cells from UCB. Transplantation of UCB unit expanded with C7 into NSG mice showed the enhancement of engraftment ability of UCB cells. These findings revealed that azole-based C7 is promising for clinical usage of expanded UCB[149].

10074-G5 is an inhibitor of c-Myc proto-oncogene which has a major role in the maintenance of glycolytic metabolism and HSC quiescence. Pharmacologically targeting of c-Myc was found to increase the mouse LSKCD34^{low} 2 fold post 7 days of treatment. Inhibition of c-Myc by 10074-G5 reduced glycolytic activity on mouse HSCs and also, 10074-G5 decreased the CDKI expressions on the expanded cells. Besides, inhibition of c-Myc upregulated the RAD51 gene which is major component of HDR modulation. The effect of 10074-G5 on human UCB cells was similar with mouse cells. It was reported that 10074-G5 induced UCB-CD34⁺ and UCB-CD133⁺ cell expansion. Moreover, the utilization of 10074-G5 along with tauroursodeoxycholic acid (TUDCA) and i-NOS inhibitor L-NIL as a cocktail induced maintenance and expansion of HSC. These findings revealed that 10074-G5 is promising for HSC expansion technologies for clinical usage and gene editing technologies[150].

MEISi-1 and MEISi-2 are inhibitors of MEIS1 gene which belongs to TALE-type class of homeobox gene family. These inhibitors were identified by in silico docking and screening method. The crucial role of MEIS1 protein was mentioned above. Briefly, MEIS1 is known to be highly expressed in bone marrow and promotes the HSC metabolism through transcriptional regulation of HIFs. Knockout of MEIS1 resulted in loss of HSC quiescence and expansion. Therefore, the inhibition of MEIS1 by small molecules was tried for HSC expansion. It was reported that MEISi-1 and MEISi-2 increased the C-kit⁺, Sca1⁺, LSK and LSKCD34^{low} populations *ex vivo* and *in vivo*. Transplantation of expanded cells with MEIS1 inhibitors into NSG mice showed the enhancement of engraftment and repopulation capacities. In addition to mouse, MEISi-1 and MEISi-2 molecules expanded UCB and mPB derived HSPCs. Besides, treatment of MEIS1 inhibitors downregulated p16, p19 and p19^{arf} CDKIs and upregulated PCNA and MCM2 S-phase genes. These findings revealed that MEIS1 inhibitors provided the mouse and human HSC expansion via upregulation of S-phase genes and downregulation of CDKIs[151].

Chrysin (C2968) is an antioxidant compound which is identified by screening of antioxidant compound library to determine the effect on HSC expansion. Usage idea of Chrysin is based

on disadvantages of ROS on cell proliferation and viability. It was reported that Chrysin induced the expansion of UCB-CD34⁺ cells (7,2 fold), CD34⁺CD38⁻CD49f⁺ cells and CD34⁺CD90⁺CD38⁻CD45RA⁻ cells. Besides, Chrysin enhanced the long-term viability. Transplantation of C2968-expanded cells into NSG mice showed the rapid engraftment at 8 weeks of transplantation. It was indicated that Chrysin may reduce ROS-mediated apoptosis and differentiation. These findings revealed that regulation of antioxidant mechanism is promising for clinical aspects of UCB-HSCs[152].

YH250 is an inhibitor of interaction of p300/Catenin. It was reported that YH250 induced HSC proliferation in mice post irradiation. YH250 was also increased the BM cycling cell percentage in S-phase and long-term repopulating cell population (LSK CD150⁺CD48⁻) Besides, treatment of YH250 provided rapid hematopoietic recovery in irradiated mice by increase in number of WBC, lymphocytes, monocytes, neutrophils, and platelets. These findings revealed that downregulation of p300/catenin by YH250 is promising for human HSC expansion[153].

Violuric acid (VA) and **1-naphthoquinone-2-monoxime (N2N1)** were identified by multidrug screening according to the criteria based on decrease in senescence and anti-proliferative profile in cells. Treatment of mouse BM cells with VA and N2N1 along with IL6, IL3, FLT3L, TPO and SCF cytokines resulted in increase in HSC maintenance and growth. Competitive repopulation assay showed the enhancement of HSC function and long-term culture initiating cells (LTC-IC). These findings revealed that VA and N2N1 is promising as novel small molecule stimulants for HSC function and expansion[154].

4E1RCat is an inhibitor of the eIF4F translation initiation complex which is involved in downstream of mTORC1 and regulation of human LT-HSC function *in vivo*. 4E1RCat was identified among 118 compounds screened depending on >1.5-fold increase in the number of human cells post 4 days of culture. Transplantation of 4E1RCat-expanded UCB cells into NSG mice showed the more than 9 fold increase in LT-HSC post 20 weeks. 4E1RCat was revealed to be promising for rapid *ex vivo* expansion of human HSCs and efficient transplantation[155].

Taken together, some of the identified small molecules will proceed to clinical trial phase, some of them are processing for clinical phase trials (shown in Table 1.1) and, many molecules are waiting to discovery for HSC expansion. Due to the advantages of small

molecules will be focus point on the HSC expansion researches and small molecule-mediated HSC expansion will remained the detail research in their side effect on clinical utilization.

Table 1.1. List of small molecules involved in regulation of hematopoiesis

Compound ID	Function	Phase Trial
SR-1	enhancement of human HSC self-renewal	Phase I/II
E478	enhancement of human HSC self-renewal	Not Yet
UM171	enhancement of human HSC self-renewal	Phase I/II
UM729	enhancement of human HSC self-renewal	Not Yet
P18IN003/P18IN011	enhancement of mouse HSC self-renewal	Not Yet
XIE18-6	enhancement of mouse HSC self-renewal	Not Yet
PGE ₂	enhancement of mouse and human HSC self-renewal and homing; reduction of apoptosis	Phase I/II
5azaD/TSA	enhancement of human HSC self-renewal	Not Yet
Garcinol	enhancement of human HSC self-renewal and homing	Not Yet
VPA	enhancement of human HSC self-renewal; reduction of HSC differentiation	Not Yet
NAM	enhancement of human HSC self-renewal and homing; reduction of HSC differentiation	Phase I/II
CHIR99021+Rapamycin	enhancement of mouse and human HSC self-renewal	Not Yet
BIO	enhancement of human HSC self-renewal	Not Yet
NR-101	enhancement of human HSC self-renewal	Not Yet
TEPA	enhancement of human HSC self-renewal	Phase I/II
DEAB	enhancement of human HSC self-renewal; reduction of HSC differentiation	Not Yet

zADfmk/zLLYfmk	enhancement of HSC self-renewal and homing; reduction of apoptosis	Not Yet
5-HT	enhancement of human HSC self-renewal; reduction of apoptosis	Not Yet
SB203580	enhancement of mouse and human HSC self-renewal and homing	Not Yet
C7 Compound	enhancement of human HSC self-renewal	Not Yet
10074-G5	enhancement of mouse and human HSC self-renewal	Not Yet
MEISi-1 and MEISi-2	enhancement of mouse and human HSC self-renewal	Not Yet
Chrysin	enhancement of human HSC self-renewal; reduction of HSC differentiation and apoptosis	Not Yet
YH250	enhancement of human HSC self-renewal	Not Yet
VA+N2N1	enhancement of mouse HSC self-renewal	Not Yet
4E1RCat	enhancement of human HSC self-renewal	Not Yet

We previously analyzed about 200 genes which are known to be knockout in HSCs *in vivo*. We primarily found that 109 genes from 200 genes increased the cell cycle activity or proliferation of HSCs following knockout of the genes. Then, 57 of these 109 genes showed increase in the expansion of stem cell pool more than 1,5 fold. After that, we selected 30 genes which have availability as small molecule inhibitor using small molecule databases. Among them 26 compound was commercially found. In addition to them, 9 different genes were identified which are associated with quiescence property from our cardiac regeneration study (shown in Figure 1.4). It was known that cardiomyocytes are similar with HSCs about cell quiescence. Therefore, we thought that these 9 cardiac quiescence factors may regulate the HSC quiescence. Eventually, we have identified 35 putative hematopoietic small molecules (HSM) targeting 35 hematopoietic and cardiogenic quiescence factors. 9 of them were tested on previous studies. 26 of them have not been previously studied for small molecule induced *ex vivo* HSC expansion. The small molecules targeting these quiescence genes are commercially available inhibitors and listed in Table 3.1.

We investigated these 26 putative HSMs for their potential roles in *ex vivo* HSC expansion comparing to previously tested 9 HSMs. We aimed to identify the HSMs and the most effective HSM cocktail which enhance the expansion of human HSCs for improvement of a GMP-grade medium.

1.4. GOOD MANUFACTURING PRACTISES

Medical products have to be produced in obvious quality standards according to determined usage and license requirements. Good Manufacturing Practices control production of medical products depending on quality standards, determined usage and license requirements in accordance with requirement of product specifications.

1.4.1. Requirement for Human Medical Products in GMP

In GMP, production processes are clearly defined. The critical steps and changes performed in production processes are validated. Qualified-educated personnel, appropriate facility-area, appropriate equipment-service, correct material labelling, approved procedures and appropriate storage-transportation are required for GMP. Whole instructions and procedures are clearly written. Whole processes are recorded to ensure that the processes are truly performed. GMP is associated with quality assurance, quality control and quality risk. Quality assurance aims to guarantee that medical products are in required quality for determined usage goal. In the scope of quality assurance, there are several criteria such as that medical products have to be developed and designed according to requirement of GMP, production and control processes have to be clear, production and recruitment of correct starting and package materials have to be organized, validations have to be done on the semi-manufacturing by required controls and other in-process controls, finished product have to be truly processed and controlled depending on determined procedures, and medical products have to be stored and handed out at appropriate conditions to provide continuance then quality during shelf-life.

Quality control involves sampling, specification, testing, organization, documentation, release procedures to determine the quality of the product. There are approved procedures, educated personnel and sufficient facilities for testing, examination and sampling of starting

and package materials, semi-manufacturing and finished products. Sampling of starting and package materials, semi-manufacturing and finished products are performed by approved personnel and methods by quality control. Test methods are validated. A new production formula or process method should be validated to show suitability of them for routine processes. Whole reports associated with all sampling, examination and test procedures are recorded. Reference sample is stored to re-investigate for starting materials and products if it is required. Quality risk is a process for examination, control and evaluation of risk on product quality. Evaluation of quality risk depends on scientific information and is associated with protection of patients.

In GMP, personnel play a critical role and they are educated about principles of GMP. The number of personnel should be sufficient. The personnel should work according to organization schema. The personnel is interested in production and storage of the medical product according to appropriate documentation, approvment of the instructions, evaluation of the production, controlling the care of department and facilities, securing the validation processes. Hygiene is important point for GMP. All personnel should be undergone a medical examination. Working in production department should be prevented from the personnel who have open wound or contagious disease. Everybody who entry to production area should wear protective clothes which appropriate to work. The facilities and equipments should be built, designed, adapted and situated depending on the work that will be done. The biological preparats or medical products that have high sensibility (eg. Penicillin etc.) should be produced in separated, independent and closed facilities to prevent cross-contamination. Starting materials or products should be prevented from another material or product. Cross-contamination can be avoided by production in separated areas, air ejection / airlock, recirculation of air, protective clothes, decontamination processes and usage of label which refers the cleaning position.

Good documentation is important for quality control and key of GMP. Good documentation aims to reveal, follow, control and record all activities which effect on the quality of medical products. There are two documentation types for GMP: instructions (guidelines, requirements) and records/reports. The documents including the instructions should be approved and signed by the authorities. The documents should not be written by hand. The hand-written documents should be clear, readable and not deleted. The changes on the data in documents should be recorded with signature and date. These changes should be done in

was that original information is read. Reports should be preserved until after one year from expiration date of the series. Raw materials, semi-manufacturing, agent should be labelled. In labelling, name of producer, raw material, test results, reports and date should be indicated.

Laboratory records include all tests data performed for guarantee suitability to specifications and standards. Appropriate laboratory facilities should be ready to use for quality units. There should be procedures which describe sampling, rejection, testing, improvement of materials and record of laboratory data. Approved specifications including impurity controls should be for effective materials. If effective material has microbiological impurity, total microbial count and action limits should be occurred. Laboratory controls should be reported and explained. Reagents and standard solutions should be prepared and labeling according to written procedures. Contamination profile should be identified for each effective material or semi-manufacturing. Contamination profile is compared to previous data to determine the changes occurred by modifications in production process. Microbiological tests should be performed for each effective material determined which of microbial quality. After analysis, the results are reported. Analysis certificate involves name of the effective material or semi-manufacturing, serial number and date of the test and release. The certificates are signed by authorized personnel and dated.

1.4.2. Special Requirements of Effective Materials Produced by Cell Culture and Fermentation

Classic fermentation principles in small molecule production and usage processes of recombinant or non-recombinant organisms are same. Production of effective material and semi-manufacturing obtained by cell culture and fermentation includes cell culture, achievement from living organisms and purification. Used raw materials (medium, tampon components) may be potent contaminants. To secure the effective material, all production phases should be controlled. To reduce the contamination risk, appropriate equipment and environmental controls are used. The process controls include maintenance of cell bank, growing and inoculation of the culture, controlling of critical points in cell culture, following of cell growth and vitality, cell isolation, purification and decontamination procedures, measuring the bio-burden and endotoxin level. Cell substrates, mediums, tampon

components are added by using closed and protective systems. Biosafety cabin or similar controlled environment is used to prevent the microbiological contamination. The personnel should wear proper clothes in the cell culture. Cell culture environment should be sterilized before use and equipments in cell culture should be cleaned and sterilized after their usage. The contamination cases are recorded. Effective safety requires remove of viruses. The processes about remove and neutralization of viruses should be validated. In addition, proper pre-secture should be taken to prevent viral contamination risk before or after remove of viruses. For more detail information, ICH (The International Conference on Harmonization) Q5A *“Quality of Biotechnological Products: Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin”* guideline.

The controls in production of effective materials that will be used in clinical experiment should be proper to improvement of drug. Process and test procedures should be flexible to allow the changes because of that the process information is increased during improvement of the clinic phase from pre-clinic phase. The production of the effective material used in clinical experiment should be documented with laboratory notebook, serial record and other proper methods. The analytical methods used for evaluation of effective material serial in clinical trials should be scientifically approved even if not validated. The data obtained during production and improvement of effective material that will be used in clinical trials should be reported and recorded in a safe system. The cell banks designed to prevent contamination and provide vitality are maintenance in proper storage conditions. ICH Q5D *“Quality of Biotechnological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological / Biological Products”* guideline is utilized for more detail information about cell banking.

1.4.3. Production of Steril Medical Products

The production of sterile products required reduction of microbial and pyrogenic contamination risk. The production of sterile products should be performed in clean area where personnel and equipments entry to passing from airlocks. Various processes including preparation of components; product preparation and storage should be realized in separated from each other clean areas. The clean areas used for production of sterile product

are categorized as various environment properties to 4 class according to EN ISO (International Organization for Standardization) 14644-1: Grade A-B-C-D.

Grade A is high risk operation area and, laminar air flow work in this area. Grade B is background of class A and it is for preparation and storage of aseptic. Grade C/D is required for less critic steps of sterile product production. Particle count and microbiological monitoring are important for GMPs. The limits are determined by authorized organizations. In the clean areas, there should be required at least personnel. All personnel in these areas should take an education about truly production of sterile products. Whole sterilization processes should be validated. There are many ways for sterilization; heat-sterilization, steam-sterilization, dry heat-sterilization, radiation-sterilization, ethylene oxide-sterilization.

GMP provide the production of medical product such as animal-derived products, allergen products, animal anti-serum products, vaccines, recombinant products, monoclonal antibody products, transgenic animal and plant products, gene therapy products and somatic & xenogeneic cell therapy products under sterile conditions by using validated and controlled materials and procedures after testing of their effect on human health. To achieve more detail information for GMP, it is utilized as a guideline ‘*Good Manufacturing Practices (GMP) Guide for Manufacturing Plants of Human Medicinal Products*’. This GMP-related section was written by utilization from this guideline[156].

1.4.4. GMP-Grade Products for HSC Expansion

Development of identified small molecule as a drug or expansion culture medium and/or growth factors have become popular approach for successful HSC transplantation in clinical area. Drug development is a long process that required many pre-clinical and post-clinical steps. As mentioned above, many small molecules are in progress for clinical trials. However, several cell culture methods including culture systems, expansion mediums or supplements have been designed for HSC expansion. Utilization of these methods in clinics is required to production under GMP conditions.

GMP-grade products that related to HSC expansion have been developed until today. For instance, StemPro™ HSC expansion medium is produced by ThermoScientific Company

under cGMP conditions for cell expansion and gene therapy. It was reported that culture of mPB-CD34⁺ cells with the serum-free StemPro™ HSC expansion medium supplemented with FLT3, SCF, IL-3 and IL-6 cytokines enhanced the expansion of Lin-CD34⁺CD90⁺CD45RA⁻ (LT-HSC) around 200 fold and Lin-CD34⁺CD45⁺ around 100 fold in short-term. Besides, xenogeneic transplantation showed the enhancement of short-term self-renewal at 8 weeks of transplantations[157]. In addition, Stemline and Stemline II (improved version of Stemline product) produced by Sigma is an optimized GMP-grade HSC expansion medium. It was reported the increase in expansion of TNC from UCB, BM and mPB[158]. Besides, Sigma and Stemgenix co-developed the GEM/Stemline medium for HSC expansion. The comparison of Stemline/GEM HSC expansion medium with other research-grade HSC expansion mediums; StemSpan H2000 and X-VIVO 15 showed that Stemline HSC expansion medium enhanced the expansion of early and committed progenitors[159]. Another expansion medium is developed as CellGenix® GMP Stem cell growth medium by CellGenix. It is optimized for expansion of HSPCs and especially natural killer cells. These medium was used for culture of induced embryonic stem cells[160]. Moreover, Miltenyi Biotech developed GMP-compliant HSC-Brew GMP Medium which is optimized for expansion of HSPCs. Another product is related to cryopreservation of HSCs. HSC-BANKER® GMP grade is produced by ZENOAQ Company for cryopreservation of HSCs[161]. In addition to expansion culture and freezing medium, there are many developed GMP-compliant culture supplements. The importance in development of expansion technologies and basic culture systems results in maintenance of the detail researches for clinical aspects of HSC transplantation.

2. AIM OF THE STUDY

In the light of the studies discussed above sections, HSCs characterized by quiescence state and cell cycle arrest are an essential therapeutic way to treat the hematological malignancies by transplantation. UCB and BM are main source of HSC transplantation, but HSCT is limited by low number of HSC resulted by their quiescence. HSC quiescence is mainly promoted by cell cycle kinetics and quiescence-related signaling pathways. Modulation of these critical regulators could present a perspective for human HSC expansion. Small molecules are new promising approach to overcome this limitation because of their characteristic properties. We have previously showed that the stem cell specific knockout of quiescence factors could induced HSC *ex vivo*. Therefore, we hypothesized that targeting to quiescence factors by putative HSMs identified as inhibitor of component in stem cell regulation pathways found by knockout studies could be induce HSC expansion *ex vivo* and *in vivo*. Therefore, we aimed to identify the most effective HSMs and HSM combinations to develop chemically-defined human HSC expansion medium for successful HSC transplantation.

3. MATERIALS

3.1. INSTRUMENTS

Cytoflex S Flow Cytometry (Beckman, Cat. No: B47903, USA), S3E Cell Sorter (BioRad, Cat. No.24L4D), Hettich® ROTINA 38/38R Centrifuge (Sigma, Cat. No: Z720119), Centrifuge (Labogene, Cat. No: 416), Mini centrifuge (Sigma, Cat. No: Sigma 1-14), EasyEights™ EasySep™ Magnet (Stemcell Technologies, Cat. No. 18103), PCR Thermal Cycler (BioRad, Cat. No:T100), LightCycler96™ Real-Time PCR System (Roche, Cat. No:12953), Nano-Drop 2000 (ThermoScientific, Cat. No:ND-2000), Carl Zeiss Inverted Microscopy (Zeiss, Cat. No:3849000464), Laminar Flow Cabinet, Incubator (ThermoScientific, Cat. No:3141), Shaking Water Bath (Witeg, Cat. No: DH.WSB03018), Vortex (Interlab, Cat. No:622.02.001), Mini Spin Centrifuge (Interlab, Cat. No:603.01.001), Autoclave, -200/+40 Refrigerators.

3.2. BLOOD SAMPLES

Umbilical Cord Blood and human mobilized Peripheral blood samples were obtained from healthy individuals by Yeditepe University and Anatolian Health Center, respectively. The severe combined immunodeficient mice and Balb/C wild-type mice were used for *in vivo* studies. All human studies were approved by the Institutional Clinical Studies Ethical Committee of Yeditepe University (Decision numbers 1067 and 944).

3.3. SMALL MOLECULES

Hematopoietic small molecules (HSMs) listed in Table 3.1 were dissolved in diverse solvents according to solubility properties of HSMs as 10mM stock concentration. Dimethyl sulfoxide (0.25 percent) was used as a positive control (DMSO, Santa Cruz Biotech, Cat. No:Sc-3590329).

Table 3.1. List of hematopoietic small molecules used in the experiments, their target proteins and solubility properties with their related companies/catalog numbers

Commercial Name of HSM	Targeted Protein	Proposed HSM	Vendors/ Catalog No	Solubility
Alexidine Dihydrochloride (tested)	Ptpmt1	HSM 1	SCBT Sc-210775A	Methanol 25mg/ml
p300/CBP Inhibitor VI	P300/CBP	HSM 2	Milipore 505298	DMSO 50 mg/ml
AS1949490	Inpp5d	HSM 3	Sigma SML1022	DMSO 20 mg/ml
RG-108	Dnmt3 α	HSM 4	Abcam ab141013	DMSO 100 mM
Garcinol (tested)	P300/CBP	HSM5	ENZO BML-GR343-0010	DMSO 25mg/ml
CHIR-99021 (tested)	GSK3 β	HSM6	Sigma SML1046	DMSO 2 mg/ml
10074-G5	C-myc	HSM 7	Sigma G3798	DMSO 10 mg/ml
Pluripotin	Tet-2	HSM 8	Sigma SML0444	DMSO 10 mg/ml
Pifithrin- α	p53	HSM 9	SCBT Sc-45050	DMSO 20 mg/ml
Ro-26-4550 Trifluoro-Acetate	Il-2	HSM 10	Tocris 1794	DMSO 28,03mg/ml
Gant61	Gli-1	HSM 11	Abcam ab120904	DMSO 50 mM
Hif-1 α Inhibitor	HIF-1 α	HSM 12	Milipore 400083	available as 25 mM in DMSO
Stemregenin-1 (tested)	AhR	HSM13	Cayman 10625	DMSO 10mM
Casin	CDC42	HSM 14	SCBT sc-397016	DMSO 100 mM

AMD3100 (tested)	CXCR4	HSM 15	Milipore 239820	available as 50 mM in H ₂ O
Ro5-3335	Runx-1	HSM 16	Milipore 219506	DMSO 50 mg/ml
SKP2-C25	Skp2	HSM 17	Milipore 506305	DMSO 5 mg/ml
bpV(hoP _{IC}) (tested)	Pten	HSM 18	SCBT sc-221377	H ₂ O
2-APB	Gjb-1	HSM 19	Sigma D9754	DMSO 20 mg/ml
Nicotinamide (tested)	Sirt1	HSM 20	SCBT CAS 98-92-0	DMSO 100mM
Tauroursodeoxy- cholic acid (TUDCA)	UI	HSM 21	Sigma T0266	Ethanol 20.40 mg/ml
SB203580 (tested)	P38 MAPK	HSM 22	ENZO BML-EI286	DMSO 50mg/ml
α -Tocopherol	UI	HSM 23	Sigma 258024	Liquid 0.950 g/ml
L-NIL	iNOS	HSM 24	Sigma I8021	H ₂ O 50 mg/ml
BIO (tested)	GSK-3	HSM 25	Sigma B1686	DMSO 5mg/ml
SKF-96365 Hydrochloride	Ca ²⁺ Entry	HSM 26	Tocris 1147	H ₂ O 100 mM
Mdivi-1	GTPase	HSM 27	Sigma M0199	DMSO 20mg/ml
(5Z)-7-Oxozeaenol	TAK1	HSM 28	Sigma 499610	DMSO 10mg/ml
SB239063	P38 MAPK	HSM 29	Sigma S0569	DMSO 11mg/ml
2-PCPA	MAO	HSM 30	Sigma P8511	Ethanol 50mg/ml
K252c	Protein Kinase C	HSM 31	SCBT CAS 85753-43-1	DMSO 25mM
BML-260	JSP-1	HSM 32	SCBT CAS 101439-76-3	DMSO 25mg/ml
TAME	APC	HSM 33	Sigma T4626	H ₂ O 40mg/ml
Meisi-1	Meis-1	HSM 34	MEINOX Cas# 446306-43-0	DMSO 10mg/ml

Meisi-2	Meis-1	HSM 35	MEINOX Cas#2250156-71-7	DMSO 10mg/ml
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*UI: Unidentified

3.4. CELL CULTURE MEDIA AND SUPPLEMENTS

Cell culture media and their supplements are provided from Invitrogen, Gibco, UK Company, Stemcell Technologies, Canada Company for expansion of primarily isolated cells. The list of cell culture components with ordered number were listed on the Table 3.2.

Table 3.2. Catalog Numbers/Vendor of cell culture mediums and their supplements used in the experiments

Materials	Vendor/Catalog Number
Roswell Park Memorial Institute Medium (RPMI) 1640 Medium	Gibco 12001-534
Iscoe's Modified Dulbecco's Medium (IMDM)	Gibco 12440053
Fetal Bovine Serum, qualified , heat inactivated, Canada origin	Gibco 12483020
StemSpan™ CC100 (100X)	Stemcell Technologies 02690
Human Recombinant Protein Thrombopoietin	Peprtech 300-18
Human Recombinant Protein Stem Cell Factor	Peprtech 300-07
Human Recombinant Protein Flt-3	Peprtech 300-19
Insulin-Transferrin-Selenium (ITS) (100x)	Thermo Fisher 41400045
Antibiotic-Antimycotic (100X)	Gibco 15240062

3.5. ISOLATION OF HUMAN UMBILICAL CORD BLOOD- AND MOBILIZED PERIPHERAL BLOOD-HEMATOPOIETIC STEM AND PROGENITOR CELLS

Umbilical cord blood and mobilized peripheral blood derived mononuclear cells (PMBCs) were isolated by using ficoll gradient centrifuge technique. Table 3.3 shows the required chemicals used for isolation with vendor and catalog numbers.

Table 3.3. Vendor /catalog number information of specific materials and chemicals used for MNCs isolation

Specific Chemicals	Vendor/Catalog Number	Aim of Utilization
Ficoll-Paque Histopaque™	Sigma 1083	Isolation of MNCs
Plastic Blood Bag T&T PED CPD/1	Kansuk 20000006204	Collection of UCB
Plastic spray-coated K2EDTA Tube	BD 367835	Collection of PB
Phosphate Buffer Saline (DPBS)	Gibco 14190250	General use

3.6. DETERMINATION OF BONA FIDE HSC MARKERS ON THE EXPANDED CELLS

Expansion levels of UCB and mPB derived MNC with small molecule effect were analyzed by flow cytometry following labelling of MNCs with surface markers. Table 3.4 shows HSPC surface markers used for the study with their vendor and catalog numbers.

Table 3.4. HSPC surface markers with their conjugated fluorochromes which were ordered from eBioscience and BD Companies

Antigens	Conjugated Fluorochromes	Catalog Number
Anti-human CD133	APC	eBioscience 12-0349-42
Anti-human CD90	FITC	eBioscience 11-0909-41
Anti-human CD34	PE	eBioscience 17-1338-42
Anti-human CD38	PE-Cyanine7	eBioscience 25-0389-42

3.7. CELL CYCLE ANALYSIS AND ANNEXIN-V APOPTOSIS DETECTION ASSAY

CD34+ cells were used for cell cycle and apoptosis analysis. The components of both assays were listed in Table 3.5 with their vendor and catalog numbers.

Table 3.5. Components of cell cycle and apoptosis assay with their vendor/catalog numbers

Specific Kits and Chemicals	Vendor/Catalog Number	Aim of Utilization
eBioscience™ Annexin V-FITC Apoptosis Detection Kit	ThermoFisher Scientific BMS500FI-20	Apoptosis Assay
Hoechst 33342 (10µg/ml)	Sigma 14533	Cell Cycle Analysis
Pyronin Y (100µg/ml)	Sigma P9172-1G	

3.8. GENOTOXIC ANALYSIS, METABOLIC ALDH MEASUREMENT AND ROS DETECTION ASSAYS ON MPB-HSPC

Expanded MNCs with HSMs were analyzed by flow cytometry following using of Apoptosis, DNA damage and cell cycle kit. In addition to that, the metabolic profiles of MNCs were determined post treatment with HSMs. Table 3.6 shows that the specialized kits for these experiments with their vendor/catalog number information.

Table 3.6. The list of specialized kits which belongs to apoptosis-DNA damage and cell cycle analysis and metabolic assays with their vendor/catalog number information

Specific Kit	Vendor/Catalog Number	Aim of Utilization
Apoptosis, DNA Damage and Cell Cycle Kit	BD 562253	Apoptosis, DNA Damage and Cell Cycle Analysis
Aldefluor Kit	Stemcell Technologies 01700	ALDH Measurement
DCFDA / H2DCFDA Cellular ROS Assay Kit	Abcam ab113851	ROS Detection

3.9. COLONY FORMING UNIT ASSAY

Colony forming unit assay is required for methylcellulose-based medium including recombinant cytokines for human MNCs and was used to detect the origin of expanded cells. The specific equipment and components for CFU assay are listed in the Table 3.7 with the vendor and catalog number information.

Table 3.7. Specific Chemicals/Equipments for CFU Assay provided by Stemcell Technologies

Specific Kit	Vendor/Catalog Number
MethoCult™ Classic	Stemcell Technologies H4434
16 gauge blunt-end needles	Stemcell Technologies 28110
3cc syringes	Stemcell Technologies 28240

3.10. CFSE PROLIFERATION ASSAY ON MPB-HSPC

Proliferation assay were performed by using CFSE (Carboxyfluorescein succinimidyl ester) fluorescent dye (CellTrace™ CFSE Cell Proliferation Kit, ThermoFisher Scientific, Cat.No: C34570).

3.11. VIABILITY ASSAY ON MPB-HSPC

The viability of expanded cells was determined by flow cytometry following labeling of the treated cells with 7AAD (7-Amino-Actinomycin D) (BD, Cat.No:559925).

3.12. GENE EXPRESSION ANALYSIS

The changes of the gene expression levels (including previously identified HSC-related gene pool, CDKI genes, S-phase genes, glycolysis-related genes, engraftment-related genes and the target genes of the protein inhibited by BML-260 and TAME HSMs) were analyzed by Real-Time Polymerase Chain Reaction (RT-PCR). Gene expression analysis was performed post cDNA synthesis following RNA isolation from expanded MNCs. Table 3.10 shows that specific kits for RNA isolation, cDNA synthesis and quantitative PCR analysis.

Table 3.8. The list of specialized kits for RNA isolation, cDNA synthesis and RT-PCR with vendor/catalog number

Specific Chemicals, Kits or other components	Brand/Catalog Number	Aim of Utilization
Invitrogen PureLink RNA Mini Kit	ThermoFisher Scientific 12183025	RNA Isolation
RevertAid First Strand cDNA Synthesis Kit	ThermoFisher Scientific K1622	cDNA synthesis
SYBR™ Green PCR Master Mix	Applied Biosystem 4309155	Real-Time PCR

For RT-PCR, predesigned human primers obtained from diverse databases (Gene Cards etc.) and literature were ordered from Sentegen and listed in Table 3.11.

Table 3.9. The sequences of human primers (5 prime to 3 prime) ordered from SENTEGEN, TURKEY

Genes	Forward Primers (5' to 3')	Reverse Primers (5' to 3')
Housekeeping Genes		
β-ACTIN	GTTGTCGACGACGAGCG	AGCACAGAGCCTCGCCTTT
RPL13a	GGCCCAGCAGTACCTGTTTA	AGATGGCGGAGGTGCAG
S-Phase and DNA Repair-related Genes		
RAD17	AAAGTGTCGCTTCAGAGGGA	TGCCTACCAGCTTTATGCCT
RAD51	CTGGTGGTCTGTGTTGAACG	CTGAGGGTACCTTTAGGCCA
MSH2	GGATCAAATGAAGGTTTTACAAGG	TTGGCAGTTTTTGTGACTCC
MCM2	CCAGGCCTCTCTTGATGTCT	TAGCCTCTCCAAGGATCAGC
XRCC1	TCATAGTCTTGCTCCCCAGC	GGCGAGAAGACCATCTCTGT
XRCC2	TCTACCTTCAAGTCGGGCAA	TAGAGTCTGCGCAGTTGGTG
ERCC1	GCAGGACATCAAACAGCCTC	CAGACCCTCCTGACCACATT
BRCA2	ACAAATAGACGAAAGGGGCA	GCCCCTTCACTTCAGCAAAT
PCNA	AAGAGAGTGGAGTGGCTTTTG	TGTCGATAAAGAGGAGGAA GC
RPA1	AGTAGAACCCATTCCCGAGC	GGCAATCCAGTGCCCTATAA
MRE11a	GGGAAGGCTGCTGTCTTGTA	CAACCAACAAAGGAAGAGG C
USP45	TCTAGAAAATGCACTTGTTTGTG	TCGACACATTGAATAACTCG
Cyclin Dependent Kinase Inhibitor Genes		
P15	TACAGGAGTCTCCGTTGGC	GTGAGAGTGGCAGGGTCTG
P16	GCCGCTTCCTAGAAGACCAG	GTGAGAGTGGCGGGGTC
P18	ACGTCAATGCACAAAATGGA	CAAATCGGGATTAGCACCTC
P19	AACCGCTTCGGCAAGAC	GTCCTGGACATTGGGGCT

P21	TGCGTTCACAGGTGTTTCTG	GTCCACTGGGCCGAAGAG
P27	AAGAAGCCTGGCCTCAGAAG	TTCATCAAGCAGTGATGTAT CTGA
P57	AGCTGCACTCGGGGATTT	AAGAAATCGGAGATCAGAG GC
Target Genes		
SKP2	ATGCCCCAATCTTGTCCATCT	CACCGACTGAGTGATAGGTG T
CDH1	AAAGGCCCATTTCTAAAAACCT	TGCGTTCTCTATCCAGAGGC T
P53	GCTTTCCACGACGGTGAC	GCTCGACGCTAGGATCTGAC
C-MYC	GGTCCTGGCAAAAGGTCA	CTGCGTAGTTGTGCTGATGT
BAX	CCCGAGAGGTCTTTTCCGAG	CCAGCCCATGATGGTTCTGA T
BCL-2	GGTGGGGTCATGTGTGTGG	CGGTTCAAGTACTCAGTCAT CC

4. METHODS

4.1. CELL ISOLATION AND CULTURE CONDITIONS

UCB samples were collected from the newborn at the birth time with parent's consent by Yeditepe University and human mPB samples were collected from healthy individuals by Anatolian Health Center. UCB and PB mononuclear cells were isolated by Ficoll-Paque (Histopaque™, Sigma, Cat.No:10831) density gradient centrifugation (1,083g/ml) method. Firstly, the blood samples were diluted as 1:1 proportion in a 15 ml falcon tube with Dulbecco's Phosphate-Buffered Saline (DPBS, Thermofisher Scientific, Cat. No:14190144) and mixed gently by inverting. Diluted blood samples were set over 15 ml Ficoll-Paque and centrifuged at 3000 rpm for 15 minutes without brake. After centrifugation, upper phase including serum was removed and cloudy interphase, also known as buffy coat, was transferred to new falcon tube. The transferred cells were washed with 3x volume of DPBS and mixed by gentle inverting. The cells were centrifuged at 1500 rpm for 5 minutes with brake and then, the supernatant was removed. The pellet was re-suspended in 10 ml DPBS and mononuclear cells (MNCs) were counted by hemocytometer to perform the appropriate experiments. UCB- and mPB-MNCs were cultured in the expansion medium which consists of Roswell Park Memorial Institute (RPMI) 1640 Medium (Gibco, Cat.No:11875085) supplemented with 2 percent FBS (Gibco, Cat.No:12483020) 1 percent PSA (10,000 units/ml penicillin and 10,000 ug/ml streptomycin and 25 µg/mL of Amphotericin B; Gibco, Cat.No:15240062) and human cytokine cocktail (StemSpan™ CC100, Stemcell Technologies, Cat.No:02690). Besides, to obtain CD34⁺ cells from MNCs, the MNCs were labelled with DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) (Thermofisher Scientific, Cat.No:D1306) and PE-conjugated CD34, (Biolegend, Cat.No:343506) according to the manufacturer's manual post isolation of the MNCs from fresh samples. The CD34⁺ cells were separated from the labelled cells by (Biorad S3E Cell Sorter, Cat. No:24L4D).

4.2. DETERMINATION OF THE EFFECT OF EACH PUTATIVE HSM ON *EX VIVO* EXPANDED-HSC CONTENT

The human UCB- and mPB-MNCs were seeded on 96 well plate at a density of 30,000 cells per well in the expansion medium. The seeded cells were treated with each of 35 putative HSMs at 0,1 μ M, 1 μ M and 10 μ M concentrations. The cells used as positive control were treated with dimethyl sulfoxide (DMSO) (0,25 percent). The treated cells were incubated in 7 days at 37°C, in 5 percent CO₂ and under normoxic conditions. The treated UCB- and mPB- MNCs were labelled with FITC-conjugated CD90, PE-conjugated CD34, PE-Cy7-conjugated CD38 (Biolegend, Cat. No:328107, 343506 and 303516; respectively) and APC-conjugated CD133 (eBioscience, Cat.No:17-1338-42) antibodies according to the manufacturer's manual (1:1000 dilution ratio). The HSC content of labelled cells were analyzed by flow cytometry (Cytoflex S, Beckman, Cat.No:B47903) (n=3).

4.3. DETERMINATION OF THE EFFECT OF SELECTED HSM ON CELL CYCLE AND APOPTOSIS PROCESSES OF EXPANDED CELLS

To be able to analyze the effect of HSMs on cell cycle, human CD34⁺ cells from UCB or mPB-MNC were sorted from MNC population by fluorescence activated cell sorting (FACs) after labelling with anti-human PE-conjugated CD34 as described above. The CD34⁺ cells were seeded in the HSC expansion medium at a density of 10,000 cells per well in 96 well-plate. The cells were treated with effective doses (shown in Table 4.1) of the p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules, determined by flow cytometry analysis, (n=3). After 4 days of the treatment, expanded HSCs were labeled with Hoechst 33342 (1ng/sample; stock: 10 μ g/ml) (Sigma Aldrich, USA, Cat. No:14533) and incubated at 37°C and 5 percent CO₂ for 30 minutes, then stained with Pyronin Y (10ng/sample; stock:100 μ g/ml) (Sigma Aldrich, USA, Cat.No:P9172-1G) and incubated at 37°C and 5 percent CO₂ for 15 minutes. Following the staining, the cells were analyzed by flow cytometry compared to DMSO control (0,25 percent).

To determine whether the HSMs display apoptotic effect on the cells, the sorted human mPB- CD34⁺ cells were seeded at a density of 10,000 cells/well on 96-well plate and treated with effective doses (shown in Table 4.1) of p300/CBP Inhibitor VI, AMD3100, 2-APB,

BML-260 and TAME molecules (n=3). After 72 hours of the treatment, the cells were collected from 96-well plates and centrifuged at 1500 rpm for 5 minutes. The pellets were re-suspended in 50 μ l of 1X binding buffer (BD Pharmingen, Cat. No:556570), then stained with 1ul of FITC Annexin V and incubated at room temperature for 15 minutes. Then 200 μ l 1X binding buffer was added onto the stained cells and labelled with 2ul of PI (Propidium Iodide) according to the modified manufacturer's manual (eBioscience, Cat. No:88-8005-74). The stained cells were analyzed by flow cytometry compared to DMSO control (0,25 percent).

Table 4.1. The effective doses of HSMs used for cell cycle analysis and apoptosis assays

HSMs	Treatment Doses	
	mPB	UCB
p300/CBP Inhibitor VI (HSM#2)	10 μ M	Not Found
AMD3100 (HSM#15)	0,1 μ M	Not Found
2-APB (HSM#19)	0,1 μ M	1 μ M
BML-260 (HSM#32)	0,1 μ M	10 μ M
TAME (HSM#33)	1 μ M	0,1 μ M

4.4. DETERMINATION OF THE EFFECT OF THE SELECTED HSM ON DNA DAMAGE, APOPTOSIS AND CELL CYCLE PROCESSES OF THE EXPANDED CELLS

The changes on cellular processes occurred by induction of HSC expansion with HSMs were evaluated with Apoptosis, DNA Damage and Cell Cycle Kit (BD, Cat. No:562253) post 7 days of treatment in three replicates. Firstly, the mPB- MNCs (at least 100,000 cells /well) were treated with effective doses of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules and 0,25 percent of DMSO (shown in Table 4.1)(n=3). Utilization of this assay designed with the inclusion of fluorescent antibodies specific for incorporated BrdU-PC5.5, which is related to cycling of cells, phosphorylated H2AX-APC, which promotes DNA repair and maintains genomic stability and cleaved PARP-PE, which functions to recruit and localize DNA repair proteins or cell cycle checkpoint factors to

DNA-damaged sites was required to antibody binding following the fixation and permeabilization of the cells according to manufacturer's protocol.

4.5. DETERMINATION OF THE EFFECT OF SELECTED HSM ON METABOLISM OF HSC

The metabolic assays were performed to determine the metabolic effect of HSMs on HSCs. For that, aldehyde dehydrogenase (ALDH) enzyme activity, which is known as non-immunological method to identify the HSPCs, was measured on expanded MNCs derived from mPB post treatment with effective doses (shown in Table 4.1) of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules and 0,25 percent of DMSO in three replicates according to manufacturer's protocol (Stemcell, Aldefluor Kit, Cat.No:01700). Briefly, 30,000 cells/well were collected and centrifuged at 300g for 5 min. The pellet was re-suspended in 200ul of ALDEFLUOR assay buffer. 1ul of ALDEFLUOR reagent was added to all samples. The cells were analyzed by flow cytometry following 45 minutes incubation at 37 °C. ALDEFLUOR™ Diethylaminobenzaldehyde (DEAB) reagent (1ul in 200ul sample) was added to control tube. The control tube was analyzed post labeling with ALDEFLUOR reagent following 4 hours of incubation with DEAB. The gating on FITC was set according to DEAB control (n=3).

To detect the Reactive Oxygen Species (ROS) production resulted by enhancement of metabolic activity, the mPB-MNCs seeded at a density of 30,000 cells/well were treated with effective doses (shown in Table 4.1) of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules and 0,25 percent of DMSO. Post 7 days of treatment, the ROS activity was measured on expanded HSPCs by flow cytometry post labelling of ROS with 2',7'-dichlorofluorescein diacetate (DCFDA) fluorogenic dye according to manufacturer's protocol (DCFDA / H2DCFDA - Cellular ROS Assay Kit, Abcam, Cat.No:ab113851). Briefly, the treated cells were stained with (20µM working concentration) DCFDA for 30 min (without washing) and then, measured by flow cytometer with excitation / emission at 485 nm / 535 nm. 50 µM Tert-Butyl Hydrogen Peroxide (TBHP) was used as a positive control following dilution from 55mM stock solution. (n=3).

4.6. DETERMINATION OF THE EFFECT OF SELECTED HSMS ON MULTI-LINEAGE ABILITY OF EXPANDED UCB CELLS

To determine the origin of the expanded cells, the human UCB-MNCs were seeded at 30,000 cells/well on 96-well plate and treated with the most effective doses (shown in Table 4.1) of 2,APB, BML-260 and TAME molecules and 0,25 percent of DMSO. After 7 days expansion, the cells were harvested and counted. After that, the cells were plated in methylcellulose-containing medium (MethoCult™ GF M3434, Stemcell Technologies, Cat.No:03444) at a density of 20,000 cells per well in 6-well plate, performed in triplicate. After 10-14 days, colonies were classified as CFU-GEMM, CFU-G/M, BFU-E and B cell colonies and counted by light microscopy (n=3).

4.7. DETERMINATION OF VIABILITY ON HSM-EXPANDED MPB HSPC

The human mPB-MNCs were seeded on 96-well plate at a density of 30,000 cells per well in the expansion medium. The seeded cells were treated with the most effective doses of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260, TAME molecules and DMSO control. After 7 days of treatment, the viability of expanded cells was determined by flow cytometry following labeling of the treated cells with 7AAD (7-Amino-Actinomycin D) (1:1000 dilution) (BD, Cat.No:559925). The count and percentage were quantified for analysis (n=4).

4.8. DETERMINATION OF THE EFFECT OF SELECTED HSM ON PROLIFERATION CAPACITY OF EXPANDED MPB HSPC

The human mPB-CD34⁺ were seeded on 30,000 cells per well in the expansion medium. The seeded cells were treated with the effective doses (shown in Table 4.1) of AMD3100, BML-260 and TAME molecules and 0,25 percent of DMSO. The treated cells were labelled with 5-(and -6) carboxyfluorescein succinimidyl ester (CFSE) (as 5µM final concentration for 15 min. at 37⁰C) (CFDA-SE, Stemcell Technologies, Cat.No:75003) and the labelling was stopped with 10 percent FBS/PBS. The sample separated at day0 was analyzed by flow

cytometry. After 1, 4 and 7 days of incubation time, the CFSE⁺ cells in labelled cells were analyzed by flow cytometry (n=2).

4.9. DETERMINATION OF THE EFFECT OF THE MOST EFFECTIVE HSM COCKTAIL FOR *EX VIVO* HSC EXPANSION

The analysis to determine the most effective mixture of HSMs for *ex vivo* HSC expansion is based on indicating each HSM effect following exclusion each HSM from all mixture. To determine the best HSM mixture, UCB-MNCs were isolated as described in section 4.1 and were treated with different HSM combinations. Then, HSC content were analyzed as described in section 4.2. By the way, each result revealed the effective new combination. We reduced the combination including seven HSMs after obtained the results. Then, ALDH and ROS level were measured post 7days of treatment with the newly prepared mixture as described in section 4.5.

4.10. DETERMINATION OF THE HUMAN HSC EXPANSION MECHANISM BY GENE EXPRESSION ANALYSIS

To determine the mechanism of HSC expansion by using the small molecules, the gene expression changes of previously the genes of CDKs, CDKIs, S-phase, glycolysis-related and engraftment-related genes were analyzed by Real-Time Polymerase Chain Reaction (RT-PCR). For that, the RNA isolation from mPB-MNCs were performed by Invitrogen PureLink RNA Mini Kit (Thermoscientific, Cat.No:12183025) according to the manufacturer's protocol post 4 days of treatment with effective doses (shown in table 4.1) of BML-260 and TAME molecules and 0,25 percent of DMSO following culture of 2×10^6 cells per well on the 6 well plate. After the RNA isolation, RNAs were converted to cDNA by using ProtoScript® First Strand cDNA Synthesis Kit (NEB, Cat.No:E6300S). The PCR conditions of cDNA synthesis process was showed in Table 4.3. For quantitative PCR analysis, GoTaq® qPCR Master Mix (Promega, Cat.No:A6002) was used with predesigned primers (shown in Table 3.9) obtained from databases (Gene Card etc.) and ordered from Sentegen Biotechnology, Turkey. qPCR reactions were conducted on LightCycler® 96 Real-Time PCR system (Roche Health Care, Cat.No:12953). The conditions of RT-PCR

was showed in Table 4.5. Data were analyzed by using $\Delta\Delta C_t$ method and GAPDH and β -Actin were used as a housekeeping gene to normalize the results.

Table 4.2. The ratio of Thermo Scientific RevertAid First Strand cDNA Synthesis Kit used for cDNA synthesis from RNA

Reagents	Volume
RNA	3 μ g
Random Primers	1 μ l
Reaction Mix 5X	4 μ l
RiboLock RNase Inhibitor (20 U/ μ L)	1 μ L
RevertAid M-MuLV RT Enzyme 200U/ μ l	1 μ l

Table 4.3. Set-up conditions for cDNA synthesis PCR

Temperature ($^{\circ}$ C)	Time
25 $^{\circ}$ C	5 min.
42 $^{\circ}$ C	60 min.
70 $^{\circ}$ C	5 min.
+4 $^{\circ}$ C	∞

Table 4.4. The used ratio of Applied Biosystems™ SYBR™ Green PCR Master Mix reagents and primers

Reagents	Volume
Syber Green 2X	7,5 μ l
Primer Forward	0,75 μ l (0,5 μ M)
Primer Reverse	0,75 μ l (0,5 μ M)
Template	2 μ l (100ng/reaction)
Distilled Water	4 μ l (up to 15 μ l)

Table 4.5. Set-up conditions for RT-PCR in Lightcycler 96

Step	Temperature (°C)	Time	Cycle
Initial Activation Step	95	120s	1
Quantification	95	15 s	55
	60	60 s	
Extansion	72	45 s	1
Melting Curve	95	10	1
	55	60	
	97*	1	
Cooling	37	30s	1

4.11. COMPARATIVE ANALYSIS OF CHEMICALLY-DEFINED MEDIUM COMPOSITIONS PREPARED WITH DIFFERENT SUPPLEMENTATIONS

To determine the effective chemically-defined basal medium including appropriate supplementations, RPMI 1640 and IMDM basal mediums were prepared as different compositions with 5 percent FBS, 10 percent FBS, 20 percent FBS and serum-free condition. These medium compositions were prepared in two types; with and without insulin-transferrin-selenium mixture. 0,1 μ M concentration of BML-260 small molecule and cytokine combinations (SCF, TPO and FLT3) were common supplements for every medium compositions (Table 4.6). Post culture of mPB-MNCs with these different medium compositions, HSPC content analysis was performed by flow cytometry following the labelling of the expanded cells with human HSC surface markers as describe in section 4.1.

Table 4.6. The chemically-defined HSC expansion medium compositions

Supplements	FBS	Cytokines (STF)	BML-260 (0,1 μ M)	ITS
RPMI/IMDM-1	SF	+	+	-
RPMI/IMDM-2	SF	+	+	+
RPMI/IMDM-3	5 percent	+	+	-
RPMI/IMDM-4	5 percent	+	+	+
RPMI/IMDM-5	10 percent	+	+	-
RPMI/IMDM-6	10 percent	+	+	+
RPMI/IMDM-7	20 percent	+	+	-
RPMI/IMDM-8	20 percent	+	+	+

4.12. STATISTICAL ANALYSIS

The statistics were analyzed by Dunnett's Multiple Comparison Test and p values was used. If the values were $p < 0.05$, this results were considered statistically significant and if $p < 0.01$ were measured statistically more significant. In addition to "2-tailed Student t test" was used to determine the level of significance.



5. RESULTS

5.1. IDENTIFICATION OF HEMATOPOIETIC SMALL MOLECULES FOR *EX VIVO* EXPANSION OF HUMAN HSPC

To develop small molecule-based human HSC expansion technique, we formed hematopoietic small molecule pool. We previously analyzed about 200 genes, which are known to be knockout in HSCs *in vivo*. We primarily found that 109 genes from 200 genes increased the cell cycle activity or proliferation capacity of HSCs following knockout of themselves. Then, 57 of 109 genes was found to increase the expansion of stem cell pool more than 1,5 fold. After that, we selected 30 genes from 57 genes according to their availability as small molecule inhibitor in ChEMBL, PubChem and etc. small molecule databases. 26 compounds, which are related to 26 of 30 selected genes, have been procured. In addition to them, we decided to investigate 9 new compounds, which was found to be related to quiescence of cardiomyocytes by our cardiac regeneration studies. The idea that HSCs display similar quiescence profile with cardiomyocytes suggested that identified quiescence factors on cardiomyocytes could regulate the HSC quiescence. Eventually, we have identified 35 putative HSM targeting 35 hematopoietic quiescence factors (Figure 5.1). 9 of them were tested on previous studies. 26 of them have not been previously studied for small molecule induced *ex vivo* HSC expansion and were tested in this study (Table 3.1).

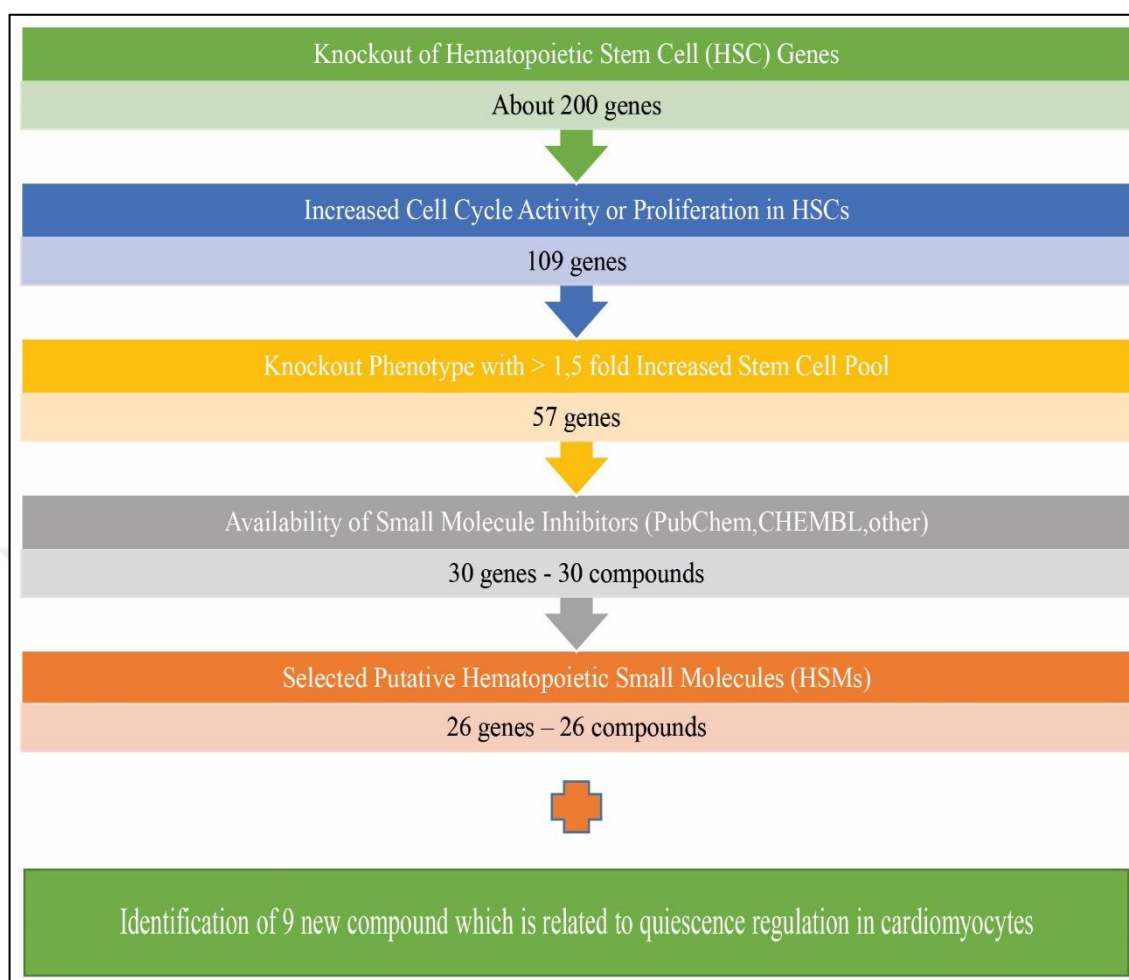


Figure 5.1. Identification strategy of hematopoietic small molecules which used for human HSC expansion

5.2. DETERMINATION OF THE EFFECTIVE HSM THROUGH ANALYSIS OF UCB-HSPC CONTENT

To determine the effective HSMs among 35 putative molecules on *ex vivo* HSC expansion, the first step was the evaluation of the changes on HSC content of UCB-MNCs post 7 days of treatment with 0,1 μ M, 1 μ M and 10 μ M doses of all HSMs. Flow cytometry analysis was performed post labelling of the HSM-expanded UCB-MNCs with human HSC characterization markers; CD34-PE, CD38-PC7, CD133-APC and ALDH-FITC. The gating

strategy used for flow cytometry analysis was schematically presented in Figure 5.2a. DMSO (0,25 percent) was used as a positive control.

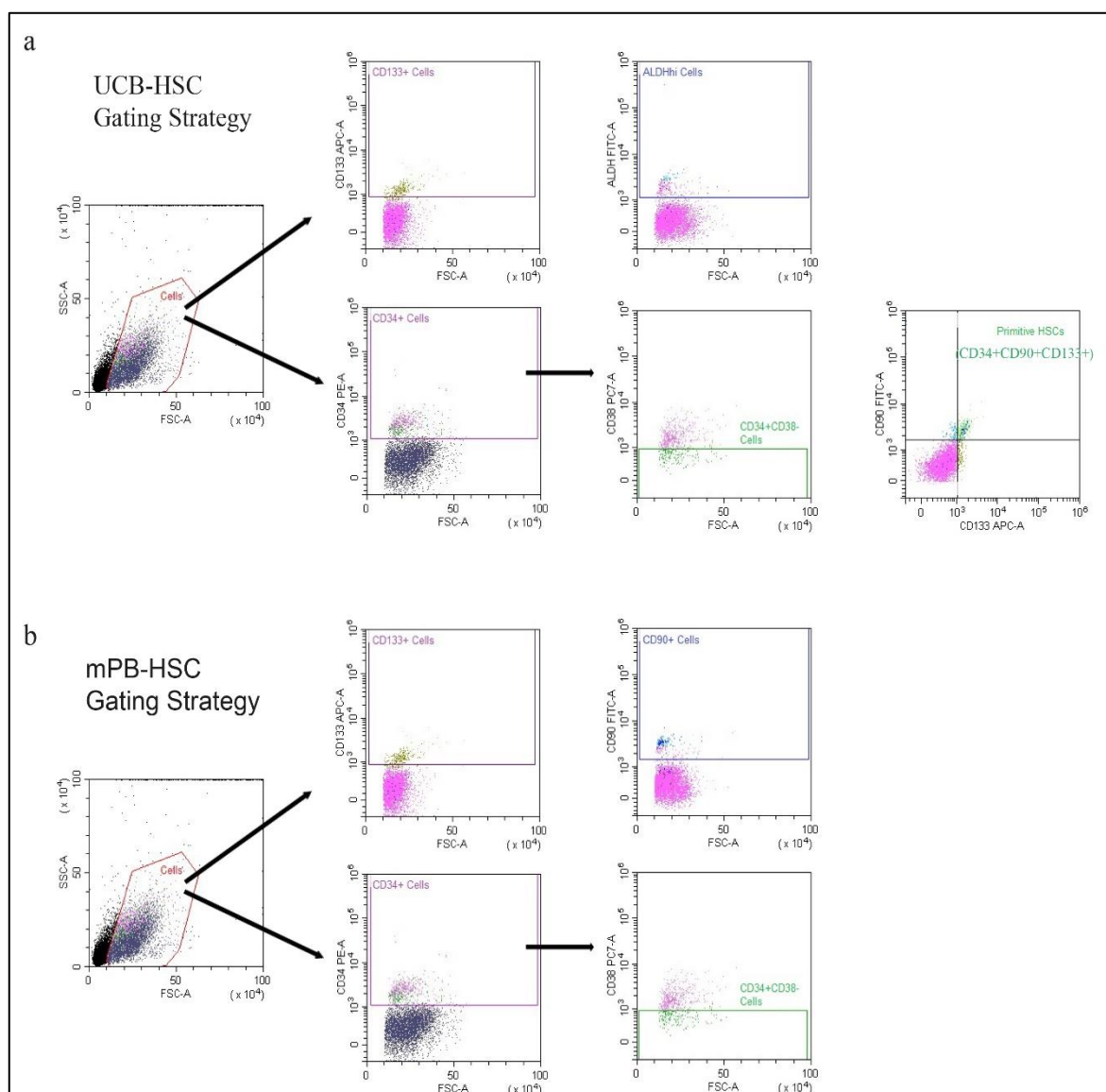


Figure 5.2. Gating strategy of flow cytometry analysis for a) UCB-HSC, b) mPB-HSC to determine HSC content on human HSPCs.

Post HSM screening, we indicated the increase in total MNC count post treatment with HSM. When we compared the average results of three different doses of HSMs to untreated sample, we found that treatments of #9, #12, #15, #16, #17, #20, #21, #22, #25, #31, #32, #33, #34 and #35 numbered molecules increased the total cell count more than 2-fold (Figure

5.3a). Then, we examined the total MNC count results of HSMs in a dose dependent manner. We noticed that while treatments of #12, #19 and #35 numbered molecules increased the total cell count as arising of the doses; on the contrary, treatments of #5, #29 and #30 numbered molecules increased the total cell count as decrease of the doses (Figure 5.3b).



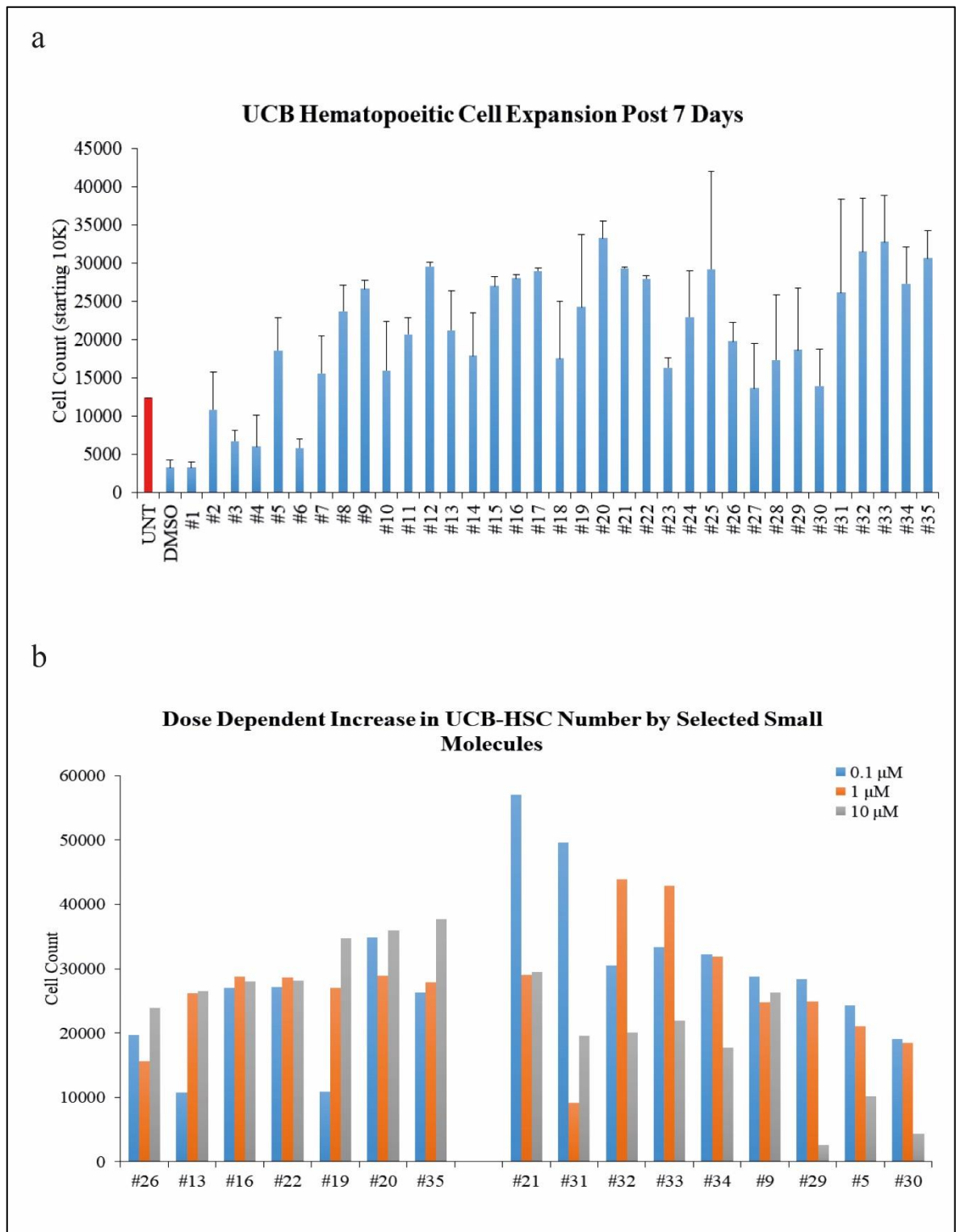


Figure 5.3. Quantification of flow cytometry analysis which performed for determination of HSM effect on total cell count of UCB-HSPCs. a) The graphic shows the average of all results which belonged to three doses of HSM, b) The graphic shows the dose dependent results of effective HSMs.

As shown in our gating strategy, CD34⁺ cell population which include HSPCs was evaluated post treatment of HSMs. Flow cytometry analysis showed that treatments of #3, #5, #8, #9, #15, #16, #18, #25, #26, #31 and #32 numbered molecules increased the CD34⁺ cell count more than 2-fold. Treatments of #10, #11, #19, #22, #23 and #33 numbered molecules increased more than 4-fold while treatments of #7, #12, #17, #24, #34 and #35 numbered molecules increased CD34⁺ cell count more than 3-fold. Besides, the enhancement effect of #13, #14 and #20 numbered molecules was more than 5-fold. Interestingly, #2 numbered molecule increased the CD34⁺ cell count more than 7-fold. Other compounds did not show any significant changes on CD34⁺ cell count (Figure 5.4a). In a dose dependent manner, #1, #4, #7, #10, #11, #13, #14, #18, #22, #23, #34 and #35 compounds were attracted the attention (Figure 5.4b). Among them, treatments of #4, #10 and #14 numbered molecules increased the CD34⁺ cell ratio as long as the dose. However, treatments of #1, #7, #11, #13, #18, #22, #23, #34 and #35 numbered molecules increased the CD34⁺ cell ratio as decrease of the doses.

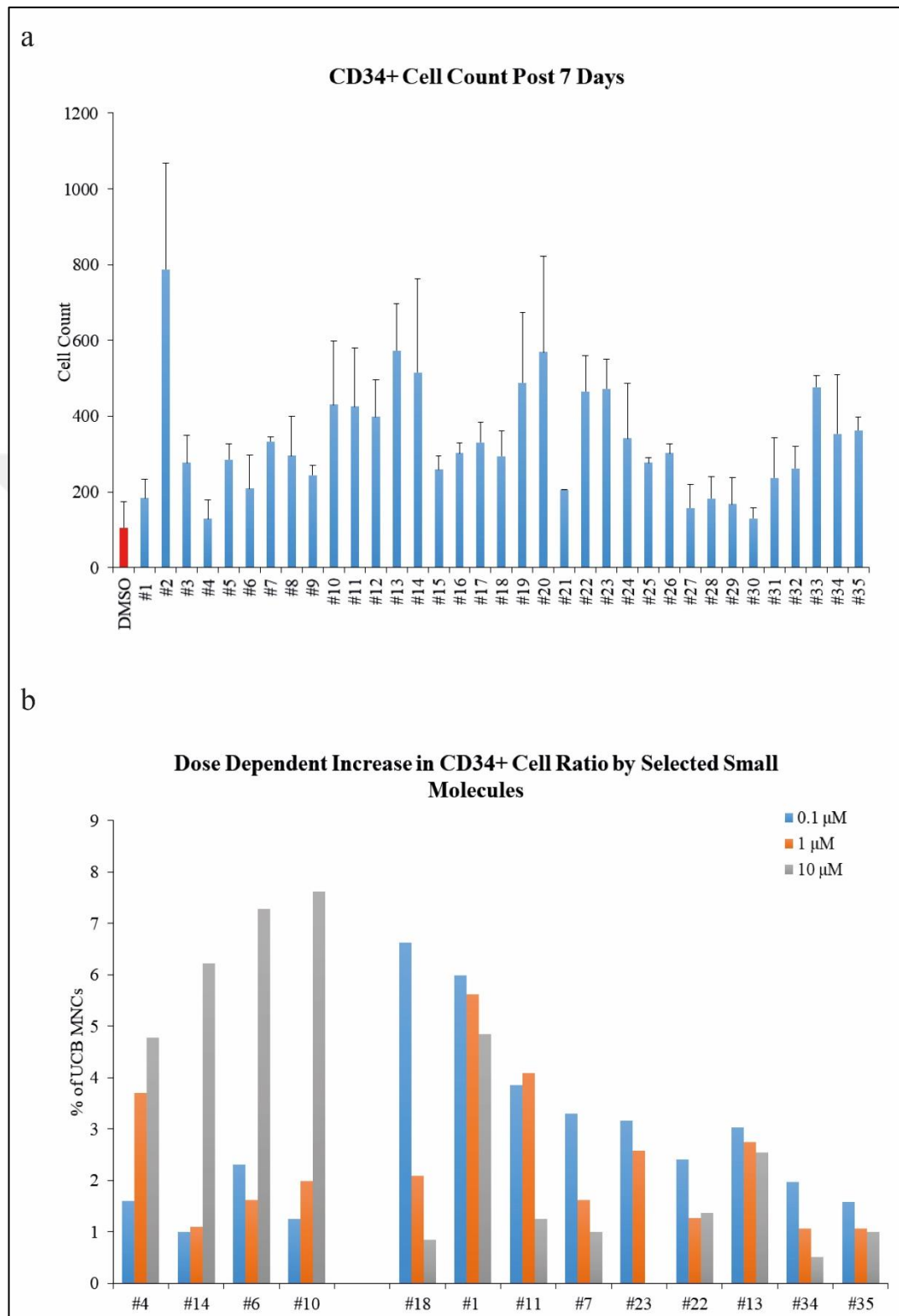


Figure 5.4. Quantification of flow cytometry analysis which performed for determination of HSM effect on CD34+ cell content of UCB-HSPCs. a) The graphic shows the average of cell count results which belonged to three doses of HSM, b) The graphic shows the dose dependent percentage results of effective HSMs.

Next, we evaluated the frequency of CD133+ cell population in HSM treated-UCB-MNCs. The treatment of our HSM pool resulted that treatments of #4, #13, #18, #19, #34 and #35 numbered molecules induced CD133+ cell expansion more than 2-fold according to average of all doses results. In addition, we found that treatments of #1 and #6 numbered molecules increased the CD133+ cell ratio more than 3-fold while treatment of #3 numbered compound increased more than 4-fold (Figure 5.5a). When it was examined the dose dependent results in details, we found that #4 and #6 numbered molecules displayed the increase pattern on CD133+ cell ratio as long as the dose. #9, #10, #16, #27 and #35 numbered molecules showed decrease pattern on CD133+ cell ratio as long as the dose (Figure 5.5b).



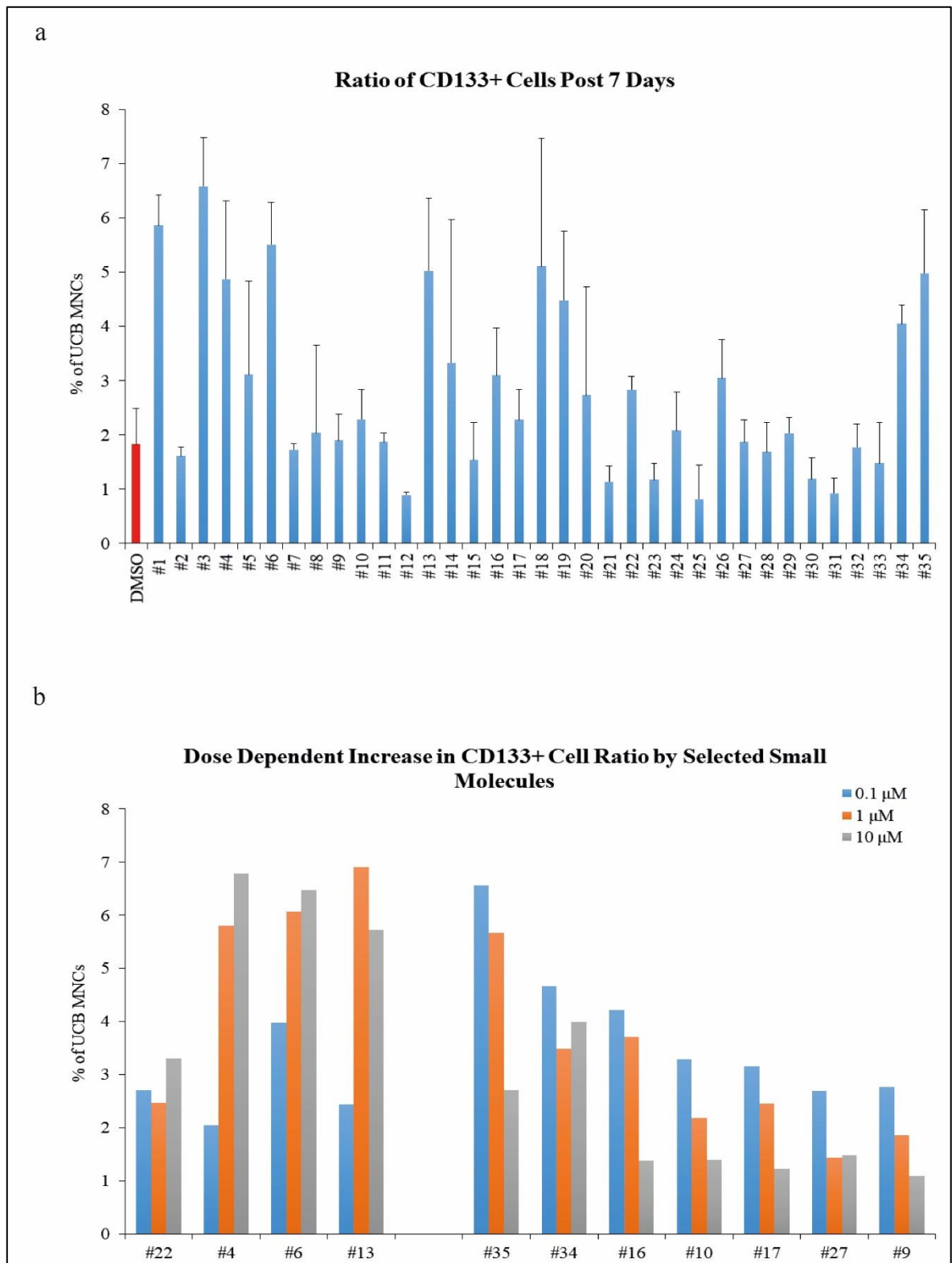


Figure 5.5. Quantification of flow cytometry analysis which performed for determination of HSM effect on CD133+ cell content of UCB-HSPCs. a) The graphic shows the average of cell ratio results which belonged to three doses of HSM, b) The graphic shows the dose dependent CD133+ cell ratio results of effective HSMs.

Then, we analyzed ALDHhi population because of the knowledge that aldehyde dehydrogenase enzyme activity is a metabolic marker for HSCs. Because, it is known that ALDH activity is high level in HSCs because of metabolically glycolysis pathway preference for energy production. ALDH enzyme activity was found to be changed post 7 days of treatment with HSMs. We determined that treatments of #9, #11, #12, #16, #22, #23, #24, #26 and #32 numbered molecules increased the ALDHhi cell count approximately 2-fold. Besides, treatments of #5, #14, #17, #18, #34 and #35 numbered molecules increased the ALDHhi cell count more than 3-fold; treatments of 13, #19, #20 and #33 numbered molecules increased more than 4-fold to 6-fold (Figure 5.6a). Moreover, we found that increase ratio in ALDHhi cell population of #1 numbered molecule treated-cells was parallel with dose increase, but increase ratio in ALDHhi population of #18, #34 and #35 numbered molecules treated –cells was parallel with dose decrease in a dose dependent manner (Figure 5.6b).

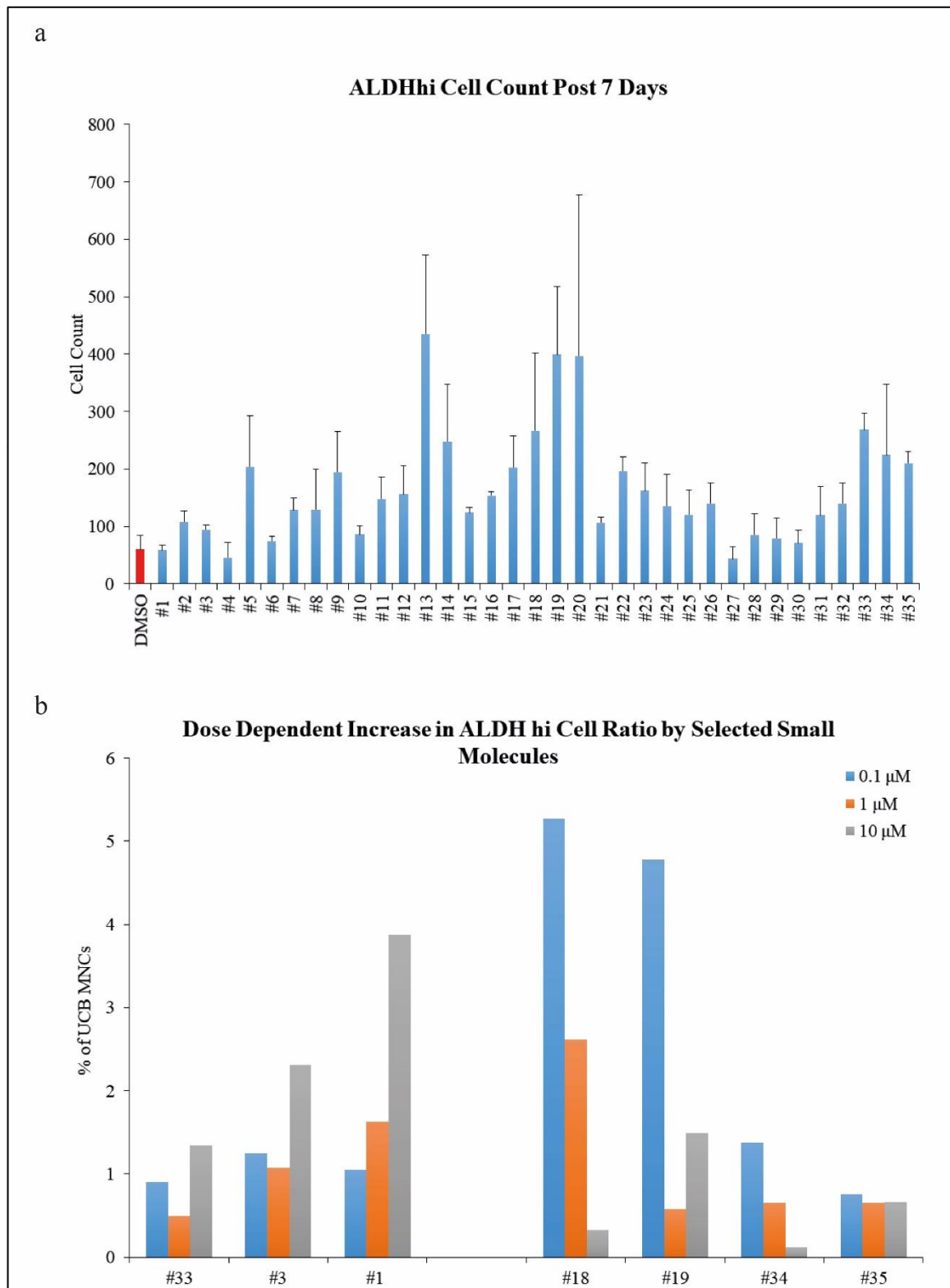


Figure 5.6. Quantification of flow cytometry analysis which performed for determination of HSM effect on ALDHhi cell content of UCB-HSPCs. a) The graphic shows the average of ALDHhi cell count results which belonged to three doses of HSM, b) The graphic shows the dose dependent ALDHhi cell ratio results of effective HSMs.

Taken together, we briefly determined that among 35 HSMs, treatment of #1 (Alexidine Dihydrochloride-previously tested molecule) numbered-molecule increased the CD133+ cell ratio more than 3-fold. Treatment of #2 (p300/CBP Inhibitor VI) numbered-molecule remarkably increased the total cell count more than 7-fold. Treatment of #5 (Garcinol-previously tested molecule) compound resulted in increase in ALDHhi cell count more than 3-fold. Treatment of #13 (Stemregenin I- previously tested molecule) molecule enhanced CD133+ cell ratio 2-fold; CD34+ and ALDHhi cell count more than 4-fold. Treatment of #15 (AMD3100- previously tested molecule) molecule increased the total cell count and CD34+ cell count more than 2-fold. Treatment of #18 (bpV(Hopic)- previously tested molecule) molecule showed 2-fold increase in CD133+ cell ratio and more than 2-fold increase in CD34+ and ALDHhi cell count. Treatment of #19 (2-APB) molecule was found to increase CD34+ and ALDHhi cell count more than 4-fold and CD133+ cell ratio more than 2-fold. Treatment of #20 (Nicotinamide- previously tested molecule) molecule increased the CD34+ and ALDHhi cell count more than 4-fold. Treatment of #32 (BML-260) molecule increased total cell count, CD34+ cell count and ALDHhi cell count more than 2-fold. Treatment of #33 (TAME) molecule had been recorded 2-fold increase in total cell count, 4-fold increase in CD34+ and ALDHhi cell count. Treatments of #34 (MEISi-1) and #35 (MEISi-2) inhibitors provided 2-fold increase in total cell count and CD133+ cell ratio, 3-fold increase in CD34+ and ALDHhi cell count.

On the light of these results, we selected these Alexidine Dihydrochloride (#1), p300/CBP Inhibitor VI (#2), Garcinol (#5), Stemregenin I (#13), AMD3100 (#15), bpV(Hopic) (#18), 2-APB (#19), Nicotinamide (#20), BML-260 (#32), TAME (#33), MEISi-1 (#34) and MEISi-2 (#35) compounds for the next experiments to investigate their role in human HSC expansion. The previously tested compounds were positive controls for our experiment.

Next, we performed the HSPC content analysis on UCB-HSPCs to confirm the previous results post 7 days of treatment at 0,1 μ M, 1 μ M and 10 μ M doses. The analysis in a dose dependent manner showed that 10 μ M concentration was the most effective dose for the CD34+ cell expansion for prominent HSMs; p300/CBP Inhibitor VI, bpV(Hopic), and BML-260 (Figure 5.7a), (Table 5.1). On the other hand, we found on average data of three doses results that treatments of p300/CBP Inhibitor VI, bpV(Hopic) and BML-260 compounds significantly increased the CD34+ cell content more than 19-fold, 6-fold and 3-

fold compared to DMSO control, respectively. Other compounds did not show any significant increase in CD34⁺ cell content (Figure 5.7b).

Table 5.1. The data of percentage and fold difference which belongs to UCB-CD34⁺ cell content quantification results

Compound Name	Effective Dose	CD34⁺ percent in MNCs compared to DMSO	Fold Difference compared to DMSO
p300/CBP Inhibitor VI	10 μ M	82,76 vs 1,46	$\approx$ 57-fold
bpV(Hopic)	10 μ M	17,83 vs 1,46	$\approx$ 12-fold
BML-260	10 μ M	11,58 vs 1,46	$\approx$ 8-fold

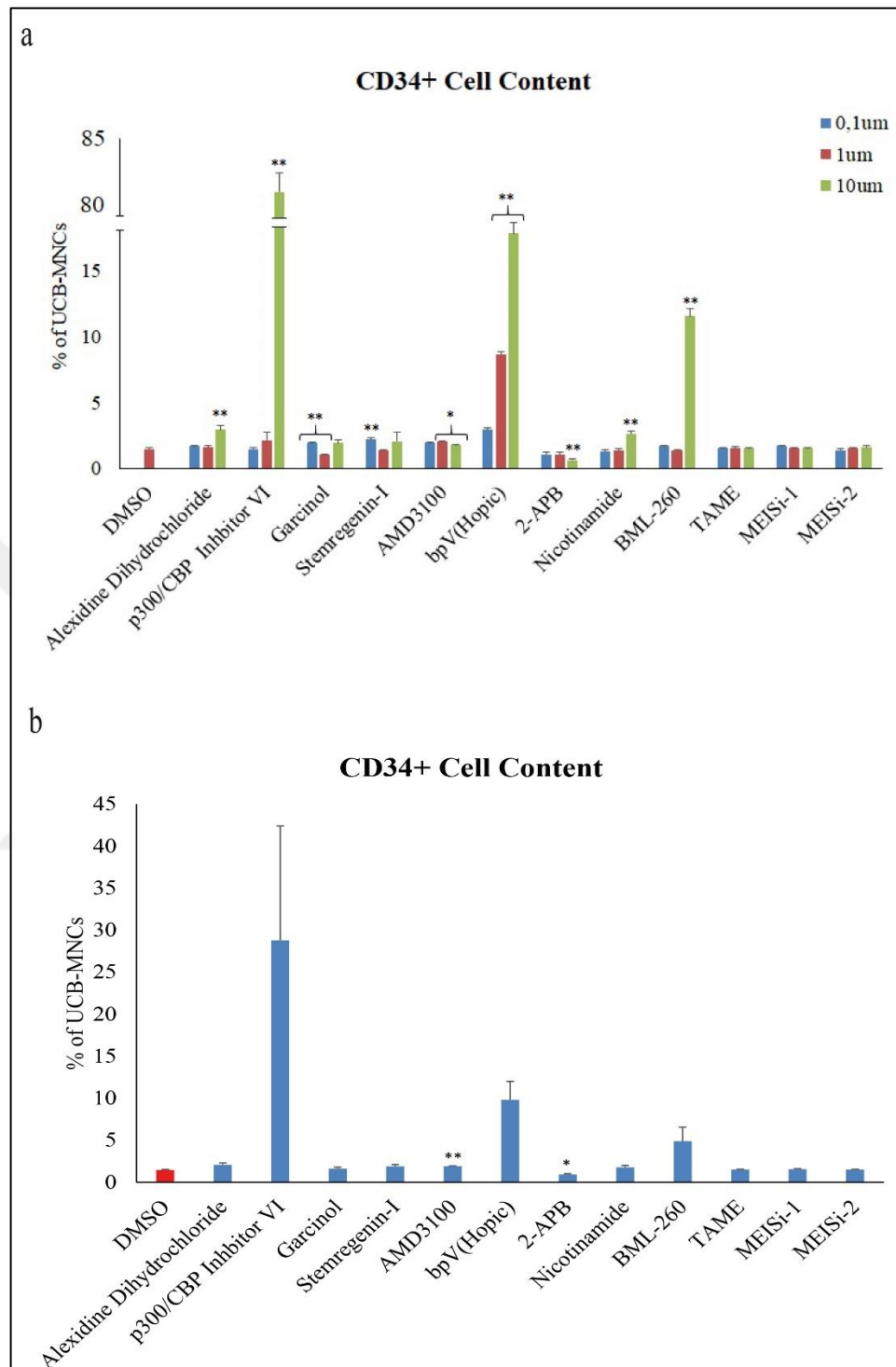


Figure 5.7. Quantification of flow cytometry analysis which performed for confirmation of the effect of selected HSMs on UCB-HSPCs. a) The graphic shows the CD34+ cell percentage in a dose dependent manner of selected HSMs (n=3, *p<0,05, **p<0,01), b) The graphic shows the average of all results which belonged to three doses of HSMs on CD34+ cell percentage (n=9, *p<0,05, **p<0,01).

Then, we analyzed the primitive HSC (CD34+CD90+CD133+ cells) content for selected HSMs to confirm the first screening results post 7 days of treatment. The analysis in a dose dependent manner showed that 10 μ M concentration was found to be prominent dose for the HSC expansion for p300/CBP Inhibitor VI, bpV(Hopic), and BML-260 compounds (Figure 5.8a) (Table 5.2). Besides, we found on average data of three doses results that treatments of p300/CBP Inhibitor VI, bpV(Hopic) and BML-260 compounds significantly increased the primitive HSC content more than 10-fold, 93-fold and 3-fold compared to DMSO control, respectively (n=9) (Figure 5.8b). Other compounds did not show significant increase in CD34+ cell content.

Table 5.2. The data of percentage and fold difference which belongs to UCB-primitive HSC content quantification results

Compound Name	Effective Dose	Primitive HSC percent in MNCs compared to DMSO	Fold Difference compared to DMSO
p300/CBP Inhibitor VI	10 μ M	1,08 vs 0,04	$\approx$ 27-fold
bpV(Hopic)	10 μ M	8,38 vs 0,04	$\approx$ 200-fold
BML-260	10 μ M	0,40 vs 0,04	$\approx$ 10-fold

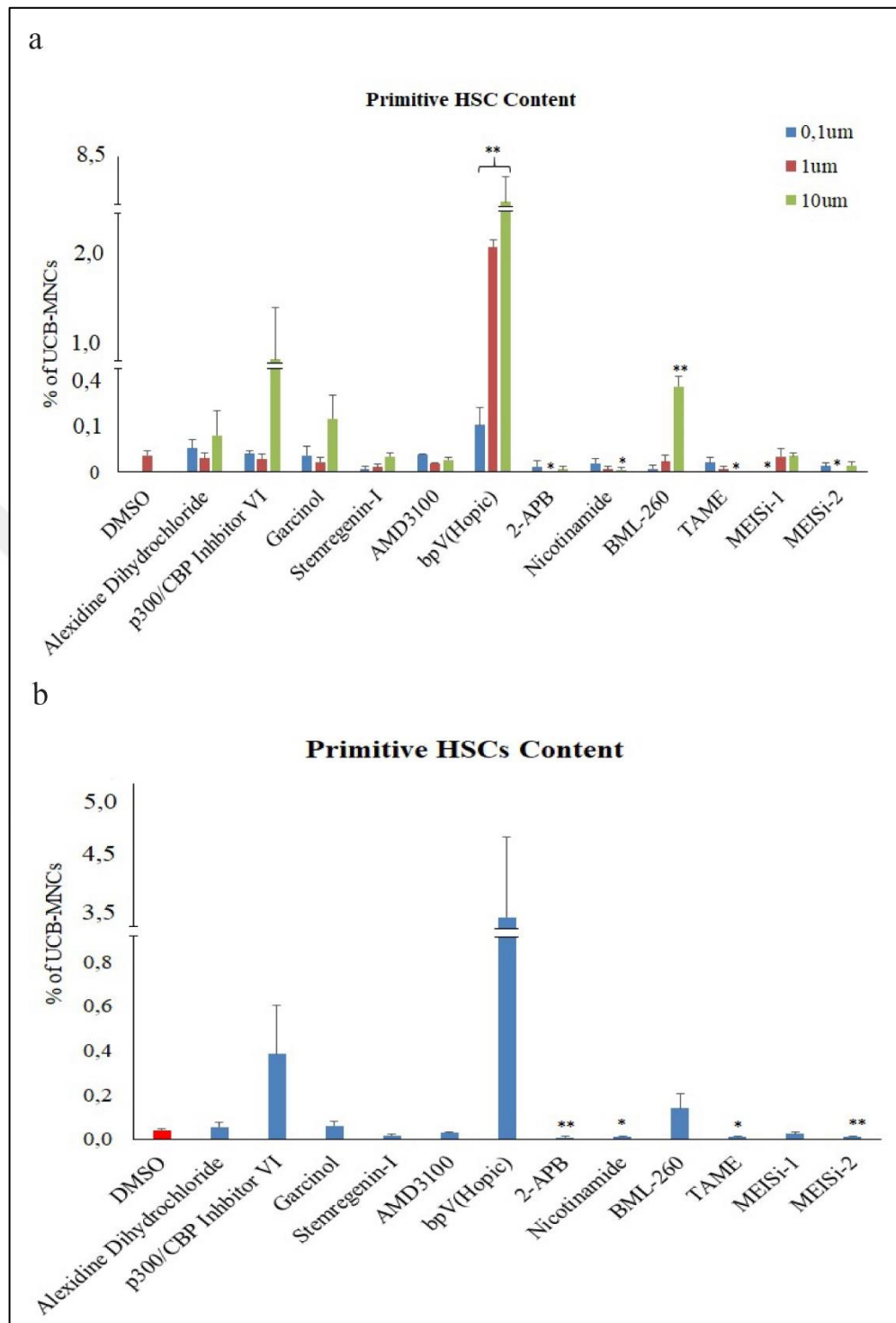


Figure 5.8. Quantification of flow cytometry analysis which performed for confirmation of the effect of selected HSMs on UCB-HSPCs. a) The graphic shows the primitive HSC content in a dose dependent manner of selected HSMs (n=3, *p<0,05, **p<0,01), b) The graphic shows the average of all results which belonged to three doses of HSMs on primitive HSC content (n=9, *p<0,05, **p<0,01).

Next, we analyzed the CD34+CD38- cell content which is important population of HSC analysis, post treatments of selected HSMs to confirm the first screening results post 7 days of treatment. The analysis in a dose dependent manner showed that 10 μ M concentration was found to be prominent dose for the HSC expansion for the compounds, too (Figure 5.9a) (Table 5.3). We also found on average data of three doses results that treatments of p300/CBP Inhibitor VI, bpV(Hopic), Nicotinamide and BML-260 compounds significantly increased the CD34+CD38- cell content more than 2-fold to 6-fold compared to DMSO control (n=9) (Figure 5.9b). Other compounds did not show significant increase in CD34+ cell content.

Table 5.3. The data of percentage and fold difference which belongs to UCB-CD34+CD38- cell content quantification results

Compound Name	Effective Dose	CD34+CD38- percent in MNCs compared to DMSO	Fold Difference compared to DMSO
p300/CBP Inhibitor VI	10 μ M	8,70 vs 0,57	$\approx$ 15-fold
bpV(Hopic)	10 μ M	8,09 vs 0,57	$\approx$ 14-fold
BML-260	10 μ M	9,12 vs 0,57	$\approx$ 16-fold
Nicotinamide	10 μ M	1,89 vs 0,57	$\approx$ 3-fold

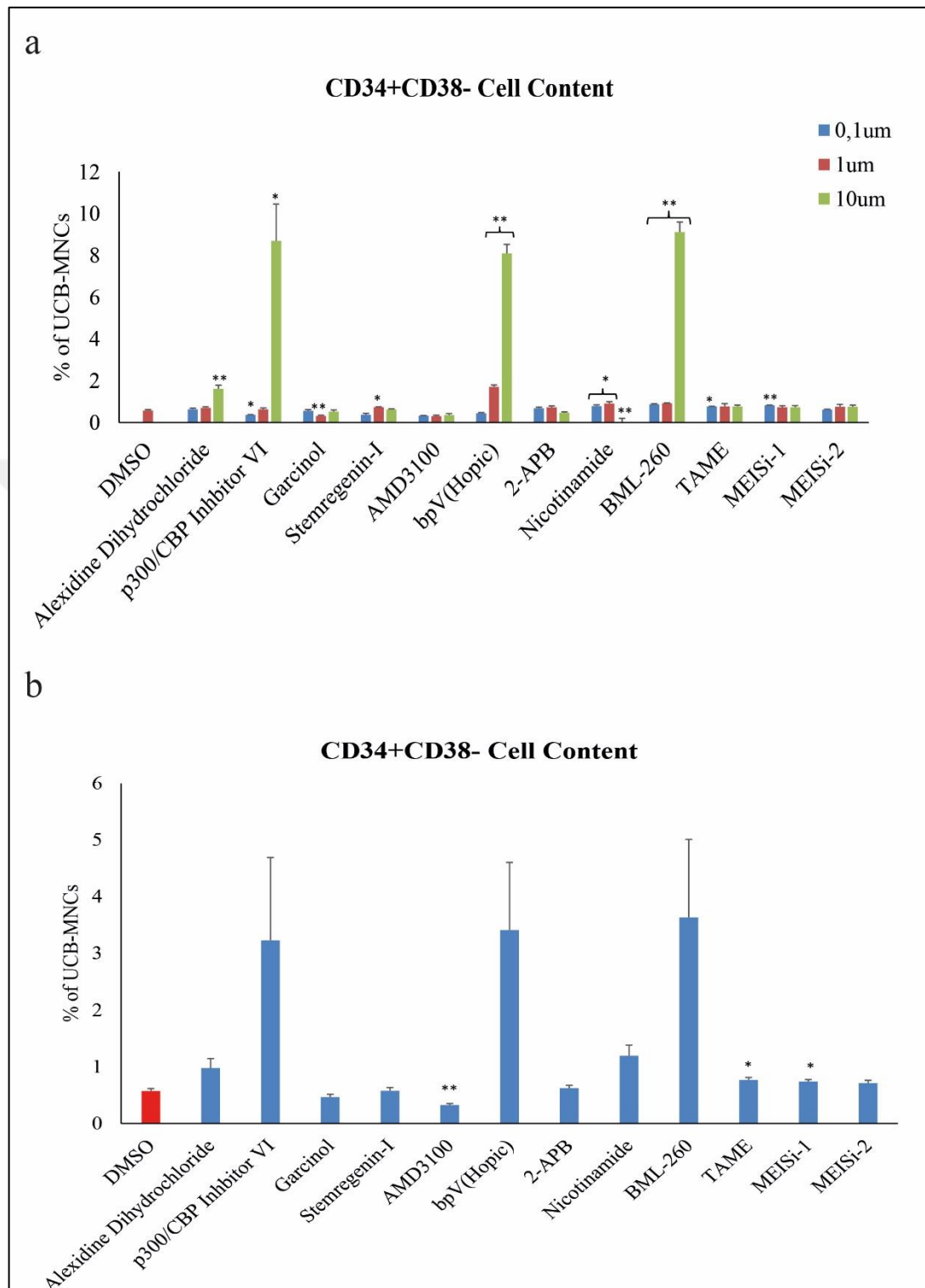


Figure 5.9. Quantification of flow cytometry analysis which performed for confirmation of the effect of selected HSMs on UCB-HSPCs. a) The graphic shows the CD34+CD38- cell content in a dose dependent manner of selected HSMs (n=3, *p<0,05, **p<0,01), b) The graphic shows the average of all results which belonged to three doses of HSMs on CD34+CD38- cell content (n=9, *p<0,05, **p<0,01).

In consequence of the first screening and confirmation results carried on UCB-HSPCs, we reported that p300/CBP Inhibitor VI and BML-260 and TAME molecules, which were not worked yet on HSC expansion, remarkably induced the increase in UCB-HSPC content compared to DMSO and previously tested control compounds.

5.3. THE EFFECT OF SELECTED HSM ON HSC CONTENT OF MPB-HSPC

Mobilized peripheral blood is another source of HSC even if their CD34⁺ cell frequency is low. We investigated the effect of the most effective HSMs, which were selected from UCB-HSC expansion study, on mobilized peripheral blood. Therefore, we treated the mPB-MNCs with 0,1 μ M, 1 μ M and 10 μ M of Alexidine Dihydrochloride, p300/CBP Inhibitor VI, Garcinol, Stemregenin I, AMD3100, bpV(Hopic), 2-APB, Nicotinamide, BML-260, TAME, MEISi-1 and MEISi-2 compounds. We performed the flow cytometry analysis for determination of HSC content post 7 days of treatment. Especially, we focused on CD34⁺, CD34⁺CD38⁻ and CD133⁺ cell populations for peripheral blood analysis according to our gating strategy (Figure 5.2b). The analysis in a dose dependent manner showed that different doses of each HSMs were the most effective for CD34⁺ cell expansion. (Table 5.4) (Figure 5.10a). We also found on the average data of three doses results about CD34⁺ cell content that treatments of p300/CBP Inhibitor VI, bpV(Hopic), Nicotinamide, BML-260, TAME, MEISi-1 and MEISi-2 molecules significantly increased the CD34⁺ cell content more than 2-fold compared to DMSO control. Other compounds did not show significant increase in CD34⁺ cell content (Figure 5.10b).

Table 5.4. The data of percentage and fold difference which belongs to mPB-CD34+ cell content quantification results

Compound Name	Effective Dose	CD34+ percent in MNCs compared to DMSO	Fold Difference compared to DMSO
p300/CBP Inhibitor VI	10 μ M	30,19 vs 0,48	$\approx$ 62-fold
bpV(Hopic)	1 μ M	1,73 vs 0,48	$\approx$ 3-fold
Nicotinamide	0,1 μ M	1,24 vs 0,48	$\approx$ 2-fold
BML-260	1 μ M	1,24 vs 0,48	$\approx$ 2-fold
TAME	1 μ M	1,57 vs 0,48	$\approx$ 3-fold
MEISi-1	0,1 μ M	1,89 vs 0,48	$\approx$ 4-fold
MEISi-2	1 μ M	1,57 vs 0,48	$\approx$ 3-fold

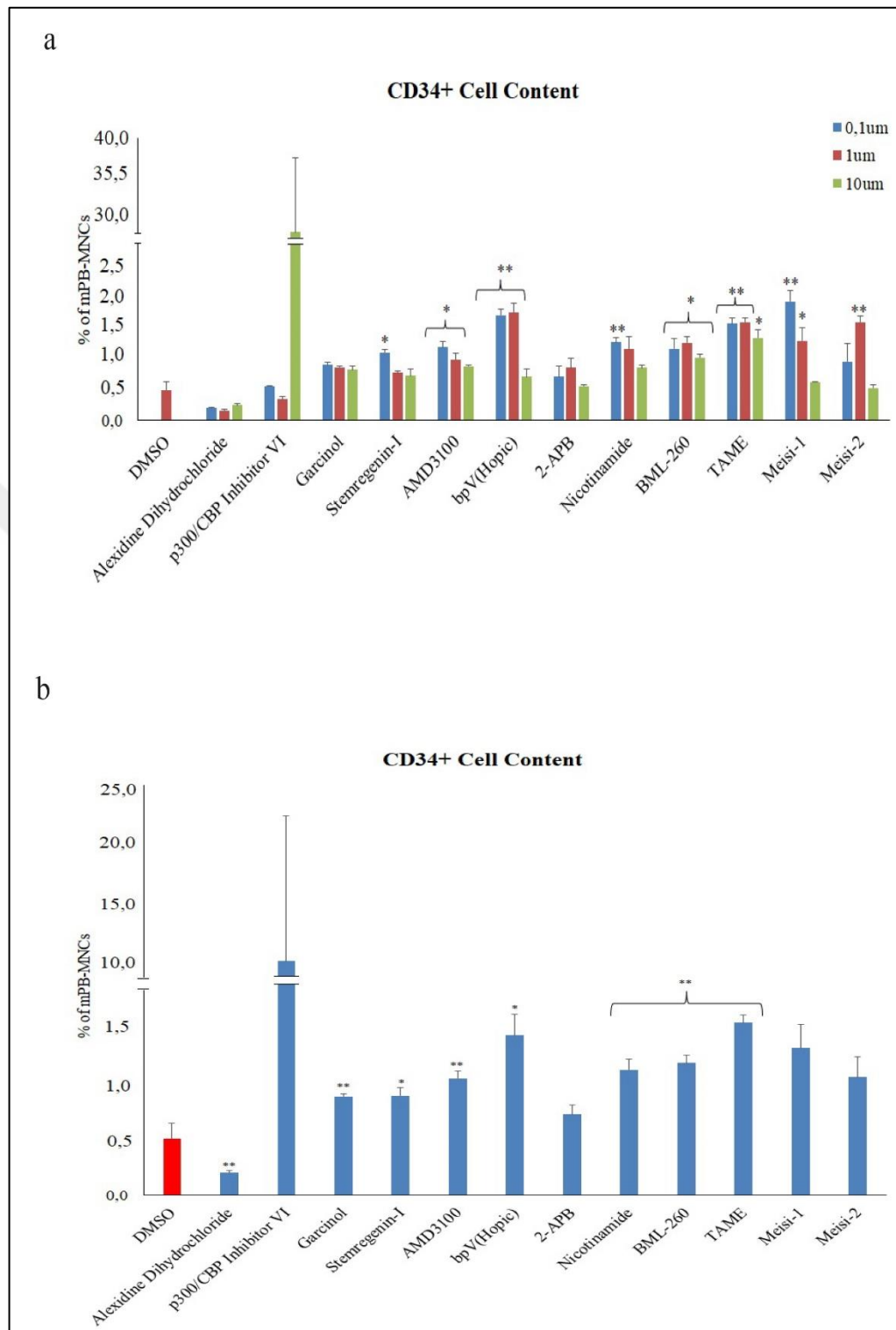


Figure 5.10. Quantification of flow cytometry analysis which performed for determination of the effect of selected HSMs on mPB-HSPCs. a) The graphic shows the CD34+ cell content in a dose dependent manner of selected HSMs (n=3, *p<0,05, **p<0,01), b) The graphic shows the average of all results which belonged to three doses of HSMs on CD34+ cell content (n=9, *p<0,05, **p<0,01).

Then, we analyzed the CD34+CD38- population post 7 days of HSM treatment. The analysis in a dose dependent manner showed that different doses of each HSMs were the most effective for CD34+CD38- cell expansion (Table 5.5) (Figure 5.11a). We also found on the average data of three doses results about CD34+CD38- cell content that treatments of p300/CBP Inhibitor VI, AMD3100, bpV(Hopic), 2-APB, Nicotinamide, BML-260, TAME, MEISi-1 and MEISi-2 molecules significantly increased the CD34+CD38- cell content more than 2-fold to 4-fold compared to DMSO control. Other compounds did not show significant increase in CD34+CD38- cell content (Figure 5.11b).

Table 5.5. The data of percentage and fold difference which belongs to mPB-CD34+CD38- cell content quantification results

Compound Name	Effective Dose	CD34+CD38- percent in MNCs compared to DMSO	Fold Difference compared to DMSO
p300/CBP Inhibitor VI	10 μ M	2,41 vs 0,21	$\approx$ 11-fold
AMD3100	1 μ M	0,50 vs 0,21	$\approx$ 2-fold
bpV(Hopic)	0,1 μ M	0,57 vs 0,21	$\approx$ 2-fold
2-APB	0,1 μ M	0,55 vs 0,21	$\approx$ 2-fold
Nicotinamide	1 μ M	0,87 vs 0,21	$\approx$ 4-fold
BML-260	1 μ M	0,86 vs 0,21	$\approx$ 4-fold
TAME	0,1 μ M	0,87 vs 0,21	$\approx$ 4-fold
MEISi-1	0,1 μ M	1,08 vs 0,21	$\approx$ 5-fold
MEISi-2	1 μ M	0,92 vs 0,21	$\approx$ 4-fold

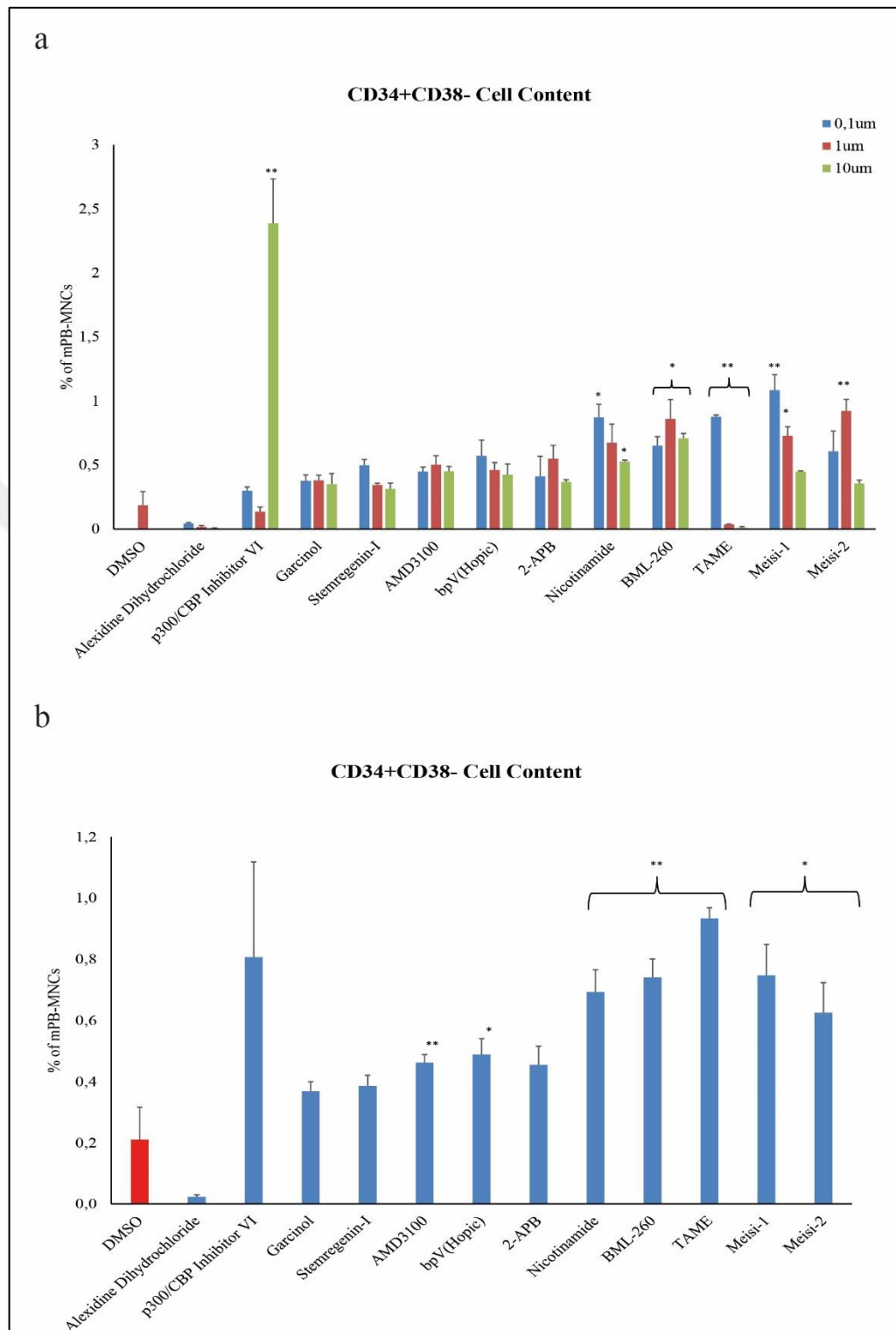


Figure 5.11. Quantification of flow cytometry analysis which performed for determination of the effect of selected HSMs on mPB-HSPCs. a) The graphic shows the CD34+CD38- cell content in a dose dependent manner of selected HSMs ($n=3$, $*p<0,05$, $**p<0,01$), b) The graphic shows the average of all results which belonged to three doses of HSMs on CD34+CD38- cell content ($n=9$, $*p<0,05$, $**p<0,01$).

Next, we analyzed the CD133+ cell population post 7 days of treatment. The analysis in a dose dependent manner showed that different doses of each HSMs were the most effective for CD133+ cell expansion (Table 5.6) (Figure 5.12a). Besides, we found on the average data of three doses results about CD133+ cell content that treatments of Alexidine Dihydrochloride, Stemregenin I, BML-260 and MEISi-1 molecules significantly increased the CD133+ cell content more than 2-fold; Garcinol, AMD3100 and bpV(Hopic) molecules increased more than 8-fold to 11-fold; MEISi-2 compound increased more than 15-fold compared to DMSO control. Other compounds did not show significant increase in CD133+ cell content (Figure 5.12b).

Table 5.6. The data of percentage and fold difference which belongs to mPB-CD133+ cell content quantification results

Compound Name	Effective Dose	CD133+ percent in MNCs compared to DMSO	Fold Difference compared to DMSO
Alexidine Dihydrochloride	10 μ M	1,95 vs 0,35	$\approx$ 5-fold
Garcinol	10 μ M	4,98 vs 0,35	$\approx$ 14-fold
Stemregenin I	0,1 μ M	1,21 vs 0,35	$\approx$ 3-fold
AMD3100	1 μ M	7,00 vs 0,35	$\approx$ 20-fold
bpV(Hopic)	10 μ M	6,20 vs 0,35	$\approx$ 17-fold
BML-260	10 μ M	1,93 vs 0,35	$\approx$ 5-fold
MEISi-1	1 μ M	0,90 vs 0,35	$\approx$ 2-fold
MEISi-2	10 μ M	1,66 vs 0,35	$\approx$ 5-fold

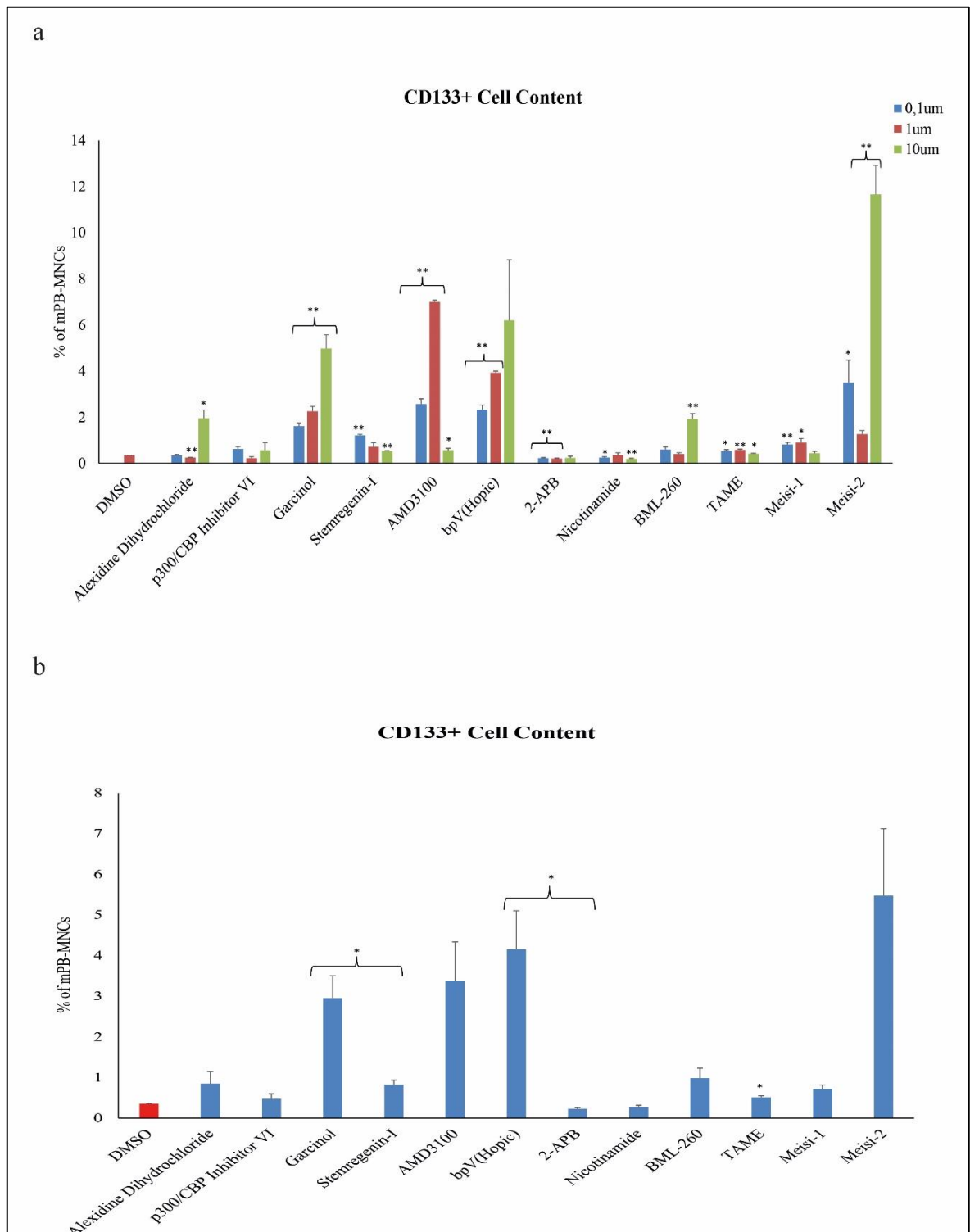


Figure 5.12. Quantification of flow cytometry analysis which performed for determination of the effect of selected HSMs on mPB-HSPCs. a) The graphic shows the CD133+ cell content in a dose dependent manner of selected HSMs (n=3, *p<0,05, **p<0,01), b) The graphic shows the average of all results which belonged to three doses of HSMs on CD133+ cell content (n=9, *p<0,05, **p<0,01).

As a consequence of these all data, we have briefly recorded that p300/CBP Inhibitor VI, BML-260, TAME, MEISi-1 and MEISi-2 HSMs induced the increase in CD34+, CD34+CD38- and CD133+ cell of mPB-HSPC content more than 2-fold to 5-fold compared to DMSO and previously tested positive control compounds. Especially, p300/CBP Inhibitor VI molecule was highly effective on CD34+ cell expansion (around 62-fold).

5.4. DETERMINATION OF THE SELECTED HSM ON METABOLIC AND ROS PRODUCTION LEVEL OF MPB-HSC

HSC characterization is mainly performed with immunophenotyping by using surface markers as mentioned above. Another identification method is based on HSC metabolism property. ALDH enzyme activity is a metabolic marker for HSCs because of their glycolysis pathway preference and HSCs are known to be ALDHhi population. Thus, we performed the ALDH measurement assay on mPB-MNCs post 7 days of treatment with the effective doses (shown in Table 4.1) of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules. We found that treatments of AMD3100 (11,10 percent), 2-APB (10,45 percent) and BML-260 (11,33 percent) treatments significantly increased the ALDHhi population ratio approximately 2-fold compared to DMSO (6,54 percent). Besides, we interestingly found that treatment of p300/CBP Inhibitor VI molecule significantly decreased in ALDHhi cell ratio (not determined) and also, TAME compound did not any significant effect on ALDH activity on the expanded mPB cells (Figure 5.13).

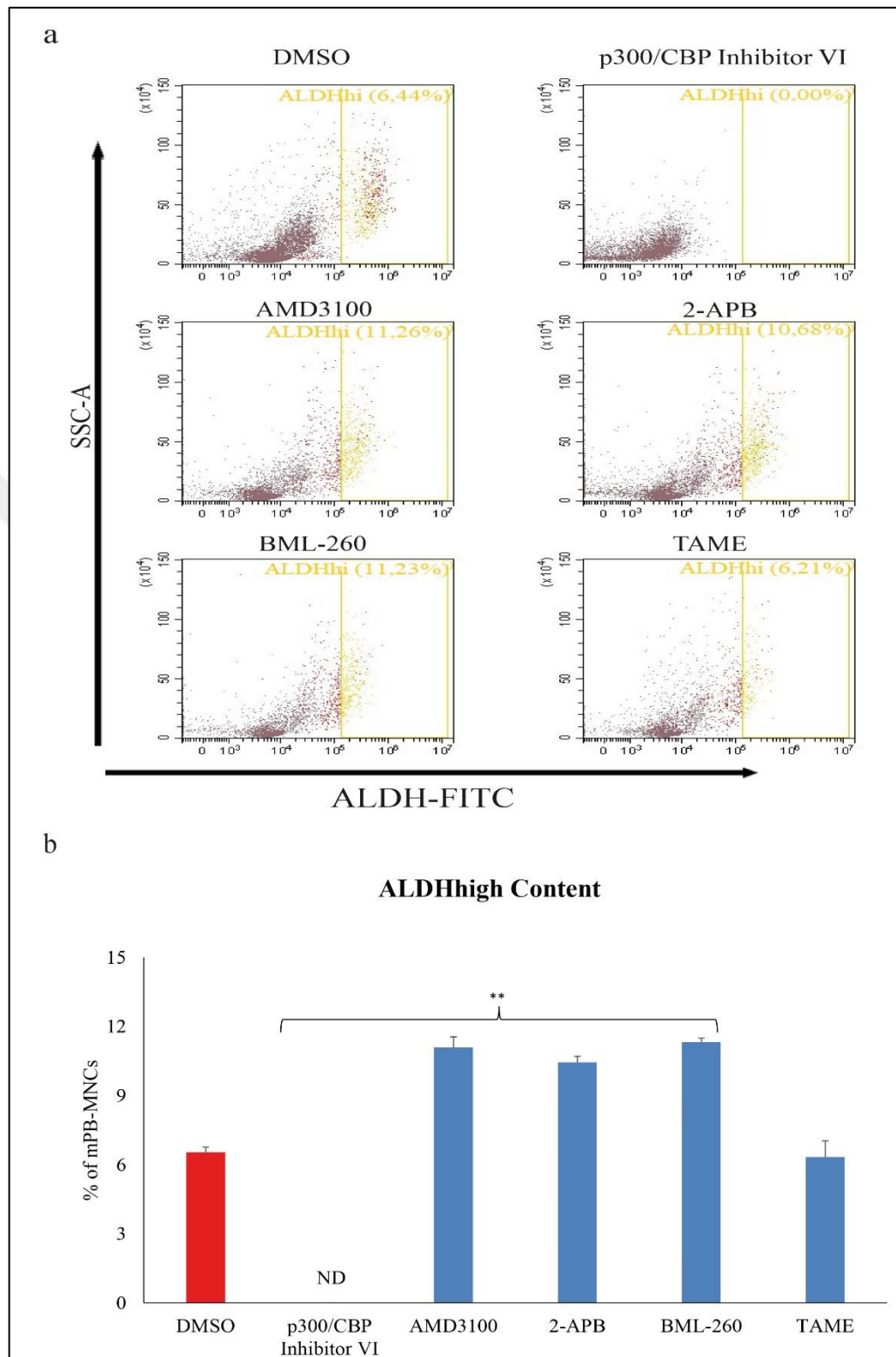


Figure 5.13. The effect of selected HSMs on metabolic profile of mPB-HSPCs post 7 days of treatment. a) The plots of flow cytometry analysis which belonged to the assay was shown, b) The quantification results were presented (n=3, **p<0,01).

The main cellular response to over metabolic activity is production of reactive oxygen species (ROS) as mentioned above. If HSCs prefer the mitochondrial respiratory for energy production instead of glycolysis during enhanced cell division, production of ROS production level is increased in parallel. Thus, we detected the ROS production on mPB-MNCs post 7 days of treatment with the effective doses (shown in table 4.1) of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules. We found that any compounds significantly did not contribute to ROS production compared to DMSO control (AMD3100: 29,55 percent, 2-APB: 28,11 percent, BML-260: 28,48 percent, TAME: 28,96 percent vs DMSO: 22,26 percent). Furthermore, p300/CBP Inhibitor VI treatment (3,34 percent) significantly reduced the ROS production compared to DMSO (22,26 percent) (Figure 5.14).

Briefly, ALDH enzyme activity showed that 2-APB and BML-260 compounds expanded the ALDH^{hi} HSC population. Besides that, ROS production was not considerably affected from HSMs. These results indicated that TAME molecule could be slightly used for HSC expansion in safe while 2-APB and BML-260 molecules could be safely used for HSC expansion.

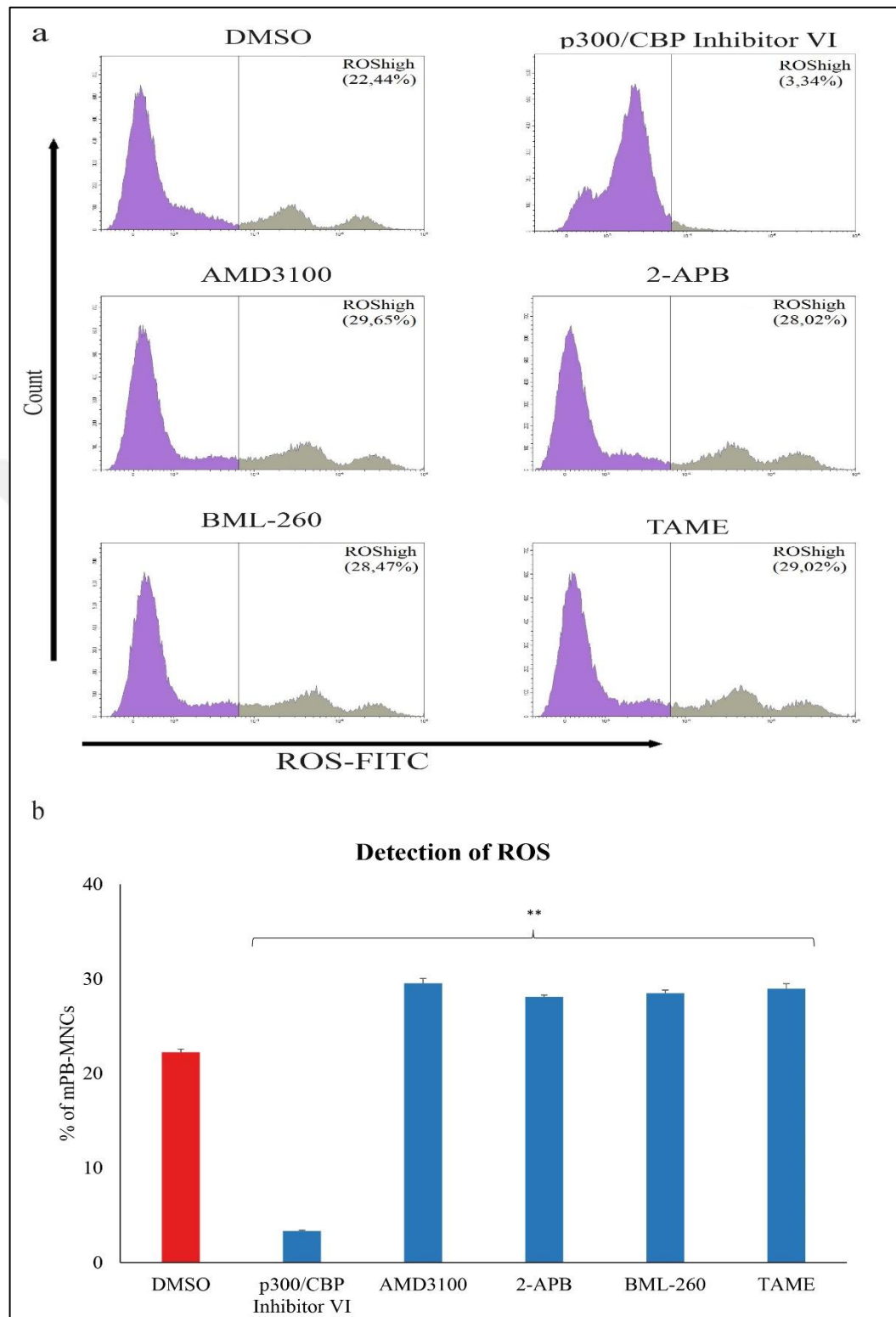


Figure 5.14. The effect of selected HSMs on senescence profile of mPB-HSPCs post 7 days of treatment. a) The plots of flow cytometry which belonged to the assay was shown, b) The quantification results were presented (n=3, **p<0,01).

5.5. THE EFFECT OF THE SELECTED HSM ON VIABILITY OF EXPANDED MPB-HSPC

Next, we evaluated the effect of HSMs on cell viability of expanded mPB-HSPCs. For this reason, we performed the cell viability of expanded mPB-MNCs with 7-AAD staining following 7 days of treatment with the effective doses (shown in table 4.1) of AMD3100, 2-APB, BML-260 and TAME molecules. We quantified the viability percentage in a time dependent manner. We found that any HSMs did not significantly change the viability percentage of the expanded cells (AMD3100: 88,3 percent, 2-APB: 85,2 percent, BML-260: 81,1 percent vs DMSO: 89,8 percent). TAME molecule treatment just slightly decreased the viability to 75 percent from 89,8 percent (compared to DMSO control) (Figure 5.15). These results reveal that BML-260 and 2-APB molecules were safer than TAME molecule.

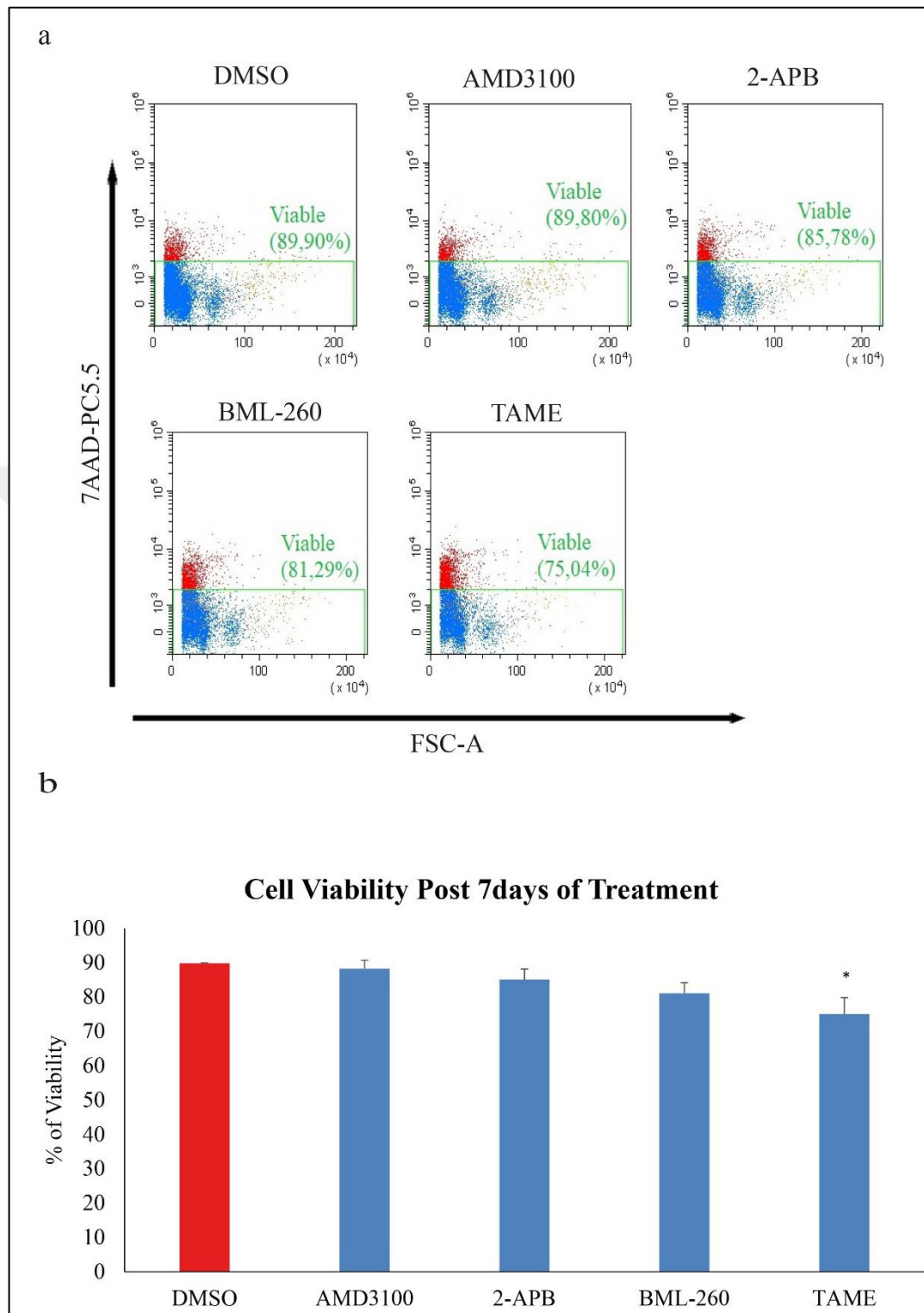


Figure 5.15. The effect of selected HSMs on viability of mPB-HSPCs. a) The plots of flow cytometry which belonged to the assay was shown, b) The quantification results were presented based on percentage and count version ($n=3$, $*p<0,05$, $**p<0,01$).

5.6. THE EFFECT OF THE SELECTED HSM ON DNA DAMAGE, APOPTOSIS AND CELL CYCLE PROCESSES OF EXPANDED MPB-HSPC

HSCs protect themselves against oxidative and genotoxic stresses through staying in quiescence state. Enhanced metabolic activity can result in oxidative stress by ROS production and thus, occurs DNA damage, senescence and apoptosis. We performed DNA damage, cell cycle and apoptosis assay to determine the effect of HSMs on expanded cells by induction of cell division post 7 days of treatment with effective doses (shown in Table 4.1) of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules. We found that treatment of TAME (7,36 percent) molecule was determined to significantly increase cycling cell percentage 3-fold by BrdU incorporation compared to DMSO (2,27 percent). Interestingly, we found that the compound significantly decreased the cycling cells ratio however p300/CBP Inhibitor VI molecule became prominent by increase in HSPC content (Figure 5.17a). Moreover, we showed the apoptotic and genotoxic effects of p300/CBP Inhibitor VI and TAME molecules more than 6-fold and 2-fold compared to DMSO control, respectively (p300/CBP Inhibitor VI: 27,18 percent, TAME: 11,55 percent vs DMSO:4,92 percent; shown in Figure 5.17b) (p300/CBP Inhibitor VI: 29,17 percent, TAME: 11,55 percent vs DMSO:4,67 percent; shown in Figure 5.17c). The results showed that TAME molecule increased the cycling cell ration but over cell division activity of TAME led the cycling cells to DNA damage and thus, apoptosis. p300/CBP Inhibitor VI molecule (45,53 percent) had apoptotic and DNA damage effect on the cells compared to DMSO (15,42 percent) (Figure 5.17d). AMD3100, 2-APB, and BML-260 treatments did not show any significant cytotoxic and genotoxic effects on mPB cells. These results reveal that 2-APB and BML-260 molecules are more advantageous comparing to p300/CBP Inhibitor VI and TAME molecules. The plots of flow cytometry were presented in Figure 5.16.

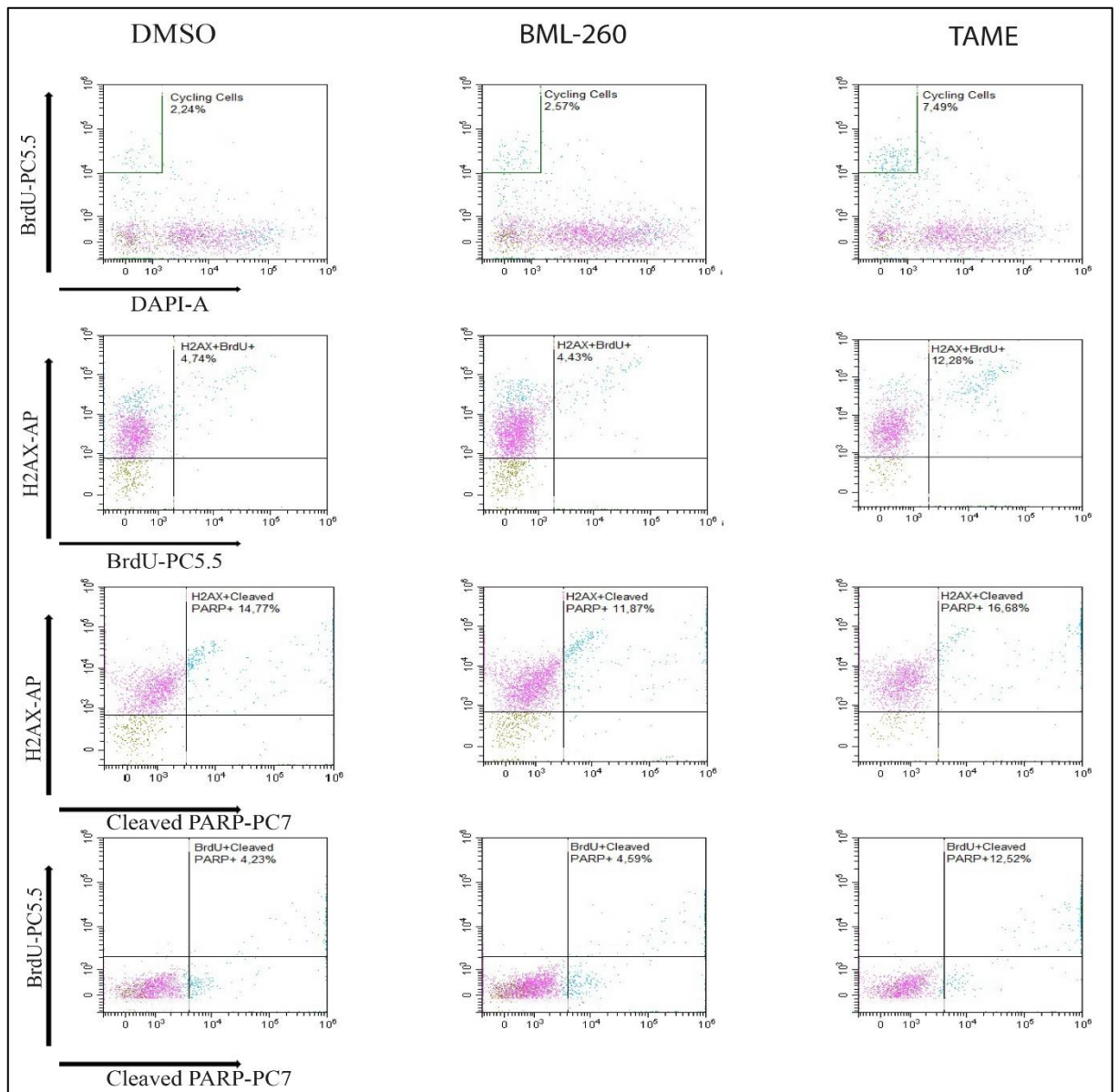


Figure 5.16. The effect of selected HSMs on DNA damage, cell cycle and apoptosis processes on mPB-HSPCs post 7days of treatment. The plots of flow cytometry analysis which belonged to the assay was shown.

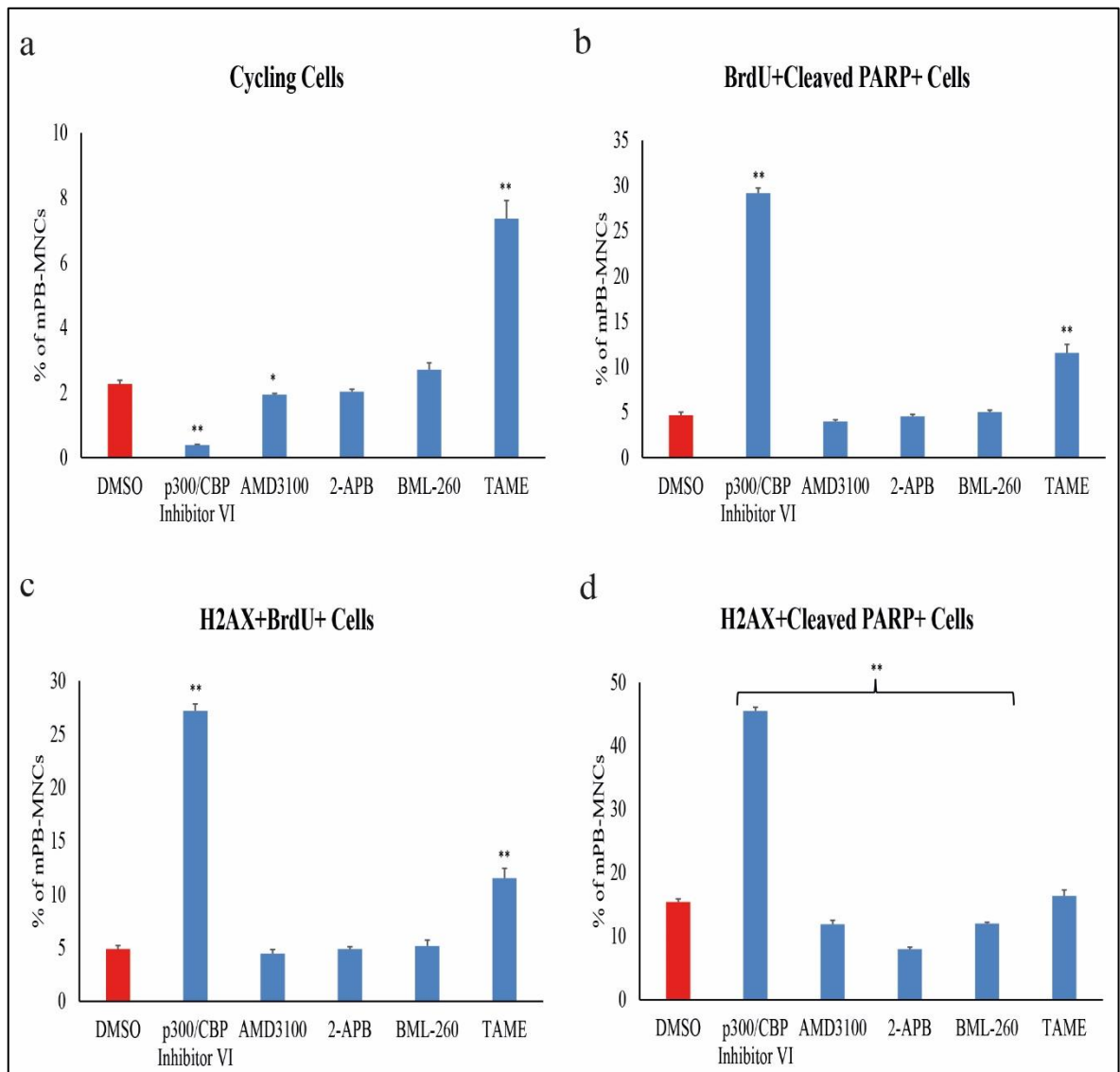


Figure 5.17. The effect of selected HSMs on DNA damage, cell cycle and apoptosis processes on mPB-HSPCs post 7 days of treatment. The quantification results of a) cycling cell percentage, b) apoptosis ratio in cycling cells, c) DNA damage ratio of cycling cells, d) the relation of DNA damage and apoptosis on the expanded cells, were presented (n=3, *p<0,05, **p<0,01).

However DNA damage, cell cycle and apoptosis assay showed the apoptosis profile, the other main apoptosis and necrosis detection assay is based on Annexin V and PI staining. We also performed the apoptosis assay post 72h of treatment with the effective doses (shown in Table 4.1) of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules on mPB-CD34+ cells. We found that p300/CBP Inhibitor VI molecule treatment

(26,11 percent) had significantly an effect on post-apoptosis compared to DMSO (6,61 percent) more than 3-fold. BML-260 compound treatment was found to significantly increase in viability of the expanded cells compared to DMSO (53,63 percent vs 43,95 percent). Besides, treatments of 2-APB and TAME molecules weren't showed any significant apoptotic or necrotic effect on mPB-CD34+ cells post treatment (Figure 5.18).

DNA damage, cell cycle and apoptosis assay and Annexin V Apoptosis Detection assay showed different results, the reason for that may be the differences between detection mechanisms of them. In Annexin V-Apoptosis detection assay, DMSO control had pre-apoptotic effect on the cells more than expected, the reason that the cells were negatively affected in the cell culture conditions. On the other hand, cytotoxicity and genotoxicity analysis with DNA damage, cell cycle and apoptosis assay was performed on mPB HSPC, but Annexin V Apoptosis Detection assay was performed on mPB-CD34+ cells. Difference between target populations may differ the results. As a conclusion, BML-260 did not have apoptotic, necrotic or DNA damage effect on the mPB-CD34+ cells and it is more safe for expanded cells. TAME molecule slightly had a negative effect on cycling cells while providing the cycling in quiescence cells.

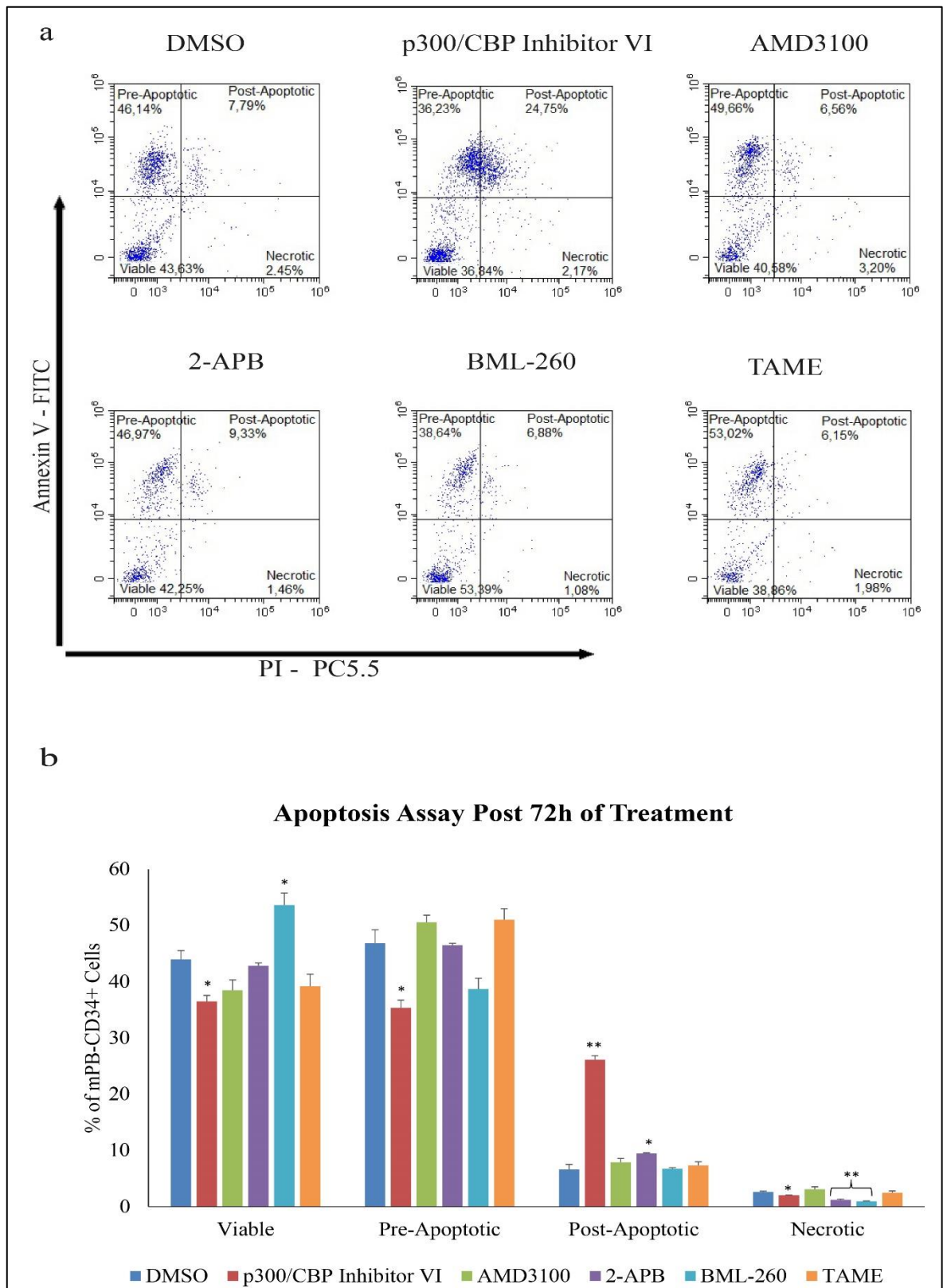


Figure 5.18. The effect of selected HSMs on apoptosis process on mPB-HSPCs post 72h of treatment. a) The plots of flow cytometry analysis which belonged to the assay was shown, b) The quantification results were presented (n=3, *p<0,05, **p<0,01).

5.7. DETERMINATION OF THE EFFECT OF THE SELECTED HSM ON CELL CYCLE PROFILE OF EXPANDED MPB- AND UCB-CD34+ CELLS

Re-entry to cell cycle is a critical point for expansion of HSCs which is characterized by cell cycle arrest resulted in cell quiescence. Hence, we performed the cell cycle analysis post 4 days of treatment with the effective doses (shown in table 4.1) of p300/CBP Inhibitor VI, AMD3100, 2-APB, BML-260 and TAME molecules on mPB and UCB-CD34+ cells. We found on mPB-CD34+ cells that treatments of BML-260 and TAME molecules significantly increased the cell ratio in G1 phase more approximately 5-fold compared to DMSO control (BML-260: 15,3 percent, TAME: 16.47 percent vs DMSO: 3,14 percent) by decrease in the cell ratio in G0 phase (BML-260: 81,42 percent, TAME: 80,17 percent vs DMSO: 94,10 percent). These compounds provided the re-entry to G1 phase from G0 phase. In addition, AMD3100 treatment (9,16 percent) was found to increase the cell ratio in G1 phase more than 3 fold compared to DMSO (3,14 percent) and treatments of p300/CBP Inhibitor VI (6,16 percent) increased the cell ratio in G1 phase approximately 2 fold compared to DMSO (3,14 percent) by decrease the cell ratio in G0 and S/G2/M phase. Treatment of p300/CBP Inhibitor VI molecule provided the re-entry to G1 phase but also, these expanded cells were arrested at G1/S checkpoint. 2-APB molecule did not show any significant effect on the mPB-CD34+ cells (Figure 5.19).

In addition to our mPB findings, the findings on UCB-CD34+ cells showed that treatments of BML-260 (28,7 percent) and TAME (27,0 percent) increased the cell ratio in S/G2/M phase more than 5-fold compared to DMSO (5,15 percent) by decrease in the cell ratio in G0 and G1 phase. Interestingly, treatment of 2-APB compound significantly increased the cell ratio in S/G2/M phase more than 5-fold however, 2-APB treatments did not affect the mPB-CD34+ cells (Figure 5.20).

Collectively, treatments of BML-260 and TAME compounds was found to significantly increase the HSC content and in parallel to this they not only remarkably induced the re-entry of mPB-CD34+ cells to cell cycle, but also induced UCB-CD34+ cells. Treatment of 2-APB molecule was efficient for re-entry of UCB-CD34+ cells to cell cycle, but not for mPB-CD34+ cells. p300/CBP Inhibitor VI compound provided the transition to G1 from G0 phase, but may be resulted in cell cycle arrest at G1/S checkpoint. Briefly, BML-260 and TAME molecules could be identified as efficient compounds for human HSC expansion.

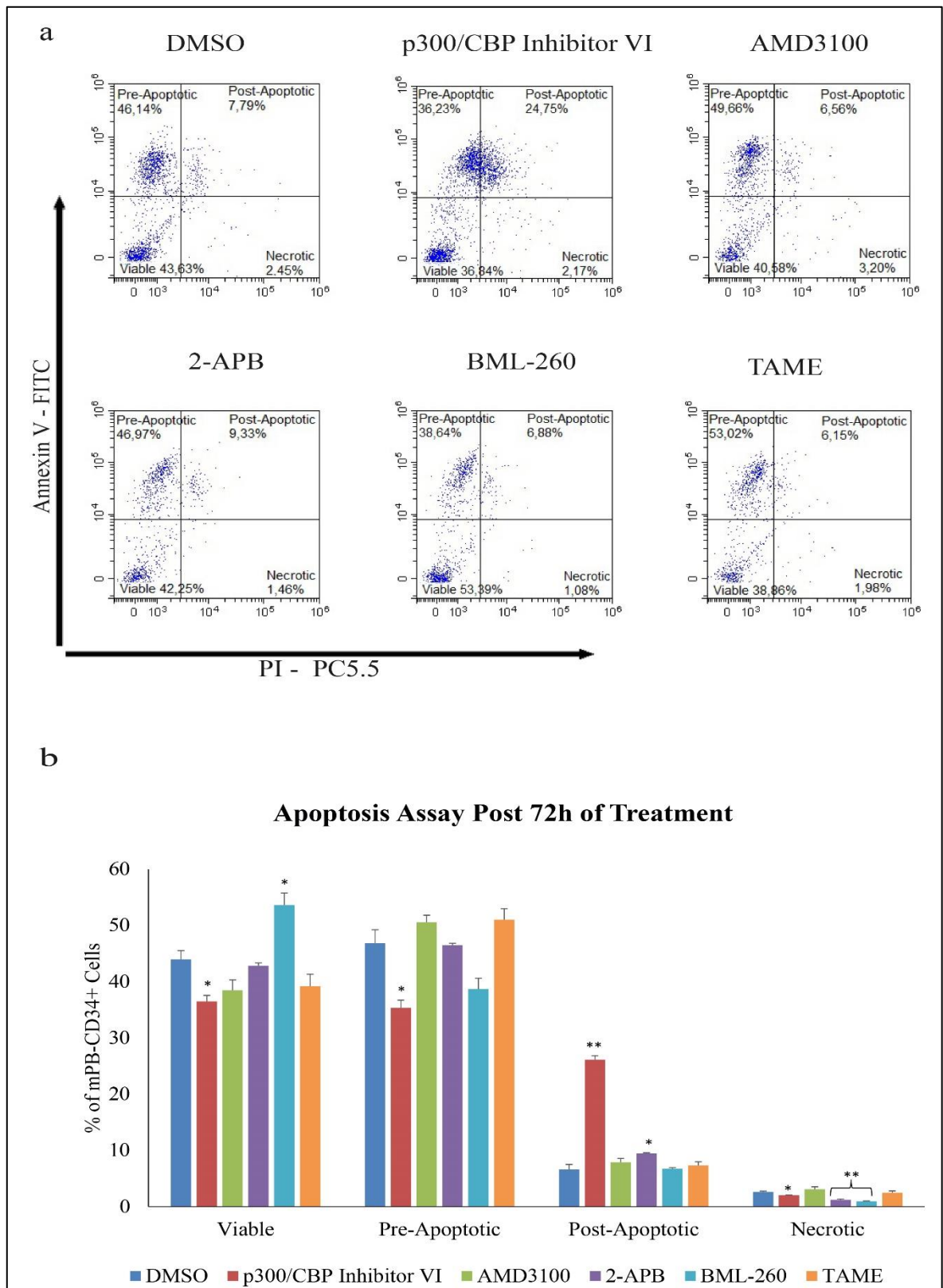


Figure 5.19. Cell cycle analysis post 4 days of treatment on HSM-expanded mPB-CD34+ cells. a) Flow plots which belonged to the assay was shown, b) The quantification results were presented (n=3, *p<0,05, **p<0,01).

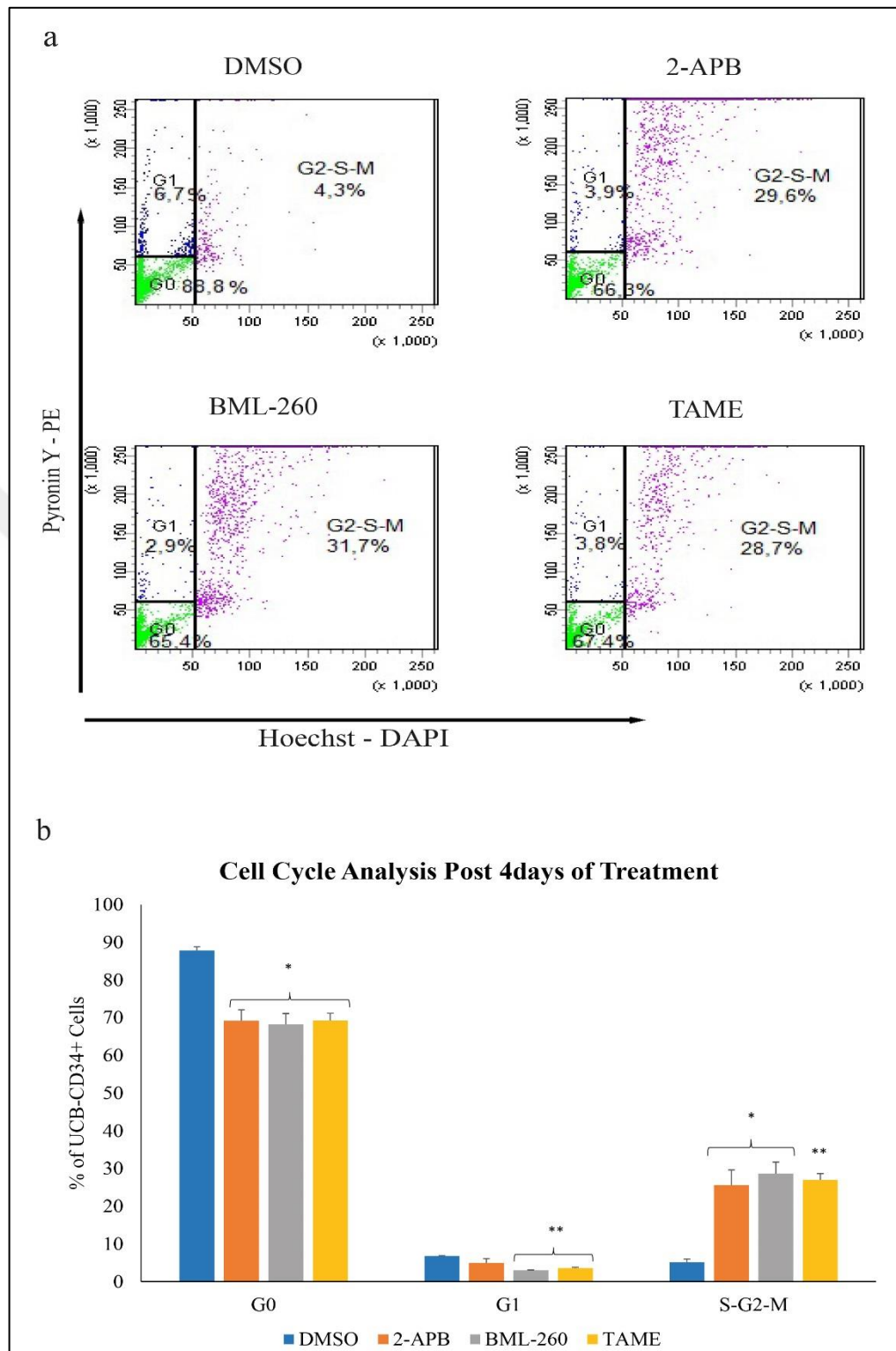


Figure 5.20. Cell cycle analysis post 4 days of treatment on HSM-expanded UCB-CD34+ cells. a) Flow plots which belonged to the assay was shown, b) The quantification results were presented (n=3, *p<0,05, **p<0,01).

5.8. COLONY FORMING UNIT ASSAY ON THE EXPANDED UCB-HSPC WITH THE SELECTED HSM

Colony Forming Unit Assay was used for determination of multi-lineage potential of expanded cells. The identification of colony type generated on special media was performed post 10-14 days of seeding of expanded UCB-MNCs with 2-APB, BML-260 and TAME compounds. We observed that all compounds increased the count of BFU-E colony, which generated from erythroid progenitors, compared to DMSO 9-fold, 2-fold and 13-fold, respectively (2-APB: 83,50 colony, BML-260: 20 colony, TAME: 121,50 colony vs DMSO: 9 colony). Likewise, they provided the increase in the count of CFU-GM colony which generated from granulocytes and macrophages more than 2-fold to 6-fold (2-APB: 62 colony, BML-260: 41,50 colony, TAME: 23,50 colony vs DMSO:9 colony). Moreover, the count of CFU-GEMM which generated from HSCs was found to be increased post treatment with 2-APB, BML-260 and TAME molecules 22-fold, 7-fold and 2,5-fold, respectively (2-APB: 22 colony, BML-260: 7 colony, TAME: 2,5 colony vs DMSO: 1 colony)(Figure 5.21).

These CFU assay results which are important almost as much as *in vivo* studies showed that 2-APB, BML-260 and TAME compounds provided the enhancement of multi-lineage potential of expanded UCB cells and also, these results confirmed the expansion capacity of HSM-treated UCB-HSC. Moreover, 2-APB and TAME molecules could be used for erythroid formation.

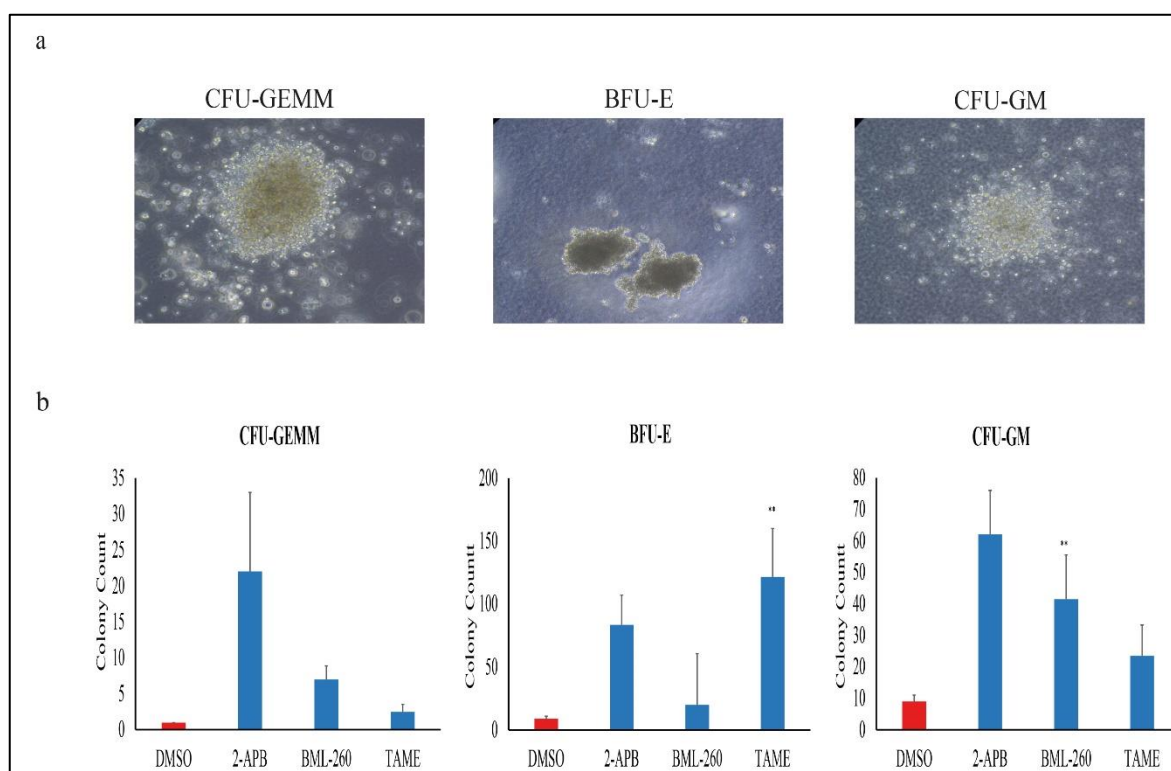


Figure 5.21. Colony Forming Unit Assay on HSM-expanded mPB- and UCB-HSPC. a) The representative images which belongs to generated colony types were shown, b) The quantitative results were presented (n=3, **p<0,01).

5.9. CFSE PROLIFERATION ASSAY ON THE EXPANDED MPB-CD34+ CELLS WITH THE SELECTED HSM

Next, we performed the CFSE proliferation assay which is based on the measurement of changes on dye amount during newly DNA synthesis in S-phase shows dividing cells to confirm the mPB-HSPC expansion. The labelled cells with CFSE were treated with effective doses (shown in Table 4.1) of AMD3100, BML-260 and TAME molecules. The CFSE fluorescence was measured on day4 and day7 and the results were compared to DMSO control and day 0 sample value. We found that while previously tested AMD3100 molecule treatment did not has any significant effect on proliferating cell ratio at day4 and day7 (Day4: 24,35 percent, Day7: 32,81 percent). Treatments of BML-260 and TAME molecules showed the effect in similar ratio with DMSO at day4 (BML-260:21,50 percent, TAME: 21,52 percent vs DMSO: 21,06 percent) but, they showed slightly proliferative effect on the HSPC

at day7 (BML-260: 39,56 percent, TAME: 34,90 percent vs DMSO: 28,16 percent),(Figure 5.22).

CFSE proliferation assay confirmed the results of previously performed assays to evaluate HSC expansion capacity of HSMs. These results showed that BML-260 molecule remarkably provided the HSPC proliferation.



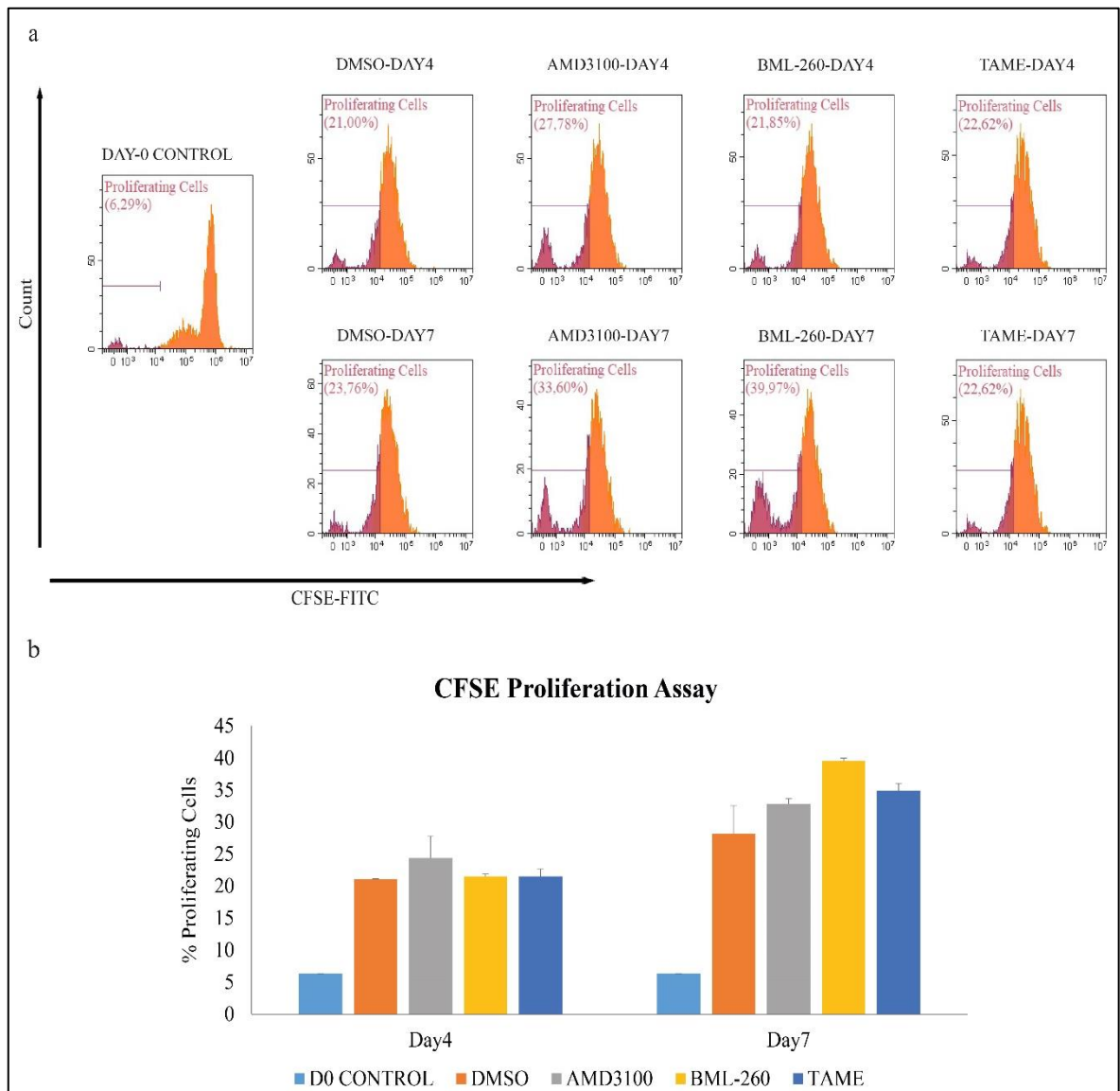


Figure 5.22. CFSE Proliferation Assay on HSM-expanded mPB-HSPCs. a) Flow plots which belonged to the assay was shown, b) The quantification results were presented. (n=3).

5.10. DETERMINATION OF CONTRIBUTION OF BML-260 AND TAME SMALL MOLECULES TO CELL CYCLE KINETICS, DNA REPAIR REGULATORS AND FUNCTIONS OF TARGET GENES

To understand what the effect mechanism of BML-260 and TAME compounds on the expansion of human hematopoietic stem and progenitor cells, we analyzed the expressions of cell cycle regulator genes (S-phase and CDKI), BML260 and TAME compound target genes and HDR-related genes on HSM-treated human mPB-HSPCs. Post analysis of CDKI expression following the effective doses of these compounds (shown in Table 4.1), we found that the expression of p19 (CDKN2D) and p27 (CDKN1B) were downregulated on BML-260-treated cells approximately 2- and 1,5-fold, respectively while the expression of p21CIP1 (CDKN1A) and p57KIP2 (CDKN1C) were upregulated compared to DMSO (0,25 percent) around 2,5- and 2-fold, respectively. Interestingly, TAME treatment just downregulated p27 expression around 4-fold while upregulated the expressions of p19, p21 and p57 CDKIs around 2-fold compared to DMSO (Figure 5.23a) (n=2).

In addition to CDKI expression profile, we found the variable gene profile on HSM-treated cells when we analyzed the expression of S-phase (MCM2, RPA1 and PCNA) and HDR-related genes comparing to DMSO. If we categorize the genes according to their function on the cells, we found that treatment of BML-260 had adjuvant effect on DNA replication and thus DNA repair mechanism by 3-fold upregulation of PCNA and also, enhanced the transition to S-phase by 2-fold upregulation of MCM2 gene. Interestingly, treatment of TAME compound did not effect on expression level of MCM2 while downregulated PCNA expression around 3-fold. There were not any remarkable changes on the expression of RPA1 which is another regulator of DNA replication post treatment of these both compounds. Moreover, treatment of BML-260 was found to be negatively regulator for DNA repair mechanism. We found that the expression of RAD51, XRCC1 and ERCC1 from HDR-related genes excepting MSH2 gene significantly declined on BML-260-treated cells. Intriguingly, MSH2 gene expression was upregulated 2-fold post BML-260 treatment. In contrast to that, TAME compound significantly upregulated RAD51 and XRCC1 genes around 2- and 4,5-fold, respectively while did not effect the expression of MSH2 and ERCC1 genes. Once for all, the expression level of RAD17 which is responsible for cell cycle arrest at G2 phase in response to DNA damage was decreased 2,5 fold on TAME-treated cells

while there was any remarkable changes on the expression post BML-260 treatment (Figure 5.23b) (n=2). These findings revealed that BML-260 molecule showed preferable effect on cell division from TAME compound by enhancement of cell cycle regulator. TAME compound showed the more adjuvant effect on DNA repair mechanism from BML-260 compound by enhancement of HDR genes.



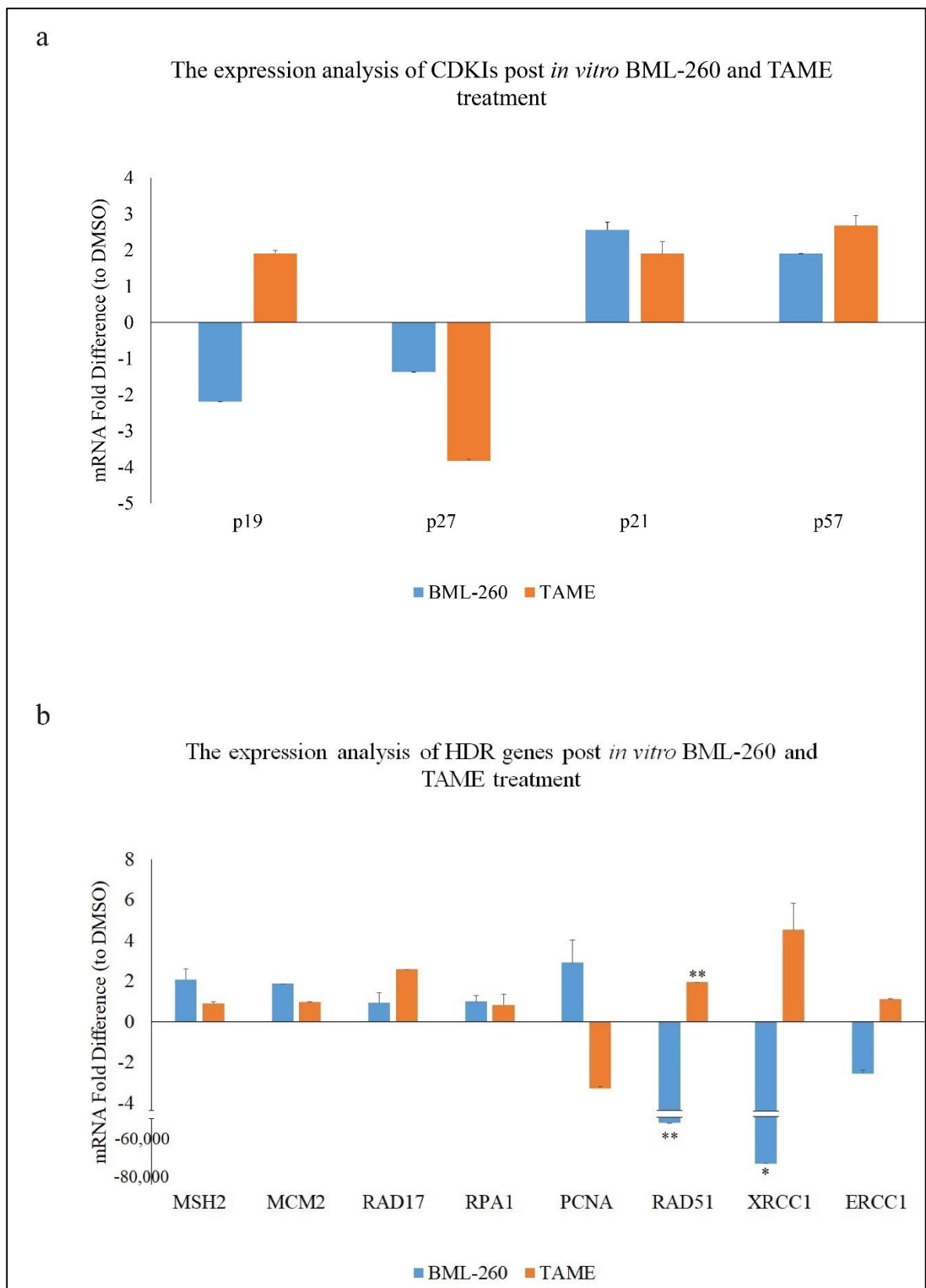


Figure 5.23. Gene expression analysis for a) CDKIs, b) HDR and S-phase on HSM-expanded mPB-HSPCs compared to DMSO by Real-time PCR; n=2; *p<0,05, **p<0,01.

Next, we determined the expression changes of targets of BML-260 and TAME inhibitors-related proteins. We found that the expression of Cdh1 which participates cell cycle regulation through composing APC/C^{Cdh1} complex and is a target of TAME, was highly downregulated in TAME-treated mPB-HSPCs (up to 4 fold). Interestingly, BML-260 treatment downregulated Cdh1 expression around 2,5-fold. Besides, Skp2 cell cycle regulator gene that is responsible for p27 degradation and regulated by APC/C^{Cdh1} for its proteosomal degradation, was analyzed and its expression was upregulated up to 4-fold in TAME-treated cells by inhibition of APC/C^{Cdh1} complex. Interestingly, the expression of Skp2 was upregulated up to 6-fold in BML-260-treated cells. The increase of Skp2 expression with BML-260 is correlated with downregulation of Cdh1 in BML-260-treated cells. On the other hand, the expression of c-Myc which is stimulated by JNK pathway and is a target of BML-260 inhibitor[162], was significantly downregulated in BML-260-treated cells as expected (up to 3-fold). Interestingly, TAME treatment slightly downregulated the expression of c-Myc around 1,5-fold. Moreover, we analyzed apoptosis-related genes since it was known that the major role of JNK pathway in apoptosis. We found that the expression of Bax (Bcl-2 associated x) which acts pro-apoptotic function regulated by p53[163], was downregulated at 2-fold in BML-260-treated cells in despite of upregulation of p53 around 2-fold. This downregulation is expected by inhibition of JNK pathway with BML-260 inhibitor. TAME treatment also did not show any significant changes in the expressions of Bax and p53. Intriguingly, TAME treatment resulted in downregulation of Bcl-2 gene expression which acts anti-apoptotic function, at 2-fold however, BML-260 treatment did not show significant change in its expression. These findings reveal that TAME treatment inhibits APC/C^{Cdh1} by downregulation of Cdh1 gene and thus, provides cell cycle progression at G1/S transition via degradation of p27 through prevention of degradation of Skp2. Unexpected downregulation of Cdh1 and upregulation of Skp2 reveal that BML-260 treatment may promote the negatively regulation of APC/C^{Cdh1} and thus, cell cycle progression. Downregulation of c-Myc could also contribute to HSPC expansion. Besides, the positive effect of BML-260 on cell viability may be related to downregulation of Bax apoptotic gene (Figure 5.24).

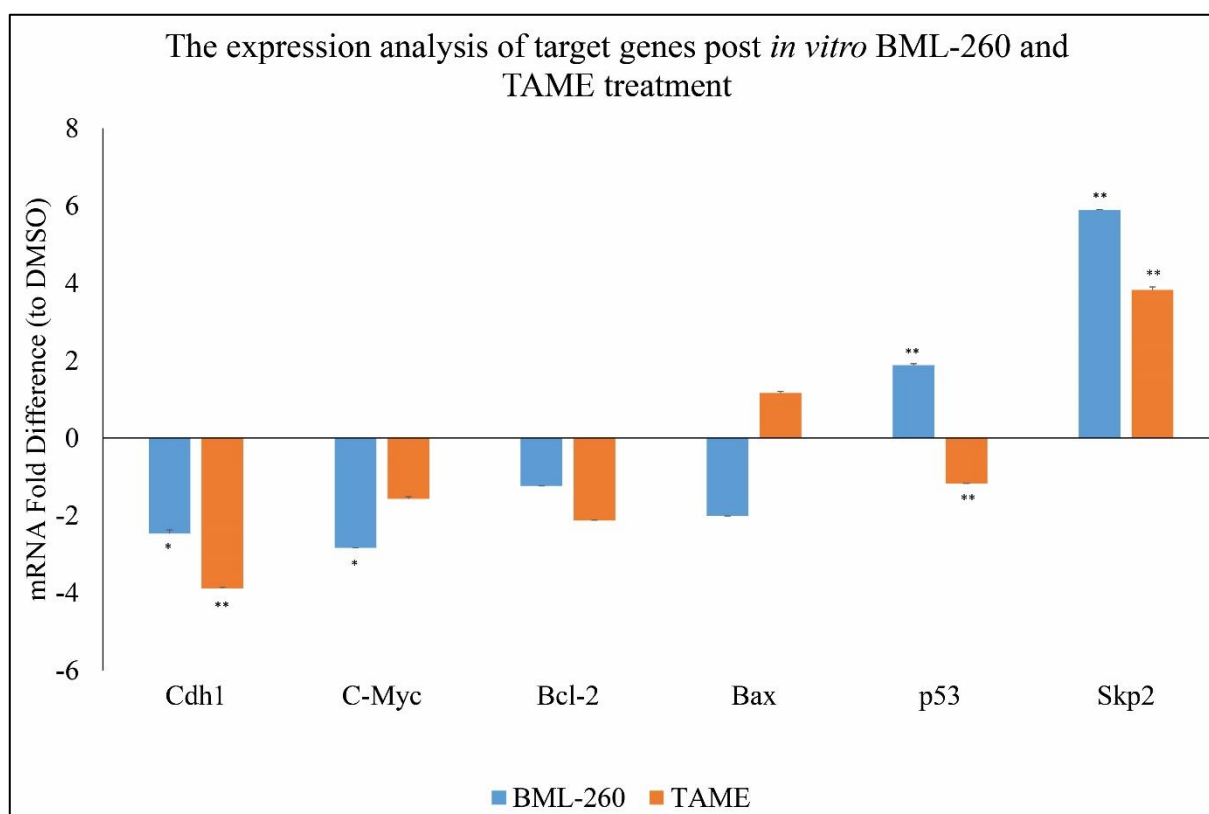


Figure 5.24. Gene expression analysis for targets of BML-260 and TAME inhibitors-related proteins on HSM-expanded mPB-HSPCs compared to DMSO by Real-time PCR; n=2; *p<0,05, **p<0,01.

5.11. COMPARATIVE ANALYSIS OF CHEMICALLY-DEFINED HUMAN HSC EXPANSION MEDIUM COMPOSITIONS ON MPB-MNC

Chemically-defined medium, which are one of many cell culture medium types and consists of RPMI 1640, DMEM, IMDM and etc., is basically used for cell culture to growth of the cells. To develop human HSC expansion medium, we compared two basal medium types RPMI 1640 and IMDM prepared with different serum concentrations such as 5 percent FBS, 10 percent FBS, 20 percent FBS and also, serum-free. BML-260 which was found to be more potent than TAME molecule, was used for small molecule supplement and also, cytokine combinations (SCF, TPO and FLT3) were used for every medium compositions. Besides, in the absence and presence of insulin-transferrin-selenium (ITS) which is known to be used for serum-free medium to reduce the serum amount was compared to determine the effective expansion medium composition (Table 4.6). Post 7days culture of mPB-HSPCs

in these different medium compositions, human HSPC content analysis was assessed. We found that any compositions of IMDM basal medium did not show any significant increase on human CD34⁺. IMDM composition supplemented with 5 percent FBS, cytokines and BML-260 molecule only increased the frequency of CD34⁺CD38⁻ cell population (Figure 5.25c and 5.25d). However, we determined that RPMI 1640 was more effective on enhancement of CD34⁺ and CD34⁺CD38⁻ cell populations. The most effective medium composition was identified as RPMI 1640 supplemented with 10 percent FBS, cytokine combinations, BML-260 and ITS. This medium composition increased the CD34⁺ cell frequency around 2-fold and CD34⁺CD38⁻ cell frequency around 25-fold (Figure 5.25a and 5.25b). These preliminary results reveal that RPMI 1640 composition supplemented with 10 percent FBS, cytokine combinations, BML-260 and ITS could be promising for development of chemically-defined human HSC expansion medium. However, the repetitive comparative analysis with basal and commercial human HSC expansion medium should be done.

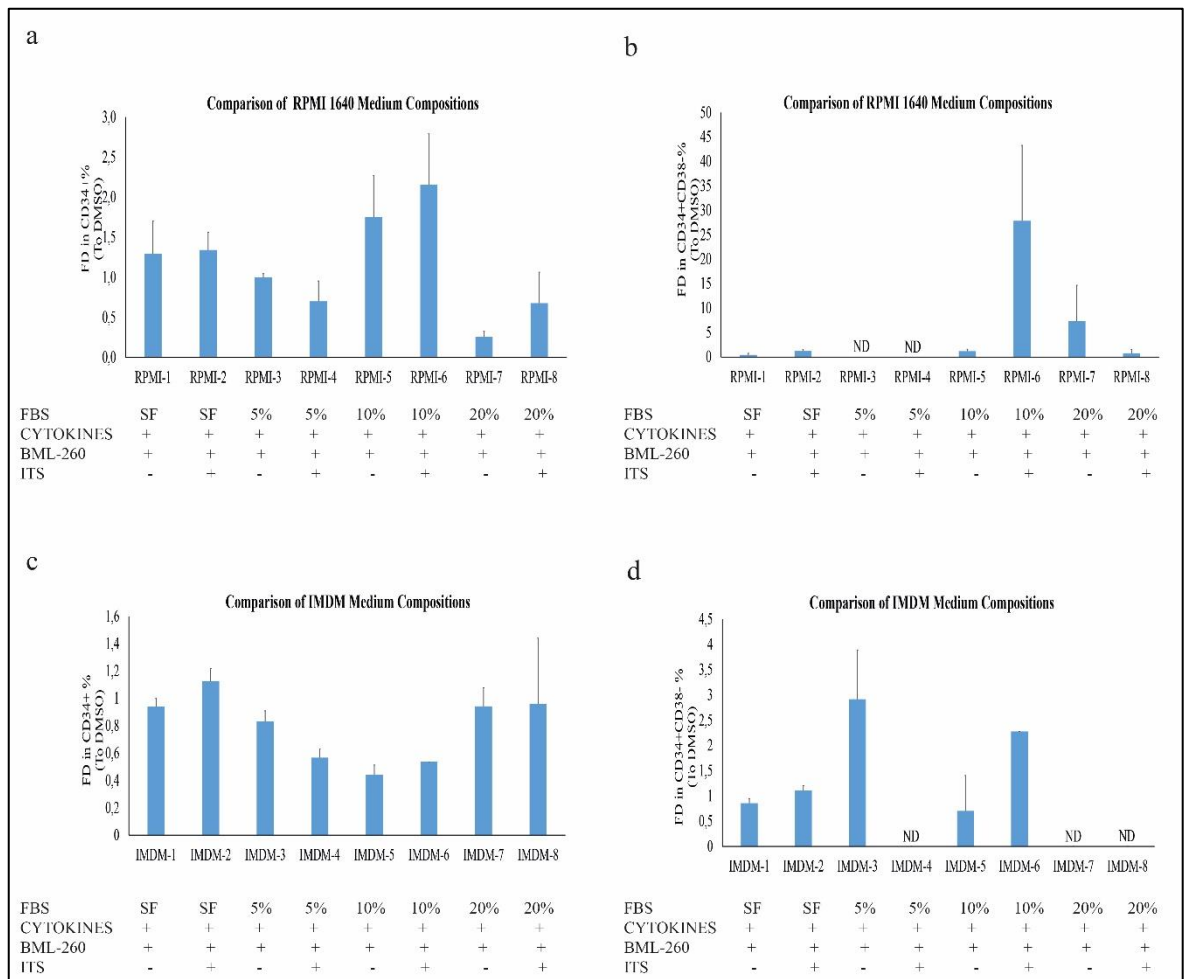


Figure 5.25. Comparative analysis of different chemically-defined HSC expansion medium which consists of various supplements by analysis of HSPC content. Post culture with RPMI 1640 basal medium compositions, the analysis of a)CD34+ b)CD34+CD38- populations and also, post culture with IMDM basal medium compositions, the analysis of c)CD34+ d)CD34+CD38- populations were shown.

5.12. DETERMINATION OF THE EFFECTIVE HSM COCKTAIL FOR HUMAN HSC EXPANSION

Cellular processes are carried out by collaboration of many pathways in a cell. HSCs have dynamic structure because of either their niche regulation or their role in hematopoiesis. Enhancement of their cell division capacity can be required for modification of many collaborative pathways. Herein, development of combinational HSM mixture, which regulates multiple modulation on the cells, could be therapeutically way for human HSC

expansion. From this point of view, we focused on the development of HSM mixture for human UCB-HSC expansion with our HSM pool. For that, we firstly prepared to different HSM mixtures with all 35 HSM. Strategy of mixture preparation using in this study was schematically presented in Figure 5.26a. We prepared to 36 mixture at 0,01 μ M, 0,1 μ M and 1 μ M working concentration and, each mixture included the all HSMs excepting one of them, respectively while the one mixture included all of them. Therefore, we determined the effect of each HSMs by this way for elimination of ineffectual HSM. We performed the trial of prepared mixtures on UCB-MNCs and HSC content was analyzed post 7 days of treatment with three doses of the mixtures.

We compared the results to DMSO and ALL mixture including all HSMs. We found that 1 μ M concentration from three working concentration was not worked well and 0,01 μ M concentration was ideally worked. The common results obtained from treatment of 0,01 and 0,1 μ M concentrations showed that #1, #3, #4, #11, #12, #18, #22, #23, #25, #26, #29, #30, #33 and #35 numbered molecules were essential for the UCB-CD34⁺ cell expansion (Figure 5.26b). Besides that, the studies based on lack of HSMs indicated that #1, #3, #4, #8, #11, #12, #15, #18, #22, #23, #25, #26, #29, #30 and #35 numbered molecules were essential for the UCB-primitive HSC expansion (Figure 5.27) for 0,01 and 0,1 μ M concentrations. The lack of these identified HSMs blocked increase in the CD34⁺ cell and primitive HSC content 1,5-fold.

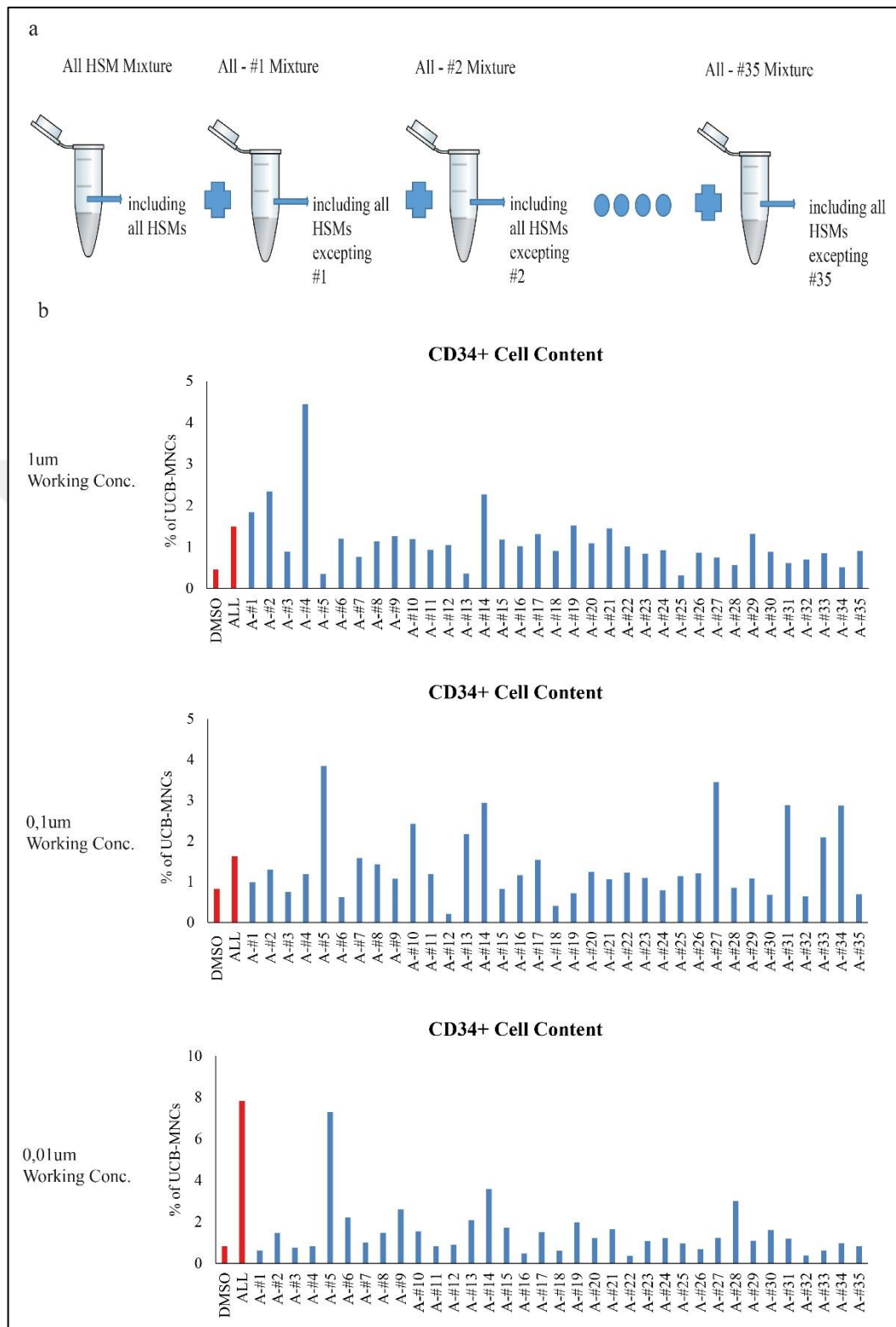


Figure 5.26. Determination of CD34+ content by flow cytometry post trial of newly prepared mixtures. a) The preparation strategy of HSM mixture was shown, b) The quantification results of CD34+ cell content analysis were shown depending on the working concentrations.

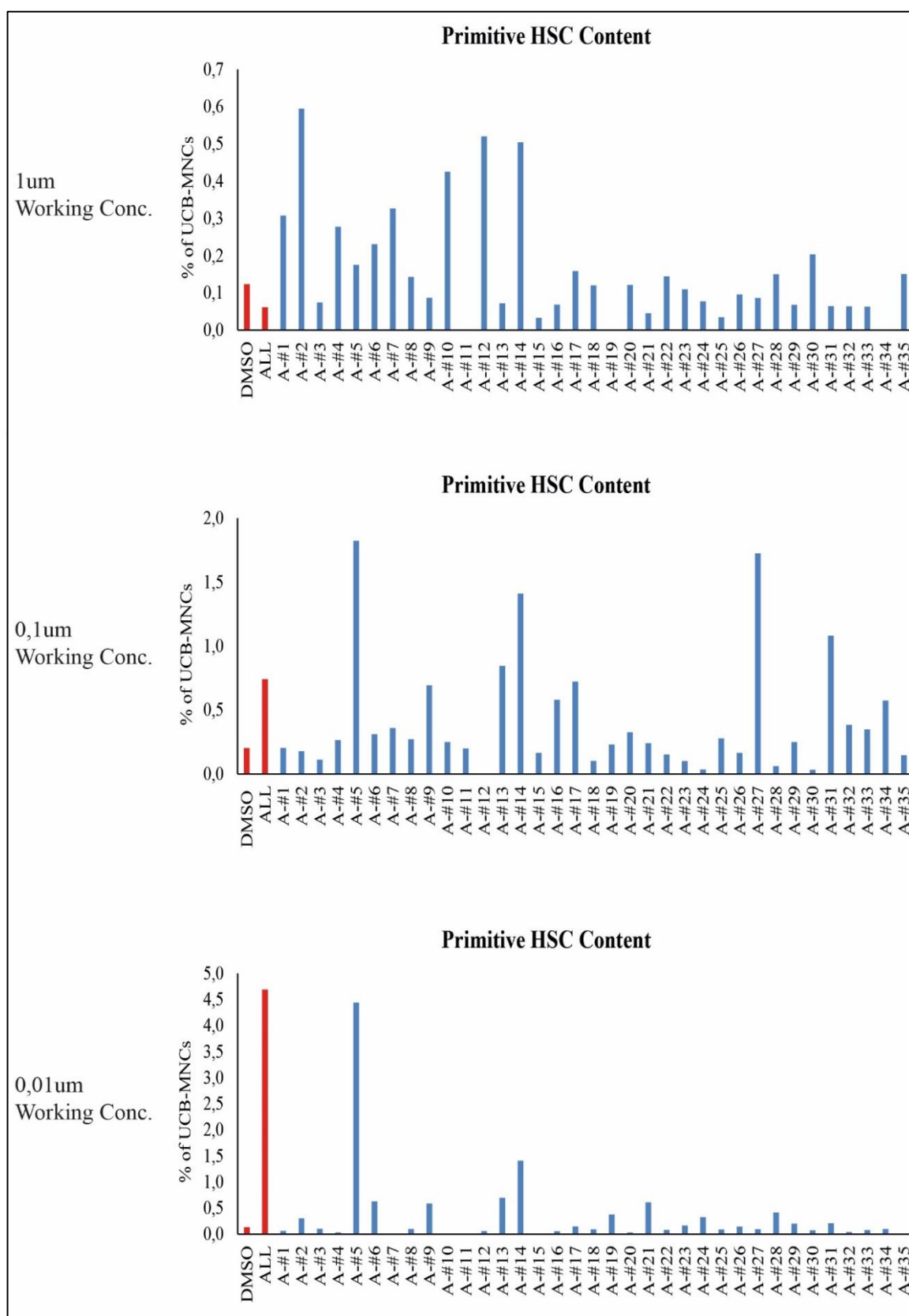


Figure 5.27. Determination of Primitive HSC content by flow cytometry post trial of newly prepared mixtures depending on the working concentrations.

The common results of CD34+CD38- cell content obtained from treatment of 0,01 and 0,1 μ M concentrations showed that #1, #3, #4, #12, #15, #16, #18, #29, #32 and #35 numbered molecules were essential for the UCB-CD34+CD38- cell expansion (Figure 5.28).

On the light of these results, we concluded that the results which belonged to 0,01 μ M and 0,1 μ M concentrations highlighted 13 HSMs including #1, #3, #4, #11, #12, #18, #22, #23, #25, #26, #29, #32 and #35 numbered molecules, to be necessary for human HSC expansion in the first step. Other HSMs were eliminated for development of mixture.



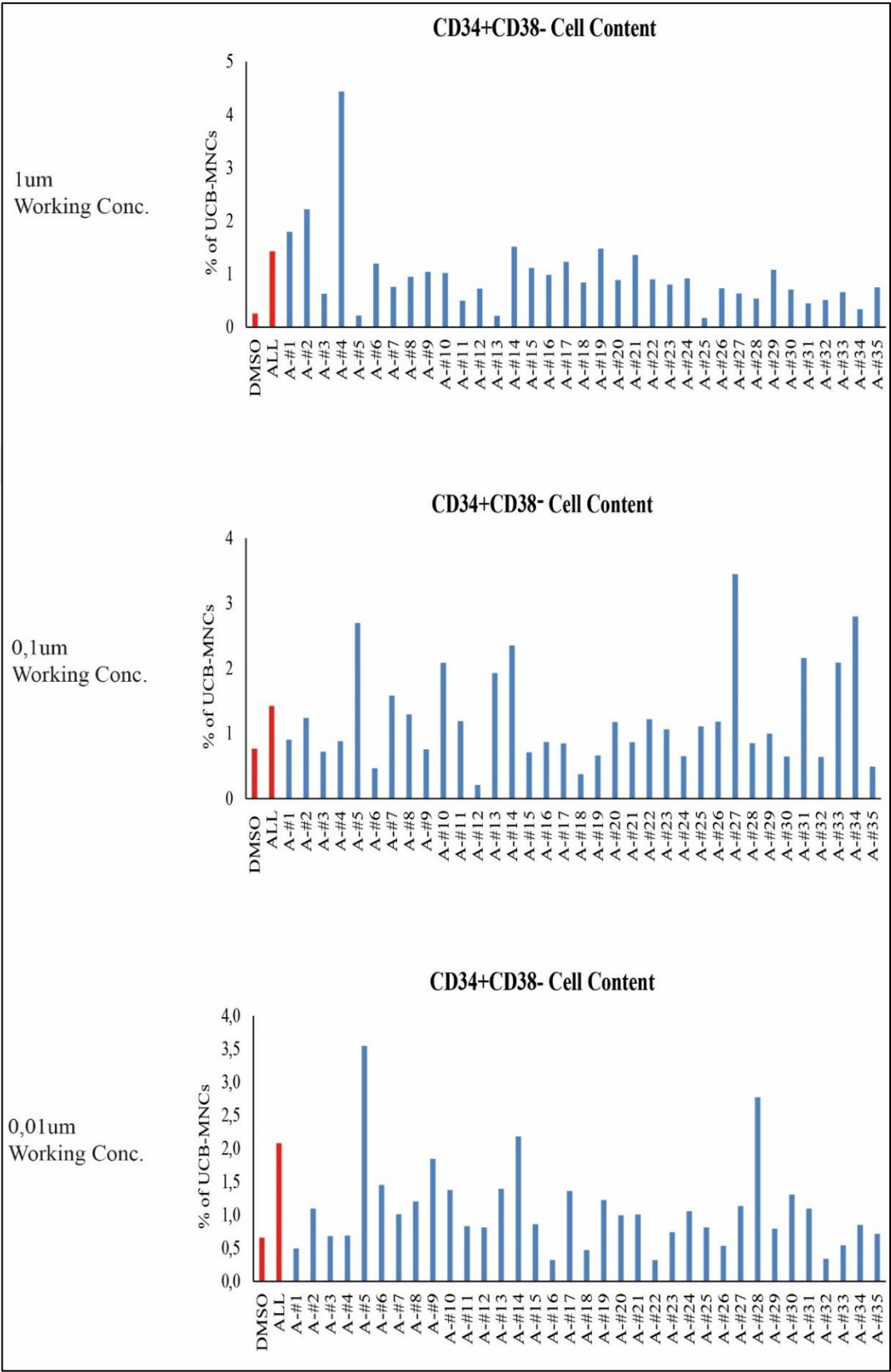


Figure 5.28. Determination of CD34+CD38- content by flow cytometry post trial of newly prepared mixtures depending on the working concentrations.

For the second elimination, the new mixtures were prepared by the way as described before. When the results were compared to results of ALL(13HSM) mixture, we found that 0,01 μ M and 0,1 μ M concentrations were worked well and according to these results, #1, #22, #23, #26, #32 and #35 numbered molecules were found to be essential for CD34⁺ cell expansion combination (Figure 5.29). Besides, #1, #11, #22, #23, #26 and #35 numbered compounds were indispensable for CD34⁺CD38⁻ cell expansion (Figure 5.30). Given these findings, we decided utilization of #1, #11, #22, #23, #26, #32 and #35 molecules to prepare the new mixtures post second elimination step.



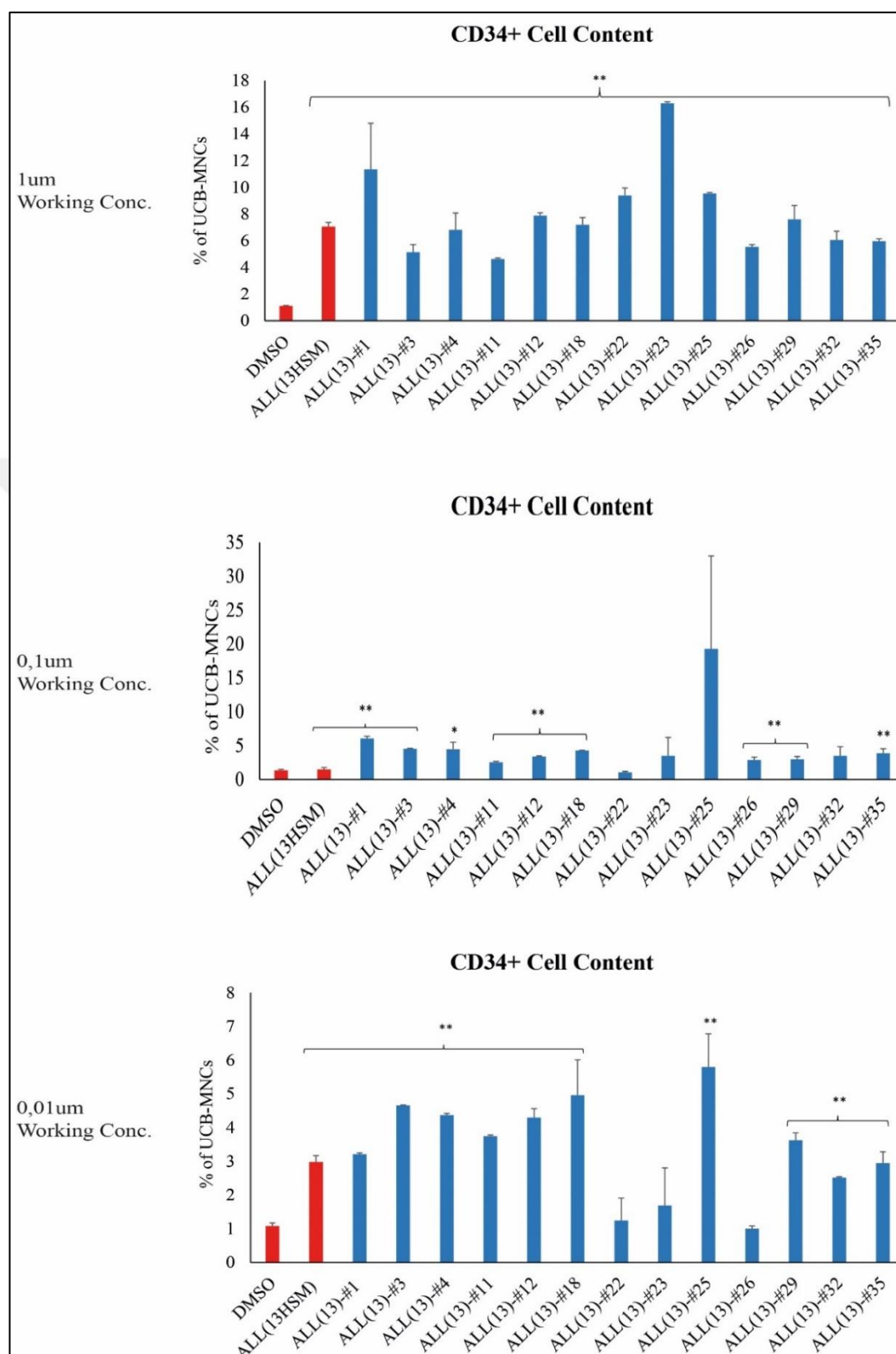


Figure 5.29. Determination of CD34+ cell content post treatment of newly identified HSM cocktail (13HSM) following first elimination depending on dose (n=3, *p<0,05, **p<0,01).

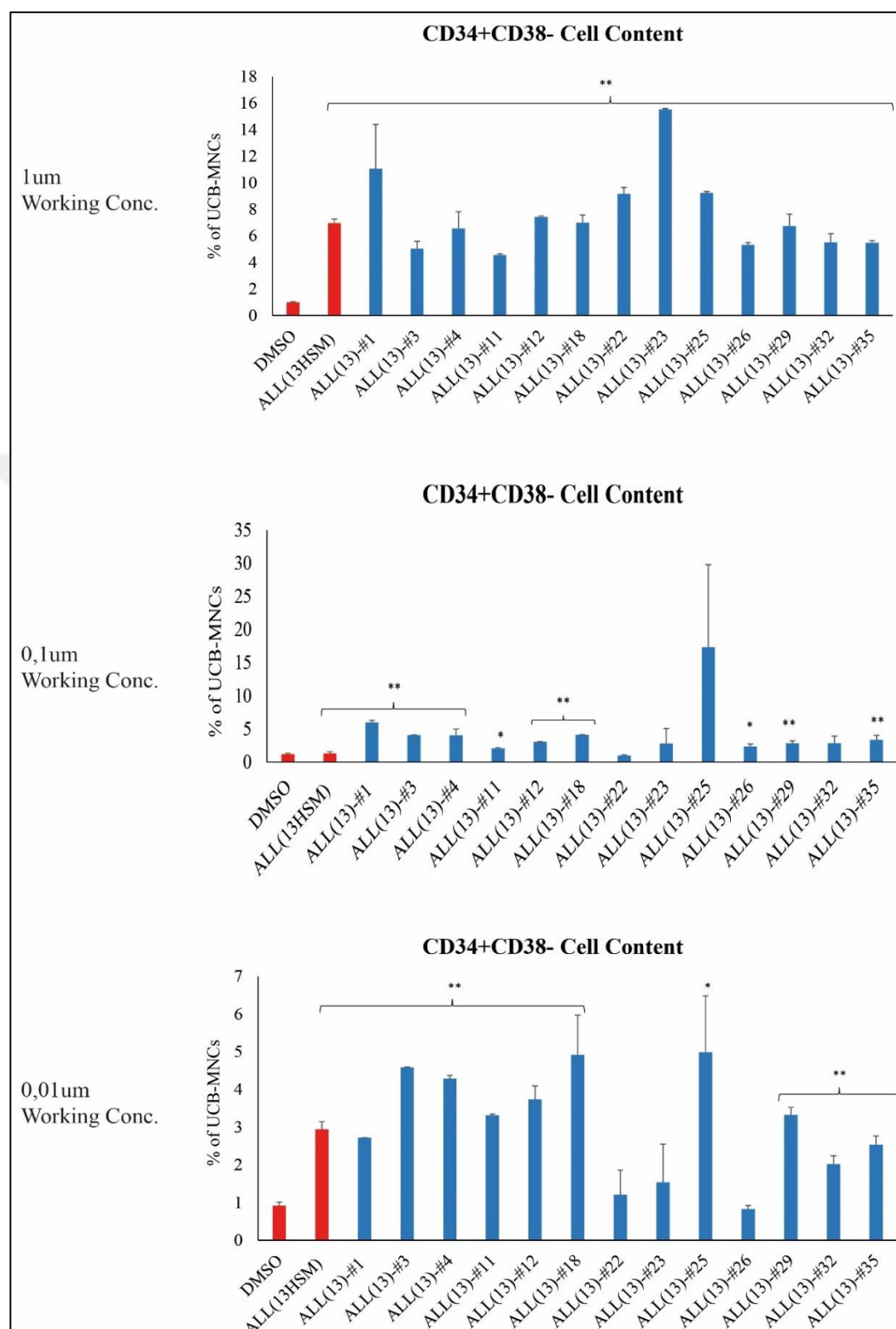


Figure 5.30. Determination of CD34+CD38- cell content post treatment of newly identified HSM cocktail (13HSM) following first elimination depending on dose (n=3, *p<0,05, **p<0,01).

We prepared the new mixtures with selected 7 HSMs and we analyzed them to perform third elimination for determination of proper mixture. According to HSC content analysis, 1 μ M of ALL(7HSM) mixture showed any increase in CD34+ and CD34+CD38- cell content like ALL(35HSM) and ALL(13HSM) mixtures. The concentration was not worked well. 0,01 μ M and 0,1 μ M concentrations of the newly prepared mixtures did not unexpectedly provided the increase depending on order of mixture (Figure 5.31). Therefore, we did not select the new HSMs for new small mixture.

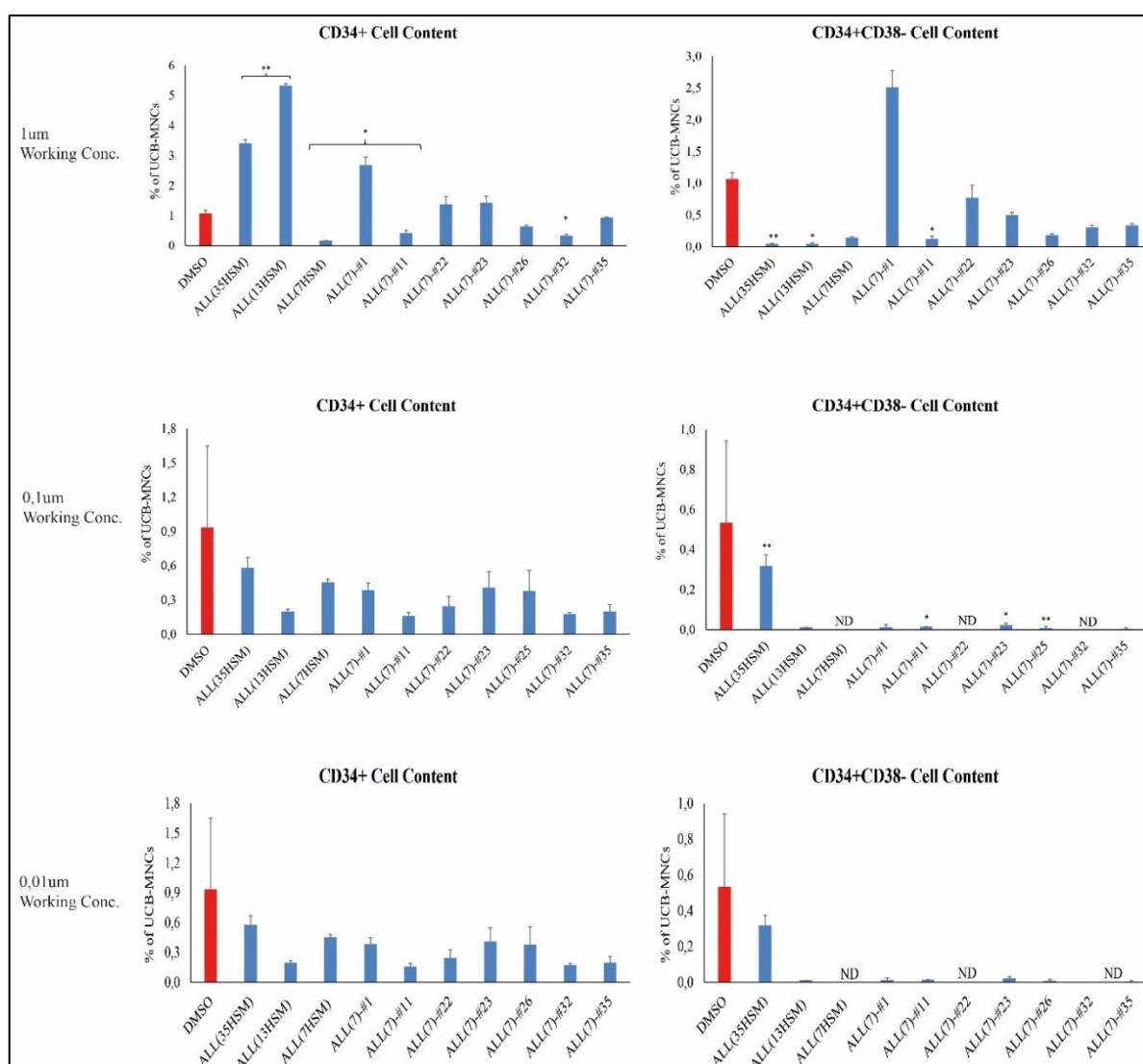


Figure 5.31. Determination of HSC content post treatment of newly identified HSM cocktail (7HSM) following second elimination (n=3, *p<0,05, **p<0,01).

Then, we wanted to analyze the mixture including 7 HSMs as a proper combination. The treated cells with three doses of the new mixture were analyzed post 7 days of treatment about HSC content. We found that any concentration of the new mixture induced CD34+ and CD34+CD38- cell expansion compared to DMSO (CD34+ cell content: 0,01 μ M: 0,22 percent, 0,1 μ M: 0,47 percent, 1 μ M: 0,06 percent vs DMSO: 0,95 percent) (CD34+CD38- cell content: 0,01 μ M: 0,09 percent, 0,1 μ M: 0,14 percent, 1 μ M: 0,05 percent vs DMSO: 0,76 percent) (Figure 5.32a). For ALDH assay, we found the supportive results with HSC content analysis; the new mixture reduced ALDHhi cell content more than 2-fold in a dose dependent manner, unexpectedly. We performed ROS detection assay to evaluate the destructive effect of the new mixture on the cells for explanation of the unexpected results. However, we found any remarkable effect on ROS production post treatment. These results were shown in Figure 5.32b. Briefly, the newly developed mixture could not be reduced to mixture including more less HSM content and the mixture as it stands was found to be unefficient for human UCB-HSC expansion. Our HSM pool could not be used for development of new HSM cocktail for human HSC expansion.

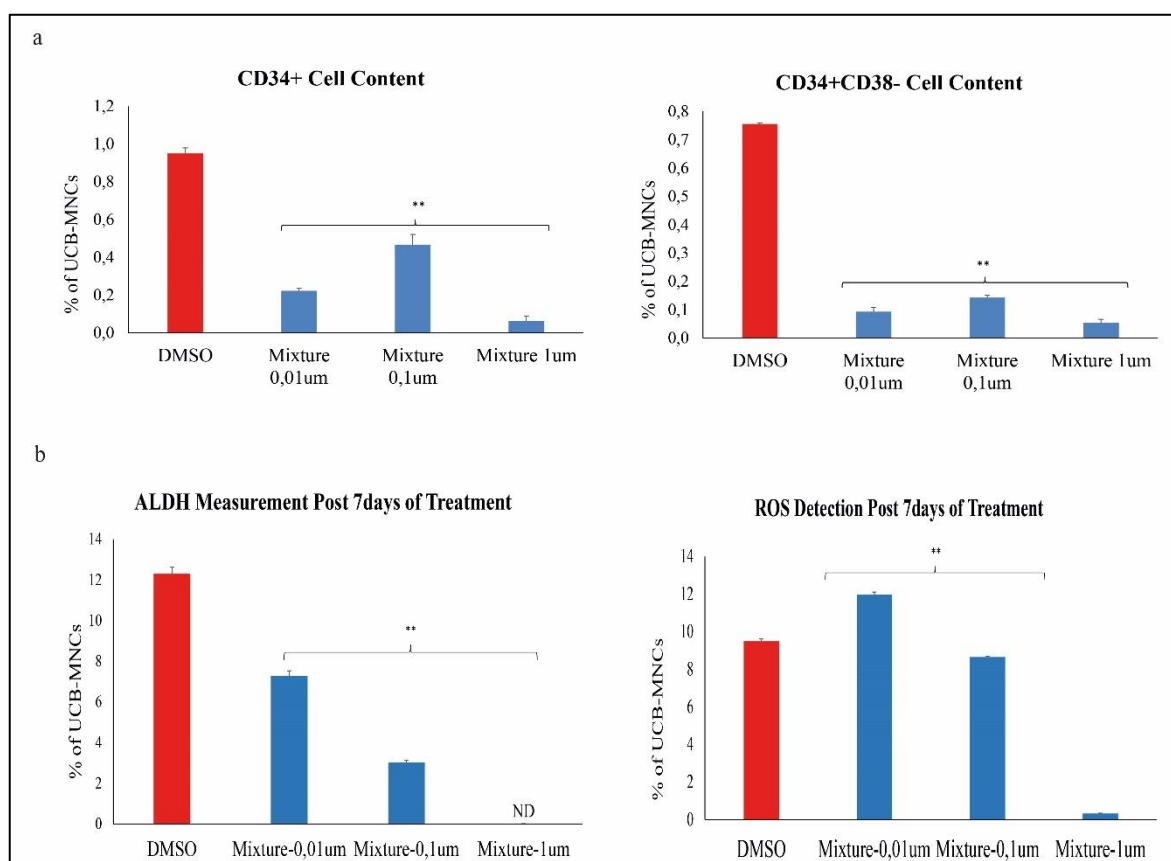


Figure 5.32. Determination of HSC Content and metabolic profile post treatment of new HSM combination, a) HSC content analysis by flow cytometry was shown, b) the quantification results of ALDH measurement and ROS detection assays were shown (n=3, **p<0,01).

Taken together, p300/CBP inhibitor VI molecule provided enhancement of mPB and UCB-HSPC content, but may be resulted in cell cycle arrest at G1/S checkpoint and DNA damage on mPB HSPCs. Besides, this molecule had apoptotic effect on the cells. 2-APB is more promising for UCB-HSC expansion, but not mPB-HSCs. Because, 2-APB specifically enhanced HSPC content, multilineage potential and the re-entry of UCB-CD34+ cells to cell cycle. Disappearance of any significant effect of 2-APB on apoptosis and DNA defect is hopeful result. TAME molecule showed the important results for human HSC expansion. TAME has similar effects with BML-260 molecule about enhancement of mPB and UCB HSPC content and multilineage potential, providing of re-entry to cell cycle and cycling. Both BML-260 and TAME molecules modulated cell cycle regulators including p27, Skp2 and Cdh1. Crosstalk between them promotes regulation of HSC cycling. However, TAME

resulted in DNA damage on mPB-HSPCs while did not effect on apoptosis or ROS production. TAME is promising according to our results but will need more detail study for safety. BML-260 molecule is the most prominent compound for human HSC expansion in this study because of their adjuvant effect on both mPB and UCB-HSPC content and multilineage potential, trigger function on re-entry to cell cycle, enhancement of viability in parallel with disappearance of any effect on apoptosis, DNA damage and ROS production. BML-260 is the most prominent compound according to this study and will required to advanced experiments for utilization in medium development. Furthermore, preliminary analysis about identification of chemically-defined HSC expansion medium reveal that RPMI 1640 is more efficient for human HSC culture and RPMI 1640 supplemented with 10 percent FBS, cytokine combinations, BML-260 and ITS composition could be promising for development of chemically-defined HSC expansion medium.

6. DISCUSSION

Hematopoietic stem cells are involved in the top of origin of adult hematopoiesis alongside their function during fetal hematopoiesis. Their vital responsibilities on blood production by high capacity of differentiation to whole blood cell types situate HSCs in the center of life of many organisms. The most amazing hallmark of HSCs is undoubtedly that they are staying on quiescent situation, nevertheless they are stem cells. Therefore, the abilities of cycling and metabolic properties, adaptation for survival against to many cellular damage, aging and death mechanisms in addition to their differentiation capacity are main topics for HSC researches.

Reconstitution of hematopoietic system have critical importance in clinical medicine. Many people in the worldwide are suffered from hematological malignancies and disorders, autoimmune disorders and also, genetic blood-related diseases[77,164]. The most promising approach of treatment of these diseases is HSC transplantation. WMDA reported that more than 50,000 people in every year are treated with HSC transplantation. For many years, bone marrow is conventionally used for HSC transplantation. However, particularly lack of HLA-matched donor, requirement of surgical intervention, and also high incidence of graft versus host diseases (GVHD) attract attention to umbilical cord blood source in despite of mobilized peripheral blood. Because, peripheral blood source has the similar disadvantages with bone marrow. Therefore, UCB is commonly used for HSC transplantation depending on their advantages such as no requirement of surgical intervention and HLA matching, and also reduced incidence of GVHD. In spite of these clinical advantages, reduced hematopoietic recovery depending on lack of sufficient HSC number for transplantation which is a major problem for UCB-HSCT. To overcome from this handicap, usage of two UCB unit have been tried for a long time, but that is not forceful solution[77]. Alongside the treatment of hematological malignancies and disorders, expansion HSC are important approach for utilization of genetically modified HSCs on genetic blood disorders such as sickle cell disease and anemia types. Thus, investigations on development of clinical-grade HSC expansion technologies have become the focus point for researchers.

The main criteria of *ex vivo* expansion of HSCs is based on symmetric cell division, enhanced self-renewal capacity without differentiation[2]. On the other hand, the main limitations of *ex vivo* expansion of HSCs are over-cycling and mutations on the cells

depending on prolonged culture[165]. These handicap and criteria have been considered for the development of *ex vivo* HSC expansion technologies. In common with identification of transcriptional and niche-related regulator of HSCs and also requirements of them in culture, utilization of cytokines/growth factors, molecular regulator pathway components and co-culture with neighbour cells involved in their microenvironment have been initial approaches. Many studies determined the most effective cytokines and combinational usage of them and showed that utilization of SCF, FLT3, TPO and IL-3,6 are sufficient and effective for HSC survival in culture. Current studies on transcriptional and molecular regulation demonstrated the role of many pathway such as Notch, Wnt, Hedgehog, Angiopoietin, hypoxia and Hox genes on HSC expansion. It was reported that overexpression of these pathway components and their ligands promotes HSC expansion and reconstitution. In addition to them, it was indicated that epigenetic control remarkably regulates HSC self-renewal, differentiation and proliferation and also, epigenetic modifications-related agents can be used for HSC expansion. Besides, the importance of niche cells and their secreted molecules revealed an approach based on co-culture of HSCs with niche cells. Furthermore, several culture systems containing the previously identified factors have been developed as 2D and 3D or liquid and perfusion systems. These techniques have been investigated and supported by clinical studies on human[3,77,79,166].

Another *ex vivo* HSC expansion technique is based on utilization of small molecules (SM) which is characterized by low molecular weight and easily diffusion from cell membrane and no immune-response. Small molecules affect many pathway components involved in regulation of self-renewal, proliferation, differentiation, apoptosis, adhesion, migration and also interaction with other cells on HSCs. In presence of these molecules cause either upregulation of target pathways or downregulation of target proteins. There are many identified small molecules, even some of them proceeded to clinical phase trials. For instance, the phase I/II trials of Stemregenin-I, UM171, Nicotinamide, PGE2 and TEPA molecules were carried out. Among them, Stemregenin-I is firstly identified SM and is highly promising for human HSC expansion. The effect mechanisms of SMs differ depending on function of their target pathways and proteins. Nicotinamide, Valproic acid and DEAB compounds induce HSC expansion by reduction of differentiation while PGE2, Nicotinamide, Garcinol and SB203580 compounds induce both HSC expansion and homing ability. Whereas, zVADfmk/zLLYfmk and 5-HT compounds result in enhanced HSC self-

renewal by reduction of apoptosis[99]. We also reported that C-myc inhibitor (10074-G5) and Meis-1 inhibitors (Meisi-1 and Meisi-2) induce mouse BM and human HSC expansion[150,151]. Taken together, SMs can replace the cytokines and other transcriptional factors for *ex vivo* HSC expansion. However, they require more detail investigation in the long-term safety.

To this end, we screened small molecule pool containing 35 different compounds. Our strategy to create hematopoietic small molecule (HSM) pool was based on analysis of role of different genes on literature, drugable property and also, availability on small molecule databases. We initially 26 compounds from 200 genes which have knockout studies in HSCs, by elimination depending on the adjuvant effect on cell cycle activity, stem cell pool and finally, availability on ChEMBL, PubChem and etc. small molecule databases. Then, we added 9 new compounds which are known to be related to quiescence of cardiomyocytes by our cardiac regeneration studies. The similarity between cardiomyocytes and HSCs about quiescence increased our 26 compounds to 35 HSMs by adding 9 new compounds. Eventually, we focused on 26 compounds among 35 HSM (9 of them, previously studied) for human *ex vivo* HSC expansion by comparison with 9 previously studied HSMs.

We hypothesized that targeting to HSC quiescence factors by putative HSMs which are known as inhibitors of component of stem cell regulation pathways could be induce HSC expansion *ex vivo* and *in vivo*. Therefore, we aimed to identify the most effective HSMs and HSM combinations to develop GMP-grade human HSC expansion medium for successful HSC transplantation and gene therapy applications. For this aim, we used two HSC sources; mobilized peripheral blood and umbilical cord blood for comparison. Depending on the first HSM screening, we determined that 12 HSMs were remarkably increase the human HSPC pool. 6 of them were previously tested and 6 of them were putative HSMs for our study. We used the previously tested molecules for comparison with our putative HSMs. We briefly determined that among HSMs, 2-APB and Meis-1 inhibitors (Meisi-1 and Meisi-2) increased the UCB-CD133+ cell ratio as well as previously tested molecules SR-I and bpV(HopIc). 2-APB and TAME compounds were higher increased CD34+ and ALDHhi cell count in UCB-MNCs compared to previously identified bpV(HopIc) and AMD3100 and as well as SR-I and Nicotinamide molecules. Besides, BML-260 and Meis-1 inhibitors (Meisi-1 and Meisi-2) were increased CD34+ and ALDHhi cell count as well as bpV(HopIc) and AMD3100. Intriguingly, BML-260, TAME and Meisi-1 inhibitors were increased UCB-

total cell count while p300/CBP inhibitor VI was highly increased comparing to other compounds. After the first screening, the determined HSMs were evaluated to confirm their effect on UCB-MNCs. The confirmation was resulted in highly increase in CD34⁺ cell, CD34⁺CD38⁻ cell and primitive HSC content post treatment of p300/CBP Inhibitor VI and BML-260 compounds comparing to DMSO. The increase in CD34⁺ cell content of p300/CBP Inhibitor VI-treated cells was higher from the increase in bpV(Hopic)-treated cells. Moreover, the increase in CD34⁺CD38⁻ cell content of BML-260-treated cells was higher from the increase in bpV(Hopic)- and Nicotinamide-treated cells. We compared all results both previously tested molecules and DMSO control by calculation of average of all doses results. When the results are evaluated based on their effective doses, their adjuvant effect on UCB-HSPC pool can be clearly indicated.

P300/CBP inhibitor VI is a potent inhibitor of p300 histone acetyltransferases less potent against CBP (cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB)-binding protein) component of p300/CBP interaction. It has been known that histone acetylation contributes to gene activation and thus, interaction between p300 and CBP is responsible for gene modification through regulation of chromatin structure[167]. The function of p300/CBP interaction in hematopoietic cell regulation is based on transcriptionally regulation of hematopoietic factors such as Gata-1, Myb and PU.1. For instance, Gata-1 regulates proliferation and differentiation of megakaryocytes and erythroid maturation and responsible for HSC quiescence and self-renewal. Besides, Gata-1 is involved in progression of differentiation through cell cycle arrest. In addition, CBP is known to be regulator of c-Myc oncogene which negatively promotes HSC proliferation[168]. Moreover, it was reported that CBP is required for self-renewal while p300 is required for differentiation in hematopoiesis[169]. These transcriptional modulation of hematopoietic factors by p300/CBP system provides the balance between self-renewal and differentiation. Potentially inhibitor effect of P300/CBP inhibitor VI on p300 component comparing to CBP and modulation of self-renewal and quiescence related protein by p300/CBP can explain that the positive regulation of p300/CBP inhibitor VI on human HSPC pool. Here, we found the highly adjuvant effect of p300/CBP inhibitor VI on mPB CD34⁺ and CD34⁺CD38⁻ cell populations comparing to bpV(Hopic) and Nicotinamide alongside UCB-HSPC pool. However, p300/CBP inhibitor VI treatment led to cell cycle arrest at S/G2/M phase on mPB-HSPC. Moreover, it was reported that interaction of p300 and CBP

play a crucial role as transcriptionally coactivator of p53-mediated apoptosis in response to genotoxic stress[170]. Recent studies showed the pro-apoptotic function of p300 rather than CBP component[171]. Binding and modulation of p53 is promoted by p300/CBP coactivators. Unlike these findings, we found that p300/CBP inhibitor VI has highly apoptotic and genotoxic effect on mPB-HSPCs. Intriguingly, we found that reduced ROS level in despite of DNA damage effect on p300/CBP inhibitor VI-treated mPB-HSPCs. Reduced ROS level have explained the metabolically inactivated cells and thus, cell cycle arrest that we detected. It was demonstrated that interaction of E2F which promotes G1/S transition in cell cycle, with p300/CBP complex by cyclin E-CDK2 regulate cell cycle progression[172]. Besides, CBP is known to be required in anaphase-promoting complex/cyclosome (APC/C) function from E3 ubiquitin ligase which controls G1 phase progression in cell cycle[173]. Since p300/CBP regulates cell cycle progression components. Cell cycle arrest on human CD34+ resulted by p300/CBP treatment may be caused by alterations on function of p300/CBP-interacted cell cycle regulators. *In vitro* and *in vivo* studies about previously identified different HAT inhibitor, GAR reported that GAR enhanced human UCB-HSC expansion[119]. Even this finding presented promising perspective for our HAT inhibitor on human HSC expansion, we did not continue with p300/CBP inhibitor VI compound for human hsc expansion on the light of our findings.

2-Aminoethoxydiphenyl borate (2-APB) is identified as an antagonist of inositol 1,4,5-trisphosphate (InsP3R) and inhibits InsP3-induced Ca^{+2} release from mouse cerebellar membranes in a concentration-dependent manner[174]. However, 2-APB is to be blocker of store-operated Ca^{+2} entry (SOCE) independently of InsP3. Moreover, it promotes the gradual release of Ca^{2+} stores through inhibiting sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs) pumps. 2-APB does not only modulate Ca^{+2} release, but also affects MagNuM ion channels which promote Ca^{+2} and Mg^{+2} ion transportation[175] Calcium metabolism involved in HSC niche regulates HSC maintenance, quiescence and self-renewal[176]. Endosteal niche where HSC localize in BM is characterized by high calcium concentrations found in osteoclasts in the niche. HSCs highly express calcium sensing receptors (CaRs) which is a G protein-coupled receptor to provide sensitivity against to calcium abundance in response to niche. Deficiency of CaR on HSC showed the reduction of HSC lodgement to endosteal niche[177]. The effect of CaRs on HSC lodgement mainly regulates HSC homing. Stimulation of CaR with its agonist Cinacalcet allowed enhancement of *in vivo*

homing to BM and CXCR4-mediated lodgement into the niche and thus, engraftment ability of HSCs which are steps of transplant engraftment[178]. Here, we show that 2-APB treatment allows *ex vivo* UCB-HSC expansion. We found that 2-APB treatment significantly increased CD34⁺, CD133⁺ and ALDHhi populations of human UCB and also, increased frequency of CD34⁺ cells at S/G2/M phase (up to 5 fold) by decreasing cell percentage at G0 and G1 phases in UCB cell cycle. However, any alterations were not detected on cycling ability of mPB-CD34⁺ cells in despite of enhancement of CD34⁺CD38⁻ and ALDHhi cell populations with 2-APB molecules. Moreover, 2-APB treatment led to enhance multi-lineage potential of UCB-MNCs. In addition, ROS production was slightly increased with 2-APB treatment. Then, we evaluated whether this increasement in ROS level of treated mPB-MNCs affect on cell viability, genotoxicity and apoptosis. 2-APB molecule did not show any effect on DNA damage, apoptosis and thus, viability in treated cells. These findings reveal that 2-APB is more promising for *ex vivo* expansion of UCB-HSC rather than mPB hematopoietic cells. 2-APB provides human mPB-HSPC pool size and maintenance.

BML-260 small molecule is an inhibitor of dual specificity phosphatase JSP-1 (DUSP22) which is involved in dual specificity phosphatase subclass of protein tyrosine phosphatase family (PTPs). PTPs are responsible for regulation of many cellular processes including cell growth, proliferation, differentiation, immune response, cell–cell and cell–matrix interactions[179]. Jsp-1 (Jnk Stimulatory Phosphatase-1) promotes activation of JNK pathway (c-Jun NH₂-terminal kinases) which is an evolutionarily conserved subclass of mitogen-activated protein (MAP) kinases and deactivation of p38 MAPK and c-Jun terminal kinase and thus, is involved in signal transduction[180]. Recent studies showed that activation of JNK pathway either play an apoptotic function or anti-apoptotic function. Pro-apoptotic effect of JNK activation is mediated by overexpression of c-Jun in neural cells. Besides, the role of c-Jun in JNK activation-dependent apoptosis was shown in mouse embryonic fibroblasts in response to ultraviolet irradiation[181]. These finding reveal that JNK pathway contribute to protect against genotoxic stress. Interestingly, Gadd45a and Gadd45b from Gadd45 proteins which promote cell growth and apoptosis in response to DNA damage and also, have a pro-apoptotic role via activation of the stress response kinases p38 and JNK protects hematopoietic cells against UV radiation-induced genotoxic stress through activation of NF-κB-dependent survival via activation of p38 and inhibition of JNK activity[182]. On the other hand, JNK pathway promotes TNF-α induced apoptosis in

response to inflammation. TNF- α which is a proinflammatory cytokine initiates activation of caspases, I κ B kinase (IKK) and JNK for apoptosis. However, NF- κ B prevents TNF- α -induced apoptosis by inhibiting caspase activation and prolonged JNK activation[163]. Besides, JNK pathway contributes to activation of mitochondrial apoptotic pathway through activation of pro-apoptotic members and inhibition of anti-apoptotic members of the Bcl2-related protein family following phosphorylation of them[180]. In hematopoietic cells, JNK pathway is activated by HPK-1 (hematopoietic progenitor kinase-1) which is highly expressed in hematopoietic tissues and a firstly identified tissue-specific kinase[183]. Caspase 3-mediated processing of HPK-1 which is involved in upstream of JNK pathway regulates monocytic differentiation[184]. JNK pathway with its signal transduction role is to be regulator of hematopoietic differentiation[185]. Besides, TGF- β signaling which regulate HSC quiescence and inhibits HSPC proliferation can be promoted through JNK pathway. JNK pathway may contribute to HSC quiescence by modulation of TGF- β signaling[186]. The role of JNK pathway in hematopoiesis makes think that how targeting of JNK pathway affect on HSC expansion. Xiong Xiao et.al showed that JNK inhibitor, JNK-IN-8 highly induced *in vitro* expansion of CD34⁺CD45RA⁻, CD34⁺CD38⁻CD45RA⁻CD90⁺, and primitive CD34⁺CD38⁻CD45RA⁻CD90⁺CD49f⁺ cells of UCB and increased *in vivo* long-term repopulation capacity of expanded cells through downregulation of c-Jun gene[187]. Here, we used BML-260 molecule which inhibits activation of JNK pathway to expand human mPB and UCB HSC. We found that BML-260 molecule induced both mPB- and UCB-derived HSPC expansion in culture. On the other hand, we analyzed whether this molecule modulates cell cycle profile of expanded cells and we found that BML-260 increased the frequency of mPB-CD34⁺ cell at G1 phase and the frequency of UCB-CD34⁺ at S/G2/M phase (up to 5 fold) through decrease in the frequency of quiescent cells (at G0 phase). Besides, we detected slight increased proliferation rate with CFSE proliferation assay. Moreover, BML-260 increased multi-lineage potential of UCB-HSPCs. In correlation with these findings, BML-260 molecules upregulated the expression of MCM2 and PCNA, DNA replication-related genes on mPB-HSPCs. Besides, the expressions of p27 from CIP/KIP CDKI family which are responsible for arrest at G1/S transition and p19 from Ink4 CDKI family were downregulated, but the expressions of p21 and p57 from CIP/KIP CDKI family were slightly upregulated. Downregulation of p27 is dependent on upregulation of Skp2 in BML-260-treated cells. It was reported that Cdh1 is tightly regulated by JNK pathway[188] and inactivation of Cdh1 is related to enhancement

of Skp2 function and thus, degradation of p27 as mentioned above[189]. Interestingly, we found the upregulation of Skp2 gene in parallel with downregulation of Cdh1. It was reported that phosphorylation of Cdh1 resulting its inactivation is promoted by JNK activation[188]. BML-260 molecule could be affect on cell cycle progression through relation between Cdh1 and Skp2 and thus, downregulation of p27. Besides, BML-260 also downregulated c-Myc expression which is responsible for HSC quiescence. On the other hand, metabolically activation of BML-260-treated cells was slightly increased detected by measurement of ROS level. In despite of slightly production of ROS, BML-260 molecule did not show any genotoxic or apoptotic effect on expanded cells. Our findings are correlated with previous studies showing the positive regulation of JNK pathway on apoptosis and relation between inhibition of JNK pathway and high response to genotoxic stress[181,182]. Furthermore, we showed increased viability on BML-260-treated cells in parallel with decreased apoptotic effect. Moreover, MSH2, DNA repair gene was also upregulated on treated cells. Interestingly, BML-260 significantly downregulated the expression of RAD51, XRCC1 and ERCC1 which are responsible for DNA repair system. In addition, the expression of pro-apoptotic Bax gene involved in JNK-mediated apoptosis[190] was downregulated in BML-260-treated cells. The downregulation of Bax and upregulation of MSH2 DNA repair gene could be related to reduced apoptotic effect of BML-260 molecule. These findings reveal that BML-260 induces *ex vivo* expansion of human mPB and UCB derived HSPC through modulation of cycling potential and BML-260 is significantly potent small molecule for human HSC expansion.

Tosyl-L-arginine methyl ester (TAME) is an inhibitor of anaphase-promoting complex/cyclosome (APC/C) which is involved in cell cycle progression[191]. APC/C from Cullin-RING E3 ligase promotes the metaphase-anaphase transition during mitosis by ubiquitination-mediated proteolysis of cell cycle regulators. APC/C function is regulated by Cdh1 and Cdc20 coactivators[192]. APC/C^{Cdh1} and APC/C^{Cdc20} play a crucial and distinct role in during cell cycle progression. APC/C^{Cdh1} regulation is associated with G1 progression, entry to S phase through degradation of cyclins and S-phase regulators including Cdc6 and G1/S transition via inhibiting SCF E3 ligase activity[193]. Furthermore, it was reported that Skp2 from SCF E3 ligases is regulated by APC/C^{Cdh1}. However, APC/C^{Cdc20} regulation is involved in mitosis. Activation of APC/C^{Cdc20} by CDK1 progresses mitosis and then, inactivation of APC/C^{Cdc20} by Emi1 (Early mitotic inhibitor 1) and SAC

(Spindle Assembly Checkpoint) and activation of APC/^{Cdh1} provides exit from mitosis[194]. The switching between APC/C^{Cdc20} and APC/C^{Cdh1} cooperates cell cycle progression. TAME inhibits the APC activation through substitution of the isoleucine-arginine (IR) tail-binding sites of Cdc20 and Cdh1 via its structural similarity with IR tail of coactivators[191]. The function of APC/C with Cdh1/Cdc20 coactivators on cell cycle regulation differs between cycling and quiescent cells. Recent studies carried out in zebrafish model organism showed that trouble-free progression of mitosis requires APC/C function in cycling cells. Loss of APC/C function in cycling cells led to mitotic arrest and apoptosis. Interestingly, loss of APC/C function resulted in re-entry to cell cycle at G1 phase and then apoptosis depending on mitotic arrest[195]. In hematopoietic cells, it was reported that downregulation of Cdh1 which is known to be highly expressed in human CD34⁺ cells, by its inhibitor Emi1 resulted in promote re-entry to cell cycle and proliferation of CD34⁺ HSCs. Besides, deficiency of Cdh1 enhanced CD34⁺ cell proliferation and reduced multi-lineage potential. The negatively regulation of Cdh1 on cell proliferation reveals that APC/C^{Cdh1} promotes self-renewal and differentiation of human CD34⁺ cells[196]. These findings and relation between Cdh1 and Skp2 reveal that modulation of APC/C complex promotes HSC cell cycle ability. Taken together, we focused on TAME inhibitor of APC/C complex and we found that TAME treatment increased both UCB-CD34⁺ cell, ALDHhi cell and total cell count and mPB-CD34⁺ and CD34⁺CD38⁻ cell population. Besides, we detected increasement of cycling cell frequency with TAME molecule by BrdU incorporation. Moreover, multi-lineage potential was enhanced in TAME-treated mPB-HSPCs. On the other hand, TAME molecule provided increase in cycling potential through re-entry to G1 in mPB-CD34⁺ cells and also, facilitates entry to S/G2/M phase in UCB-CD34⁺ cells. APC/C-Cdh1 regulates cell cycle arrest through degradation of Cdk activators, such as cyclins, and the accumulation of Cdk inhibitors including p27 with proteasomal degradation of Skp2[189]. According to gene expression analysis, we found that the expression of p27 CDKI was downregulated in mPB-HSPCs with inhibition of APC-mediated proteolysis. In parallelly, the expression of Skp2 was upregulated in TAME-treated cells in addition to downregulation of Cdh1 gene. This result is correlated with literature as expected. TAME inhibitor provided HSC proliferation through increased Skp2 function-dependent p27 degradation with inhibition of Cdh1 activation. Besides, downregulation of c-Myc which is related to HSC quiescence[197] in TAME-treated cells could contribute to human HSC expansion. However, the expression of p53 CDKI was upregulated in treated cells.

Moreover, we found slight increase of ROS level on TAME-treated mPB-HSPCs and in spite of that, TAME molecule displayed apoptotic and genotoxic effect on expanded cells. The apoptotic effect of TAME molecule may be dependent on p53-mediated apoptosis. Besides, anti-apoptotic gene Bcl-2 was downregulated in TAME-treated cells. This could play a role in its apoptotic effect. Interestingly, we found the upregulation of RAD17, RAD51 and XRCC1 (DNA repair-related genes) and downregulation of PCNA which is responsible for DNA replication. These findings reveal that TAME molecule increased human mPB and UCB HSPC expansion through notably inhibition of Cdh1 and thus, modulation of cell cycle kinetics, but its side effects on expanded cells are remarkable point.

Furthermore, we aimed to improve HSM cocktail to generate simultaneously effect on multiple mechanisms of UCB-HSC expansion. For that, we could reduce our 35 HSM pool to seven HSM that determined as effective for human UCB cell expansion. Next, we analyzed the proliferative effect of small molecule cocktail including seven HSM on the cells. Nevertheless, we did not show any expansion function of the final SM mixture on UCB populations. Therefore, we did not improve HSM mixture among our HSM pool depending on the absence of synergistic effect of our 35 HSM on UCB cells.

On the other hand, comparative analysis of chemically-defined medium including RPMI 1640 basal medium supplemented with cytokine combinations (SCF, TPO and FLT-3), 10 percent FBS, ITS and BML-260 molecule was found to be most effective medium composition for human mPB HSC expansion and culture according to the preliminary data. These preliminary data should be supported with repetitive comparative analysis and also, the effective medium composition should be validated with detail investigations.

On the light of these all findings, we determined that BML-260 and TAME molecules are promising for both human mPB- and UCB-derived HSC *ex vivo* expansion. BML-260 and TAME contribute to human HSC expansion through modulation of cell cycle kinetics. BML-260 molecule is more potent to expand human HSC with no cytotoxic and genotoxic effect rather than TAME molecule. To improve the utilization of these small molecules, these molecules require more detail *in vivo* investigations for determination of their safety. On the other hand, the chemically-defined HSC medium composition including RPMI 1640 basal medium supplemented with cytokine combinations (SCF, TPO and FLT-3), 10 percent FBS, ITS and BML-260 molecule could be promising for development of chemically-defined human HSC expansion medium post detail investigations and validations.

7. CONCLUSION AND FUTURE PERSPECTIVE

In this study, we have reported that BML-260 and TAME small molecules from our HSM pool including 35 HSM induced *ex vivo* expansion of both human mPB- and UCB-derived hematopoietic stem cells by targeting of quiescence factors. The treatments of these molecules also induced multi-lineage potential of expanded cells. Gene expression analysis resulting upregulated Skp2-dependent p27 downregulation by downregulation of Cdh1 which is responsible for degradation of Skp2, is consistent with induced cell cycle profile of quiescent human CD34⁺ cells. However, BML-260 targets inhibition of JNK pathway. Thus, the expression analysis of JNK pathway-related genes needs to be performed to detect main effect mechanism of BML-260. Genotoxicity and apoptosis results showed that BML-260 molecule did not have any genotoxic or apoptotic effect on expanded mPB hematopoietic cells, but TAME molecule slightly had. These analysis needs to be performed to detect the effect of these molecules on UCB-derived HSPC. Furthermore, determination of the engraftment and repopulation ability of BML-260 and TAME-expanded cells is required for pre-clinical outcome. Transplantation studies on immunodeficient SCID mice are ongoing. Briefly, BML-260 and TAME molecules are promising for *ex vivo* expansion of both human mPB and UCB-HSPCs and BML-260 is more potent small molecule for human HSC expansion rather than TAME.

This study contributes to small molecule-based HSC expansion technologies. BML-260 and TAME molecules could be utilized for human HSC expansion and development of GMP-grade chemically defined human HSC expansion medium based on adding small molecules on basal cell medium (RPMI, IMDM etc.) including human HSC cytokines (Flt3, SCF, TPO, IL3 and IL6). Further studies about detail safety investigations including genotoxicity, senescence and mutagenicity due to prolonged culture, will contribute to indicate safety of these molecules for use in research. Besides, the repetitive comparative studies with basal medium types and commercial HSC expansion media will contribute to generation of medium composition including HSMs, cytokines and other appropriate supplements. Besides, clinical trials following *in vitro* and *in vivo* investigations will contribute to utilization of this molecules and medium that is planned to develop in clinical area.

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APPENDIX A: ETHICAL APPROVAL FORM

All human studies were approved by the Institutional Clinical Studies Ethical Committee of Yeditepe University, Istanbul, Turkey in 2019 (Decision Numbers: 1067 and 944) (Figure a1 and a2).





T.C. YEDİTEPE ÜNİVERSİTESİ

Sayı : 37068608-6100-15- 1719

Konu: Klinik Araştırmalar
Etik kurul Başvurusu hk.

11/07/2019

İlgili Makama (Esra Albayrak)

Yeditepe Üniversitesi Genetik ve Biyomühendislik Bölümü Doç. Dr. Fatih Kocabaş'ın sorumlu araştırmacı olduğu **“İyi Üretim Uygulamaları Çerçevesinde Kordon Kanı Kökenli Hemotopoitik Kök Hücre Kültürü”** isimli araştırma projesine ait Klinik Araştırmalar Etik Kurulu (KAEK) Başvuru Dosyası **(1688)** kayıt Numaralı KAEK Başvuru Dosyası, Yeditepe Üniversitesi Klinik Araştırmalar Etik Kurulu tarafından **10.07.2019** tarihli toplantıda incelenmiştir.

Kurul tarafından yapılan inceleme sonucu, yukarıdaki isimi belirtilen çalışmanın yapılmasının etik ve bilimsel açıdan uygun olduğuna karar verilmiştir (**KAEK Karar No: 1067**).



Prof. Dr. Turgay ÇELİK
Yeditepe Üniversitesi
Klinik Araştırmalar Etik Kurulu Başkanı

Yeditepe Üniversitesi 26 Ağustos Yerleşimi, İnönü Mahallesi Kayışdağı Caddesi 34755 Ataşehir / İstanbul

T. 0216 578 00 00 www.yeditepe.edu.tr F. 0216 578 02 99

Figure a1. Ethical approval form for umbilical cord blood used in this study



T.C. YEDİTEPE ÜNİVERSİTESİ

Sayı : 37068608-6100-15- 1592

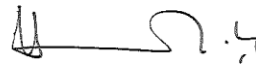
Konu: Klinik Araştırmalar
Etik kurul Başvurusu hk.

31/01/2019

İlgili Makama (Neslihan Meriç)

Anadolu Sağlık Merkezi Hastanesi Hematoloji Anabilim Dalı Prof. Dr. Zafer Gülbaş'ın sorumlu olduğu **“Lösemi Kök Hücrelerinde MEIS Ekspresyon Profiline Belirlenmesi ve MEIS İnhibitörlerinin Etkisinin İncelenmesi”** isimli araştırma projesine ait Klinik Araştırmalar Etik Kurulu (KAEK) Başvuru Dosyası (**1556** kayıt Numaralı KAEK Başvuru Dosyası), Yeditepe Üniversitesi Klinik Araştırmalar Etik Kurulu tarafından **30.01.2019** tarihli toplantıda incelenmiştir.

Kurul tarafından yapılan inceleme sonucu, yukarıdaki isimi belirtilen çalışmanın yapılmasının etik ve bilimsel açıdan uygun olduğuna karar verilmiştir (**KAEK Karar No: 944**).



Prof. Dr. Turgay ÇELİK
Yeditepe Üniversitesi
Klinik Araştırmalar Etik Kurulu Başkanı

Yeditepe Üniversitesi 26 Ağustos Yerleşimi, İnönü Mahallesi Kayışdağı Caddesi 34755 Ataşehir / İstanbul

T. 0216 578 00 00 www.yeditepe.edu.tr F. 0216 578 02 99

Figure a2. Ethical approval form for human mobilized peripheral blood used in this study