DESIGNING SELECTIVE SMALL MOLECULE INHIBITOR SCREENING THROUGH BH3 PROFILING

by

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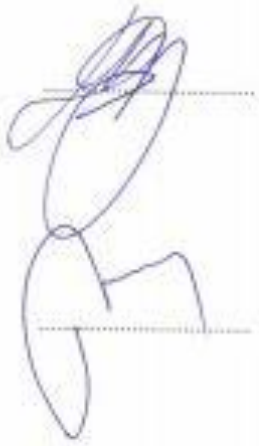

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ABSTRACT

DESIGNING SMALL MOLECULE INHIBITOR SCREENING BY BH3 PROFILING

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Molecular Biology, Genetics and Bioengineering, MSc Thesis, July 2018

Thesis Supervisor: Prof. Hüveyda Başağa

Key Words: Mcl-1, apoptosis, mitochondria, BH3 Profiling, library screening

Apoptotic cell death is a crucial programmed mechanism for homeostasis of tissue. Mitochondrial apoptotic cell death is governed by BCL-2 protein family members. Aberrations in mitochondrial-mediated apoptosis lead to impaired cell death signaling in response to cellular damage and thereby promotes cancer cell survival. Upon with selectively interacting antiapoptotic BCL-2 proteins, BH3-only BCL-2 proteins play a crucial role in governing cellular damage. All anti-apoptotic members of the BCL-2 protein family share a BH3 death domain, thus BH3 profiling enables us to assess the apoptotic blocks caused by cancer cells. The myeloid cell leukemia-1 protein encoded by MCL-1 gene and is an anti-apoptotic member of a BCL-2 protein family. It is known as a survival molecule for the cancer cells and overexpression of it confers a resistance against chemotherapeutic drugs for the treatment of several types of cancer. These findings point out that the significance of the small molecule inhibitor for MCL-1.

We have designed a small molecule inhibitor screening with the aid of the automated pipetting system, epMotion5070, through BH3 profiling method to monitor priming to cell death in the context of MCL-1 dependence. We optimized the epMotion5070 by using a library, consisting of kinase inhibitors (80 molecules) for designing small molecule inhibitor screening. Then, we aimed to trigger mitochondrial apoptotic cell death via MCL-1 inhibitor molecules on non-small lung cancer cell lines (H-23, H-1975) by screening a library (1280 molecules), which is accomplished by BH3 profiling. In this study, we identified specific small molecules for the inhibition of MCL-1.

ÖZET

BH3 PROFİLLEME YÖNTEMİ İLE KÜÇÜK MOLEKÜL İNHİBİTOR TARAMA SİSTEMİNİN DİZAYN EDİLMESİ

SEVDE NUR KARATAŞ

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Anahter kelimeler:miyeloid lösemi,apoptoz,mitokondri,BH3 profillemesi,BCL-2 protein ailesi

Programlanmış hücre ölümü mekanimanızması hücre iç ortamında normal dengenin korunması için oldukça kritik bir mekanizma olarak bilinmektedir. Mitokondri aracılığı ile düzenlenen hücre ölümü mekanizması BCL-2 protein ailesi tarafından kontrol edilmektedir. Bu mekanizmada gerçekleşen anormallikler hücre ölümünü yöneten sinyallerlerde ciddi deformasyonlara sebep olmaktadır. Hücrenin zarar gördüğü durumlarda, mekanizmadaki deformasyon kanser hücrelerinin hayatta kalmasını tetiklemektedir. Sadece BH3 bölgesine sahip BCL-2 proteinlerinin, mitokondriyal hücre ölümüne entegrasyonu antiapoptotic BCL-2 protein etkileşimi ile gerçekleşmektedir. Tüm anti-apoptotik proteinler BH3 bölgesine sahiptir ve bu yüzden BH3 profilleme yöntemi apoptotik proteinlerin bloklanmasını gözlemlemek için oldukça etkili bir yöntemdir. Miyeloid hücre lösemisi proteini MCL-1 geni tarafından kodlanır ve BCL-2 ailesinin antiapoptotik bir üyesidir. MCL-1 kanser hücrelerinin sağ kalımı için önemli olup, daha çok üretilmesi, kanser hücrelerinde kemoterapik ilaçlara karşı direnç geliştirmektedir. Tüm bulgular MCL-1 proteininin hücre ölümünde yerinin önemli olduğunu belirtmektedir. Amacımız, BH3 profilleme metodu çerçevesinde, otomatik pipetleme yardımıyla küçük molekül inhibitörü tarama sistemidizayn ederek hücre ölümü cevabını/eğilimini indüklemektir. Bu amaç doğrultusunda, otomatik pipetleme cihazı 80 molekülden oluşan kütüphane kullanılarak optimize edilmiştir. Ardından 1280 molekülden oluşan kütüphane, BH3 profilleme yöntemi ile taranmıştır. MCL-1 proteinini inhibe edebilecek spesifik moleküller belirlenmiştir.

I dedicate this thesis to my mother

Şirvan Savsun

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LIST OF ABBREVIATIONS

3D	3 Dimension
3′-UTR	3′-Untranslated Region
A-1210477	a potent and selective MCL-1 inhibitor with Ki
ABT-199	Bcl-2-selective inhibitor with Ki
ABT-737	a BH3 mimetic inhibitor of Bcl-xL, Bcl-2 and Bcl-w with EC50
AIF	Apoptosis-Inducing Factor
ALL	Acute Lymphoblastic Leukemia
AML	Acute myeloid leukemia
APAF-1	Apoptotic Protease Activating Factor 1
AsPC-1	Human Pancreatic Cancer Cells
ATCC	American Type Culture Collection
BAX	BCL2-Associated X Protein
BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-Extra Large
BCR-ABL	Breakpoint Cluster Region protein-Abelson Murine Leukemia viral oncogene homolog 1
BFL-1	Bcl-2-related Protein A1
BH3	Bcl-2 Homology 3
BID	BH3 domain-only Death Agonist Protein
BM-1197	Specific dual inhibitor of Bcl-2 and Bcl-xL with Ki
BME	2-Mercaptoethanol
BML-259	(CAS 267654-00-2), a potent Cdk5/p25 and Cdk2 inhibitor
BSA	Bovine Serum Albumin
BxPC3	Pancreatic Cancer Cell Line
Caspases	Cysteine Proteases with Aspartate Specificity
CED-3	Cell Death Protein 3
CED-4	Cell Death Protein 4

CLL	Homo Sapiens Colon Colorectal Carcinoma
CO_2	Carbon Dioxide
DBP	Dynamic Dynamic BH3 profiling
DISC	Death-Inducing Signaling Complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene-Diamine-Tetra-Acetic acid
EGTA	Ethylene Glycol Tetraacetic Acid
ELISA	Enzyme-Linked Immune Absorbance Assay
Endo G	Endonuclease G
ERK	(Extracellular Receptor Kinase)
FACS	Fluorescence-Activated Cell Sorting
FADD	Fas-Associated Death Domain
Fas	TNF Superfamily Receptor 6
FASL	Fas Ligand
FBS	Fetal Bovine Serum
FCCP	Carbonilcyanide p-triflouromethoxyphenylhydrazone
H-1975 and H-23	Non-small Lung Cancer Cell Lines
H1299	Non- small-cell Lung Carcinoma Cell Line
HCT116	Human Colon Cancer Cell Line
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
Human SCLC cells	Small-cell Lung Carcinoma
IAP	Inhibitor of Apoptosis
iBH3	Intracellular BH3
JC-1	Mitochondrial Membrane Potential Dependent Fluorescent Dye
Jurkat T cell	Leukemia Cell Lines
KCL	Potassium Chloride
KOH	Potassium Hydroxide
MCL-1	Myeloid Cell Leukemia 1
MEFs	Mouse Embryonic Fibroblasts
MiaPaCa-2	Human Pancreatic Cancer Cells
MIM1	Mcl-1 Inhibitor

miRNA	micro Ribonucleic Acid
ML-1	Myeloblastic Leukemia Cell Line
MM66	Human Uveal Melanoma Cell Line
MOMP	Mitochondrial outer membrane permeabilization
MP41	Human Melanoma Cell Line
mRNA	Messenger RNA
Navitoclax (ABT-263)	Potent Inhibitor of Bcl-xL, Bcl-2 and Bcl-w
NCI-H929	Myeloma Cell Line
NHL	Non-Hodgkin Lymphoma
nM	Nano Molar
non-SCLC	Non-Small Cell Lung Cancer
PBS	Phosphate-Buffered Saline
PC-3	Human Prostate Cancer Cell Line
PCR	Polymerase Chain Reaction
Pen-Strep	Penicillium Streptomycin
PEST	Proline (P), Glutamic Acid (E), Serine (S), and Threonine (T)
RCF -G	Relative Centrifugal Force
RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
S63845	Small Molecule MCL1 Inhibitor
S-44563	Potential inhibitor of BCL-2/BCL-XL
Smac	Second Mitochondria-Derived Activator of Caspase
STAT3	Signal Transducer and Activator of Transcription 3
t-BID	Translocated BID
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
Triciribine	Cancer Drug
µM	Micro Molar
UMI-77	Selective Mcl-1 Inhibitor
VEGH	Vascular Endothelial Growth Factor

1. INTRODUCTION

1.1. Apoptosis

Apoptosis is an evolutionary preserved mechanism that allows facilitating tissue homeostasis and response to an external signal in metazoans [1]. Due to the fact that the key role of apoptosis, either failure of the apoptotic machine or aberrant changes might be critical for the cell fate [2]. Chromatin condensation, nuclear fragmentation, breaking of cellular fragments and blebbing of the plasma membrane are included in the cellular hallmark of apoptosis [3]. Caenorhabditis Elegans has been used to demonstrate the cell death mechanism. Previous studies showed that CED-3 and CED-4 trigger apoptosis in C. Elegans. CED-3, cysteine protease, is called caspase. Cysteinyl aspartate-specific proteases are responsible for activation of certain members of downstream target caspases [4]. Apoptosis is taking place through activation of initiator, inhibitor, and effector caspases. Effector caspases are existed as inactivate zymogens and cleavage of the certain substrates through caspases cause the formation of apoptotic body [2].

Apoptotic pathways are operated by two molecular programs are known as an extrinsic and intrinsic pathway. The extrinsic pathway is guided by transmembrane receptors, Fas, and Tumor necrosis family. Death receptor signals stimulate death caspases 8 and 10, which are able to cleave death substrates [1].

The extrinsic pathway is categorized into two pathways, based on the kind of signaling molecules and receptors. The extrinsic pathway is mediated by death receptors and these death receptors have specific ligands such as; Fas Ligand (FASL) or Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) on the plasma membrane [4, 5]. Binding of either FASL to FAS receptor or TRAIL to death receptor 4 and 5 leads to conformational change on their structure. The conformational change causes the formation of DISC complex [6].Upon engagement by FADD, self-cleavage of procaspase 8 results in its activation. There are two main paths as consequences of activation of caspase 8. In type 1 cell, activation of caspase 8 allows activation of downstream effector

caspases, caspase 3 and caspase 7 that are responsible for activation of pro-death substrates, leading cellular changes characteristic of apoptosis [7, 8]. Initiation of apoptosis is propagated by activation of mitochondrial pathway in the cell, which is called type 2 cell. The signal from death receptors drives mitochondrial outer membrane permeabilization [9]. Upon cleavage of pro-apoptotic BCL-2 member BID by caspase 8, translocated t-BID promotes mitochondrial membrane permeabilization to release cytochrome c [8].

In contrast to the extrinsic pathway, the intrinsic pathway, which is induced by intracellular death signals, depends on mitochondrial depolarization and permeability [6]. Mitochondrial outer membrane permeabilization (MOMP) triggers the releasing of apoptogenic factors, including cytochrome c, that begins caspase activation and thereby initiates apoptotic process [10]. BCL-2 family proteins are responsible for the regulation of MOMP and thereby the cell fate is determined by the interaction between proapoptotic and antiapoptotic BCL-2 proteins. Cytochrome c engages with protein apoptotic protease activating factor 1 (APAF-1) and procaspase 9 and apoptosome complex is formed. By triggering caspase 3 and caspase 7, downstream caspases are activated. Therefore, the mitochondrial-mediated apoptotic process is committed [6].

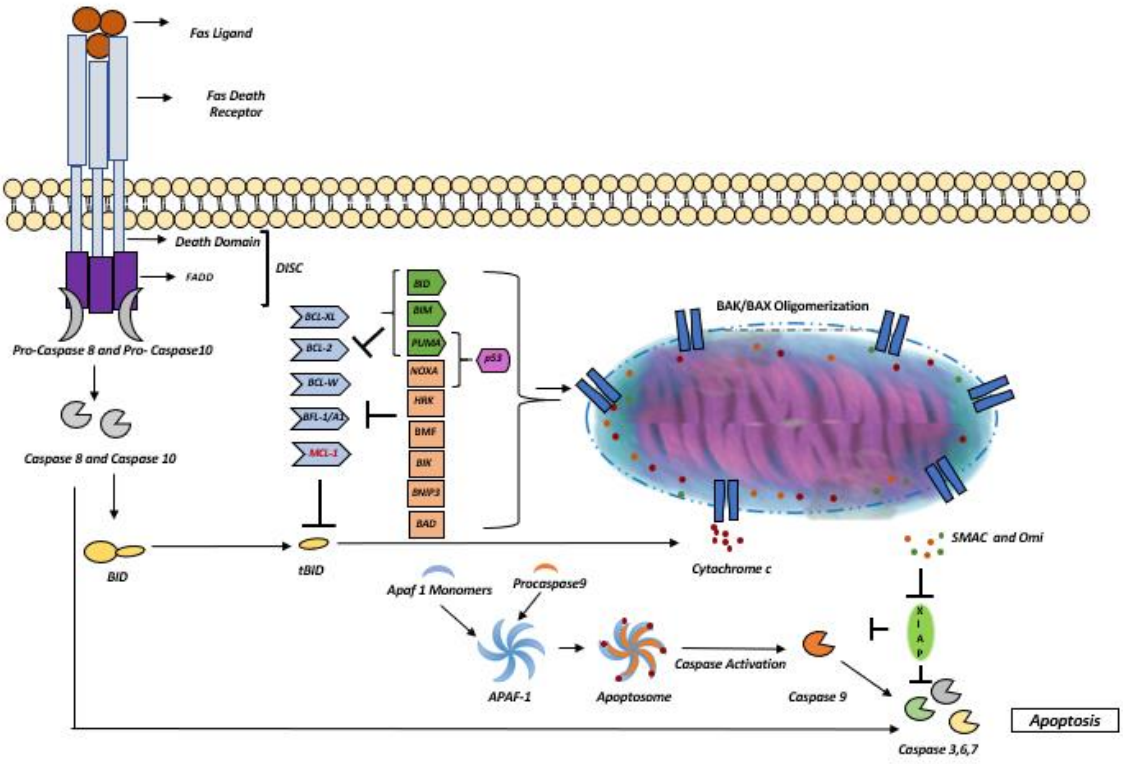


Figure 1. 1 The two major pathways involved in apoptosis regulation.

1.1.1. BCL-2 Family Proteins

BCL-2 family proteins play a crucial role on mitochondrial-mediated apoptotic pathway since members of BCL-2 family operate mitochondrial permeabilization and the release of several mitochondrial intermembrane space proteins into the cytosol. BCL-2 was identified as an oncogene in follicular lymphomas at the t(14;18) chromosomal breakpoint [1]. BCL-2 family proteins are classified as the multi-domain proapoptotic, the BH3 only proapoptotic and the multidomain pro-survival proteins. Although they belong to distinct groups, all BCL-2 family proteins share at least one BCL-2 homology domain (BH 1-4 homology) [11].

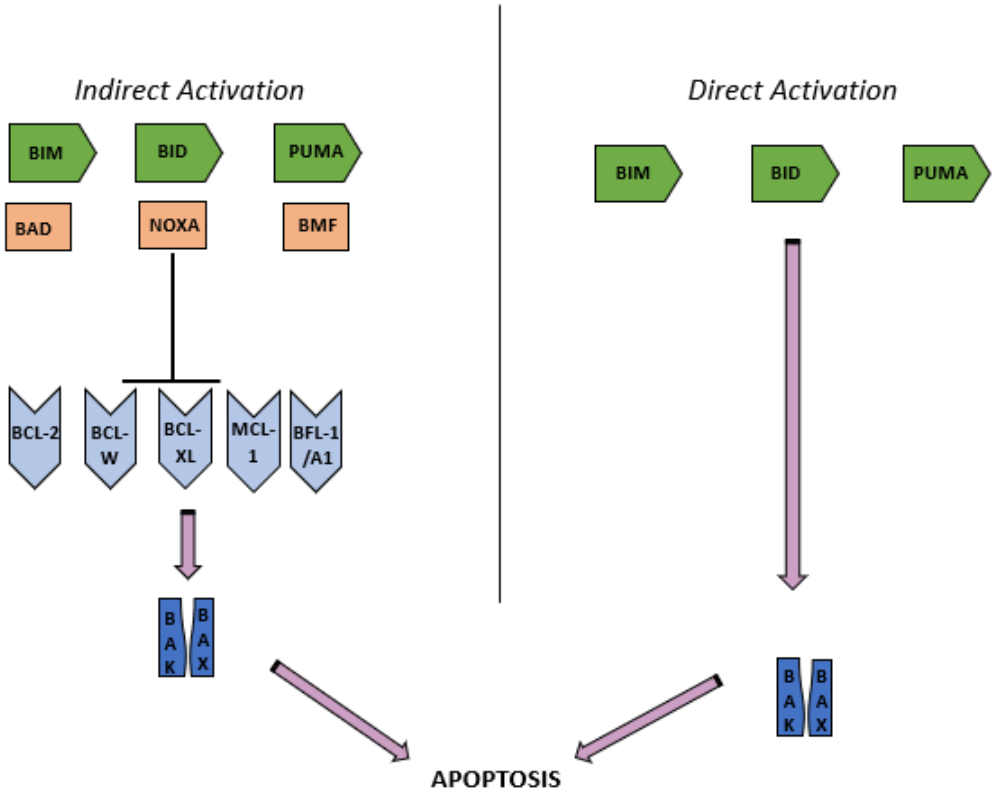


Figure 1. 2 Two models for the activity of BH3 only proteins in apoptosis.

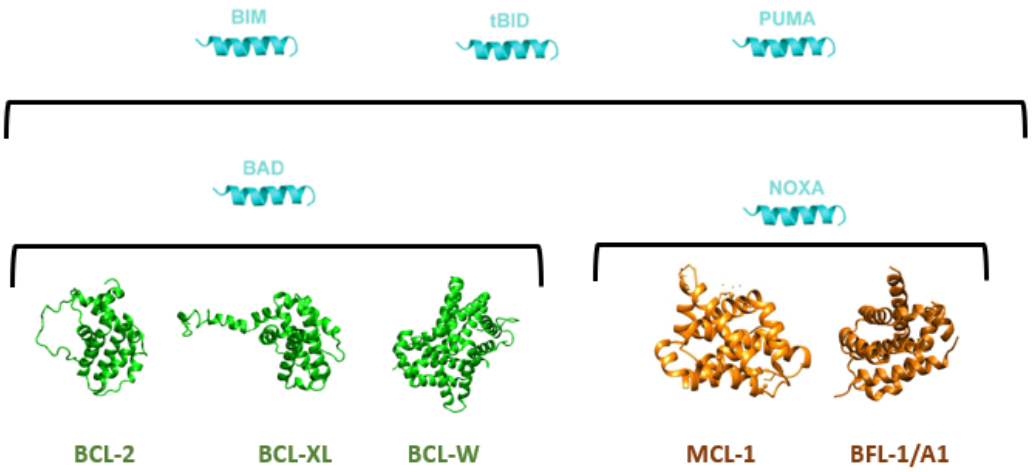


Figure 1. 3 Selective binding among BCL-2 family proteins.

1.1.2. Pro-apoptotic Proteins

The pro-apoptotic proteins possess either multidomain or only BH3 domain. The effector proteins BAX and BAX, involves BH 1-3 domains, operates the commitment of mitochondrial cell death either directly or indirectly. Numerous proteins, that facilitate the activation state of BAX and BAK, are called activator proteins. BIM, BID, PUMA, and NOXA act as direct activators in case of BAX and BAK oligomerization, while BAD, NOXA and HRK which, indirectly induce apoptosis by exerting their function on antiapoptotic proteins, are called sensitizers [12]. The activation of BAX and BAK act as a gate, leading a conformational change on the mitochondrial outer membrane and thereby operates MOMP. During permeabilization, intermembrane space proteins are released into the cytoplasm to induce apoptosis [10, 13]. In addition to these proteins, BOK is described as a responsive element to ER stress [14]. BH3 only proteins, including BIM, BID, BAD, BIK, NOXA, HRK, PUMA, which possess only BH3 death domain, transmit multiple cell death signals to mitochondria[1].

1.1.3. Pro-survival Proteins

The pro-survival proteins, including BCL-2, BCL-XL, BCL-W, MCL-1, BFL-1, and BCL-B, play a key role in the survival mechanism for the cancer cells. Cell type environment, activation level, and cell type are critical factors for cellular survival

mechanisms and the expression of pro-survival BCL-2 proteins relies on these factors [12]. They block the core apoptotic mechanism by either precluding them from activating BAX or BAK or by binding effector proteins after their activation to expose BH3 domains [14].

The BH3 domain, which is included in all BCL-2 family proteins, has an essential role for pro-death function. It is consisting of 20 amino acids amphipathic alpha-helix and only 3 amino acid is evolutionary conserved [14]. Structurally, BH1-3 regions are formed by helices 2, 3, 4 and 5. The conserved part of the BH3 domain mainly comprises hydrophobic cleft. The antiapoptotic proteins domains (BH1-4), including BH1, BH2, and BH3 constitute a hydrophobic groove that binds to the hydrophobic cleft of pro-apoptotic proteins [15]. The crucial interaction begins at intracellular membranes and proceeds through mitochondrial outer membrane [11].

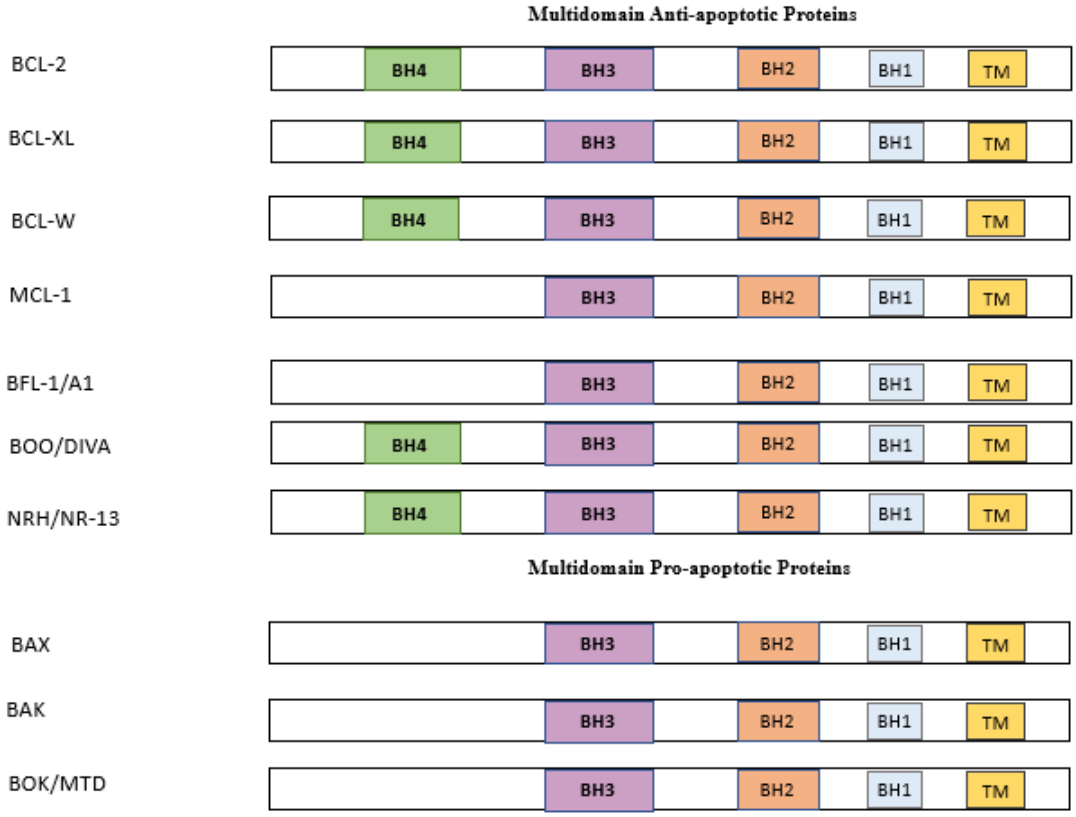


Figure 1. 4 BH3 domain conservation among antiapoptotic BCL-2 family proteins.

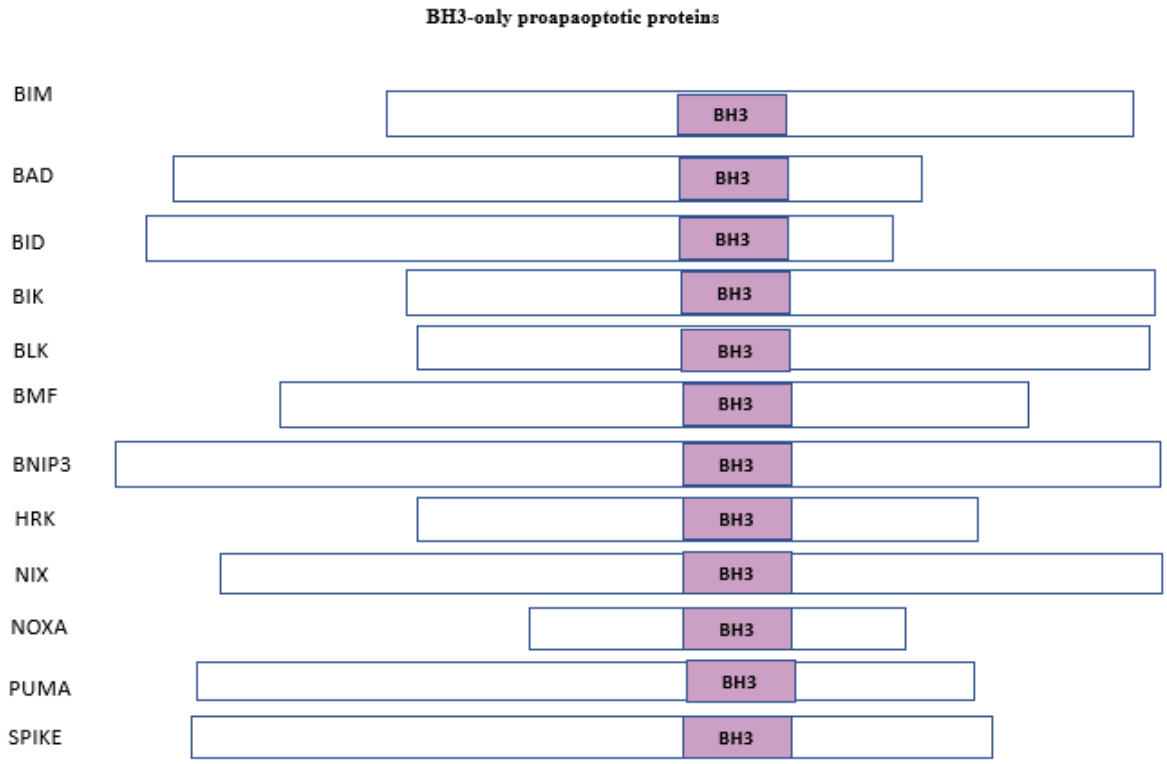


Figure 1. 5 BH3 domain conservation among BH3 only proapoptotic BCL-2 family proteins.

1.2. Priming Classes in Apoptosis

The cells might be influenced by chemotherapeutic agents in a different way according to the activation of some components in the apoptotic pathway. Priming can be described as a comparison mechanism depending on the tendency of committing the cell death, MOMP like a cliff and some cells are closer to the cliff comparing the others. Thereby, physiological features of these cells, that are defined as ‘’primed’’, are notably distinct from’’unprimed’’ cells since they tend to commit the cell death [16]. Priming feature basically facilitates the rate of mitochondrial outer membrane permeabilization. Several factors, including genetic background, perturbations in metabolism, might impact on priming levels of the cell [16]. Priming levels are categorized into three distinct groups. The principle of BH3 Profiling relies on measuring apoptotic priming levels of the cell.

Types of Priming

Class A cells are defined as competent although it is involved in the unprimed category. While activation of BIM and BID operates Class A priming, class A cells have no response to sensitizer peptides. Even though BAK and BAX oligomerization normally proceeds, they are not triggered by any pro-apoptotic proteins [17].

Class B has some abnormalities in functions of BAX and BAK, and thereby it is defined as incompetent cells. Since the lack of BAK and BAX, there is no formation of the pore on their mitochondrial outer membrane. Therefore, it is not possible to release apoptogenic factors by mitochondria even if exposing high doses BIM and BID [17].

In contrast to Class A, Class C primed cells have a response to not only activator but also sensitizer class peptides. In addition to pore formation by BAX and BAK, they might be activated by pro-death proteins. Class C primed cells are categorized by their response to the sensitizer peptides. While Class C MCL-1 is sensitive to BIM, BID, PUMA, BMF, and NOXA, it has no positive response to BAD and HRK. BIM, BID, PUMA, BMF, and BAD are effective peptides to acquire a positive response to Class 2 BCL-2 proteins [17].

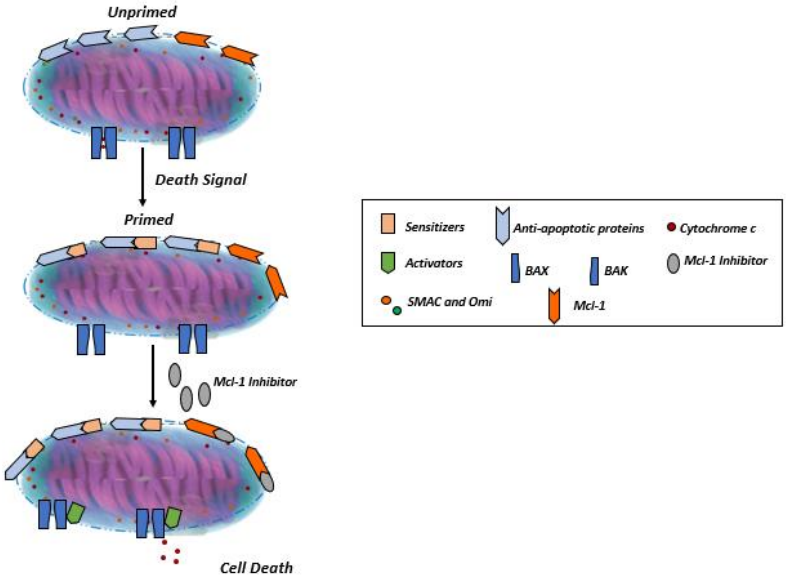


Figure 1. 6 Apoptotic Priming.

1.3. Mitochondrial Outer Membrane Permeabilization (MOMP)

Mitochondrial outer membrane permeabilization determines the cell fate and it is called a point of no return. In the absence of caspase activation, cells are still able to go through the apoptotic cell death [10].

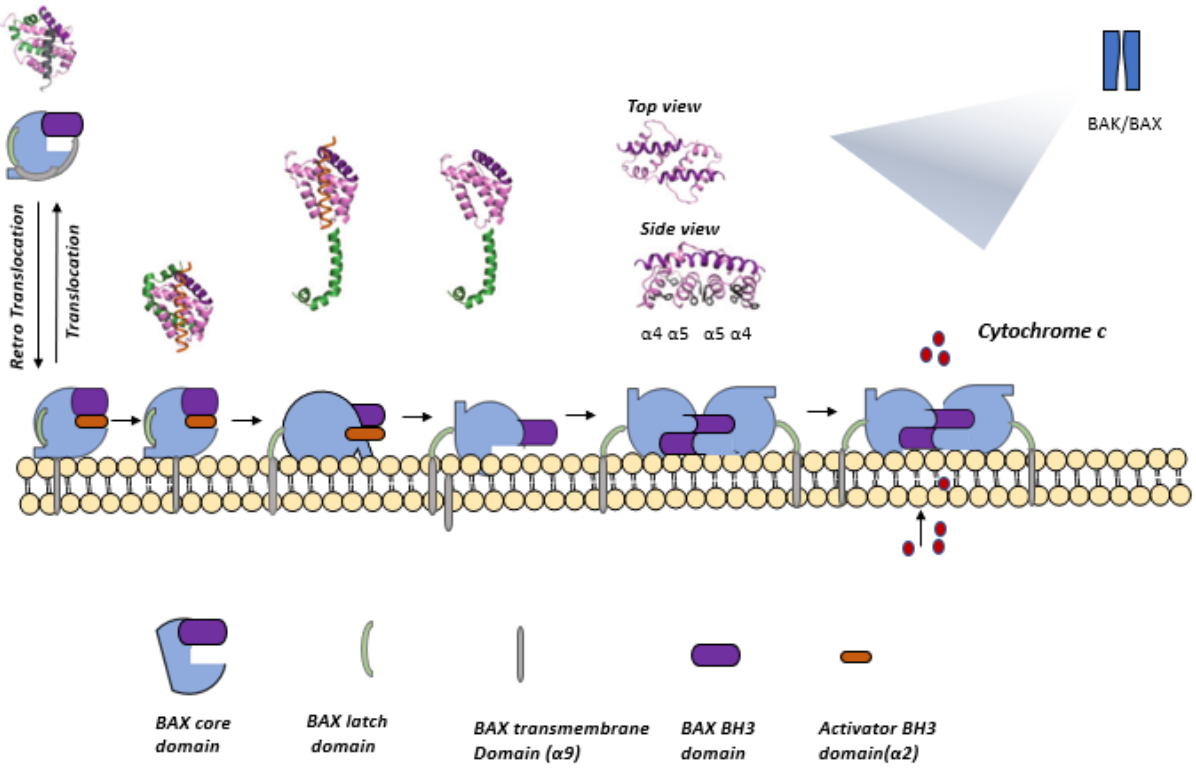


Figure 1. 7 Mitochondrial Outer Membrane Permeabilization through Bax/Bak Oligomerization.

1.3.1. Caspase-Independent Cell Death

There are several theories to demonstrate the molecular mechanism of caspase-independent cell death. One of these models proposes that AIF and endonuclease G might have a contribution on caspase-independent cell death mechanism [10]. Several perturbations in mitochondria might be described as a second model for caspase-independent cell death.

1.3.2. Mitochondria-dependent Caspase Activation

After mitochondrial outer membrane permeabilizes, a bunch of intermembrane space proteins are diffused from mitochondria. Binding of cytochrome c to APAF-1 leads to conformational change and activation of caspases. The following interaction between APAF-1 and procaspase-9 creates an apoptosome complex. Upon with activation of caspase 9 in apoptosome complex, caspase 3 and 7 trigger mitochondrial cell death. In the regulation of cell death, cytochrome c is an essential component to maintain the apoptotic process. However, other intermembrane space proteins, including Omi and Smac, have a key role in the apoptotic stimulation. Omi and Smac are responsible for induction of caspase activation through blocking of caspase inhibitors. Indeed, endonuclease G and AIF operates the DNA cleavage. In contrast to cytochrome c, the apoptotic process is able to maintain in the absence of Omi and Smac [10]. It suggests that other IAPs are suppressed by another inhibitory molecules [18].

1.4.BH3 Profiling

The mitochondrial-mediated cell death has a crucial role on cell fate since the mitochondria might operate the response to several perturbations. To measure these changes in mitochondria at the molecular level, BH3 profiling, which has been developed by Letai Laboratory was introduced as a functional tool that utilizes either peptides or small molecules to trigger mitochondrial depolarization [19].

The BH3 profiling method is based on interactions between BCL-2 family and how these interactions impact the mitochondrial cell death. The BCL-2 family proteins facilitate mitochondrial outer membrane permeabilization which is called a point of no return. Due to the depolarization, caused by mitochondria, is a significant indicator to evaluate the response of the cell against chemotherapeutic agents, it is possible to predict the tendency of the cell for the mitochondrial apoptotic cell death, upon with measuring mitochondrial outer membrane permeabilization [19, 21]. The principle of monitoring the changes in mitochondria is accomplished by the emission of fluorescent dye (JC-1). JC-1 is normally fluoresced green as a monomer while it fluoresces red in aggregation formation. It is able to accumulate in a matrix of mitochondria during polarization because of its lipophilic cation. While either BH3 peptides or small molecules are introduced as an input,

depolarization of outer membrane begins and results in releasing intracellular membrane space proteins and JC-1 dye. Thereby, upon measuring the red fluorescent, we are able to evaluate the potential of the outer membrane [22]. Recently, there are five main types of BH3 profiling, including ELISA based profiling (enzyme-linked immune absorbance assay), plate-based assay via JC-1, JC1- FACS based assay, intercellular BH3 and Dynamic BH3. Distinctions between BH3 profiling methods enable to work with both heterogeneous and homogenous cell populations. Type of the population that needs to be analyzed, determines the choice of BH3 profiling method [20].

1.4.1 Types of BH3 Profiling

1.4.1.1. ELISA Based Profiling

ELISA based BH3 Profiling is notably an efficient method to predict the amount of cytochrome c in mitochondria. After either BH3 peptides or small molecule exposure, the high amount of releasing cytochrome c from mitochondria points out that the cell is more primed since the proportion of cytochrome c is associated with priming. Even though the method is highly influential, detection of intermembrane space proteins requires antibodies and immunostaining methods [20]. These requirements have some disadvantages, including low throughput, labor-intensive, expensive, and they do not allow real-time measurement [17].

1.4.1.2. Whole Cell-Based BH3 Profiling Assay

The whole cell-based BH3 profiling assay is designed to overcome the limitations of ELISA based method. Plate-based JC-1 BH3 profiling is the best-modified tool for cell lines and homogenous samples since it allows to get a bulk response instead of single cell measurement. Loss of membrane potential triggers releasing of cytochrome c from intermembrane space [17]. In order to measure the loss of mitochondrial membrane potential, the JC-1 fluorescent dye is used as a probe which has a dual emission. While JC-1 as a monomer fluoresces green, accumulation of JC-1 in the matrix forms JC-1 aggregates and gives red fluorescent [17, 23]. To exposure either BH3 peptides or small molecules into mitochondria, the plasma membrane permeabilization is required. Digitonin enables integration of BH3 peptides into mitochondria and causes permeabilization without disrupting membrane potential. During permeabilization, the mitochondrial stabilization is accomplished by the components of MEB Buffer. Upon with optimal concentration of digitonin, cell suspension are stained through JC-1 and

placed in 384 well plate. By integration of BH3 peptides, the second emission peak is detected at 590 nM and thereby diminishing in the red fluorescence is measured [17, 24].

1.4.1.3. FACS based BH3 Profiling

FACS based BH3 profiling is a novel method to aim to separate distinct populations in heterogeneous samples. This method offers a platform to detect mitochondrial response for a viable population. Single cells are tagged on cell surface markers, washed, permeabilized and exposed to BH3 peptides respectively. One of the critical points is considering the laser that excites the dye. In the manner of JC-1, 488 or 561 nm lasers are able to excite JC-1 dye. To analyze different populations, subpopulations need to be gated and profiled. The population of the profile is set between forwarding and side to scatter. Another critical point is setting the cytometer voltage [17].

1.4.1.4. IBH3 Profiling Assay

Intercellular BH3 profiling is a suitable method for heterogeneous cell population [16]. Upon with formaldehyde, the fixation step provides to prevent peptide exposure. Fixation as distinct from plate-based BH3 profiling protects the cell structure and enables mitochondrial bound proteins to be stained. iBH3 profiling is designed for measuring retained JC-1 surroundings MOMP rather than fluorescent dye [20]. Through cell surface markers, cells are stained with surface markers to distinguish cell populations. After the permeabilization step by digitonin, BH3 peptides are exposed at a certain concentration. Lastly, fixation is done by formaldehyde. Incubation with cytochrome c antibody allows the detection of retained cytochrome c. iBH3, which relies on a flow cytometer, allows the analysis of primary samples weeks after fixation [16].

1.4.1.5. Dynamic Profiling Assay

Pre-treatment with a drug to sample is introduced as a novel step in dynamic profiling assay in addition to the intercellular iBH3 method. The method proposes to display differences in priming states of the cells. To consider the chemotherapeutic response in vivo, dynamic BH3 profiling might act as biomarker [20]. It has been reported that DBP, when performed on breast cancer cell lines, could predict the response to chemotherapy [25].

1.5.MCL-1

MCL-1 belongs to the BCL-2 family protein, was revealed from human myeloid leukemia cell line. There is a sequence similarity between the carboxyl terminus of both MCL-1 and BCL-2 and thereby it is a BCL-2 homolog gene. Even though numerous similarities exist including sequence, location and functional activity between MCL-1 and BCL-2, their expression levels are not equal at the same phase of differentiation. While expression of MCL-1 rises, any considerable change is not observed in the expression of BCL-2 [26]. Although MCL-1 is largely located in mitochondria, nucleus, cytoplasm, and endoplasmic reticulum comprise low levels of anti-apoptotic MCL-1 [28]. The function of the MCL-1 varies from localization to localization. While mitochondrial MCL-1 acts as a survival factor, a nuclear localization of MCL-1 decelerates cell cycle [29]. It was identified that expression of MCL-1 has a crucial role on postponement in cell death, in Chinese hamster ovary and hematopoietic cells. Since the deletion of MCL-1 gene declines the number of mature B cells as well as T cells, it is described as a critical factor for B and T cell development [26].

There are some distinctions between MCL-1 and the other BCL-2 members. In contrast BCL-2 and BCL-xl, MCL-1 has only BH1, BH2 and BH3 domain and N-terminal extension, which comprises two PEST domains and phosphorylation sites. N-terminal of MCL-1 containing PEST domains regulates the protein stability of MCL-1, mitochondrial localization, and antiapoptotic activity [29]. MCL-1, which is regulated by transcriptional, posttranscriptional and proteasomal degradation, is distinguished from the others by having a short lifetime. Particularly, alternative splicing might reveal proapoptotic forms of MCL-1 at the post-transcriptional level [30, 31]. Similar to the Bcl-2 protein family members besides from Bid and Bad, a transmembrane domain at the C terminus might support the interaction between MCL-1 and family members. It is estimated that the hydrophobic cleft, including BH1, BH2 and BH3 and transmembrane support anti-apoptotic activity of MCL-1.

2. OBJECTIVE OF THE STUDY

MCL-1 has been shown to be a major chemoresistance factor and survival in hepatocellular carcinoma [42], myeloid leukemia [43,44], lymphoblastic leukemia [45], multiple myeloma [46], and lung cancer [47]. Due to the knocking out MCL-1 was corresponded with numerous side effects and disruption of some crucial roles including, B and T cell development, the best approach would be a targeting interaction of MCL-1 with proapoptotic BCL-2 proteins [48,49]. Some clinical drugs including ABT 737 and ABT 263, have been developed to repress anti-apoptotic function of certain proteins such as; Bcl-XL, Bcl-W, and BCL-2, whereas these drugs are not able to inhibit MCL-1. ABT 737 was the first molecule which targets BH3 binding groove of BCL-XL. It is decided that ABT-737 is particularly effective for small cell lung cancer and lymphoma cells. However, due to the lack of oral viability of ABT-737, ABT 263 (navitoclax) was developed to compensate for the disadvantages of the ABT-737. ABT 263, which is analog of ABT-737, contrıbuted to development of clinical studies for BCL-XL/BCL-2 inhibitors [37,38]. S-44563 was identified as a potential inhibitor of BCL-2/BCL-XL in uveal melanoma cells (MP41, MM26, and MM66) [33,39]. In the context of MCL-1, BM-1197 is introduced as another inhibitor for blockage of BCL-2/BCL-XL in only MCL $^{-/-}$ MEFs[40]. While ABT-199 (Venetoclax), which is able to inhibit BCL-XL/BCL-2 in non-Hodgkin lymphoma (NHL), acute myeloid leukemia (AML), and ALL cell lines, utilization of either daunorubicin or cytarabine with Venetoclax triggers apoptosis in U937 and AML samples in the context of Venetoclax resistance [41]. Even though these drugs have a potential to achieve inducing apoptosis in somehow, specific MCL-1 inhibition through protein-protein interaction paves the way for clinical trials of inhibition of MCL-1. In early studies, ERK and lipoxygen inhibitors were introduced as a positive upstream molecule since ERK and lipoxygenase were the upstream element of MCL-1 in case of the acute ML-1 myeloblastic leukemia cell line and MiaPaCa-2 and APC-1 pancreatic cancer cells respectively [50,51].

Additionally, vascular endothelial growth factor (VEGH), which is a critical molecule for expression of MCL-1, was achieved by cyclin-dependent kinase inhibitor seliciclib in multiple myeloma [52]. In the context of human endometrial and cervical cancer tissues and non-SCLC tissues, it was proved that activation of STAT3 is interrelated MCL-1 overexpression. This finding points out that the significance of MCL-1 as a drug candidate to restore the apoptotic cell death mechanism in clinical studies for the treatment of cancer [53,54].

In the case of the MCL-1 inhibition through non-selective molecules would not be feasible and result in efficient consequences. Thereby, a novel and highly selective candidate MCL-1 inhibitors are reasonable to prevent such consequences [55]. In BCL-2 protein family, NOXA selectively binds to MCL-1 to inhibit its apoptotic activity in CLL cells and human SCLC cells [55, 56, 57]. Based on the MCL-1 NOXA interaction, celastrol proved that MCL-1 degradation with the aiding of NOXA in PC-3 human prostate cancer cell line [58]. It is found that stabilized MCL-1 BH3 helix is selectively able to target MCL-1 in OMP2 multiple myeloma and Jurkat T cell leukemia cell lines [59]. Another study revealed that MIM1 is a potential inhibitor to prevent the inhibition of BAK and BAX, caused by MCL-1 trigger caspases in MCL-1- rescued, BCR-ABL(p185)-transformed, Arf-null, MCL-1-deleted, B-lineage ALL (p185+ $Arf^{-/-}$ MCL-1del B-ALL) cells [60]. All these studies emphasize that peptide derivatives, NOXA, may be utilized as an MCL-1 inhibitor.

The studies, based on NOXA-mediated targeting of MCL-1 claims that inhibitor GSI XII overcomes ABT-737 resistance in breast cancer cell lines [61].TW37, that is also an inhibitor of BCL-2 proteins, has a role in NOXA mediated inhibition of MCL-1 in H1299 non-SCLC cell line [62].

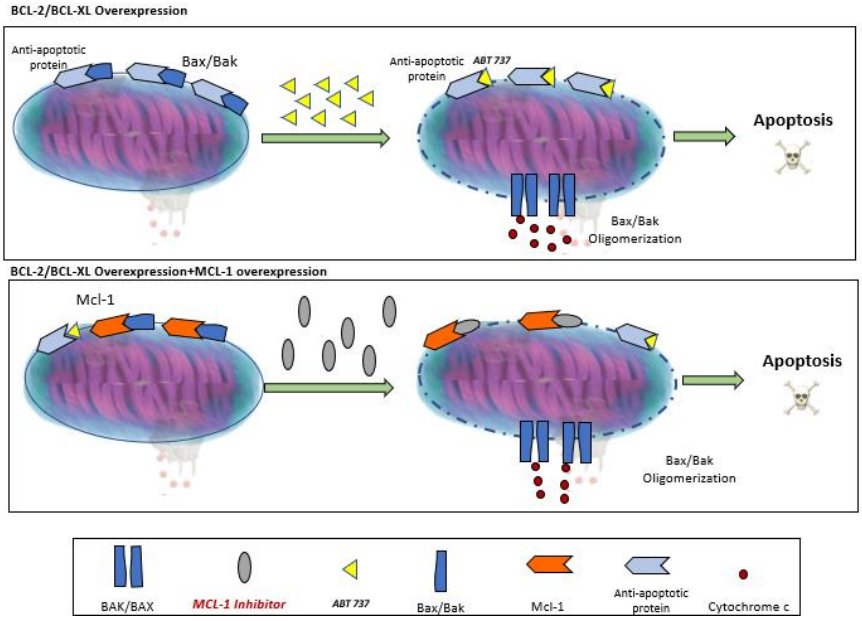


Figure 2. 1 Selective inhibitor molecule for MCL inhibition.

Drug	Cell Line/tissue	Concentration
Celastrol-dependent NOXA upregulation and MCL-1 degradation	PC-3 human prostate cancer cell line	1 μM-2 μM
Stable MCL-1BH3helix	OPM2multiplemyeloma and Jurkat T-cell leukemia	20 μ M-40 μ M
MIM1	MCL-1 rescued p185+ Arf−/− MCL-1del B-ALL cells	5 μM-20 μM
Secretase inhibitor (GSIXII)	BT549, MDAMB231, and MCF-7 breast cancer cell lines	10 μM
TW-37	H1299 non-small cell lung cancer cell line 10	10 μM
Gossypol	NB4leukemia cell line	2.5 μM-40 μM
Compound 7 (PDBID: 4WGI) N	Not Available	Not Available

Table 2. 1 Non-selective inhibitors for MCL-1 (adapted from [63]).

Drug	Cell Line/tissue	Concentration
miR-101, miR-148, miR-153, miR-193a, miR518, miR-582, miR-681, miR-876-3P, miR-886-3P, miR-892b	HCT-116 colon cancer cell line	Transfection with 50nM
miR-193b	Malme-3 M, MeWo, SK-MEL-2, and SK-MEL-28melanoma cell lines	Transfection with 5nM
Compound 6h	SMMC-7721human liver cancer cell line and MCF-7 human breast adenocarcinoma cell line	10 μM
Maritoclax	K562, Raji, and multidrug-resistant HL60/VCR cancer cell lines	1 μM-2 μM
Maritoclax	HL60 acute myeloid leukemia cell line	2 μM
Maritoclax	NCI-H460 human lung cancer cell line	3 μM
UMI-77	BxPC3 pancreatic cancer cell line	4 μM
A-1210477	NCI-H929myeloma cell line and NHL BCL2High cell lines	3 μM-10 μM
S-63845	Hematological cancer-derived cell lines, in vitro and in vivo acute myeloid leukemia models, solid tumor-derived cell lines and against tumor cells of mice	10nM–10Minvitro and 12.5–25 mg/kg in vivo

Table 2. 2 Selective inhibitor for MCL-1 (adapted from [63]).

Besides from NOXA mediated targeting and mimetics of NOXA, MCL-1 inhibiton was targeted via miRNAs. Numerous miRNAs are considered as potential molecules to induce

apoptosis by MCL-1 inhibition in HCT-116 colon cancer cell line. In the presence of ABT-263, MCL-1 mRNA and protein stability were raised and thereby, it was hard to degrade MCL-1. However, miRNA targeting overcame this problem in ABT-263 treatment [64]. It has been shown that mi-RNA 193 might be used for treatment in several melanoma cell lines to ABT -737 through binding of 3 UTR MCL-1 [65].

The structure-guided design approach plays a key role in the development of MCL-1 inhibitor. S1(3-thiomorpholin-8-oxo-8H-acenaphtho[1,2-b]pyrrole-9-carbonitrile) was described as a potent inhibitor to disrupt the interaction of MCL-1/BCL-2 with proapoptotic proteins [66].

Maritoclax, isolated from Streptomycetes, was reported as a novel compound for the blockade of MCL-164. The distinction of Maritoclax from the others is a selective molecule for MCL-1 instead of BCL-2/BCL-XL in large granular lymphocyte leukemia. In the literature, there were some contradictory results. However, this molecule has a potential to be a novel molecule for MCL-1 inhibition [67].

As another drug, UMI-77 has an inhibitory impact on MCL-1/BAX interaction by utilizing BH3 binding groove of MCL-1 in a BxPC3 pancreatic cancer cell line [68].

Currently, A-1210477, derived from the indole-2 carboxylic acid core, was identified as a potent molecule to induce apoptosis and the concentration range is 3-10 uM in NCI-H929 myeloma cell line [69].

Lastly, S63845 was considered as a most potent MCL-1 inhibitor at 10 nM to10 uM in hematological derived cancer cell lines and particularly AML models [63].

3. MATERIALS&METHODS

3.1.Materials

3.1.1. Chemicals

Chemicals utilized in this thesis are given in Appendix A.

3.1.2. Equipment

Equipment utilized in this thesis is given in Appendix B.

3.1.2.1. Multichannel Pipetting Robot (EPMotion5070)

Small molecule library (1280 molecules) was screened through pipetting robot (epMOtion5070-Eppendorf), which is already optimized for BH3 Profiling method. Because the number of small molecules that were screened is in thousands, epMotion was used to speed up the process by its automated system.

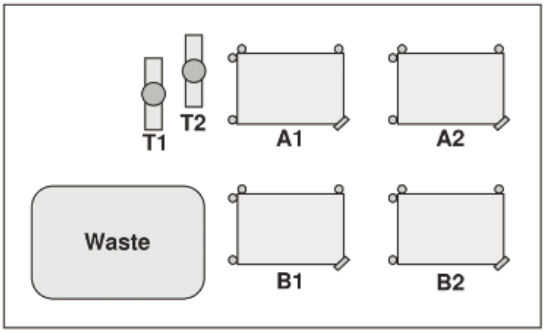


Figure 3. 1 Worktable (adapted from epMotion5070 manual).

A1 -50 ul Filter Tips

A2-96 PCR Well Plate

B1- Cells in MEB 1 X and Staining Solution

B2- 384 Costar Well Plate

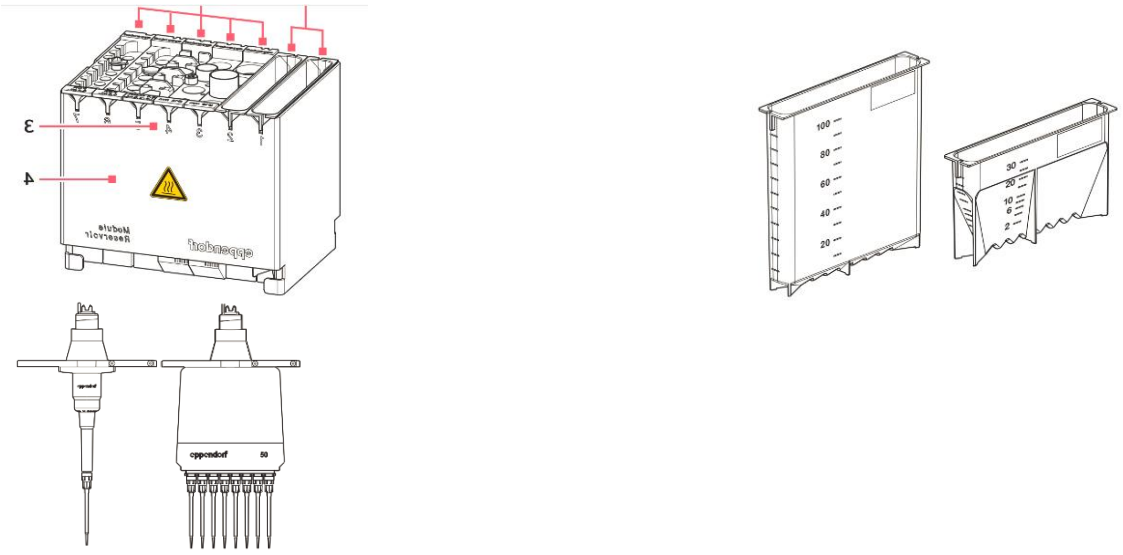


Figure 3. 2 Reservoir Rack, reservoirs (30ml,100ml) and dispensing Tools (adapted from epMotion5070 manual).

3.1.3. Solutions and Buffers

Profiling Buffer (MEB Buffer 1X)

150mM Mannitol, 10mM HEPES, 50mM KCL, 5 mM Succinate Acid, 0.1% BSA were dissolved in 100 ml ddH2O for 250 ml MEB Buffer 1X. 0.02 mM EDTA and 0.02mM EGTA were added to the solution. The pH of the solution was adjusted to 7.5 +/-0.1 with KOH. DDH2O was added up to 250 ml. The solution was filtered through 0.22-micron filter and stored at 4^{o}C.

3.1.4. Preparation of Staining Solution

For 5 ml staining solution, 5 ul Oligomycin (20 mg/ml), 100 ul JC-1(100 uM), 10 ul 2 -mercaptoethanol (5M), 4485 ul MEB Buffer 1X were respectively added into 15 ml falcon tube. Since the JC-1 is light-sensitive, the solution should be stored in the dark.

Oligomycin

50 mg Oligomycin was dissolved in 1mL DMSO. The concentration of the stock solution was 20 mg/ml in DMSO. The solution was stored at -20^{o}C.

JC-1 Dye

10mM JC-1 was dissolved in 1mL DMSO. The concentration of the stock solution was 100uM/ml in DMSO. The solution was stored at -20^{o}C.

FCCP

10mM FCCP was dissolved in 1mL DMSO. The concentration of the stock solution was 1mM/ml in DMSO. The solution was stored at -20^{o}C.

2-Mercaptoethanol (BME)

5 M 2-Beta-mercaptoethanol was diluted in ddH_2O.

3.1.5. Growth Media

RPMI: RPMI 1640 growth medium supplemented with 10% heat-inactivated fetal bovine serum(FBS) and 1% Pen-Strep (100U/ml Penicillium and 100ug/ml Streptomycin.

Freezing Medium: H-1975 and H-23 (non-small lung cancer cell lines were frozen in heat-inactivated FBS containing 20% sterile DMSO.

3.1.6. Mammalian Cell Lines

H-1975 (independent on MCL-1), H-23 (dependent on MCL-1) non-small lung cancer cell lines were used to investigate the impacts of the MCL-1 inhibitors.

3.2.Methods

3.2. 1. Mammalian Cell Culture

3.2.1.1. Maintenance of the cell lines

H-23 and H-1975 were grown in sterile cell culture flask through RPMI 1640 and incubated at 37^{o}C with 5% CO2. The cells were passaged (1:4 ratio) to a new cell culture flask at the confluence of 80 %.

3.2.1.2. Cryopreservation of the cells

In the freezing medium (80% FBS,20% DMSO), the cell was frozen for further use. 2-6 x106 cells were counted and centrifuged at 300 G for 5 minutes. The supernatant was discarded, pelleted cells were resuspended in 1 ml freezing medium and transferred into labeled cryovial. The cryovials are stored in Mr. Frosty, which was found in -80 ^{o}C. In the next day, they were placed into a liquid nitrogen tank for a long-term storage.

3.2.1.3. Thawing Mammalian Cells

Cryovial was taken from the liquid nitrogen tank and thawed in a water bath (37^{o}C). 9 ml RPMI 1640 was added into 15 ml falcon tube and frozen cells were gently transferred into 15 ml falcon tube drop by drop. Cells were spanned down at 300 G for 5 minutes in order to remove DMSO. They were resuspended in RPMI1640 medium. Cells were placed into sterile tissue culture flask (T-25) and incubated at 37 ^{o}C with 5 % CO_2.

3.2.2. BH3 Profiling Method

BH3 Profiling method was performed by BH3 Profiling Plate-based protocol (Laboratory Manual by Jeremy Ryan). JC-1 plate based BH3 profiling was done and small molecule library LOPAC (1280 molecules) was used in this assay. Cells were resuspended in MEB Buffer (150 mM Mannitol, 10 mM HEPES – KOH pH 7.5, 50 mM KCL, 0.02 mM EGTA, 0.02 mM EDTA, 0.1 % BSA, 5 mM Succinate). Small molecule inhibitors were permeabilized in staining solution (5 mM β-mercaptoethanol, 10 µg/ml Oligomycin, 1uM JC-1). Small molecule inhibitors in four different concentrations (10 uM, 1uM, 100 nM,

10nM) and cells were respectively transferred to 384 well plate. JC-1 fluorescence was analyzed at 545 nm excitation and 590 nm emission through Spectramax Gemini multi-plate spectrofluorometer after 90 minutes incubation of small inhibitor molecules at 30 ^{o}C[17].

The method, which is described above was used to optimize manual BH3 profiling protocol for the robot. Since the number of small molecules that we are going to screen is high, epMotion provide saving time by its automated system. Moreover, the pipetting robot enables to perform almost all steps of the screening including all liquid works from dilutions to the transfer of cells. Another point that we take advantage of is the precision of the experiments because there are 1280 small molecules that are going to be tested in different concentrations, in different cell lines with their replicates. At the same time, working volumes are considerably small. Additionally, the variations resulted from the unprecise manual transfer may affect the reliability of volume transfers. All these conditions indicate that providing the precision of the results is difficult when it is manually performed. However, the liquid transfer-robotic system provides a precision in an automated manner.

3.2.2.1. Optimization

Dimethyl Sulfoxide, sample inhibitor library (80 molecules) and non-small lung cancer cell lines (H-23 and H-1975) were purchased from ATCC. Sample inhibitor library was diluted in DMSO and screening was performed by BH3 profiling method on those cell lines with the help of epMotion5070.

3.2.2.2. Preparation of Main Stocks through Serial Dilution of Small Molecules

Small molecule library is composed of 80 small molecules in DMSO at 10 mM concentration and the volume of each small molecule is 25 ul. To dilute these inhibitors, DMSO was used as a solvent. It was purposed that each inhibitor must be dissolved in DMSO at 4 distinct concentrations (10uM, 1uM,100nM and 10 nM). Each small molecule was manually transferred to the first column of 96 PCR well plate for storage. 96 PCR well plate containing inhibitors was placed into labware B2. To transfer DMSO as a solvent, a reservoir rack, that involves a reservoir (30 ml), was located in labware B1. 25 ml DMSO was manually added to the reservoir. 90 ul DMSO was added to other eight columns by pool command as an initial step of serial dilution. Then, 10 ul of each inhibitor was transferred into the second column, which contains 90 ul DMSO, with an aiding of

the multichannel epMotion pipette. After mixing each inhibitor with DMSO, 10 ul from each molecule was transferred to another column. This process was repeated for seven times without tip change since each 96 PCR well plate involves 16 distinct inhibitors at four different concentrations. As a result of the serial dilution, sources plates were prepared as main stocks for small molecules.

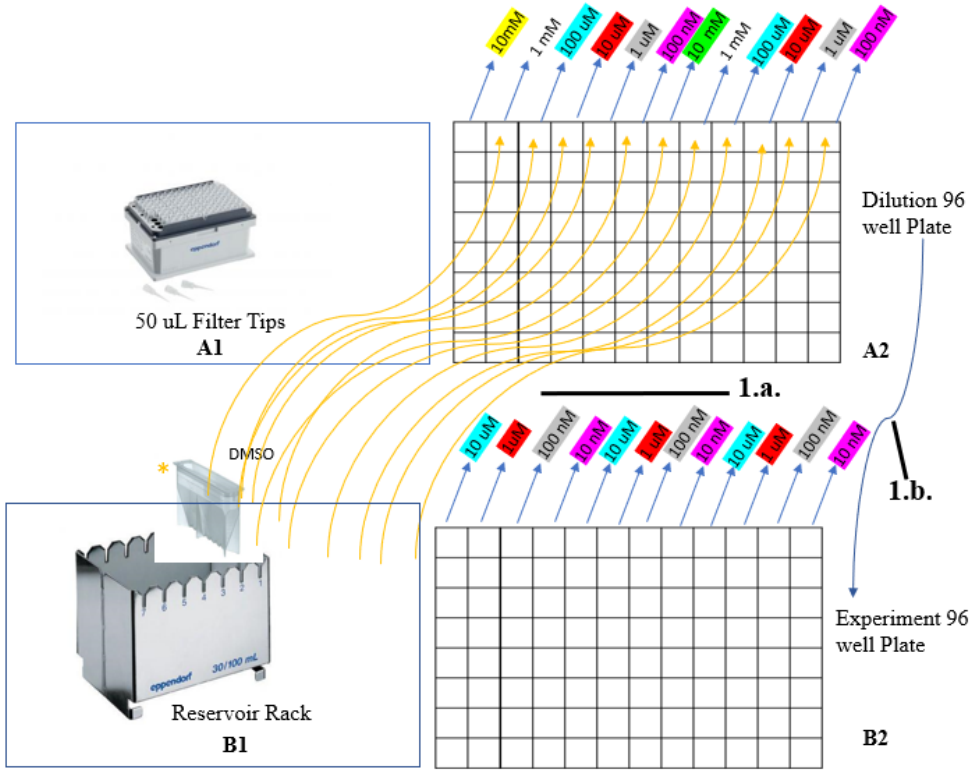


Figure 3. 3 Serial Dilution and Transferring Inhibitors to the 96 well Experiment Plate.

3.2.2.3. Transferring Inhibitors to the 96 well Experiment Plate

The main stock plate and the experiment plate were placed in labware A2 and B2 respectively. 12 ul of each small molecules were transferred from the main stock plate to the experiment plate through the reagent transfer command of epMotion. In each transfer step, the command for mixing was available before aspiration of the molecules and 50 ul filter tips were changed to prevent contamination.

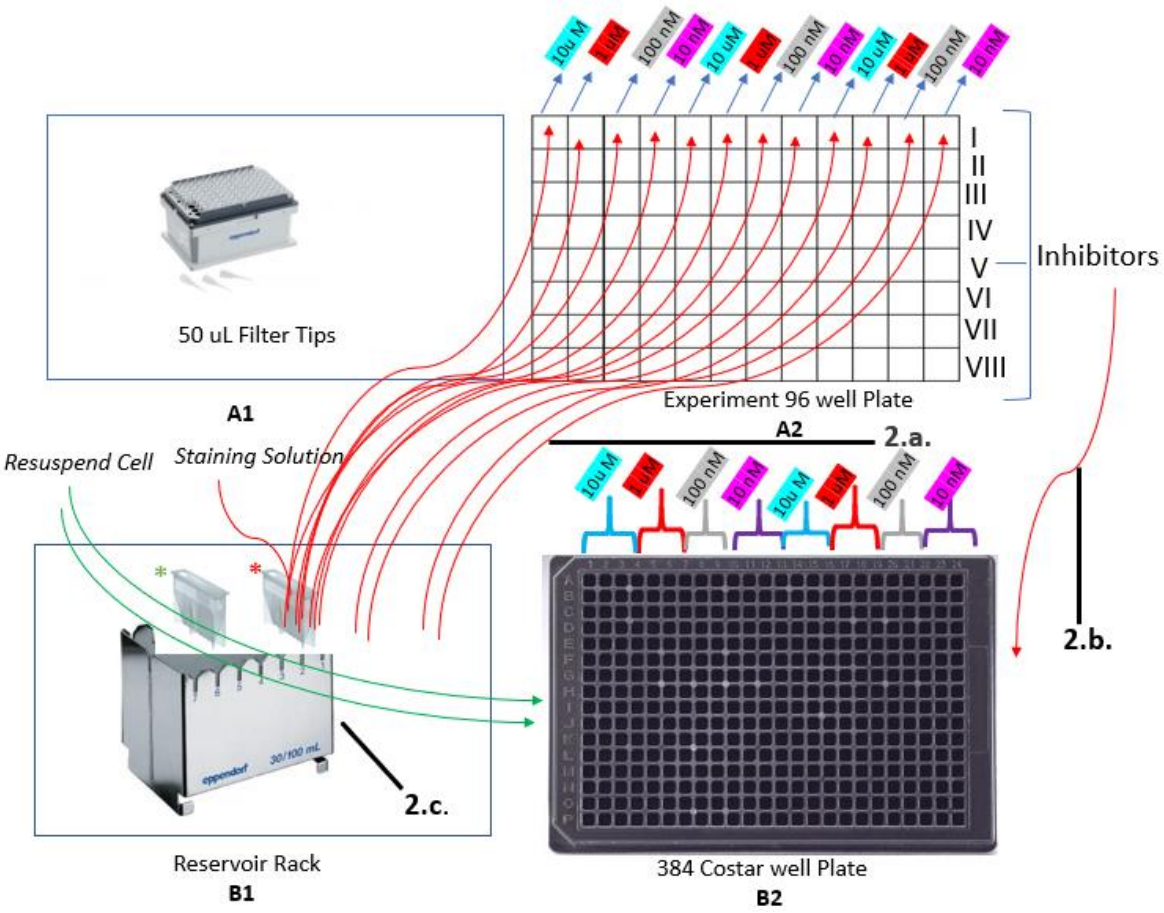


Figure 3. 4 BH3 Profiling Method through epMotion 5070.

3.2.2.4. BH3 Profiling Method through epMotion 5070

BH3 Profiling method through epMotion was categorized in two basic steps.

Transferring Staining Solution to Inhibitors

50 µl filter tips were placed in labware A1. Reservoirs 1 and 2, including 4 ml staining solution and resuspend non-small lung cancer cells, were respectively placed in reservoir rack in the labware B1. 24 µl of staining solution was aspirated by a multichannel pipette of epMotion 5070 from reservoir 1 and dispensed to the experiment plate at A2, which have small molecule inhibitors. This procedure was repeated twice since the final volume of each small molecule must be 60 µl. In each transfer step, staining solution was dispensed from the top and thereby same 50 µl filter tips are utilized for each column of the 96 well PCR plate.

Transfering Inhibitors in Staining Solution from experiment plate to 384 Well Plate

After addition of the staining solution to small molecule inhibitors in the previous step, the solution, consisting of small molecules and staining solution, was mixed three times at 15 ul mixing volume. Then, 15 µl solutions in each well were aspirated from the experiment plate and dispensed to the 384 well plate. The filter tips of the multichannel pipetting tool were changed for each column.

Transfering Resuspend Cells to the 384 well plate

Cells were detached from the surface of the cell culture flask (T-75). Then, they were centrifuged to remove trypsin at 300 G for 5 minutes. MEB 1X was used to wash the cells at 300 G for 5 minutes. Cells were resuspended in 4 ml MEB 1X and transferred into the reservoir. Before the aspiration of the resuspended cells, they were mixed three times at 15µl mixing volume. 15 µl of resuspended cells were transferred into 384 well plate for each column.

As a final step, DMSO and FCCP, which were negative and positive control respectively, were manually added into 384 well plate. Then 384 well plate was incubated at 30^{o}C for 90 minutes.

3.2.2.5. Procedures for Multichannel Pipetting Robot (EPMotion5070)

Serial Dilution Command

Dilution command operates the creation of dilution series. The reagent which needs to be diluted was transferred from well to well by the multichannel pipetting tool. First, the diluent was transported from the source to the destination plate and then dilution command was defined. Dilution command was consist of 3 stages with 1:10 dilution in each case.

Source Destination

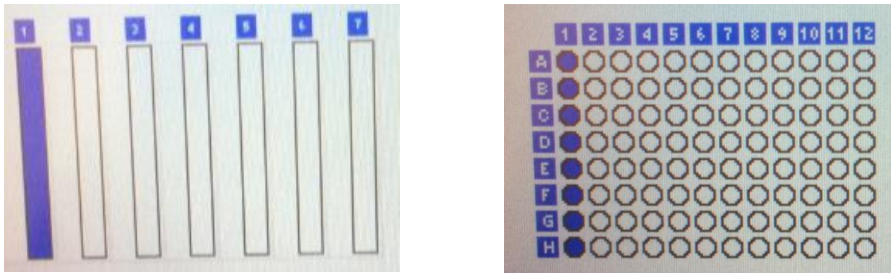
Figure 3. 5 Serial Dilution

Sample Transfer Command

A sample is transferred from one position to another position.

Reagent Transfer Command

The reagent transfer command allowed a transfer from one labware as a source to numerous labware as a destination. To speed up the library screening, staining solution transfer was accomplished by the reagent transfer command. 16 distinct inhibitors at 4 concentrations were able to be screened.



Source Source

Destination

Figure 3. 6 Reagent Transfer Command

4. RESULTS

4.1.Optimization Results

Sample library screening was performed at the Sabanci University Molecular Biology Genetics and Bioengineering Department, utilizing the Enzo (80 Molecules) for the optimization of pipetting robot (epMotion5070 - Eppendorf). We proposed to make sure the precision of the pipetting robot. Thereby, the same inhibitor was utilized with the same cell line H-23) and BH3 profiling assay was performed twice in four different concentrations.

Non-small lung cancer cells were considerably primed in response to small kinase inhibitor molecule Apigenin. In the second repeat, the cell was primed as well as in the first repeat in the presence of Apigenin.

In the presence of Y27632, H-23 cell line was primed like kinase inhibitors Apigenin. The percent of mitochondrial potential loss showed similarity in first and second measurements. In the context of Y-27632, the gap between the measurements was unexpectedly widen, the reason could be that the values of positive an negative controls were distinct from each other for each experiment. While the standard deviation was tolerable, consistency between the experimental repeats was preserved.

According to our results, the experimental set up was succeeded by pipetting robot for the BH3 profiling methods due to both the first and second repeats seem consistent.

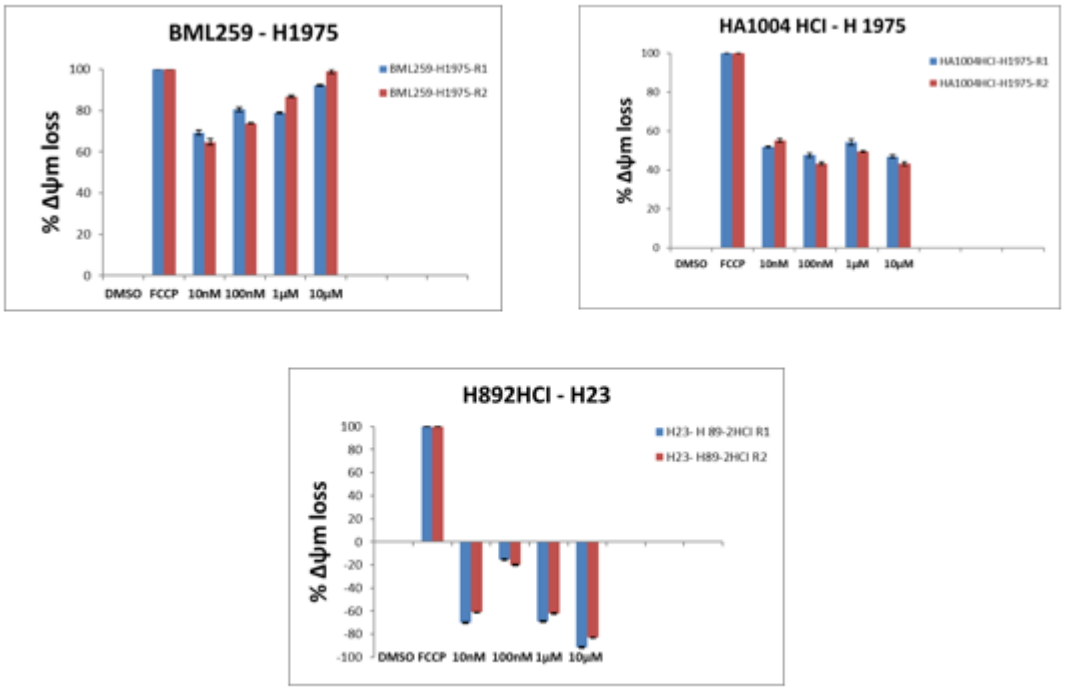


Figure 4. 1 BH3 profiles of non-small lung cancer cell lines (H-23, H-1975) in response to Triciribine, BML-259, H 89-2HCI and HA 1004 HCL for the optimization of pipetting robot.

As we expected, each response showed the similarity as a percentage of mitochondrial potential loss and standard deviation seemed quite small for each inhibitor in response to Triciribine, HA 1004 HCI, and BML-259.

Contrary to BML 259, Triciribine and, HA1004HCI, we found that H 89-2HCI led to mitochondrial hyperpolarization. The coherence between the first and second measurements was preserved.

4.2. Results for Actual Small Molecule Library Screening (1280 molecules)

Mitochondrial apoptotic priming level determines the cell fate and facilitates the tendency of undergoing apoptosis [38]. BH3 profiling method is a predictive approach to measure the changes in proapoptotic signaling [25]. Several anti-apoptotic proteins play a crucial role on chemoresistance mechanism. Particularly, MCL-1 confers a resistance to either targeted therapy or chemotherapy. Thereby, we further investigated the mitochondrial cell death priming status of the cells through BH3 profiling in non-small lung cancer cell line panel. We screened 1280 small molecules through BH3 profiling with the aid of the automated pipetting system and obtained different profiles in the manner of mitochondrial membrane potential loss. DMSO and FFCP were used as negative and positive controls respectively.

While some drugs caused hyperpolarization, the others led to mitochondrial depolarization. Depolarization percentage was calculated by the formula'' 1- ((Sample-Positive Control (FCCP))/ (Negative Control-Depolarization))'' for each inhibitor at four different concentrations. The variety of responses point out that BH3 profiling is an effective method for the screening of large numbers of molecules on cell lines. We performed a BH3 profiling method on H-23 cell line known to be Mcl-1 dependent. After selecting the candidate Mcl-1 inhibitors, we will use the H-1975 as a negative control cell line, known to be Mcl-1 independent. We showed that randomly selected results from our screen.

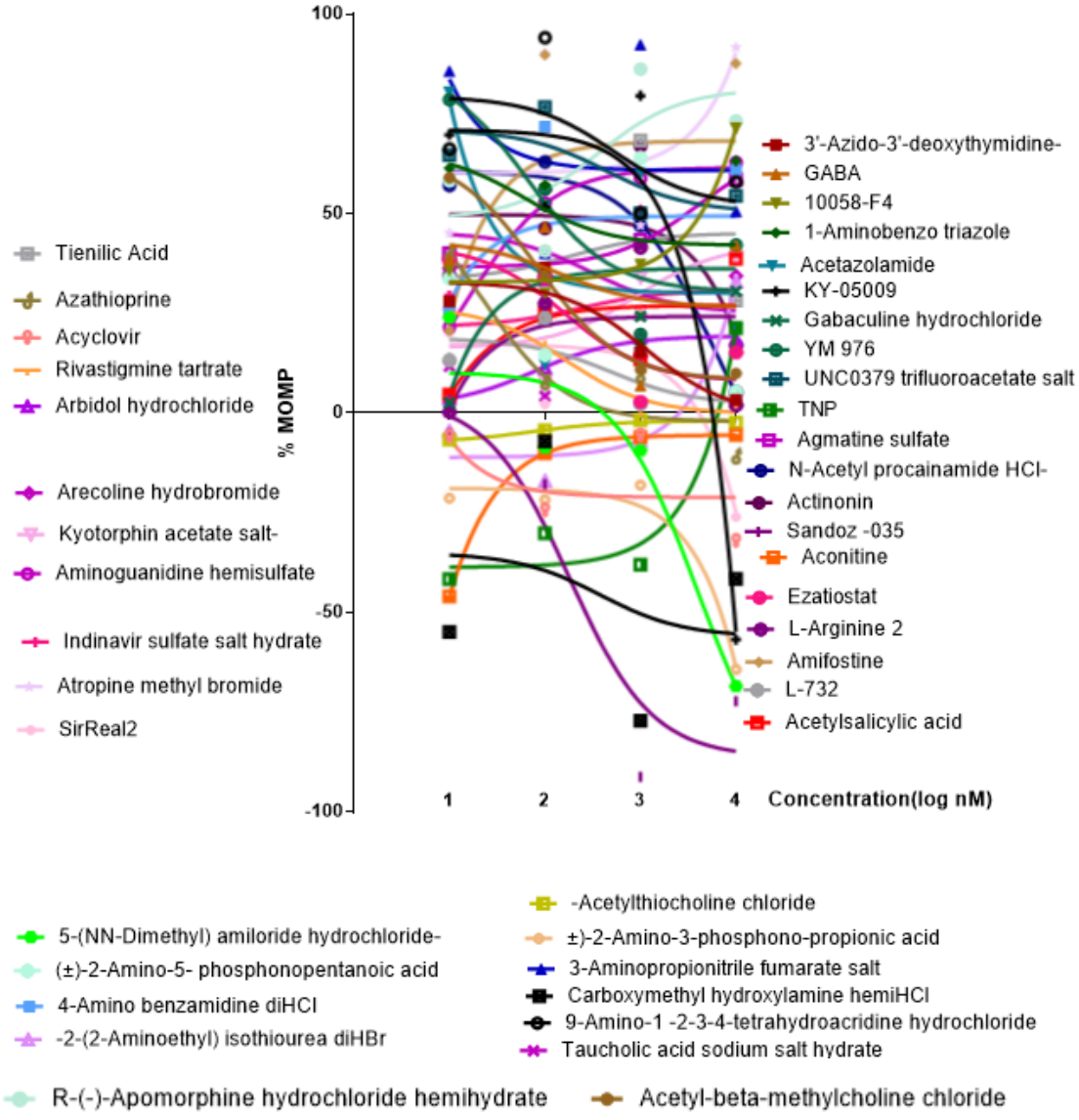


Figure 4. 2 Dose-response curves of 44 distinct small molecules in non-small lung cancer cell line H-23 line at 90 minutes post-treatment.

We analyzed the depolarization percentage of each inhibitor using non-linear regression. Several small molecules depolarized the mitochondria and caused mitochondrial outer membrane potential loss. Each inhibitor was tested in four different concentrations. On the other hand, they led to mitochondrial depolarization besides from 3-Aminopropionitrile fumarate salt, (+)2 Amino-5-phosphonopentanoic acid, 9 amino 1,2,3,4 tetrahydroacridine hydrochlorides, Amifostine, KY-05009, which have distinct responses in different concentrations.

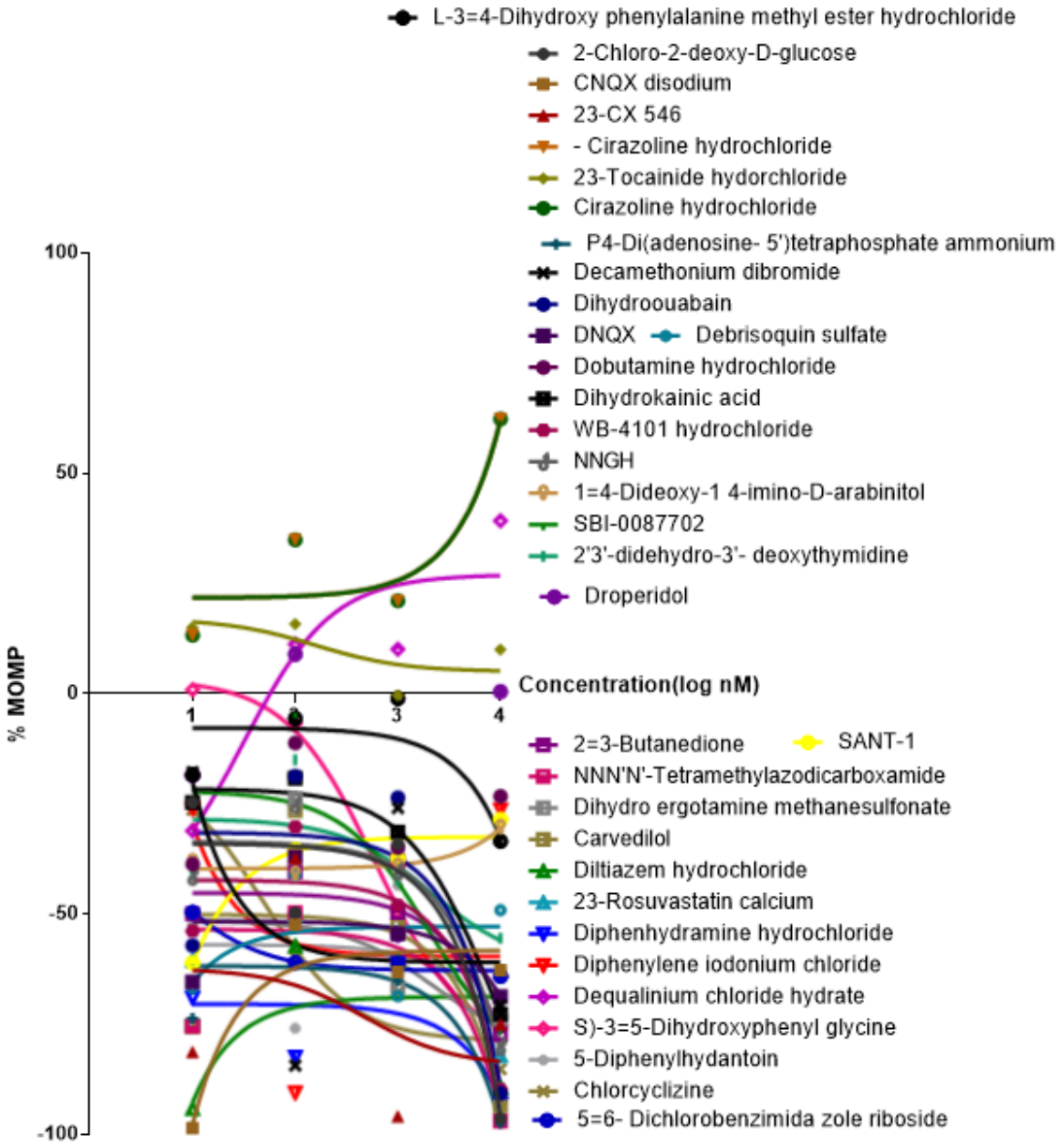


Figure 4. 3 Dose-response curves of 34 distinct small molecules in non-small lung cancer cell line H-23 line at 90 minutes post-treatment.

While non-small lung cancer cells (H-23) mostly have hyperpolarized membrane potential voltage, a few small molecules succeed in mitochondrial depolarization and caused membrane potential loss. In response to Carvedilol, Cirazoline hydrochloride and dequalinium chloride hydrate, primed cells (H-23) were having sufficient mitochondrial depolarization whereas some molecules led to very low or none mitochondrial depolarization in primed cells (H-23).

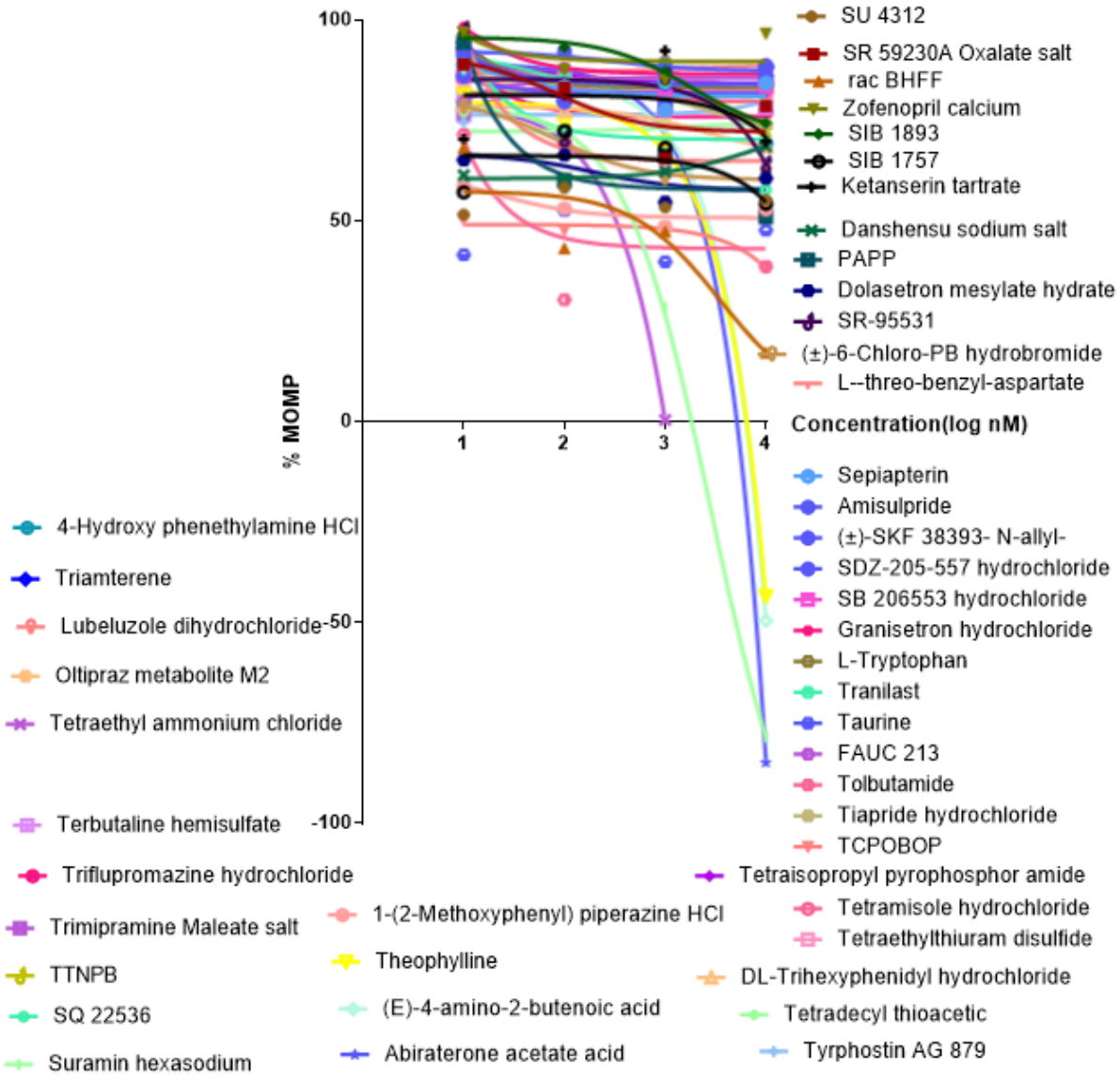


Figure 4. 4 Dose-response curves of 47 distinct small molecules in non-small lung cancer cell line H-23 line at 90 minutes post treatment.

Abiraterone acetate acid, Theophylline, Suramin hexasodium caused mitochondrial outer membrane potential loss at high concentrations (10µM, 1µM, 100nM), whereas mitochondrial hyperpolarization was observed at low concentration (10nM). However,

the other small molecules gave a positive result and led to mitochondrial depolarization at each distinct concentration. Even the depolarization rate was above 50 percent.

Due to we obtained different responses in the context of mitochondrial outer membrane potential loss, we categorized these responses in five profiles.

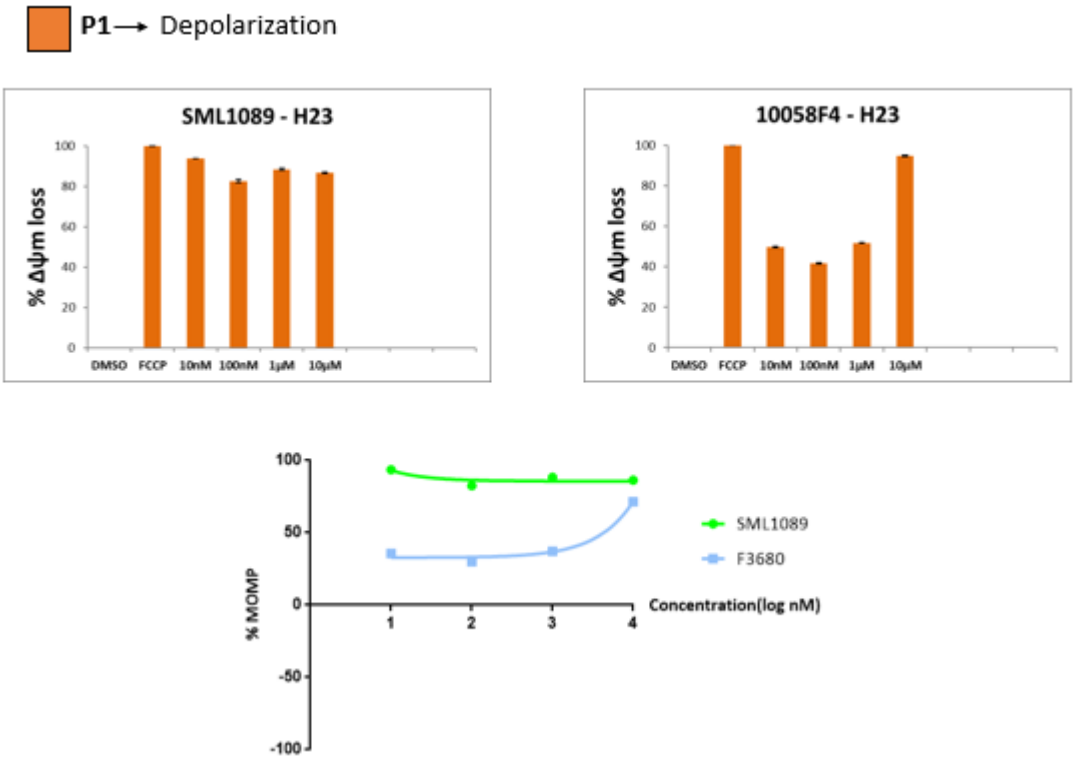


Figure 4. 5 BH3 profiles of non-small lung cancer cell line (H-23) in response to SML 1089 and F 3680.

We categorized the molecules, which led high mitochondrial outer membrane potential loss in each concentration in profile 1. SML1089 and F 3680 caused mitochondrial depolarization since mitochondrial potential loss percent was high for each concentration in the bar chart. We found that these drugs might be specific molecules for the inhibition of MCL-1. Due to SML1089 caused more permeabilization of the mitochondrial outer membrane the mitochondrial cell membrane than F3680 at 10 nM, SML1089 would be a potent molecule for targeting of MCL-1.

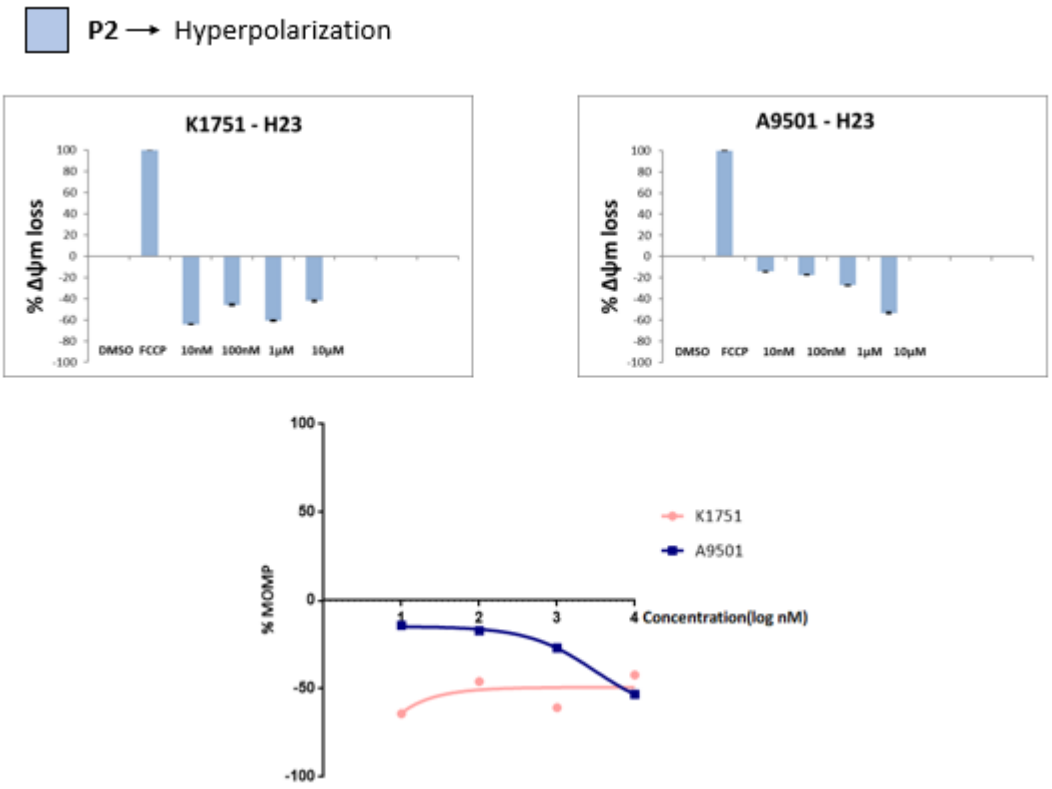


Figure 4. 6 BH3 profiles of non-small lung cancer cell line (H-23) in response to K 1751, A 9501.

We grouped small molecules that led to a mitochondrial hyperpolarization in profile 2. The mitochondrial potential loss was quite low in response to K1751 and A9501. It means that they caused mitochondrial hyperpolarization and that is why these drugs seem not be a specific molecule for the inhibition of MCL-1. In the lower part of figure 19, we showed the nonlinear regression analysis of A9501 and K1751. While the hyperpolarization percent of A9501 was directly proportional to the concentration increase, H23 cell line had different responses at distinct concentrations in the presence of K1751.

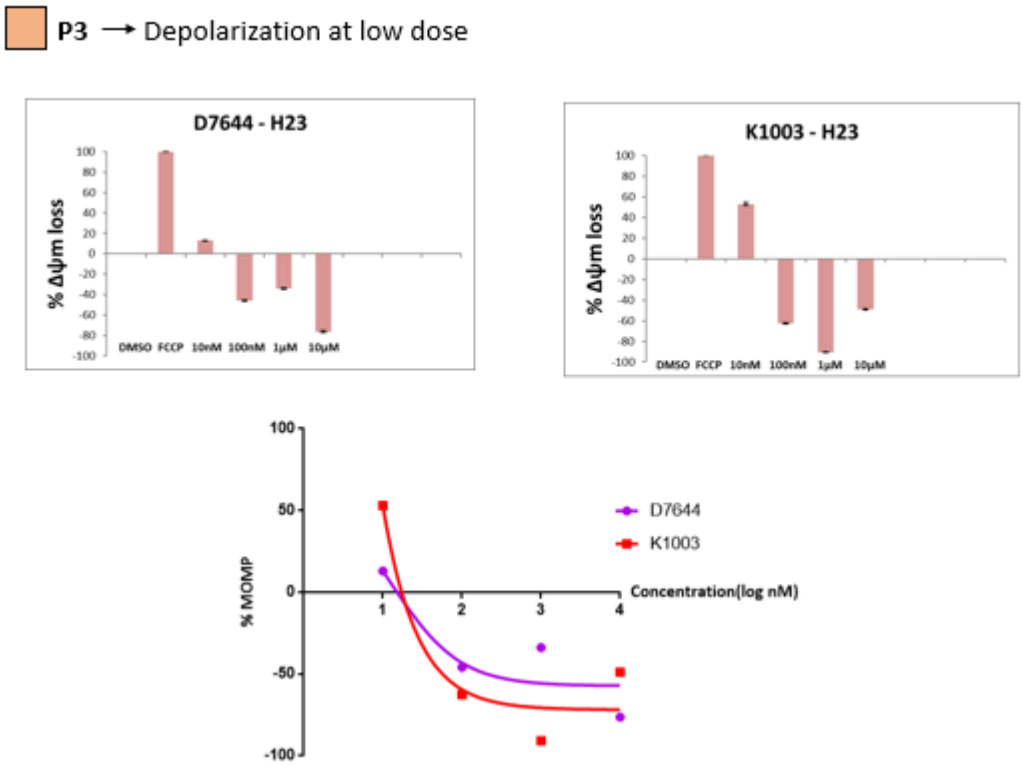


Figure 4. 7 BH3 profiles of non-small lung cancer cell line (H-23) in response to D 7644 and K 1003.

In the profile 3, we categorized the inhibitors that caused high mitochondrial potential loss only at a low dose (10 nM). H23 cell line was primed in response to D7644 and K1003 at only 10 nM concentration. K1003 seems a potent molecule rather than the D7644 because mitochondrial potential loss percent, which is caused by K1003, was more than the mitochondrial potential loss, caused by D7644.

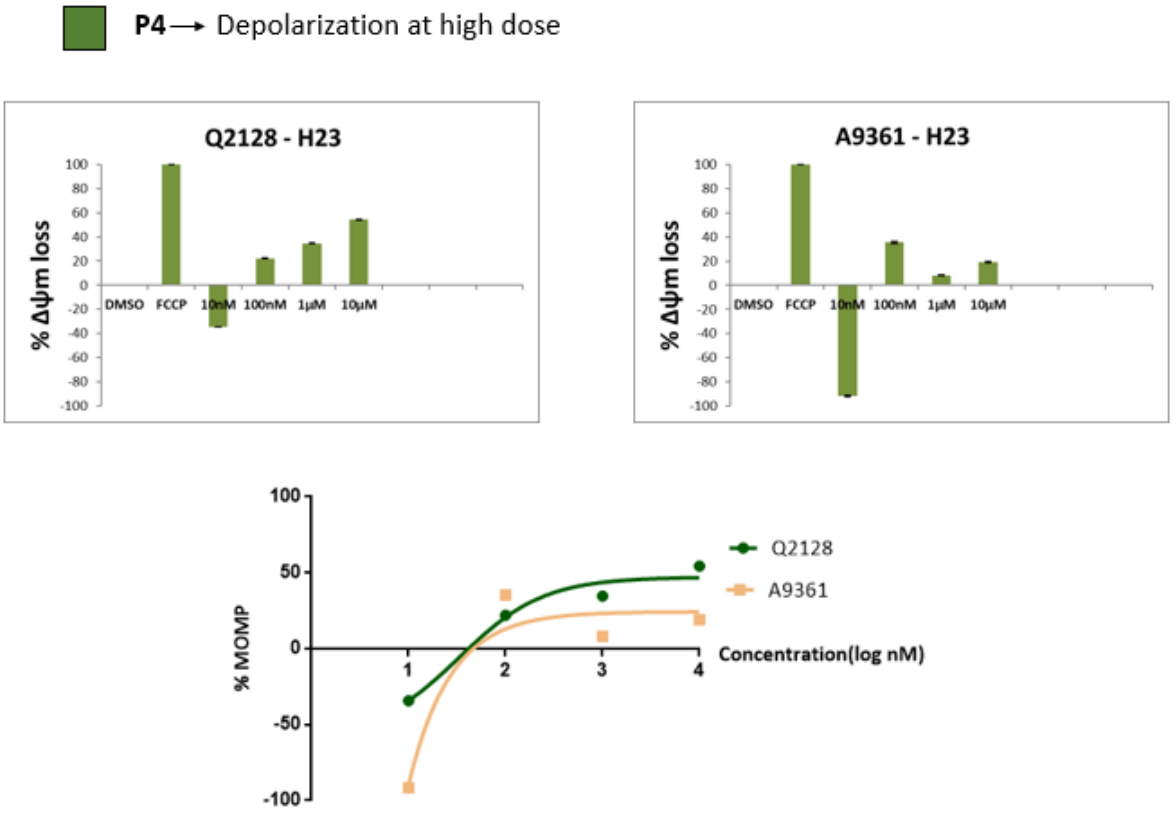


Figure 4. 8 BH3 profiles of non-small lung cancer cell line (H-23) in response to Q2128 and A9361.

The other profile (P3) involves the small molecules which triggered mitochondrial depolarization only at high dose (10µM, 1µM,100 nM). In the response to Q2128 and A9361, the mitochondrial outer membrane of the cells was permeabilized. Even though we observed the mitochondrial depolarization in response to A9361 at high doses, depolarization percent was low compared to the Q2128. Moreover, the depolarization rate was directly proportional to the concentration increase in the presence of Q2128 only at high dose. Thereby, Q2128 seems more potent small molecule for MCL-1 inhibition.

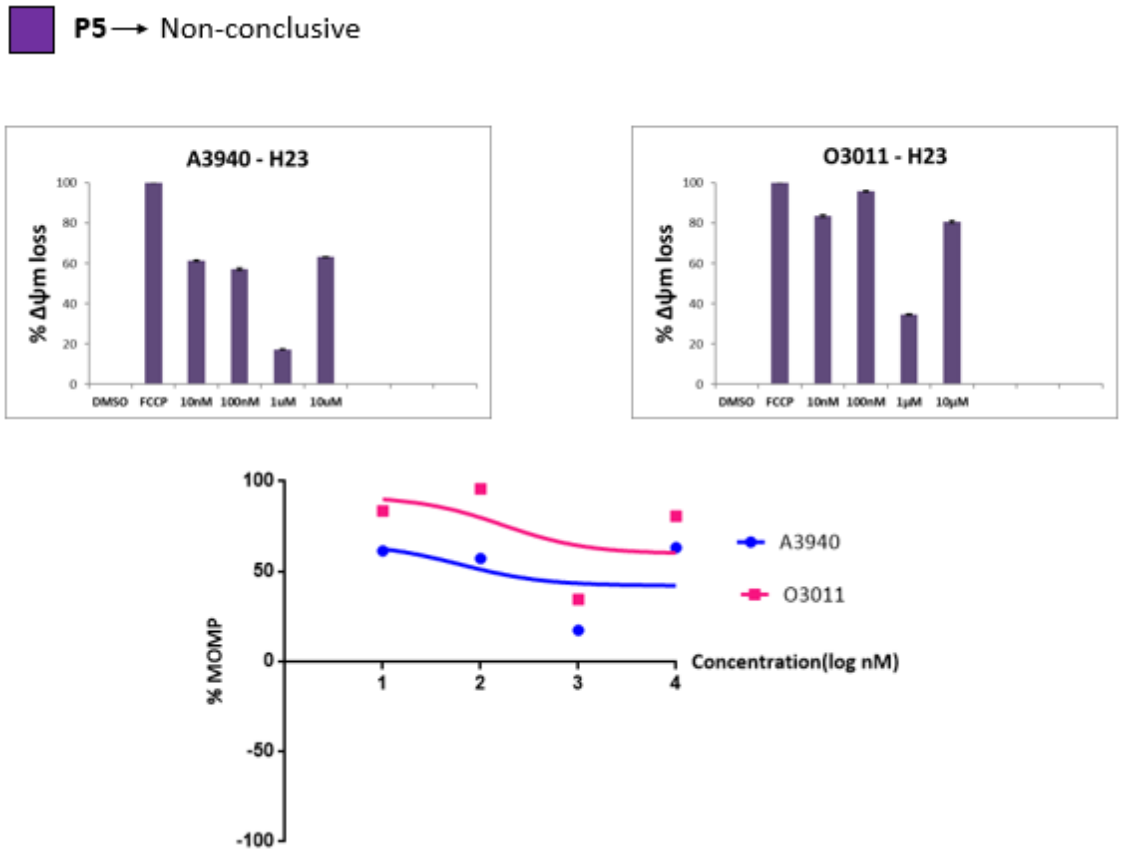


Figure 4. 9 BH3 profiles of non-small lung cancer cell line (H-23) in response to A3940 and O3011.

In profile 5, even though the molecules led to mitochondrial depolarization, there was no direct proportion to the concentration due to the increase in concentration. To comment on this profile in detail, first we need to utilize these inhibitors on the H1975 cell line. If we observe the mitochondrial depolarization, we might perform cell death assays as further studies.

To sum up, we have counted the small molecule inhibitors and categorized these five distinct profiles into three basic groups, including depolarization, hyperpolarization and non-exclusive responses. We summarized the percentage of each group in the pie chart.

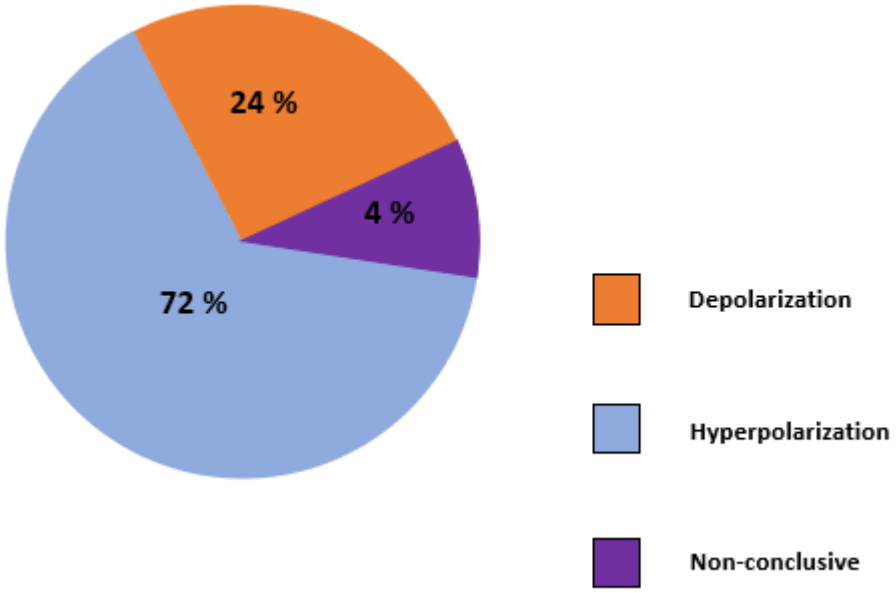


Figure 4. 10 The percentage of each group in the small molecule inhibitor library.

4.3. EC50 Values

The EC50 values of the molecules were determined by nonlinear regression analysis.

The Name of the Inhibitor	*EC50*
Acetyl-beta-methylcholine chloride	82.71
3'-Azido-3'-deoxythymidine-	1119
GABA	148.2
10058-F4	~ 162449
1-Aminobenzo triazole	62.2
9-Amino-1-2-3-4-tetrahydroacridine hydrochloride	531
KY-05009	~ 6.517e+007
Gabaculine hydrochloride	~ 0.004333
YM 976	66.26
UNC0379 trifluoroacetate salt	444.5
R-(-)-Apomorphine hydrochloride hemihydrate	354.6
Carboxymethyl hydroxylamine hemiHCl	379.4
5-(NN-Dimethyl) amiloride hydrochloride-	3977
Azathioprine	23.18
Acyclovir	0.00015
Rivastigmine tartrate	163.7
Sandoz -035	179.8
±)-2-Amino-3-phosphono-propionic acid	~ 7.347e+007
L-Arginine 2	0.00003378
Ezatiostat	159.9
L-732	479.6
Acetylsalicylic acid	4.6
Aconitine	1.581
-Acetylthiocholine chloride	72.57
TNP	~ 4.161e+007
-2-(2-Aminoethyl) isothiourea diHBr	~ 9.914e+006
4-Amino benzamidine diHCI	0.0000339
3-Aminopropionitrile fumarate salt	0.00003796
Acetazolamide	~ 0.006471
Amifostine	0.00006785
Aminoguanidine hemisulfate	16.47
Agmatine Sulfate	3909
Arbidol hydrochloride	78.21
Kyotorphin acetate salt-	904.3
Arecoline hydrobromide	311.6

SirReal2	~ 1.693e+007
Atropine methyl bromide	1326000000
Indinavir sulfate salt hydrate	230.3

The Name of the Inhibitor	***EC50 Values***
N-Acetyl procainamide HCl-	5593
Tienilic Acid	183.3
Actinonin	~ 4.032e+008
2-Chloro-2-deoxy-D-glucose	~ 7.073e+006
CNQX disodium	~ 0.004335
23-CX 546	482.5
Cirazoline hydrochloride	~ 6.086e+006
Tocainide hydrochloride	158.3
Cirazoline hydrochloride	~ 6.086e+006
P4-Di(adenosine- 5')tetraphosphate ammonium	~ 2.441e+006
Decamethonium dibromide	0.00005588
Dihydroouabain	~ 1.379e+007
DNQX	83270000
Dihydrokainic acid	9592
WB-4101 hydrochloride	~ 502298
NNGH	30840
1=4-Dideoxy-1 4-imino-D-arabinitol	~ 2.111e+007
SBI-0087702	2190
2'3'-didehydro-3'- deoxythymidine	2069
Debrisoquin sulfate	0.00003632
SANT-1	1.675
5-6- Dichlorobenzimida zole riboside	6.479
L-3-4-Dihydroxy phenylalanine methyl ester hydrochloride	~ 1.937e+007
2-3-Butanedione	38552
NNN'N'-Tetramethylazodicarboxamide	~ 8.595e+009
Dihydro ergotamine methanesulfonate	2062
Carvedilol	~ 3.633e+006
Diltiazem hydrochloride	0.00003305
Diphenhydramine hydrochloride	~ 1.856e+007
Diphenylene iodonium chloride	~ 4.421e-006
Dequalinium chloride hydrate	31.02
S)-3=5-Dihydroxyphenyl glycine	609.6
Chlorcyclizine	49.83
SR 59230A Oxalate salt	81.62
rac BHFF	3481
Zofenopril calcium	0.00001448
SIB 1893	1874
SIB 1757	~ 7.796e+007
Ketanserin tartrate	4018000000
Danshensu sodium salt	7163
1-(2-Methoxyphenyl) piperazine HCl	20.57

The Name of the Inhibitor	***EC50***
PAPP	~ 0.06996
Dolasetron mesylate hydrate	196.9
SR-95531	~ 1.943e+006
(±)-6-Chloro-PB hydrobromide	81.14
L--threo-benzyl-aspartate	~ 0.004506
Suramin hexasodium	2866
SQ 22536	~ 1.171
Sepiapterin	18.57
Amisulpride	~ 4.764e-006
(±)-SKF 38393- N-allyl-	~ 9.584e-006
SDZ-205-557 hydrochloride	307.7
SB 206553 hydrochloride	~ 0.007837
Granisetron hydrochloride	4.163
L-Tryptophan	~ 0.001471
Tranilast	57.42
FAUC 213	~ 0.008860
Tolbutamide	~ 0.006004
Tiapride hydrochloride	1794
TCPOBOP	~ 1.207e+006
Tetraisopropyl pyrophosphor amide	56.91
Tetramisole hydrochloride	57.42
Tetraethylthiuram disulfide	~ 1.813e+007
DL-Trihexyphenidyl hydrochloride	2263
Theophylline	~ 3.389e+007
(E)-4-amino-2-butenoic acid	~ 7.033e+007
Tetradecyl thioacetic	1675
Abiraterone acetate acid	~ 7.068e+007
Tyrphostin AG 879	~ 5.712e+006
Tetraethyl ammonium chloride	4776
Terbutaline hemisulfate	31.11
Triflupromazine hydrochloride	~ 0.004672
Trimipramine Maleate salt	~ 0.007004
Oltipraz metabolite M2	499.4
TTNPB	1860000000
Lubeluzole dihydrochloride	1204
Triamterene	~ 0.009184
Estetrol	1285
Tetrahydrozoline hydrochloride	830.4
N-p-Tosyl-L-phenylalanine chloromethyl ketone	~ 0.004617
(6R)- 5- 6 7 8Tetrahydro-L-biopterin dihydrochloride	~ 5.898e-006
cDPCP	14.3

The Name of the Inhibitor	***EC50***
Theobromine	~ 2.158e+007
(±)-Taxifolin	~ 5.561e+007
CYM-5520	39288
Nedocromil	116
Entecavir	~ 1.612e+010
Pivmecillinam	~ 0.004345
RU-SKI 43 maleate	~ 0.004345
DL-Thiorphan	~ 2.091e+007
(+-)-alpha-Lipoic Acid	1190
Tulobuterol hydrochloride	1.296
Trazodone hydrochloride	37.69
BAY 61-3606 hydrochloride hydrate	~ 0.007705
Triamcinolone	265.9
S(-)-Timolol maleate	9125
Triprolidine hydrochloride	173.5
Tyrphostin AG 112	681
Tyrphostin 1	898.5
Tyrphostin 23	2438
Na-p-Tosyl-L-lysine chloromethyl ketone HCl	381.7
Pifithrin-μ	424.3
Enclomiphenehydrochloride	290.5
1-3-Dipropyl-8-p-sulfophenyl xanthine	201.8
Gardiquimod	~ 0.004281
VU0420373	49.95
Amoxapine	246.1
Aurora-A Inhibitor I	2097000000
Arecaidine propargyl ester hydrobromide	8.036
Aminobenztropine	~ 7.754e+006
S(-)-Atenolol	~ 0.009875

Table 4. 1 EC 50 values of randomly selected screening results.

5. DISCUSSION

Cancer cells are governed by apoptotic inhibitions and proliferative signals. The mitochondrial apoptotic pathway relies on mitochondrial outer membrane permeabilization that is driven by interactions between BCL-2 family proteins. This family has a dual role in cellular homeostatic activities. BCL-2 family proteins provide the elimination of damaged cell from organism under hemostatic conditions whereas, in the case of cancer, their overexpression induces tumor development, chemoresistance, and metastasis. The network between BCL-2 family proteins decides how mitochondrial outer membrane permeabilization proceed. Once the abundance of pro-apoptotic proteins exceeds the binding capacity of antiapoptotic, the cell fate is determined as a suicide. Even though BCL-2 proteins are categorized into three groups, all of them possess BH3 death domain [10, 18]. BH3 profiling assay was used to determine the chemosensitivity of cancer cells and predict the ability of small molecules to trigger mitochondrial depolarization [17, 20]. Since the BH3 domain of antiapoptotic proteins has a hydrophobic groove and its groove is deeper than pro-apoptotic proteins, targeting protein-protein interaction is a feasible approach for this study. We proposed finding a selective small molecule to inhibit MCL-1 through BH3 profiling method with the aid of automated system [10, 18]. To adapt the manual BH3 profiling to pipetting robot, we designed an optimization protocol through the commands of the pipetting robot. We performed the optimization protocol through a sample molecule inhibitor library which is consist of 80 kinase inhibitors. The effect of each inhibitor was tested twice to make sure the precision of the pipetting robot and critical points at each step of the method were properly considered.

After the optimization step, we screened actual small molecule library (1280 molecules) at four different concentrations through BH3 profiling method with the aid of pipetting robot and investigated the ability of distinct inhibitors in the context of mitochondrial outer membrane permeabilization. BH3 profiling results have shown that a variety of the inhibitors result in distinct responses. While most of the molecules caused mitochondrial hyperpolarization, the others succeed in depolarizing mitochondria. To predict the

optimal concentration range of the candidate molecule, the inhibitors were utilized at four different concentrations (10 µM,1 µM,100 nM,10 nM). While the percent of mitochondrial outer membrane potential loss was high at 10 µM,1 µM and 100 nM concentrations in response to abiraterone acetate acid, theophylline, Suramin hexasodium, the response was declined at 10nM. However, there were some inhibitors that have a potential to depolarize the mitochondria at low concentrations rather than the high concentrations. To confirm the ability of the candidate molecules for mitochondrial depolarization, we are going to repeat the experiment three times. The negative control cell line (H-1975) will be utilized to makes sure whether the candidate molecules are potentially MCL-1 specific or not. If the candidate molecules lead to hyperpolarization of mitochondria, it means that the candidates would be notably MCL-1 specific molecules.

Our initial results indicate that BH3 profiling assay is a functional method to provoke a response from mitochondria against chemotherapeutic molecules. Our results point out that the potential of utilizing BH3 Profiling method as a unique precision tool in medicine.

In summary, we targeted pro-survival protein MCL-1 because it is known as a survival and chemoresistance factor. We screened 1280 small molecule inhibitors to find a specific small molecule for the inhibition of MCL-1. We categorized our results in five profiles. We are planning to perform a BH3 profiling assay through the candidate molecules, leading to mitochondrial depolarization, on H-1975 cell line. In order to confirm the specificity of the candidate small molecules to MCL-1, we need to apply the inhibitors our negative control cell line (H-1975), which is known as an MCL-1 independent.

6. REFERENCES

1. Proteins B-F, Checkpoints C, Death C. BCL-2 Family Proteins: Critical Checkpoints of Apoptotic Cell Death. 2007;13(24):7254-7264. doi:10.1158/1078-0432.CCR-07-1598
2. Kuida K, Haydar TF, Kuan C, et al. Reduced Apoptosis and Cytochrome c – Mediated Caspase Activation in Mice Lacking Caspase 9. 1998;94:325-337.
3. Bredesen DE, Cousins N. Programmed Cell Death Mechanisms in Neurological Disease. 2008:173-186.
4. Ashkenazi A, Dixit VM. Death Receptors : Signaling and Modulation. 1998;281(August):1305-1309.
5. Green DR, Llambi F. Cell Death Signaling. 2016.
6. Oral O, Akkoc Y, Bayraktar O, Gozuacik D. Physiological and pathological significance of the molecular cross-talk between autophagy and apoptosis Histology and. 2016:479-498. doi:10.14670/HH-11-714
7. Defining Characteristics of Types I and II Apoptotic Cells in Response to TRAIL. 2002;4(6):551-557. doi:10.1038/sj.neo.7900270
8. Hao Z, Mak TW. Type I and Type II Pathways of Fas-mediated Apoptosis Are Differentially Controlled by XIAP. 2018;(May):63-64.
9. Li H, Zhu H, Xu C, Yuan J. Cleavage of BID by Caspase 8 Mediates the Mitochondrial Damage in the Fas Pathway of Apoptosis. 1998;94:491-501.
10. Tait SWG, Green DR, Silke J, Meier P, Chan FK. Mitochondrial Regulation of Cell Death. 2014. doi:10.1101/cshperspect.a008706
11. Czabotar PE, Lessene G, Strasser A, Adams JM. Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. *Nat Rev Mol Cell Biol*. 2014;15(1):49-63. doi:10.1038/nrm3722
12. Peperzak V, Slinger E, Ter Burg J, Eldering E. Functional disparities among BCL-2 members in tonsillar and leukemic B-cell subsets assessed by BH3-mimetic profiling. *Cell Death Differ*. 2017;24(1):111-119. doi:10.1038/cdd.2016.105
13. Delbridge ARD, Strasser A. The BCL-2 protein family, BH3-mimetics and cancer therapy. *Cell Death Differ*. 2015;22(7):1071-1080. doi:10.1038/cdd.2015.50
14. Bhola PD, Letai A. Mitochondria-Judges and Executioners of Cell Death Sentences. *Mol Cell*. 2016;61(5):695-704. doi:10.1016/j.molcel.2016.02.019
15. Letai AG. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer*. 2008;8(2):121-132. doi:10.1038/nrc2297
16. Spring C. To Prime , or Not to Prime : That Is the Question. 2016;LXXXI.
17. Ryan J, Letai A. BH3 profiling in whole cells by fluorimeter or FACS. *Methods*. 2013;61(2):156-164. doi:10.1016/j.ymeth.2013.04.006

18. Kalkavan H, Green DR. MOMP , cell suicide as a BCL-2 family business. *Nat Publ Gr*. 2017;25(1):46-55. doi:10.1038/cdd.2017.179

19. Manuscript A. NIH Public Access. 2014;61(2):617-632. doi:10.1016/j.ymeth.2013.04.006.BH3

20. Butterworth M, Pettitt A, Varadarajan S, Cohen GM. BH3 profiling and a toolkit of BH3-mimetic drugs predict anti-apoptotic dependence of cancer cells. *Br J Cancer*. 2016;114(6):638-641. doi:10.1038/bjc.2016.49

21. Manuscript A. NIH Public Access. 2013;332(2):202-205. doi:10.1016/j.canlet.2011.12.021.BH3

22. Ryan J, Montero J, Rocco J, Letai A. IBH3: Simple, fixable BH3 profiling to determine apoptotic priming in primary tissue by flow cytometry. *Biol Chem*. 2016;397(7):671-678. doi:10.1515/hsz-2016-0107

23. Deng J, Carlson N, Takeyama K, Cin PD, Shipp M, Letai A. Article BH3 Profiling Identifies Three Distinct Classes of Apoptotic Blocks to Predict Response to ABT-737 and Conventional Chemotherapeutic Agents. 2007;(August):171-185. doi:10.1016/j.ccr.2007.07.001

24. Ryan JA, Brunelle JK, Letai A. Heightened mitochondrial priming is the basis for apoptotic hypersensitivity of CD4+ CD8+ thymocytes. *Proc Natl Acad Sci*. 2010;107(29):12895-12900. doi:10.1073/pnas.0914878107

25. Wang MO, Vorwald CE, Dreher ML, et al. HHS Public Access. 2016;27(1):138-144. doi:10.1002/adma.201403943.Evaluating

26. Zhou BP, Qian L, Kozopas KM, Craig RW. Mcl-1, a Bcl-2 Family Member, Delays the Death of Hematopoietic Cells Under a Variety of Apoptosis-Inducing Conditions. 2018;89(2):630-643.

27. Yang T. The Intracellular Distribution and Pattern of Expression of Mcl-1 Overlap with, but Are Not Identical to, Those of Bcl-2. 1995;128(6):1173-1184.

28. Kozopas KM, Yang TAO, Buchan HL, Zhou P, Craig RW. MCLI , a gene expressed in programmed myeloid cell differentiation , has sequence similarity to BCL2. 1993;90(April):3516-3520.

29. Germain M, Duronio V. The N Terminus of the Anti-apoptotic BCL-2 Homologue MCL-1 Regulates Its Localization and Function *. 2007;282(44):32233-32242. doi:10.1074/jbc.M706408200

30. Colosetti P, Herrant M, Jacquel A, Marchetti S, Belhace N. Cleavage of Mcl-1 by caspases impaired its ability to counteract Bim-induced apoptosis. 2004:7863-7873. doi:10.1038/sj.onc.1208069

31. Opferman JT. Unraveling MCL-1 degradation. 2006:1260-1262. doi:10.1038/sj.cdd.4401978

32. Bingle CD, Craig RW, Swales BM, Singleton V, Zhou P, Whyte MKB. Exon Skipping in Mcl-1 Results in a Bcl-2 Homology Domain 3 Only Gene Product That Promotes Cell Death *. 2000;275(29):22136-22146. doi:10.1074/jbc.M909572199

33. Lin KH, Winter PS, Xie A, et al. Targeting MCL-1 / BCL-X L Forestalls the Acquisition of Resistance to ABT-199 in Acute Myeloid Leukemia. 2016;(June):1-10. doi:10.1038/srep27696

34. Nguyen M, Marcellus RC, Roulston A, et al. Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. *Proc Natl Acad Sci U S A.* 2007;104(49):19512-19517. doi:10.1073/pnas.0709443104

35. Chen L, Fletcher S. Mcl-1 inhibitors: a patent review. *Expert Opin Ther Pat*. 2017;27(2):163-178. doi:10.1080/13543776.2017.1249848

36. Bruncko M, Oost TK, Belli BA, et al. Studies Leading to Potent , Dual Inhibitors of Bcl-2 and. (847):1-5.

37. Tse C, Shoemaker AR, Adickes J, et al. ABT-263 : A Potent and Orally Bioavailable Bcl-2 Family Inhibitor. 2008;(9):3421-3429. doi:10.1158/0008-5472.CAN-07-5836

38. Shoemaker AR, Mitten MJ, Adickes J, et al. Human Cancer Biology Activity of the Bcl-2 Family Inhibitor ABT-263 in a Panel of Small Cell Lung Cancer Xenograft Models. 2008;14(11):3268-3278. doi:10.1158/1078-0432.CCR-07-4622

39. Loriot Y, Mordant P, Dugue D, et al. Radiosensitization by a novel Bcl-2 and Bcl-X L inhibitor S44563 in small-cell lung cancer. *Cell Death Dis*. 2014;5(9):e1423-13. doi:10.1038/cddis.2014.365

40. Bai L, Chen J, Mceachern D, et al. BM-1197 : A Novel and Specific Bcl-2 / Bcl-xL Inhibitor Inducing Complete and Long-Lasting Tumor Regression In Vivo. 2014;9(6):1-13. doi:10.1371/journal.pone.0099404

41. Niu X, Wang G, Wang Y, et al. HHS Public Access. 2015;28(7):1557-1560. doi:10.1038/leu.2014.72.Acute

42. Fabregat I. Dysregulation of apoptosis in hepatocellular carcinoma cells. 2009;15. doi:10.3748/wjg.15.513

43. Glaser SP, Lee EF, Trounson E, et al. Anti-apoptotic Mcl-1 is essential for the development and sustained growth of acute myeloid leukemia. 2012:120-125. doi:10.1101/gad.182980.111.120

44. Aichberger KJ, Mayerhofer M, Krauth M, et al. Identification of mcl-1 as a BCR / ABL-dependent target in chronic myeloid leukemia (CML): evidence for cooperative antileukemic effects of imatinib and mcl-1 antisense oligonucleotides. 2018;105(8):3303-3312. doi:10.1182/blood-2004-02-0749.Supported

45. Koss B, Morrison J, Perciavalle RM, et al. Requirement for antiapoptotic MCL-1 in the survival of BCR-ABL B-lineage acute lymphoblastic leukemia. 2018;122(9):1587-1599. doi:10.1182/blood-2012-06-440230.The

46. Derenne S, Monia B, Dean NM, Taylor JK. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x L is an essential survival protein of human myeloma cells. 2018;100(1):194-200.

47. Zhang H, Guttikonda S, Roberts L, et al. Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. *Oncogene*. 2010;30(16):1963-1968. doi:10.1038/onc.2010.559

48. Sarosiek KA, Letai A. HHS Public Access. 2017;283(19):3523-3533. doi:10.1111/febs.13714.Directly

49. Vo T, Ryan J, Carrasco R, et al. NIH Public Access. 2013;151(2):617-632. doi:10.1016/j.cell.2012.08.038.Relative

50. Townsend KJ, Trusty JL, Traupman MA, Eastman A, Craig RW. Expression of the antiapoptotic MCL1 gene product is regulated by a mitogen activated protein kinase-mediated pathway triggered through microtubule disruption and protein kinase C. 1998.

51. Tong W, Ding X, Witt RC, Adrian TE. Lipoxygenase Inhibitors Attenuate Growth of Human Pancreatic Cancer Xenografts and Induce Apoptosis through the Mitochondrial Pathway 1. 2002;1(September):929-935.

52. Gouill S Le, Podar K, Amiot M, et al. NEOPLASIA VEGF induces Mcl-1 up-regulation and protects multiple myeloma cells against apoptosis. 2017;104(9):2886-2893. doi:10.1182/blood-2004-05-1760.Supported

53. Chen C, Hsieh F, Lieblein JC, et al. Stat3 activation in human endometrial and cervical cancers. 2007:591-599. doi:10.1038/sj.bjc.6603597

54. Weerasinghe P, Garcia GE, Zhu Q, et al. Inhibition of Stat3 activation and tumor growth suppression of non-small cell lung cancer by G-quartet oligonucleotides. 2007;(9):129-136.

55. Willis SN, Chen L, Dewson G, et al. Proapoptotic Bak is sequestered by Mcl-1 and Bcl-x L , but not Bcl-2 , until displaced by BH3-only proteins. 2005;(Adams 2003):1294-1305. doi:10.1101/gad.1304105.Interactions

56. Nakajima W, Hicks MA, Tanaka N, Krystal GW, Harada H. Noxa determines localization and stability of MCL-1 and consequently ABT-737 sensitivity in small cell lung cancer. 2014;5(2):e1052-10. doi:10.1038/cddis.2014.6

57. Baou M, Kohlhaas SL, Butterworth M, et al. O riginal A rticles Role of NOXA and its ubiquitination in proteasome inhibitor-induced apoptosis in chronic lymphocytic leukemia cells. 2010;95(9):1510-1518. doi:10.3324/haematol.2010.022368

58. Dai Y, Desano J, Tang W, et al. Natural Proteasome Inhibitor Celastrol Suppresses Androgen-Independent Prostate Cancer Progression by Modulating Apoptotic Proteins and NF-kappaB. 2010;5(12). doi:10.1371/journal.pone.0014153

59. Sensitizer A. HHS Public Access. 2011;6(8):617-632. doi:10.1038/nchembio.391.The

60. Cohen NA, Stewart ML, Gavathiotis E, et al. NIH Public Access. 2013;19(9):1175-1186. doi:10.1016/j.chembiol.2012.07.018.A

61. Séveno C, Loussouarn D, Bréchet S, Campone M, Juin P, Barillé-nion S. g - Secretase inhibition promotes cell death , Noxa upregulation , and sensitization to BH3 mimetic ABT-737 in human breast cancer cells. *Breast Cancer Res*. 2012;14(3):R96. doi:10.1186/bcr3214

62. Varadarajan S, Vogler M, Butterworth M, Dinsdale D, Walensky LD, Cohen GM. Evaluation and critical assessment of putative MCL-1 inhibitors. *Cell Death Differ*. 2013;20(11):1475-1484. doi:10.1038/cdd.2013.79

63. Timucin AC, Basaga H, Kutuk O. Selective targeting of antiapoptotic BCL-2 proteins in cancer. 2018;(May):1-30. doi:10.1002/med.21516

64. Doi K, Liu Q, Gowda K, et al. Maritoclax induces apoptosis in acute myeloid leukemia cells with elevated Mcl-1 expression. 2014;(August):1077-1086.

65. Chen J, Zhang X, Lentz C, et al. Short Communication miR-193b Regulates Mcl-1 in Melanoma. *AJPA*. 2011;179(5):2162-2168. doi:10.1016/j.ajpath.2011.07.010

66. Zhang Z, Song T, Zhang T, et al. A novel BH3 mimetic S1 potently induces Bax / Bak-dependent. doi:10.1002/ijc.25484

67. Doi K, Li R, Sung S, et al. Discovery of Marinopyrrole A (Maritoclax) as a Selective Mcl-1 Antagonist that Overcomes ABT-737 Resistance by Binding to and Targeting Mcl-1 for Proteasomal Degradation *. 2012;287(13):10224-10235. doi:10.1074/jbc.M111.334532

68. Abulwerdi F, Liao C, Liu M, et al. NIH Public Access. 2015;13(3):565-575. doi:10.1158/1535-7163.MCT-12-0767.A

69. Leverson JD, Zhang H, Chen J, et al. Potent and selective small-molecule MCL-1 inhibitors demonstrate on-target cancer cell killing activity as single agents and in combination with ABT-263 (navitoclax). 2015;6(1):e1590-11. doi:10.1038/cddis.2014.561

70. Fry DC, Vassilev LT. Targeting protein-protein interactions for cancer therapy. 2005:955-963. doi:10.1007/s00109-005-0705-x

71. D MDFP. Why cancer cells have a more hyperpolarised mitochondrial membrane potential and emergent prospects for therapy. 2015:1-42.

72. Sarosiek KA, Ni Chonghaile T, Letai A. Mitochondria: Gatekeepers of response to chemotherapy. *Trends Cell Biol*. 2013;23(12):612-619. doi:10.1016/j.tcb.2013.08.003

7. APPENDICES

APPENDIX A-Chemicals

Chemicals and Media Components	Supplier Company
Mannitol	Sigma
EDTA	Sigma
EGTA	Sigma
Succinic Acid	Sigma
BSA (IgG Free)	VWR
HEPES	VWR
Potassium chloride	Sigma
Potassium hydroxide	Sigma
Digitonin	Sigma
FCCP	Sigma
Oligomycin	Sigma
JC-1	VWR
Trypan blue solution	Thermofisher Scientific
pH 4.0 buffer solution	Merck Millipore
pH 7.0 buffer solution	Merck Millipore
RPMI 1640	Gibco
Trypsin	Pantech
Ethanol	Sigma
Fetal Bovine Serum	Pantech
DMSO	Sigma
Distilled Water	Merck Millipore
2-Mercaptoethanol	Sigma
Hydrocholoric acid	Sigma
PBS	Pantech

APPENDIX B-Equipment

Equipment	Supplier Company
Automated Cell Counter	EVE
Autoclave	HiClave HV-110, Hirayama,
Isolab,	
Centrifuge	5418R, Eppendorf 5702, Eppendorf 5415R, Eppendorf Allegra X-15R, Beckman
Balance	Eppendorf
CO_2 Incubator	Binder
Deep Freeze	Thermofischer Scientific
Eppendorf Motion 5070 Pipetting Robot	Eppendorf Motion 5070
Filters (0.22 uM and 0.45 uM)	Merck Millipore
Freezing Container	Thermo Fischer
Hemocytometer	Neubauer Improved, Isolab
Ice Machine	AF20, Scotsman Inc.
Incubator	BE300, Memmert
Laminar Flow	HeraSafe HS15, Heraeus,
Liquid Nitrogen Tank	Taylor-Wharton, 300RS
Magnetic Strirrer	SB162, Stuart
Microliter Pipettes	Eppendorf
Microscope	CK40, Olympus
pH Meter	Mettler Toledo
Refrigerator	Bosch Arcelik
SpectroFluorometer	Gemini SoftmaxPro
Vortex	VWR
Water Bath	New Brunswick