ANTICANCER ACTIVITY OF CHLORPROMAZINE ON U266 MULTIPLE MYELOMA CELL LINE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

ALİYE EZGİ GÜLEÇ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOCHEMISTRY

AUGUST 2017

Approval of the thesis:

ANTICANCER ACTIVITY OF CHLORPROMAZINE ON U266 MULTIPLE MYELOMA CELL LINE

submitted by **ALİYE EZGİ GÜLEÇ** in partial fulfillment of the requirements for the degree of **Master of Science in Biochemistry Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver _____
Dean, Graduate School of Natural and Applied Sciences

Prof. Dr. Bülent İçgen _____
Head of Department, Biochemistry

Assoc. Prof. Dr. Can Özen _____
Supervisor, Biotechnology Dept., METU

Assist. Prof. Dr. Salih Özçubukçu _____
Co-Supervisor, Chemistry Dept., METU

Examining Committee Members:

Prof. Dr. Bülent İçgen _____
Environmental Engineering Dept., METU

Assoc. Prof. Dr. Can Özen _____
Biotechnology Dept., METU

Assist. Prof. Dr. Urartu Özgür Şafak Şeker _____
Institute of Materials Science and Nanotechnology (UNAM), Bilkent University

Assist. Prof. Dr. Salih Özçubukçu _____
Chemistry Dept., METU

Assist. Prof. Dr. Harun Koku _____
Chemical Engineering Dept., METU

Date: 24.08.2017

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: ALİYE EZGİ GÜLEÇ

Signature:

ABSTRACT

ANTICANCER ACTIVITY OF CHLORPROMAZINE ON U266 MULTIPLE MYELOMA CELL LINE

Güleç, Aliye Ezgi

M.S., Department of Biochemistry
Supervisor: Assoc. Prof. Dr. Can Özen
Co – Supervisor: Assist. Prof. Dr. Salih Özçubukçu
August 2017, 62 pages

Multiple Myeloma (MM) is the second most common hematological malignancy caused by malignant growth of plasma B cells. It accounts for 10% of deaths from blood cancers. Although introduction of new drugs has significantly increased the success of chemotherapy, MM remains as an incurable disease with a high relapse rate.

Drug repositioning, finding new uses for approved drugs, is a frequently used strategy for the discovery of new chemotherapeutic agents. Since already approved drugs are used, the cost and time spent are considerably reduced.

Chlorpromazine (CPZ), which has been used in several drug repositioning studies against different cancer types, is an FDA-approved antipsychotic drug mainly used in the treatment of schizophrenia.

In this work, we aim to investigate the anticancer effect and mechanism of CPZ on U266 MM cell line. Firstly, CPZ`s *in vitro* inhibitory effect at various doses and time points was studied. Then three different apoptosis studies and cell cycle analysis were conducted. Combination potential of CPZ with cisplatin was also investigated during this study.

CPZ showed both dose- and time- dependent growth inhibitory effect on U266 MM cell line with an IC_{50} value of 22.4 ± 0.9 μM. Apoptotic effect of CPZ was indicated by activation of Caspase-3 and change in the cell membrane asymmetry. In addition, CPZ treatment induced cell cycle arrest at G_2/M phase. Its combination with cisplatin was moderately synergistic.

Based on our preliminary findings, therapeutic potential of CPZ for MM treatment can be investigated with in-depth mechanistic studies and *in vivo* experiments.

Keywords: Multiple myeloma, drug repositioning, chlorpromazine, apoptosis, cell cycle arrest

ÖZ

KLORPROMAZİN' İN U266 MULTİPL MİYELOM HÜCRE HATTI ÜZERİNDEKİ ANTİ-KANSER AKTİVİTESİ

Güleç, Aliye Ezgi

Yüksek Lisans, Biyokimya Bölümü
Tez Yöneticisi: Doç. Dr. Can Özen
Ortak Tez Yöneticisi: Yar. Doç. Dr. Salih Özçubukçu
Ağustos 2017, 62 sayfa

Multipl Miyelom (MM), plazma B hücrelerinin habis büyümesinin neden olduğu ikinci en yaygın hematolojik malignitedir. Kan kanserinden ölümlerin %10' unu oluşturmaktadır. Yeni ilaçların kullanılmaya başlanması, kemoterapi başarısını önemli ölçüde arttırmış olsa da, yüksek nüks oranı MM' in tedavisi mümkün olmayan bir hastalık olarak kalmasına sebep olmaktadır.

İlacın yeniden konumlandırılması, onaylanmış ilaçların yeni kullanımlarının keşfedilmesi, yeni kemoterapötik ajanların keşfi için sıklıkla kullanılan bir stratejidir. Zaten onaylanmış ilaçlar kullanıldığından maliyet ve harcanan zaman önemli ölçüde azalmaktadır.

Çeşitli kanser türlerine karşı yeniden konumlandırılma çalışmalarında kullanılmış olan Klorpromazin (CPZ), birincil olarak şizofreni gibi rahatsızlıkların tedavisinde kullanılan bir FDA onaylı antipsikotik ilaçtır.

Bu çalışmada, CPZ'nin U266 MM hücre hattı üstündeki antikanser etkisinin ve mekanismasının araştırılması amaçlanmıştır. İlk olarak, CPZ' nin doza ve zamana bağlı inhibe edici ve sitotoksik etkisi çalışılmıştır. Daha sonra üç farklı apoptoz çalışması ve hücre döngüsü analizi gerçekleştirilmiştir. Ayrıca CPZ'nin sisplatin ile kombinasyon potansiyeli de bu çalışma sırasında araştırılmıştır.

CPZ U266 MM hücre hattı üzerinde doza ve zamana bağlı büyüme inhibe edici etki göstermiştir ve IC_{50} değeri 22.4 ± 0.9 uM olarak belirlenmiştir. Apoptozun indüksiyonu Kaspaz-3' ün aktivasyonu ve hücre zarı asimetrisindeki değişim ile gösterilmiştir. Buna ek olarak, CPZ tedavisi hücre döngüsünün G_2/M fazında durmasına sebep olmuştur. Sisplatin ile kombinasyonu orta derecede sinerjistiktir.

Ön bulgularımız doğrultusunda, MM tedavisi için CPZ'nin terapötik potensiyeli derinlemesine mekanik çalışmalar ve *in vivo* deneylerle araştırılması gerekmektedir.

Anahtar Kelimeler: Multipl miyelom, ilaç yeniden konumlandırma, klorpromazin, apoptoz, hücre döngüsü arresti

To my beloved parents,

without whom none of my success would be possible

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude and thanks to my advisor Dr. Can Özen for guiding and supporting me over the years. You have set an example of excellence as a researcher, mentor, instructor, and role model.

I would like to express my deepest gratitude to my co-advisor Dr. Salih Özçubukçu for his support and understanding. I am also grateful to the rest of my thesis committee members Dr. Bülent İçgen, Dr. Harun Konu and Dr. Urartu Özgür Şafak Şeker for their valuable criticism and advices.

I would also like to thank all of my dear colleagues, Hepşen Hazal Hüsnügil, Selin Gerekci, Heval Ataş and İpek Zeynep Durusu for their valuable contributions, help and lovely friendship. I am grateful them for creating the most enjoyable lab environment and always cheering me up when I was down. Studies of this thesis would not be possible without them.

I would also like to thank Doğancan Tigan and Seyit Ahmet Kuzucanlı for their friendship and emotional support.

I would like to thank my dearest Yuki who set an example of excellence for supporting me, guiding me and giving me endless love. I could not be able bare these times without his presence.

I wish to thank METU BAP for financial support as LTP grant.

Last but not least, I owe my deepest gratitude to my parents, Serap and Ömer Güleç. Thank you for supporting me and encouraging me in all aspects of my life. Your endless support and love gave me the motivation to complete my study. You are the most precious gifts of life!

TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ABC: ATP Binding Cassette Membrane Transporters

CaM: Calmodulin

Cis: Cisplatin

CPZ: Chlorpromazine

DMSO: Dimethyl Sulphoxide

DNA: Deoxyribonucleic Acid

FDA: Us Food and Drug Administration

ICD-O-3: International Classification of Diseases for Oncology, Third Edition

Interleukin 6: IL-6

IGF-1: Insulin-like growth factor 1

MAPK: Mitogen Activated Protein Kinase

MM: Multiple Myeloma

MMP: Mitochondrial Membrane Potential

MRP1: Multidrug Resistance-associated Protein 1

MXR: Mitoxantrone Resistance Protein

DPBS: Dulbecco's Phosphate-Buffered Saline

PDRGFR: Platelet-Derived Growth Factor Receptor

Pgp: P-glycoprotein

Phts: Phenothiazine

PI-3K: Phosphatidylinositol-3-Kinase

PKC: Protein Kinase-C

SEER: Surveillance, Epidemiology, and End Results

TNF-α: Tumor Necrosis Factor alpha

VEGF: Vascular Endothelial Growth Factor

WHO: World Health Organization

CHAPTER 1

INTRODUCTION

1.1. CANCER

1.1.1. Definition

Cancer is a complex disease which arises when abnormal cells divide in an uncontrolled fashion and accumulate in the body. In normal conditions, the body has several integrated control mechanisms to achieve the balance between proliferation and death of the cells. Normal cells undergo programmed cell death (apoptosis) when they are old or damaged. When the normal control mechanism of the body stops working, cancer (abnormal) cells continue to divide and form new cells which are also abnormal or damaged. Extra cells formed by uncontrolled division of cancer cells may form a mass of tissue which is called as tumor [1].

1.1.2. Cancer Classification

Cancer can develop nearly in any tissue of the body and there are more than 100 types of cancers. Cancers can be classified based on two characteristics: the origin and the tissue type. International Classification of Diseases for Oncology, Third Edition (ICD-O-3) is accepted as the international standard for the classification and nomenclature of histologies and ICD-O-3 classifies cancer into six major categories based on the tissue type: carcinoma, sarcoma, myeloma, leukemia, lymphoma, and mixed type cancers [2].

1.1.3. Hallmarks of Cancer

Although there are more than 100 types of cancers and each cancer type has its unique features, there are several common features to all cancer types. All cancer cells share six traits or "hallmarks" that collectively lead malignant growth. These hallmarks are; self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis (programmed cell death), limitless replicative potential, induced angiogenesis, and tissue invasion and metastasis as represented in Figure 1 [3].

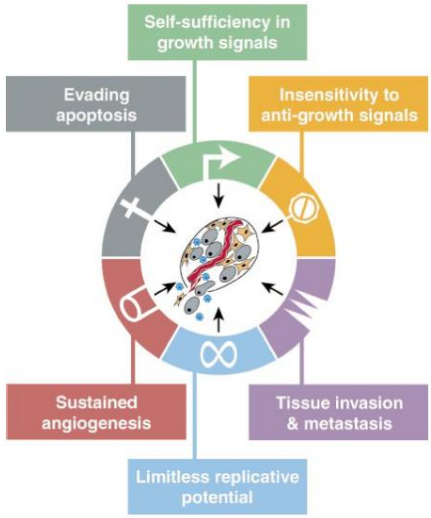


Figure 1. Hallmarks of Cancer [3].

One of the most important hallmarks of the cancer is evasion of apoptosis. Apoptosis is an ordered and orchestrated cell death which is activated to clear damaged, old or unwanted cells from a normal tissue. Cancer cells have acquired different mechanisms to evade apoptosis, thus every defect or abnormality of apoptotic pathways also provides an interesting target of cancer treatment [3].

1.1.4. Major Drug Resistance Mechanisms and Relapse

Drug resistance is an event in which cancer cells become tolerant to anticancer treatment [4]. Cancer cells adapt different mechanism to resist anticancer agents. Unfortunately, most of the cancer patients develop drug resistance at some point during treatment which frequently results in relapse. Drug resistance and high relapse rate remain as a major obstacle of cancer therapy. Understanding and identifying the mechanisms that enable cancer cells to develop resistance give researchers an opportunity to improve treatment strategies [5].

Drug resistance can be divided into two classes with respect to response of tumor to initial therapy; innate and acquired resistance [5]. As can be seen in Figure 2, cancer cells develop resistance through distinctive mechanisms such as alterations of uptake, efflux, and metabolism of drugs [6]. Most drugs use surface transporters for entry to cells. Thus, cancer cells can acquire resistance through mutations that alter activity or expression of those transporters. Increased drug efflux is frequently observed in cancer cells and this is achieved through increased expression of ATP binding cassette (ABC) membrane transporters. In addition, elevation of P-glycoprotein (Pgp), multidrug resistance-associated protein 1 (MRP1), and mitoxantrone resistance protein (MXR) are known to be correlated with cancer chemoresistance to various drugs. Many anticancer drugs such as cytarabine require metabolic activation in order to exert their cytotoxic activity. Cancer cells downmodulate the enzyme which are involved in metabolic activation to interfere with cytotoxic effect of drugs. Additionally, inactivation of anticancer drugs to prevent them from exerting their cytotoxic effect is another mechanism of drug resistance. For instance, glutathione (a powerful anti-oxidant) protects the cells against the harmful effects of reactive oxygen species and it is frequently conjugated to drugs in order to inactivate them. Many cancers have a dependency on an oncogene which are mostly targeted molecules in cancer therapy such as BCR-ABL targeted by imatinib. However, in long-term use these targeted drugs lose their effect due to occurrence of mutation on

their targets [5]. Amplification of alternative oncogenes or activation of alternative survival pathways are the other mechanisms used by cancer cells (Figure 2) [6].

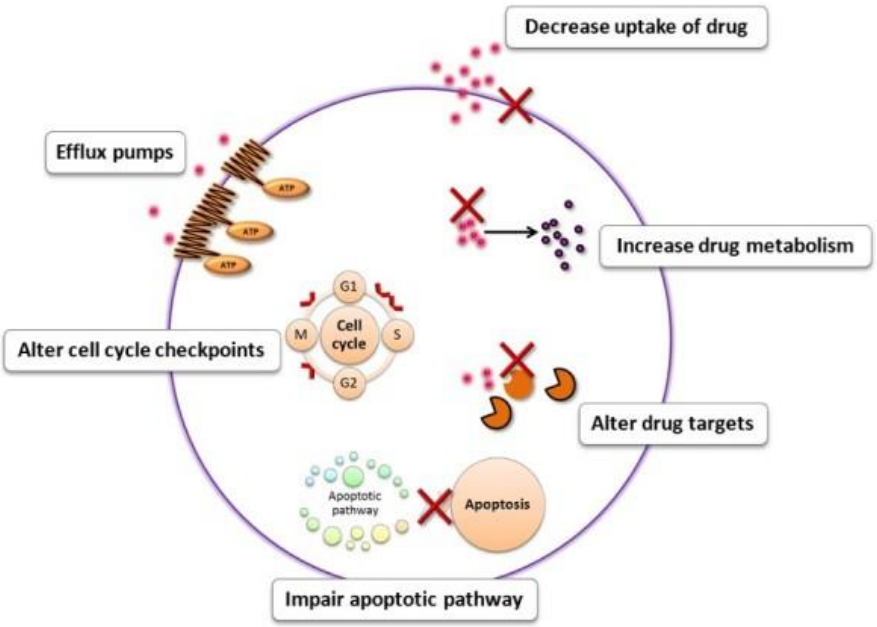


Figure 2. Major Drug Resistance Mechanisms [7].

1.2. MULTIPLE MYELOMA

1.2.1. Definition

Multiple Myeloma (MM) is a type of blood cancer which is caused by malignant growth of plasma B cells in the bone marrow. Plasma cells are differentiated, maturated form of B cells, a type of white blood cells [8]. While normal plasma cells have a function in recognition and removal of pathogen from the body by producing antibodies (Ab), MM cells produce M protein which is an abnormal form of Ab (Figure 3). This abnormal Ab, M protein, is not able to attack pathogens. Thus, it is not needed by the body. As a matter of fact, M proteins build up in the bone marrow,

lead to osteolytic lesions in the bone, cause the blood to thicken, and damage kidneys [9]. Proliferation of malignant plasma B cells is driven by various factors such as tumor necrosis factor (TNF) alpha, interleukin 6 (IL-6) and so on. Bone pain and fractures, anemia, frequent infections, and renal failure are the common symptoms of MM [8].

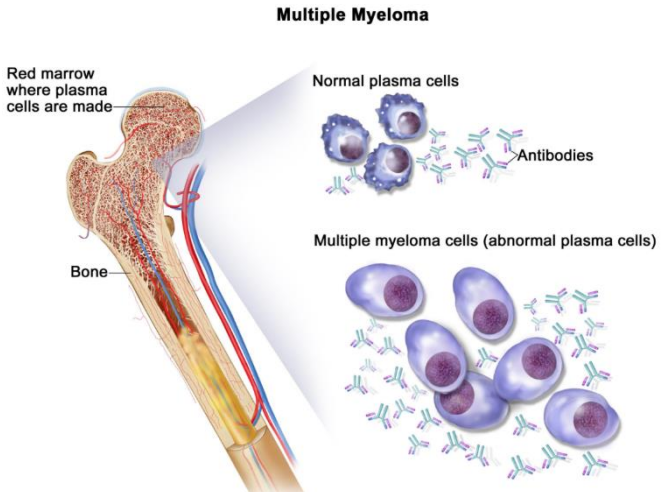


Figure 3. Normal Plasma Cells vs MM Cells [9].

1.2.2. Epidemiology

MM is a disease that is more common in the elderly with 70 as median age at diagnosis [8]. It is the second most common hematological malignancy which accounts for at least 10% of deaths from blood cancers and at least 1% of deaths from all cancers [10]. According to Surveillance, Epidemiology, and End Results (SEER) data incidence adjusted rates from 1992 to 1998, overall incidence of MM is 4.5 per 100,000 per year, while it is 4.2 per 100,000 per year among whites and 9.3 per 100,000 per year among blacks. According to international mortality data, MM is most commonly seen in Northern Europe, North America, Australia, and New Zealand. Additionally, the lowest rates are in Japan, Yugoslavia, and Greece [11].

Around three thousand people each year are diagnosed with MM in Turkey. Male to female ratio of MM is 1.4 to 1.0. Survival range is 1-10 years with 5-year survival rate of 46.6% [12].

1.2.3. Pathogenesis

Abnormalities related with apoptotic mechanisms, bone marrow microenvironment, signaling pathways, and cell cycle related molecules underlie the pathogenesis of MM [8,11].

Soluble factors produced in the bone marrow microenvironment have been reported to induce proliferation and malignant transformation. As shown in the Figure 4, Insulin-like growth factor 1 (IGF-1), IL-6, IL-2, IL-15, Wnt3A, platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor (VEGF), and TNF-α are some of those soluble factors. IL-6 is the most widely studied soluble factors among all [13].

IL-6 is a cytokine which functions in growth and maturation of B cells and the differentiation of T cells. With regard to MM, IL-6 supply is thought to be supported by both autocrine and paracrine mechanisms. Several studies have shown that some MM-derived cell lines (U-266, OCI-My3 and 2) produce IL-6 mRNA and secreted IL-6 levels are detectable in supernatant fluid of the cell lines. Altogether, these results indicate the existence of autocrine mechanism. On the other hand, proliferation of some MM cell lines (MM-S1, MM-A1, MM-Y1 and MM-C1) is induced by IL-6 and these cell lines possess IL-6 receptor while they do not express IL-6 mRNA indicating a paracrine mechanism. In paracrine mechanism, bone marrow stromal cells appear to be the major source of IL-6 supply. Additionally, macrophages, osteoblasts, osteoclasts, fibroblasts, and monocytes produce and secrete IL-6 to support MM cell growth [14].

IL-6 is both a growth factor and an anti-apoptotic factor. Stimulation by IL-6 induce two pathways; the JAK-STAT pathway and the Ras-MAP kinase pathway. Anti-apoptotic proteins Mcl-1 and Bcl-XL are upregulated through activation of JAK-2 / STAT3 pathway, while ELK-1, AP-1 and NF-IL-6 are upregulated through Ras-MAP kinase pathway. Additionally, NF-kappaB and IL-6 mediate Bcl-2, Mcl-1 and Bcl-XL increase. Overall, induction of apoptosis is prevented and MM proliferation is enhanced via activation of these pathways. Constitutive activation of STAT3 that occurs independent of IL-6 is also an important factor in MM pathogenesis [15].

IL-1 upregulation also results in increased production of IL-6. MM cells are able to produce and secrete VEGF. When stromal and endothelial cells are exposed to VEGF, IL-6 secretion is upregulated leading to a further stimulation of MM cells. IGF signals through the phosphatidylinositol-3-kinase (PI-3K) pathway, stimulates growth of MM cell directly, and increases responsiveness to IL-6 through mitogen activated protein (MAP) kinase [11].

Bone marrow microenvironment consisting of fibroblasts, stromal cells, osteoblasts, and osteoclasts has a considerable role in MM pathology. Stromal cells derived from marrow samples of MM patients produce higher levels of IL-6 *in vitro*. Generally, plasma cells of MM patients strongly express CD56 which is a cell adhesion molecule belonging to the immunoglobulin superfamily. CD56 is believed to play an important role in MM homing and cell adhesion to the marrow. In most of the patients, MM cells expressed higher levels of adhesion molecules lymphocyte function-associated antigen (LFA)-3, LFA-1 (CD11a), and very late antigen-4 (VLA-4). Also, angiogenesis is enhanced in MM patients due to increased vascular permeability and endothelial cell proliferation via VEGF [11].

Increased cyclin D1 expression, hyper-methylation of cyclin-dependent kinase regulatory gene p16, mutations of the ras oncogene, and loss of p53 have a major effect on cell cycle regulation of MM cells. Nearly one-third of MM patients have

elevated levels of cyclin D1 and these patients have a higher proliferative rate. Although the t(11;14)(q13;q32) translocation, which juxtaposes the immunoglobulin heavy chain promoter and the cyclin D1 gene, is seen in almost 25% of MM patients, it is not typically associated with a worse prognosis. Selective methylation of p15 and p16, which are important cell cycle suppressors, occurs as frequently as 67% and 75% of cases, respectively. Finally, c-myc protein and c-myc RNA are overexpressed in approximately 25% of MM patients [11].

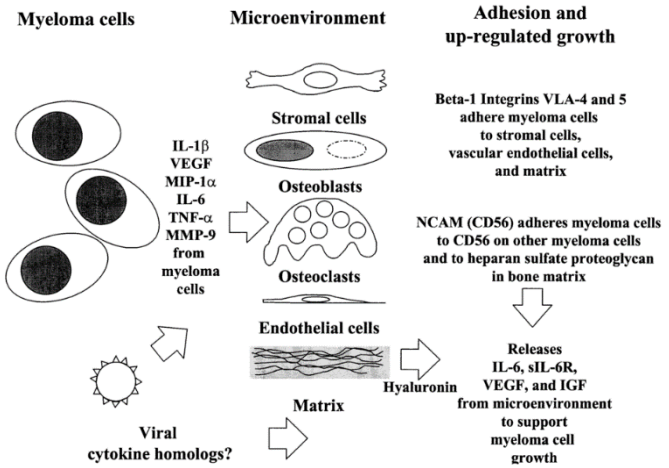


Figure 4. MM Pathology [11].

1.2.4. History of MM and Its Treatment

Samuel Solly reported first well-documented case of MM belonging to a 39-year-old woman Sarah Newbury in 1844. She developed fatigue and bone pain from MM. Inflammation was the first diagnosis of Solly, and he tried to treat disease with rhubarb pill and infusion of orange peel. 4 years after the onset of symptoms, autopsy revealed that the bone marrow was replaced by a red substance [16].

The best known case of MM belongs to 45-year-old man, Thomas Alexander McBean. He developed fatigue and with his own words his "body linen was stiffened

by his urine." His physician, Thomas Watson, started treatment with steel and quinine because his repetitive pains. Although treatment resulted in rapid improvement, his pain recurred and despite the variety of therapies, he died. His autopsy revealed soft, brittle, readily fractured ribs and a red colored gelatin form substance in the bones. Round or oval cells which are one-half to twice of an average blood cells with one or two nuclei was observed during histological examinations. His urine sample examinations by Dr. Henry Bence Jones who was a chemical pathologist reveal the presence of an abnormal protein in the urine. This abnormal protein identified as 'hydrated deutoxide of albumen' and later called as Bence Jones protein. Bence Jones protein, which is a urinary tract immunoglobulin free light chain, is in use for diagnosis of MM [16].

In 1947, Nils Alwall observed a reduction of serum globulin, a decrease in bone marrow plasma cells, and an increase in serum hemoglobin of a MM patient treated with urethane. Urethane became a standard MM therapy for over 15 years [16].

In 1966, Holland and his colleague carried out a randomized trial with 83 MM patients who received urethane or a placebo containing cherry and cola-flavored syrup. According to the results of trial, there was no difference between urethane and placebo groups in terms of improvement and survival [16].

Melphalan is a chemotherapeutic drug that belongs to the nitrogen mustard alkylating agents class. In 1958, Blokhin et al reported that three of six patients with MM benefitted from melphalan [16].

In 1962, Corticosteroids were firstly tested in a comparison of the effect of prednisone and a placebo which was carried out by R. Maas. Maas reported a significant decrease in serum globulin and increase in hematocrit but no difference in survival compared with a placebo [16].

In 1970, Alexanian et al led a randomized trial with 183 MM patient in which the classic regimen of melphalan plus prednisone (MP) was established. This trial revealed that survival was 6 months longer with MP compared to melphalan as a single agent. MP remained as the principal strategy of MM treatment [16].

First successful syngeneic bone marrow transplantation for MM was performed by Osserman et al in 1982 [16].

Autologous bone marrow transplantation was first reported by McElwain and Powles in 1983. The brief history of MM history is shown below in Figure 5 [16].

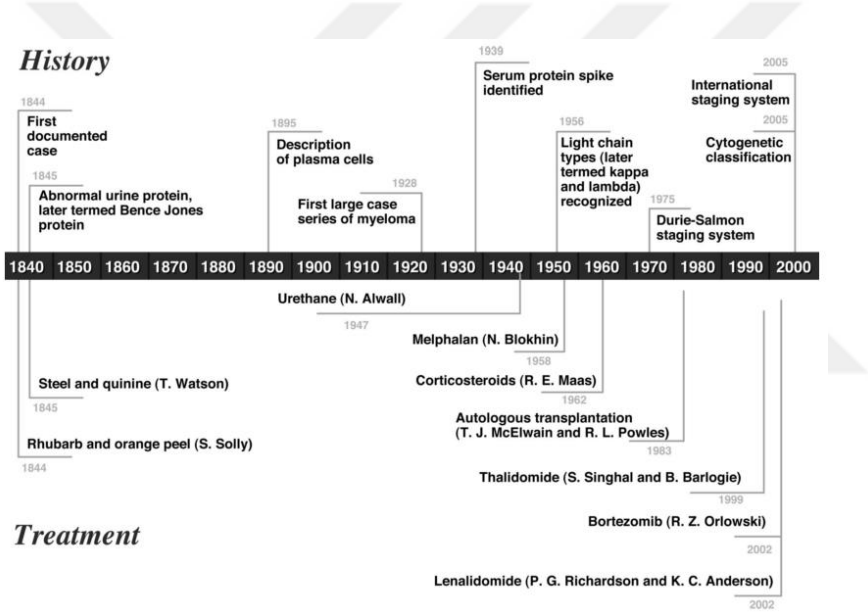


Figure 5. Timeline of MM Treatment from 1844 to 2008 [16].

1.2.5. Current Therapy of MM

Current therapy of MM consists of 4 options including new drugs, chemotherapy, corticosteroids, and stem cell transplantation [10].

1.2.5.1. New Drugs

In the past decade, several types of non-chemotherapy drugs have emerged as an option for MM treatment. New drugs can be divided into 3 categories as immunomodulatory drugs, proteasome inhibitors, and monoclonal antibodies.

Immunomodulatory drugs use body's own immune system to fight malignancies. Thalidomide was the first immunomodulator that showed a significant effect on MM patients. Thalidomide was introduced to the market as a sedative by Chemie Grünenthal, a German pharmaceutical company and it was famous as a treatment for pregnancy morning sickness. In 1961, it was realized that it was related with severe teratogenic malformations when it was taken in the first trimester between days 35 and 49 after the last menstrual period. Although it was taken off the market in most countries at the end of 1961, nearly 10.000 infants had already been affected. Arkansas study, which was confirmed by many other studies, showed that thalidomide was a successful candidate in all phases of MM. Lenalidomide and pomalidomide are the other immunomodulatory drugs which are currently used in MM treatment [12].

Proteasome, which breaks down proteins in the cell when they are produced in excess amount, is highly active in MM cells. Thus, proteasome inhibitors induce MM cell death by leading proteins to build up in the cells. Bortezomib is the first specific proteasome inhibitor shown to be suitable for clinical use. Julian Adams and his colleagues designed and developed bortezomib which is a boronic acid dipeptide. Preclinical studies showed that it was a potent cytotoxic and growth inhibitory agent. Robert Orlowski led the initial clinical study using bortezomib against advanced hematologic malignancies. Ixazomib and carfilzomib are the other proteasome inhibitor that are currently used in MM treatment [12].

Monoclonal antibodies are proteins that are made by identical immune cells to attack specific cells. Monoclonal antibodies that are designed to specifically target antigens on the surface of cancer cells can also be used in MM treatment. Daratumumab and elotuzumab are the commercially available monoclonal antibodies which are currently used for MM treatment [12,16].

1.2.5.2. Chemotherapy

Chemotherapy is the use of medicines to stop or slow down the growth of cancer cells. Melphalan, cyclophosphamide, doxorubicin, liposomal doxorubicin, and panobinostat are the chemotherapeutic agents that are used currently for MM treatment [12].

1.2.5.3. Corticosteroids

Corticosteroid drugs are the synthetic analogues of steroid hormones that are produced in the adrenal cortex of vertebrates. In general they are used to provide relief for inflamed areas of the body. Dexamethasone and prednisone are the corticosteroid drugs used in MM treatment [12].

1.2.5.4. Stem Cell Transplantation

Stem cell transplantation has been shown to be not curative, but can be performed to prolong life in some MM patients [12].

Even though emerging of new drugs leads remarkable advancements in MM treatment, there is still no way to cure MM completely.

1.2.6. Drug Resistance and Combination Therapy

In MM patients, interaction of plasma cells with bone marrow microenvironment which consist of bone marrow stromal cells, osteoblasts, endothelial cell, and extracellular matrix promotes proliferation, migration, and drug resistance of malignant cells. Moreover, mutations in *TP53, ACTG1, RB1, PRDM1, CYLD, TRAF3, BRAF, FAM46C, DIS3, NRAS,* and *KRAS* genes were shown to have an essential role in drug resistance of MM cells [17].

Combination therapy, which is the use of anticancer drugs in combination, is the most commonly used approach to break resistance in cancer cells. The drugs given in combination are chosen in a way that each of them targets a different biochemical mechanism in cancer cells to allow for synergistic effect [5].

1.2.7. U266 MM Cell Line

U266 cell line is a B lymphocyte established from blood sample of 53 year old male with IgE-secreting myeloma in 1968. U266 cell line has been shown to produce IL-6 mRNA and have IL-6 receptor cell surface expression [18]. In addition, there are several studies established growth promoting effect of IL-6 on U266 cell line [15,19]. U266 cells demonstrate an increase in DNA synthesis upon exogenous IL-6 addition, while antisense IL-6 oligomer inhibits their growth [19]. Even though these studies indicate an autocrine signaling in which U266 cells produce IL-6 to induce their proliferation, neither bioactivity assays nor direct secretion assays like ELISA were not able to detect IL-6 secretion to supernatant from U266 cells. Rapid rate of IL-6 internalization may interfere with the detection of secreted IL-6 meaning that although there is an autocrine signaling, it may not be possible to detect secreted IL-6 without blockage of IL-6 receptors [1414,19,20].

One of the most important features of U266 is to have a single *p53* mutated allele (inactivating mutation at exon 5) [18]. Stable expression of wild type *p53* in U266 cells resulted in significant suppression of IL-6 gene expression and cell growth [21]. In addition, U266 MM cells are shown to be resistant to Fas-mediated apoptosis although they express high levels of Fas antigen on their surface [22]. IL-6-dependent U266 cells activate Stat3 constitutively and express anti-apoptotic protein Bcl- xL in high levels. Blocking IL-6 receptor signaling from Janus kinases to Stat3 seems to inhibit Bcl- xL expression and induce apoptosis and this evidence proves remarkable contribution of constitutively activated Stat3 to MM pathogenesis [23].

1.3. DRUG REPOSITIONING

According to US Food and Drug Administration (FDA), drug development process involves five steps; discovery and development, preclinical research, clinical research, FDA review, and finally FDA Post-Market Safety Monitoring. During the discovery and development step, high number of compounds are screened and experiments conducted to gather information related to their absorption, mechanisms of action, best dosage, side effects and so on. Preclinical research step is composed of *in vitro* and *in vivo* stages which are conducted to provide detailed information on dosing and toxicity levels. Clinical researches begin immediately after successful completion of preclinical research step. Clinical research step involves four phases of trials that are conducted on people and informed consents must be provided from the patients who are involved in clinical trials. Safety, efficacy, dosage, and side effect assessments are carried out during this step. If drug developers are able to complete all these steps successfully, the new drug application can be submitted to FDA review. During FDA review step, a review team thoroughly examines whole story of the applied drug. If new drug application is accepted, the new drug now is able to skip to the last step. The true safety of a drug can only be observed over months, even years. Thus, last step of FDA review involves the gathering of complete information about safety of drug during product's lifetime in the marketplace [24]. Less

than 20% of drugs are able to complete the drug development steps, approximately 14 years are needed for completion of a single drug development and its cost is around US$2 billion. Thus, de novo drug discovery is a high risk and high cost process [25].

Drug repositioning is identification of new therapeutic indications for existing drugs and it is considered as the more logical, effective route for drug development. Since the drug is already approved and marketed, drug repositioning bypasses the safety concern which in turn reduces the cost and time needed for drug development (Figure 6) [26].

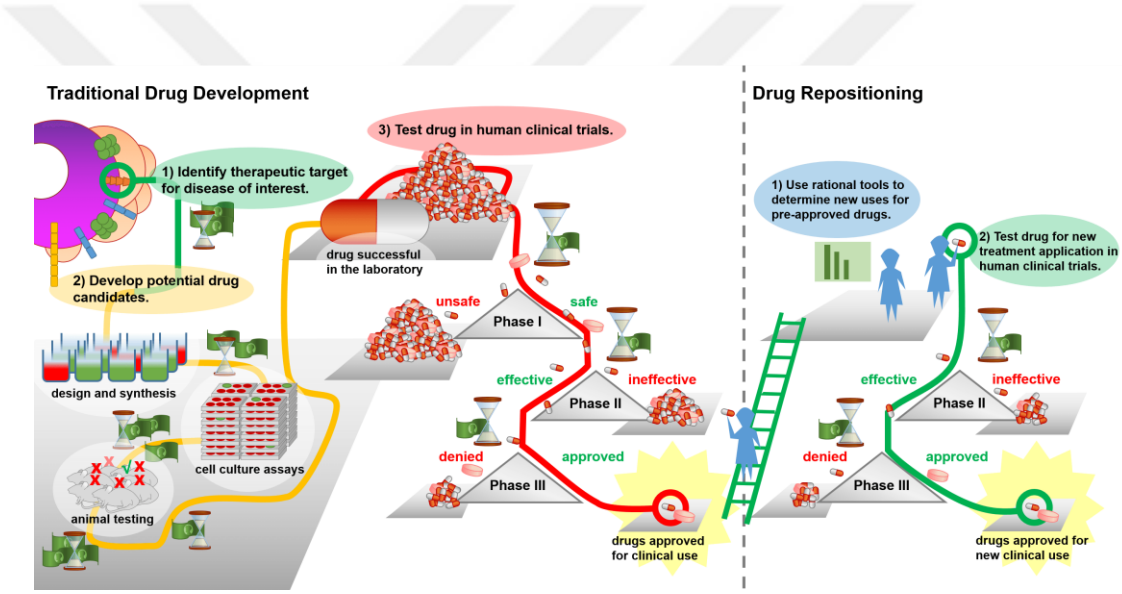


Figure 6. Traditional Drug Development vs Drug Repositioning [26].

As explained in Figure 7, there are six different repositioning methods which can be use drug itself, shared target, shared pathway or side effects of drug as start point. Most of the successfully repositioned drugs have been identified through serendipitous observation methodology which is the first method of repositioning. High-throughput screening of approved drugs for inhibition of disease phenotype or disease target is forms the second and third methods. Also, sometimes new role of a

target can be found in another disease which enables its drug to be repositioned. Similar to new role of target in another disease, a pathway may be found to be important in another disease. Thus, the drug with shared target pathway may be repositioned in new disease. Finally, unexpected side effects may be observed during clinical trials which lead to repositioning [25].

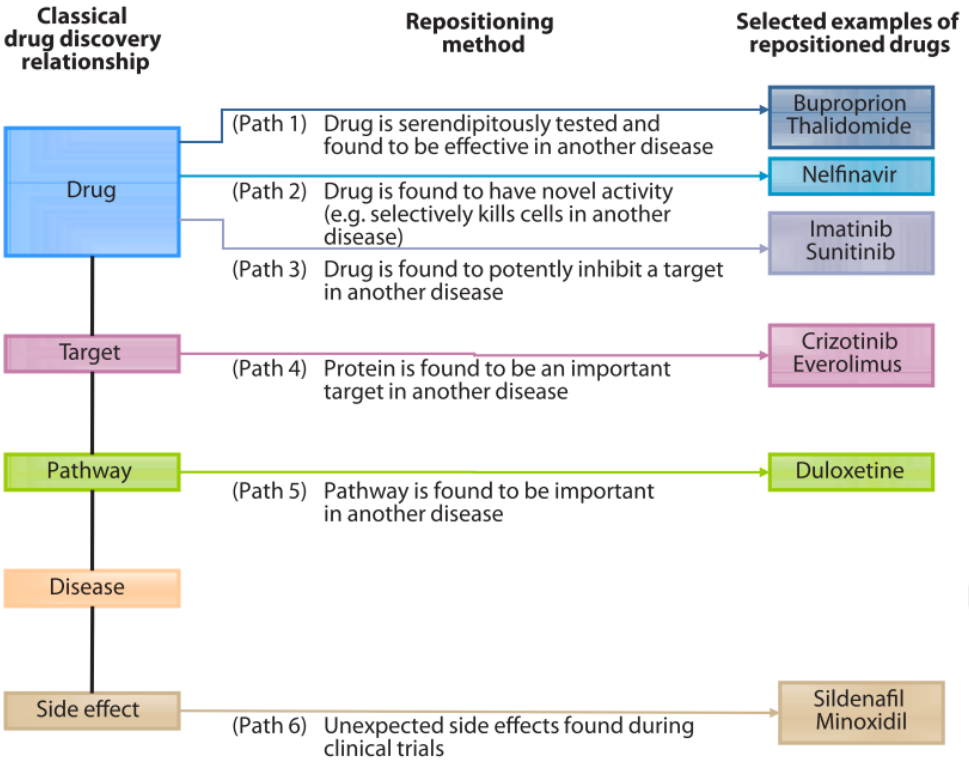


Figure 7. Potential Avenues of Drug Repositioning [25].

There are numbers of successful examples for repositioned drugs such as itracanozole, nelfinavir, digoxin, nitroxilin, asprin, trastuzumab, thalidomide, dexamethasone and so on [25].

Some breast cancers express high amounts of human epidermal growth factor receptor 2 (*HER2*), so they are named as *HER2* positive breast cancer. Trastuzumab, which is one of the first targeted drugs developed, is a fully humanized monoclonal

antibody directed against *HER2* and it is a standard therapy for *HER2* positive breast cancer patients. Later it has been understood that HER2 is implicated in other severe form of cancer such as *HER2* positive metastatic gastric cancer and several studies showed that trastuzumab is also effective in *HER2* positive metastatic gastric cancer cells [25].

Dexamethasone, which is a corticosteroid drug, was firstly used as a treatment option for inflammatory and autoimmune diseases. In 1972, a research group found glucocorticoid receptor on a cloned myeloma line from a Balb/c mouse. Further studies showed that dexamethasone induced myeloma cell death [27].

1.4. PHENOTHIAZINES

Phenothiazine (Phts) is a thiazine-class heterocyclic compound with a formula of $S(C_6H_4)_2NH$. And it was initially synthesized in 1883 by Bernthsen. Chlorpromazine, thioridazine, and prochlorperazine are well known examples of phts. The timeline of the most important phts (from 1876 to 2009) is explained below in Figure 8 [28].

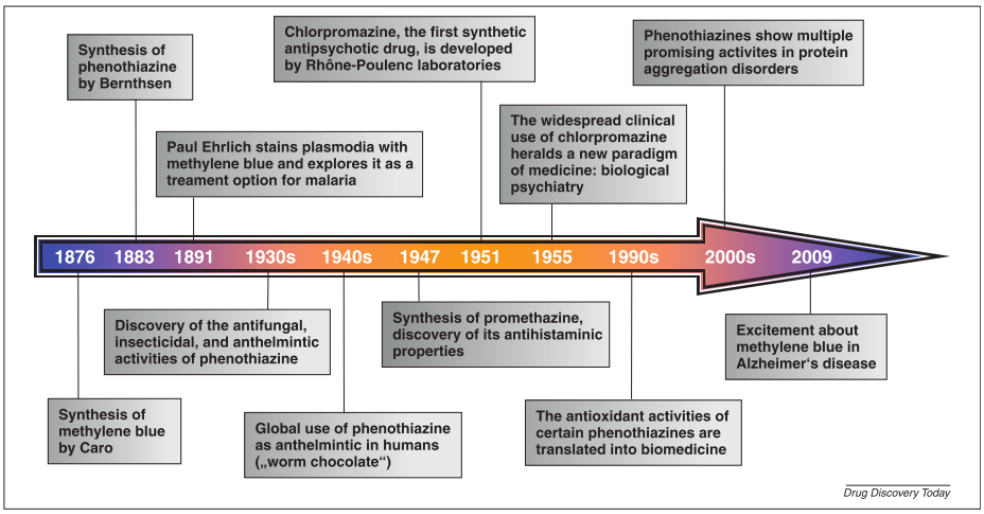


Figure 8. Timeline of The Most Important Developments of Phenothiazines [28].

Phts exhibit diverse range of biological activity and form the largest class of antipsychotic drugs. They inhibit various receptors of central nervous system with a most known inhibitory activity on dopaminergic receptors. Adrenergic, serotonin, histamine, muscarinic and GABA-ergic receptors are also known to be inhibited by phts [29].

Phts are highly membrane permeable thanks to their hydrophobic nature. Having a strong affinity to lipid bilayers of cell membrane in neurons, they easily pass through the blood-brain barrier and used as neuroleptic drugs due to this ability [29].

Calmodulin (CaM), Protein Kinase-C (PKC) and Pgp are proteins also inhibited by the phts. Inhibition effect on these proteins form the basis of their anti-proliferative, apoptotic and multi drug resistance inhibitory activities [29].

CaM is a multifunctional, widespread, calcium-binding protein. CaM is one of the major regulator of calcium-dependent signaling pathways. CaM accomplishes its regulatory function by interacting with different proteins. cGMP, cAMP, CaM-dependent protein kinase, ATPase and phospholipase A_2 are some of the CaM-dependent enzymes. Phts block the CaM activity which in turn inhibits the activity of these CaM-dependent enzymes. Cell proliferation inhibition effect of phts is correlated with its inhibition effect on CaM-dependent enzymes [29].

Pgp, an ABC family protein, functions as an active efflux transporter. Bioavailability of several vital therapeutic agents are lower by its function and it is one of the most important mechanisms for drug resistance mechanisms. According to data available in the literature, the CaM-activated enzymes are involved in the phosphorylation of Pgp which is a fundamental step of its efflux function. Several studies showed that CaM-activated enzymes inhibition by phts, as well as inhibition of the PKC by phts, leads to decreased Pgp phosphorylation and decreased Pgp function. [29,30].

A number of studies demonstrated that phenothiazine derivatives have antiproliferative potential and they can be used to reverse multidrug resistance in various cancer types such as colon, lung and breast cancers as well as melanoma, glioblastoma, leukemia, and lymphoma. It was shown that antitumor activity was accomplished by targeting Wnt, MAPK and retinoic acid signaling pathways; and altering the expression levels of downstream signaling molecules [31,32].

1.4.1. Chlorpromazine

Chlorpromazine (CPZ) is a FDA-approved, marketed antipsychotic medication used to treat disorders such as schizophrenia, bipolar disorder, attention deficit hyperactivity disorder, nausea and vomiting, anxiety before surgery, and hiccups that do not improve following other measures. It has also been used as a sedative and antiemetic. The structure of CPZ can be seen below in Figure 9 [33].

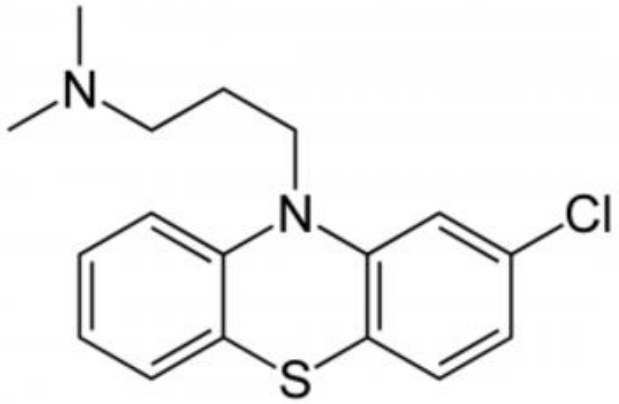


Figure 9. Chemical Structure of Chlorpromazine [33].

1.4.1.1. Synthesis and History

A French pharmaceutical company Rhône-Poiulenc synthesized CPZ in 1950 while they were trying to develop antihistamine drugs with effects on the central nervous

system. After completion of laboratory tests in rats, CPZ was selected for assessment in humans within several other drugs developed [27].

During human assessments, Laborit realized that CPZ was inducing calmness without sedation which was an indication of use in psychiatry. After this realization, Jean Delay and Pierre Deniker who were two psychiatrists of St Anne's Hospital in Paris used CPZ in patients with mania and schizophrenia. They observed that CPZ was very successful as antipsychotic medication and they published their first report based on CPZ in 1952. Over the following years, its use as antipsychotic spread and by 1956 CPZ was being widely prescribed by psychiatrists in both Europe and North America. CPZ listed as essential drug by the World Health Organization (WHO) [27].

1.4.1.2. Mechanism of Action

CPZ works via blockage of post-synaptic D2 dopamine receptors. CPZ occupies the D2 dopamine receptor which in turn inhibits the binding of dopamine to the receptor and the subsequent signal transduction. Dopamine antagonism accounts for the antipsychotic and anti-emetic function of CPZ. Additionally, CPZ is antagonists of H1 Histamine and α-1-adrenergic receptors [34]. Figure 10 explains the mechanism of action of CPZ as an antipsychotic agent [35].

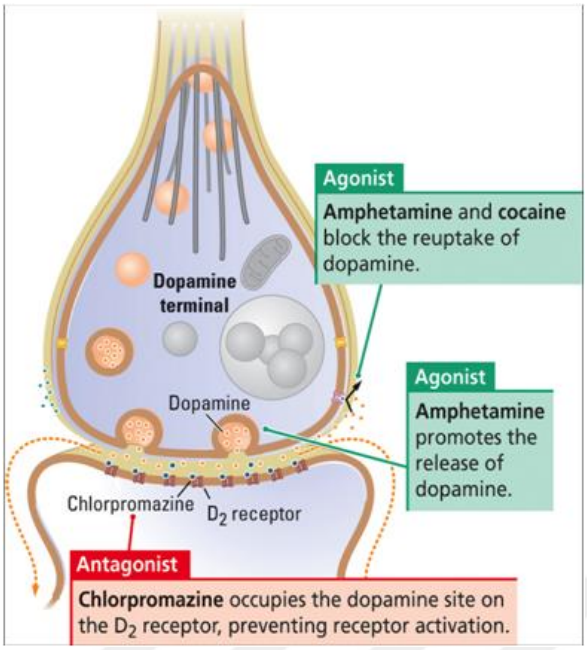


Figure 10. How Chlorpromazine Works [35].

1.4.1.3. Anticancer Effect

There are several studies demonstrating anticancer potential of CPZ in various cancer types. First repositioning studies started with the recognition of its antiproliferative properties and its function in antibiotic resistance reversal [36].

In 2001, Bebawy et al showed that CPZ reversed resistance to vinblastine by modulation Pgp [37].

In 2007, CPZ was reported as a specific inhibitor of the mitotic kinesin KSP/Eg5. It is shown that CPZ leads mitotic arrest and accumulation of monopolar spindles and it works synergistically with pentamidine [38].

In 2009, Lialiaris et al investigated the genotoxic, cytotoxic, and cytostatic potential of CPZ, alone or in combination with mitomycin C. CPZ was shown to increase

chemotherapeutic effectiveness of mitomycin C [39]. In another study that was also conducted in 2009, CPZ was reported to increase antiproliferative effect of tamoxifen both in the antiestrogen-sensitive breast cancer cell line and in tamoxifen-resistant cell line through an estrogen receptor-mediated mechanism [36].

In 2011, CPZ was shown to suppress tumor growth *in vivo* in mouse model of hepatocellular carcinoma [40].

In 2013, Lim et al demonstrated that CPZ inhibited cell proliferation and long-term clonogenic survival of U-87MG glioma cells. Also, it triggered autophagy via inhibition of Akt/mTor pathway without induction of apoptosis in the same cell line [41].

In 2015, Lee et al reported that CPZ induces apoptosis in colorectal cancer in a p53-dependent manner and JNK activation was shown to be crucial for induction of apoptosis. Also, it is shown that CPZ increases p53 acetylation, which is known to increase transcriptional activity of p53, in time- and dose-dependent manner. Induction of CPZ-mediated apoptosis was reported to be attenuated by sirtuin-1 silencing. Sirtuin-1 is a deacetylase which is known to involve in deacetylation of p53. Additionally, the study suggest that CPZ is an sirtuin-1 inhibitor as CPZ leads increased degradation of sirtuin-1 [42].

In 2017, Oliva et al investigated the effect of CPZ on chemoresistant patient-derived glioma stem cells and chemoresistant human glioma cell lines. CPZ was reported to inhibit cytochrome c oxidase activity only in chemoresistant cells, not in chemosensitive cells [43].

1.5. AIM OF THE STUDY

The aim of this study was to investigate the anticancer effect and mechanism of CPZ on U266 MM cell line based on our preliminary findings from potency screening studies. This is the first in-depth cytotoxicity and apoptosis study of CPZ on MM.

CHAPTER 2

MATERIALS AND METHODS

2.1. CHEMICALS

Chlorpromazine, trifluoperazine and haloperidol were purchased from Alfa Aesar (Lancashire, United Kingdom). Cisplatin was purchased from Sigma-Aldrich (Taufkirchen, Germany). Chlorpromazine and trifluoperazine stocks were prepared in MilliQ water, haloperidol in DMSO as 10 mM stocks, and stored at -20 ^{0}C. Cisplatin was prepared in 0.9% NaCl as 1 mM stock, and. stored at room temperature.

2.2. CELL CULTURE

Human myeloma U266 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin, 1% (v/v) non-essential aminoacids and 2.5 μg/mL plasmocin prophylactic as suspension culture, and incubated at 37 ^{o}C in 5% CO_2. For western blot analysis and one of dose response studies, cell density was $5x10^5$ cells/mL at final volume. For all of the other assays, cell density was $1x10^5$ cells/mL at final volume. DMSO concentration in the culture medium for haloperidol treated cells and their controls fixed as 2% (v/v).

2.3. CELL VIABILITY ASSAY

Potency screening, dose response, time response, and combination assays were performed using CellTiter-Blue Cell Viability Assay (Promega / Madison, USA).
For all of these assays, 100 μL of untreated and treated cells with their blank control were seeded in 96-well plates ($1x10^4$ or $5x10^4$ cells/well) with five technical replicates. After treatment, 20 μL of the assay reagent was added to each column and incubated for 4h at 37 ^oC in 5% CO_2. Fluorescence signals were measured at 555 nm excitation and 595 nm emission using SpectraMax Paradigm Multi-Mode Microplate Reader (Molecular Devices / Sunnyvale, USA). Data analysis was done by normalizing RFU values of treated cells to untreated cells.

2.3.1. Potency Screening

Five different drug groups were selected for first potency screening as follows: antipsychotics (Chlorpromazine, Trifluoperazine, and Haloperidol), nonsteroidal anti-inflammatory drugs (Oxaprozin, Flurbiprofen, Dexketoprofen, and Diclofenac), pain killer (Tramadol), anti-depressant (Trazadone) and anti-epileptic (Valproic Acid). Cell treatment with dissolved drug formulations of selected drugs was carried out at 100 μM (24h) for each compound.

For the second screening assay, cells were treated with pure forms of chlorpromazine (CPZ), trifluoperazine (TFP) and haloperidol (HLP) at 25 μM and 100 μM for 24 h.

2.3.2. Dose and Time Response Study

For dose response study, two different cell densities ($1x10^4$ or $5x10^4$ cells/well) were used to assess dose-dependent effect of CPZ on U266 MM cell line. Cells were treated with 9 doses of CPZ, which are serially diluted from 10 mM CPZ stock with

sterilized MilliQ water, for 24 h. 1 μM, 5 μM, 10 μM, 15 μM, 20 μM, 25 μM, 50 μM, 75 μM, 100 μM doses were used for $1x10^4$ cells/well cell density, while 20 μM, 50 μM, 55 μM, 60 μM, 65 μM, 70 μM, 80 μM, 90 μM, 100 μM doses were used for $5x10^4$ cells/well cell density. IC50 value of CPZ was calculated on GraphPad (La Jolla, USA) Prism v6.0 using non-linear curve fitting, where x-axis is log values of these concentrations and y-axis is viability percentages at these concentrations.

For time response study, cells with a cell density of $1x10^4$ cells/well were treated with 12.5 μM CPZ for 12 h, 24 h, and 48 h. Normalization was done separately for each time point by normalizing RFU values of treated cells to untreated cells.

2.3.3. Combination Study

For combination study, cells with a cell density of $1x10^4$ cells/well were treated separately with single drugs (only cisplatin and only CPZ) and combined doses of CPZ and cisplatin (Cis) for 24 h. Combination of 9 doses was performed by crossing 3 different doses of each drug (40, 25, 20 μM for CPZ and 25, 20, 15 μM for Cis). For each combined dose, Combination Index (CI) value was calculated using CompuSyn software (ComboSyn Inc. / Paramus, USA) according to viable cell percentages. As indicated in the Figure 11, combined doses with CI values > 1.10 show antagonism, CI values between 0.90 - 1.10 show additivity, and CI values < 0.90 show synergism.

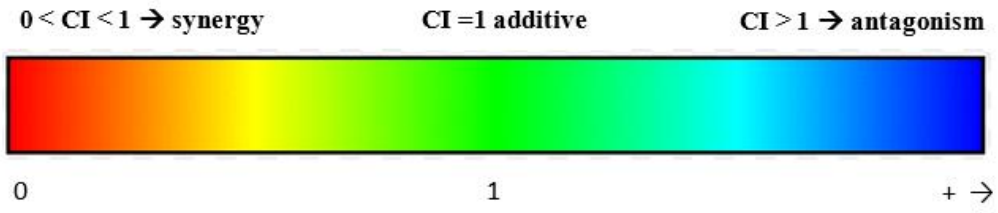


Figure 11. Combination Index Color Scale

In antagonism, a drug decreases the effectivity of another drug leading a decreased total effect when these interfering drugs are used in combination. Synergism means that when drugs are used in combination, the total effect becomes greater than the sum of individual effects of drugs. On the other hand, additivity means that the drugs that are used in combination does not enhance or reduce the effectivity of each other. Thus, the total effect that occurs with combination is similar to sum of individual effects of drugs.

2.4. APOPTOSIS ASSAYS

Three apoptosis assays were conducted to evaluate mitochondrial membrane potential, caspase-3 activation, and change in cell membrane asymmetry of cells. MitoScreen Flow Cytometry Mitochondrial Membrane Potential Detection Kit, PE Active Caspase-3 Apoptosis Kit, and PE Annexin V Apoptosis Detection Kit I was supplied from BD Biosciences (San Diego, USA). Kit protocols were applied as recommended. Analysis of these assays was performed by using Accuri C6 flow cytometer (BD Biosciences / San Diego, USA).

2.4.1. Mitochondrial Membrane Potential Assay

For mitochondrial membrane potential assay, cells were treated with 20 μM CPZ for 24 h. Fluorescence signals of gated samples stained with JC-1 were measured on FL1-H channel at x-axis, and FL2-H channel at y-axis. Color compensation was set by subtracting 17 % of FL1 from FL2. Data analysis was done by normalizing gated depolarized and polarized state values for untreated and treated cells.

2.4.2. Caspase-3 Activity Assay

For caspase-3 activity assay, cells were treated with 20 μM CPZ for 24 h. Fluorescence signals of gated samples incubated with PE conjugated anti-active

caspase-3 antibody were measured on fluorescence intensity histogram, where x-axis represents FL2-A channel, and y-axis represents cell count. An intensity threshold was set to determine caspase-3 activity percentage.

2.4.3. Cell Membrane Asymmetry Assay

For cell membrane asymmetry assay, cell treatment was performed with 20 μM CPZ for 24 and 48 h. Since this assay has ability to show both early and late apoptotic cells, two time points were used. Fluorescence signals of gated samples stained with 7-AAD and PE conjugated Annexin V were measured on FL2-H channel at x-axis, and FL3-H channel at y-axis respectively. Color compensation was set by subtracting 1.5% of FL3 from FL2 and 21% of FL2 from FL3. Cell population percentages of untreated and treated cells in live, early apoptosis, and late apoptosis stages were determined from quadrants on fluorescence intensity dot plots.

2.5. CELL CYCLE ARREST ANALYSIS

For cell cycle assay, cells were treated with 20 μM CPZ for 24 h. Treated samples were fixed with 70% ethanol after a cold DPBS (Biological Industries / Cromwell, USA) wash and kept for 2 h on ice. Following centrifugation at 800 g for 5 min and DPBS (Biological Industries / Cromwell, USA) wash, 5 μL propidium iodide (25 μg/mL) and 90 μL RNase (3 mg/mL) was added. After completing the volume to 300 μL with DPBS (Biological Industries / Cromwell, USA), cells were incubated at 37 ^{o}C for 30 min. Then, cell cycle analysis was also performed on Accuri C6 flow cytometer (BD Biosciences / San Diego, USA). Fluorescence signals of gated samples stained with propidium iodide were measured on fluorescence intensity histogram, where x-axis represents FL2-A channel, and y-axis represents cell count. Borders of G0/G1, S, and G2/M phases were determined on the histogram, and their normalized values were plotted for untreated and treated cells.

2.6. FLOW CYTOMETRY

Three apoptosis assays and cell cycle analysis were performed on Accuri C6 flow cytometer (BD Biosciences / San Diego, USA). For all flow cytometry analysis, gating was performed so that cell debris, cell clumps and doublets were excluded. Ten thousand events were collected into the gate at medium speed of fluidics (35 μML/min).

For cell cycle analysis, FSC-A channel at x-axis and FSC-H channel at y-axis was chosen and gating was performed on that pot to gate single cell population.

2.7. CONFOCAL LASER SCANNING MICROSCOPY

Confocal Lase Scanning microscopy was used to support flow cytometry data in PE Annexin V Apoptosis Detection Apoptosis Assay. Images were obtained on Zeiss LSM 510, Confocal Laser Scanning Microscope using a PlanNeofluar 40x/1.3 Oil DIC objective.

Confocal Laser Scanning Microscopy images were obtained as qualitative results. U266 cells were treated with 20 μM CPZ for 24 hours. The cell number in each flask was set to $1x10^5$ cells/ml with complete medium. After 24 hours treated and untreated cells were stained with PE-conjugated annexin V and 7-AAD as previously described. After staining cells were first fixed before visualization. Stained cells were seeded on lams and covered with autoclaved coverslips (Marienfield, Germany). Annexin V was excited with 488 nm laser and visualized through a 515 nm emission filter, shown in green. 7-AAD was excited with 488 nm laser and emitted through a 590 nm emission filter, shown in red.

2.8. STATISTICAL ANALYSIS

For IC50 determination, curve fitting was performed by using variable slope with log(concentration) vs viability curve of GraphPad Prism. For time response, mitochondrial membrane potential, cell membrane asymmetry, and cell cycle assays, statistical significance of results were determined using GraphPad Prism one-way ANOVA with Bonferroni's Post-hoc Test for selected pairs. Statistical significance of cytotoxicity and caspase-3 activity assay results were determined using unpaired t-test with two-tails. Data were represented as Mean ± SEM. Significance of differences was marked on the figures with asterisks. All statistical tests were conducted with 95% confidence interval.

CHAPTER 3

RESULTS

3.1. POTENCY SCREENING

3.1.1. Potency Screening of Drug Formulations

For potency screening, cell treatment with 100 μM dissolved drug formulations of chlorpromazine (CPZ), trifluoperazine (TFP), haloperidol (HLP), oxaprozin (OXA), flurbiprofen (FLP), dexketoprofen (DKP), diclofenac (DCF), tramadol (TRM), trazadone (TRZ), and Valproic Acid (VLP) was performed. As can be seen in Figure 12, antipsychotics drugs were more potent than other drug groups. Mean and Standard Deviation (SD) values of technical replicates for viability percent of cells treated with each drug is indicated in Table 1.

Table 1. Mean and SD Values for Viability of Cell Treated with Each Drugs

	CPZ	TFP	HLP	OXA	FLP	DKP	DCF	TRM	TRZ	VLP
Mean	4	1	7	74	135	83	99	91	70	99
SD	1	1	3	3	5	2	1	7	5	2

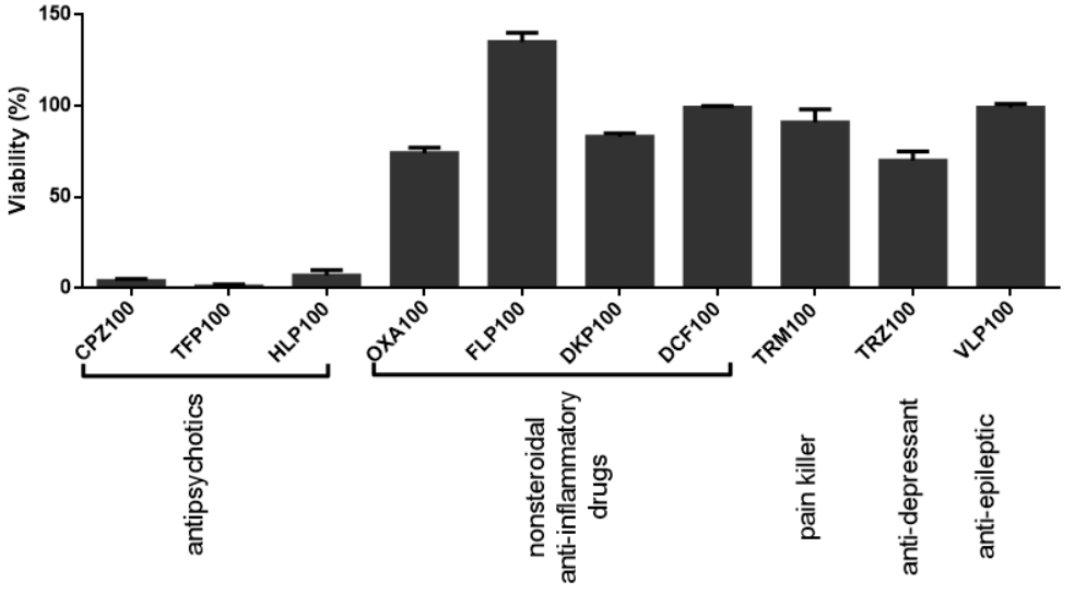


Figure 12. Potency screening of drug formulations at 100 µM (24 h). CPZ, TFP, HLP, OXA, FLP, DKP, DCF, TRM, TRZ, and VLP refer to chlorpromazine, trifluoperazine, haloperidol, oxaprozin, flurbiprofen, dexketoprofen, diclofenac, tramadol, trazadone, and Valproic Acid respectively. Each column represents the Mean ± SD of technical replicates.

3.1.2. Potency Screening of Pure Compound Forms

Three different antipsychotics chlorpromazine (CPZ), trifluoperazine (TFP) and haloperidol (HLP) were tested. Treatment was performed with two different doses (25 and 100 µM) of compounds. As can be seen in Figure 13, CPZ and TFP were more potent than HLP. Mean and Standard Deviation (SD) values of technical replicates for viability percent of cells treated with each drug is indicated in Table 2.

Table 2. Mean and SD Values for Viability of Cell Treated with Each Drugs

	CPZ (100 μM)	CPZ (25 μM)	TFP (100 μM)	TFP (25 μM)	HLP (100 μM)	HLP (25 μM)
Mean	0	39	0	6	26	89
SD	0	1	0	0	1	3

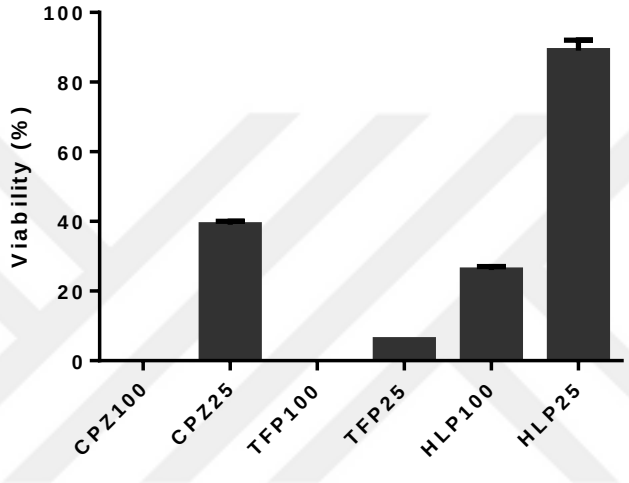


Figure 13. Potency screening of three different antipsychotic drugs at 25 μM and 100 μM (24 h). CPZ, TFP, and HLP refer to chlorpromazine, trifluoperazine, and haloperidol, respectively. Each column represents the Mean ± SD of technical replicates.

3.2. CHLORPROMAZINE HAS DOSE- AND TIME-DEPENDENT GROWTH INHIBITORY EFFECT

Two sets of dose response studies (one with $1x10^5$ cells/mL and other with $5x10^5$ cells/mL cell density at final volume) were performed to evaluate the efficacy of CPZ, and dose-response curves were generated to calculate half maximal inhibitory concentration (IC_{50}) of CPZ by treating cells with doses ranging from 1 μM to 100

μM. IC_{50} at $1x10^5$ cells/mL cell density was calculated as 22.4 ± 0.9 μM, while it was calculated 57.29 ± 0.9 μM at $5x10^5$ cells/mL cell density (Figure 14).

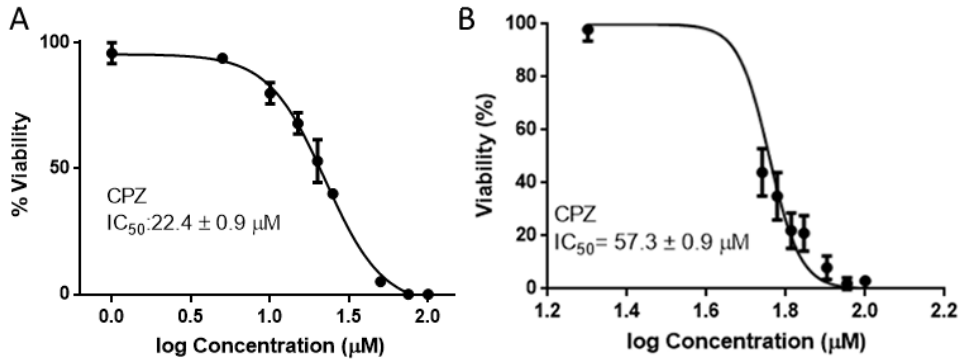


Figure 14. Dose-response curves for 9 doses of CPZ ranging from A) 1 μM to 100 μM at cell density of $1x10^5$ cells/mL B) 20 μM to 100 μM at cell density of $5x10^5$ cells/mL at final volume. Data represents the Mean ± SEM (n=3).

Following dose-response studies, time response studies were also conducted to determine whether CPZ shows time-dependent growth inhibitory effect on U266 MM cell line. Cells were treated with 12.5 μM CPZ for 12, 24, 48 h and viability of the samples were measured by using CellTiter Blue cell viability assay. As indicated in Figure 15, CPZ treatment leads to a time-dependent decrease in the viability of U266 MM cells.

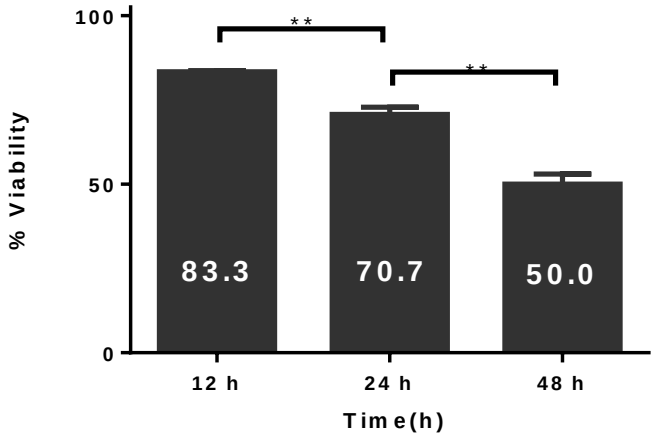


Figure 15. Time-dependent inhibitory effect of CPZ on U266 cell viability. Cells were treated with 12.5 µM CPZ; and cell viability was measured at 12, 24 and 48 hours as indicated. Asterisks ** denote statistical significance at $p<0.01$ (n=3).

Results mentioned above indicates that CPZ treatment leads a decrease in viability of U266 MM cells.

Decrease in viability can be achieved in two ways; stopping the division of cells (inhibition of proliferation, anti-proliferative effect) and cell death or both ways. Thus, apoptosis assay and cell cycle arrest assay were conducted afterwards to determine which mechanism activated with CPZ treatment.

3.3. APOPTOSIS ASSAYS

As indicated in the hallmarks of cancer part of introduction chapter, cancer cells share common traits and escaping from apoptosis is one of these common traits. Cancer cells evolve different mutations to evade apoptosis and accumulation of these mutations in turn leads to malignant transformation. Since these defects leads the

malignant formation itself, it is a good target for the treatment of cancer [44]. In addition, apoptosis is not destructive to surrounding healthy tissue since it is a non-inflammatory cell death. For this reason, assessment of apoptosis was chosen type of cell death. Three different apoptotic markers were examined; change in mitochondrial membrane polarization status, activation of caspase-3 and phosphatidylserine (PS) translocation to outer leaflet of the membrane accompanied by the loss off cellular membrane (membrane fragmentation). As indicated in Figure 16, there are 2 pathways of apoptosis; intrinsic and extrinsic pathways of apoptosis. Mitochondrial membrane depolarization observed in only intrinsic pathway of apoptosis, while caspase-3 activation and changes in cell membrane asymmetry observed in both pathways.

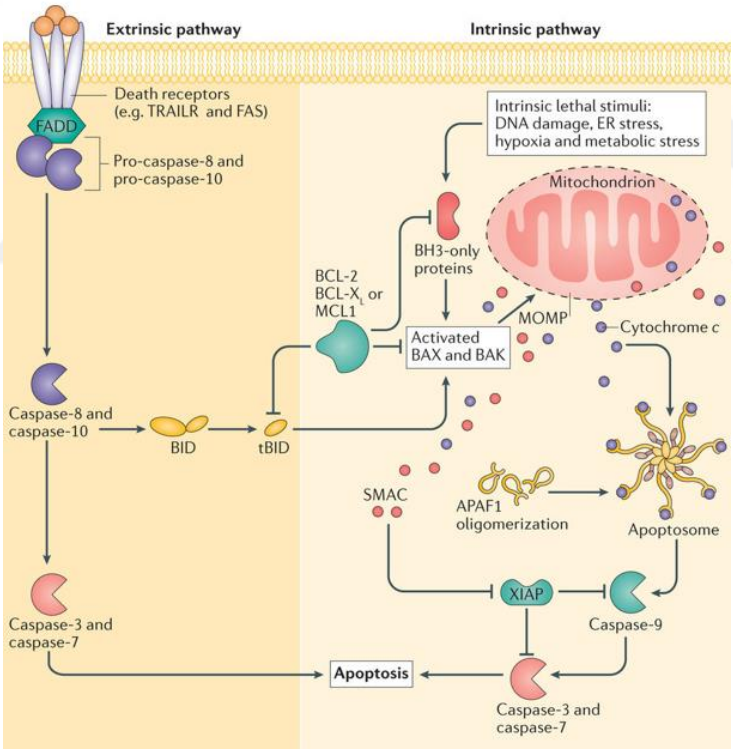


Figure 16. Extrinsic and Intrinsic Pathway of Apoptotic Cell Death [45].

3.3.1. CPZ Treatment Does Not Induce Mitochondrial Membrane Depolarization

Depolarization of mitochondrial membrane is a marker of intrinsic apoptosis pathway. It is often, but not always, observed at early stages of apoptotic cell death. JC-1, is a lipophilic dye, was used to determine effect of CPZ treatment on mitochondrial membrane potential. JC-1 exhibits potential-dependent accumulation in mitochondria which leads a shift in fluorescence emission from green to red. When it is in mitochondria (red), it forms aggregates, and it exists in monomer form in cytoplasm (green). As a result, mitochondrial depolarization is indicated by a decrease in the red/ green fluorescence intensity ratio [46].

When mitochondrial membrane becomes depolarized, JC-1 remains in the cytoplasm as monomers which have lowered red fluorescence. This decrease in the red fluorescence is used to evaluate the change in the mitochondrial membrane potential [46].

The cells were treated with 20 μM CPZ for 24 hours and stained with JC-1 dye and analyzed by flow cytometer. As indicated in the Figure 17, CPZ treatment did not induce mitochondrial membrane depolarization of U266 MM cells, since 96% of the U266 MM cells remained in the polarized mitochondrial membrane state.

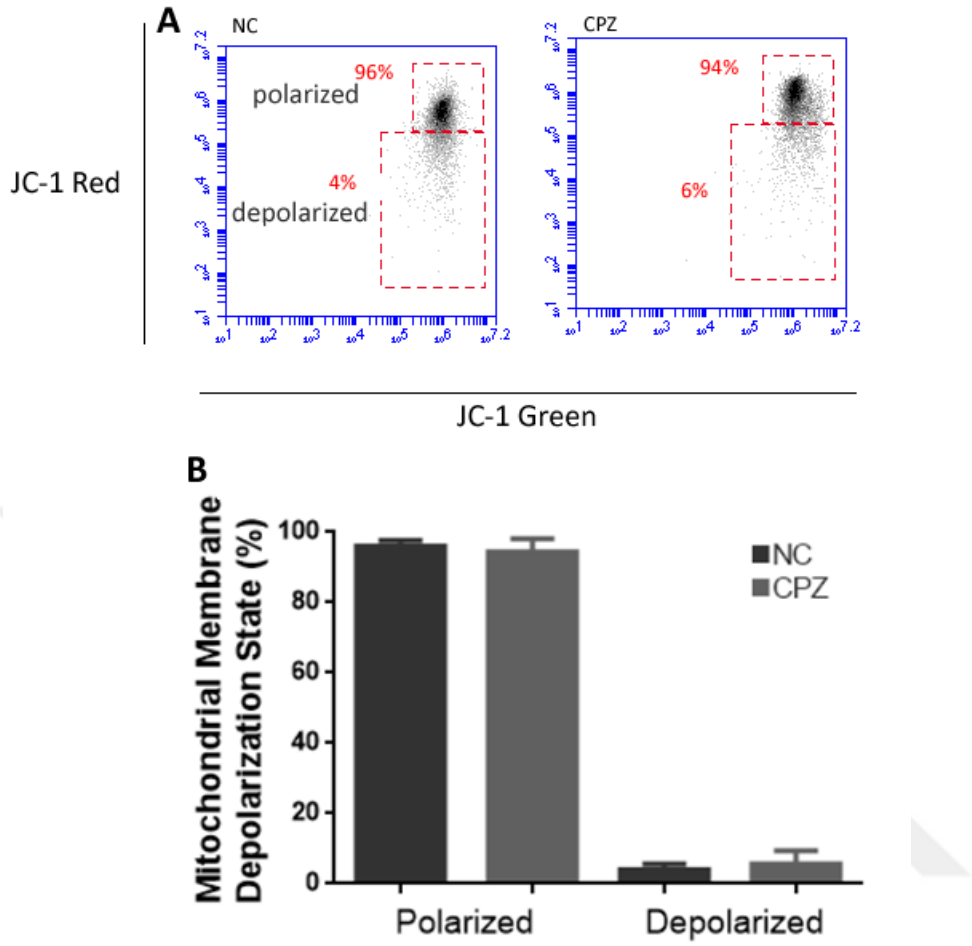


Figure 17. Effect of 20 µM CPZ treatment on mitochondrial membrane potential at 24 h Representative flow cytometry fluorescence intensity dot plots of untreated cells (NC) and 20 µM CPZ treated cells (CPZ) stained with JC-1. Fluorescence intensity values for polarized and depolarized states was gated and labeled accordingly. B) Bar plots of normalized mitochondrial membrane polarization state values for untreated and CPZ-treated cells (n=3). Error bars indicate the standard error of mean. Untreated and CPZ-treated samples are not significantly different (p>0.05).

This negative JC-1 result may indicate that CPZ treatment is not inducing intrinsic pathway of apoptosis.

3.3.2. CPZ Treatment Induces Caspase-3 Activation

Caspases are enzymes that are activated in a cascade during apoptosis. They are proteases that are synthesized in zymogen form (inactive form) containing a prodomain. They activate each other by cleaving the prodomain. Activation of caspase-3 is a key event that occurs at early stages of apoptosis; and observed in both intrinsic and extrinsic apoptotic pathways. Detection of activated caspase-3 is possible with a use of antibody that specifically recognizes cleaved (active) form of caspase-3. Therefore, caspase-3 activity was tested in order to observe whether CPZ treatment induce apoptosis of U266 MM cells

U266 cells were treated with 20 μM CPZ for 24 hours. PE labeled Anti-Active Caspase-3 antibody was used to label activated caspase-3. Then, cells were analyzed by flow cytometer (Figure 18). When compared with the untreated samples, significant increase in caspase-3 activity was observed in CPZ-treated cells (17.7% vs. 36.5%).

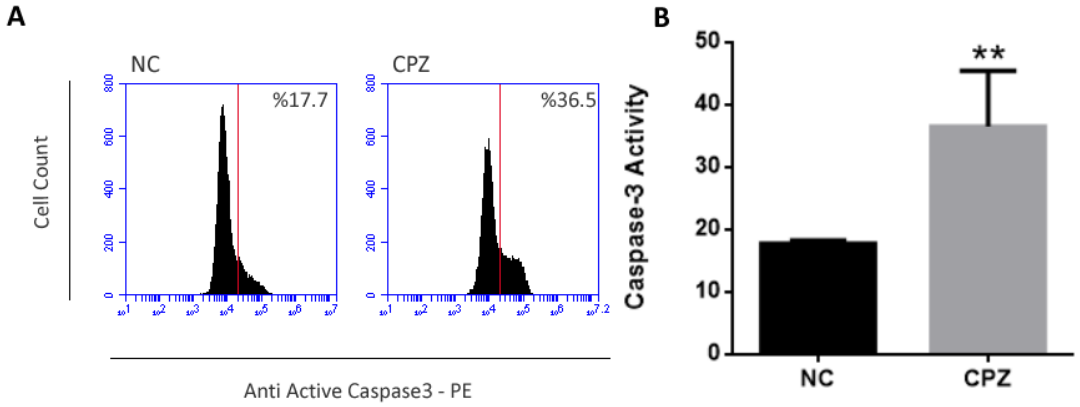


Figure 18. Effect of CPZ treatment (20 µM, 24 h) on caspase-3 activation in U266 cells. A) Flow cytometry fluorescence intensity histograms of untreated cells (NC) and 20 µM CPZ-treated cells (CPZ) stained with anti-active Caspase 3-PE antibody. Intensity threshold for caspase-3 activity is indicated in the left panel. B) Bar plot of corresponding histograms are placed on the right. Asterisks ** denote statistical significance at p<0.01 (n=3).

Activation of caspase-3 indicates that CPZ induces apoptosis of U266 MM cells.

3.3.3. CPZ Treatment Changes Cell Membrane Asymmetry

Loss of plasma membrane asymmetry is one of the early markers of apoptosis. PS is membrane phospholipid which locates in the inner leaflet of plasma membrane in healthy cells. Flippase is an ATP-dependent enzyme that is responsible for keeping PS in the inner leaflet of plasma membrane by translocating them from the outer leafle. Since apoptosis is an ATP-dependent process, ATP is consumed during apoptosis. Due to ATP consumption, flippase in not able to work and keep PS in the inner leaflet of plasma membrane under apoptotic conditions leading to membrane asymmetry changes in apoptotic cells [47]. Detection of PS in the outer surface of the membrane is possible with use of a molecule that has high affinity for PS such as Annexin V. Fluorescently labeled Annexin V binds to the cells with exposed PS; and serves as a probe for detection of apoptosis.

Loss of cellular membrane integrity is another characteristic of apoptotic cells which happens in later stages of apoptosis. When the membrane is fragmented, the cellular contents and DNA will be exposed. Detection of exposed DNA is possible with use of DNA-binding dye such as 7-AAD which is normally cell membrane impermeable and therefore not able to stain DNA of healthy cells. Use of 7-Amino-Actinomycin (7-AAD) together with Annexin V enables recognition of apoptotic cells at different stages. Healthy cells are both Annexin V and 7-AAD negative; early apoptotic cells are Annexin V positive but 7-AAD negative; and late apoptotic cells are Annexin V and 7-AAD positive.

U266 cells were incubated with 20 μM CPZ for 24 and 48 hours; and stained with Annexin V-PE / 7-AAD probes. As seen in the Figure 19-B, CPZ treatment caused significant decrease in viable cell population at 24 h. On the other hand, significant increase in early apoptotic cell populations was observed after treatment. As the cytotoxic effect of CPZ increased in time, the difference between untreated and CPZ-treated cells became more prominent at 48 h (Figure 19-A&B). Almost 80% of the cell population consist of early and late-apoptotic cells at 48h (Figure 19-A&B).

As seen in Figure 19-C, live cells are not stained with any of the dyes, while in early apoptotic cells only the membrane is stained with Annexin V (green) but not with 7AAD (red) and late apototic cells were stained with both AV and 7AAD.

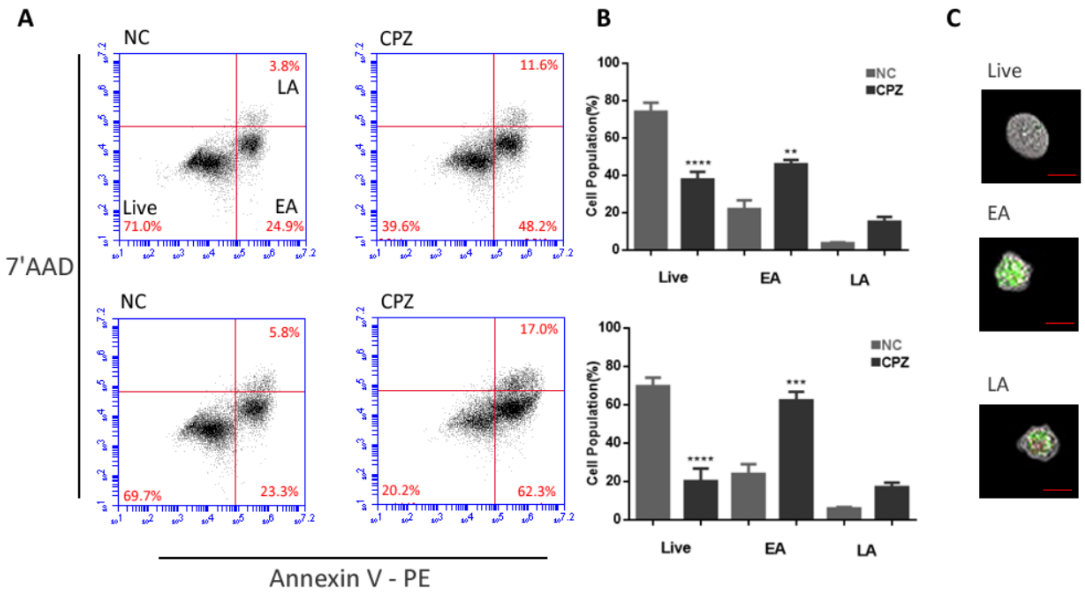


Figure 19. Analysis of U266 cell membrane asymmetry after 20 µM CPZ treatment for 24 h and 48 h. U266 cells were stained with Annexin V-PE and 7-AAD; and analyzed at 24 h and 48 h. A) Dot plots of Annexin V-PE vs 7-AAD-stained cells gated as live, early apoptotic (EA) and late apoptotic (LA) quadrants as shown in the upper-left plot. B) Cell population bar graphs of corresponding dot plot quadrants. Asterisks **, *, and **** denote statistical significance between control and treatment populations at p<0.01, p<0.001, and p< 0.0001 respectively (n=3). C) CLSM images of Live, early apoptotic and late apoptotic cells. Scale indicates 10 µm.**

Annexin V& 7AAD staining data is another indicator of U266 cell that are undergoing apoptosis led by CPZ treatment.

3.4. CPZ TREATMENT LEADS TO ACCUMULATION OF U266 MM CELLS AT G_2/M PHASE

As mentioned earlier, anti-proliferative effect of CPZ treatment could be the other explanation for decreased cell viability after treatment. To analyze whether CPZ

treatment has such anti-proliferative effect, cell cycle progression of U266 cells was analyzed by propidium iodide (PI) staining. PI is a nucleic acid binding dye, so that RNase treatment was performed to get rid of RNA content of cells and signal noise caused by RNA content. In this situation, PI only binds to DNA and PI binding leads to a concomitant florescence intensity that is proportional to the amount of DNA present in the cells. Cells in G_0/G_1 phase have 2n amount of DNA. When they begin to replicate in S phase, DNA amount increases from 2n to 4n which corresponds to increasing PI signal. Finally at G_2/M phase, cells have 4n amount of DNA which in turn leads a doubled PI signal compared to cell in G_0/G_1 phase.

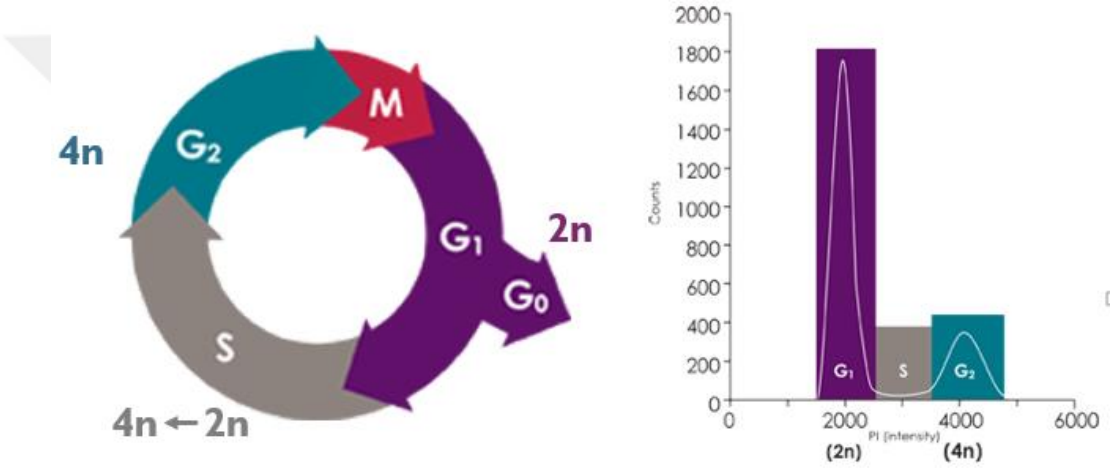


Figure 20. Cell cycle phases and illustrative cell cycle progression plot [48].

U266 cells were treated with 20 μM CPZ for 24 hours; and stained with PI. Cell cycle distribution of PI-labeled cells was analyzed with flow cytometry. Data were represented as flow cytometry histograms and the bar plots of corresponding cell populations in each phase of the cell cycle (Figure 21).

Percentages of cell populations at G_0/G_1, S and G_2/M phases for untreated and CPZ-treated cells were determined as 50.5% vs. 44.7%, 21.1% vs. 16.5% and 28.5% vs. 38.8%, respectively. CPZ treatment resulted in significant increase in G_2/M

population. These results indicated that CPZ treatment leads U266 MM cells to accumulate at G_2/M phase. Thus, U266 MM cells were arrested at G_2/M phase of cell cycle upon CPZ treatment.

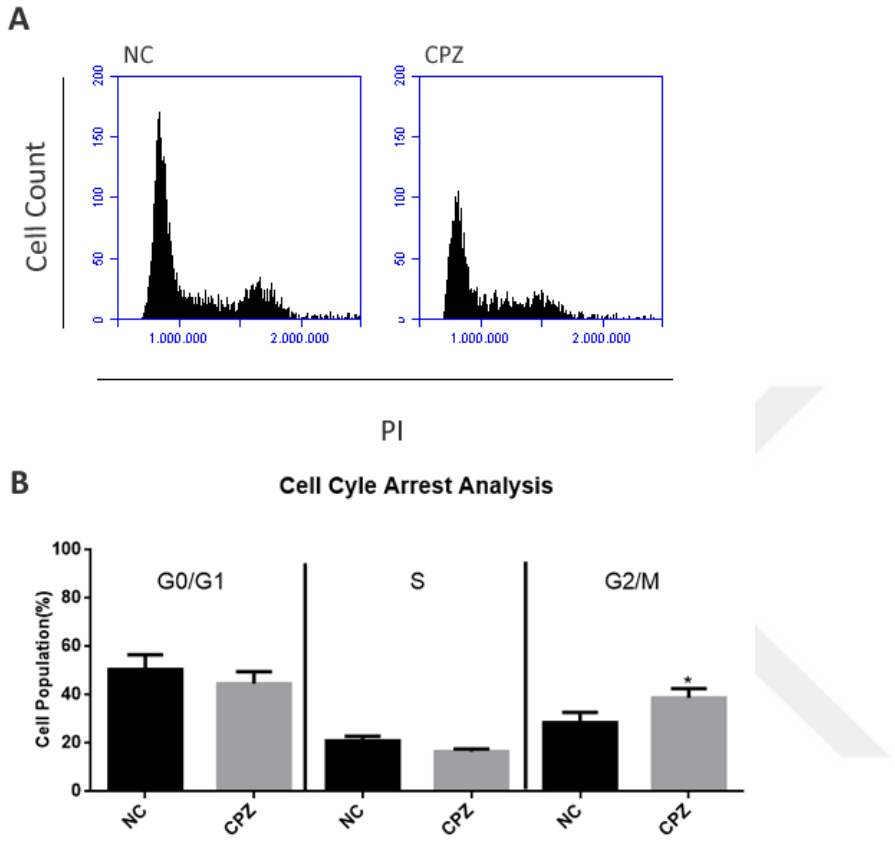


Figure 21. Cell cycle analysis of untreated U266 cells (NC) and tretaed with 20 µM CPZ (CPZ) for 24 h. A) Cell-cycle distribution of propidium iodide (PI)-labeled cells represented as flow cytometry fluorescence intensity histograms. The peaks in the illustration correspond to the G_1/G_0, S and G_2/M phases of the cell cycle. B) Bar plots representing the percentages of cell population in each phase of the cell cycle, for untreated and CPZ-treated cells. Asterisk * denotes statistical significance at $p<0.05$ (n=3).

3.5. CPZ-CIS COMBINATION IS MODERATELY SYNERGISTIC

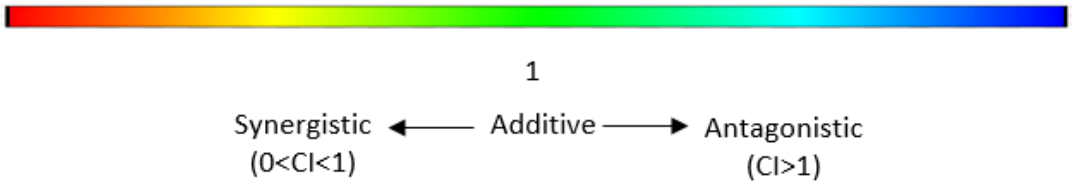
Cytotoxic effect of CPZ, Cis and CPZ-Cis combinations at 24 h was analyzed by CellTiter Blue cell viability assay. Combination potential of the drugs was represented as Combination Index (CI) values which were calculated using the CompuSyn software as described in Methods section. In this context, CI < 0.9 indicates synergism, 0.9 < CI < 1.1 indicates additivity, CI > 1.1 indicates antagonism.

Firstly, bortezomib and CPZ combination was planned to be conducted. However, combination studies could not be completed due to stability problem of bortezomib. Since it is a modified dipeptidyl boronic acid analogue, it has really low stability after reconstution. Therefore, we decided to use cisplatin, which is currently used in the treatment of late-stage MM patients, in combination studies instead of bortezomib.

As can be seen in Table 3 (color scale indicates the effect of CI value given), CPZ-Cis combination was slightly synergistic when CPZ was used in 40 μM concentration. Synergistic effect was lost with decreased CPZ concentration and effect of combination became additive.

Table 3. Combination Potential of CPZ & Cis. Color Scale indicates the effect corresponding the given CI values.

Cis/CPZ	40 µM	25 µM	20 µM
25 µM	0.74	0.90	0.85
20 µM	0.75	0.88	0.85
15 µM	0.79	0.84	0.91



CHAPTER 4

DISCUSSION

Drug repositioning is a more logical and effective route for drug development due to reduced cost and time needed for the development process. Lately, it has become highly popular strategy as a drug development route, especially for cancer treatment [49]. In this study, we aimed to reposition CPZ as an anticancer drug against MM. Currently, CPZ is an FDA-approved antipsychotic drug mainly used in the treatment of schizophrenia and bipolar disease. Consistent with the previous studies on anticancer potential of CPZ, our results showed that CPZ has dose- and time-dependent growth inhibitory effect on U266 cell growth and it induces both apoptosis and cell cycle arrest of U266 MM cells [40–42]. Although anticancer potential of CPZ has been studied in various cancer types, this is the first in-depth cytotoxicity and apoptosis study on MM.

Half maximal growth inhibitory concentration (IC_{50}) of CPZ was found as 22.4 ± 0.9 μM at a cell density of $1x10^5$ cells/mL while it was found as 57.29 ± 0.9 μM at $5x10^5$ cells/mL cell density. There is almost 3-fold increase in IC_{50} value with a 5- fold increase in cell density. This result proves that IC_{50} is a cell density-dependent value and varying cell densities should not be used. For that reason, cell density was kept same in all the following experiments. Having an IC_{50} value of 22.4 μM makes CPZ highly potent since 22.4 μM is in low micromolar (μM) range. Additionally, time-response studies showed that even 12.5 μM CPZ treatment lead to a significant decrease in U266 cell viability, and the decrease in cell viability was time-dependent. Similar results for assessment of CPZ potency against cancer types such as breast cancer, colorectal cancer, and glioma cells exist in the literature. Oliva et. al.

determined the IC_{50} of CPZ as 13.12 μM on TMZ-resistant UTMZ glioma cells [43]. In another study, IC_{50} of CPZ was determined as 11.6 μM on HCT116 cells with MTT assay [42]. Also, Mello et. al. calculated the IC_{50} of CPZ 14 μM on Lucena 1 and K562 cells [50].

Chemotherapeutic drugs can be very harmful not only to cancer cells, but also to healthy cells. Also, serum levels of a drug is an important factor to determine drug dosage so that drug concentrations can be maintained within a target range. Thus, it is important to consider toxicity of CPZ on healthy cells within its IC_{50} range and its serum levels. According to the literature, CPZ had no inhibitory effect on viability of normal (healthy) human lymphocytes in the range of 1 to 40 μM [51]. Moreover, Lee et. al. investigated the toxicity of CPZ by treating zebrafish embryos with 25 μM and 50 μM CPZ for 48h. This study showed that CPZ-treated zebrafish embryos yielded similar survival rate with wild type zebrafish embryos which is an indicator of low toxicity at least up to 50 μM [42]. CPZ steady-state serum levels have been showed to have a 20-fold variation in 25 patients receiving repeated oral doses of CPZ (100 mg/ 12h) and single intramuscular dose of CPZ (50mg) [52]. This study suggests that there are large inter-individual variations in the extent of CPZ treatment and further in-depth studies for pharmacokinetics of CPZ is required.

Decreased cell viability can be caused either by inhibition of cell proliferation or cell death or both. Thus, we conducted 3 different apoptosis assays and cell cycle progression analysis for the assessment of mechanism behind the decrease in cell viability following the dose- and time-response studies. According to results that were gathered, CPZ did not induce depolarization of mitochondrial membrane while it induced activation of caspase-3 and changes the plasma membrane asymmetry. Moreover, CPZ treatment induce cell cycle arrest at G_2/M phase. As mentioned in the introduction section, cancer cells evolve mutations to escape from apoptosis. Thus, targeting the induction of apoptosis is the logical way to push cancer cells to cell death. Also, apoptosis is a non-inflammatory cell death type meaning that it does

not destroy the healthy cells surrounding the tumors [53]. Therefore, apoptosis assays are the typical cell death assays that are commonly used in drug development studies.

First apoptotic assay that was conducted was mitochondrial membrane depolarization assay which works through staining with JC-1 dye. As it can be seen in the Figure 17, CPZ treatment did not induce mitochondrial depolarization of U266 MM cells. JC-1 staining negative result is not surprising considering that CPZ inhibits opening of mitochondrial permeability transition (MPT) pores [54]. Various factors such as Ca^{2+}, reactive oxygen species (ROS), inorganic phosphates can promote MTP pore opening. When MPT pores are opened, cytoplasmic substance can move into mitochondrial membrane non-selectively leading depolarization of mitochondrial membrane. MPT pore opening is an event occurred during intrinsic pathway of apoptosis [55] . Thus, inhibition of MPT pores keeps mitochondrial membrane in polarized state. Consequently, apoptotic function of CPZ treatment seems to work through extrinsic pathway of apoptosis. Extrinsic pathway of apoptosis is triggered by activation of death receptors via stimulation of respective ligands. Death receptors share a common domain of death domain and they are members of TNF receptor superfamily. There are 6 human death receptors: TNF-R1, Fas/APO-1, TRAIL-R1 (DR4), TRAIL-R2 (DR4), DR3, and DR6. Their targets respectively are TNF, FasL, TRAIL and TL1A [56]. Since U266 MM cells have shown to be resistant against Fas-mediated apoptosis, CPZ treatment may activate the extrinsic pathway of apoptosis by triggering other death receptors [22]. For more accurate determination, activation state of caspase-8 and caspase-9 can be assessed by using respective activation assays. Caspase-8 and caspase-9 are initiator caspases. Caspase-8 is only activated in extrinsic pathway of apoptosis, while caspase-9 is only activated in intrinsic pathway of apoptosis [53].

Capase-3 activation occurs during apoptosis regardless of the pathway activated. It is the executioner caspase; and both of caspase-8 and caspase-9 activate caspase-3. Once activated, caspase-3 cleaves cellular target proteins leading to loss of

membrane asymmetry, DNA fragmentation, degradation of nuclear proteins, and formation of apoptotic bodies that finally up taken by phagocytes [53]. Following mitochondrial membrane depolarization assay, activation of caspase-3 and changes in plasma cell asymmetry were examined by PE conjugated anti active caspase-3 and PE conjugated Annexin V & 7-AAD staining. As can be seen in the Figures 18 and 19, CPZ treatment leads caspase-3 activation and change the asymmetry of plasma membrane of U266 MM cells. CPZ-induced apoptosis has been shown in several cell lines such as K562, Lucena 1, HCT116 [42,50]. Furthermore, Yde et. al. observed that use of CPZ in combination with tamoxifen, increased the apoptotic potential of tamoxifen [57].

As mentioned earlier, decreased U266 MM cell viability upon CPZ treatment can also be driven by an antiproliferative effect. Therefore, we also tracked the cell cycle progression with PI staining. According to the gathered results, CPZ treatment lead a significant increase in G_2/M cell population ($p=0.0310$) meaning that U266 cells were arresting at that stage upon CPZ treatment. According to the literature, cell cycle arrest at G_2/M phase less well tolerated by the cells which in turn leads to increased apoptotic outcome[58]. This evaluation provides a therapeutic advantage for CPZ which causes G2/M arrest in U266 cells. Effect of CPZ on cell cycle progression has been studied before. Oliva et. al. showed that CPZ treatment leads G_2/M phase arrest on U251 cell line, while it leads G_1 phase arrest on UTMZ cell line [43]. Moreover, it has been determined that CPZ treatment induce cell cycle arrest at G_2/M phase in rat C6 glioma cells [59].

Combination chemotherapy is the most widely used procedure of cancer treatment and it is the administration of at least two different anticancer drugs in combination. The rationale behind the combinatorial usage is decreased possibility of drug resistance development. Since drugs that are working in different mechanisms will attack the cell in different aspects, the like hood of resistance development will be lowered. Consequently, the combination potential of CPZ with cisplatin was tested.

Cisplatin is a chemotherapeutic drug that is currently used in MM treatment at late stage. As can be seen in the Table 1, CPZ-cis combination was moderately synergistic. But the synergistic effect was lost with the decreased concentrations of CPZ.

CHAPTER 5

CONCLUSION

This study showed that CPZ has a dose- and time-dependent growth inhibitory and cytotoxic effect on U266 MM cell line with a IC_{50} value of 22.4 ± 0.9 μM. Apoptosis assay conducted for the determination of activation of caspase-3 and changes in cell membrane asymmetry proved that CPZ treatment is able to induce apoptosis of U266 MM cells. On the other hand, negative result for mitochondrial membrane depolarization indicates that apoptosis induction by CPZ treatment does not work through intrinsic pathway of apoptosis. In addition, we tracked cell cycle progression by flow cytometer following PI staining. According to the results, CPZ induced the accumulation of G_2/M cell population which is an indicator of cell cycle arrest at G_2/M phase of cell cycle. CPZ-cisplatin combination potential was also assessed during this study and CPZ-cisplatin combination was found as moderately synergistic.

According to previous studies, CPZ is not cytotoxic to normal lymphocytes and it has a low toxicity in zebrafish embryo. Moreover, CPZ is an FDA-approved, cheap drug. Considering our findings and literature studies, CPZ becomes a powerful candidate for further in-depth mechanistic studies and *in vivo* experiments.

REFERENCES

1. Institute, N. C. What is Cancer. (2015). Available at: http://www.cancer.gov/cancertopics/what-is-cancer.
2. Cancer Classification. Available at: https://training.seer.cancer.gov/disease/categories/classification.html.
3. Hanahan, D., Weinberg, R. A. & Francisco, S. The Hallmarks of Cancer Review University of California at San Francisco. Cell 100, 57–70 (2000).
4. Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N. & Sarkar, S. Drug resistance in cancer: An overview. Cancers (Basel). 6, 1769–1792 (2014).
5. Zahreddine, H. & Borden, K. L. B. Mechanisms and insights into drug resistance in cancer. Front. Pharmacol. 4 MAR, 1–8 (2013).
6. Nikhil, C. M. & Avet-Loiseau, H. NIH Public Access. Clin. Cancer Res. 17, 1234–1242 (2013).
7. Chai, S., To, K. K. & Lin, G. Circumvention of multi-drug resistance of cancer cells by Chinese herbal medicines. Chin. Med. 5, 26 (2010).
8. Tosi, P., Gamberi, B. & Giuliani, N. Biology and treatment of multiple myeloma. Biol. Blood Marrow Transplant. 12, 81–6 (2006).
9. Plasma Cell Neoplasms. (2017). Available at: https://www.cancer.gov/types/myeloma/patient/myeloma-treatment-pdq.
10. Bergin, K., McQuilten, Z., Moore, E., Wood, E. & Spencer, A. Myeloma in the Real World: What Is Really Happening? Clin. Lymphoma Myeloma Leuk. 17, 133–144.e1 (2016).
11. Dispenzieri, A., Lacy, M. Q. & Greipp, P. R. Disorders, Hematologic Malignancies: Multiple Myeloma and Related Plasma Cell. Springer-Verlag 13, (Springer New York, 2004).
12. Rajkumar, S.V. Patient education: Multiple myeloma treatment (Beyond the

Basics). (2017). Available at: http://kanserveyasam.org/kanser-hakkinda/kanser-turleri/adan-zye-kanser-turleri/m/multipl-miyelom/.

13. Naymagon, L. & Abdul-Hay, M. Novel agents in the treatment of multiple myeloma: a review about the future. J. Hematol. Oncol. 9, 52 (2016).
14. Lauta, V. M. A Review of the Cytokine Network in Multiple Myeloma Diagnostic , Prognostic , and Therapeutic Implications. Am. Cancer Soc. 6, 2440–2452 (2003).
15. Gadó, K., Domján, G., Hegyesi, H. & Falus, A. Role of Interleukin-6 in the Pathogenesis of Multiple Myeloma. Cell Biol. Int. 24, 195–209 (2000).
16. Kyle, R. A. & Rajkumar, S. V. Multiple myeloma ASH 50th anniversary review Multiple myeloma. Blood 111, 2962–2971 (2008).
17. Miguel, J. F. S. Editorial: Introduction to a series of reviews on multiple myeloma. Blood 125, 3039–3041 (2015).
18. U266B1 [U266] (ATCC® TIB-196™). Available at: https://www.lgcstandards-atcc.org/Products/All/TIB-196.aspx?geo_country=tr#specifications.
19. Barut, B. Zon, L. I., Cochran, M. K., Paul, S. R., Chauhan, D., Mohrbacher, A., Fingeroth, J & Anderson, K. C. Role of Interleukin 6 in the Growth of Myeloma Derived Cell Lines. Leuk. Res. 16, 951–959 (1992).
20. Schwab, G., Siegall, C. B., Aarden, L. A., Neckers, L. M. & Nordan, R. P. Characterization of an Interleukin-6-Mediated Autocrine Growth Loop in the Human Multiple Myeloma Cell Line, U266. Blood J. 77, 587–593 (1991).
21. Rowley, M., Liu, P. & Van Ness, B. Heterogeneity in therapeutic response of genetically altered myeloma cell lines to interleukin 6, dexamethasone, doxorubicin, and melphalan. Blood 96, 3175–80 (2000).
22. Landowski, T. H., Gleason-Guzman, M. C. & Dalton, W. S. Selection for drug resistance results in resistance to Fas-mediated apoptosis. Blood 89, 1854–61 (1997).
23. Catlett-falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A., Savino, R., Ciliberto, G., Moscinski, L., Ferna´ndez-Luna, J. L., Nunnez,

G., Dalton, W.S., & Jove, R. Constitutive Activation of Stat3 Signaling Confers Resistance to Apoptosis in Human U266 Myeloma Cells. Immunity 10, 105–115 (1999).

24. The Drug Development Process. (2015). Available at: http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm.
25. Li, Y. Y. & Jones, S. J. Drug repositioning for personalized medicine. Genome Med. 4, 27 (2012).
26. Sagers, J. Re-Engineering Cures for the Big Data Age: Precision Medicine and Computational Drug Repositioning. (2016). Available at: http://sitn.hms.harvard.edu/flash/2016/re-engineering-cures-big-data-age-precision-medicine-computational-drug-repositioning/.
27. Gan, F., Cao, B., Wu, D., Chen, Z., Hou, T. & X Mao, X. Exploring old drugs for the treatment of hematological malignancies. Curr. Med. Chem. 18, 1509–1514 (2011).
28. Ohlow, M. J. & Moosmann, B. Phenothiazine: The seven lives of pharmacology's first lead structure. Drug Discov. Today 16, 119–131 (2011).
29. Jaszczyszyn, A., Gasiorowski, K., Swaitek, P., Malinka, W., Ciezlik-Boczula, K., Petrus, J. & Czarnik-Matusewicz, B. Chemical structure of phenothiazines and their biological activity. Pharmacol. Reports 64, 16–23 (2012).
30. Srivalli, K. M. R. & Lakshmi, P. K. Overview of P-glycoprotein inhibitors : a rational outlook. Brazilian J. Pharm. Sci. 48, 353–367 (2012).
31. Qi, L. & Ding, Y. Potential antitumor mechanisms of phenothiazine drugs. Sci. China Life Sci. 56, 1020–1027 (2013).
32. Fond, G., Macgregor, A., Attal, J., Larue, A., Brittner, M., Ducasse, D. & Capdevielle, D. Antipsychotic drugs: Pro-cancer or anti-cancer? A systematic review. Med. Hypotheses 79, 38–42 (2012).
33. Haddad, P., Kirk, R. & Green, R. Chlorpromazine, the first antipsychotic medication: history, controversy and legacy. British Association for Psychopharmacology (2016). Available at:

https://www.bap.org.uk/articles/chlorpromazine-the-first-antipsychotic/.

34. Allen, H. Y., Everitt, Z. & Judd, A. Chlorpromazine Hydrochloride.

35. How Chlorpromazine Works. The Biology of Schizophrenia (2014). Available at: http://www.schizophrenia.com/research/slide17.htm.

36. Yde, C. W., Clausen, M. P., Bennetzen, M. V., Lykkesfeldt, A. E., Mouritsen, O. G. & Guerra, B. The antipsychotic drug chlorpromazine enhances the cytotoxic effect of tamoxifen in tamoxifen-sensitive and tamoxifen-resistant human breast cancer cells. Anticancer. Drugs 20, 723–35 (2009).

37. Bebawy, M., Morris, M. B. & Roufogalis, B. D. Selective modulation of P-glycoprotein-mediated drug resistance. Br. J. Cancer 85, 1998–2003 (2001).

38. Lee, M. S. Johansen, L., Zhang, Y., Wilson, A., Keegan, M., Avery, W., Elliott, P., Borisy, A. A. & Keith, C. T. The novel combination of chlorpromazine and pentamidine exerts synergistic antiproliferative effects through dual mitotic action. Cancer Res. 67, 11359–11367 (2007).

39. Lialiaris, T. S., Papachristou, F., Mourelatos, C. & Simopoulou, M. Antineoplastic and cytogenetic effects of chlorpromazine on human lymphocytes in vitro and on Ehrlich ascites tumor cells in vivo. Anticancer. Drugs 20, 746–51 (2009).

40. Chen, M., Yang, W. R., Lin, K., Liu, C., Liu, Y., Huang, K., Chang, P. M., Lai, J., Hsu, C., Chao, K., Kao, C. & Huang, C. F. Gene Expression-Based Chemical Genomics Identifies Potential Therapeutic Drugs in Hepatocellular Carcinoma. PLoS One 6, (2011).

41. Shin, S. Y., Lee, K. S., Choi, Y., Lim, H. J., Lee, H. G., Lim, Y. & Lee, Y. H. The antipsychotic agent chlorpromazine induces autophagic cell death by inhibiting the Akt/mtor pathway in human U-87MG glioma cells. Carcinogenesis 34, 2080–2089 (2013).

42. Lee, W., Lee, W., Cheng, C., Chen, K., Chou, C., Chung, C., Sun, M., Cheng, H., Ho, M. & Lin, C. Repositioning antipsychotic chlorpromazine for treating colorectal cancer by inhibiting sirtuin 1. Oncotarget 6, 27580–95 (2015).

43. Oliva, C. R., Zhang, W., Langford, C., Suto, M. J. & Griguer, C. E.

Repositioning chlorpromazine for treating chemoresistant glioma through the inhibition of cytochrome c oxidase bearing the COX4-1 regulatory subunit. Oncotarget 8, 37568–37583 (2017).

44. Wong, R. S. Y. Apoptosis in cancer : from pathogenesis to treatment. J. Exp. Clin. Cancer Res. 30, 87–101 (2011).
45. Ichim, G. & Tait, S. W. G. A fate worse than death: apoptosis as an oncogenic process. Nat. Rev. Cancer 16, 539–548 (2016).
46. BDbiosciences. Flow Cytometry Mitochondrial Membrane Potential Detection Kit Instruction Manual. 1–20 (2013).
47. Segawa, K. & Nagata, S. An Apoptotic ‘ Eat Me ’ Signal : Phosphatidylserine Exposure. Cell 25, 639–650 (2015).
48. Image Cytometry for Cell Cycle Analysis. Nexcelom Bioscience Available at: http://www.nexcelom.com/Applications/fluorescent-assays-3-image-cytometry-for-cell-cycle-analysis.php.
49. Lee, H., Kang, S. & Kim, W. Drug repositioning for cancer therapy based on large-scale drug-induced transcriptional signatures. PLoS One 11, 1–17 (2016).
50. De Mello, J. C., Moraes, W. V., Watashi, C. M., Silva, D. C., Cavalcanti, L. P., Franco M. K., Yokaichiya, F., Araujo, D. R. & Rodrigues, T. Enhancement of chlorpromazine antitumor activity by Pluronics F127/L81 nanostructured system against human multidrug resistant leukemia. Pharmacol. Res. 111, 102–112 (2016).
51. Zhelev, Z., Ohba, H., Bakalova, R., Hadjimitova, V., Ishikawa, M., Shinohara, Y. & Baba, Y. Phenothiazines suppress proliferation and induce apoptosis in cultured leukemic cells without any influence on the viability of normal lymphocytes: Phenothiazines and leukemia. Cancer Chemother. Pharmacol. 53, 267–275 (2004).
52. Dahl, S. G. Plasma Level Monitoring of Antipsychotic Drugs Clinical Utility. Clin. Pharmacokinet. 11, 36–61 (1986).
53. Elmore, S. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35,

495–516 (2007).

54. Furuno, T., Kanno, T., Arita, K., Asami, M. & Utsumi, T. Roles of long chain fatty acids and carnitine in mitochondrial membrane permeability transition. Biochem. Pharmacol. 62, 1037–1046 (2001).

55. Kim, J. S., He, L. & Lemasters, J. J. Mitochondrial permeability transition: A common pathway to necrosis and apoptosis. Biochem. Biophys. Res. Commun. 304, 463–470 (2003).

56. Walczak, H. Death Receptor – Ligand Systems in Cancer, Cell Death, and Inflammation. Cold Spring Harb Perspect Biol. 5, 1–18 (2013).

57. Lee, W., Lee, W., Cheng, C., Chen, K., Chou, C., Chung, C., Sun, M., Cheng, H., Ho, M. & Lin, C. The antipsychotic drug chlorpromazine enhances the cytotoxic effect of tamoxifen in tamoxifen-sensitive and tamoxifen-resistant human breast cancer cells. Oncotarget 20, 27580–95 (2015).

58. Tyagi, A. K., Strongly, S. & Human, S. To Arrest or Not To G 2 -M Cell-Cycle Arrest. 8, 3311–3314 (2002).

59. Shin, S. Y., Kim, C. G., Kim, S. H., Kim, Y. S., Lim, Y. & Lee, Y. H. Chlorpromazine activates p21Waf1/Cip1 gene transcription via early growth response-1 (Egr-1) in C6 glioma cells. Exp. Mol. Med. 42, 395–405 (2010).