INDUCTION OF APOPTOSIS AND CELL CYCLE ARREST ON U266 MULTIPLE MYELOMA CELL LINE BY PROCHLORPERAZINE

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ABSTRACT

INDUCTION OF APOPTOSIS AND CELL CYCLE ARREST ON U266 MULTIPLE MYELOMA CELL LINE BY PROCHLORPERAZINE

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Multiple myeloma (MM) is a plasma cell neoplasm accounting for 1% of all malignancies and 13% of hematological malignancies. Despite the introduction of potent anticancer agents, MM remains as an incurable disease. High frequency of relapses and acquisition of resistance to current chemotherapy create a need for the development of novel agents for MM treatment.

Prochlorperazine (PCP) is an FDA-approved phenothiazine drug, mainly used for the treatment of chemotherapy-associated nausea and vomiting. In addition, PCP was studied as a potent antitumor agent on various cancers such as melanoma, glioblastoma, colon and breast cancers. The aim of this study was to investigate the anticancer effect and mechanism of PCP on U266 MM cell line.

We first studied the anticancer potential of PCP on U266 cell line at various doses and time points. Next, three flow cytometric apoptosis assays; JC-1, Caspase 3 and PE Annexin V-7 AAD were performed. PCP's effect on cell cycle was examined with propidium iodide staining. As a part of the study, anticancer potential of cisplatin-PCP combination was also investigated.

PCP exhibited dose- and time-dependent inhibitory effect on U266 cell viability. IC50 of PCP was calculated as 21.8 ± 0.8 μM. It was demonstrated that PCP exerted cytotoxic action through inducing apoptosis. No change in mitochondrial membrane potential was observed with JC-1 MMP assay which suggested activation of extrinsic apoptotic pathways by PCP. Cell cycle analysis indicated that exposure of U266 cells to PCP resulted in cell cycle arrest at G_2/M phase. It was also demonstrated that PCP-cisplatin combination exhibited additive effect. These results indicated that PCP has potent anticancer activity alone and in combination with cisplatin on U266 MM cells. Further in-depth mechanistic studies and *in vivo* experiments are warranted to evaluate its therapeutic potential.

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

ÖZ

PROKLORPERAZİN'İN U266 MULTİPL MİYELOM HÜCRE HATTINDAKİ APOPTOTİK VE HÜCRE DÖNGÜSÜ ARRESTİ ETKİSİ

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Multipl miyelom (MM) plazma hücresi tümörü olup, bütün malignitelerin %1'ini ve hematolojik malignitelerin %13'ünü oluşturmaktadır. Yeni nesil başarılı kemoterapi ilaçlarına rağmen, hala tedavisi mümkün olmayan bir hastalıktır. Sıklıkla karşılaşılan nüksler ve kemoterapi ilaçlarına karşı geliştirilen direnç sebeplerinden dolayı MM tedavisinde kullanılacak yeni ilaçların geliştirilmesi önem taşımaktadır.

Proklorperazin (PCP) FDA onaylı antiemetik bir ilaç olup sıklıkla kemoterapiye bağlı bulantı ve kusma tedavilerinde kullanılmaktadır. Bunun dışında, Proklorperazin'in melanom, glioblastom, kolon ve meme kanseri hücre hatları üzerinde etkili bir antikanser ajanı olduğu gösterilmiştir. Bu çalışmada, proklorperazin'in U266 multipl miyelom hücre hattındaki antikanser etki ve mekanizmalarının çalışılması amaçlanmıştır.

İlk olarak, PCP'nin U266 hücre hattı üzerindeki doz ve zamana bağlı antikanser potansiyeli çalışılmıştır. PCP'nin apoptotik etkisi üç ayrı akış sitometri apoptoz çalışması ile gösterilmiştir. PCP'nin hücre döngüsü üzerindeki etkisi PI boyası ile

analiz edilmiştir. Son olarak, Sisplatin-PCP kombinasyonun antikanser potansiyeli araştırılmıştır.

Yapılan çalışmada, PCP'nin U266 MM hücre canlılığını doz ve zamana bağlı olarak inhibe ettiği gösterilmiştir. PCP IC50 değeri 21.8 ± 0.8 μM olarak hesaplanmıştır. PCP sitotoksik etkisinin apoptoz aktivasyonu aracılığıyla olduğu gösterilmiştir. JC-1 MMP analizinde elde edilen sonuçlar doğrultusunda mitokondriyal membran potansiyelinin değişmediği görülmüş ve PCP'nin ekstrinsik apoptoz yolaklarını aktive edebileceği önerilmiştir. Hücre döngüsü arresti çalışmaları sonucunda PCP'nin U266 hücre döngüsünü G2/M aşamasında durdurduğu gösterilmiştir. Son olarak, Sisplatin-PCP kombinasyonun hücre canlılığı üzerinde ilave etkisi etkisi olduğu bulunmuştur. Çalışmada elde edilen sonuçlar, PCP'nin potent bir U266 antikanser ajanı olduğunu göstermektedir. Bu doğrultuda, etki mekanizmalarının daha kapsamlı ve detaylı çalışılması ve *in vivo* çalışmalarının yapılması, PCP'nin terapötik potansiyelinin araştırılması açısından önem taşımaktadır.

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

To my beloved family,

who always support me in all aspects of my life

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LIST OF ABBREVIATIONS

AML: Acute Myeloid Leukemia

AMPK: 5' AMP-activated Protein Kinase

BM: Bone Marrow

BMSC: Bone Marrow Stromal Cells

CaM: Calmodulin

CML: Chronic Myeloid Leukemia

CNS: Central Nervous System

CPZ: Chlorpromazine

CTZ: Chemoreceptor Trigger Zone

Cyt C: Cytochrome C

DCEP: Dexamethasone, Cyclophosphamide, Etoposide, Cisplatin

DMSO: Dimethyl Sulphoxide

DNA: Deoxyribonucleic Acid

DOX: Doxorubicin

FDA: Food and Drug Administration

ECM: Extracellular Matrix

EGFR: Epidermal Growth Factor Receptor

GABA: Gamma-aminobutyric Acid

H_2O_2: Hydrogen Peroxide

IFN-γ: Interferon Gamma

IGF-1: Insulin-like Growth Factor 1

IL-6: Interleukin 6

IMiDs: Immunomodulatory Imide Drugs

MDRi: Multi Drug Resistant Inhibitor

MIC: Minimum Inhibitory Concentration

MM: Multiple Myeloma

MMGI: Multiple Myeloma Genetics Initiative

MMP. Mitochondrial Membrane Potential

MRP-1: Multidrug Resistance-associated Protein 1

MT: Metallothionein

mTOR: Mammalian Target of Rapamycin

NFκB: Nuclear Factor Kappa-light-chain-enhancer of Activated B cells

PARP: Poly ADP Ribose Polymerase

PBS: Phosphate-buffered Saline

P-gp: P-glycoprotein

PCP: Prochlorperazine

Phts: Phenothiazines

PKC: Protein Kinase C

PS: Phosphatidylserine

TGF-β: Transforming Growth Factor Beta

TFP: Trifluoperazine

TNF-α: Tumor Necrosis Factor α

VEGF: Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

1.1. CANCER

Cancer is a disease which arises when the cells begin to divide in uncontrollable fashion. Normally, cells are programmed to die when they are old or become damaged. However, cancer cells continue to divide and form new cells which are also abnormal or damaged. These cells can divide without stopping and eventually form tumors.

Cancer can start almost anywhere in the body. There are more than 100 types of cancer. Cancers can be classified according to the site of origin or the tissue type. International Classification of Diseases for Oncology, Third Edition (ICD-O-3) is taken as the international standard for the classification and nomenclature of histologies. According to ICD-O-3, there are six major categories of cancers based on tissue type: carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed type cancers [1].

Cancer cells are different from the normal cells in number of ways. All cancer cells share 6 common traits or “hallmarks” that collectively dictate malignant growth. These capabilities are; self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, induced angiogenesis, and tissue invasion and metastasis [2]. Increased research about cancer through the last decade suggested two additional hallmarks; deregulation of cellular energy metabolism and evasion of immune destruction [3].

These acquired characteristics of cancer cells provide a basis for drug resistance mechanisms. Resistance and relapse are the major challenges in cancer treatment. Understanding and identifying the mechanisms that enable cancer cells to develop resistance to therapy will enable researchers to improve treatment strategies.

Drug resistance is classified into two categories: innate and acquired [4]. Cancer cells acquire resistance through different mechanisms including alteration of drug targets, transport and metabolism and deregulation of apoptotic pathways (Figure 1) [5].

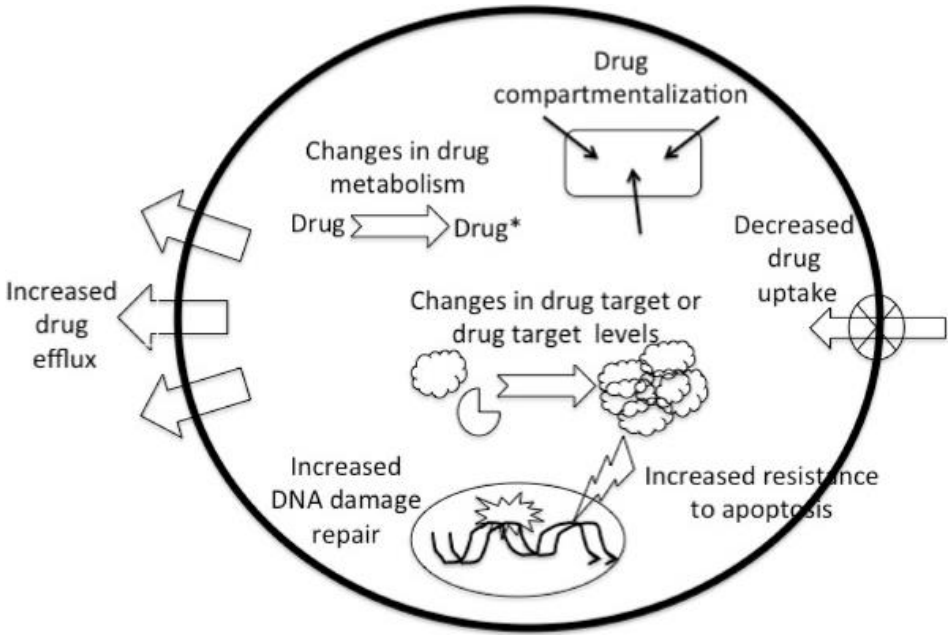

Figure 1. The major cellular drug resistance mechanisms [5]

Most of the drugs are taken into cells through cell surface receptors and transporters. Decreased levels of transporters or mutations which impair their activity, therefore, will result in decreased drug uptake. On the other hand, increase in drug efflux is also frequently observed in cancer cells. Elevation in levels of transporters such as P-glycoprotein (P-gp, or MDR1) and multidrug resistance-associated protein 1 (MRP-1) has been associated with resistance to various drugs [4]. For example, P-gp is responsible for the transport of doxorubicin, taxol and vincristine; and elevated P-gp levels has been linked to resistance against these agents. Most of the anticancer agents are metabolized to their active forms by enzymes within the cells.

Downregulation or inactivation of drug metabolizing enzymes is another way of cancer cells to inactivate drugs. For example, cisplatin, and other platinum-based drugs, is inactivated by binding to metallothionein (MT).

Targeting certain oncogenes with anticancer agents is one of the successful cancer treatment strategies. Examples include imatinib targeting BCR/ABL tyrosine kinase in chronic myeloid leukemia (CML), and gefitinib and erlotinib targeting the epidermal growth factor receptor (EGFR) tyrosine kinase domain in non-small cell lung carcinoma [6]. Mutations in the target proteins might prevent drug binding, therefore, the effectiveness of drug will be lowered.

Deregulation of apoptotic signals is another way of cancer cells to achieve resistance. Normally, cells monitor the extracellular and intracellular environments through several cell surface receptors and intracellular signaling pathways; and determine whether they live or die according to pro-survival or apoptotic signals. Apoptosis results from activation of extrinsic (death receptor-mediated) or intrinsic (mitochondria-mediated) pathway. Extrinsic pathway is activated when ligands such as FAS and TNF-α bind to their corresponding receptors. Intrinsic pathway, on the other hand, activated through DNA damage, hypoxia or oxidative stress. Depolarization of mitochondrial membrane and subsequent cytochrome c release into cytosol are the characteristics of intrinsic pathway. Caspases are the effector proteins function in both pathways. Caspase-3 -executer caspase- is common to both pathways, while initiator caspases Caspase-8 and Caspase-9 are activated in extrinsic and intrinsic pathways, respectively (Figure 2).

Intrinsic pathway is regulated by Bcl-2 family proteins. This family is divided into two groups: pro-apoptotic (e.g. Bax, Bad) and anti-apoptotic (e.g. Bcl-2, Bcl-x_L). Another protein, p53, also regulates the activation of Bcl-2 family proteins. P53 is activated in response to DNA damage, which in turn activates pro-apoptotic Bcl-2 proteins. Cancer cells develop resistance to apoptosis through deregulations of these pathways. Loss of tumor suppressor p53 function is the most common strategy which

is observed in more than 50% of the cancers. Other ways include increasing the expression of anti-apoptotic regulators or decreasing the pro-apoptotic ones [6].

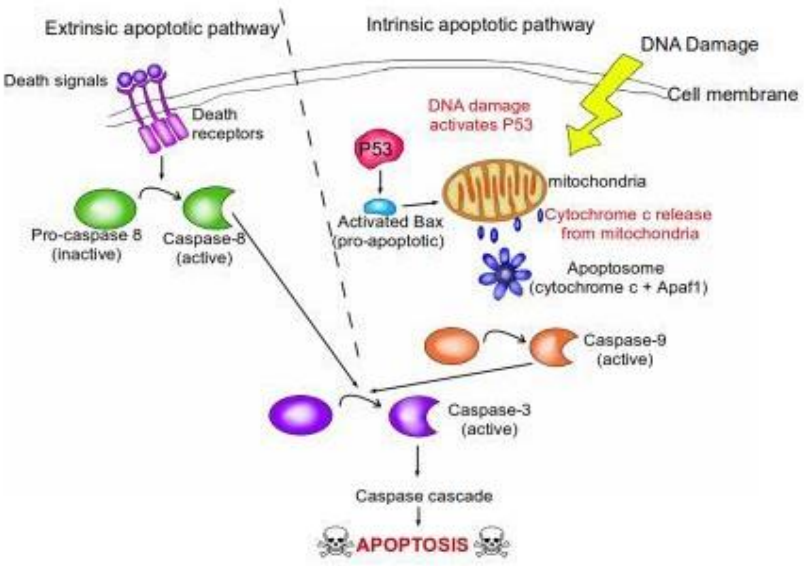

Figure 2. Overview of extrinsic and intrinsic apoptotic pathways [6]

Another way of cancer cells to become resistant involves DNA damage repair mechanisms. Many anticancer agents induce DNA damage directly or indirectly. For example, cisplatin induces direct damage by formation of DNA adducts. Cell cycle arrest due to DNA damage allows cells to repair cisplatin-induced damages in the DNA. In this context, combination of DNA damaging agents with the inhibitors of DNA damage response machinery is one way of overcoming this resistance [7].

Development of cross resistance is another problem that limits the effectiveness of current therapies. It happens when the development of resistance to one drug leads to resistance of another drug. When the first therapy fails due to development of resistance by increasing the levels of drug transporters, this situation will affect the efficacy of the following treatments. In this case, treatment options should include the use of drug that is not recognized by transporters. Use off efflux blockers is another strategy to overcome resistance and increase the effectiveness of follow-up treatments [7].

1.2. MULTIPLE MYELOMA

Multiple myeloma is a B cell tumor that develops in the plasma cells found in bone marrow (Figure 3). Damaged plasma cells transform into multiple myeloma cells through a multi-step process. The primary role of plasma cells is to produce antibodies in response to foreign antigens. Likewise, MM cells produce abnormal antibodies called M proteins. M proteins outnumber the functional antibodies, accumulate in the blood and urine, and trigger damage in bone marrow, kidney and other organs [8].

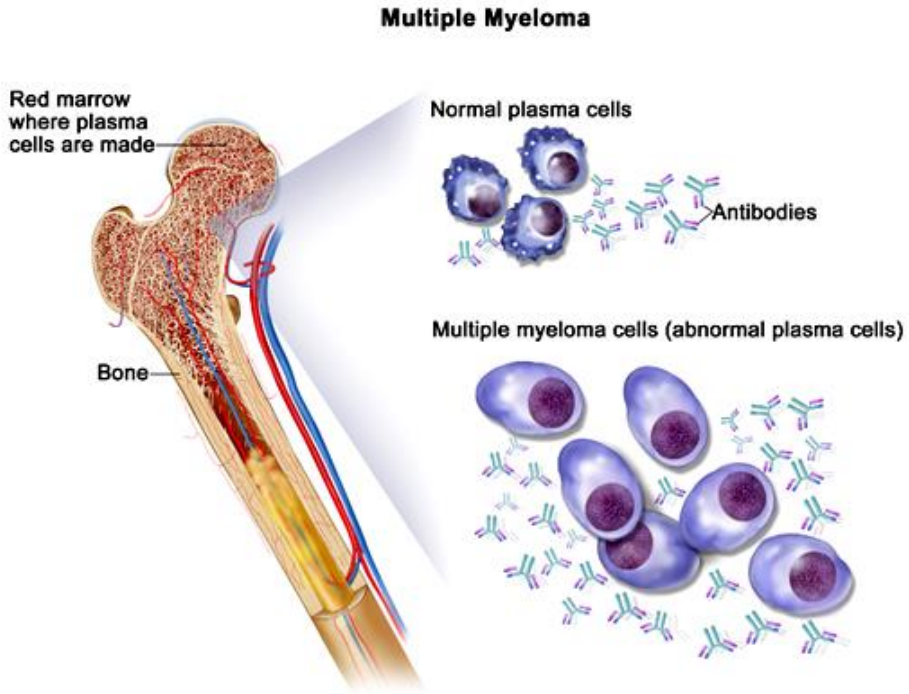

Figure 3. Comparison of healthy plasma cells and MM cells in bone marrow [8]

MM is the second most common (13% of all) hematological cancer; and accounts for 1.8% of all new cancer cases and 2.1% of all cancer-related deaths worldwide. Statistical models stated that rates for new myeloma cases have been rising on average 0.8% each year over the last 10 years. In 2016, it is estimated that there will be 30,330 new cases of myeloma. Five-year relative survival rate is approximately 50%. MM is most frequently diagnosed among people aged 65-74. Men have higher incidence of MM than women [9].

MM is a slow progressing disease, and patients at early stages of MM are often presenting no visible signs. MM symptoms and complications, although they may vary by patient, include fracture and lesions in bone, low blood count (anemia), impaired immunity, hypercalcemia and renal failure. The exact causes of MM are still unclear. In 2006, a progressive genome-mapping program called Multiple Myeloma Genetics Initiative (MMGI) was launched to improve the understanding of disease progression and to accelerate the development of new therapies [10]. Although MM still remains as an incurable cancer, survival rates are improving by the introduction of new treatment strategies.

MM treatment options include drug therapies, stem cell transplantation, radiation therapy and surgery. Five drug classes are currently used for the treatment of myeloma; immunomodulatory drugs, proteasome inhibitors, chemotherapy, histone deacetylase inhibitors and steroids. They are generally administered as double or triple combinations. New drugs and combinations are constantly tested to improve the patient outcome and the survival rate [11]. In recent years, introduction of agents such as proteasome inhibitor bortezomib; and immunomodulatory drugs (IMiDs) thalidomide and lenalidomide has changed the management of myeloma (Figure 4). The use of these drugs is associated with better outcomes such as extended remission and survival time.

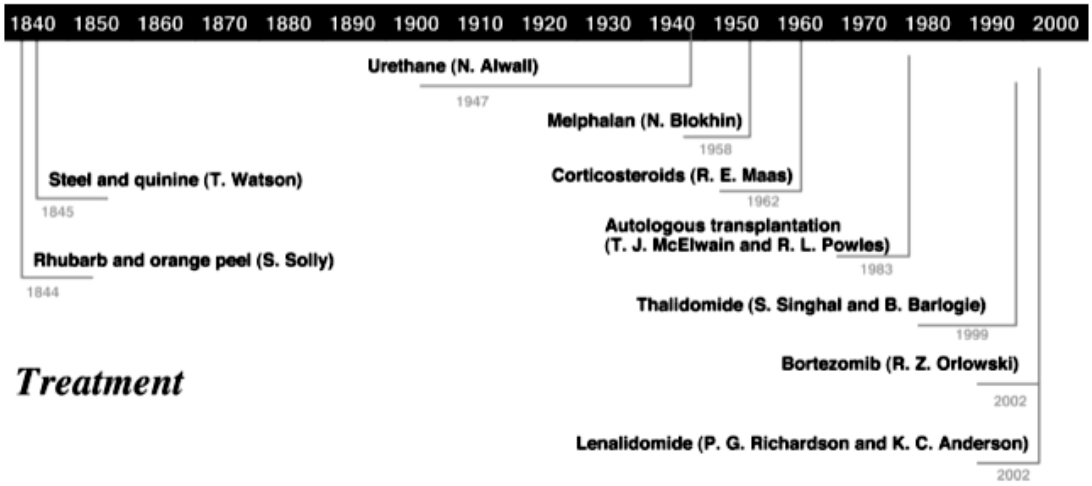

Figure 4. Timeline depicting the treatment of MM [11]

MM arises due to certain genetic changes that occur during differentiation of B cells into plasma cells. A chromosomal translocation of oncogene immunoglobulin heavy chain gene on chromose 14 (IgH translocation) was observed in more than half of the cases [12]. The resulting overexpression of oncogene causes abnormal cellular proliferation. Hyperdiploidy which is the trisomy of odd numbered chromosomes (3, 5, 7, 9, 11, 15, 19 and 21) was also encountered in MM. As myeloma develops, further mutations occur such as Ras mutation which also contribute to malignant growth of MM cell.

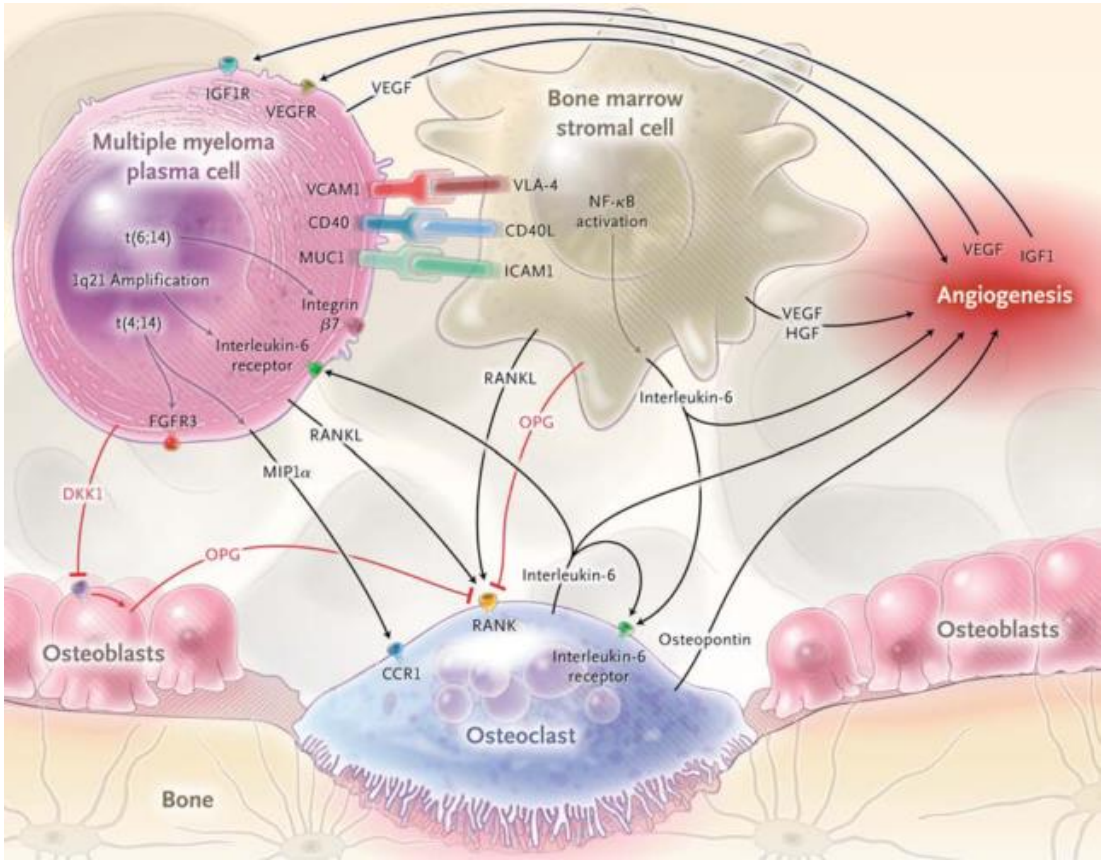

Figure 5: Interaction between plasma cells and bone marrow in MM [13]

Bone marrow (BM) microenvironment is crucial for the growth and survival of MM cells (Figure 5). The mutations caused changes in the expression levels of adhesion molecules and cell surface receptors (e.g. integrins, cadherins, selectins) that affect

the interactions of MM cells with the stromal cells and extracellular matrix (ECM) proteins in tumor microenvironment. The adhesion of MM cells to stromal or hematopoietic cells resulted in the autocrine or paracrine secretion of several cytokines and growth factors including IL-6 and IL-10, VEGF, TNF superfamily proteins and TGF-β. They mediate tumor growth, survival, migration and drug-resistance properties of MM cells [11]. In addition, adhesion of MM cells to ECM proteins resulted in the upregulation of cell-cycle regulatory and anti-apoptotic proteins. The upregulation of proangiogenic factors such as vascular endothelial growth factor (VEGF) enhances the angionesis in BM microenvironment.

Since MM cells are dependent on their interactions with the tumor microenvironment, new MM drugs are developed to target and disrupt this interaction. Proteasome inhibitors and immunomodulatory drugs exert their anti-myeloma activity through inhibition of signaling pathways that are important for MM growth and survival.

Bortezomib, the first proteasome inhibitor approved for MM treatment, inhibits MM signaling by targeting NFκB and its downstream pathways. Bortezomib inhibits cell cycle progression, growth ad DNA damage repair in MM cells; and induces both Caspase-8 and Caspase-9 dependent apoptosis and heat shock protein response [14]. Moreover, Bortezomib targets BM microenvironment; prevents adhesion and interaction of MM cells with stromal cells. In addition, Bortezomib affects the cytokine feedback loops mediated by IL-6, VEGF, IGF-1 and TNF-α, therefore affect the survival of MM cells. Downregulation of angiogenesis and inhibition of DNA repair mechanisms are the other anti-myeloma activities of Bortezomib [11].

Immunomodulatory agents Thalidomide and Lenalidomide affects MM through different mechanisms. They may directly interact with MM or stromal cells in the microenvironments and prevent adhesion of MM cells and prosurvival cytokine secretion. Caspase-8 mediated apoptosis induction is also one mechanism of action of IMiDs. Secondly, they exhibit anti-angiogenesis activity through inhibition of TNF-α and NFκB signaling pathways. They also stimulate an enhanced immune

response to myeloma cells by T cells and natural killer (NK) cells, via induction of IL-2 and IFN-γ secretion [14]. Most recently, the E3 ubiquitin ligase cereblon and its targets the Ikaros transcription factors IKZF1 and IKZF3 have been identified as the molecular target of lenalidomide.

Despite the promising therapy options, MM still remains as an incurable disease due to high frequency of relapses. Different treatment strategies are utilized with same or different drugs to treat relapsed or refractory myeloma. Combination therapy with dexamethasone and either bortezomib or lenalidomide is one of the treatment of choice for those patients [13]. Dexamethasone, cyclophosphamide, etoposide and cisplatin (DCEP) combination was also one of the successful treatment options for relapsed or refractory MM patients [15]. Autologous transplantation is another option for patients who did not undergo transplantation at diagnosis.

Resistance to initial chemotherapy is another common problem observed in MM therapy. The resistance to initial treatment with bortezomib, thalidomide or lenalidomide could be overcomed with the combination of lenalidomide, bortezomib, and dexamethasone. The efficacy of this combination could be enhanced by the addition of cyclophosphamide, melphalan, or doxorubicin [13].

1.2.1. U266 CELL LINE

U266 cell line has a mutant p53 alelle with a codon 161 mutation. In addition, U266 cells express high levels of anti-apoptotic protein Bcl-xL which makes these cell inherently resistant to apoptosis. As a multiple myeloma cell line, U266 cells are dependent on various cytokines to grow and survive. Interleukin-6 (IL-6) signaling is essential for U266 cells which involves JAK-STAT proteins; and a STAT family member Stat3 is constitutevely activated in U266 cells. It was demonstrated that the inhibition of IL-6 signaling through JAK-STAT pathway inhibits Bcl-xL expression and induces apoptosis [16]. Moreover, U266 cells are resistant to Fas-mediated mediated apoptosis regardless of the high expression of Fas receptors which was attributed to high expression levels of Bcl-xL antiapoptotic protein [16].

IL-6 promotes myeloma cell growth through autocrine and paracrine feedback loops. Major IL-6 producers in BM microenvironment are bone marrow stromal cells (BMSCs). The adhesion of MM cells to BMSCs involves NF-κB signaling; and is the driving factor for IL-6 production by BMSCs [17]. Having a mutant p53 allele, U266 cells can also provide an autocrine source of IL-6. It was demonstrated that stable expression of wild-type (wt) p53 in U266 cells caused decrease in IL-6 gene expression, induced cell cycle arrest and significant gowth inhibition in U266 cells [18]. Addition of exogenous IL-6 restored the growth of myeloma cells.

1.3. DRUG REPURPOSING

Cancer remains as one of the leading causes of mortality worldwide. Governments and pharmaceutical research companies invest tremendous resources for the development of new cancer drugs. The launch of a single drug to market takes approximately 13 years and costs around US $1.8 billion. Moreover, only 5% of prospective anticancer agents are entering Phase I clinical trials and approved by FDA [19]. Due to the high cost and high failure rates, alternatives for *de novo* drug discovery are required.

Drug repurposing is the identification of new therapeutic indications for already approved drugs. This strategy has many advantages over traditional *de novo* drug discovery. Since the safety of the drug has already been proven by preclinical and clinical studies, its repositioning will be more efficient, cost-effective and less time-consuming. Pharmaceutical companies prefer this strategy since repurposing minimizes the risk of clinical and post-marketing failures of drugs. Patients also benefits from repurposing that might reduce safety risks and speed up successful access to treatment.

Drug repositioning is either performed as on-target (the known activity is associated with different clinical application) or off-target (a new activity is discovered which led to novel applications). Thalidomide is a well-known example for off-target repurposing [20]. Both computational and experimental methods are used for drug

repositioning. Pharmacological, genomic, phenotypic, chemical, and clinical information from various data sources are gathered with computational methods to select candidate compounds, which will later be studied with *in vitro* and *in vivo* experimental methods.

Drug repurposing has a broad range of application in oncology. Table 1 summarizes some of the successful repurposed drugs [19].

Table 1. Original and new anticancer indications of repurposed drugs

Drug	Original Indication	New anticancer Indication
Thalidomide	Antiemetic for pregnancy	Multiple myeloma
Aspirin	Analgesic, antipyretic	Colorectal cancer
Valproic acid	Antiepileptic	Leukemia, solid tumors
Celecoxib	Rheumatoid arthritis	Colorectal cancer, lung cancer
Statins	Myocardial infarction	Prostate cancer, leukemia
Metformin	Diabetes mellitus	Breast, adenocarcinoma, prostate, colorectal cancer
Rapamycin	Immunosuppressant	Colorectal cancer, lymphoma, leukemia
Methotrexate	Acute leukemia	Osteosarcoma, breast cancer, Hodgkin lymphoma
Zoledronic acid	Anti-bone resorption	Multiple myeloma, prostate, breast cancer
Leflunomide	Rheumatoid arthritis	Prostate cancer
Wortmannin	Antifungal	Leukemia
Minocycline	Acne	Ovarian cancer, glioma
Vesnarinone	Cardioprotective	Oral cancer, leukemia
Thiocolchicoside	Muscle relaxant	Leukemia, Multiple myeloma
Nitroxoline	Antibiotic	Bladder, breast cancer
Noscapine	Antitussive, antimalarial, analgesic	Multiple cancer types

Thalidomide is one of the successful examples for drug repurposing strategy. It was originally used as antiemetic for morning sickness in pregnancy in 1950s [21]. However, severe teratogenic side-effects were observed in newborns infants such as phocomelia; and thalidomide was removed from the market in 1961. Thalidomide regained interest back in 1990s as antineoplastic agent due to discovery of its anti-inflammatory, immunomodulatory and anti-angiogenic properties. After series of successful clinical trials, thalidomide was approved by FDA for MM treatment. More potent analogues of thalidomide such as lenalidomide were also developed. The introduction of Thalidomide and its analog Lenalidomide is considered as a major breakthrough in MM treatment. They are currently used as frontline therapy for MM. They also show promising results for other hematological malignancies such as acute myeloid leukemia (AML) as well as some solid tumors [22].

Metformin is another successful example for drug repositioning strategy. The drug has been widely used as first line therapy for type 2 diabetes for more than 30 years [23]. Metformin posses anti-hyperglycemic activity which accounts for its antidiabetic action. At molecular level, metformin was demonstrated to activate AMP-activated protein kinase (AMPK) pathway. mTOR is a serine/threonine kinase involved in cancer cell survival, proliferation and growth, and is negatively regulated by AMPK. This property led researchers to evaluate efficacy of metformin in cancer patients prescribed with this drug [19]. There is substantial *in vitro* and *in vivo* preclinical evidence showing the antitumor potential of metformin through inhibition of cellular proliferation. It has also been observed that metformin activates the T cell mediated immune response against cancer cells. Epidemiologic data have demonstrated decreased cancer incidence and mortality in cancer patients taking metformin. The therapeutic potential of metformin in prostate, breast, endometrial, and pancreatic cancers is currently being evaluated in several clinical trials.

1.4. PHENOTHIAZINES

Phenothiazines are group of drugs that possess diverse biological activities. They are primarily used as antipsychotics due to their dopaminergic receptor blockade activity. Phenothiazine derivatives had been used for different purposes before they were utilized as antipsychotics (Figure 6) [24].

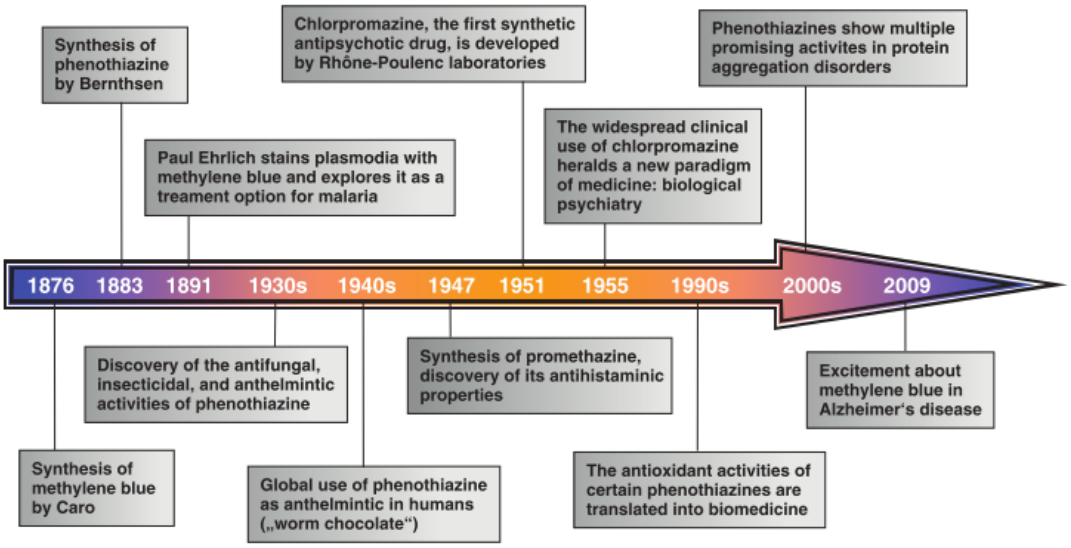

Figure 6. Timeline of important developments with regard to phenaothiazines

Phenothiazines (Phts) are dopamine receptor antagonists. They also interact with other receptors in central nervous system (CNS) such as serotonin, α-adrenergic, muscarinic and GABA-ergic receptors. However, the strongest affinity is for dopaminergic receptors, because the structure of dopamine and phenothiazines are similar to some extent (Figure 7) [25].

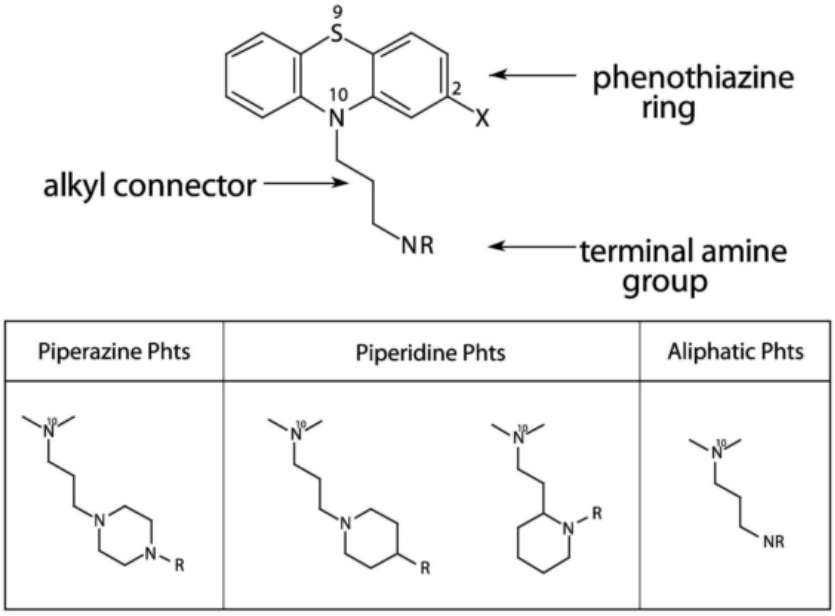

Figure 7. General structure of phenothiazines [25]

Due to their hydrophobic nature, Phts can pass thorough lipid bilayers which enables them to cross blood-brain barrier and to be used as neuroleptic drugs. The intensity of neuroleptic action is determined by the lipophilicity and the structure of the substituents in the side chain. The activity is the highest at Phts with piperazine group and lowest at Phts with aliphatic side chain. Therefore, piperazine phenothiazines possess the strongest antipsychotic activity.

Phts are also inhibitors of Calmodulin (CaM), Protein Kinase-C (PKC) and P-glycoprotein (P-gp). These properties account for their anti-proliferative, apoptotic and multidrug resistance (MDR) inhibitory activities [26].

CaM is a calcium-binding protein and regulates calcium-dependent signaling pathways by interacting with several other proteins. cGMP, cAMP, CaM-dependent protein kinase, ATPase and phospholipase A_2 are some examples to CaM-activated enzymes. Phts inhibit these enzymes' activities through inhibition of CaM [27]. Several studies showed that the antiproliferative effects of phenothiazines is correlated with their CaM-inhibiting properties.

Multidrug Resistance (MDR) is defined as the ability of the cancer cells to develop resistance against certain drugs or structurally-related drug groups; and it is one of the major problems in cancer chemotherapy. Cancer cells mainly achieve this resistance through increased drug efflux by Pgp. Phenothiazines are MDR inhibitors. They increase cellular sensitivity to cytotoxic drugs (they restore the drug sensitivity of neoplastic cells), mainly by a strong inhibition of the Pgp-dependent mechanism of MDR [28]. Several *in vitro* studies showed that trifluoperazine, chlorpromazine and prochlorperazine significantly reversed MDR at the IC_{50} concentrations of 1 to 10 μM [29]. Two possible mechanisms are proposed for the MDR-reversal by Phts; direct interaction with P-gp and modulation through interaction with membrane phospholipids [30]. Trifluoperazine inhibits MDR by directly interacting with Pgp, whereas Fluphenazine achieves that by interacting with membrane phospholipids and increasing the fluidization of the membrane [25].

Antitumor potential of phenothiazine derivatives (trifluoperazine, chlorpromazine, thioridazine, fluphenazine and prochlorperazine) was previously on several cancers such as colon, lung and breast cancers as well ass melanoma, glioblastoma, leukemia and lymphoma. The mechanism of antitumor potential of some phenothiazine derivatives was also studied. It was demostrated that they exert their antitumor activity mainly by targeting Wnt, MAPK and retinoic acid signaling pathways; and altering the expression levels of downstream signaling molecules [31].

Another mechanism was proposed for Phts antitumor activity in a study conducted by Zong et. al. [32]. They showed that phenothiazine drug trifluoperazine (TFP) decreased cell viability and induced cell death in small cell lung carcinoma (SCLC). This effect was mediated through phenothiazine-induced lysosomal dysfunction rather than apoptosis induction. Moreover, this effect of Phts was reported to be independent of p53 status and not affected by resistance to chemotherapeutic agents. These findings represented a novel contex-dependent activity of Phts.

1.4.1. PROCHLORPERAZINE

Prochlorperazine (PCP) is an FDA-approved antiemetic drug that is currently used in the treatment of chemotherapy-induced nausea and vomiting.

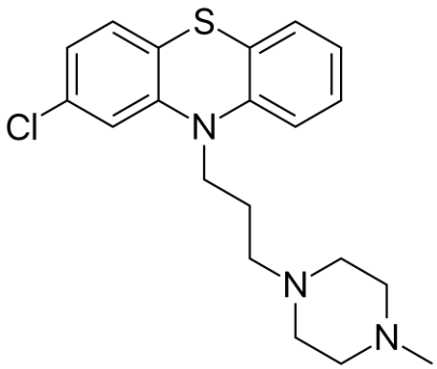

Figure 8. Prochlorperazine molecular structure [33]

PCP is a synthetic phenothiazine derivative. The phenothiazine class drugs are mostly used as antipsychotics as indicated in the previous section. However, PCP is primarily used as antiemetic agent rather than an antipsychotic drug. Due to its antiemetic properties, PCP is also used for the vertigo treatment.

As a phenothiazine class drug, PCP has a dopamine receptor blocking activity. PCP block dopaminergic D_2 receptors in the CNS which accounts for its antipsychotic and antiemetic properties. Blockade of dopamine D_2-receptor in the chemoreceptor trigger zone (CTZ) of the brain by PCP prevents chemotherapy-induced emesis [34]. Moreover, PCP causes sedation, muscle relaxation, and hypotension by blocking alpha (1)-adrenergic receptors, anticholinergic and alpha-adrenergic receptors [35].

PCP was also demonstrated to have antimicrobial activity against both Gram positive and Gram negative bacteria [36]. The minimum inhibitory concentration (MIC) of PCP was determined by agar dilution method, which ranged from 25 to 200 mg/ml with respect to most of the strains. Due to this property, PCP is included in the antibacterial chemotherapeutics group of drugs.

In addition to its primary clinical use as an antiemetic drug, PCP also act as potent antitumor agent. Prochlorperazine was shown to have an inhibitory effect on melanoma, glioblastoma, colon and breast cancers [37]–[40]. Its anticancer mechanisms mainly attributed to calmodulin inhibition and multidrug resistance reversal properties.

It was indicated that PCP inhibited DNA synthesis in B16 Melanoma cells [37]. Significant reduction in [^{3}H] Thymidine uptake was observed during the first two hours of PCP treatment, and this effect was maintained at 48 h. IC50 of PCP was found as 13 μM at 48 h while the IC50 of W7, a more specific calmodulin antagonist, was 40 μM. Inhibitory effect of the agents on cell proliferation was reversed with addition of extracellular calmodulin. Therefore, the anticancer mechanism of action was attributed to PCP's calmodulin inhibition ability.

PCP's inhibitory effect on glioblastoma cells was also studied [38]. In this study, cell viability analysis was performed with MTT and clonogenic assays. PCP and several other phenothiazine derivatives were tested at doses in the range of 1 to 10 μM. PCP was indicated as a potent anti-glioblastoma agent with IC50 < 10 μM. They also studied the anticancer mechanism of PCP on glioblastoma cell line. It was reported that LC3-II protein (autophagy marker) was significantly upregulated in cells treated with PCP, which suggested the induction of autophagy pathway by PCP.

In a different study, PCP's antiproliferative effect on tamoxifen-resistant breast cancer cells was reported [39]. It was indicated that resistance to endocrine therapy is a common problem observed in estrogen receptor (ER)-positive breast tumors. Novel agents and treatment strategies are needed in the field to overcome this resistance. Herein, phenothiazines was reported as potent anticancer agents that inhibits proliferation of tamoxifen resistant breast cancer cells. MCF-7 cells were treated with increasing doses of PCP (1 to 10 μM) and cell viability was analyzed with methylene blue staining. Resistance was associated wth increased expression levels of cyclin E2 protein. PCP treatment at indicated doses also reduced cyclin E2 levels in MCF-7 cell line. PCP's anticancer mechanisms was suggested in relation to

inhibition of calmodulin and prostaglandin synthesis. Since they both have potential impact on estrogen receptor (ER) function and their inhibition by PCP was suggested to alter response to endocrine therapy.

Multidrug reversal properties of phenothiazine agents were demonstrated in various studies. In this context, MDRi activity of PCP was also studied. It was demonstrated that PCP is an effective doxorubicin-efflux blocker [41]. Co-incubation of DOX-resistant P388 leukemia cells with 1 μM PCP and DOX increased the retention and effectiveness of doxorubicin. The combination of PCP and DOX was shown to have synergistic effect on doxorubicin-resistant colon cancer cell lines [40]. In this study, combination potential of PCP was investigated. Various combinations of PCP (1.5 - 40 μM) and DOX (0.5 – 5 μM) were tested; and moderate to high synergistic effect was observed with CI < 1. It needs to be highlighted that the MDR reversal property makes PCP a good candidate for the treatment of drug resistant cancers.

PCP's anticancer potency has been demonstrated on several human cancer cell lines as indicated above. To our knowledge, there is no study in the literature reporting the potency of PCP on multiple myeloma.

1.5. AIM OF THE STUDY

The aim of this study is to investigate the anticancer potential and mechanism of prochlorperazine on U266 multiple myeloma cell line.

CHAPTER 2

MATERIALS AND METHODS

2.1. CHEMICALS

Prochlorperazine (P9178) and Hydroxyzine (H8885) were purchased from Sigma-Aldrich. Cisplatin (10471) was purchased from Alfa Aesar. Prochlorperazine stock solutions (10 mM) were prepared in DMSO and stored at -20ºC. Cisplatin stock solutions (1 mM) were prepared in 0.9% NaCl and stored at room temperature. For combination studies only, Prochlorperazine was dissolved in 100% MeOH as 1.5 mM stock solutions. The final DMSO and methanol concentrations used in the assays were fixed to 2%.

2.2. CELL CULTURE

U266 Multiple myeloma cells were maintained in RPMI-1640 growth medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% non-essential amino acid solution (Sigma-Aldrich) and 2.5 µg/mL plasmocin prophylactic (InvivoGen). The cells were cultured in T-25 and T-75 sterile tissue culture flasks at 37 ºC temperature and 5% CO_2 with humidified Thermo Scientific (USA) incubator.

2.3. CELL VIABILITY ASSAYS

2.3.1. CellTiter Blue Cell Viability Assay

Compound and drug screening assays, potency (IC_{50}) determination, time-response and combination assays were completed using CellTiter Blue Cell Viability Assay (Promega, USA). The cells were treated with drug molecules at a density of 100.000 cells/mL and incubated for 12, 24 and/or 48 hours (depending on the assay) at previously mentioned cell culture conditions. 96-well microplates were used; and five technical replicates were prepared for each measurement. Cells were incubated with assay reagent for additional 4 h at the end of treatment durations. Fluorescent reads were taken with SpectraMax® Paradigm® Multi-Mode Microplate Reader. Excitation and emission wavelengths were 555 nm and 595 nm, respectively. IC50 values were calculated on GraphPad (La Jolla, USA) Prism v5.0 using non-linear curve fitting model.

U266 cells incubated with 2% DMSO (and 2% MeOH for combination studies only) was used as negative control and denoted as untreated. Cell viability of control (untreated cells) was set to 100%; and the viability of PCP-treated cells was normalized according to viability of control.

2.3.2. Multitox-Fluor Multiplex Cytotoxicity Assay

Cell viability and cytotoxicity assessments after drug treatment were done with Multitox-Fluor Multiplex Cytotoxicity Assay (Promega, USA). The assay enables the simultaneous measurement of the relative number of live and dead cells in cell population. Drug treatment and cell incubation conditions were the same as the CellTiter Blue Cell Viability Assay. At the end of incubation time, cells were added with assay reagent and incubated for another 3 h. Excitation/emission parameters for viability and cytotoxicity were 400 nm/505 nm and 485 nm/525 nm, respectively.

2.4. COMPOUND SCREENING

Compound screening was performed in two-steps. At first step, antiemetic drugs -hydroxyzine, flunarizine, cinnarizine, cetirizine, doxylamine and metoclopramide- were purchased as pharmaceutical formulations; and tested at 100 μM concentrations on U266 cells. Hydroxyzine and flunarizine stock solutions (10 mM) were prepared in water, others were prepared in DMSO. For the second part of the study, active compounds of the two drugs -hydroxyzine and prochlorperazine- were purchased and dissolved in water and DMSO, respectively. They were prepared as 10 mM stock solutions; and serial dilutions were made to obtain lower doses. The cells were incubated with two doses of the selected drugs (25 μM and 100 μM) for 24 hours. Viability of the cells were analyzed with CellTiter Blue cell viability assay.

2.5. APOPTOSIS ASSAYS

The apoptosis assays were purchased from BD Biosciences (San Diego, USA). Protocols were adapted according to manufacturer's recommendation. For all apoptosis assays, the cells were incubated in 6-well cell culture plates with a final cell density of 100.000 cells/mL. Analyses were performed with Accuri C6 Flow Cytometer (Accuri Cytometer, Ann Arbor, MI, USA). For each analysis, a total of 10.000 events were collected with flow cytometer. Cells were gated to exclude cell debris. Color compensation corrections were performed whenever necessary.

2.5.1. JC-1 Mitochondrial Membrane Potential Assay

The BD™ Mitoscreen Kit was utilized for the detection of changes in the mitochondrial membrane potential (MMP). The cells were treated with 15 μM PCP and incubated in 5% CO_2 incubator at 37 °C for 24 h. At the end of treatment duration, 100.000 cells were collected and washed with PBS, then incubated with 500 μL JC-1 staining solution for 15 min at 37 °C incubator at dark. After incubation, cells were washed twice with 1X assay buffer. Finally, they were resuspended in 500 μL 1X assay buffer and analyzed by flow cytometer.

2.5.2. Caspase-3 Assay

BD™ PE Active Caspase-3 Apoptosis Kit was used to analyze caspase-3 activation status within the cells. The cells were treated with 15 μM PCP and incubated in 5% CO_2 incubator at 37 °C for 24 h. Then, they were washed with PBS and resuspended in cold BD CytoFix/Cytoperm solution and incubated on ice for 20 min. After washed with BD Perm/Wash solution, cells were resuspended in 25 μL Perm/Wash solution and 5 μL PE Rabbit Anti- Active Caspase-3 antibody. The cells were incubated for 30 min at RT in the dark. Finally, samples were added with 500 μL Perm/Wash buffer and analyzed with flow cytometer within 1 hour.

2.5.3. AnnexinV / 7-AAD Assay

PE Annexin V Apoptosis Detection Kit I was used to detect phosphatidylserine (PS) exposure to outer surface of the plasma membrane. Cells were treated with 15 μM PCP and incubated in 5% CO_2 incubator at 37 °C for 24 h and 48 h. After incubation time, cells were washed with PBS and resuspended in 200 μL 1X Binding Buffer. 100 μL of the cell solution was transferred into a new Eppendorf; and cells were added with 5 μL of PE Annexin-V and 5 μL of 7-AAD staining solutions. After incubation of 15 min at RT in the dark, 400 μL 1X Binding Buffer was added to cell solutions. Analysis was performed with flow cytometer within 1 hour.

2.6. CELL CYCLE ANALYSIS

Cells were incubated with 15 μM PCP for 24 h. After incubation time, cells were washed twice with cold PBS. Then, samples were resuspended in cold PBS; and ice-cold absolute EtOH was added dropwise onto cell solution. The cells were kept on ice for 2 h for fixation. Following this step, cells were washed with PBS; then Propidium Iodide (PI) with a final concentration of 25 μg/mL and RNase solution with a final concentration of 3 mg/mL were added to samples. Lastly, the volume was completed to 200 uL with PBS and the cells were incubated at dark at 37 ºC for 30 min before flow cytometry analysis.

Gating with the flow cytometer was performed so that cell debris, cell clumps and doublets were excluded. Analysis of population histogram and cell cycle phases were done with flow cytometer.

2.7. COMBINATION STUDIES

The combination potential of PCP with a chemotherapeutic drug cisplatin was tested by using CellTiter Blue Cell Viability Assay. Cells were treated with alone and various combinations of the drugs for 24 h. PCP doses were determined as 15, 20, 25 and 30 µM; and Cisplatin doses were 20 and 40 µM.

The combination analyses were done with CompuSyn software (ComboSyn Inc., Paramus, USA). Outcome of the drug combinations were represented as combination index (CI) equation which was generated by Chou-Talalay. The Chou-Talalay method for drug combination is based on the median-effect equation, derived from the mass-action law principle. In this context, CI < 0.9 indicates synergism, 0.9 < CI < 1.1 indicates additivity, and CI > 1.1 indicates antagonism (Table 2).

Table 2. Degree of synergism and antagonism based on CI calues

CI	**Description**
< 0.1	Very Strong Synergism
0.1–0.3	Strong Synergism
0.3–0.7	Synergism
0.7–0.85	Moderate Synergism
0.85–0.90	Slight Synergism
0.90–1.10	Nearly Additive
1.10–1.20	Slight Antagonism
1.20–1.45	Moderate Antagonism
1.45–3.3	Antagonism
3.3–10	Strong Antagonism
> 10	Very Strong Antagonism

2.8. STATISTICAL ANALYSIS

Statistical significance of results for multitox, time-response, cell cycle arrest and Annexin-V assays were analyzed using GraphPad Prism one-way ANOVA with Bonferroni's multiple comparison post-test module. Unpaired t-test with two-tails was applied to analyze the significance of the results for the remaining experiments. Significance of differences were marked on the figures with asterisks.

CHAPTER 3

RESULTS

3.1. Preliminary Studies

Based on the successful repurposing of antiemetic drug Thalidomide for MM treatment, we aimed to investigate the anticancer potential of various antiemetic agents in this study.

Drug candidates were selected based on certain criteria. First one was the novelty. We cross-checked the literature and chose candidates that were not studied with MM before. Structural similarity to compounds with previously demonstrated anticancer activities was another important criterion. In this context, we selected our candidates based on the structural similarity to drugs that were studied by our group. Clofazimine, an anti-leprosy drug, was shown to be a potent anticancer agent with cell-cycle arrest and apoptosis induction activities on U266 multiple myeloma cell line. We take the Clofazimine molecular structure as a template and concentrated on compounds with tricyclic core structure. The last but not least determinant factors were the availability in pure form and the price of the drug. Since we could not purchase the all antiemetic drugs, we narrowed our list down according to price and solubility of the compunds. Solubility was important since compounds were applied to cells in solution form. We paid attention that the selected drugs were soluble in either DMSO or water which are the solvents commonly used in cell culture studies.

According to criteria considered in prelimary studies, we selected seven drug candidates and then tested their potency on U266 multiple myeloma cell line.

3.2. Potency Screening of Antiemetic Drugs

As a result of preliminary study, six antiemetic drug formulations were screened for their effect on the viability of U266 multiple myeloma cells. Cell viability was measured with CellTiter Blue cell viability assay. Basically, the assay is based on cellular reduction of resazurin dye to the fluorescent product resorufin. Viable cells maintain the metabolic capacity to perform this reduction. On the other hand, nonviable cells, lose the ability to reduce indicator dye; so they do not generate a fluorescent signal.

Drugs were applied to cells at a concentration of 100 μM for 24 hours. Cinnarizine and cetirizine exhibited moderate cytotoxicity with the cell viability of 76% and 83%, respectively. Doxylamine and metoclopramide, on the other hand, hardly had an effect on the viability of U266 cells. Hydroxyzine and flunarizine were found to be more active than the others (Figure 9).

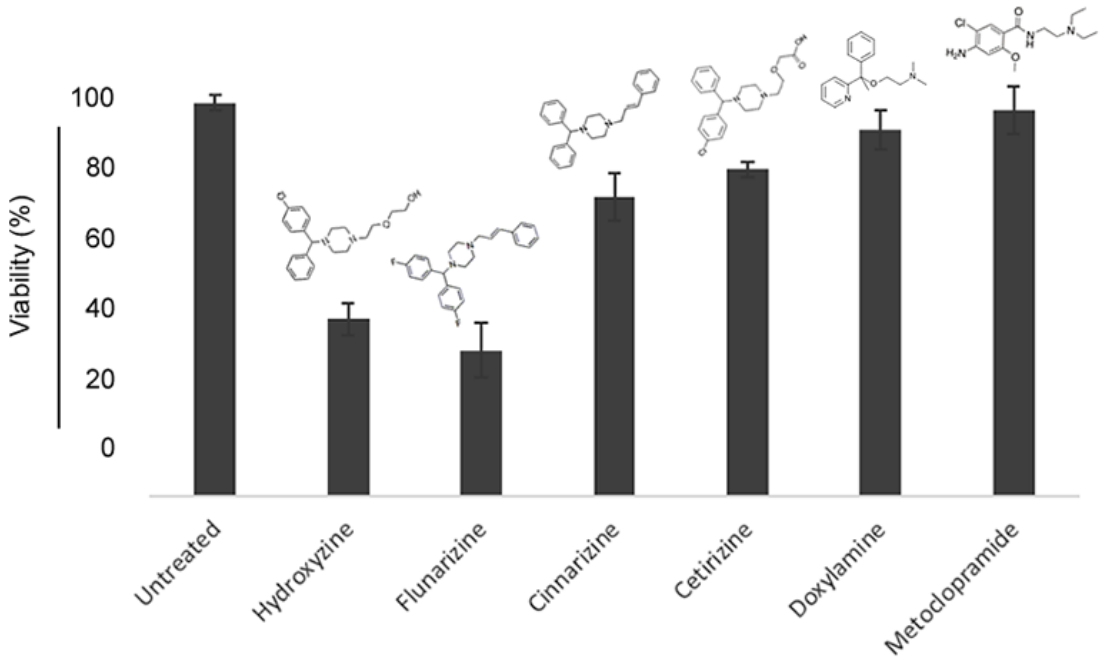

Figure 9. Screening of the selected antiemetic drug formulations (100 μM, 24 h) for their effect on the viability of U266 cells. Compound structures are shown above the corresponding bars.

First part of the screening studies narrowed down the candidates to two possible hits, hydroxyzine and flunarizine. Flunarizine, however, had already been shown to significantly decrease the viability of U266 multiple myeloma cells [42]. For the second part of the preliminary studies, hydroxyzine and prochlorpeazine were tested. We did not include prochlorperazine to first screening, because its pharmaceutical formulation was not provided in Turkey. However, its active compound form is relatively cheap, so we decided to purchase and test it.

Compounds were tested at two doses for the second screening; as 25 μM and 100 μM (Figure 10). When used as compound forms, both drugs decreased the viability of U266 cells. Prochlorperazine was found to be more active than hydroxyzine. Therefore, we selected the PCP for further *in vitro* characterization.

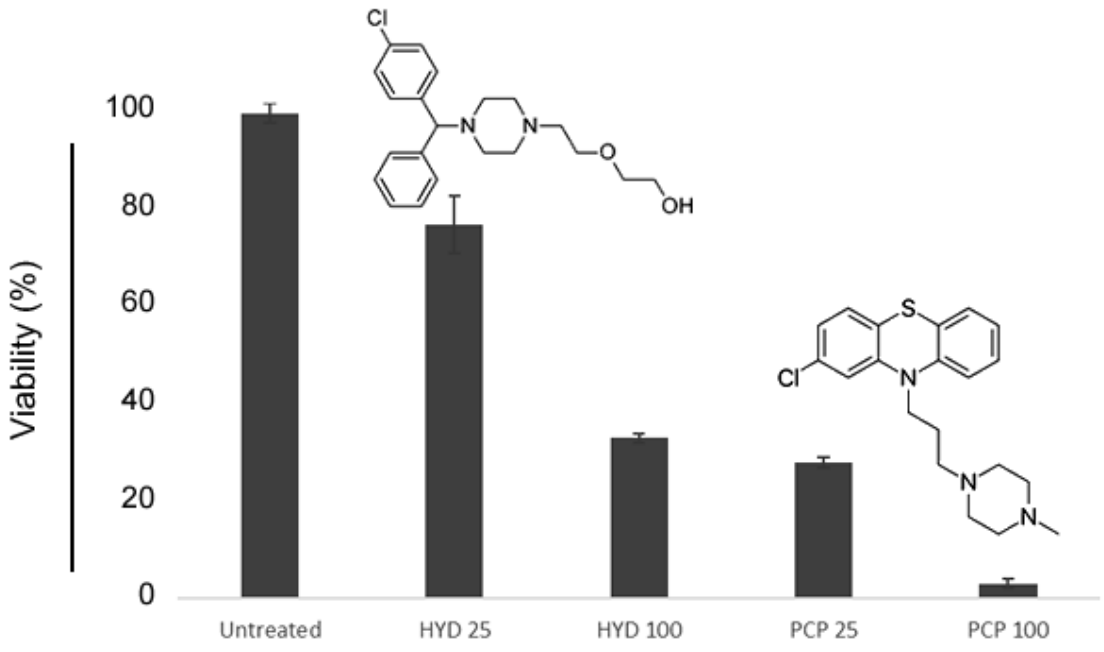

Figure 10. Effect of antiemetic drugs on viability of U266 cells. Bar plot showing the viability of cells treated with 25 μM and 100 μM of Hydroxyzine (HYD) and Prochlorperazine (PCP) for 24 h.

3.3. Prochlorperazine Exerts Cytotoxic Effects on U266 Multiple Myeloma Cell Line

To evaluate the efficacy of PCP, a dose response curve was generated by using ten different doses in 1-100 µM range after 24 h treatment. Half-maximal inhibitory concentration (IC50) of prochlorperazine was calculated as 21.8 ± 0.8 µM (Figure 11A).

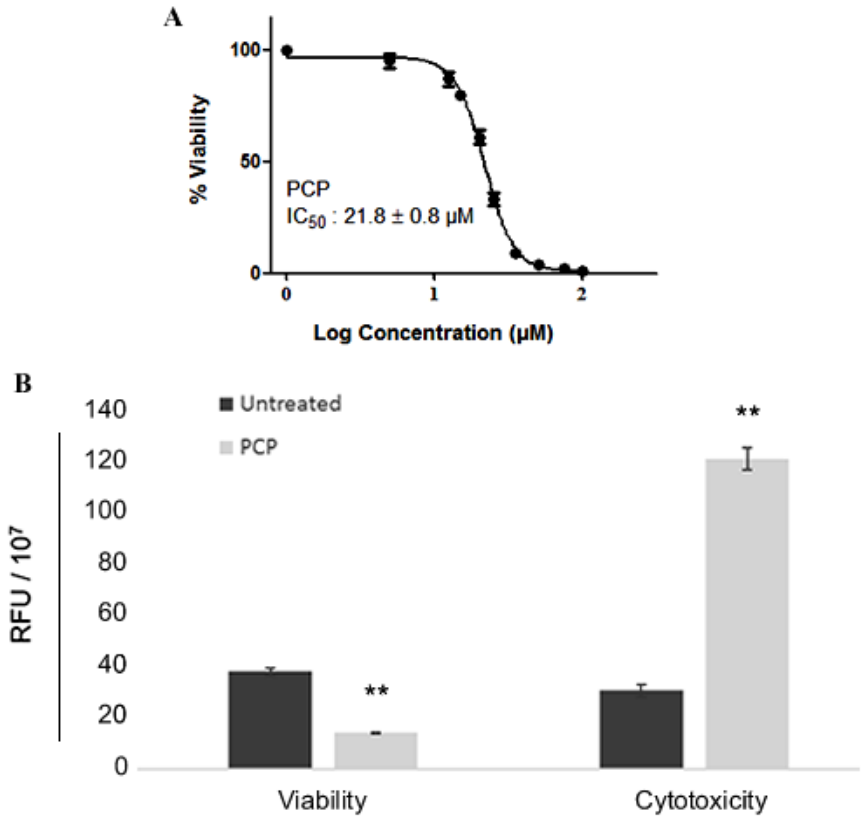

Figure 11. Effect of Prochlorperazine on Viability of U266 cells. A) Dose-dependent cytotoxicity and potency (IC50) determination of prochlorperazine at 24 h. Concentration range for PCP was 1 to 100 µM (n=3). B) Measurement of relative amount of live and dead cells completed with MultiTox-Fluor Multiplex Cytotoxicity Assay. Cell viability and cytotoxicity were measured simultaneously after 15 µM prochlorperazine treatment for 24 h. Error bars indicate the standard error of mean. Asterisks ** denote statistical significance at $p<0.01$ (n=2).

Effect of PCP treatment on cell viability and cytotoxicity was also analyzed with MultiTox-Fluor Multiplex Cytotoxicity Assay. With this assay, it is possible to measure the relative number of live and dead cells in the population. Basically, it detects the products of two different proteases; one is for cell viability, other is for the cytotoxicity. These products have different excitation and emission spectra which makes the simultaneous measurement possible.

15 μM PCP treatment for 24 hours resulted in a significant reduction in viable cell population and significant increase in dead cell population (Figure 11B).

3.4. Prochlorperazine Inhibits the Cell Growth in A Time-Dependent Manner

The time-dependent inhibitory effect of PCP on U266 cells was also tested. The cells were incubated with 15 μM PCP for 12, 24 and 48 hours. Viabilities were measured with CellTiter Blue cell viability assay. As shown in Figure 12, U266 cells treated with PCP exhibited a decrease in viability in a time-dependent manner. Cell viabilities at 12, 24 and 48 hours were measured as 93%, 69% and 24%, respectively.

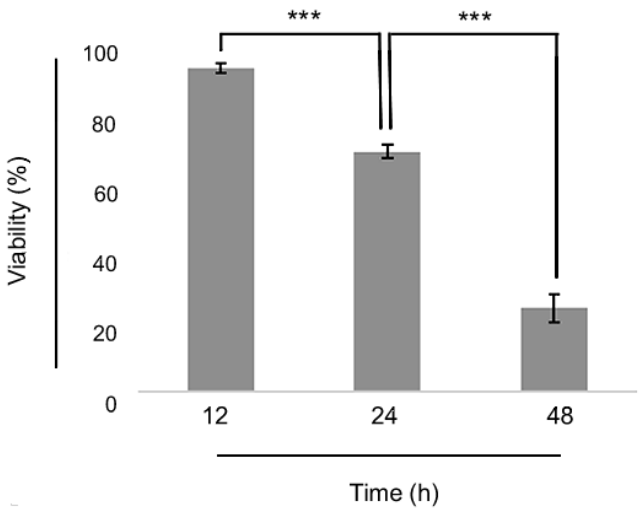

Figure 12. Time-dependent inhibitory effect of Prochlorperazine on U266 cell viability. Cells were treated with 15 μM Prochlorperazine; and cell viability was measured at 12, 24 and 48 hours as indicated. Asterisks *** denote statistical significance at $p<0.001$ (n=3).

3.5. Apoptosis Assays

In the view of obtained cytotoxic effects, we aimed to find that whether apoptosis is the underlying mechanism for PCP-induced cytotoxicity. To investigate this possibility, three different apoptotic markers were examined; change in mitochondrial membrane potential, activation of Caspase-3 and PS translocation to outer leaflet of the membrane accompanied by loss off cellular membrane.

3.5.1. Prochlorperazine Treatment Does Not Induce Mitochondrial Membrane Depolarization

Mitochondrial membrane depolarization is often, but not always, observed at early stages of apoptosis. JC-1 assay was used to investigate whether PCP treatment induces depolarization of mitochondrial membrane. JC-1 is a lipophilic fluorochrome used as an indicator for the polarization state of the mitochondrial membrane. JC-1 exists in aggregate or monomer forms with different fluorescence properties. Both forms exhibit green fluorescence. In healthy cells with polarized mitochondrial membrane, JC-1 is taken into mitochondria, and forms aggregates which causes higher levels of red fluorescence emission. 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.

The cells were treated with 15 μM PCP for 24 hours and stained with JC-1 dye and analyzed by flow cytometer (Figure 13). Results showed that PCP treatment did not induce mitochondrial membrane depolarization at 24 h.

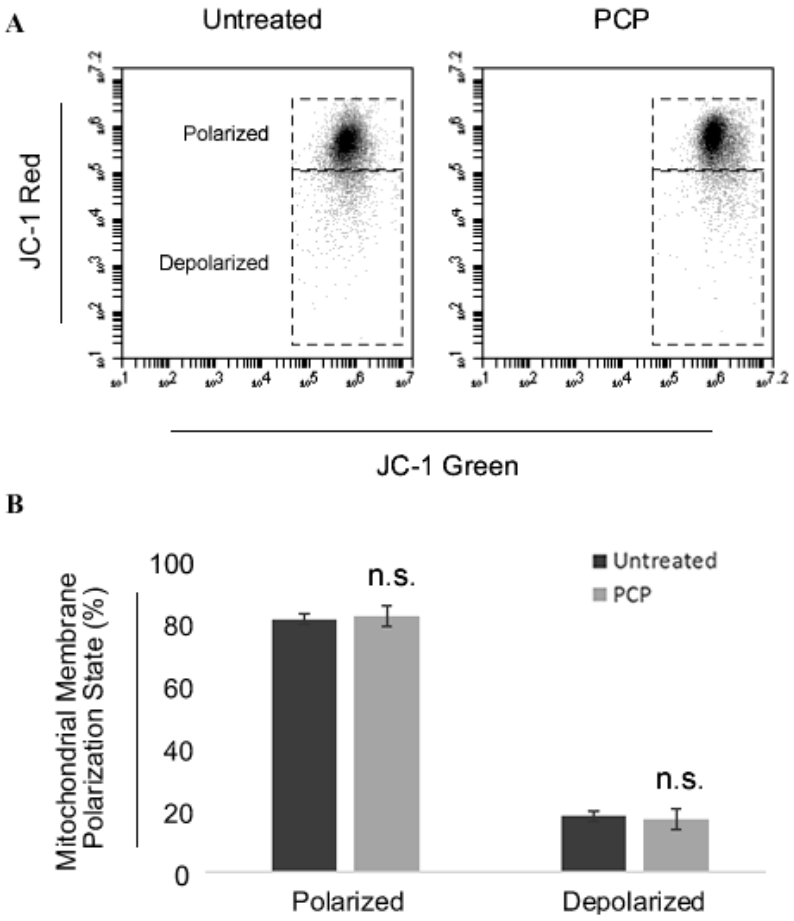

Figure 13. Effect of 15 μM Prochlorperazine treatment on mitochondrial membrane potential at 24 h. A) Representative flow cytometry fluorescence intensity dot plots of cells stained with JC-1. Fluorescence intensity values for polarized and depolarized states was gated and labeled accordingly. B) Bar plots of normalized mitochondrial membrane polarization state values for untreated and prochlorperazine-treated cells (n=3). Error bars indicate the standard error of mean. Untreated and PCP-treated samples are not significantly different ($p>0.05$).

JC-1 assay was repeated at 12 h and 48 h with 15 μM Prochlorperazine. The results were the same; no change in the mitochondrial membrane potential was observed (Figure 17).

3.5.2. Prochlorperazine Induces Caspase-3 Dependent Apoptosis

Caspase-3 enzyme is synthesized as inactive pro-enzyme and activated by proteolytic cleavage in cells undergoing apoptosis. Activation of Caspase-3 is a key event 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.

U266 cells were treated with 15 μM PCP for 24 hours. PE labeled Anti-Active Caspase-3 antibody was used and cells were analyzed by flow cytometer (Figure 14). When compared with the untreated samples, significant increase in Caspase-3 activity was observed in PCP-treated cells (22% vs. 45%).

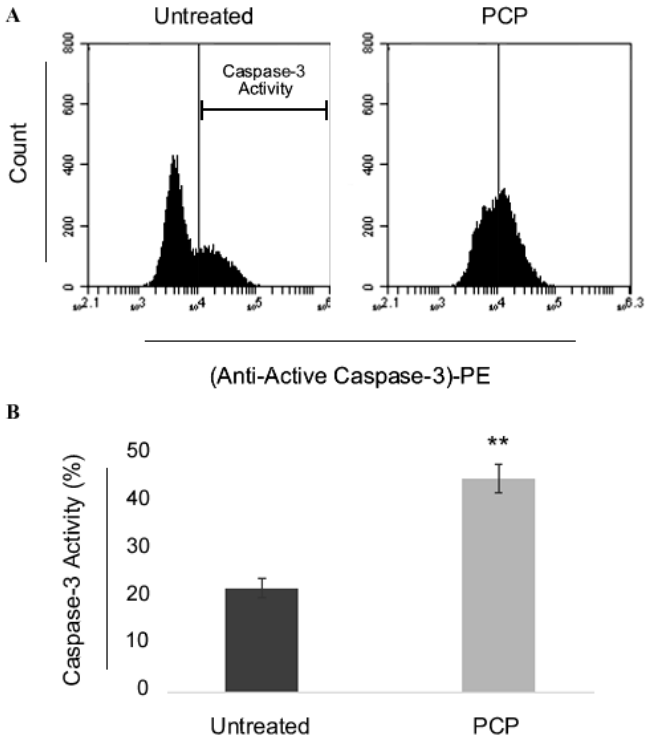

Figure 14. Effect of Prochlorperazine treatment (15 μM, 24 h) on caspase-3 activation in U266 cells. A) Flow cytometry fluorescence intensity histograms of cells stained with anti-active Caspase 3-PE antibody. Intensity threshold for caspase-3 activity is indicated in the upper-left panel. B) Bar plots of corresponding histograms. Asterisks ** denote statistical significance at $p<0.01$ (n=3).

3.5.3. Prochlorperazine Treatment Changes Cell Membrane Asymmetry

Loss off plasma membrane asymmetry is one of the earliest events in cells undergoing apoptosis. Phosphatidylserine (PS) is membrane phospholipid which locates in the inner leaflet of plasma membrane in healthy cells. Membrane asymmetry changes in apoptotic cells; and translocation of PS from inner to outer leaflet is observed. 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 is another characteristic of apoptotic cells which happens in later stages of apoptosis. When membrane is disintegrated, the cellular contents and DNA will be exposed. Detection of exposed DNA is possible with use of DNA-binding dye such as 7-AAD. 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 15 μM PCP for 24 and 48 hours; and stained with Annexin V-PE / 7-AAD probes. As seen in the Figure 15 and Table 3, PCP treatment caused significant decrease in viable cell population at 24 h (67% control vs. 34% treatment). On the other hand, significant increase in early apoptotic and late apoptotic cell populations was observed. As the cytotoxic effect of PCP increased in time, the difference between untreated and PCP-treated cells became more prominent at 48 h. Almost 85% of the cell population was consisted of early and late-apoptotic cells at 48h.

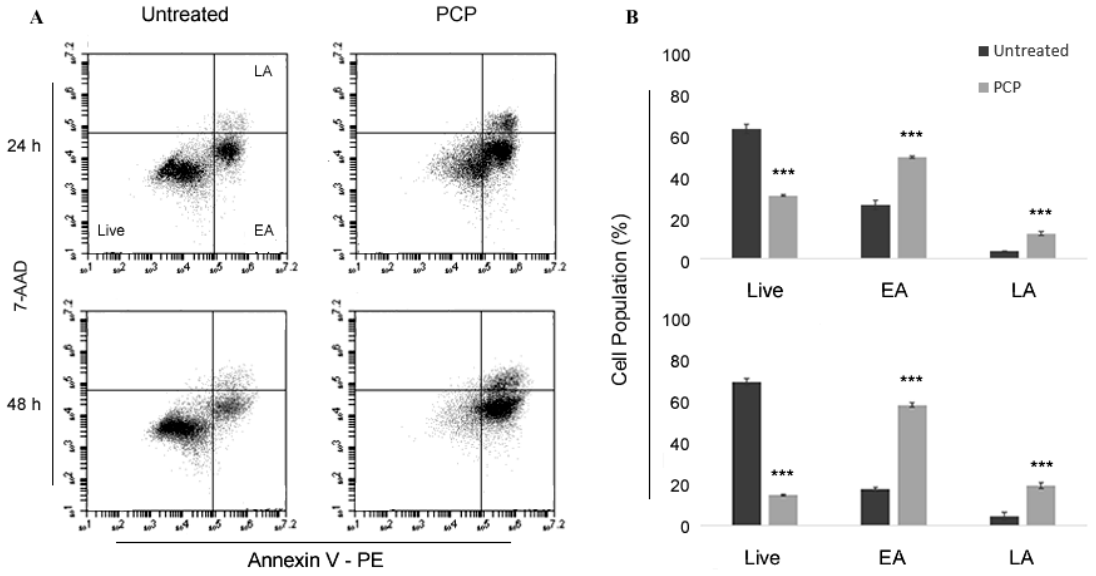

Figure 15. Analysis of U266 cell membrane asymmetry after 15 μM PCP treatment for 24 h and 48 h. U266 cells were stained with Annexin V-PE and 7-AAD; and analyzed at 24 h and 48 h. A) Dot plots of Annexin V-PE vs 7-AAD-stained cells gated as live, early apoptotic (EA) and late apoptotic (LA) quadrants as shown in the upper-left plot. B) Cell population bar graphs of corresponding dot plot quadrants. Asterisks *** denote statistical significance between control and treatment populations at $p<0.001$ (n=3).

Table 3. Percentages of cells at live and apoptotic stages

	24 h Treatment		
	Live	Early Apoptotic	Late Apoptotic
Untreated	68 ± 1	28 ± 1	4 ± 0
PCP	34 ± 1	53 ± 1	13 ± 1
	48 h Treatment		
	Live	Early Apoptotic	Late Apoptotic
Untreated	75 ± 2	19 ± 1	5 ± 2
PCP	16 ± 1	63 ± 1	21 ± 2

3.6. Prochlorperazine Induces G2/M Cell Cycle Arrest on U266 Cell Line

The effect of PCP treatment on cell cycle progression was analyzed by propidium iodide (PI) staining. PI is an DNA-intercalating agent and used to evaluate DNA content in the cell cycle analysis. PI binding and concomitant florescence intensity are proportional to the amount of DNA present in the cells. In this context, DNA content in the S phase will be higher than G0/G1 phase since DNA replication occurs during S phase. Likewise, DNA content in G2/M phase will be higher than S phase and will be twice as high as that of cells in the G0/G1 phase.

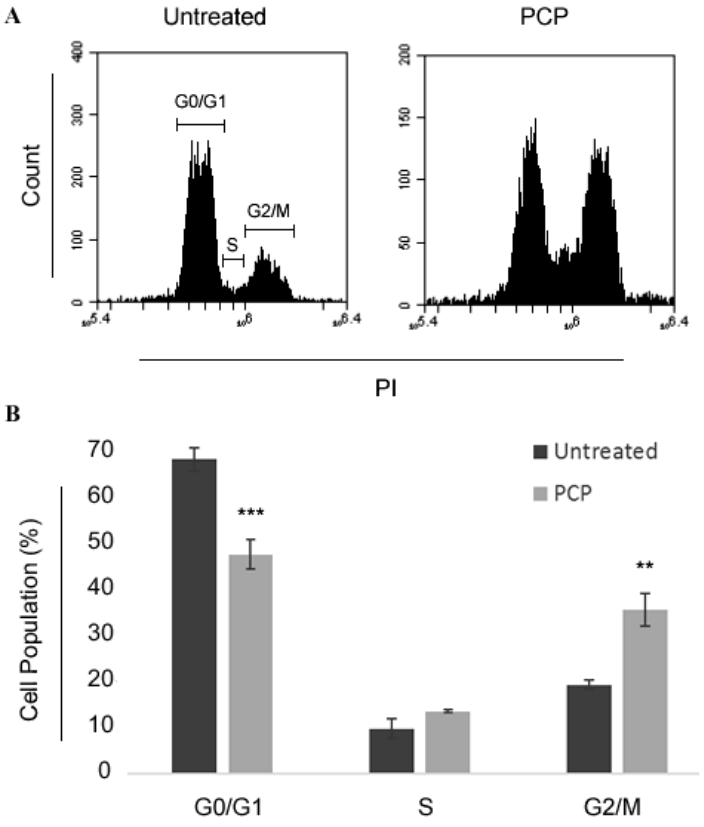

Figure 16. Cell cycle analysis of U266 cells tretaed with 15 μM PCP for 24 h. A) Cell-cycle distribution of propidium iodide (PI)-labeled cells represented as flow cytometry fluorescence intensity histograms. The peaks in the illustration correspond to the G1/G0, S and G2/M phases of the cell cycle. B) Bar plots representing the percentages of cell population in each phase of the cell cycle, for untreated and prochlorperazine-treated cells. Asterisks ** and *** denote statistical significance at $p<0.01$ and $p<0.001$, respectively (n=3).

U266 cells were treated with 15 μM PCP 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 16).

Percentages of cell populations at G0/G1, S and G2/M phases for untreated and PCP-treated cells were determined as 66%vs. 46%, 10% vs. 13% and 19% vs. 35%, respectively. Prochlorperazine treatment resulted in significant decrease in G0/G1 population accompanied with significant increase in G2/M population. These results indicated G2/M arrest associated with PCP treatment.

3.7. Prochlorperazine-Cisplatin Combination is Additive

Cytotoxic effect of PCP, Cisplatin (Cis) and PCP-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.

At first, four combinations of the drugs were evaluated. Table 4 shows the combination cytotoxicity and combination index values of corresponding drug combinations. All four combinations tested here were found to be additive with CI values of 1.0 and 1.1.

Table 4. Combination Effect of Cisplatin and Prochlorperazine

PCP (μM)	Cis (μM)	Combination Cytotoxicity (%)	Combination Index (CI)
20	40	87 ± 2	1.0 ± 0.1
20	20	84 ± 3	1.0 ± 0.1
15	40	65 ± 4	1.1 ± 0.2
15	20	61 ± 4	1.1 ± 0.1

Another six combinations with different doses -Cis 15 μM with PCP 15, 20 and 25 μM; and Cis 10 μM with PCP 20, 25 and 30 μM- were also tested as a part of combination studies. Four of these combinations were found to be additive; and two of them exhibited antagonistic effect (Table 5).

CHAPTER 4

DISCUSSION

In this work, PCP induced apoptosis and cell cycle arrest on U266 resistant multiple myeloma cell line was reported. Our results showing the cytotoxic effect and mechanism of PCP are consistent with the previous studies on antitumor potential of PCP and phenothiazine-derivative drugs in the literature [26].

The viability of U266 cells was significantly decreased by Prochlorperazine in dose and time-dependent manner. PCP was found to be effective in low micromolar (μM) range; and IC50 value of PCP on U266 cells was calculated as 21 ± 0.8 μM. Similar results for PCP potency were reported on melanoma, glioblastoma, colon and breast cancer cell lines [37]–[40]. Krishan et al. showed that concentrations of >20 μM PCP significantly decreased the viability of doxorubicin-sensitive SW620 and doxorubicin-resistant SW620/Ad300 human colon cancer cells [40]. The cytotoxicity of PCP on other cancers was studied with MTT and clonogenic assays and reported IC50 values were in the range of 5 to 15 μM [37]–[39].

It is important to address the effect of PCP on healthy cells. To our knowledge, there is no study reporting the PCP cytotoxicity against healthy blood cells. On the other hand, effects of other phenothiazine derivatives on the viability of normal lymphocytes were previously studied [43]. Phenothiazines at concentrations in the range of 1 to 40 μM were tested on normal lymphocytes; and no inhibitory effect was observed in viability at 24 hours' incubation time. Based on this study, it is safe to assume that PCP also would not affect the viability of heathy blood cells. Serum/plasma concentration of PCP is another issue that needs to be addressed to interpret the *in vivo* potency of the drug. Approximately 2.5 μM PCP was detected in the serum after 24 h of PCP administration (i.v.) at maximum tolerated dose of 180

mg/m^2 [44]. Although, this concentration is quite lower than the calculated IC50 value in this study; additional *in vivo* experiments are required in order to evaluate the clinical potential of PCP for MM treatment.

We also reported that cytotoxic effects of PCP are mediated through apoptosis. This is the first study showing the apoptotic effect of PCP on a cancer cell line. We showed significant Caspase-3 activation, PS translocation to outer leaflet and loss of plasma membrane integrity on U266 cells after PCP treatment. On the other hand, PCP did not have an effect on mitochondrial membrane potential. Studies showed that phenothiazine derivatives induce apoptosis on various cancer cell lines. Phenothiazine-induced apoptosis involved activation of Caspase-3, PARP cleavage, PS exposure on the outer leaflet of the plasma membrane and DNA fragmentation [43], [45]–[49]. On the other hand, phenothiazines exhibited different effects on mitochondrial membrane polarization. The influence of Trifluoperazine, Thioridazine and Chlorpromazine on MMP was not uniform and reported to be dose-dependent [49]–[51]. In this context, we may assume that PCP induced apoptosis in U266 cells in a mitochondria-independent mechanism, probably through activation of death receptors. Indeed, activation of extrinsic apoptototic pathways in U266 cell line by several anticancer agents was previously reported [52]. In this work, we only studied the activation status of Caspase-3 which is the initiator caspase common in both intrinsic and extrinsic pathways. Additional markers such as Caspase-8 and Caspase-9 activation or cytochrome c release should be analyzed in future studies to test the hypothesis of extrinsic apoptotic pathway activation by PCP.

We showed that in addition to its cytotoxic potential, PCP also exhibits anti-proliferative effects on U266 cells. PCP arrests cell cycle of U266 multiple myeloma cells at G2/M phase. To our knowledge, this is the first study showing PCP-induced cell cycle arrest in a cancer cell line. PCP is a phenothiazine derivative; and the effect of phenothiazines on cell cycle arrest was previously studied. G2/M arrest in mouse leukemia and U1810 human non-small cell lung carcinoma (NSCLC) cell lines by Trifluoperazine was reported [53]. In a different study, Chlorpromazine-induced G2/M arrest in C6 rat glioma cells was shown [54]. In this study, decrease in

expression levels of cell-cycle related proteins (Cyclin A, Cyclin D1 and Cyclin B1); but increase in the levels of p21 -a cyclin-dependent kinase inhibitor- was demonstrated. They found a link between ERK and JNK MAPK pathways and Egr-1 directed p21 activation; which was thought to be the mechanism of CPZ-induced cell cycle arrest. This study may provide a basis for future studies to decipher the cell cycle arrest mechanism of PCP. On the other hand, PCP is also a calmodulin antagonist; and G0/G1 cell cycle arrest by various calmodulin antagonists was reported [37], [55], [56]. However, this does not show that our results are contradicting with the literature. Calmodulin levels changes throughout the cell cycle; start to increase at G1/S transition and reached a maximum level at G2/M phase. It is stated that, when calmodulin antagonists are added to cells at S phase, G2/M arrest will be observed [57]. In this context, we can say that results reported in this study agree with the literature.

U266 cells have mutant p53 gene with a codon 161 mutation [58]. Studies reported that a pattern of G2/M arrest followed by apoptosis was observed in cells having mutant p53 [59], [60]. We mentioned that PCP, probably through inhibition of calmodulin, arrested cells at G2/M phase. We also showed that PCP induced apoptosis in U266 cells. These observations are consistent with the literature. Therefore, it can be assumed that these events may also be relevant to the activation of apoptotic pathways in U266 cells by PCP.

The use of two or more chemotherapeutic agents in combination has proven to be an effective strategy for cancer therapy. In this study, we tested the combination potential of PCP with a chemotherapeutic drug cisplatin which is currently used in MM treatment. We showed that PCP-Cis combination exhibits additive effect on viability of U266 multiple myeloma cell line.

There are several studies in the literature indicating the combination potential of phenothiazine derivatives with commonly used anticancer drugs [61]–[64]. Combination of chlorpromazine and trifluoperazine with antitumor antibiotic bleomycin augmented the inhibitory effects of bleomycin against B16 melanoma and

L1210 leukemia cells [61], [62]. Another study showed that chlorpromazine-pentamidine combination was synergistic which was attributed to dual mitotic action [63]. Whellan et al. showed that the use of DNA-alkylating agent temazolomide (5-15 μM) in combination with perphenazine (5 μM) exhibited additive effect in all tested combinations [64]. There is also one study in the literature showing the synergistic effect of PCP and doxorubicin combination on P388 and SW620 drug-resistant colon cancer cell lines [40]. In this study, various combinations of doxorubicin (1 to 5 μM) and PCP (1.5 to 40 μM) were found to have moderate-to-high synergism with CI values ranging from 0.2 to 1.0.

The observed additive combinatory effect of PCP and Cis might be attributed to multidrug resistance inhibition (MDRi) activity of phenothiazine-derivative compounds [28]. Significant MDR reversal activities of PCP and other phenothiazines (TFP, CPZ) had been reported at the *in vitro* IC50 concentrations of 1 to 10 μM [29]. Pajak et al. demonstrated that MDRi inhibition by PCP was dose-dependent; and PCP was more powerful than Verapamil as MDR inhibitor at concentrations of 4 to 40 μM [65]. In addition to this, Phase I clinical and pharmacokinetic studies showed that PCP was an effective doxorubicin efflux blocker in drug-resistant cells [44]. Based on these studies, it can be assumed that PCP augments the cytotoxic effect of cisplatin in U266 cells by acting as a cisplatin efflux blocker.

The reason why we observed additive effect rather than synergism with PCP-Cis combinations might be due to drug regimen schedule. For instance, additive or synergistic inhibitory effect was observed when paclitaxel was given to cells before cisplatin or they were administrated concomitantly. On the contrary, when cells were treated with cisplatin first, antagonism was observed [66]. In this context, change in drug regimen schedule might improve the cytotoxic effect of PCP-Cis combinations so that synergism would be achieved.

CHAPTER 5

CONCLUSION

In this study, we reported dose-and time-dependent cytotoxic effect of PCP on U266 resistant MM cell line. Flow cytometry analyses performed with anti-Caspase-3 antibody and AnnexinV / 7-AAD dyes confirmed that PCP executes its cytotoxic activity through apoptosis. Most importantly, PCP treatment did not have an effect on mitochondrial membrane potential which suggest that PCP might be involved in the activation of extrinsic apoptotic pathways on U266 cells. PCP also induced G2/M cell cycle arrest in addition to apoptosis. Moreover, PCP-cisplatin combation was shown to have an additive effect on U266 cell viability which might be attributed to its MDRi ability.

Our study represents the first *in vitro* evidence of PCP-induced apoptosis and cell cycle arrest on U266 cells. MDRi ability also makes PCP a good candidate for combination therapy. Based on the results of the present study, further *in vitro* mechanistic studies and animal experiments are warranted to evaluate the therapeutic potential of PCP.

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APPENDIX

SUPPLEMENTARY DATA

1. Combination Studies

Combination potential of Prochlorperazine and Cisplatin was studied with cell viability assay. Cells were treated with indicated doses of PCP, Cis and PCP-Cis combinations for 24 h. After the initial combination screening, additional doses were tested as indicated in Table 5.

Combination Index (CI) value was calculated using the CompuSyn software. The outcome of combinations was evaluated according to CI values; as CI < 0.9 (synergism), 0.9 < CI < 1.1 (additive), CI > 1.1 (antagonism).

Table 5. Combination Effect of Cisplatin and Prochlorperazine

PCP (μM)	Cis (μM)	Combination Cytotoxicity (%)	Combination Index (CI)
25	15	83	1.1
20	15	68	1.2
15	15	42	1.4
30	10	86	0.9
25	10	72	1.1
20	10	63	1.1

Four out of six combinations were additive. Only PCP 20 μM – Cis 15 μM and PCP 15 μM – Cis 15 μM combinations caused antagonism.

2. JC-1 Mitochondrial Membrane Potential Assay

The effect of 15 uM PCP treatment on mitochondrial membrane potential was also studied at 12 h and 48 h.

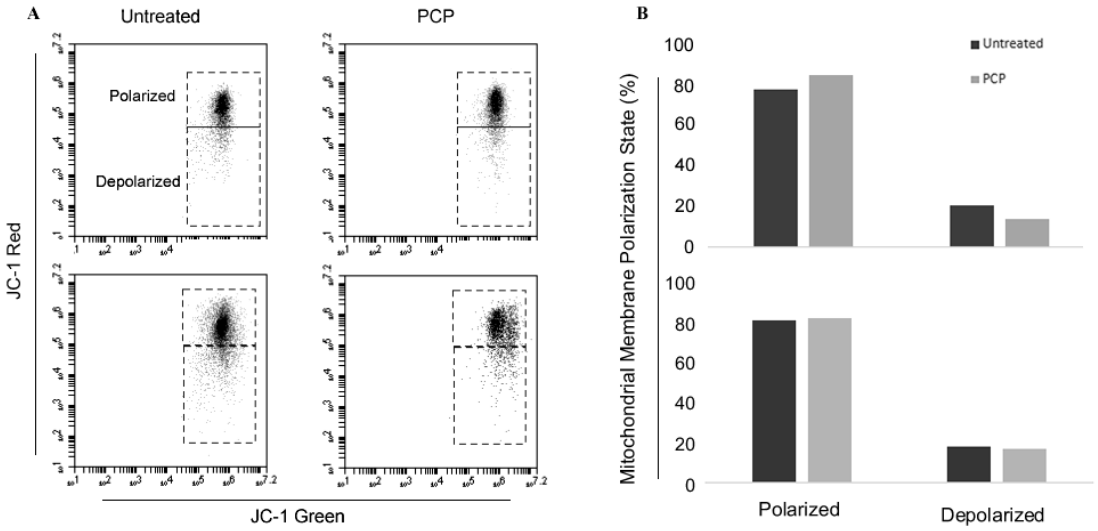

Figure 17. Effect of 15 uM PCP treatment on mitochondrial membrane potential at 12 h and 48 h. A) Representative flow cytometry fluorescence intensity dot plots of cells stained with JC-1. Fluorescence intensity values for polarized and depolarized states was gated and labeled accordingly. B) Bar plots of normalized mitochondrial membrane polarization state values for untreated and prochlorperazine-treated cells.

As demonstrated in Figure 17, the mitochondrial membrane potential of control group (untreated) and PCP-treated cells was not different from each other. These finding are consistent with those obtained with 24 h treatment. It can be concluded that PCP did not have an effect on mitochondrial membrane potential at tested time points.