

ANTICANCER EFFECT AND COMBINATION POTENTIAL OF  
CLOFAZIMINE IN MULTIPLE MYELOMA

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İPEK ZEYNEP DURUSU

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CLOFAZIMINE IN MULTIPLE MYELOMA**

submitted by **İPEK ZEYNEP DURUSU** in partial fulfillment of the requirements  
for the degree of **Doctor of Philosophy in Biotechnology Department, Middle  
East Technical University** by,

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

Assoc. Prof. Dr. Çağdaş Devrim Son  
Head of Department, Biotechnology

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

Prof. Dr. Ufuk Gündüz  
Co-Supervisor, Biology Dept., METU

**Examining Committee Members:**

Assoc. Prof. Dr. Çağdaş Devrim Son  
Biology Dept., METU

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

Prof. Dr. Mayda Gürsel  
Biology Dept., METU

Prof. Dr. Muhit Özcan  
Hematology Dept., Ankara University Medical Faculty

Assoc. Prof. Dr. Meltem Kurt Yüksel  
Hematology Dept., Ankara University Medical Faculty

**Date:** 12.02.2016

**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: İPEK ZEYNEP DURUSU**

**Signature:**

## ABSTRACT

### ANTICANCER EFFECT AND COMBINATION POTENTIAL OF CLOFAZIMINE IN MULTIPLE MYELOMA

Durusu, İpek Zeynep

PhD, Department of Biotechnology

Supervisor: Assoc. Prof. Dr. Can Özen

Co – Supervisor: Prof. Dr. Ufuk Gündüz

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Multiple Myeloma (MM) is a malignant neoplasm of bone marrow plasma B cells with high low survival rates. Clofazimine (CLF) is a FDA-approved leprostatic, anti-tuberculous and anti-inflammatory drug. CLF has shown to have growth suppression in various cancers such as hepatocellular, lung, cervix, melanoma, esophageal, colon, leukemia, neuroblastoma and breast.

Objective of this study was to investigate anticancer effect and mechanism of Clofazimine on U266 Multiple Myeloma cell line. The relative cell viability of a panel of hematological cell lines (Jurkat, U266, Namalwa, K562, HL60) treated with CLF is screened. Dose and time response effect of CLF on U266 cell line is evaluated and anti-cancer effect of CLF combination treatment is examined using CellTiter Blue assay. For apoptosis JC-1, Caspase 3 and PE Annexin V-7 AAD assays are performed.

CLF (10  $\mu$ M, 24 h) showed growth suppression on all cell lines, highest on U266 (72%). It has an IC<sub>50</sub> value of  $9.87 \pm 0.9$   $\mu$ M and has synergistic effect in combination with cisplatin. Cell cycle analysis indicates accumulation of cell

population at S phase. CLF has apoptotic effect on U266 cells as, mitochondrial membrane depolarized cells 44, 67.8, 80.2 percent increased, caspase 3 positive cells 19.1, 41.3, 43,2 percent increased and early apoptotic cells 18.9, 39.3, 62,9 percent increased compared to control group at 12, 24 and 48 h respectively. Clofazimine has potent anticancer effect on U266 MM cell line. Clofazimine in vivo studies, alone and in combination with cisplatin, are warranted to evaluate its therapeutic potential for MM treatment.

Keywords: Multiple myeloma, clofazimine, apoptosis, synergism

## ÖZ

### MULTİPL MİYELOM'DA CLOFAZİMİNE'İN APOPTOTİK ETKİLERİ VE KOMBİNASYON POTANSİYELİ

Durusu, İpek Zeynep

Doktora, Biyoteknoloji Bölümü

Tez Yöneticisi: Doç. Dr. Can Özen

Yardımcı Tez Yöneticisi: Prof. Dr. Ufuk Gündüz

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Multipl Miyelom (MM), çok düşük sağ kalım oranlarına sahip kemik iliğindeki plazma B hücrelerinin habis uru olmanın gelişimi ile oluşur. Clofazimine (CLF) FDA tarafından onaylanmış, lepra ve tüberküloz tedavisinde kullanılan ve yüksek dozlarda antiinflamatuvar etki gösteren anti-mikrobiyel bir ilaçtır. CLFün, lösemi, meme, akciğer, serviks, melanoma, özafagus, kolon, nöroblastoma ve hepatoselüler kanserde apoptoz yada antiproliferatif etkisi gösterilmiştir. Bu çalışmanın amacı MM hücre hattı U266 üzerinde CLFün antikanser etki ve mekanizmasının incelenmesidir.

HL60, Namalwa, K562, Jurkat ve U266 hücre hatları üzerinde CLFün sitotoksik etkileri CellTiterBlue hücre proliferasyon testi ile ölçüldü. CLFün IC50 değeri doz ve zamana bağlı etkisi ve sisplatin ile sinerjistik etkisi hesaplandı. Apoptotik etkileri üç farklı test ile araştırıldı. Kaspaz-3 enzim aktivitesindeki değişimler, mitokondri zar potansiyelindeki bozulmalar ve apoptotik hücre popülasyonu değişimleri akım sitometrisinde belirlendi. Hücre döngüsü profilleri PI boyası ile analiz edildi.

CLF (10  $\mu$ M, 24 h) U266da en yüksek sitotoksik etkiyi gösterdi. CLFün IC50 dozu  $9.87 \pm 0.9 \mu$ M olarak doz ve zamana bağlı etkisi olduğu gösterildi. Sisplatin ile CLF kombinasyonu ayrıca sinerjik bir etki gösterdi. CLF, S aşamasında hücre döngüsünü dururdu. CLF ile 12, 24 ve 48 saat muamele edilen U266 hücrelerinin sırasıyla 44,



67.8, 80.2 kat mitokondri zar potansiyelinde bozulma, kaspaz 3 enzim aktivitesinde ise kontrole göre yüzde 19.1, 41.3, 43,2 oranında artış ve erken apoptotik hücre popülasyonunda kontrol grubuna oranla 18.9, 39.3, 62,9 kat artmıştır. Sonuçlar CLFun, p53 mutant dirençli U266 hattı üzerinde potansiyel anti-kanser etkisi gösterildi. CLF in vivo çalışmaları MMda tek başına ve cisplatin ile kombine edildiğinde terapötik etkisi olabileceğini göstermektedir.

Anahtar Kelimeler: Multipl miyelom, clofazimine, apoptoz, sinerji

To my precious family ...

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## LIST OF ABBREVIATIONS

B663: Clofazimine  
BID: BH3-interacting Domain  
BSA: Bovine Serum Albumin  
cAMP: Cyclic Adenosine Monophosphate  
CLF: Clofazimine  
CLL: Chronic Lymphocytic Leukemia  
CRAC: Calcium Release-activated Ca<sup>2+</sup>  
Cyt C: Cytochrome C  
DCEP: Dexamethasone, Cyclophosphamide, Etoposide, Cisplatin  
DISC: Death-inducing Signaling Complex  
DMSO: Dimethyl Sulphoxide  
DNA: Deoxyribonucleic Acid  
EP2: Prostaglandin E2 Receptor 2  
H<sub>2</sub>O<sub>2</sub>: Dihydrogen Dioxide  
HCC: Hepatocellular Carcinoma  
HSCT: Hematopoietic Stem Cell Transplantation  
IAP: Integrin-associated Protein  
Kv1.3: Voltage Gated Potassium Channel 1.3  
MDR: Multi Drug Resistant  
MM: Multiple Myeloma  
MOMP: Mitochondrial Outer Membrane Permeabilization  
MSC: Mesenchymal Stem Cell  
NFAT: Nuclear Factor of Activated T-cells

NHL: Non Hodgkin Lymphoma  
OS: Overall Survival  
PARP: Poly ADP Ribose Polymerase  
PBS: Phosphate-buffered Saline  
PFS: Progression-free Survival  
PGE2: Prostaglandin E2  
PGs: Prostaglandin E2  
PLA2: Phospholipase A2  
PTP: Permeability Transition Pore  
PTX: Paclitaxel  
ROS: Reactive Oxygen Species  
siRNA: Small Interfering RNA  
VGIC: Voltage Gated Ion Channel  
WHO: World Health Organization





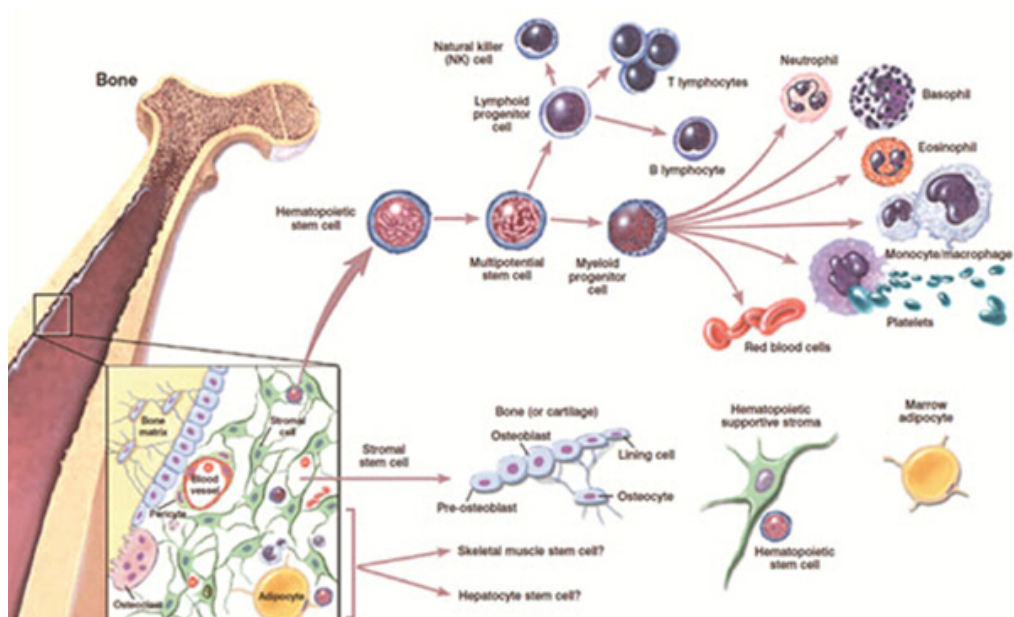
## **CHAPTER 1**

### **INTRODUCTION**

#### **1.1 HEMATOLOGICAL MALIGNANCIES**

Hematopoiesis is the process of blood cells to form and mature. All different types of blood cells origin in the bone marrow from a pluripotent hematopoietic stem cell. The stem cell undergoes a series of developmental steps and differentiates into mature cells. They then had specific roles in the body. (Figure 1)

Hematopoietic neoplasms are are cancers of the blood-forming organs such as leukemia, lymphoma, and multiple myeloma. They arise from the errors in the genetic information of the immature blood cell. As a result, the cell's development is arrested. It does not mature further, but continues to proliferate. Nearly errors of every stage of the hematopoietic process can become a distinct type of cancer. Hematological malignancies have two different origins as myeloid and lymphoid cell lines. The myeloid cell line under normal conditions produces granulocytes, erythrocytes, macrophages, thrombocytes and mast cells; the lymphoid cell line produces NK, B, T, and plasma cells. Lymphomas, lymphocytic leukemias, and myeloma are occurred from the lymphoid line, acute and chronic myelogenous leukemia, myeloproliferative diseases and myelodysplastic syndromes are myeloid in origin.<sup>1</sup>



**Figure 1. Hematopoiesis**

## 1.2 MULTIPLE MYELOMA

Multiple myeloma (MM) is malignant neoplasm of bone marrow plasma B cells. It results in increased levels of monoclonal protein and it also suppressed levels of other immunoglobulin proteins. MM accounts for 1% of all cancers, 13% of all hematologic malignancies, and 20% of deaths related hematological malignancies.<sup>2</sup> Although there are significant advances in the treatment of MM, it still has very high morbidity and low survival rates.<sup>3</sup> MM survival rates, which have historically ranged from 3 to 5 years, can now exceed 10 years due to the advent of high-dose therapy with autologous stem cell transplantation in combination with novel chemotherapeutic agents.<sup>4</sup>

There is an increasing trend in medical research on tumor biology, genetic and molecular mechanisms of tumor growth and survival, and defined the impact of the microenvironment in cancer pathogenesis. As a result some fatal cancers a few decades ago consequence of these advances, can now be treated effectively, with prolonged survival. For MM, some drugs enter the market targeting the tumor in its



microenvironment. Proteasome inhibitor bortezomib and the immunomodulatory drugs (IMiDs) thalidomide and lenalidomide are the important treatments in MM's initial, consolidation, maintenance, and salvage therapies. They markedly improved patient outcome.

In the past 10 years Lenalidomide, a second-generation synthetic derivative of glutamic acid and thalidomide analogue, has been used to treat both inflammatory disorders and cancers. Lenalidomide, *in vitro* has three main activities: inhibition of angiogenesis, direct anti-tumor effect, and immunomodulatory effect. *In vivo*, it induces tumor cell apoptosis. On a molecular level, Lenalidomide interacts with the ubiquitin E3 ligase Cereblon and it targets this enzyme to degrade the Ikaros transcription factors IKZF1 and IKZF3. This is a novel mechanism that lenalidomide re-targets the activity of an enzyme.<sup>5</sup> Lenalidomide is used to treat MM by disrupting cellular interactions and adherence of MM to stromal constituents. It decreases growth factors as IL-6, and induces apoptosis of the neoplastic cells to block disease progression. Hematopoiesis dysregulation and increased inflammatory cytokine near bone marrow microenvironment contributes to impaired immune effector of the cell function. Lenalidomide treatment in MM, as similar to MDS and B-CLL, directly reverses T-cell defects.<sup>6</sup>

Bortezomib has a mechanism as reversible inhibitor of the 26S proteasome. It becomes undetectable after 72 hours and the proteasome activity inhibited recovers. Bortezomib has a boronic acid group that binds and forms a complex with the active site of threonine hydroxyl group in the  $\beta$ 5-subunit. It blocks the chymotrypsin-like activity of the proteasome. This proteasome is responsible for the cell-death inducing capabilities.<sup>7</sup> The anti-cancer mechanisms of bortezomib include: inhibition of NF $\kappa$ B and its anti-apoptotic target genes, upregulation of proapoptotic proteins (e.g., Noxa, I $\kappa$ B), down-regulation of expression of proteins involving in DNA repair pathways, suppression of some anti-apoptotic proteins (e.g., Bcl-XL, Bcl-2, and STAT-3), and induction of pro-apoptotic Unfolded Protein Response (UPR) and endoplasmic reticulum (ER) stress. Bortezomib has potent chemo-/radio-sensitizing effects. When used in combination with potential chemotherapies, it can overcome drug resistance

in tumors. Bortezomib has been successful in improving clinical outcomes, but relapse may occur in patients who respond initially.<sup>7</sup>

New agents after thalidomide, lenaliomide, and bortezomib are currently investigated in clinical trials. More recently, using monoclonal antibodies (mAbs) to target antigens expressed on the surface of MM cells a targeted approach to MM treatment has emerged. MAbs on clinical trials kill MM cells via the host's immune system or by promoting apoptosis. They appear to have generally improved tolerability when compared with current available treatments. However these mAbs are costly and new future data from detailed studies are needed to see the effect on long-term survival and also quality of life in patients with MM.<sup>8</sup>

However, MM remains an incurable disease. Patients who were successfully treated, eventually relapse. It always requires further therapy. For patients with relapsed or refractory MM treatment options include hematopoietic stem cell transplantation, enrollment into a clinical trial for a new regimen or rechallenge of the previous therapeutics. Only limited numbers of patients are eligible for transplantation. Most of the patients do not have the eligibility criteria to participate in clinical trials. The dexamethasone, cyclophosphamide, etoposide, and cisplatin (DCEP) antitumor activity has been proven as an effective regimen for relapsed MM patients after high-dose chemotherapy and autologous transplantation. A recent research reported that the DCEP regimen in relapsed or refractory MM patients exhibited a 58.3 % overall response rate. It has a median response duration of 9 months. It also offered the chance for a successful peripheral blood stem cell mobilization for patients who were eligible for autoHSCT. The National Comprehensive Cancer Network guidelines (NCCN) recommend DCEP as the preferred salvage chemotherapy.<sup>8,9</sup>

### **1.3 U266 CELL LINE**

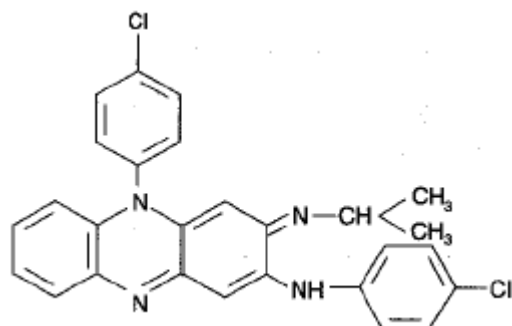
The U266 cell line contains a single mutant p53 allele. Wild-type (wt) p53 stable expression in U266 cells resulted in suppression of interleukin-6 gene expression. Concomitant suppression of cell growth could be restored by addition of exogenous

IL-6. Expression of wt p53 leads to protection from doxorubicin (Dox)- and melphalan (Mel)-induced apoptosis and cell cycle arrest. The addition of IL-6 resulted in cell cycle progression and blocked p53-mediated protection from apoptosis.<sup>10</sup> U266 cells express high levels of the anti-apoptotic protein Bcl-x and exhibit constitutive activation of STAT3. Blocking STAT3 by using JAK inhibitor results in reduced Bcl-x expression and enhanced sensitivity to Fas mediated apoptosis in the IL6-dependent line. Therapeutic approaches to interrupt this pathway may result in improved drug response.<sup>11 12</sup> Constitutively active STAT3 was implicated in the induction of resistance to apoptosis<sup>11</sup>, possibly through the expression of Bcl-xL and cyclinD1.<sup>13</sup>

#### 1.4 CLOFAZIMINE

Riminophenazines are active phenazine compounds wherein a substituent (R) has been included in the 'imino' part of the molecule. History dates back to 40s when this group of compounds was derived from lichens. Lichens constituted an important source against tuberculosis with several active compounds like usnic acid, roccellic acid and diploicin (obtained from the lichen *Buellia canescens*). Several derivatives of diploicin led Barry and colleagues to the discovery of anilinoaposafranine, which was highly active against tubercle bacilli but had severe toxicity. After this discovery, Barry' s group prepared several structural modifications (R-substitution) mainly at the (NH) imino group; with the name ' rimino' compounds. Barry and colleagues synthesize 2-chloro-anilino-5-*p*-chlorophenyl-3,5-dihydro-3-isopropyl iminophenazine. It was initially called B663 and, later, Lamprene or Clofazimine. In 1957 International Tuberculosis Conference in New Delhi, people witnessed the great excitement which Dr. Barry announced of this discovery as a very powerful drug to cure tuberculosis once and for all.<sup>14-19</sup>

The molecular structure of clofazimine, contains an isopropylimino group at position two of the phenazine nucleus, is shown in Figure 2.



**Figure 2. Clofazimine molecular structure**

Clofazimine is mainly used in the treatment of lepromatous leprosy, lepromatous leprosy and dapsone-resistant lepromatous leprosy complicated by erythema nodosum leprosum, and still has been included as an anti-leprosy medicine in the current WHO Model Lists of Essential Medicines. While it is used in its original capacity in tuberculosis, it was found to be more efficient against another *Mycobacteria leprae* and with other leprosy-causing species of *Mycobacteria*. In 59 years of treatment history they discovered it as a broad-range antibacterial agent, and its main side effect immunosuppression. This side effect is now a new application as a general anti-inflammatory agent, and found beneficial in treatment of infectious skin diseases caused by microorganisms.<sup>20</sup>

## 1.5 PHARMOKINETICS OF CLOFAZIMINE

Clofazimine is very lipophilic and distributes primarily into fatty tissues, besides cells of the mononuclear phagocyte system. Clofazimine administration to mice with a dose of 25 mg/kg body weight (28 days), the average concentrations in the fat, spleen, lungs and plasma were respectively ~ 4000, 800 and 80 mg/kg and 3 mg/L.<sup>18</sup>

The plasma clofazimine concentration achieved upon leprosy treatment is 1  $\mu\text{M}$ .<sup>21</sup> However in experimental animals the higher doses up to 10  $\mu\text{M}$  in plasma were also tolerable.<sup>22</sup> Clofazimine accumulates in tissues and the concentrations in organs vary from 10  $\mu\text{M}$  up to 500  $\mu\text{M}$ .<sup>21</sup> So far, no transporter mechanism has been shown to

efflux the clofazimine out of cells, and the drug molecules have seemed to accumulate in subcellular compartments. Clofazimine possesses an unusually long pharmacokinetic half-life of up to 70 days associated with extensive accumulation of the drug in the body did not follow classical pharmacokinetics models in which an elimination process, such as hepatic metabolism or renal excretion, plays a major role in lowering plasma concentration.<sup>23</sup>

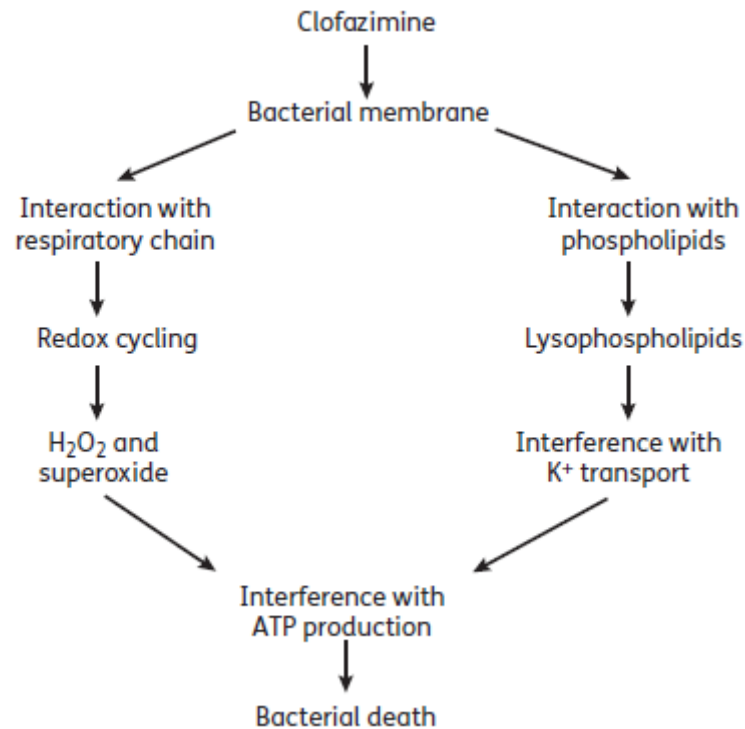
## 1.6 CLOFAZIMINE MECHANISM OF ACTION

Clofazimine has therapeutic efficacy in various chronic inflammatory disorders, non-microbial and including discoid lupus erythematosus, pustular psoriasis, Melkersson–Rosenthal syndrome, granuloma annulare, necrobiosis lipoidica and cutaneous lesions in systemic lupus erythematosus.<sup>24</sup> Lately, it is found that clofazimine holds therapeutic promise, encompassing non-cutaneous autoimmune disorders (multiple sclerosis and type I diabetes mellitus).

CLF has an immunomodulatory action which T lymphocytes play a major role in the activation of this effect. Two major mechanisms suggested for this action as firstly targeting plasma membrane  $K^+$  transport. This acts on both sodium–potassium exchanger,  $Na^+$ ,  $K^+$ -ATPase, and Kv1.3 potassium channel. This causes to have an inhibitory effect on the activation and also proliferation of T lymphocytes.<sup>25</sup> It has been showed that CLF selectively blocked the Kv1.3 channel activity. This is by perturbing of the calcium-release activated calcium channel. This change then led to the inhibition of the calcineurin-NFAT signaling pathway. CLF also was found to be effective in human T cell-mediated skin graft rejection blocking in an animal model. Together, these results show that CLF is a promising immunomodulatory drug candidate for treating a variety of autoimmune disorders.<sup>25</sup> It was shown that CLF exhibits apoptosis-inducing activity in macrophages as cells exhibited a marked decrease in metabolic activity and showed shrinkage in cell size, indicating cell death. In CLF-treated macrophages and THP-1 cells significant enhancement of caspase-3 activity was observed. Apoptosis-inducing activity of CLF in macrophages, may also be responsible for the antibacterial properties of CLF.<sup>26</sup> CLF

massively accumulates in macrophages forming insoluble, intracellular crystal-like drug inclusions (CLDIs). Macrophages sequester CLF in CLDIs. Phagocytized CLDIs did not lead to mitochondrial destabilization and apoptosis. CLDIs change immune signaling response pathways. They downstream of Toll-like receptor (TLR) ligation and enhance interleukin-1 receptor antagonist (IL-1RA) production. They both dampened NF- $\kappa$ B activation, tissue necrosis factor alpha (TNF $\alpha$ ) production. They also decreased TLR expression levels. CLDIs undergo active phagocytosis without any cytotoxicity. CLF is suggested as a novel, macrophage targeted therapeutic approach for treating inflammatory diseases.<sup>27</sup>

Since 1998, *in vitro* and *in vivo* anticancer effects of CLF has also been studied on a large variety of cancer types such as hepatocellular, lung, cervix, melanoma, esophageal, colon, leukemia, neuroblastoma and breast cells. CLF has been shown to reverse multidrug-resistance (MDR) in various cell lines<sup>28-33</sup>, have tumoricidal properties due to increases in the cell membrane lysophospholipid content<sup>33</sup>, inhibit respiratory function and energy metabolism in yeast and in transformed fibroblasts, inhibit tumor energy metabolism of a chemo-resistant human non-small-cell bronchial-carcinoma cell line<sup>34</sup>, inhibit both respiratory function and tumor energy metabolism, activate the intrinsic apoptotic pathway in a Kv1.3-dependent and a Bax/Bak-independent way<sup>35,36</sup>, make poly (ADPribose) polymerase (PARP) cleavage<sup>35</sup>, phospholipase A2-mediated oxidative and nonoxidative mechanisms<sup>33</sup> and finally inhibit Wnt signaling pathway<sup>37</sup>.



**Figure 3. Antimicrobial mechanisms of clofazimine.**

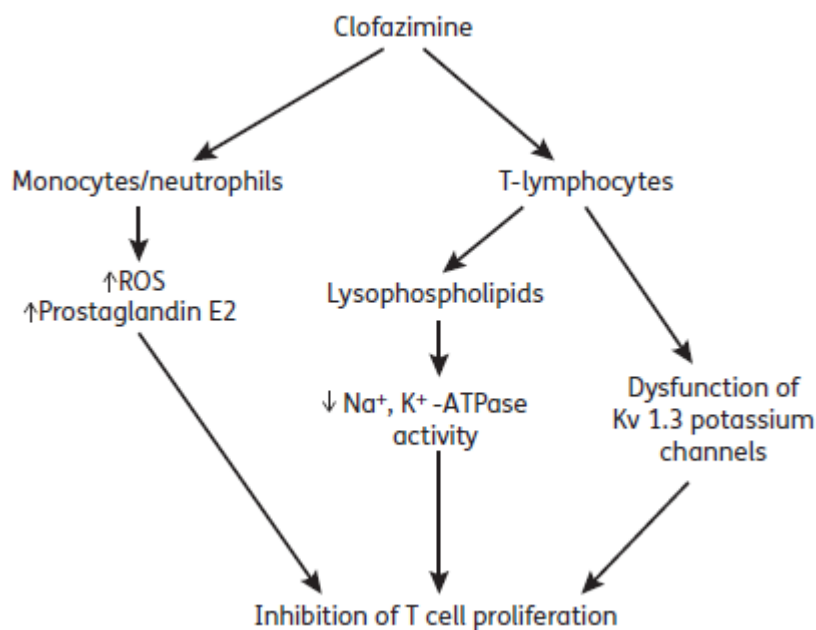
### 1.6.1 Na<sup>+</sup> AND K<sup>+</sup>-ATPASE

Na<sup>+</sup>, K<sup>+</sup>-ATPase exchanges 3 sodium ions for 2 potassium ions, enabling cells to accumulate potassium intracellularly, that is essential for sustaining activities like resting membrane potential, enzyme activities involved in cellular metabolism, active transport of nutrients, biosynthesis of macromolecules and cell proliferation.<sup>38</sup> Treatment of T lymphocytes with 0.6 micromolar concentration of clofazimine for 60 min caused inhibition of the mitogen-activated increase in Na<sup>+</sup>, K<sup>+</sup>-ATPase activity. At the same concentration, clofazimine caused significant inhibition of the proliferative responses of T lymphocytes. Clofazimine induced decrease in T lymphocyte Na<sup>+</sup>, K<sup>+</sup>-ATPase activity by increased arachidonic acid.<sup>39</sup> This event may be a result of the increased activity of phospholipase A<sub>2</sub> (PLA<sub>2</sub>), and direct acid hydrolysis of membrane phospholipids ester linkages.<sup>40</sup> In either case, the lysophospholipids are formed.

## 1.6.2 ROS AND PROSTAGLANDIN E2

Clofazimine could indirectly interfere with the proliferation of T cells. It can promote the release of E-series prostaglandins (PGs), especially PGE<sub>2</sub>, and ROS from neutrophils and monocytes.<sup>41</sup> Production of PGE<sub>2</sub> results in cyclooxygenase which is the substrate for Arachidonic acid released from membrane phospholipids. Adenylyl cyclase couples with EP2 receptors on T cells, then PGE<sub>2</sub> initiates the production of antiproliferative 3'-5'-cyclic adenosine monophosphate (cAMP). Arachidonic acid is also an activator of NADPH oxidase. It was also known as the ROS-generating system of phagocytes.<sup>42</sup>

Clofazimine causes T lymphocyte activation and proliferation. Its mechanisms are summarized in Figure 4.<sup>20</sup>



**Figure 4. Immunosuppressive activity mechanism of clofazimine**

## 1.6.3 KV 1.3 POTASSIUM CHANNELS

CLF was previously shown to be a potent Kv1.3 channel blocker.<sup>25,32,35,36,43</sup> Kv 1.3 is a voltage-gated, delayed rectifier K<sup>+</sup> ion channel. They regulate membrane potential and Ca<sup>2+</sup> signalling in effector memory T cells. It is identified that



clofazimine is interfered with  $\text{Ca}^{2+}$  signalling in T cells activated with phorbol myristate acetate (PMA)/ionomycin, by antagonism of the Kv 1.3 potassium channel and involved in transcriptional activation of the gene encoding interleukin 2 (IL-2).  $\text{K}^+$  efflux interference and failure of the efficient membrane repolarization response necessary to drive  $\text{Ca}^{2+}$  influx by calcium release-activated  $\text{Ca}^{2+}$  channels are the functions of this ion channel. Suppression of the cytosolic  $\text{Ca}^{2+}$  oscillation is necessary for activation of the calcineurin-nuclear factor of activated T cells (NFAT) signalling pathway (initiates transcriptional activation of the IL-2 gene).<sup>25</sup>

Direct inhibitors of mitoKv1.3 are very attractive novel chemotherapeutic agents. This was exemplified for chronic lymphocytic leukemia (B-CLL), a common leukemia with limited treatment options: importantly, the effects were obtained independently of the prognostic factors (ZAP70, CD38 and hyper-somatic mutation). Clofazimine very efficiently triggered death of B-CLL cells, while surprisingly the drug had almost no effect on B or T cells isolated from healthy subjects or on T cells isolated from the same B-CLL patient. The relatively high sensitivity of B-CLL cells compared with non-malignant lymphocytes was explained by an increased, constitutive oxidative stress in cancer cells compared to non-malignant cells. This hypothesis is consistent with the finding of increased ROS production and a marked mitochondrial depolarization in B-CLL cells after treatment of the tumor cells and inhibition of Kv1.3 with clofazimine. Studies indicate that inhibition of Kv1.3 is an attractive novel target to be explored for its potential to induce death of a variety of tumor cells. Since membrane permeable inhibitors of Kv1.3 activate the intrinsic apoptotic pathway, also in the absence of Bax and Bak, they might be effective in many malignancies that are resistant to treatment due to an up-regulation of anti-apoptotic Bcl-2/Bcl-xL or a lack of pro-apoptotic Bax/Bak proteins.<sup>44</sup>

Kv1.3-deficient mice were generated and show an increase in the platelet count, a finding that would be consistent with a role of Kv1.3 in apoptosis of platelets or precursors. However, the lack of Kv1.3-expression seems to be compensated in most cells of these mice by up-regulation of Kv1.1 and of a chloride channel.<sup>44</sup>

Kv1.3 is characterized by a selective and potent pharmacology, leading to specific pharmacological targeting. Since potassium channels may play a pivotal role in tumor cell proliferation, these proteins should be considered when designing new cancer treatment strategies.<sup>45</sup>

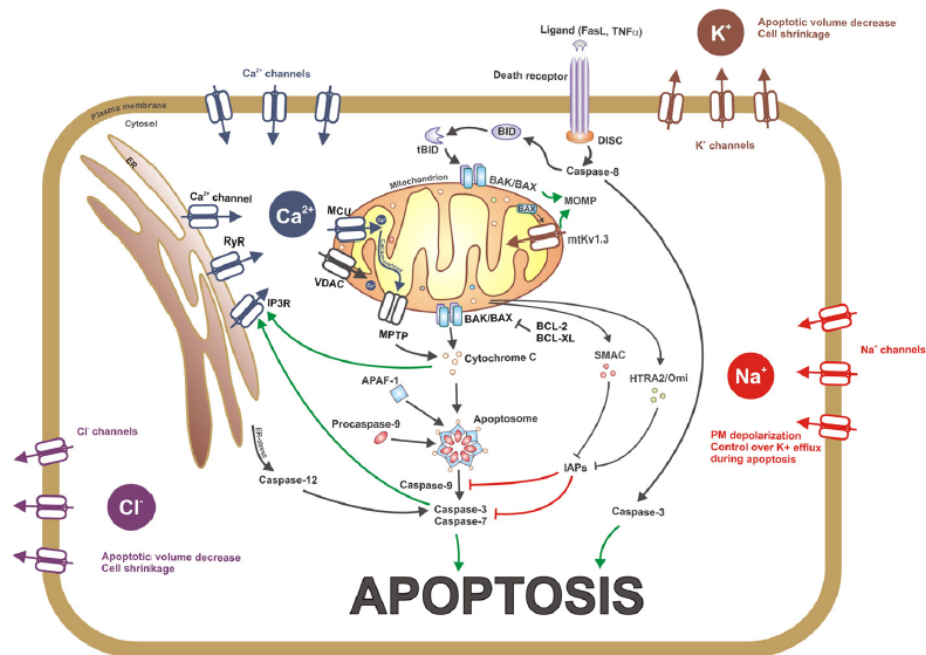
## **1.7 APOPTOSIS AND KV1.3**

The apoptotic machinery is highly complex which involves a lot of molecular players. Depending on the mechanism of initiation, apoptosis signaling pathways can be divided into two main pathways: the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathway. Although both pathways have different initiators and mechanisms, these two pathways are closely interrelated.

Intrinsic pathway is initiated with DNA damage, ER stress etc. These stimuli induce changes in mitochondrial membrane permeability. This causes the release of proapoptotic factors from the intermembrane space. These pro-apoptotic proteins include second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI (SMAC/DIABLO), cytochrome c (Cyt C), high-temperature requirement protein A2 (HTRA2/Omi) and others. Cyt C, once released into the cytoplasm, binds to apoptotic protease activating factor 1 (Apaf-1) and dATP to form apoptosome and triggers the activation of initiator caspase-9. Active Caspase 9 then activates effector caspase-3.

Extrinsic pathway is induced by extracellular pro-apoptotic signals or ligands that activate death receptors which trigger apoptosis by caspase-dependent pathway. Ligands bind to death receptors which initiate the formation of multiprotein complex dubbed death-inducing signaling complex (DISC). This results in activation of initiator caspase-8. Active caspase 8 activates caspase-3, and triggers caspase-dependent apoptosis. Caspase-8 mediates the cleavage of Bcl2-protein family member BH3-interacting domain death agonist (BID), resulting in a pro-apoptotic truncated BID (tBID), inducing subsequent MOMP, release of CytC from

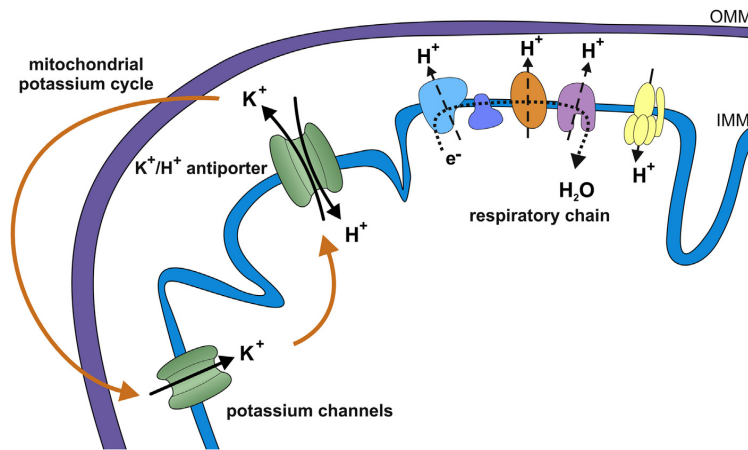
mitochondria and triggering caspase-9-dependent apoptosis. Major types of ion channels involved in apoptosis regulation are represented in Figure 6.<sup>46</sup>



**Figure 5. Ions and ion channels in the regulation of apoptosis.**<sup>46</sup>

Kv1.3, as well as other Kv channels (Kv1.1 and Kv1.5), was localized both at plasma membrane and in the inner mitochondrial membrane. Kv1.3 is active in the inner mitochondrial membrane even at negative resting potential ( $-180$  mV). In normal conditions, K<sup>+</sup> channels located to the inner mitochondrial membrane mediates an inward K<sup>+</sup> flux from the cytosol to the mitochondrial matrix. The electrochemical gradient of this ion as shown in Figure 7.<sup>44</sup>

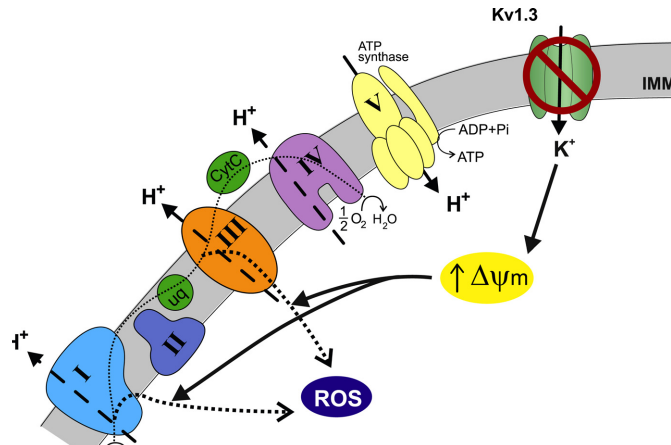
This positive flux is compensated by the efflux of protons (H<sup>+</sup>) mediated by the respiratory chain and by the K<sup>+</sup>/H<sup>+</sup> antiporter, to avoid volume changes and depolarization.<sup>47</sup> Kv1.3 is located on the inner mitochondrial membrane. Since no targeting sequence is present in the Kv1.3 protein, the molecular mechanisms of mitochondrial targeting of Kv1.3 are still unknown.<sup>44</sup>



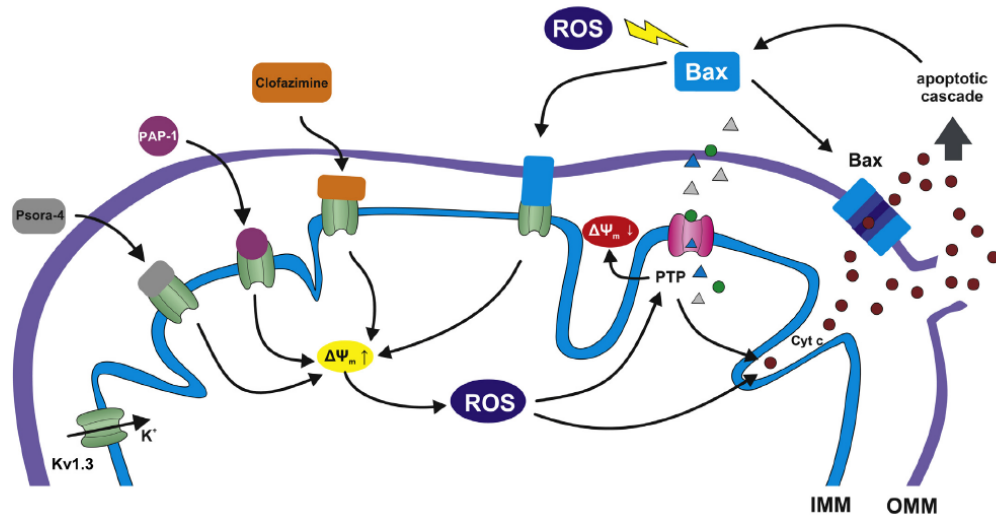
**Figure 6. Mitochondrial potassium channel**

MitoKv1.3 is a voltage-dependent  $K^+$  channel that mediates an inward  $K^+$  flux to the mitochondrial matrix and participates to the  $K^+$  cycle. Inhibition of mitochondrial potassium channels blocks the  $K^+$  entrance into the mitochondrial matrix leading to hyperpolarization of the inner mitochondrial membrane as shown in Figure 7.

The mitochondrial respiratory chain is regulated by the membrane potential  $\Delta\psi$ , the ATP/ADP ratio and phosphorylation of the complexes I and IV. Hyperpolarization of the inner mitochondrial membrane changes the redox state of the respiratory chain complexes. This increases single electron leakage at complexes I and III to molecular oxygen and increases superoxide anion production. Complex III-dependent ROS is formed by depolarization of inner mitochondrial membrane. The membrane impermeable superoxide anion is released to either the mitochondrial matrix space or to the inter-membrane space when produced at complex I or III.



**Figure 7. MitoKv1.3 and ROS production**



**Figure 8. MitoKv1.3 induced apoptotic cell death**<sup>44</sup>

Inhibition of mitoKv1.3 by Bax results in several dramatic changes of mitochondrial physiology, in particular the release of cytochrome c from mitochondria, an initial hyperpolarization of the inner mitochondrial membrane that is followed by a depolarization and the production of ROS. The initial hyperpolarization of the inner mitochondrial membrane is consistent with the inhibition of Kv1.3 and results in an increased release of ROS that oxidize the permeability transition pore (PTP) finally resulting in depolarization. ROS are also involved in the release of cytochrome c, possibly by a change of the oxidation state of cardiolipin that binds to Kv1.3, but

other, yet unknown mechanisms seem to be also important in the release of cytochrome c by inhibition of Kv1.3. In summary, the model of mitoKv1.3 and Bax interactions predicts that the positively charged lysine of Bax plugs the pore of the channel, and thereby initiates mitochondrial changes during apoptosis. The inactivation of mitoKv1.3 by Bax, toxins or pharmacological inhibitors results in an active change of mitochondria physiology, i.e. hyperpolarization of the mitochondrial membrane and ROS release (Figure 8). Kv1.3 has been shown to be expressed in brain, lung, thymus, spleen, lymph node, fibroblasts, lymphocytes, tonsils, macrophages, microglia, oligodendrocytes, osteoclasts, platelets, liver, skeletal muscle, in hippocampal neurons, astrocytes and brown and white fat. Furthermore, Kv1.3 was shown to be expressed in various cancers, such as lymphoma, melanoma, glioma, breast, prostate, gastric and colon cancer. Finally, Kv1.3 was shown to be expressed in the prostate and breast cancer cell lines PC3 and MCF7, respectively, and lymphoma and leukemia cells even in mitochondria. As described above, Kv1.3 is a direct target of Bax and mediates its pro-apoptotic effects. Thus, drugs that inhibit mitoKv1.3 should induce cell death mimicking Bax interaction, even in cells overexpressing anti-apoptotic Bcl-2-like proteins.

## **1.8 Kv1.3 AND CELL PROLIFERATION**

Several VGIC have been found playing important roles during the cell cycle by their expression level. Opening of the voltage-gated Na<sup>+</sup> and/or Ca<sup>++</sup> channel causing depolarization of the membrane, move positive charges from the extracellular space to the cytoplasm. This event appears to promote transition from the G0/G1 phase to the S phase of the cell cycle.

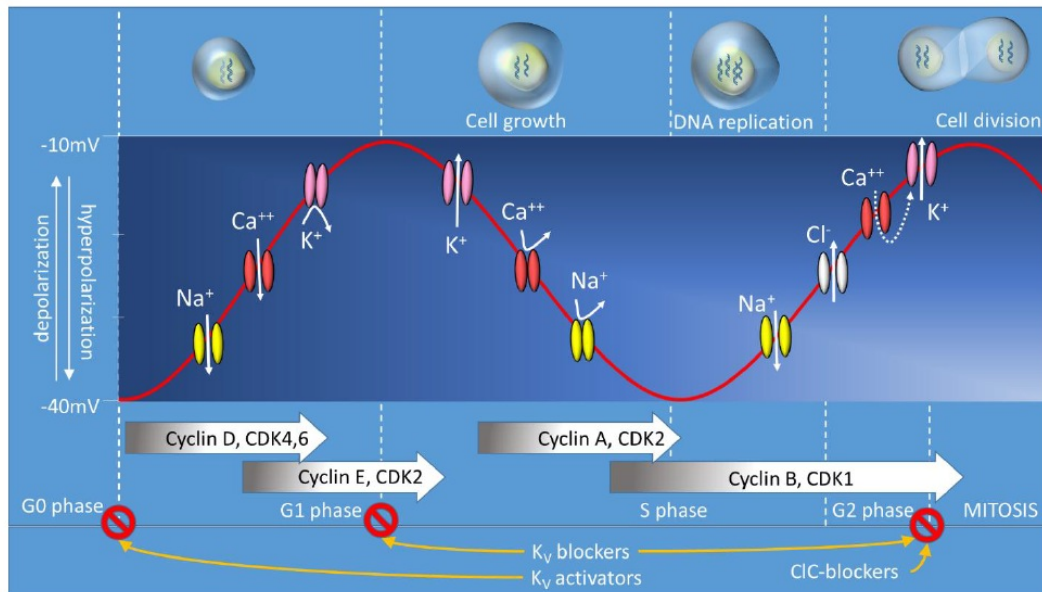
In contrast, membrane potential during the S phase tends to repolarize due to the opening of K<sup>+</sup> channels and/or the closing of Ca<sup>++</sup> and/or Na<sup>+</sup> channels. Mitosis is associated with more depolarized membrane potential compared to cells in the rest of the S phase. This is possible due to increased cytosolic Na<sup>+</sup> and/or Ca<sup>++</sup>. Depolarization and augmented Ca<sup>++</sup> entry will allow stimulation of Ca<sup>++</sup>-activated K<sup>+</sup> channels and activation of Cl<sup>-</sup> channels. Both K<sup>+</sup> and Cl<sup>-</sup> outward currents are

responsible for water leaving the cytoplasm, which leads to a cell shrinkage before cell division. Chronic application of K<sup>+</sup> channel blockers (e.g., Kv11.1 blocker E4031) or openers (Kv11.1 opener NS1634) leads to changes of the membrane potential in the opposite direction, but both type of drugs cause arrests of the cell cycle in the G<sub>0</sub>/G<sub>1</sub> phase, while blockade of the VGIC arrests the cell cycle in the G<sub>2</sub> phase. This suggests that oscillation of the membrane potential is a fundamental event that promotes progression of the cell cycle. (Figure 9) Clofazimine (5 μM) also suppresses calcium signaling in the human leukemia Jurkat T cell line.<sup>25</sup>

During proliferation, in the early stages of G<sub>1</sub> phase or during the G<sub>1</sub>/S transition, Kv members may be important during the cell cycle progression. This is the case for Kv1.3 and Kv1.5. The use of pharmacological tools suggests that the mechanism may involve cyclin-dependent kinase (CDK) inhibitors, such as p21 and p27.<sup>45</sup>

Kv1.3 inhibition significantly increased expression level of p21 and decreased the expression level of Cdk4 and cyclin D3. Both change in expression levels inhibit G<sub>1</sub> to S transition. The *in vivo* anti-proliferative effect of Mgtx which is a Kv1.3 selective blocker (IC<sub>50</sub>: 2nM) was confirmed in an independent work, in an orthotopic melanoma model using B16F10 cells.<sup>35</sup>

Pro-proliferative properties of Kv1.3 channel shows aberrant expression in many tumors. The expression of Kv1.3 correlates with a certain grade of malignancy in several types of tumors, as in lung, glioma, breast, colon, and prostate cancers. Kv1.3 abundance in prostate cancers is inversely correlated with tumorigenicity and no apparent correlation between Kv1.3 expression and degree of malignancy in gliomas was found.<sup>45</sup>



**Figure 9. Schematic representation of possible involvement of different VGIC activity during the cell cycle of cancer cells.**

## 1.9 CLOFAZIMINE AND CANCER

Clofazimine's anticancer activity was first described in 1993 by Rensburg who have shown that clofazimine as well as a derivative, B669, are potent inhibitors of various cancer cell lines including T24; a human transitional carcinoma cell line FaDu; a human squamous carcinoma cell line, HeLa; a human cervix carcinoma cell line, and PLC; a hepatocellular carcinoma cell line which has intrinsic multidrug resistance and two primary cultures and a noncarcinoma cell line. The treatment of FaDu cells with clofazimine and B669 was related with enhanced activity of phospholipase A2, as evidenced by increased release of radiolabeled arachidonate and lysophosphatidylcholine from membrane phospholipids. They found that the tumoricidal properties of clofazimine and B669 are because of increases in the lysophospholipid content of cell membranes.<sup>33</sup>

In 1994 Rensburg showed the potential of the clofazimine and B669, at therapeutically relevant concentrations, to reverse P-glycoprotein-mediated multidrug-resistance (MDR) in a human lung cancer cell line (H69/LX4) *in vitro*.<sup>28</sup>



Clofazimine and B669 at minimally cytotoxic concentrations of 1 and 0.5 µg/ml were equally effective in restoring sensitivity to vinblastine, doxorubicin, daunorubicin and mitomycin C in the H69/LX4 cell line.

In 1994 Sri-Pathmanathan showed that Clofazimine inhibit respiratory function and hence energy metabolism in yeast and in transformed fibroblasts. They examined the effect of Clofazimine on the energy metabolism of a chemoresistant non-small-cell bronchial-carcinoma cell line (WIL) and determined growth inhibition rate *in vitro* and *in vivo*. Clofazimine had a direct cytotoxic effect *in vitro* with an ID<sub>50</sub> of 10.2 pM. Clofazimine reduced the tumor size one third that of controls when administered to athymic mice bearing WIL as a subcutaneous xenograft. They suggest Clofazimine selectively inhibits tumour energy metabolism.<sup>34</sup>

In 1996 Myer et al. showed that differential expression of a permeability glycoprotein (P-gp) in human myeloleukaemia K562 cells grown in the presence of doxorubicin rised to subclones with varying degrees of resistance to other anti-tumour drugs such as vinblastine and daunorubicin. Subclones K562/MMB, MMG and MMF with MDR nature were produced from the parental (K562IP) cell line. When these subclones were pre-incubated with the chemosensitizing agents clofazimine and B669 at the remarkably low concentration of 0.06,µg/ml effectively subverted MDR in all three K562 subclones.<sup>48</sup>

In 1998 Ruff *et al.* published the first phase II study results of clofazimine, in unresectable and metastatic hepatocellular carcinoma. They showed objective response rate of 10% (three patients), as well as 43.3% (13 patients) disease stabilization for up to 20 months, and an overall median survival of 13 weeks. Clofazimine is very well tolerated, has minimal toxicity, and they suggest that oral clofazimine may be useful in the treatment of hepatocellular carcinoma.<sup>29</sup>

At the same year in 1998 Rensburg *et al.* investigated *in vitro* the effects of the membrane-stabilizing agent, α-tocopherol (25 mg/ml), on the chemosensitizing interactions of cyclosporine A (5 mg/ml), verapamil (2 mg/ml), clofazimine (1

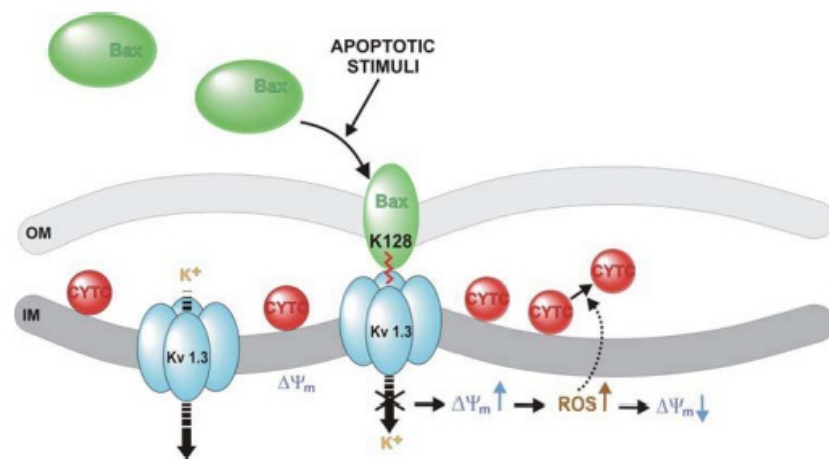
mg/ml), B669 (0.5 mg/ml) and GF120918 (0.015 mg/ml) with a P-glycoprotein-expressing human lung cancer cell line (H69/LX4). In cell proliferation assay, all the agents restored the sensitivity of H69/LX4 cells to doxorubicin and vinblastine.<sup>49</sup>

In 1999 Falkson published the second phase II study which was undertaken as it was hoped that clofazimine would add to the therapeutic effect of doxorubicin without increasing toxicity. Seven patients were treated with clofazimine to gain experience with the tolerance of the drug with HCC. 7 patients who received clofazimine alone had not an objective response to treatment. Out of 21 patients who received clofazimine plus doxorubicin, 9 had stable disease, 18 had progression of their disease as best response. The median survival time is found as 7 weeks, relevant to the patient discriminants. The absence of response and the poor overall median survival time of the study patients mediate against further investigation of other combinations of clofazimine and doxorubicin in patients with HCC.<sup>30</sup>

In 2001 Niekerk *et al.* showed the effects of nine new tetramethylpiperidine (TMP)-substituted phenazines on the growth of two human hepatocellular carcinoma cell lines (PLC and HepG2) a human esophageal cancer cell line (WHCO3), and three human colon cancer cell lines (CaCo2, COLO 320DM and HT29). They compared to those of clofazimine, B669 and five standard chemotherapeutic agents. The efficacy of compounds suggests that these agents may be useful in the treatment of intrinsically resistant cancers such as liver and colon cancer.<sup>31</sup>

In 2008 Szabo *et al.* showed that mouse and human cells that are transfected with siRNA or genetically deficient in Kv1.3, suppress Kv1.3-expression resisted apoptosis induced by several stimuli (including Bax over-expression). Retransfection of Kv1.3 or a mitochondrial-targeted Kv1.3 restored cell death. Incubation of isolated Kv1.3-positive mitochondria with recombinant Bax, t-Bid, or toxins that bind to and inhibit Kv1.3 forms reactive oxygen species, releases cytochrome c, and triggered hyperpolarization and marked depolarization. Kv1.3-deficient mitochondria were resistant to Bax, t-Bid, and the toxins. They suggest that Bax mediates cytochrome c release and mitochondrial depolarization in lymphocytes, by its

interaction with mitochondrial Kv1.3. They proposed the model for action of mitochondrial Kv1.3 during apoptosis as in Figure 10. Bax inhibits Kv1.3 via interaction of lysine 128 with the channel pore vestibule which results in hyperpolarization of the IMM. Hyperpolarization interferes with respiration and triggers release of ROS, which favors detachment of cytochrome c. Release of cytochrome c is mediated by oxidation of membrane lipids, activation of PTP might be caused by oxidation of cysteine residues. They established a direct link in lymphocytes between proapoptotic machinery in the cytoplasm and mitochondrial potassium conductance, indicating a channel-blocking action of Bax.<sup>50</sup>



**Figure 10. The model for action of mitochondrial Kv1.3 during apoptosis**

In 2012 Leanza *et al.* showed that Clofazimine reduced tumor size by 90% in an orthotopic melanoma mouse model *in vivo*. Injection of clofazimine into mice shown to effectively eliminate a melanoma *in vivo* did not result in any significant change of blood cell counts. Clofazimine activated the intrinsic apoptotic pathway also in the absence of Bax and Bak, a result in agreement with the current mechanistic model for mitochondrial Kv1.3 action. They demonstrated that PAP-1, Psora-4 and clofazimine induce apoptosis, in a Kv1.3-dependent and a Bax/Bak-independent way, in three cancer cell lines expressing mitoKv1.3. Clofazimine effectively suppresses the *in vivo* growth of a transplanted mouse melanoma. They provide a novel approach for the development of drugs that induce apoptosis independently of

Bax and Bak. They showed that clofazimine treatment did not induce histologically detectable changes in the lungs, small intestine, brain, heart, kidney, liver and spleen and appears to be non-toxic. Also they did not detect a gross deletion of lymphocytes in clofazimine-treated mice, although the effect of the drug on lymphocyte sub-populations. PAP-1, Psora-4 and clofazimine makes poly (ADPribose) polymerase (PARP) cleavage, an event downstream of cytochrome c release and caspase-3 activation, only in cells expressing Kv1.3. When these agents were used in combination with MDRi PARP was cleaved. On the other hand, MgTx and ShK did not induced caspase-9 or caspase-3 activation, cytochrome c release, mitochondrial depolarization or PARP cleavage, indicating that inhibition of plasma membrane Kv1.3 is not enough to trigger cell death. All their data indicate that membrane-permeant Kv1.3 inhibitors activate intrinsic apoptotic signaling pathways, and suggest that this action is achieved by specifically targeting mitochondrial Kv1.3. *In vivo*, they showed that intraperitoneal injection of clofazimine in an orthotopic melanoma B16F10 mouse model reduced 90%, tumor size without causing visible side-effects as assessed by histology.<sup>35</sup>

In 2013 Leanza *et al.* showed that Clofazimine might represent a novel treatment for B-CLL. It is characterized by a clonal accumulation of mature neoplastic B cells which are resistant to apoptosis. Clofazimine, an inhibitor of both Kv1.3 and multidrug resistance was also effective alone at a concentration of 10 uM. They also showed that B or T cells isolated from healthy subjects and human peripheral blood monocytes were resistant to these stimuli. Pathologic B cells showed higher channel expression and were sensitive to treatment. However healthy cells, which expressed less Kv1.3 protein, were resistant to the drugs. As Clofazimine ‘bypass’ the action of Bax, which is a critical apoptotic factor believed to be neutralized by Bcl-2, it can also induce apoptosis in the case of Bcl-2 overexpression. They found out that mtKv1.3 inhibitors are potent and selective inducers of B-CLL apoptosis via the intrinsic pathway. They kill B-CLL cells while sparing the residual T cells although the same patient, and act on B-CLL cells independently of current prognostic factors. They showed that a synergistic action of Kv1.3 expression level and of altered redox state in the malignant B-CLL cells accounts for the selective action of the drugs.<sup>36</sup>

In 2014 Koval *et al.* perform in silico docking of the 1100-large FDA approved drugs library. Fourteen candidate compounds with higher scores were selected for further *in vitro* testing using combination of the conventional TopFlash transcriptional readout assay and the GTP-binding assay, the latter especially suitable for monitoring of the early steps in Wnt signaling. Out of 14 candidates, they found four that specifically inhibited the Wnt signal transduction. However, in all cases suppression occurs at the levels of the cascade lower than the ligand–receptor interaction. They continued to work with clofazimine and report the specific anti-cancer activity of clofazimine affecting Wnt-dependent triple-negative breast cancer cells. They investigated its effects on the HTB19 triple-negative breast cancer, growth of which was shown to be dependent on the Wnt pathway and estimated the IC50 value of clofazimine for the growth the HTB19 cells, within the range of 3–6 mM—similar to the effect of clofazimine on the inhibition of the Wnt pathway in HEK293T cells.<sup>37</sup>

Szabo *et al.* in 2015 done experiments on B-CLL and healthy B cells cultured on a mesenchymal stromal cells (MSC) monolayer, in order to reproduce the lymph node niche and to let the cells receive different proliferative and anti-apoptotic signals produced by the MSC. Co-culturing with MSC cells partially reduced Kv1.3 activity in B-CLL. MSC cells release signals that modulate channel expression and/or activity in the pathologic cells. Adhesion of B-CLLs on MSC is known to favour their survival, proliferation, and tissue retention and a mutual interaction exist between B-CLL and MSC. Changes in Kv1.3 expression level alone do not alter the sensitivity of the cells to apoptosis-inducing Kv1.3 inhibitors while a higher basal ROS production in the B-CLL cells is required to be able to pass the critical threshold necessary to selectively kill the pathologic cells by ROS-inducing agents. The changes in Kv1.3 expression in leukemic cells upon incubation with PLX4720 or by co-culture with MSC are significant but do not lead to complete abolishment of channel expression and activity, therefore the cells remain sensitive to Kv1.3 inhibitors. Co-culturing of B-CLL with MSC decrease the ability of the drugs to induce cell death, in accordance with the known anti-apoptotic action of MSC. Clofazimine, is able to kill to a significant extent the pathologic cells even under this

condition, and leaves unaffected the healthy MSC cells. This work provides evidence that Kv1.3 expression and activity is upregulated in B-CLL cells with respect to B cells from healthy subject possibly by B-RAF mediated signalling pathway. Clofazimine as a selective, potent apoptosis induced agent of B-CLL cells even in the presence of mesenchymal stromal cells. The general view is emerging that modulation of ion channel activity in B-CLL cells might be a strategy to pursue for therapeutical purposes.<sup>43</sup>

### **1.10 COMBINATION POTENTIAL OF CLOFAZIMINE**

Upto date there are two publications done on combination studies with Clofazimine and a drug. First one is the phase II study published in 1999. Falkson and his colleagues stated that there is not a significant response and overall median survival time of the study patients are poor but emphasize the need for further investigation of other combinations of clofazimine and doxorubicin in patients with HCC. Seven patients were treated with clofazimine with HCC and none of the 7 patients who received clofazimine alone had an objective response to treatment. From 21 patients received clofazimine plus doxorubicin, 9 showed no change (had stable disease) while the remaining 18 had progression of their disease as best response. The median survival time of 7 weeks found in this study is not unexpected, relevant to the patient discriminants.<sup>30</sup>

The second study was published in 2015 by Koot *et al.* They used Riminocelles (lipopolymeric micelle) co-encapsulating with synergistic fixed-ratio combination of paclitaxel (PTX) and clofazimine (B663). Although they showed the synergistic effect of PTX and B663 the delivery system they used was not as successful as they planned. CI values for PTX:B663 ratios with a greater proportion of B663 were found to be synergistic ( $CI < 1$ ) at all simulated  $f_a$  levels (0.1–0.9) at the intrinsic Pgp-expressing colorectal adenocarcinoma (COLO 320DM, ATCC CCL-220). The trend is for synergy to be greater at lower  $f_a$  levels. They used the MDR effect of Clofazimine to increase the effect of PTX. They concluded that lipopolymeric

micellular delivery system requires improvement so as to maintain and selectively deliver the fixed-ratio drug combination.<sup>51</sup>

### **1.11 AIM OF THE STUDY**

The aim of the present study was to investigate the apoptotic and antiproliferative effect of Clofazimine against human multiple myeloma cell line.





## **CHAPTER 2**

### **MATERIALS AND METHODS**

#### **2.1. MATERIALS**

##### **2.1.1. CHEMICALS**

Clofazimine (C8895), Camptothecin (C9911) and Cisplatin (C2210000) were purchased from Sigma-Aldrich. Clofazimine and Camptothecin (10 mM stock solutions) were prepared in 0.1% dimethyl sulphoxide (DMSO) and stored at -20<sup>0</sup>C. Clofazimine was prepared in 100% EtOH for combination studies (DMSO inactivates cisplatin). Cisplatin (1Mm stock solution) was prepared in 0.9% NaCl and stored at room temperature. The final DMSO concentration in the culture media never exceeded 0.2% (v/v).

##### **2.1.2. CELL CULTURE**

HL60 (Acute promyelocytic leukemia), Namalwa (Burkitt lymphoma), K562 (Chronic myelogenous leukemia), Jurkat (T cell leukemia) and U266 (Multiple myeloma) cells were kindly provided by Prof. Dr. Yusuf Baran from Department of Molecular Biology and Genetics, Izmir Institute of Technology, İzmir.

All cells were cultured in RPMI-1640 growth medium containing 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in 5% CO<sub>2</sub> incubator.

## **2.2. METHODS**

### **2.2.1. CELL VIABILITY ASSAYS**

Cell line screening, potency (IC<sub>50</sub>) determination, time-response and combination assays were completed using CellTiter Blue Cell Viability Assay (ThermoFisher Scientific). For each measurement, five technical replicates were used.

Cells were treated at a density of 1.000.000 cells/ml in screening and 100.000 cells/ml in all the other experiments. At the end of the treatment duration, assay reagent was added and plates were incubated for additional 4 h. Fluorescence was measured with excitation wavelength at 555 nm and emission wavelength at 595 nm with SpectraMax® Paradigm® Multi-Mode Microplate Reader. IC<sub>50</sub> values were calculated on GraphPad (La Jolla, USA) Prism v5.0 using non-linear curve fitting model.

### **2.2.2. COMBINATION INDEX**

Effects of drugs combination used in this study were evaluated using the combination index equation which was based on the multiple drug-effect equation of Chou-Talalay<sup>52</sup>. CI<1 indicates synergism, CI=1 indicates additive effect, and CI>1 indicates antagonism. The analysis was done with CompuSyn software (ComboSyn Inc., Paramus, USA).

Time-dependent IC<sub>50</sub> value (drug concentration inhibits cell growth by 50 %) and time response of clofazimine were determined by CellTiter Blue assay. Cells were treated with ten different doses of Clofazimine for 24 hours. IC<sub>50</sub> doses were calculated by Graphpad Prism 5.0 software. U266 cells were treated with increasing doses of clofazimine (2, 5, 10 µM) at 3 different time points (12, 24 and 48 hours).

This theory provides algorithms for computer simulation of synergism and/or antagonism. This can be at any effect and concentration/dose level, as shown in the CI plot and isobologram, respectively. (Appendix B) <sup>52</sup>

### **2.2.3. FLOW CYTOMETRY**

Cell cycle analysis, proliferation and apoptosis assays were performed on BD Biosciences (San Diego, USA) Accuri C6 flow cytometer. Ten thousand events were recorded for each measurement.

### **2.2.4. ANTIPROLIFERATIVE EFFECT**

Clofazimine (10  $\mu$ M) treated U266 cells were stained with 10  $\mu$ M Carboxyfluorescein succinimidyl ester (CFSE) for 15 min at 37°C. Cells were then added to each condition and were cultured for 3 days. The CellTrace™ reagents readily diffuse into cells and bind covalently to intracellular amines, which results in stable, well-retained fluorescent staining that can be fixed with aldehyde fixatives. Excess unconjugated reagent passively diffuses to the extracellular medium. It then can be quenched with complete media and washed away. Data acquisition and analyses were performed with an Accuri C6 Flow Cytometer (Accuri Cytometer, Ann Arbor, MI, USA).

The Proliferative Index (the sum of the cells in all generations divided by the calculated number of original parent cells), was used to compare samples treated with clofazimine, cisplatin and untreated samples. This data was obtained by using Proliferation Wizard Basic Mode analysis of ModFit LT software ver.3.0 (Verity Software House, USA).

## **2.2.5. CELL CYCLE ANALYSIS**

Cells were treated with 10  $\mu$ M CLF for 24 h and harvested. After cold PBS wash, samples were fixed with 70 % ethanol and kept on ice for 2 h. Following centrifugation at 800 g for 5 min, cells were washed with PBS and stained with 25  $\mu$ g/mL propidium iodide. Then waited 30 min at 37°C. Staining solution added that contains 3 mg/mL RNase. Samples were finally analyzed on flow cytometer.

Cells were gated to exclude cell debris, cell clumps and cell doublets. The proportion of cells with hypodiploid DNA and cell cycle phases were analyzed by ModFit Software Program (Verity Software, Topsham, ME, USA).

## **2.2.6. APOPTOSIS ASSAYS**

### **2.2.6.1. DETECTION OF MITOCHONDRIAL MEMBRANE POTENTIAL**

Energy released during the oxidation reactions in the mitochondrial respiratory chain is stored as a negative electrochemical gradient across the mitochondrial membrane and generally occurred during apoptosis. Mitochondria depolarization is one of the first events occurring in apoptosis. In addition to apoptosis, changes in the mitochondrial membrane potential have also been described during necrosis (depolarization) and cell cycle arrest (hyperpolarization).

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimidazolcarbocyanine iodide) is a lipophilic fluorochrome that is used to evaluate the status of the mitochondrial membrane potential change. The fluorescence outflow range of JC-1 is reliant on its focus. JC-1 can exist in two unique states as aggregate and monomers. Each has distinctive emission spectra. JC-1 aggregates and monomers both display fluorescence in the green part of the range. It is measured in the Green (FL-1) channel on flow cytometers.

When live cells are incubated with JC-1, the dye penetrates the plasma membrane of cells as monomers. Uptake of JC-1 into mitochondria is driven by the mitochondrial membrane potential change. JC-1 is rapidly taken up by mitochondria and healthy mitochondria is polarized. This increases the concentration gradient of JC-1. Then leads to the formation of JC-1 aggregates in the mitochondria. JC-1 aggregates show a red spectral shift resulting in higher levels of red fluorescence emission measured in the FL-2 channel on flow cytometers.

Detection of mitochondrial membrane potential was analyzed by Accuri C6 Flow Cytometer (Accuri Cytometer, Ann Arbor, MI, USA) using the JC-1 probe. After treatment with 10  $\mu$ M Clofazimine for 12, 24 and 48 h, cells are washed with PBS and 5 $\mu$ l of the JC-1 staining solution (Becton Dickinson) incubated in 5% CO<sub>2</sub> incubator at 37°C for 20 min protected from light. After washing two times with buffer solution, cells were analyzed by flow cytometer.

#### **2.2.6.2. DETECTION OF ACTIVE CASPASE-3**

The caspase family plays an important role in apoptosis and inflammation. Caspase-3 is a key protease that is activated during the early stages of apoptosis. It is synthesized as an inactive pro-enzyme like other members of the caspase family, and processed in cells undergoing apoptosis by cleavage by another protease and/or self-proteolysis. Detection of active caspase-3 by flow cytometry was used to assess apoptosis induced by 10  $\mu$ M Clofazimine. U266 cells were treated with Clofazimine for 12, 24 and 48 h. The cells were washed twice with PBS and resuspended in Cytotfix/Cytoperm solution (Becton Dickinson) for 20 min on ice. After two washes with Perm/Wash Buffer (Becton Dickinson) at room temperature, the pellets were resuspended in FITC-conjugated monoclonal active caspase-3 antibody (Becton Dickinson) containing Perm/Wash buffer and incubated for 30 min at room temperature. Each sample was then washed with Perm/Wash buffer and then analyzed by flow cytometry.

### **2.2.6.3. PE ANNEXIN V/7AAD ASSAY**

Apoptosis is characterized by certain morphologic features as internucleosomal cleavage of DNA. One of the earliest features of apoptosis is the loss of plasma membrane. The membrane phospholipid phosphatidylserine (PS) is moved from the inner to the outer leaflet of the plasma membrane in apoptotic cells. Annexin V is a 35-36 kDa Ca<sup>2+</sup> dependent phospholipid-binding protein with a high affinity for PS. PS is exposed to the external cellular environment. Annexin V can be conjugated to fluorochromes like Phycoerythrin (PE). This is a sensitive probe for flow cytometric analysis of apoptotic cells.

In the earlier stages of apoptosis externalization of PS occurs, PE Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes.

Flow cytometric analysis of cell apoptosis was performed according with the PE Annexin V/7AAD assay to the manufacturer's recommendation (BD Biosciences). U266 cells ( $1 \times 10^5$  cells/well) in all apoptosis assays were cultured at T25 flasks and incubated for 12, 24 and 48 h with 10 $\mu$ M Clofazimine. Then, cells were washed with cold PBS and resuspended in 100  $\mu$ l 1X binding buffer, followed by addition of 5  $\mu$ l PE Annexin V-PE and 5  $\mu$ l 7-AAD. The cells were incubated for 15 min at room temperature in the dark. Finally, 400  $\mu$ l 1X binding buffer were added to the cells, which were analyzed by flow cytometry. A total of at least 10.000 events were collected and analyzed by Accuri C6 Flow Cytometer (Accuri Cytometer, Ann Arbor, MI, USA).

### **2.2.7. CONFOCAL IMAGING**

Confocal microscopy was used to support flow cytometry data in apoptosis studies. Images were obtained on Zeiss LSM 510, Laser Scanning Confocal Microscope using a PlanNeofluar 40x/1.3 Oil DIC objective.

To evaluate apoptosis, U266 cells were first stained with both PE-conjugated annexin V (AV) and 7-Amino-actinomycin (7AAD). Confocal microscopy experiments were performed to determine how Clofazimine induces apoptosis in U266 cells. In early apoptotic cells only the membrane is stained with AV but not with 7AAD. AV was excited with 488 nm laser and visualized through a 515 nm emission filter, shown in green. 7AAD was excited with 488 nm laser and emitted through a 590 nm emission filter, shown in red.

Clofazimine induced U266 cells also stained with JC1 dye. The membrane-permeant JC-1 dye is used to monitor apoptosis with regards to mitochondrial health. JC-1 dye has potential-dependent accumulation in mitochondria, indicated by a fluorescence shift from ~529 nm green emission to ~590 nm red emission. Mitochondrial depolarization is shown by the decrease in the red/green fluorescence intensity ratio. The potential-sensitive color shift is because of the concentration-dependent formation of red fluorescent J-aggregates and green monomers.

#### **2.2.8. STATISTICAL ANALYSIS**

Statistical significance of results was analyzed using GraphPad Prism one-way ANOVA with Tukey post test module or unpaired t-test with two-tails. Significance of differences was marked on the figures with asterisks.





## CHAPTER 3

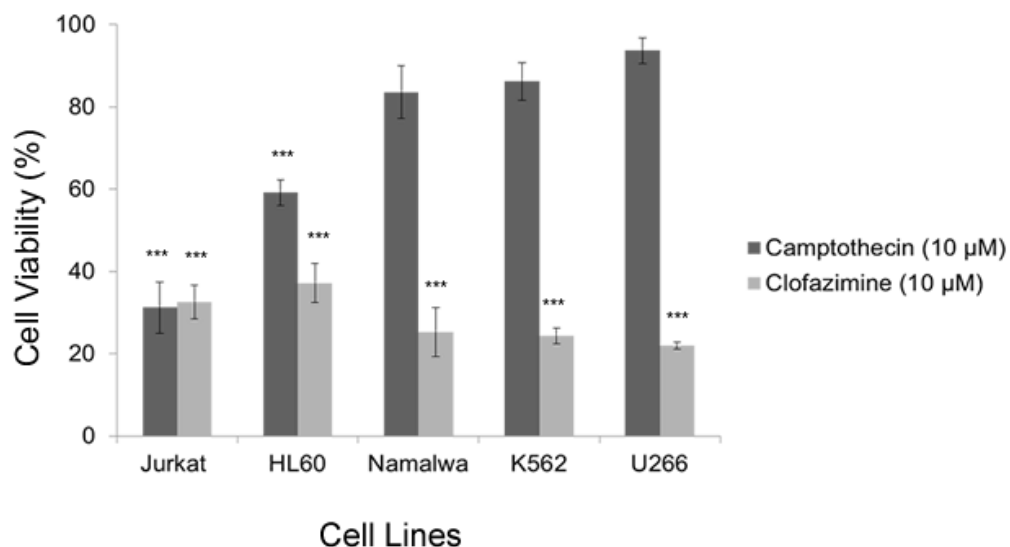
### RESULTS

#### 3.1. CLOFAZIMINE INHIBITS THE GROWTH OF HEMATOLOGICAL CELL LINES.

The relative cell viability of a panel of hematological cell lines (Jurkat, U266, Namalwa, K562, HL60) treated with CLF is shown in Table 1 and Figure 11. The susceptibility to 10  $\mu$ M CLF after 24 h treatment was determined. Treatment with CLF significantly resulted in reduced viability in all cell lines, viability ranging between 28-38%. Although all cell lines viability decreased, U266 (Multiple Myeloma) showed the highest growth suppression with  $72 \pm 6$  % ( $p < 0.001$ ).

**Table 1. Percent viability of the cells treated with 10  $\mu$ M clofazimine or 10  $\mu$ M camptothecin.**

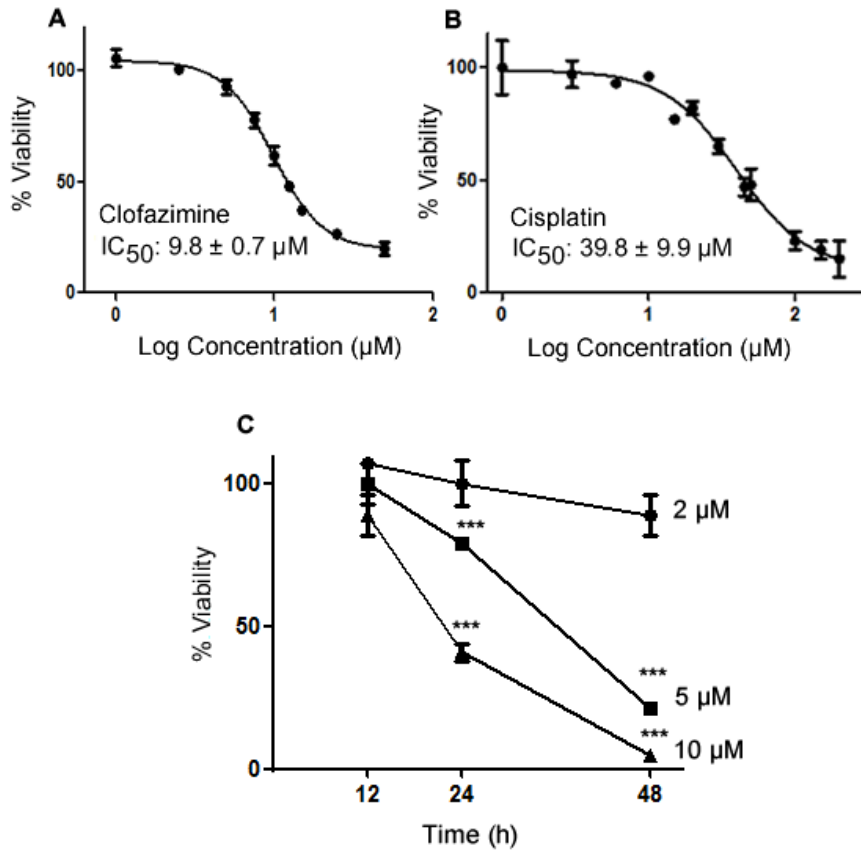
	Untreated	Jurkat	HL60	Namalwa	K562	U266
Camptothecin	100	33 $\pm$ 6	60 $\pm$ 3	89 $\pm$ 7	86 $\pm$ 5	94 $\pm$ 5
Clofazimine	100	38 $\pm$ 6	29 $\pm$ 6	30 $\pm$ 6	30 $\pm$ 5	28 $\pm$ 6



**Figure 11** Effect of clofazimine and camptothecin on viability of five different hematological cell lines as determined by CellTiter Blue assay. Jurkat, U266, Namalwa, K562, HL60 cells were treated with 10 μM clofazimine or 10 μM camptothecin for 24 hours. Asterisk denotes statistical significance at  $p < 0.001$  (n=4).

### **3.2. INHIBITORY EFFECT OF CLOFAZIMINE IS DOSE AND TIME-DEPENDENT**

Fifty percent inhibition of proliferation dose ( $IC_{50}$ ) of CLF was determined in 1-50 μM range using ten different dose values.  $IC_{50}$  value of CLF was calculated as  $9.87 \pm 0.9$  μM. The time-response relationship with regard to the viability in three different doses (2, 5, 10 μM) of CLF at three different time points (12, 24 and 48 hours) is shown in Figure 12. CLF had both dose and time dependent inhibitory effect on U266 cell line ( $p < 0.001$ ).



**Figure 12. Dose and time response of CLF's growth suppression effect on U266 cells. Dose potency (IC<sub>50</sub>) determination of A) clofazimine and B) cisplatin. C) Time-response relationship of Clofazimine treatment (2, 5 and 10 μM) at 12, 24, 48 h respectively. Asterisks denote statistical significance at p<0.001 (n=3).**

### 3.3. CLOFAZIMINE EXHIBITS SYNERGISTIC EFFECT IN COMBINATION WITH CISPLATIN

Both CLF and cisplatin alone are able to inhibit proliferation of U266 cells. To investigate whether CLF could enhance the anti-tumor effect of cisplatin, combination effect was examined. Four different concentrations of CLF (2, 5, 10, 15  $\mu\text{M}$ ) and a single dose of cisplatin (20  $\mu\text{M}$ ) were evaluated by CellTiter Blue assay after 24 h treatment. Table 2 shows combined cytotoxicity and Combination Index (CI) values. Using CLF and cisplatin together was found to have synergistic effect in all tested combinations.

**Table 2. Combination Index (CI) for Clofazimine and Cisplatin Combination. Inhibitory effect of CLF, cis and CLF-cis combination at 24 h was determined by cell viability assay (n=3).**

CLF ( $\mu\text{M}$ )	Cis ( $\mu\text{M}$ )	Combination Cytotoxicity (%)	Combination Index (CI)
2	20	53 $\pm$ 5	0.61 $\pm$ 0.06
5	20	72 $\pm$ 5	0.52 $\pm$ 0.05
10	20	84 $\pm$ 4	0.48 $\pm$ 0.01
15	20	89 $\pm$ 2	0.46 $\pm$ 0.03

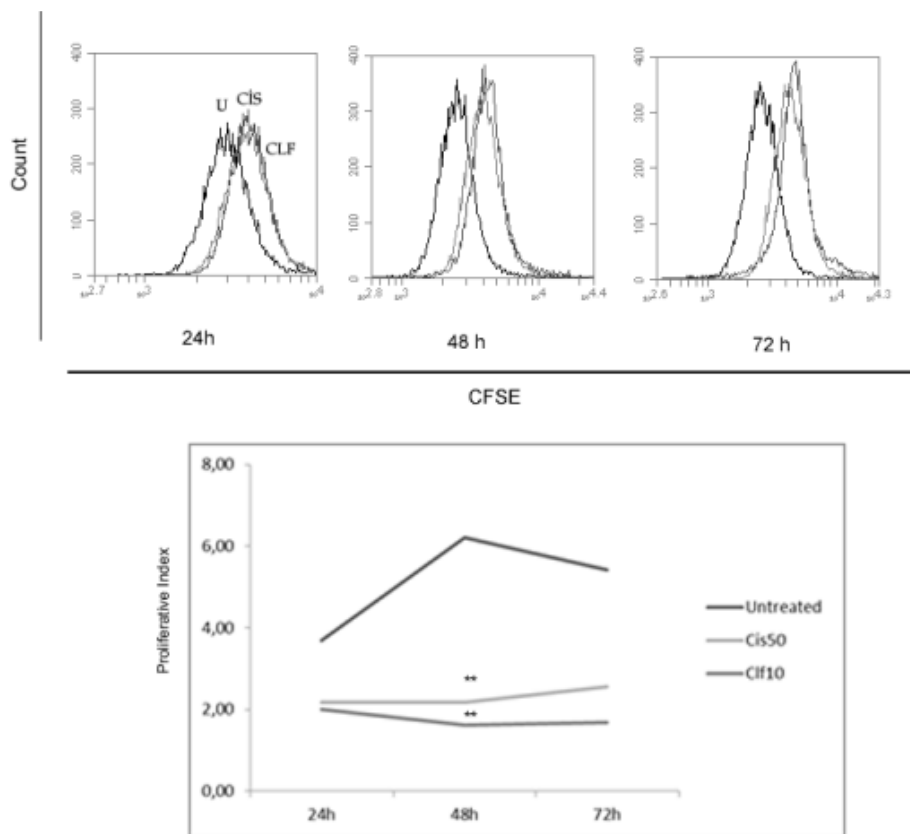
### 3.4. CLOFAZIMINE HAS ANTIPROLIFERATIVE EFFECT

U266 cells were labelled with CFSE and were cultured for 24, 48 and 72 h; after culture, cells were harvested and analysed by flow cytometry. Cell division is characterized by sequential halving of CFSE fluorescence, with equally spaced peaks on a logarithmic scale; each peak indicates the division cycle number. Similar results of CellTiter Blue colorimetric assay were obtained using CSFE methodology, which accurately confirmed rate of proliferation of U266 cells, in different periods of treatment with 10  $\mu\text{M}$  Clofazimine. Figure 15 presents histograms acquired using

ModFitLT 2.0 software. Proliferation index as in Table 3 showed a significant decrease in the population of U266 cells at both Cisplatin and Clofazimine treatment.

**Table 3. Proliferative Index of Clofazimine**

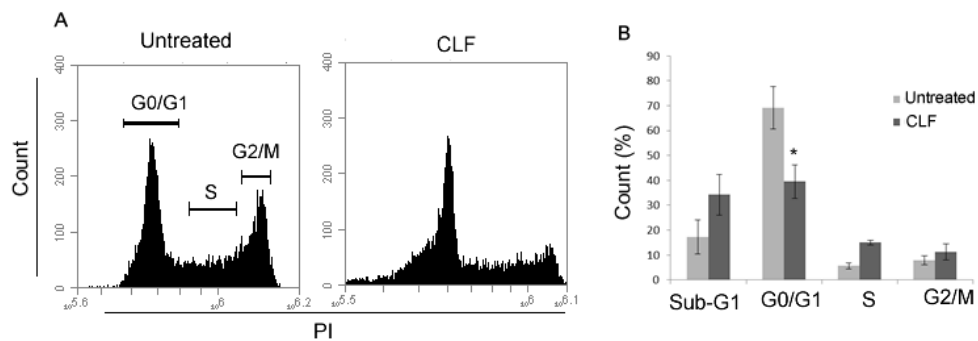
	24h	48h	72h
Untreated	3.69 ± 0.38	6.21 ± 0.42	5.42 ± 0.8
CIS 50uM	2.18 ± 0.46	2.16 ± 0.18	2.55 ± 0.63
CLF 10uM	2.01 ± 0.49	1.63 ± 0.15	1.68 ± 0.1



**Figure 13. U266 cell proliferation was assessed after 2 and 3 days analyzing the dilution of CFSE.**

### 3.5. CLOFAZIMINE-TREATED CELLS ARE ARRESTED AT S PHASE

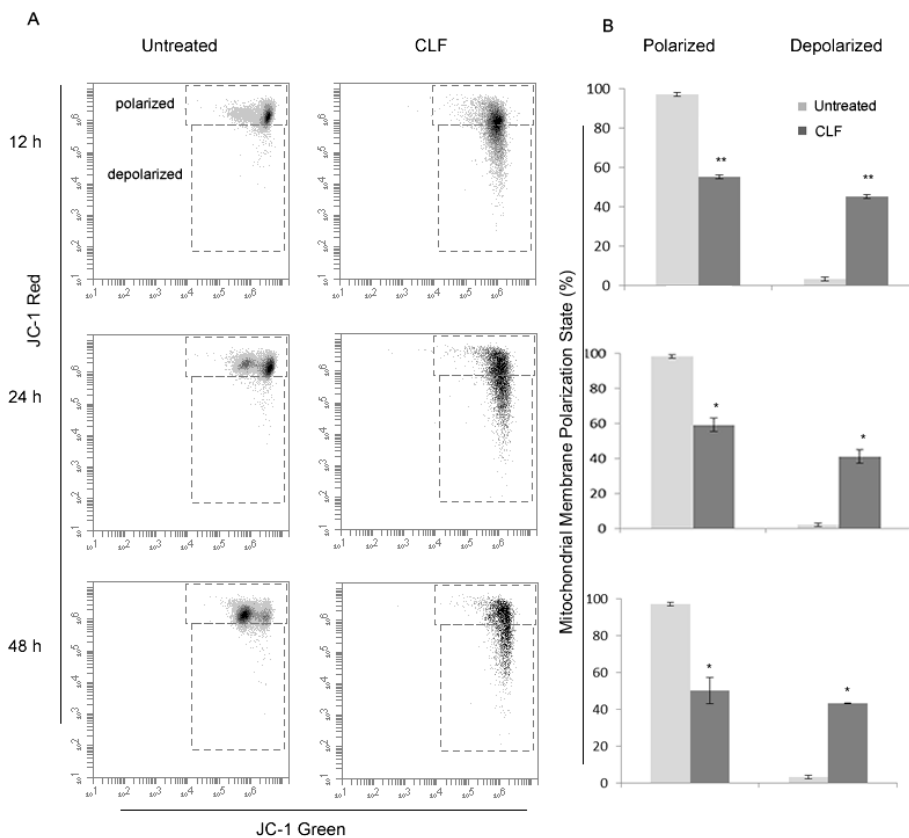
The effect of CLF on cell cycle progression in U266 cell line was studied. Cells were treated with either 0.1 % DMSO alone or 10  $\mu$ M CLF. After 24 h of treatment, cells were labeled with PI and analyzed by flow cytometry. A representative histogram for the U266 cells is shown in Figure 16, and the data obtained are summarized as bar plots of normalized count values of each phase for untreated and clofazimine-treated cells. CLF caused an increase in S phase and associated decrease in G1 phase cell population. CLF-treated cells were found apoptotic after 24 h, as detected in a prominent sub-G1 apoptotic peak. These results were also confirmed using the PE Annexin V-7AAD apoptosis assay.



**Figure 14. Cell-cycle analysis of U266 cells treated with 10  $\mu$ M Clofazimine treated (24 h). A) Flow cytometry fluorescence intensity histograms of cells stained with propidium iodide. Intensity ranges for corresponding cell-cycle phases (G0/G1, S and G2/M) were labeled. B) Bar plots of normalized count values of each phase for untreated and clofazimine-treated cells. Asterisk denotes statistical significance at  $p < 0.05$  ( $n=3$ ).**

### **3.6. CLOFAZIMINE DEPOLARIZES MITOCHONDRIAL MEMBRANE**

In view of the obtained growth-inhibitory effects, we were interested in determining whether CLF also induced apoptosis in U266 cell line. Depolarization of the mitochondria is one of the first events in apoptosis. During the redox reactions in the mitochondrial respiratory chain, a negative electrochemical gradient across the mitochondrial membrane is formed. JC-1 is a lipophilic and cationic dye that can selectively enter into the mitochondria. It reversibly change color from green to red as the membrane potential increases. The JC-1 flow cytometry and confocal microscopy experiments showed that 10  $\mu$ M CLF results in the depolarization of mitochondria in U266 cell line. CLF at 10  $\mu$ M induced the loss of mitochondrial membrane potential in U266 cells at 12, 24 and 48 hours from  $42 \pm 1\%$  to  $39 \pm 3\%$  and  $40 \pm 0,1\%$  respectively, relative to control. (Figure 17) Confocal micrographs also supported flow cytometry data. (Figure 18)



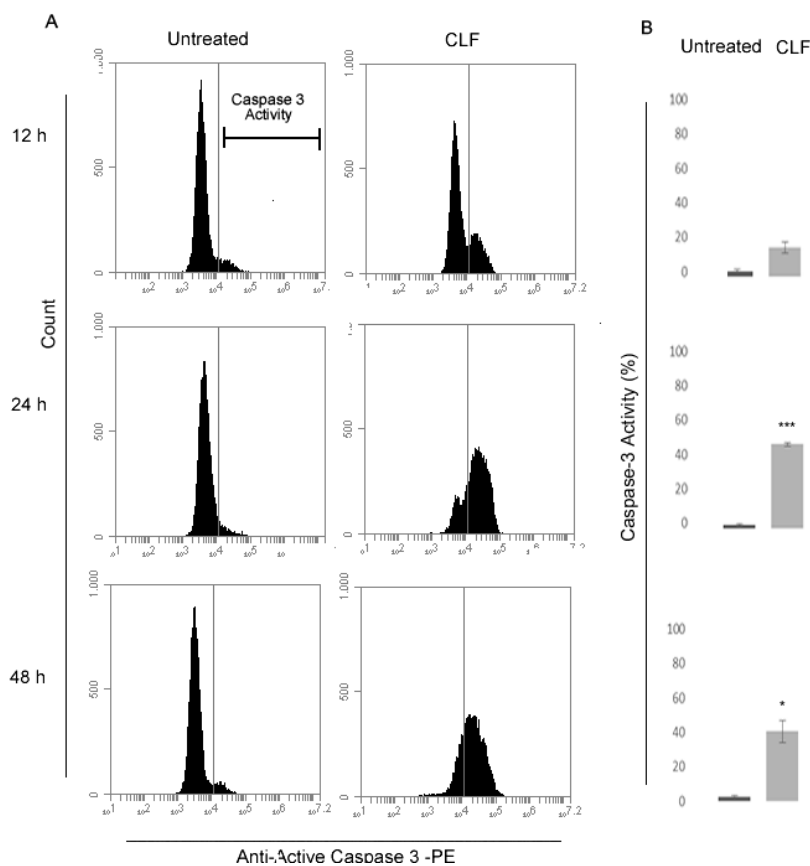
**Figure 15. Effect of 10  $\mu$ M Clofazimine on mitochondrial membrane potential at 3 different treatment durations (12, 24 and 48 h). A) Flow cytometry fluorescence intensity dot plots of cells stained with JC-1. Gated fluorescence intensity values for polarized and depolarized states were labeled. B) Bar plots of normalized mitochondrial membrane polarization state values for untreated and Clofazimine-treated cells. Asterisks \* and \*\* denote statistical significance at  $p < 0.05$  and  $p < 0.01$ , respectively. (n=3)**

### 3.7. CLOFAZIMINE ACTIVATES CASPASE-3

The caspase family of cysteine proteases plays an important role in apoptosis. Caspase-3 is an important protease activated during the early stages of apoptosis. Caspase 3 is synthesized as an inactive pro-enzyme. In cells undergoing apoptosis they processed by self-proteolysis and/or cleavage by another protease. We detected active caspase-3 by flow cytometry to assess apoptosis induced by CLF. U266 cells were treated with 10  $\mu$ M Clofazimine for 12, 24 and 48 h. The cells were treated



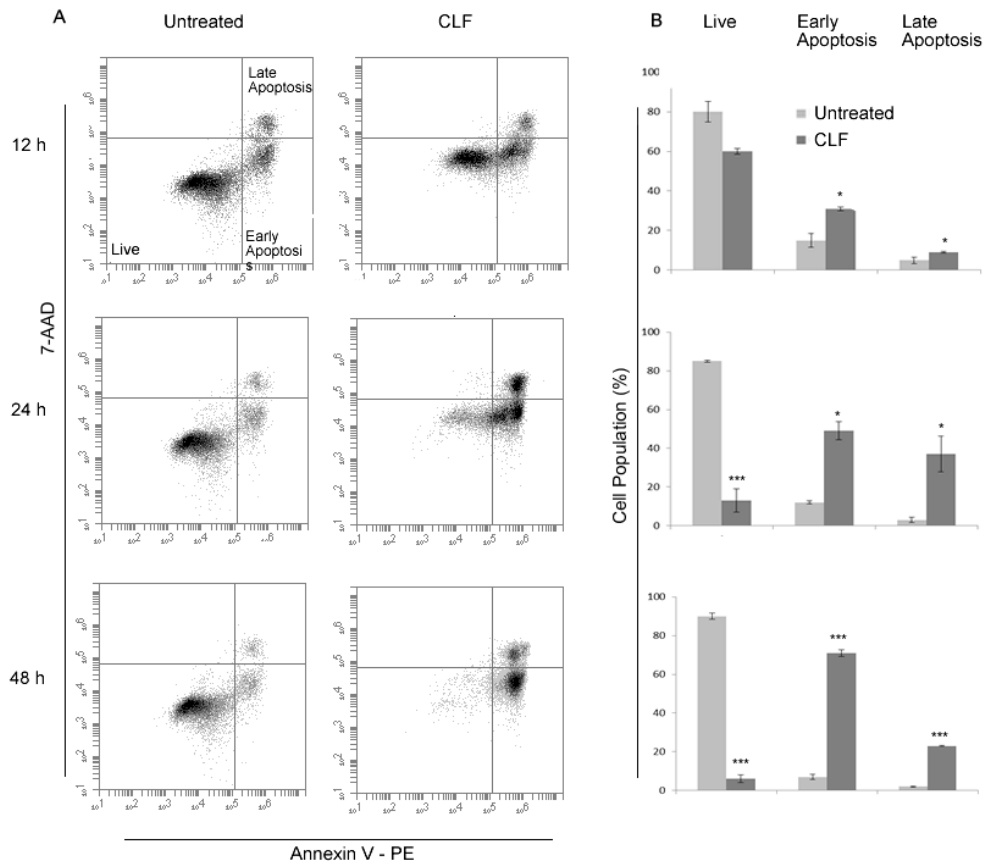
according to the manufacturer's protocol and stained with the FITC-conjugated monoclonal active caspase-3 antibody and analyzed by flow cytometry. Caspase-3 is activated in CLF-treated U266 cells. Caspase-3 positive cells were  $3\pm 1\%$  in the control group and increased in the CLF-treated group to  $17\pm 3\%$ ,  $49\pm 1\%$  and  $41\pm 7\%$  at 12, 24 and 48 h, respectively as shown in Figure 18.



**Figure 16. Effects of Clofazimine (10  $\mu\text{M}$ ) treatment on caspase activation of U266 cells at 12, 24 and 48 h. A) Flow cytometry fluorescence intensity histograms of cells stained with anti-active caspase-3 PE are shown. Intensity threshold for caspase-3 activity is indicated in the upper-left panel. B) Bar plots of corresponding histograms are placed on the right. Asterisks \* and \*\*\* denote statistical significance at  $p < 0.05$  and  $p < 0.001$ , respectively. (n=3)**

### **3.8. CELL MEMBRANE ASYMMETRY CHANGES AFTER CLOFAZIMINE TREATMENT**

The effect of the CLF on apoptosis was also evaluated using Annexin V PE/7-Aminoactinomycin D (7-AAD) staining through flow cytometry. Phosphatidylserine (PS) translocation to the outer leaflet of cellular membrane is the key step in the early stages of apoptosis. Annexin V selectively binds to PS and apoptotic cells are identified. 7-AAD intercalates in double-stranded DNA with a high affinity for GC-rich regions. It is a red fluorescent and live-cell impairment chemical compound. The cells that do not stain with either Annexin V or 7-AAD are alive and shown in histograms lower left region; the cells that stain with only Annexin V are in the stage of early apoptosis and reside in lower right region while the cells that stain with both reagents are nonviable late apoptotic/necrotic cells and scatter in upper right region. Apoptosis of U266 cells with 10  $\mu$ M CLF was detected and quantified at 12, 24 and 48 hours with AnnexinV-PE/7-AAD. Cell population in early apoptosis increases 16 to 63% at all time points in CLF-treated cells relative to the control while 4 to 34% increase is observed for late apoptosis as shown in Table 4 and Figure 17. Confocal micrographs also supported flow cytometry data. (Figure 19)



**Figure 17. Cell membrane asymmetry of U266 cells with treated with 10 uM clofazimine was detected and quantified at 12, 24 and 48 hours with Annexin V-PE/7-AAD stained. A) Histograms of Annexin V-PE vs 7-AAD signal gated as live, early apoptotic and late apoptotic quadrants as shown in the upper-left plot. B) Cell population bar graphs of corresponding dot plot quadrants (n=3). Asterisks \* and \*\*\* denote statistical significance between control and treatment populations at  $p < 0.05$  and  $p < 0.001$ , respectively.**

**Table 4. Percent of the cells at live and apoptotic stages**

	12h Treatment		
	Live	Early Apoptosis	Late Apoptosis
Untreated	80,2±5	14,55±4	5,1±2
Clofazimine	59,55±1	30,85±1	9,45±0

	24h Treatment		
	Live	Early Apoptosis	Late Apoptosis
Untreated	85±1	12,1±1	2,75±1
Clofazimine	12,9±6	48,6±5	36,75±9

	48h Treatment		
	Live	Early Apoptosis	Late Apoptosis
Untreated	90,25±2	7,3±1	2,4±0
Clofazimine	6,2±2	70,55±2	22,9±0

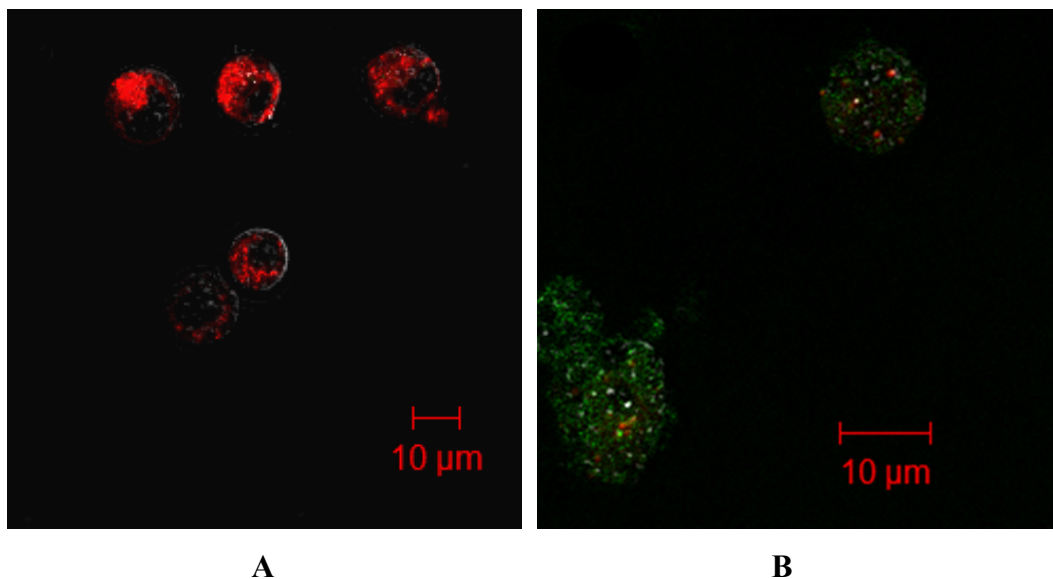
### 3.9.CONFOCAL IMAGING

Confocal images were obtained as quantitative results. U266 cells were treated with 10  $\mu$ M Clofazimine for 24 hours. The cell number in each flask was set to 10000 cell/ml with complete medium. After 24 hours treated and untreated cells were stained with FITC-conjugated annexin V (AV) and 7-Amino-actinomycin (7AAD) as previously described. After staining cells were first fixed before visualization. We seeded stained cells on lams and covered with autoclaved coverslips (Marienfield, Germany) . In Figure 18 U266 cells were stained with both FITC-conjugated annexin V (AV) and 7-Amino-actinomycin (7AAD). In early apoptotic cells only the membrane is stained with AV (green) but not with 7AAD (red). Apototic cells were stained with both AV and 7AAD.

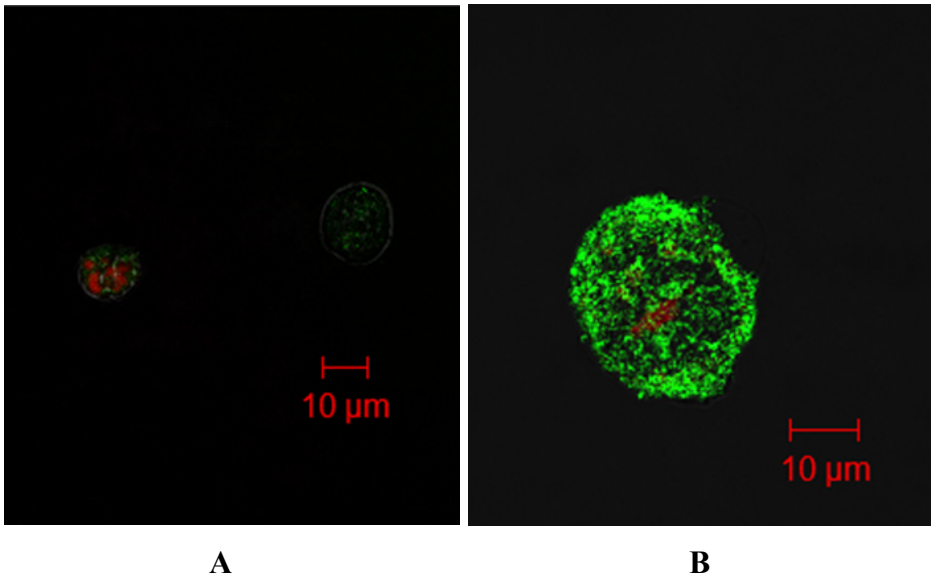
In Figure 19 U266 cells stained with JC1 dye. U266 cells were treated with 10  $\mu$ M Clofazimine for 24 hours. The cell number in each flask was set to 10000 cell/ml

with complete medium. After 24 hours treated and untreated cells were stained with JC-1 dye. After staining cells were first fixed before visualization. We seeded stained cells on lams and covered with autoclaved coverslips (Marienfield, Germany). Red or yellow spots (aggregated form of JC-1) in cytoplasm show mitochondria with higher membrane potential and weak green fluorescence represents monomer of JC-1 at sites of low membrane potential.

Images were obtained in Laser Scanning Confocal Microscopy (LSCM) using PlanNeofluar 40x/1.3 Oil DIC objective (Zeiss LSM 510, Middle East Technical University Central Laboratory, Molecular Biology and Biotechnology Research Center) (bar = 5.00  $\mu\text{m}$ ).



**Figure 18.** CSLM images of of mitochondrial membrane potential (JC-1 staining) of cells (PlanNeofluar 40x/1.3 Oil DIC objective). A) JC1 aggrretates in the untreated control cells (2X scan zoom). B) JC1 monomers in the CLF treated cells (4X Scan zoom).



**Figure 19. CSLM images of apoptotic cells (PlanNeofluar 40x/1.3 Oil DIC objective). A) Early apoptotic U266 cell (right) and a late apoptotic U266 cell (left) with a leaky plasma membrane (2X Scan zoom). B) A late apoptotic U266 cell (3X Scan Zoom).**

## CHAPTER 4

### DISCUSSION

MM is still an incurable disease and patients will eventually relapse or become refractory despite introduction of novel agents in recent years.<sup>9</sup> We showed that CLF has anti-proliferative, apoptotic, and synergistic combination effects on a resistant MM cell line U266. Our results on the anticancer effect of CLF on U266 are consistent with previous studies in the literature.

CLF has dose and time-dependent inhibitory effect on U266 cells. We found the IC<sub>50</sub> value of CLF as 9.87 μM. Previous studies indicated similar potency values. For example, IC<sub>50</sub> of CLF was found in 1-10 μM range, for non-small cell bronchial-carcinoma cells, rat hepatoma cells, murine cytotoxic T lymphocytes and breast carcinoma cells.<sup>34, 37, 53</sup>

CLF has been safely used since 1960's on leprosy patients. *In vivo* cancer studies performed on experimental animals also show a high safety profile. It was shown that a pre-treatment of B-CLL cells with 10 μM CLF caused a selective apoptosis while it did not affect healthy T and B lymphocytes.<sup>36</sup> In a related study conducted by Szabo, CLF was able to kill B-CLL cells and left healthy mesenchymal stem cells (known to inhibit apoptosis) viable.<sup>43</sup> In addition, injections of CLF at therapeutic doses (5 μg/g mouse) did not cause a significant change in the blood cell count in mice.<sup>36</sup>

Plasma CLF concentration in leprosy patients was found approximately 1 $\mu$ M. In experimental animals 10  $\mu$ M plasma concentration was found tolerable. Since CLF tends to accumulate in tissues, drug concentration in different organs was shown to vary from 10  $\mu$ M up to 500  $\mu$ M.<sup>54,55</sup>

Cisplatin has been in use as a part of effective chemotherapy regimens for relapsed MM patients. For the first time, we showed that cisplatin-CLF combination has a synergistic effect on U266 MM cell line.

To our knowledge, CLF-cancer drug combination was previously studied in two different works. Ruff and his colleagues tested CLF in combination with doxorubicin in patients with hepatocellular carcinoma and found objective response rate of 10%, as well as 43.3% disease stabilization for up to 20 months, and an overall median survival of 13 weeks.<sup>29</sup> The second study by Koot *et al* found *in vitro* synergistic effect between CLF and paclitaxel. However, this outcome was not translated to *in vivo* studies in which the drug combination was encapsulated in a lipopolymeric micelle.<sup>51</sup>

Resistance to cytotoxic drugs is the major cause of failure to successful treatment of disseminated neoplastic disease. U266 cells used in our study have mutant p53 (at A161T) showing resistance to cytotoxic agents such as platinum analogs.<sup>56-58</sup> CLF shown to inhibit multi drug resistance.<sup>28,34,48,51</sup> CLF inhibits the *mdr 1* gene product, p170-glycoprotein.<sup>28,33</sup>

Our results indicate that multidrug resistance inhibitor property of CLF is an important feature to combine it with especially platinum analog chemotherapeutics like cisplatin in relapse MM treatment.

To our knowledge, this work is the first cell cycle analysis study conducted on CLF. CLF was previously shown to be a potent Kv1.3 channel blocker.<sup>25,32,35,36,43</sup> One possible mechanism for CLF's cell cycle arrest ability might be related to the voltage-gated K<sup>+</sup> channels, including Kv1.3, which are involved in proliferation and



cell cycle progression. Other K<sup>+</sup> channel modulators have been also shown to cause cell cycle arrest.<sup>45,59-61</sup> More specifically, Leanza showed that Kv1.3 inhibition significantly increased expression level of p21, a cyclin-dependent kinase inhibitor. It also decreased the expression level of Cdk4 and cyclin D3. These changes in the expression levels inhibited G1 to S transition.<sup>35,62</sup> Since CLF has multiple molecular targets including DNA, phospholipases and phagocytosis related proteins<sup>63</sup>, cell cycle arrest mechanism can be related to pathways other than those involving Kv1.3.<sup>64</sup>

Our study shows increased sub-G0/G1 population of clofazimine-treated U266 cells indicated an apoptotic outcome in cell cycle analysis experiments. To provide further support for this observation, we conducted three additional apoptosis assays. All assays performed showed intrinsic apoptosis hallmarks. CLF depolarizes mitochondrial membrane, increases caspase 3 activity and disturbs plasma membrane symmetry. This is the first study showing the apoptotic effect of CLF on a MM cell line.

In ideal chemotherapeutic regimens induction of apoptosis is a highly desirable outcome. It is known that the cytotoxic effects of many forms of chemotherapy are mediated through apoptosis.<sup>56-58</sup> As in the case of cell cycle arrest, apoptotic response to CLF treatment in U266 cells may also be linked to its Kv1.3 blocking activity. CLF induced apoptosis in Kv1.3 expressing cells such as Kv1.3 transfected murine cytotoxic T lymphocyte cell line (CTLL-2), human T cell line (Jurkat), human osteosarcoma (SAOS-2) and mouse melanoma (B16F10), but not in Kv1.3-negative cells.<sup>36,64</sup> The knock-down of Kv1.3 channels by siRNA technique inhibits Jurkat T cells apoptosis induced by the Kv1.3 inhibitors.<sup>36</sup>

Leanza *et al.* found that CLF induced apoptosis involved activation of caspase-9 and Caspase 3 (not caspase-8), release of mitochondrial cytochrome c, and increase of mitochondrial ROS production, depolarization of the inner mitochondrial membrane and cleavage of poly-ADP ribose-polymerase (PARP).<sup>36</sup> One very important aspect of this work was that, CLF induced apoptosis in a Bax/Bak independent way. Since

Bax/Bak pathway is highly correlated with apoptosis evasion by cancer cells, this finding indicated therapeutic potential of CLF especially in drug-resistant malignancies.<sup>35</sup> Wnt pathways is another likely mechanism for apoptosis based on the previous finding of Koval *et al.* in CLF-treated HTB19 triple-negative breast cancer cells.<sup>37</sup> All these biomarkers and pathways may also be relevant in U266 cells and should be checked in future studies.

## **CHAPTER 5**

### **CONCLUSION**

In this study, CLF significantly inhibited the growth of U266, HL60, Namalwa, K562 and Jurkat cell lines. In U266 cells, CLF inhibitory effect is both time and dose dependent. It induced S-phase cell cycle arrest and apoptosis. Apoptosis suggested occurring intrinsically via mitochondrial membrane permeabilization and caspase 3 activation. The increased early and late apoptotic cell populations were accompanied with the increased sub-G1 population in the cell cycle analysis.

Despite introduction of novel agents in recent years, MM is still an incurable disease and patients will eventually relapse or become refractory. We provided new evidence that CLF alone has apoptotic and growth inhibitory effect on a p53 mutant resistant MM cell line. We also showed that CLF in combination with cisplatin has potent synergistic effect. Combined therapy employing CLF together with chemotherapeutics seems to be a possible future therapeutic approach for MM.



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## **APPENDICES**

### **APPENDIX A**

#### **SUPPLEMENTARY DATA**

##### **A1. QUANTITATIVE REVERSE TRANSCRIPTION PCR**

Quantitative reverse transcription PCR (qRT-PCR) is a powerful, sensitive, and quantitative assay for the detection of RNA levels. It is frequently used in the expression analysis of single or multiple genes, and expression patterns for identifying infections and diseases.

Total RNA from Namalwa, Cem, Jurkat, Ramos, K562 and U266 cells was extracted with the PerfectPure RNA Cell & Tissue Kit (5 Prime) following the manufacturer's recommendations and stored at -80 °C until used. 1 µg of total RNA was used for cDNA synthesis by using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR in a final 20-µL reaction volume was performed by using 50 ng of cDNA of each sample with triplicates using the FastStart Universal SYBR Green Master (Roche) on a Rotor-Gene 6000 (Corbett) real-time PCR cyclers. To allow standardization of the amount of cDNA added to each PCR reaction and to minimize the effects of individual difference on the results, quantitative PCR for the housekeeping gene  $\beta$ -actin was performed for each sample. We used real-time PCR to simultaneously detect and quantify Kv1.3 gene expression. The following primer sets were used for amplification: Kv1.3\_FP (5' ACGACCCCACTTCAGGTTTC 3'),

Kv1.3\_RP (5' CCACGTGCATGTACTGGGAT 3'),  $\beta$ -actin\_FP (5' CCAACCGCGAGAAGATGA 3') and  $\beta$ -actin\_RP (5' CCAGAGGCGTACAGGGATAG 3').

PCR conditions were: 3 min 95 °C; 20s 95 °C, 20s 55 °C, and 40s 72 °C (40 cycles). Copy numbers per volume of the samples were determined with the help of a calibration curve of standards with a known number of copies. The resulting concentrations were standardized to the amount of the  $\beta$ -actin gene. The standardized values further normalized to the Jurkat cell Kv1.3 expression by using  $\Delta\Delta$ CT method (comparative method) for relative quantification.

Five cell lines are tested to detect Kv1.3 mRNA expression level. According to literature Jurkat, HL60 cells overexpress, K562 does not express Kv1.3 channels whereas there were no study on Kv1.3 expression in NAMALWA and U266 cell line. A high level of Kv1.3 mRNA was detected in Cem and Jurkat cells and lower Kv1.3 mRNA level was detected in other cell lines as shown in Figure 20 and 21.



**Figure 20 Expression of voltage-gated Kv1.3 channel in five cell lines. Total RNA isolated and reverse transcribed to cDNA. A 242 bp fragment was**

amplified from RNA from all cells. All bands that appeared were of the correct size. Kv1.3 was very highly expressed except K562.

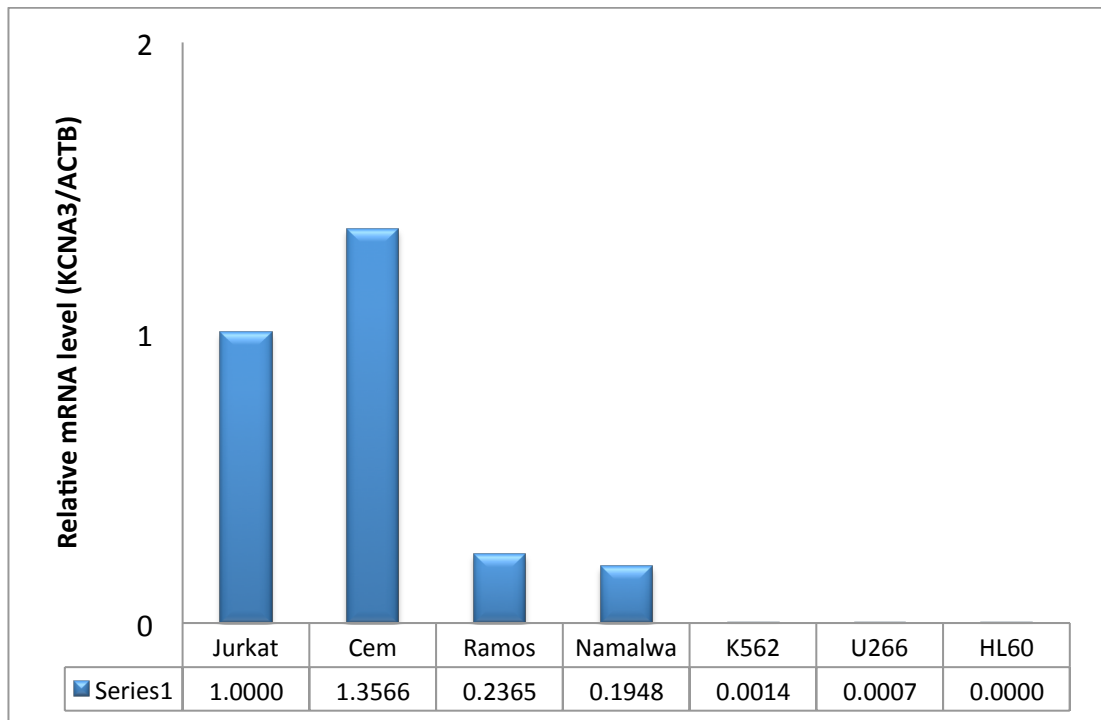


Figure 21 The relative Kv1.3 mRNA expression level normalized to B-Actin was measured by real-time PCR.

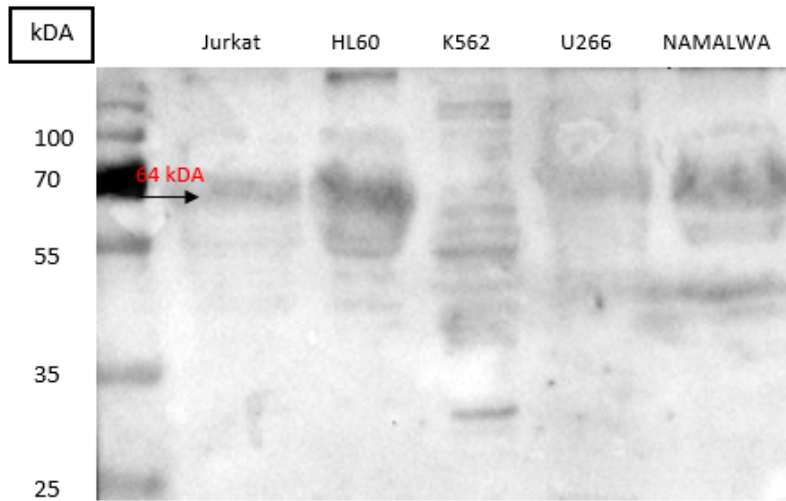
## A2. WESTERN BLOT

The lysates of cells were analyzed by Western blot to detect Kv1.3 protein level. Jurkat, HL60, U266 and Namalwa have Kv1.3 expression at 64 kDa where K562 has very low expression. (Figure 22) Our monoclonal Kv1.3 Ab had nonspecific interactions with other proteins on the membrane. It is difficult to observe transmembrane proteins (like Kv1.3 ion channel) with Western Blot technique. Because they are found in low number in the cells; and we need to start with at least 10 million cells for isolation, we need to enrich membrane fraction (both cell membrane and mitochondrial membrane) to obtain a reasonable amount of protein. Still, it is quite difficult to detach protein from the membrane since it has 7 transmembrane domains. According to our experiences we can confidently say that Western Blot is not an easily applicable technique for ion channel proteins.

Leanza showed correlation between the protein expression of the Kv1.3 channels and susceptibility to death upon treatment with staurosporine, C2-ceramide and cisplatin. They also showed higher protein expression of Kv1.3 significantly correlated with lower cell survival upon treatment with clofazimine.<sup>32</sup>

It was also found out that K<sup>+</sup> channel expression at the protein level varied to a much bigger extent than at the mRNA level in case of all the studied channels, indicating significant post-transcriptional regulation of K<sup>+</sup> channel expression.<sup>32</sup> This may explain the difference in our results in Kv1.3 protein and RNA expression level.

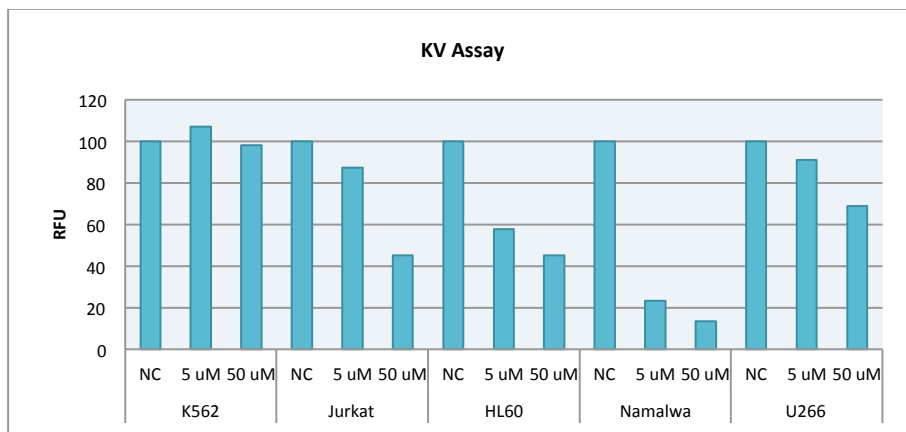




**Figure 22. Western blotting analysis of Kv1.3 protein expression. Kv1.3 antibody was used to detect Kv1.3 expression.**

### A3. K<sup>+</sup> ION FLUX ASSAY

Clofazimine blocks K ion flux in dose-dependent manner in Jurkat and Namalwa. The flux of thallium (Tl<sup>+</sup>) through K<sup>+</sup> channels was monitored in the presence and absence of 5 and 50 μM Clofazimine. Clofazimine inhibited the flux of thallium in a concentration-dependent manner except K562. (Figure 23) The sensitivity of measuring the flux of thallium must be done at a quick and sensitive instrument. We used SpectraMax® Paradigm® Multi-Mode Microplate Reader. Although we obtained results compatible with literature the sensitivity of the assay is very low and the errors are so high to make a correlation. We plan to use patch clamp experiments in the future to find the effect of Clofazimine on Kv channels so we do not repeat this experiment.

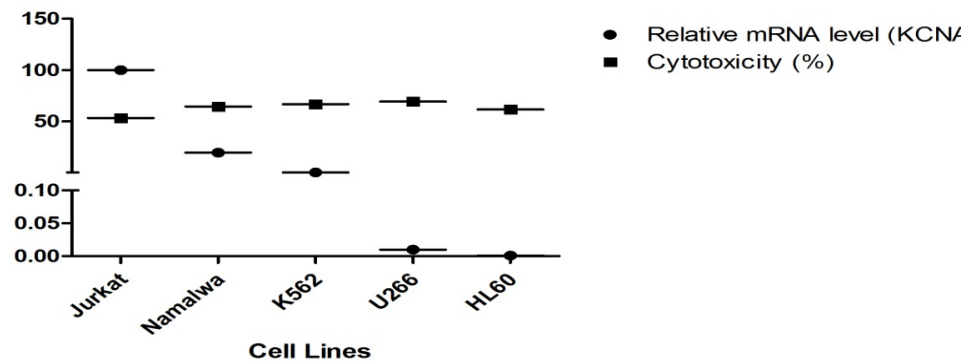


**Figure 23 Clofazimine inhibit thallium flux through potassium channels. Various concentrations of Clofazimine were tested using the thallium (Tl<sup>+</sup>) flux assay.**

#### A4. CORRELATION BETWEEN KV1.3 MRNA LEVELS AND CYTOTOXICITY

According to the Kv1.3 mRNA levels and effect of 10 uM Clofazimine on cell viability we could not find a correlation between both. (Figure 24) Our cytotoxicity is mostly because of apoptosis induced by Clofazimine as shown by our results. Leanza *et al.* showed that K<sup>+</sup> channel expression at the protein level varied to a much bigger extent than at the mRNA level in case of all the studied channels, indicating significant post-transcriptional regulation of K<sup>+</sup> channel expression. They also showed that Clofazimine induces apoptosis via Kv1.3 channels by silencing Kv1.3 channels. By this correlation analyses we showed that Clofazimine do not cause apoptosis only with Kv1.3 channels.

**Correlation Between Kv 1.3 mRNA Level and % Cytotoxicity**



Pearson r	-0.8949
95% confidence interval	-0.9931 to -0.05950
P value (two-tailed)	0.0403

**Figure 24. Correlation chart between Kv1.3 mRNA levels and cytotoxicity percentage.**

## APPENDIX B

### EXPERIMENT PROTOCOLS

#### B1. CELL CYCLE ANALYSIS PROTOCOL

##### Solutions

##### Cisplatin Stock Solution

- 10 mM, -20°C, in DMSO

##### Clofazimine Stock Solution

- 10 mM, -20°C, in DMSO

##### 1 mM Cisplatin Solution

- 10 uL 10 mM stock Cmt + 90 uL DMSO → 1 mM Cis

##### 1 mM Clofazimine Solution

- 10 uL 10 mM stock Clf + 90 uL DMSO → 1 mM Clf

##### Samples

Negative Control: 15 uL DMSO + 1.485 uL cell culture

Positive Control: 15 uL 1 mM cisplatin + 1.485 uL medium

Test Sample: 15 uL 1 mM clofazimine + 1.485 uL cell culture

##### Procedure

1. Transfer 3600K cells to an eppendorf
2. Centrifuge at 400 g / 5 min / RT and remove supernatant
3. Add 3 mL medium, resuspend the pellet and transfer to 50 mL falcon
4. Complete volume to 36 mL using fresh medium

5. Incubate the cells for 6-12-24 h at 37°C in 6 well plate with corresponding drug
6. Transfer content of each well to an Eppendorf
7. Transfer 4.5 mL 30% EtOH to eppendorfs drop wise with continuous vortexing
8. Centrifuge at 400 g / 5 min / RT and remove supernatant
9. Add 1mL PBS to each Eppendorf
10. Keep cells for at least 2 h at 4°C
11. Centrifuge at 300 g / 5 min / RT and remove supernatant
12. Resuspend pellet in 5 mL PBS
13. Wait for 30 sec and centrifuge at 300 g / 5 min / RT
14. Resuspend pellet in PI staining solution
15. Incubate 37°C for 10 min
16. Analyze with flow cytometry
  - Set reading FL2 (PI)
  - Count 10000 event

## **B2. CFSE PROTOCOL**

### **Solutions**

#### CFSE stock solution

5 mM (20 uL aliquots), -20°C, in DMSO

#### CFSE stock 2 solution

500 uM (10 uL aliquots), -20°C, in DMSO

### **Samples**

Negative Control: Cell Culture with 1 % DMSO

Positive Control: 50 uM Cisplatin

Test Sample: 10 uM Clofazimine

### **Procedure**

1. Take 500 K cells
2. Wash with pre-warmed PBS
3. Resuspend pellet with 500 uL PBS (final concentration:  $10^6$  cells/mL) in 2 mL eppendorf
4. Take 0.5 uL from stock 2 and put it to the side of the eppendorf
5. Immediately vortex upside down (vortex at slow speed for 15 seconds)
6. Incubate at dark at 37°C for 15 min
7. Add 1 mL of pre-warmed medium
8. Incubate cells at 37°C for 5 min
9. Centrifuge at 400 g / 5 min / RT and remove supernatant
10. Resuspend pellet with 5 mL medium (final concentration:  $10^5$  cells/mL) in T25
11. Take 200 uL cell suspension at indicated time points: 0h, 24h, 48h, 72h
12. Analyze with flow cytometry
  - Set reading FL1
  - Count 10000 event

### **B3. JC-1 PROTOCOL**

#### **Solutions**

##### 1X Assay Buffer Preparation

- Warm 10X Assay Buffer (60 mL) in 37<sup>0</sup>C
- Dilute 10X A.B. 1:10 in ddH<sub>2</sub>O (2.5 mL A.B + 22.5 mL ddH<sub>2</sub>O) → 25 mL Assay Buffer (for 3 sample, 3 time points)
- Stir the solution for 5 min

##### JC-1 Working Solution Preparation (Should be used immediately!)

- Warm 1X Assay Buffer to 37<sup>0</sup>C
- Mix 1485 uL A.B. + 15 uL JC-1 stock solution (for 3 sample, 1 time point)
- Pipette up & down to fully dissolve

#### **Samples**

Negative Control: Cell Culture with 1 % DMSO

Positive Control: 50 uM Cisplatin

Test Sample: 10 uM Clofazimine

#### **Procedure**

1. Count and take 600 K cells and centrifuge at 400 g / 5 min / RT
2. Resuspend pellet with 6 mL of corresponding drug solution (final conc. 100 K cells /mL)
3. Incubate the cells for 24-48-72 hr at 37°C (in T25 cell culture flasks)
4. Take 1.2 mL from cell culture flask (120.000 cells)
5. Centrifuge at 400 g / 5 min / RT, remove supernatant
6. Add 500 uL JC-1 solution to the sample
7. Pipette up&down to disrupt clumps
8. Incubate cells for 15 min at 37°C in incubator
9. Centrifuge at 400 g / 5 min / RT, remove supernatant
10. Add 1 mL of 1X Assay Buffer, centrifuge at 400 g / 5 min / RT
11. Add 500 uL of 1X Assay Buffer
12. Centrifuge at 400 g / 5 min / RT
13. Resuspend cells in 500 uL Assay Buffer
14. Analyze cells with flow cytometry : Set reading FL2 and FL3, Count 10000 event

## **B4. ANNEXIN-V / 7-AAD PROTOCOL**

### **Solutions**

#### **1X Binding Buffer**

- 1:10 dilution with sterile distilled water
- Warm to 37 °C

### **Samples**

Negative Control: Cell Culture with 1 % DMSO

Positive Control: 50 uM Cisplatin

Test Sample: 10 uM Clofazimine

### **Procedure**

1. Count and take 500 K cells
2. Centrifuge at 400 g / 5 min / RT
3. Resuspend pellet with 5 mL of corresponding drug solution (final conc. 100 K cells /mL)
4. Incubate the cells for 24,48 and 72 hr at 37°C (in T25 cell culture flasks)
5. Take 1 mL from cell culture flask (200.000 cells)
6. Centrifuge at 400 g / 5 min / RT to remove medium
7. Wash cells twice with 1 ml cold PBS (400 g / 5 min)
8. Resuspend cells in 200 uL 1X Binding Buffer
9. Transfer 100 µl of the solution ( $1 \times 10^5$  cells) to 1.5 mL eppendorf
10. Add 5 µl of PE Annexin V and 5 µl 7AAD
11. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark
12. Add 400 µl of 1X Binding Buffer to each tube
13. Analyze by flow cytometry within 1 hr



## **B5. CASPASE-3 PROTOCOL**

### **Solutions**

- Ab- 2 mL
- 1X CytoFix/Cytoperm - 65 mL
- 10X Perm/Wash - 65 mL - 1:10 dilution with sterile distilled water
- Warm to 37 °C

### **Samples**

Negative Control: Cell Culture with 1 % DMSO

Positive Control: 50 uM Cisplatin

Test Sample: 10 uM Clofazimine

### **Procedure**

1. Count and take 2000 K cells
2. Centrifuge at 400 g / 5 min / RT
3. Resuspend pellet with 20 ml medium 10%FBS
4. Centrifuge 400 g 5 min RT
5. Resuspend 500 uL PBS
6. Centrifuge 400 g 5 min RT
7. Resuspend 500 uL cold BD CytoFif/Cytoperm
8. Incubate 20 min on ice
9. Centrifuge 400 g 5 min RT
10. Resuspend 500 uL BD Perm/wash at RT
11. Centrifuge 400 g 5 min RT
12. Add 25 uL 1X BD Perm/wash and 5 uL Caspase AntiB
13. Incubate 30 min RT
14. Add 500 uL BD Perm/wash
15. Analyse flow cytometer

## APPENDIX C

### COMPUSYN REPORT

**Drug: Cisplatin (cis) [uM]**

**Drug: Clofazimine (clf) [uM]**

**Drug Combo: Combo1 (uM) (cis+clf)**

**Data for Drug: cis [uM]**

**Dose Effect**

**20.0 0.2**

**50.0 0.48**

**2 data points entered.**

**X-int: 1.72335**

**Y-int: -2.4568**

**m: 1.42559**

**Dm: 52.8877**

**r: 1.00000**

**Data for Drug: clf [uM]**

**Dose Effect**

**2.0 0.19**

**5.0 0.4**

**10.0 0.64**

**15.0 0.7**

**4 data points entered.**

**X-int: 0.83389**

**Y-int: -0.9830 +/- 0.06130**

**m: 1.17883 +/- 0.07123**

**Dm: 6.82160**

**r: 0.99637**

**Data for Non-Constant Combo: uM (cis+clf)**

**Dose cis Dose clf Effect**

**20.0 2.0 0.53**

**20.0 5.0 0.72**

**20.0 10.0 0.84**

**20.0 15.0 0.89**

**4 data points entered.**

### Dose-Effect Curve

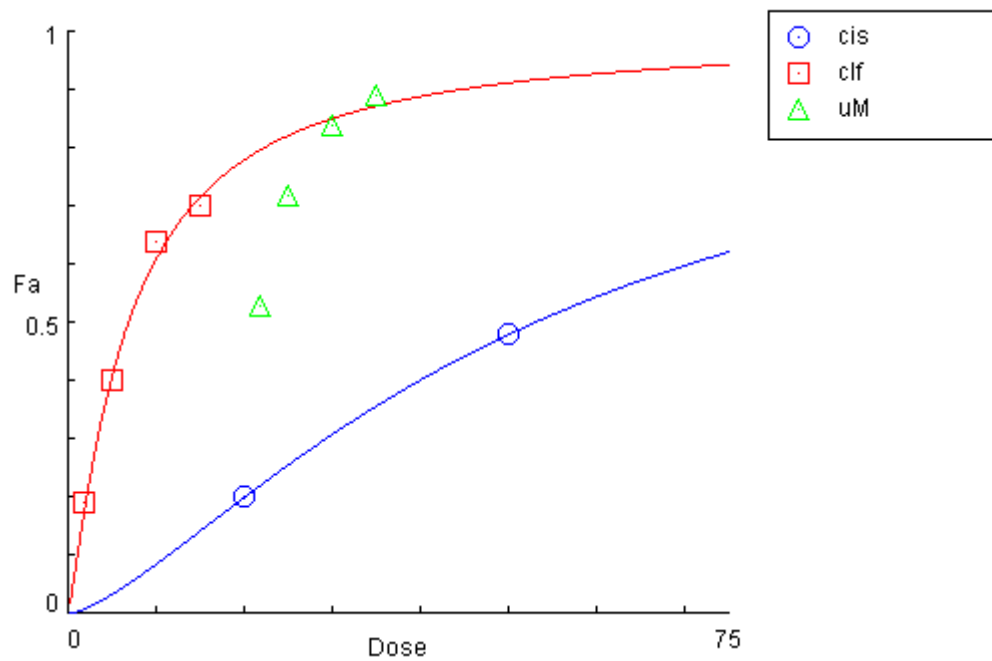


Figure 25. Dose Effect Curve of Clofazimine and Cisplatin Combination

### Median-Effect Plot

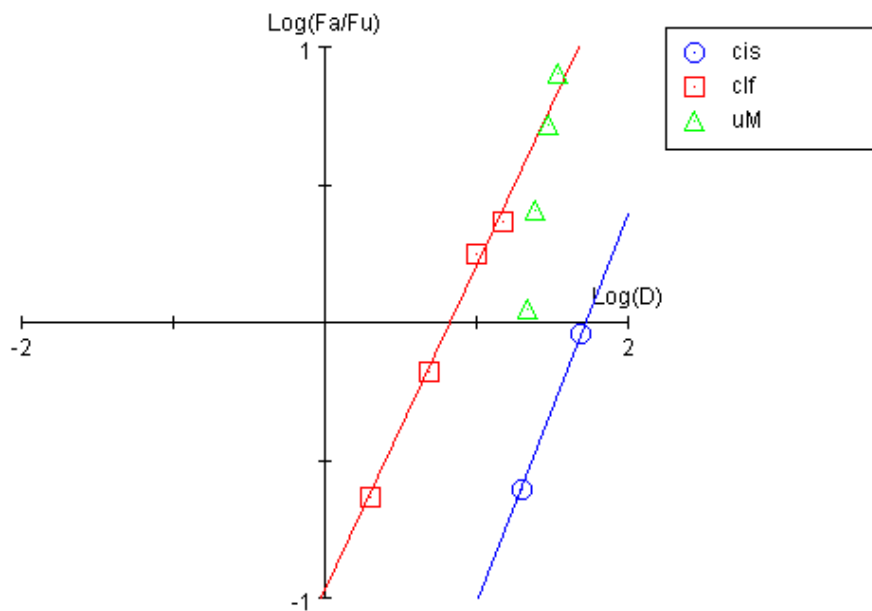


Figure 26. Median Effect Plot of Clofazimine and Cisplatin Combination

**CI Data for Non-Constant Combo: uM (cis+clf)**

Dose cis	Dose clf	Effect	CI
20.0	2.0	0.53	0.61237
20.0	5.0	0.72	0.52392
20.0	10.0	0.84	0.47726
20.0	15.0	0.89	0.46046

**DRI Data for Non-Constant Combo: uM (cis+clf)**

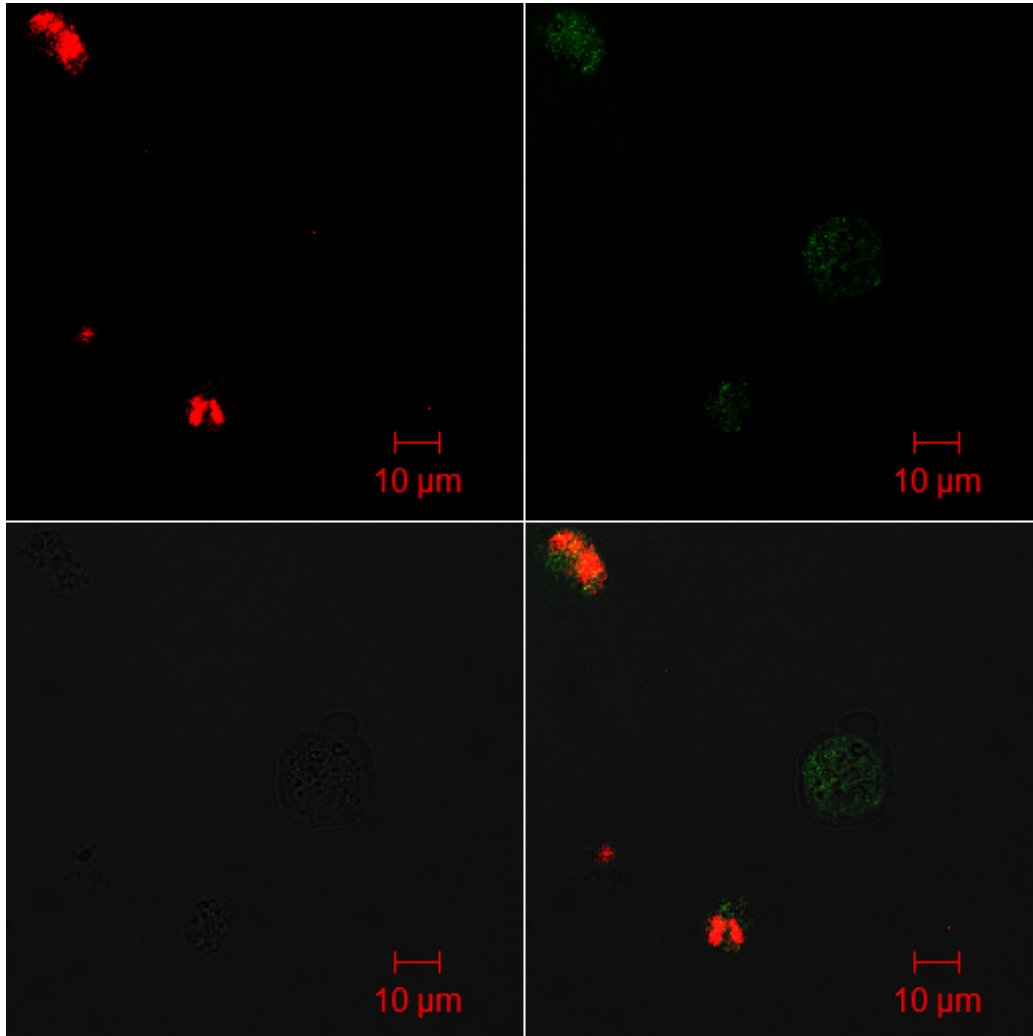
Fa	Dose cis	Dose clf	DRI cis	DRI clf
0.53	57.5381	7.55350	2.87690	3.77675
0.72	102.584	15.1997	5.12918	3.03994
0.84	169.247	27.8480	8.46236	2.78480
0.89	229.236	40.1917	11.4618	2.67945

**Summary Table**

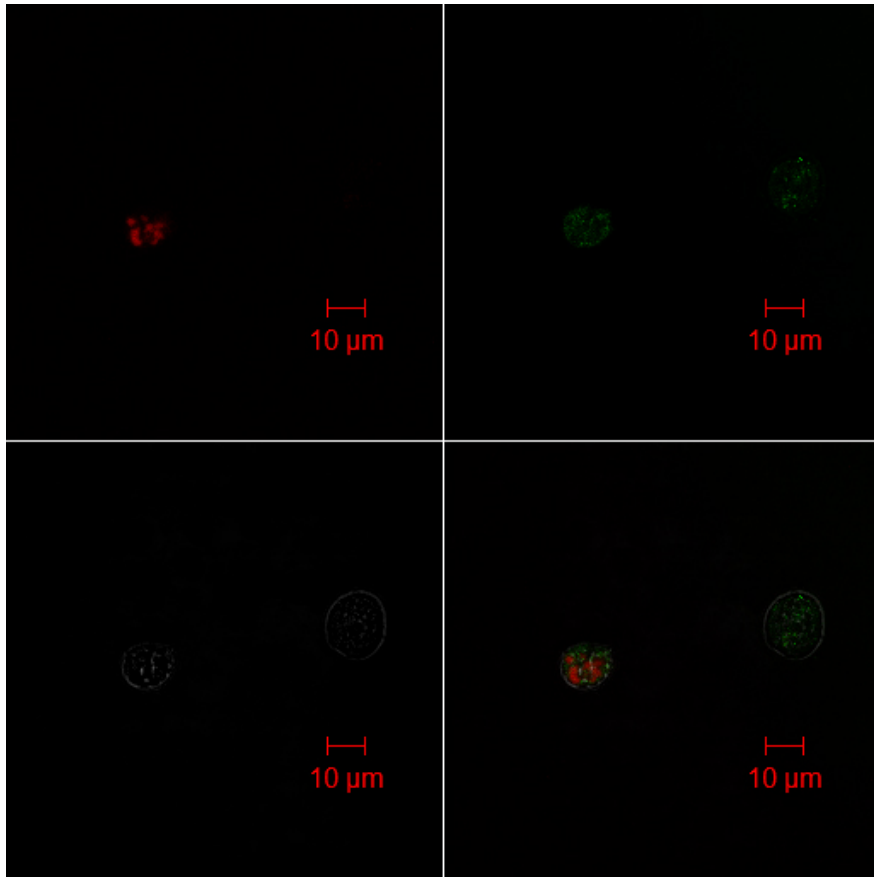
Drug/Combo	Dm	m	r
Cis	52.8877	1.42559	1.00000
Clf	6.82160	1.17883	0.99637

## APPENDIX D

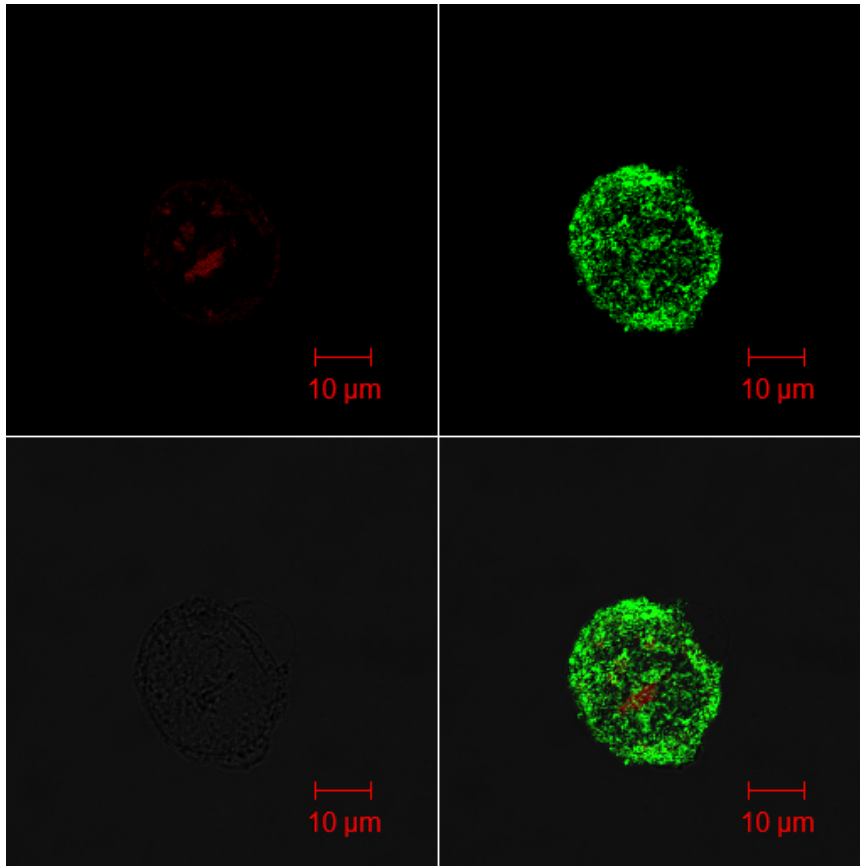
### FLOURESCENCE MICROSCOPY IMAGES



A



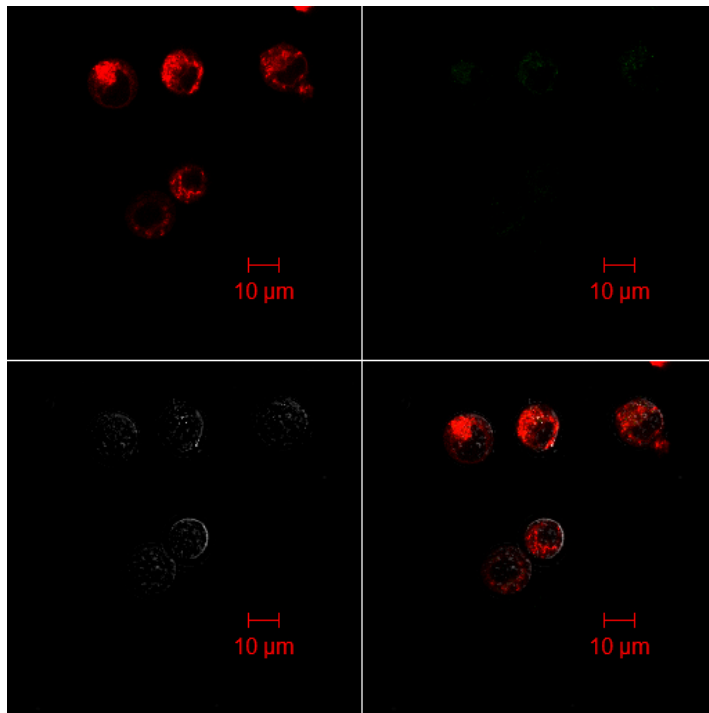
**B**



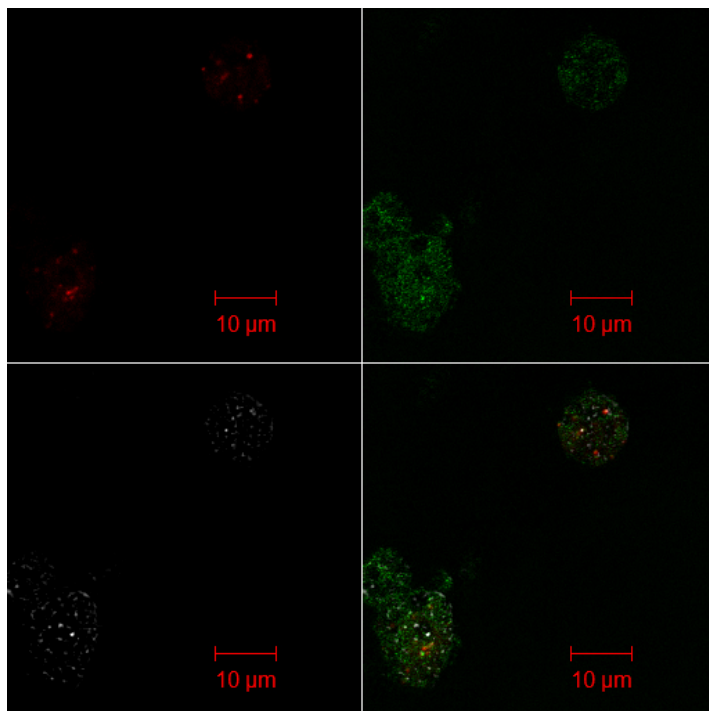
C

**Figure 27. Fluorescence images of apoptotic cells stained with Annexin V-7AAD (PlanNeofluar 40x/1.3 Oil DIC objective). A and B) Early apoptotic U266 cell and a late apoptotic U266 cell with a leaky plasma membrane (2X Scan zoom). C) A late apoptotic U266 cell (3X Scan Zoom).**





**A**



**B**

**Figure 28** CSLM images of of mitochondrial membrane potential (JC-1 staining) of cells (PlanNeofluar 40x/1.3 Oil DIC objective). **A)** JC1 aggreates in the untreated control cells (2X scan zoom). **B)** JC1 monomers in the CLF treated cells (4X Scan zoom).



## **CURRICULUM VITAE**

**IPEK ZEYNEP DURUSU**

**Esat Caddesi 102/12**

**Ankara-TURKEY**

**ipekdurusu@yahoo.com**

**Tel: +905325216172**

**Fax: +903124383723**

**Date of Birth: 20.11.1977**

### **EDUCATION AND QUALIFICATIONS**

**2007- 2008 University of Minnesota Cancer Center - Junior Scientist**

**Skills:** Human cell culture, flow cytometry, electroporation, MLL, siRNA

**2005- Continue Middle East Technical University, Ankara, Turkey**

**PhD in Biotechnology Department**

**1999- 2001 Middle East Technical University, Ankara, Turkey**

**Master of Science in Biotechnology Department**

**Thesis:** Optimization of Callus Culture and Regeneration Systems of Turkish Wheat Cultivars.

**Skills:** Molecular Biology, Biochemistry, Cell Culture, Gene Transfer via particle bombardment, FISH

**1994 – 1998 Hacettepe University, Ankara, Turkey**

## **Bachelor of Science in Biology**

### **WORK EXPERIENCE:**

**2007- 2008 University of Minnesota Cancer Center- Junior Scientist ( 6 months )**

**2004- Turkish Society of Hematology Executive Manager**

**2002-2004 Sales Representative, ROCHE**

**2002 Biologist, GENOM, Molecular Biology Laboratory**

### **SKILLS:**

- **Teamwork:** I have been involved in various team projects within both academic and non-academic environments
- **Communication:** As a sales representative I had to demonstrate knowledge of the product and convey up-to-date literature information to be able respond effectively to the customer queries.
- **Computer:** Good knowledge of Microsoft Windows XP, Microsoft Office XP (Word, Excel, PowerPoint, Access), Minitab, Cytovision, regularly use internet and e-mail
- **Languages:** Fluent English (TOEFL: 240)

### **CERTIFICATE SKILLS**

- Sales Dynamics- Roche 2002
- Sales Management- Roche 2002
- Key Account Management- Roche 2004
- Good Clinical Practice –Sanofi-Aventis 2005
- Health Services Management- Hacettepe University 2005

### **ACTIVITIES AND INTERESTS:**

- Attended many Bailo Tango workshops and classes organized by METU Couple Dance Society since 2000
- Compete in many sports, particularly squash and horseback riding

#### **PUBLICATIONS:**

- İpek Z. Durusu. Middle East Technical University. Master of Science Thesis. Ankara. December 2001. ‘Optimization of Callus Culture and Regeneration Systems of Turkish Wheat Cultivars’. Thesis Book.
- İpek Z. Durusu, Fahriye Ertuğrul, Prof.Dr. Hüseyin Avni Öktem, Prof.Dr. Meral Yücel. ‘Callus Culture and Plant Regeneration in Turkish Wheat Cultivars’. XII. Biotechnology Congress Ayvalık – Turkey 17-21 October 2001. Abstract book and poster presentation.
- İpek Z. Durusu, Fahriye Ertuğrul, Prof.Dr. Hüseyin Avni Öktem, Prof.Dr. Meral Yücel. ‘Callus Culture and Plant Regeneration in Turkish Wheat Cultivars’. Second Balkan Botanical Congress, İstanbul – Turkey. 14-18 May 2000. Abstract book and poster presentation
- Erdeş E, Durusu İ, Gizlenci Ö, Coşar İ, Danışman T, Kunt KB, Özen C. Leiurus abduhbayrami (Scorpiones: Buthidae) Ham Venom ve Peptit Fraksiyonunun Sitotoksik ve Antimikrobiyal Etkisinin Araştırılması, 22. Ulusal Biyoloji Kongresi. Eskişehir, Türkiye. 23-27 June 2014. Abstract book and poster presentation
- İpek Durusu, Hazal Hüsnügil, Heval Ataş, Can Özen. Cytotoxic Effect of Kv1.3 Potassium Channel Blocker Clofazımına on Hematological Cancer Cell Lines. 5th International Congress on Leukemia Lymphoma Myeloma. Istanbul Turkey. 21-23 May 2015. Abstract book and poster presentation

- İpek Zeynep Durusu, Hazal Hepşen Hüsniğil, Ayşenur Biber, Heval Ataş, Can Özen. Clofazimine'in Multipl Miyelom Üzerinde Terapötik Etkisi 41. Ulusal Hematoloji Kongresi. Antalya. 21-24 Ekim 2015  
Abstract book and oral poster presentation
- Ayşenur Biber, İpek Durusu, Can Özen. Anticancer Effect and Mechanism of Imipramine, a Tricyclic Antidepressant, on Jurkat Cells, 2015. IV. International Congress of the Molecular Biology Association of Turkey. Ankara Turkey. 27-29 November 2015.  
Abstract book and poster presentation