OSMAN OĞUZ

THE ROLE OF CERAMIDE METABOLISM IN APOPTOSIS TRIGGERED BY RESVERATROL AND THE THERAPEUTIC POTENTIAL OF RESVERATROL IN PH+ ACUTE LYMPHOBLASTIC

AGU 2019

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A THESIS
SUBMITTED TO THE DEPARTMENT OF
BIOENGINEERING
AND THE GRADUATE SCHOOL OF ENGINEERING AND
SCIENCE OF ABDULLAH GUL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

By
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ACCEPTANCE AND APPROVAL

M.Sc. thesis titled The Role of Ceramide Metabolism in Apoptosis Triggered by Resveratrol and The Therapeutic Potential of Resveratrol in Ph+ Acute Lymphoblastic Leukemia and prepared by Osman Oğuz has been accepted by the jury in the Bioengineering Graduate Program at Abdullah Gül University, Graduate School of Engineering & Science.

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(Thesis Defense Exam Date)

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APPROVAL:

The acceptance of this M.Sc. thesis has been approved by the decision of the Abdullah Gül University, Graduate School of Engineering & Science, Executive Board dated ….. /….. / …….. and numbered ……..……. .

…….. /…….. / ……..

(Date)

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ABSTRACT

THE ROLE OF CERAMIDE METABOLISM IN APOPTOSIS TRIGGERED BY RESVERATROL AND THE THERAPEUTIC POTENTIAL OF RESVERATROL IN PH+ ACUTE LYMPHOBLASTIC LEUKEMIA

Osman Oğuz

MSc. in Bioengineering

Supervisor: Assist. Prof. Dr. Aysun Adan

December 2019

The mechanisms underlying the growth inhibitory effect of resveratrol on Ph + ALL cells were investigated with regard to targeting of ceramide metabolism and changes in BCR-ABL expression. Growth inhibition and apoptotic effects of resveratrol, SK inhibitor (SKI II), GCS inhibitor (PDMP), SPT inhibitor (myriocin) and resveratrol-inhibitor combinations were investigated by MTT cell proliferation test, Annexin-V/PI staining, caspase-3, PARP expression and cytochrome c release by western blot, while cytostatic effect was investigated by flow cytometry. The effect of resveratrol, inhibitors and combinations on BCR-ABL protein expression was determined by western blot. The effect of resveratrol on SPT, SK-1/2, GCS protein expression was determined by western blot. In both cell lines resveratrol and resveratrol with SKI II and PDMP suppressed cell growth, triggered apoptosis and arrested the cell cycle at S phase. Resveratrol: myriocin combination showed cell-specific effects on cell growth and cell cycle, but triggered apoptosis in both cells. Resveratrol and combinations generally increased cytochrome-c release, caspase-3 cleavage and PARP cleavage, but cell-specific changes were also detected. Resveratrol decreased the expression of SK-1 / SK2 and GCS in both cells and increased SPT expression. While resveratrol, SKI II and PDMP decreased BCR-ABL expression and myriocin increased BCR-ABL expression. Resveratrol: SKI II and resveratrol: PDMP caused increases in BCR-ABL, while resveratrol: myriocin reduced BCR-ABL expression. As a result, resveratrol suppressed cell growth and triggered apoptosis on Ph + ALL by regulating ceramide metabolism and BCR-ABL expression.

Keywords: Ph + ALL, resveratrol, glucosyl ceramide synthase, serine palmitoyl transferase, sphingosine kinase

ÖZET

RESVERATROL'ÜN PH+ AKUT LENFOBLASTİK LÖSEMİDE TERAPÖTİK POTANSİYELİ VE RESVERATROL TARAFINDAN TETİKLENEN APOPTOZDA SERAMİD METABOLİZMASININ ROLÜ

Osman Oğuz

Biyomuhendislik Bölümü Yüksek Lisans

Tez Yöneticisi: Dr. Ögr. Üyesi Aysun Adan

Aralık 2019

Proje ile resveratrol'ün, Ph+ ALL hücreleri üzerindeki büyümeyi inhibe edici etkisinin arkasında yatan mekanizmalar, seramid metabolizmasının hedeflenmesi ve BCR-ABL ifadesindeki değişimler ile ilişkilendirilerek araştırılmıştır. Resveratrol, SK inhibitörü (SKI II), GSS inhibitörü (PDMP), SPT inhibitörü (myriocin) ve resveratrol: inhibitör kombinasyonlarının Ph+ ALL hücreleri üzerindeki büyümeyi durdurucu ve apoptotik etkileri MTT hücre çoğalması testi, Aneksin-V/PI boyaması, kaspaz-3, PARP ifadeleri ve sitokrom c salınımı western blot ile, sitostatik etki ise akım sitometresi (PI boyaması) ile araştırılmıştır. Resveratrol ve sfingolipid metabolizması inhibitör kombinasyonlarının BCR-ABL protein ifadesi değişimleri western blot ile belirlenmiştir. Resveratrol'ün SPT, SK-1/2, GSS protein ifadeleri üzerindeki etkisi western blot ile belirlenmiştir. Her iki hücre hattında resveratrol ve resveratrol: SKI II ve resveratrol: PDMP ile kombinasyonları hücre büyümesini baskılamış, apoptozu tetiklemiş ve hücre döngüsünü S fazında tutmuştur. Resveratrol: myriocin kombinasyonu ise hücre büyümesi ve hücre döngüsü üzerinde hücreye özgü etkiler gösterirken apoptozu her iki hücrede tetiklemiştir. Her iki hücre tipinde resveratol ve kombinasyonları sitokrom-c salınımını, kaspaz-3 kesimini ve PARP kesimini genel olarak arttırmakla beraber hücreye özgü değişimler de saptanmıştır. Resveratrol her iki hücrede SK-1/SK2 ve GSS ifadesini azaltırken SPT ifadesini arttırmıştır. Resveratrol, SKI II ve PDMP BCR-ABL ifadesini azaltırken myriocin arttırmıştır. Resveratrol: SKI II ve PDMP kombinasyonları BCR-ABL üzerinde artışlara neden olurken resveratrol: myriocin kombinasyonu BCR-ABL ifadesini azaltmıştır. Sonuç olarak, resveratrol seramid metabolizmasını ve BCR-ABL ifadesini düzenleyerek Ph+ ALL üzerinde hücre büyümesini baskılamış ve apoptozu tetiklemiştir.

Anahtar kelimeler: Ph+ ALL, resveratrol, glukosil seramid sentaz, serin palmitoil transferaz, sfingozin kinaz

Acknowledgements

First of all, I would like to express my sincere thanks to my advisor Assist. Prof. Dr. Aysun Adan for supporting me academically and for being a role model with her knowledge and humanity.

I would like to thank my labmates Nur Şebnem Ersöz, İrem Sultan Dilbaz, Hande Nur Şahin, Kardelen Gökçen, Melisa Tecik for their help, support and friendship during this research.

I would like to thank AGU administration which provides great opportunities in terms of laboratory facilities to conduct this research.

This thesis is supported by TUBITAK with project number 315S248 within the context of ‘’3001-Starting R&D Projects Funding Program’’.

Osman Oğuz

Table of Contents

List of Figures

List of Tables

To Labmates

Chapter 1

1. Introduction

Acute lymphoblastic leukemia (ALL) occurs as a result of abnormal accumulation of the lymphoid progenitor cells in the bone marrow, blood and extramedullary regions. ALL consists of a B-cell precursor lineage (B-ALL) and a T-cell precursor lineage (T-ALL) subtypes. ALL is subdivided into these subtypes based on morphological, immunophenotypic, cytogenetic and chromosomal properties. Both types are caused by structural chromosomal changes, changes in the number of copies in DNA, and sequence mutations that cause leukomogenesis. 80% of ALL cases are seen in children, but the results are more severe when seen in adults [1]. Different chromosomal abnormalities such as t (9; 22) BCR-ABL and t (4; 11) MLL–AF4 have been considered as hallmarks and contributed to it's ALL's classification. Philadelphia chromosome positive ALL (Ph + ALL,) is characterized by the presence of the *BCR-ABL* fusion gene generated by a reciprocal translocation between chromosome 9 and chromosome 22, t (9; 22). The presence of *BCR-ABL* is associated with poor prognosis in ALL [2]. Although there are promising treatment strategies including multi-agent conventional chemotherapy, allogeneic stem cell transplantation and tyrosine kinase inhibitors (TKIs) in ALL, there are challenges such as toxicity, development of drug resistance in the clinic [3-5]. Therefore, the discovery and targeting of novel signaling pathways and the potential of natural products such as resveratrol in cancer treatment have been studied extensively in cancer.

Bioactive sphingolipids are a family of lipids that play important roles in cellular functions such as cell growth, division, metastasis and apoptosis, and

include important members such as ceramide, sphingosine-1-phosphate (S1P) and glucosyl ceramide (GC). The functions controlled by sphingolipids are directly related to the onset, progression and response to anticancer treatments. Ceramide, synthesized by the *de novo* synthesis pathway (serine palmitoyl transferase (SPT) is the main regulated enzyme), is the central molecule of sphingolipid metabolism and plays an important role in triggering apoptosis. On the other hand, the conversion of ceramide to S1P and /or GC by the sphingosine kinase (SK) enzyme and/or glucosyl ceramide synthase enzyme (GCS) triggers the proliferation of cancer cells. Therefore, ceramide metabolism (anabolism/catabolism) and regulation of the enzymes involved in this metabolic pathway, such as SPT, SK and GCS, have therapeutic importance [6].

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a natural phytoalexin found in many different plant species, especially grapes, peanuts and blueberries. In the literature, the anticarcinogenic potential and responsible mechanisms of resveratrol effect have been defined in many types of leukemia and solid cancers [7]. However, there are limited studies investigating the therapeutic potential and working mechanisms of resveratrol in Ph + ALL.

With this project, the therapeutic potential of resveratrol in Ph + ALL was investigated in relation to targeting ceramide metabolism and regulation of BCR-ABL. Moreover, the potential of resveratrol and its combinations with SPT inhibitor, SK inhibitor and GCS inhibitor as a new combination therapy approach was investigated.

1.1Acute Lymphoblastic Leukemia

ALL originates from malignant hematopoietic B- and T-lineage lymphoids that have genetic abnormalities including mutations, aneuploidies and translocations in the genes regulating cell growth, division, differentiation and other important cellular processes [3-5]. ALL is characterized by the accumulation of these malignant, immature lymphoid cells within the bone marrow, peripheral blood and extramedullary sites such as spleen. Common symptoms seen in ALL include bone marrow related anemia, leukopenia,

thrombocytopenia, fever, weight loss, easy bleeding, fatigue and brusing [1]. Diagnosis is carried out by detecting 20% or more lymphoblasts in the peripheral blood or bone marrow. To be able to confirm the diagnosis, morphological analysis, immunophenotyping, flow cytometry and cytogenetic analysis are commonly used [2]. Although ALL is the most commonly seen childhood leukemia (80%), it can be seen in adolescents and young adults with severe consequences and lower survival rates due to the heterogeneity differences of the disease [8].

Chromosomal alterations, changes in DNA copy number, tumor-promoting secondary somatic mutations and sequence mutations that drive leukemogenesis have become important hallmarks in the classification, pathogenesis and biomarker identification of ALL based on comprehensive genetic studies including whole exome/genome sequencing, transcriptome analysis and genomic microarrays [9]. The majority of the genes involved in ALL pathogenesis include transcriptional regulators, lymphoid signaling molecules and tumor suppressor genes related to lymphoid development. (Table 1.1.1). For instance, mutated *PAX5* and *IKZF1* genes drive progressive development of B-ALL (Table 1.1.2). Tumor suppressor genes like *RB1* and *CDKN2A/CDKN2B* are altered by deletions and translocations in B-ALL [10].

Transcriptional regulators	PAX5, IKZF1, EBF1, LEF1
Tumor suppressor	CDKN2A, CDKN2B, RB1, TP53
Lymphoid signaling	BTLA, CD200 TOX
Transcriptional regulators and coactivators	TBL1XR1, ERG
Chromatin structure and epigenetic regulators	CTCF, CREBBP

Table 3.1.1 Identified important genes involved in ALL pathogenesis [10].

ALL is divided into two main categories, B-cell lymphoblastic leukemia/lymphoma and T-cell lymphoblastic leukemia/lymphomas based on Health Organization (WHO) revision in 2016, (Table 1.1.4) [1].

B-cell lymphoblastic leukemia
B-cell lymphoblastic leukemia based on t (9;22) (q34; q11.2) *[BCR-ABL1]*
B-cell lymphoblastic leukemia based on t(v;11q23) [*MLL* rearranged]
B-cell lymphoblastic leukemia based on t (12;21) (p13; q22) [*ETV6-RUNX1*]
B-cell lymphoblastic leukemia based on t (1;19) (q23; p13.3) [*TCF3-PBX1*]
B-cell lymphoblastic leukemia based on t (5;14) (q31; q32) [*IL3-IGH*]
B-cell lymphoblastic leukemia based on intrachromosomic amplification of chromosome 21 (iAMP21)
B-cell lymphoblastic leukemia based on translocations related tyrosine kinases or cytokine receptors (Ph+ like ALL)
B-cell lymphoblastic leukemia based on hyperdiploidy
B-cell lymphoblastic leukemia based on hypodiploidy
T-cell lymphoblastic leukemia
Early T-cell precursor lymphoblastic leukemia

Table 1.1.5 ALL is divided into two main categories based on WHO [1].

B-ALL accounts for 75-80% while T-ALL accounts for 20-25% of all ALL types. The subtypes of B-ALL have been classified based on recurrent genetic abnormalities including hypodiploidy, hyperdiploidy, t (9; 22) (BCR-ABL1), T (v; 11q23) (MLL rearranged) and BCR-ABL1-like (Ph like) ALL (Table 1.1.6) [11]. These cytogenetic abnormalities are related to the diagnosis status of both pediatric and adult B-ALL (reference, Table 1.1.7). For instance, **Ph** + ALL is known as the most common subtype of B-ALL cases (20-25% of all B-ALL) indicated in this classification with poor prognosis.

Risk evaluation	**Cytogenetic anomalies**	**Clinical significance**	**Incidence Children/ Adult**
Good	Hyperdiploidy (>50 chromosomes)	Favorable prognosis	25-30%/ 7-8%
	(12;21) (p13; q22) [*ETV6-RUNX1*]	Favorable prognosis	25%/ 0-4%
Intermediate	t (1;19) (q23; p13.3) [*TCF3-PBX1*]	Intermediate prognosis	1-6%/ 1-3%
	t (5;14) (q31; q32) [*IL3-IGH*]	Intermediate prognosis	infrequent
Poor	**t (9;22) (q34; q11.2) *[BCR-ABL1]***	**Poor prognosis**	**1-3%/ 25-30%**
	t(v;11q23) [*MLL* rearranged]	Poor prognosis	1-2%/ 4-9%
	Hypodiploidy (<44 chromosomes)	Poor prognosis	6%/ 7-8%

Table 1.1.8 Prevalent Cytogenetic Abnormalities in B-ALL

T-ALL is commonly observed at older age with dominancy in male sex and has poorer outcomes compared to B-ALL [12]. T-ALL is characterized by mutations and deletions in the *PHF6* tumor suppressor gene, which accounts for 16% of all T-ALL cases in children and 38% in adults [13]. In addition to *PHF6* gene mutation, activating *NOTCH1* mutations, *LMO2*, *MYB*, *WT1* and *PTEN* gene mutations, rearrangements of transcription factors like *TLX1*, *LYL1*, *TAL1* and *MLL* were also observed in T-ALL [14]. Early T-cell precursor ALL (ETP-ALL) is a subtype of T-ALLs distinguished by different cell surface markers with poor prognosis. These cells do not have CD1a and CD8 expressions while having weak CD5 expression and one or more myeloid-associated or stem cells associated markers [15].

ALL is a treatable disease, especially in the pediatric population with a success rate of around 90%. However, this rate is around 30-35% in adults despite following the same treatment approaches [16]. This is due to the fact that adults are more intolerant and resistant to chemotherapy and having risky genetic subtypes, mutations and epigenetic changes frequently [17].

Chemotherapy has been used as the standard cure for all ALL types with different phases including the steroid pre-phase, the induction therapy, the consolidation and maintenance phases and central nervous system (CNS) prophylaxes. In different clinical setups, different combinations of drugs with different mechanisms of action have been used to remove the majority of the malignant ALL cells and prevent drug resistance [18].

In the steroid pre-phase therapy, corticosteroids are used. Moreover, genetic and prognostic characterization of the disease such as the presence of *RAS and CREBBP* mutations might affect the therapy in the steroid pre-phase. In induction therapy which is the first stage of ALL treatment, vincristine, methotrexate (MTX), anthracyclines including doxorubicin, daunorubucin, cytarabine are commonly used to provide normal blood cell production. Although this therapy provides a high rate of complete remission (CR), it causes severe side effects in children [19]. At the end of induction therapy, consolidation and maintanance therapy is given and eliminates residual leukemia cells. Various combinations of cytotoxic drugs used in the induction therapy are administered at high doses (like MTX and cytrabine). Hyper-CVAD (hyperfractionated Cyclophosphamide, Vincristine, Doxorubicin and Dexamethasone) is one of the most used protocols in this phase. After consolidation therapy, maintenance therapy lasts for 1-2 years and daily 6-mercaptopurine (6-MP) and weekly methotrexate (MTX) are given to patients. Maintenance therapies are also strengthened by combining vincristine and steroids. Although maintenance therapy can provide CR, some obstacles including infection may result in death. In addition, a study showed that long-term maintenance therapy and high doses of 6-MP led to the development of secondary malignancies. Since chemotherapy-resistant cells might still remain following chemotherapy, allogeneic stem cell (allo-SCT) transplantation plays a significant role in eradicating remaining resistant cells. For patients at the relapse phase, especially for children, allogeneic stem cell (allo-SCT) transplantation is the backbone of the consolidation therapy. However, complications such as infertility, growth retardation, metabolic diseases and

secondary malignant neoplasms may occur after transplantation. Therefore, allo-SCT should be introduced to patients in the high-risk group if possible. The purpose of CNS prophylaxis is to prevent CNS relapse of the disease. Two main protocols which are intrathecal injection or high intravenous dose of MTX or Cytarabine (also used intrathecal, usually with steroids) are use are used to overcome the blood-brain barrier: d in order to overcome the blood-brain barrier. CNS irradiation might be another option both for ALL adults and childhood. With this CNS relapse rate could be reduced [18-20]. In addition to conventional chemotherapeutic approach and AlloSCT, targeted therapies such as immunotherapy, signaling pathway inhibition and CAR-T cell therapy have been revolutionized the therapy in ALL in favor of personalized medicine.

Immunotherapy has made progressions in the treatment of ALL in recent years. This treatment targets antigens such as CD20, CD22, and CD19 which are commonly found on the surface of B cells. These surface markers are targeted by naked monoclonal antibodies and antibody-drug combination (Table 1.1.9) [21].

Targeted Surface Antigen	**ALL Subtype**	**Monoclonal Antibodies or antibody-drug combination**
CD19	B-ALL	Blinatumomab
CD20	B-ALL	Rituximab, ofatumumab, obinituzumab
CD22	B-ALL	Inotuzumab ozogamicin, epratuzumab, moxetumomab pasudotox

Table 1.1.10 Immunotheraputic Targets in ALL [22].

The detailed understanding of ALL molecular biology have given opportunities to target and design specific molecules for altered signaling pathways including PI3K/Akt/mTOR, BCR-ABL and JAK/STAT (Table 1.1.11) [23].

Signaling Pathway	Inhibitor	Function of Inhibitors
JAK/STAT	Ruxolitinib	JAK1/JAK2-JAK-STAT
	Pacritinib	Inhibitor of FLT3-ITDs and JAK2, JAK2V617F
mTOR	Sirolimus,	Immune suppressive
	AZD8055	Phosphorylation of mTORC1
	Rapamycin	PI3K/mTOR inhibition
MEK	Pimasertib	Selective to MEK1/2
	GSK690693	Inhibition of apoptosis in sensitive ALL cells
AKT	MK-2206	Inhibition of the PI3K/Akt pathway
	Gefitinib	EGFR (Epidermel Growth Factor Receptor) inhibitor
PI3K	Idelalisib	Effective in p53 mutation carrier patients
	Volasertib	Inhibits PLK1 (Polo Like Kinase 1)

Table 1.1.12 Inhibitors targeting altered signaling pathways in ALL [23].

Chimeric antigen receptor-modified (CAR) T cells possess genetically engineered receptors that specifically target cell surface antigen of the cancer cell of interest. Autologous CAR-T cells are obtained by using patient's own native $CD4^+$ or $CD8^+$ T cells to use the potential of both innate and adaptive immunities. Obtained T cells are activated *in vitro* with an anti-CD3 mAb with or without an anti-CD28 mAb and then genetically modified to express the CAR by using gene delivery systems such as retroviral or lentiviral transfection. The transferred genes are inserted into the cell membrane of T cell [24]. Engineered T cells are treated *in vitro* with suitable cytokines to induce cell expansion and proliferation to obtain effective dose for therapeutic activity. Antigen targeting is the most critical step for CAR-T cell development which is similar to antigen-antibody interaction. Therefore, antigen amount on the surface of cancer cell and the affinity power of the receptor on T cells are important parameters for effective therapy. CAR- T cell therapy has significant potential for both solid and hematological cancers (Receptor affinity and extracellular domain modifications affect tumor recognition by ROR1-specific chimeric antigen

receptor T cells). The potential of CAR-T cells in ALL is more convenient for B-ALL. CD19 is an attractive target for CAR-T cell therapy against B-ALL and clinical studies showed that CAR-T cell targeting CD19 in B-ALL led to CR and partial remission in children and adults [21]. In another clinical study, patients were treated with fludarabine or cyclophosphamide before giving adoptive CAR-T cell and CR was around 90% [25]. CD22 is another potential target for CAR-T cell in B-ALL with two recent clinical trials to overcome the deficiency of anti- CD19 therapy CD22 is another potential target for CAR-T cell, and recently, two different anti-CD22 agents have been tested against B-ALL in clinical trials to overcome the deficiency of anti- CD19 therapy [26].

1.1.1 Philadelphia Positive Acute Lymphoblastic Leukemia

Ph + ALL is characterized by an unbalanced translocation between *ABL* gene located on chromosome 9 and *BCR* gene located on chromosome 22 which results in the formation of *BCR-ABL* fusion gene with abnormal tyrosine kinase (TK) activity. BCR-ABL fusion gene is predominantly found in chronic myeloid leukemia (CML), however, the presence of BCR-ABL is also a major pathogenicity factor in B-ALL (25%) [27]. Newly formed *BCR-ABL* gene is responsible for the malignant transformation of the cells. BCR-ABL with different molecular sizes (p190, p210, p230*)* can be produced due to the different breakpoint regions in the *BCR* and *ABL* gene [28]. Translocation commonly occurs between exons 1, 13/14 or exon 19 of *BCR* and a 140-kb region of *ABL1* between exon 1b and 2 in all BCR-ABL positive hematological cancers such as CML, ALL and some AML cases [29]. The majority of the Ph+ ALL cases possess p190 kda protein, however, p210 kda protein can be also detected occasionally [28]. Exon 1 of *BCR* and exon 2 of *ABL* are fused to produce p190 BCR-ABL in ALL, which is also called minor breakpoint BCR (*m-BCR*) (Figure 1.1.1.1) [29].

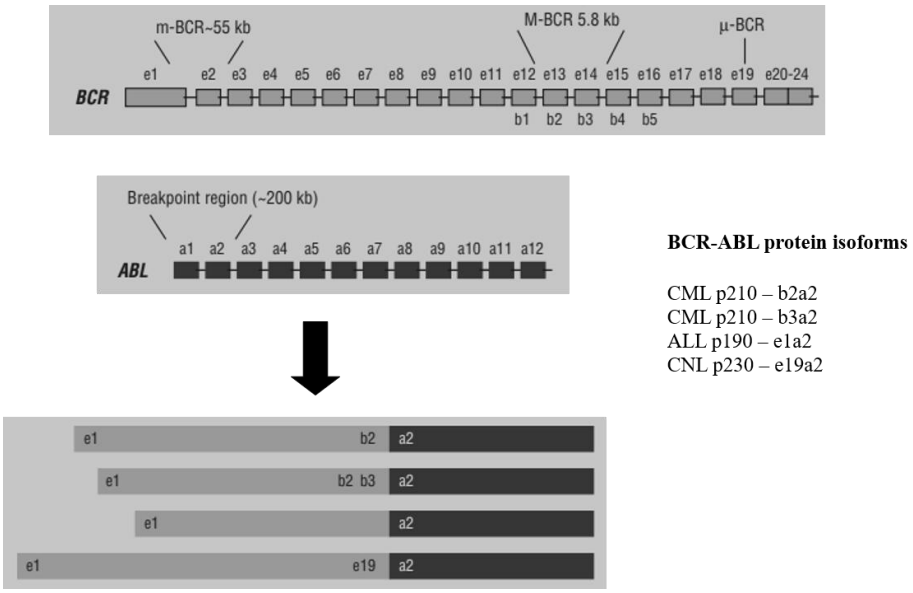


Figure 1.1.1.1 Formation of BCR-ABL molecules with different molecular weights based on the breakpoints on BCR and ABL genes [29].

The resulting p190 BCR-ABL oncoprotein have several structural domains which determine its function. These domains include Serine/Threonine domain, src homology domains (SH1, SH2, SH3), nuclear localization signal (NLS) and actin binding domain from ABL (Figure 1.1.1.2) [28].

Constitutively active tyrosine kinase BCR-ABL protein (p190) can modulate several downstream signaling pathways including PI3K/AKT and JAK/STAT5 pathways responsible for Ph+ ALL pathogenesis and further lymphoid development. STAT5 has important roles in cell proliferation and B-cell development. Deregulated STAT5 promotes the survival of leukemia cells in malignant precursor B-cells and is also activated in Ph + ALL cells. STAT5 can be activated either by JAK2 via phosphorylation or by BCR-ABL1 [30]. The PI3K / AKT / mTOR signaling pathway has many functions in the cells such as cell growth, differentiation and suppression of apoptosis. The altered PI3K/AKT/mTOR signaling pathway is often associated with leukemogenesis, and continuous activation of this pathway induces cell proliferation and the inhibition of apoptosis. Activating PIK3CA (PI3K-alpha) and inactivating PTEN (negative regulator of AKT) mutations are frequently seen in T-ALL although they are also present in Ph + ALL [31-35].

The BCR-ABL oncogenic pathway also activates RAS-mediated signaling that might also activate mitogen activated protein kinase (MAPK)/extracellular signal reductase kinase (ERK) 1/2/ (MEK) pathway, which is responsible for abnormal cell proliferation. (Figure 1.1.1.3) [36-37].

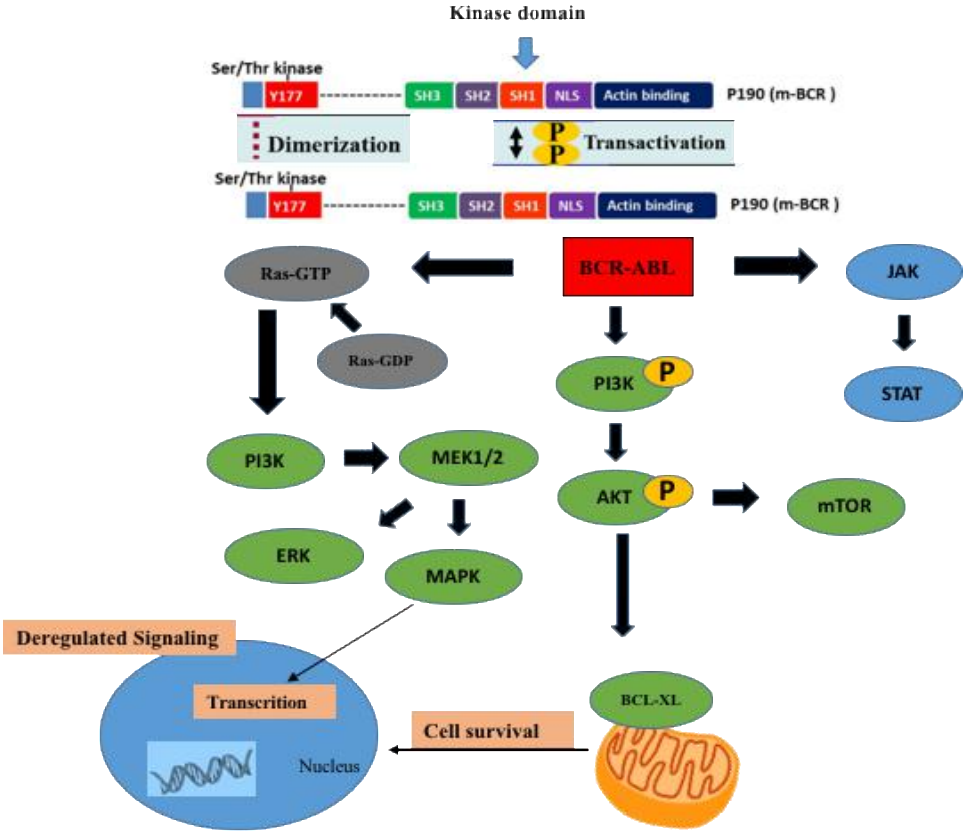


Figure 1.1.1.2 BCR-ABL oncogenic pathway activates several downstream signaling pathways related to leukemogenesis. The Structure of BCR-ABL (p190) protein. NLS: Nuclear localization signal, SH: Src homology domain [38].

Despite the presence of the BCR-ABL as a major driver for leukemia formation, it has been confirmed that BCR-ABL Ph+ ALL shows biological heterogeneity due to the secondary chromosomal abnormalities, further epigenetic changes, mutations on BCR-ABL and chromosome copy number changes (such as trisomy 8, isochromosome 17) especially following chemotherapy. Molecular pathway related abnormalities such as p53 pathway

mutations, deficiency of p16INK4A/ARF and BCR-ABL independent activation of LYN, AKT, STAT5 pathways are also involved in disease formation [39-40].

1.2 Ph + ALL Therapy

The incidence of Ph+ ALL increases with age reaching around 50% in patients above 60 years. Historically, intensive chemotherapy adapted from pediatric ALL protocols was given to adult patients as a sole therapy which led to very poor outcomes such as short remission period and lower overall survival (OS) (<20%). [41-42]. Although CR was observed in patients receiving intensive chemotherapy, relapse was a major challenge for the patients who died within 6- 11 months after treatment. Therefore, allogeneic stem cell transplantation (Allo-SCT) was thought as an effective treatment method in adult patients in the presence of suitable matched donors with increased OS (35-55%). If chemotherapy and AlloSCT were given together, significant success was observed with improved CR rates. However, finding available matched donors and decision for the number of AlloSCT trials represent major limitations [43-46]. The understanding of the molecular mechanism of Ph+ ALL and the role of BCR-ABL oncoprotein in leukemogenesis has opened the way of using TKIs which target TK activity of BCR-ABL. In addition to chemotherapy, AlloSCT, TKIs and combinational therapies, chimeric antigen receptor-modified (CAR) T cell therapy makes treatment approaches modernized [18]. Moreover, the development of unique agents including inotuzumab (CD22 monoclonal antibody conjugated to the cytotoxic antibiotic calicheamicin) and blinatumomab (bispecific T cell engager anti-CD3 and CD19 antibody construct, resulted in a serial lysis of B cells by redirecting CD3+ T cells toward CD19+ B-ALL cells) have beneficial potentials for clinical usage especially for relapsed and/or refractory (R/R) Ph+ B-ALL [47-48].

1.2.1 Selective TKIs

The introduction of the first generation TKI, imatinib, which targets ATP binding domain of BCR-ABL to block its TK activity, into adult Ph+ ALL therapy had modest and unstable results, however, its combination with standard chemotherapy was safe and resulted in CR rates between 91% and 98% and OS rate reaching up to 50% (Treatment of Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia). Imatinib has been given to the patients together with chemotherapy by two general approaches which are simultaneous and successive methods [49-56]. Even though these combination therapies have antileukemic effects, the development of imatinib resistance throughout the therapy stays as a major problem. This resistance is divided into *BCR-ABL* dependent and independent mechanisms. *BCR-ABL* dependent mechanism is associated with the multiplication of *BCR-ABL* gene and point mutations in BCR-ABL that might be mutations at threonine 315 [T315] [57-59] and phenylalanine 317 [F317], at the Src homology 2 (SH2) binding site and at the ATP-binding pocket (in SH1 domain). The most common mutation leading to conversion of glutamic acid to lysine at codon 255 (E255K) occurs principally after imatinib administration. Some mutations, particularly ATP-binding pocket mutations, are more resistant to imatinib and patients having these mutations are generally described with worse prognosis [60]. BCR-ABL independent mechanisms are related to failure in drug uptake and efflux, altered alternative signaling pathways that promote abnormal cell proliferation and survival. For instance, the presence of BCR-ABL might increase multidrug resistance protein (MDR, PgP) expression which pumps imatinib out, therefore, intracellular concentration of imatinib is decreased [61]. The presence of secondary resistance to imatinib has resulted in the development of a number of second- and third-generation TKIs to overcome the resistance. Most common second generation TKIs are nilotinib and dasatinib. Nilotinib shows higher binding affinity for BCR-ABL and greater activity compared to imatinib. Moreover, it overcomes resistance to mutations that imatinib causes [62-64]. In clinical studies from independent centers, nilotinib has been administrated to the patients

in combination with chemotherapy and showed promising results with increased CR and OS rates [65-66]. Dasatinib, a second-generation TKI, inhibits both active and inactive forms of BCR-ABL and is 325-fold more effective than imatinib. It overcomes most of the imatinib-resistant kinase domain mutations [67-68]. Dasatinib introduced into Ph+ ALL patients with resistance or intolerance to imatinib as single agent and showed some initial activities. In a study, 7 out of 10 patients treated with dasatinib achieved CR and 8 patients showed significant cytogenetic response [62[. In another study, 78% of 46 patients having BCR-ABL positive kinase domain mutations and patients (20%) carrying T315I showed remarkable hematologic and cytogenetic responses after dasatinib treatment [69]. However, observed results were short-lived and progression free survival was around maximum 3 months. Therefore, combination of dasatinib with chemotherapeutic regimens have been investigated in various clinical studies. In a study, combination of dasatinib with chemotherapy hyper-CVAD: in 35 Ph+ ALL patients resulted in 94% CR and extended life span up to 2 year [70].

Patients, who relapse after therapy with imatinib, often develop kinase domain mutations responsible for imatinib resistance. T315I mutation, responsible for up to 75% of cases of acquired kinase mutations at the time of relapse, is known to be less sensitive to all first- and second-generation TKIs [71]. Therefore, third generation TKIs such as ponatinib have been specifically designed to overcome most of the kinase domain mutations, including T315I [72, 73]. Ponatinib introduced into Ph + ALL patients as single agent or in combination with chemotherapy. However, ponatinib in combination with intensive chemotherapy has shown the highest anti-leukemia activity. For example, ponatinib in combination with hyper-CVAD in Ph+ ALL was more effective than dasatinib in combination with hyper-CVAD (reference). Ponatinib did not only overcome T3151 mutation, but also resulted in higher CR [74]. Currently, the standard treatment protocol includes the combination of a TKI with chemotherapy or corticosteroids. The major problem in combination setups

due to the lack of randomized trials evaluating the advantage of one TKI over the others is which TKI should be preferred.

1.3 Sphingolipid Metabolism

Sphingolipids are the major structural components of the eukaryotic cell membranes with members including ceramide (Cer), sphingosine (Sph), sphingosine-1-phosphate (S1P), ceramide-1-phosphate (C1P) and glucosylceramide (GC). In addition to their roles in the membranes, they have been found to regulate various important cellular functions such as cell growth, differentiation, senescence, apoptosis and inflammation by regulating intracellular signaling pathways [6, 75]. Sphingolipid metabolism (anabolism/catabolism) is a complex pathway in which different enzymes subjected to strict regulation are involved. All pathways involved are connected to each other and Cer is the central molecule in both anabolic and catabolic reactions. Ceramide is synthesized by two main pathways which are *de novo* pathway and hydrolysis of complex lipids such as sphingomyelin (SM). The *de novo* pathway begins with the condensation of serine and palmitoyl-CoA to produce 3-keto-dihydrosphingosine, which is catalyzed by serine palmitoyl transferase (SPT). The production of sphingolipids starts at endoplasmic reticulum (ER) where heterodimeric phosphate-bound SPT localized. In mammals, SPT consists of two large subunits (SPTLC1 and SPTLC2) [76]

3-keto-dihydrosphingosine is reduced to form dihydrosphingosine (sphinganine) which is converted to dihodroceramide (dhCer) and Cer by Cer synthases (CerS) via N-acetylation reaction. Six Cer synthase genes (CerS1-6) have been identified to synthesize Cers with different chain lengths [77, 78]. The resulting Cer is primarily used for the synthesis of SM by adding phosphocholine headgroup from phosphatidylcholine by SM synthases with the generation of diacylglycerol (DAG). Ceramide can be also phosphorylated to C1P by ceramide kinase (CK) and converted to glucosyl or galactosylceramide. In the hydrolytic pathway of Cer synthesis, SM is cleaved by sphingomyelinases (SMases) to produce phosphocholine and Cer. Ceramide can be also released

through the hydrolysis of glycosphingolipids, glucosylceramide or galactosylceramide by the function specific beta-glucosidases and galactosidases such as glucosyl ceramide synthase (GCS). GCS transfers glucose molecules to ceramide to produce glucosylceramide (GC). This enzyme is found in golgi apparatus in which ceramide is glycosylated and transported by ceramide transport proteins [79-80].

Cer is metabolized to Sph by ceramidases (CDases) and Sph can be recycled to synthesize Cer by CerS or S1P by sphingosine kinases (SK). SKs have two isoforms called SK-1 and SK-2 which are encoded by *SPHKL1* and *SPHKL2*, respectively. SK-1 and SK-2 have similarity in their protein sequences except for Ser225 phosphorylation site which is conserved and required for SK-1 activation [79]. SK-1 is located in cytoplasm under normal conditions. However, the presence of growth factors and cytokines might alter its localization from cytoplasm to plasma membrane. SK-2 is normally located in the nucleus and cytoplasm. However, its location can be changed to ER during cellular responses where S1P phosphatases is located. S1P is available to regenerated Sph by S1P phospahateses (Figure **1.3.1.1.1)**.

1.3.1 Sphingolipid Metabolism in Cancer

Sphingolipid metabolism and the roles of sphingolipids have been extensively investigated in cancer. In particular, Cer, Sph and their phosphorylated forms affect many physiological and pathological conditions such as regulation of fever and sugar metabolism and cancer in the cell and they act as a secondary messenger to determine the cell fate [81-84].

The intracellular balance between sphingosine (or S1P) and ceramide is crucial for the cells to determine either they survive or die, which is called ‘’sphingolipid rheostat’’ [85]. If this balance is disrupted due to external factors towards ceramide, intrinsic or extrinsic apoptosis is activated [82, 85]. On the other hand, the conversion of Cer by CDases to Sph is associated with cell proliferation and division. Morever, S1P directly or indirectly by binding to G-protein coupled receptor (GPCRs) induces PI3K and PLC (Phospholipase C)

pathways to induce cell proliferation and division. [86-88]. Therefore, Cer is considered as an apoptotic lipid while Sph and S1P act as antiapoptotic molecules.

In glioblastoma cell lines, the association between Cer and Fas-mediated extrinsic apoptosis was investigated and Cer was responsible for the downregulation of FLICE inhibitory protein (FLIP), negative regulator of Fas-FasL signaling [89, 90]. In a study, serum-levels of C16 ceramide and S1P have become a diagnostic marker for hepatocellular carcinoma [91]. In the study performed in glioblastomas, S1P was observed 9-fold higher and Cer was observed 5-fold lower compared to normal gray matter [92]. SK-1 has been upregulated in many cancers and SK-1 inhibition has reduced proliferation, angiogenesis and metastasis and increased apoptosis by using pharmacological inhibitors or genetic silencing [93]. S1P and sphingolipid pathway played an important role in the pathogenesis and resistance of ovarian cancer. In addition, the conversion of ceramide to S1P, GC and SM in ovarian cancer has a mitogenic effect and inhibits apoptotic pathway [94].

In a study conducted in hepatocellular carcinoma, melatonin increased the amount of ceramide by regulating ceramide synthesis pathways and inhibition of SPT with myriocin inhibited melanin-related autophagy [95].

SK-1 overexpression resulting in increased S1P levels inhibited apoptosis in NIH3T3 fibroblasts and HEK293 kidney cells [96]. Similarly, overexpression of SK-1 and S1P production has been proven to cause cell proliferation in many cancer types. SK/S1P/S1PR pathway modulates pro-survival cellular responses via autocrine and paracrine manner by activating GPCR family S1P receptor 1-5 (S1PR1-5) [97]. S1P inhibited intrinsic apoptotic pathway activation by inhibiting cytochrome c and Smac /DIABLO release from mitochondria in AML cells [98]. In non-small cell lung cancer, S1P was found to activate the oncogenic signal by activating PI3K [99].

It was determined that MOLT-4 T-ALL cells were arrested at the G0 /G1 phase due to the accumulation of ceramide produced by SM hydrolysis after exposure to serum starvation [100]. In neuroblastoma cells, dihydroceramide

arrested the cell cycle progression at G0/G1 [101]. In a study, ceramide arrested G1/S transition by dephosphorylating p21 and Rb through p53 dependent and independent manner [102, 103]. In addition, several studies have shown that ceramide affects autophagy by regulating autophagy related players [104]. For instance, melatonin increased ceramide levels via *de novo* and salvage patyway which led to autophagy related cell death in hepatocarcinoma cells. In this study, SPT inhibition prevented autophagy while SPT inhibition induced cell death [95]. Ceramide caused cell cycle arrest by dephosphorylating Rb gene, activating p21 inhibitor, and inhibiting cyclin dependent kinase 2 (CDK2) in breast cancer [105]. S1P has been found to have an important role in cell migration and matrix metalloproteinase-9 expression, also induce Epithelial-Mesenchymal Transition (EMT) in breast cancer [106]. In another study, S1P and S1P receptors were found to be positive regulators of angiogenesis and metastasis in breast cancer cells [107]. In human glioblastoma cells, S1P initiated metastasis by secreting matrix metalloproteinase to degrade extracellular matrix [108]. Ceramide increased sensitivity of chemoresistant breast cancer cells to chemotherapy [109].

Abnormal GCS expression in cancer is associated with prognosis. Inhibition of GCS, either molecularly or pharmacologically, eliminated resistance to chemotherapy. For instance, upregulated MDR1 expression is associated with overexpressed GCS in breast, ovary, cervical and colon cancer cells. Targeting GCS by genetically reversed drug resistant these cancer cells to doxorubicin [110].

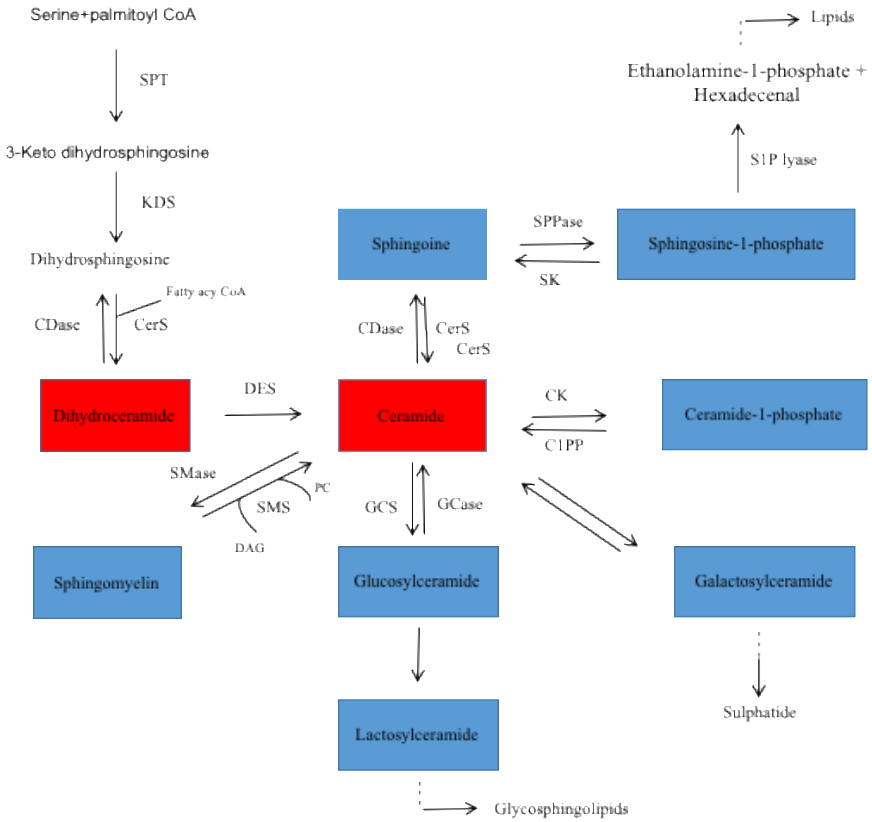


Figure 1.3.1.1.1 Sphingolipid metabolism pathways (*de novo* and salvage). Anti-apoptotic sphingolipids are highlighted in blue. Apoptotic sphingolipids are indicated in red [6].

1.3.1.1 Effect of Sphingolipid Metabolism in Leukemia

The effect of sphingolipid metabolism in leukemia has been investigated intensively as compared to solid tumors. In T-ALL cells, dihydroceramides increased retinoid-induced cytotoxicity [111] and inhibition of sphingomyelin synthase (SMS) increased the amount of Fas-associated ceramide and triggered caspase-9 activation in human Jurkat leukemia cells [112]. SMS and glycosyl ceramide synthase (GCS) activities have made AML and CML patients resistant to chemotherapy by decreasing ceramide levels and increasing leukemic blasts [113]. Thus, inhibition of SMS or GCS may be a therapeutic approach in chemoresistant hematological malignancy. It was found that modulation of pro-apoptotic and pro-survival sphingolipids could contribute to overcome chemoresistance in HL-60 leukemia cells [114]. Inhibiting GCS

and SK-1 increased sensitivity resistant CML cells to nilotinib and resulted in cell death [115]. The treatment of U937 leukemia cells with Bcl-2 family inhibitors and GCS inhibitor PDMP led to synergistic effect on cell death and PDMP treated imatinib resistant CML cells underwent cell death [116].

Disruption of sphingolipid rheostat toward S1P by SK-1 overexpression made K-562 cells imatinib resistant. However, suppression of SK-1 expression increased sensitivity to imatinib [117]. In chemosensitive HL-60 cells, doxorubicin and etiposide treatment caused SK-1 inhibition and Cer accumulation. On the other hand, in doxorubin and etiposide resistant HL-60 cells, SK-1 activated and Cer levels decreased, which inhibited apoptosis through the prevention of cytochrome c release from mitochondria [118]. Interleukin-6 (IL-6) activated SK in human multiple myeloma cells resulted in upregulation of Mcl-1 which promotes cell proliferation and survival [119]. SKI-II, SK-1 inhibitor, inhibited the cell growth and caused apoptosis in U937 and HL-60 AML cells by increasing intracellular ceramide level. The results of this study suggest that SKI-II may be a novel therapeutic agent in AML cells [120]. Tamoxifen and its metabolite caused cell death by blocking ceramide glycosylation, ceramide hydrolysis and SK1 activity in AML cell lines and AML patient samples [121]. In ALL, SK-2 has been shown to play an oncogenic role and modulates the regulation of the MYC oncogene. In the mouse model of ALL, SK-2 has caused the development of leukemia. However, the inhibition of SK-2 pharmacologically prolonged the survival of mouse [122].

Enzyme	Malignancy	Malignancy Effect
Glucosylceramide synthase	AML	Higher expression in cells resistant to chemotherapy
Ceramide synthase	AML	Blocked FLT3 signaling
Acid ceramidase	AML	Regulated Mcl-1 expression post-transcriptionally.
Sphingosine kinase 1	AML ALL	Upregulated in patients Caused drug resistance
Sphingosine kinase 2	ALL	Accelerated B-ALL disease by increasing Myc expression

Table 1.3.1.1.1 The role of sphingolipid enzymes in leukemia [123].

1.4 Targeting Sphingolipid Metabolism

Sphingolipids have prominent roles for the determination of the cell fate and differences in expression levels of anti-apoptotic and pro-apoptotic lipids have been observed in many cancer cells. Dysregulations in sphingolipid metabolism might cause drug resistance. Thus, targeting sphingolipid metabolism has been paid attention in cancer therapy. Different approaches can be used to target sphingolipid metabolism including using synthetic ceramide analogs and small molecule inhibitors which increase ceramide anabolism and prevent its conversion into antiapoptotic sphingolipid specimens. For instance, tumor promoting S1P effect can be eliminated by using SK inhibitors or by inactivating S1P receptor. Moreover, additional approaches might be used to reactivate some genes such as SMase and S1P phosphatase that are suppressed in cancer cells. The combination strategies including sphingolipid metabolism inhibitors and conventional cytotoxic chemotherapeutic agents to increase ceramide production have been studied in cancer [124]. Tamoxifen and sphingosine analog (FTY720) combination synergistically triggered apoptotic cell death as compared to each agent alone in drug-resistant ovarian cancer

[125]. Using SPT inhibitor (myriocin) and SK inhibitors reduced tumor volume in merkel cell carcinoma [126]. Vincristine resistant HL-60 AML cells underwent apoptosis after treatment with P-glycoprotein inhibitors and C6-ceramide analog. Apoptotic event was associated with cytochrome c release and mitochondrial ROS production [127]. Acid ceramidases can be a target due to their contribution to metastasis and chemotherapy resistance. Therefore, targeting acid ceramidases by synthetic inhibitors may be a promising therapeutic strategy [128]. For instance, inhibition of acid ceramidases increased ceramide levels and decreased S1P. Terefore, this strategy prevented cell proliferation in melanoma cells as compared to normal skin cells [129]. Targeting GCS as pharmacologically or genetically is another strategy to induce cell death or overcome drug resistance. In sorafenib resistant hepatoma cells, GSC inhibition increased the sensitivity to sorafenib [130]. In cervical carcinoma cells using SK-2 inhibitor (ABC294640), apoptosis and cell cycle arrest in G1/S phase was induced [131]. Moreover, SK-1 inhibition with the novel inibitors induced apoptosis in breast and prostate cancer cells [132].

1.5 Resveratrol and Its Potential in Cancer

Resveratrol, firstly isolated from white hellebore plant in 1940s, is an important polyphenol produced by plants under stress conditions such as microbial and fungal infections for protection and it is commonly found in grapes, peanuts and berries [133-135]. Processed plant products include large quantities of resveratrol as well. The presence of resveratrol (0.1–14.3 mg/L) in red wine has been related to a terminology called ‘’French Paradox’’, which shows why Southern French people consuming a lot of red wine have very low rate of heart diseases despite having very rich saturated fat based diet [136].

Resveratrol regulates cell survival, metabolism, stress, cell aging and immune function by activating the *SIRT1* gene, a member of the sirtuin family proteins in mammals. Thus, resveratrol has a potential for the treatment of the diseases resulting from abnormal metabolism, inflammation and cell cycle disorders via SIRT activation [137].

Resveratrol, has been investigated as a therapeutic agent in pre-clinical models of many diseases including cancer, cardiovascular diseases, diabetes, and neurological disorders. Resveratrol has many biological properties, such as the elimination of total free hydroxyl groups, which is an indicative anti-oxidant feature of resveratrol [138]. Therefore, the anti-oxidant effect of resveratrol protects cells from oxidative stress caused by hydrogen peroxide and provides intracellular and extracellular redox balance in C6 glioma cells [139]. Resveratrol eradicated radicals that *Helicobacter pylori* caused in gastric cancer [140]. Furthermore, resveratrol significantly reduces lipid oxidation, and prevents the formation and accumulation of toxic side products [141].

Resveratrol is used as a chemotherapeutic and chemopreventive agent due to its anti-inflammatory, anti-proliferative, pro-apoptotic and anti-oxidant properties. The chemopreventive and chemotherapeutic effects of resveratrol has been demonstrated both *in vitro* and *in vivo* for all stages of cancer which are initiation, promotion and progression by targeting multiple different signaling pathways based on the cancer type (**Figure 1.4.1.1)** [142,143]. In one study resveratrol significantly prevented proliferation, migration and invasion in ovarian cancer by targeting glycolysis and inhibiting mTOR activation and increasing caspase-3 [144]. Resveratrol targeted and downregulated EGFR which is overexpressed in human lung cancer [145]. Furthermore, resveratrol has a growth suppressive effects on EGFR/Her-2 positive and negative ovarian cancer cells [146]. Resveratrol demonstrated its pro-apoptotic and anti-proliferative effects by regulating the expression of Bcl-2 family proteins in human cervical Hela cells [147]. In addition, resveratrol activated caspase-3 and caspase-9 together with p53, which is responsible for cell survival and cell cycle regulation. Resveratrol triggered cell death through mitochondrial related and caspase independent apoptosis in prostate cancer cells [148]. Resveratrol in combination with histone deacetylases inhibitor induced the inhibition of angiogenesis, cell cycle arrest, apoptosis and autophagy activation in cancer [149]. Resveratrol caused both apoptosis and G2/M transition cell cycle arrest in

a dose and time dependent manner in oral squamous cell carcinoma by upregulating cyclin A2-B1 proteins [150]. There are important studies in which resveratrol has a different pivotal role in cancer cells such as resveratrol induced ROS related ER stress which leads to apoptotic cell death. For instance, resveratrol triggered enhanced ROS and ER stress that inhibits cell growth in melanoma cells [151]. Cell cycle activators aurora protein kinase (AURKA) and PLK1 were inhibited by resveratrol in breast cancer. Resveratrol prevented G1/S transition and also increased the *BRCA1* gene expression [152]. Another role of resveratrol is to regulate inflammation and immune response by affecting the nuclear factor κB signaling, which was observed in U937 myeloid cells, jurkat lymphoid cells and Hela cells [153]. Additionally, resveratrol inhibited tumor growth in human colon cancer cell by blocking IGF-1R/Akt/Wnt pathways and activating p53 [154]. Resveratrol caused apoptosis and also inhibited the PI3K/Akt pathway, which regulates cell differentiation, growth and proliferation in prostate cancer cells [155]. Moreover, PTEN/AKT is commonly activated in prostate cancer, therefore, resveratrol regulated PTEN/AKT pathway by dephosphorylating AKT [156]. In one study, the use of resveratrol in combination with PI3K/Akt/mTOR inhibitors showed an important treatment approach in human glioma cells [157]. The combination of resveratrol with other chemotherapeutic agents *in vitro* cancer models has reduced drug resistance and made tumor cells susceptible to drugs [158]. The combination of resveratrol and cisplatin triggered synergistically autophagy-related apoptosis in A549 cells [159]. In another study, the combination of resveratrol and 5-fluorouracil inhibited STAT3 and AKT signaling pathways and led to S phase arrest in colorectal cancer cells. In addition, this combination therapy prevented EMT [160]. Resveratrol and rapamycin which inhibits rapamycin related mTOR/AKT activation resulted in cell death in bladder cancer. Therefore, these data suggested that resveratrol and rapamycin combination might be promising treatment approach [161]. In one study, resveratrol in combination with cisplatin increased the DUSP1 (Dual specifity phosphatse 1) expression which is associated with NF-κB pathway and Cox-2 inhibition in prostate cancer cell line

[162]. Moreover, conjugated nanoparticles including resveratrol and docetaxel together caused cell death by downregulating anti-apoptotic proteins and reversed MDR in prostate cancer cells [163].

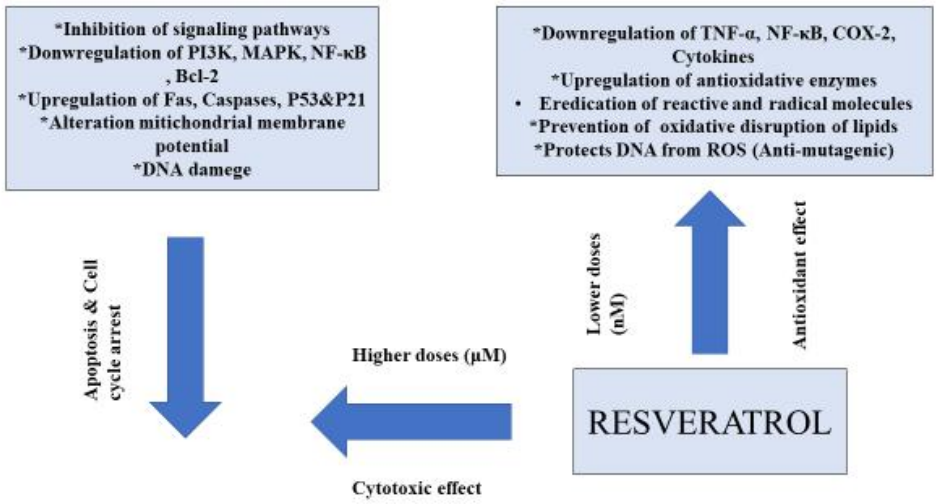


Figure 1.5.1 Molecular effects of resveratrol based on its concentrations. Resveratrol has been shown to possess different effects on the cell based on its concentrations. Higher concentration generally have apoptotic and antiproliferative effects while lower concentrations have antioxidative effects [164].

1.5.1 Effect of Resveratrol on Leukemia

The therapeutic potential of resveratrol and its mechanisms of action have been also investigated on different types of hematological cancer despite less studies are present in the literature as compared to solid tumors. Resveratrol-mediated cell death was found to be related to the proteolytic cleavage of caspase substrate poly (ADP-ribose) polymerase (PARP) and CD95 signaling in HL60 AML cells [165]. Moreover, resveratrol in HL60 cells induced cell death in a dose dependent manner by release of cytochrome c from the mitochondria followed by caspase-9 and caspase-3 activation [166]. Resveratrol decreased cell viability, suppressed DNA synthesis and reduced anti-apoptotic Bcl-2 protein expression in HL60 AML cells, which resulted in

growth inhibition and suppression of the cell cycle [167]. Resveratrol also initiated apoptosis via FasL-associated ASK1 / JNK signaling in HL-60 AML cells [168]. It was found that resveratrol caused PARP and caspase-3 cleavage which is responsible for apoptotic cell death in OCI/AML3 AML cells and it arrested the cells at the S phase [169]. Resveratrol, reduced phosphorylated STAT3 levels which resulted in the regulation of Bcl-2 and Bax protein levels in Kasumi-1 AML and SUP-B15 ALL cell lines [170]. Lower doses of resveratrol caused erythroid differentiation in AML cells while increasing doses of resveratrol decreased the expression of genes that allow differentiation [171].

Resveratrol induced S phase arrest in T-ALL cells and Fas or FasL blocking did not affect resveratrol-induced death. In addition, the use of caspase family inhibitors did not alter resveratrol induced death [172]. Resveratrol induced irreversible growth inhibition in ALL cells by causing DNA fragmentation and G1 arrest [173]. The mechanism of cell death triggered by resveratrol in ALL cells was related to activation of caspases and independent of Fas [174]. Resveratrol initiated apoptosis effectively by altering mitochondrial membrane potential in T-ALL cells [175]. Cell proliferation has been suppressed in resveratrol treated T-ALL cells through PARP and caspase-3 cleavage [176]. Resveratrol decreased miRNA expression such as miR196b and miR-1290 in SUP-B15 Ph + ALL cells, which caused growth and migration inhibition [177]. Resveratrol not only stimulated apoptosis but also stimulated autophagy, which was related to Akt / mTOR inhibition and p38-MAPK activation in T-ALL cells [178].

Resveratrol also induced apoptosis in CML cells in a caspase-dependent manner and also triggered erythroid differentiation in imatinib-sensitive and resistant CML cells [179]. Resveratrol treatment resulted in apoptosis and an increase in the amount of cell in the S phase in K562 CML cells [173]. Resveratrol induced apoptosis in K562 cells by decreasing the expression of genes such as *Bcl-xL*, *Bcl-2*, *Cyclin D1*, *Mcl-1* and STAT5. Resveratrol also induced ER stress in these cells, initiated cell cycle arrest and apoptosis [180]. Furthermore, resveratrol inhibited PI3K, Akt, mTOR

phosphorylation, then caspase-3 was activated [181]. Interestingly, autophagic markers were modulated at some doses of resveratrol and an increase was detected in the expression of LC3B-II, which is an autophagic marker in AML cells [171].

The therapeutic effect of resveratrol on various chronic lymphocytic leukemia (CLL) cells was demonstrated by the suppression of cell cycle at G2 / M phase and stimulation of apoptosis [182].

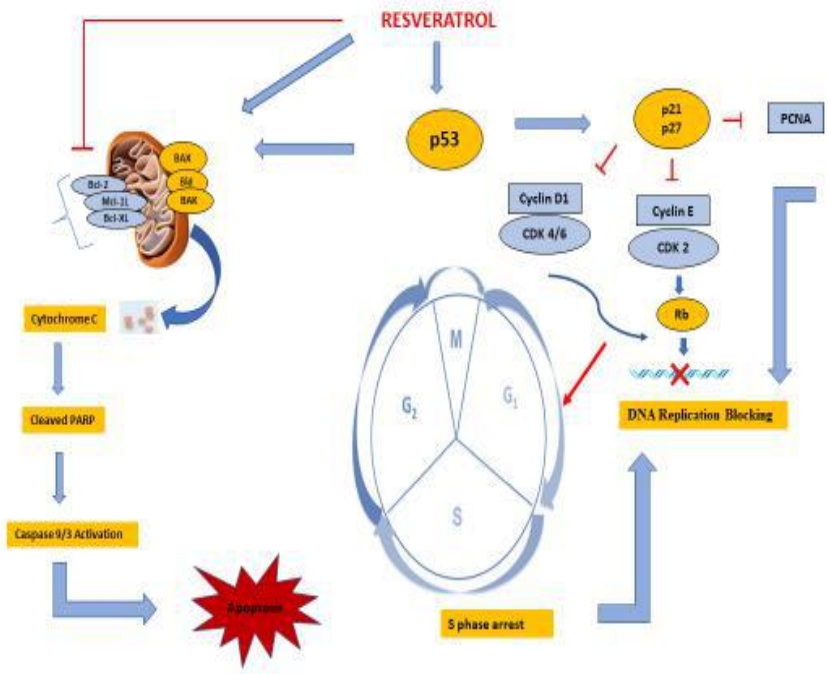


Figure 1.5.1.1 The therapeutic effect of resveratrol on cell cycle and apoptosis has been demonstrated in solid cancers and leukemia. Yellow colors are negative regulators of apoptosis and cell cycle, while blue colors are positive regulators of apoptosis and cell cycle [183].

1.5.2 Resveratrol Targets Sphingolipid Metabolism

It has been shown that resveratrol alters sphingolipid metabolism as a mechanism of its therapeutic features. The accumulation of ceramide in the cell results from various stresses such as chemotherapy and initiates cellular apoptosis. Resveratrol regulated ceramide synthesis by upregulating SPT, which is responsible for *de novo* ceramide synthesis in prostate cancer [184] Resveratrol increased the amount of ceramide in MCF-7 breast cancer cells and

myeloid leukemia cells by downregulating the enzyme SK-1 [185] Resveratrol induced the production of *de novo* ceramide in K562 cells and reduced anti-apoptotic SK-1 and GCS expression [186]. Resveratrol stimulated not only apoptosis but also autophagy by regulating sphingolipid metabolism in human gastric cancer cells. The increase in the amount of ceramide could be also related to increased expression of sphinomyelinases [187]. In a study, resveratrol increased the amount of ceramide and dihydroceramide resulting in cell death in gastric cancer cells [188]. The inhibition of SK-1 and GCS by using pharmacological inhibitors, PDMP and SKI II, respectively together with increasing doses of resveratrol induced synergistic cytotoxic effect and an increase in the amount of ceramide in K562 CML cells [186].

Chapter 2

2. Material and Method

2.1 Chemicals

Stock solutions for resveratrol (Sigma Aldrich), SPT inhibitor (Myriocin, Cayman Chemical), SK inhibitor (SKI II, Cayman Chemical) and GCS inhibitor (PDMP, Cayman Chemical) were prepared in dimethylsulfoxide (DMSO) as specified by the manufacturer and stored at -20ºC. The defined working concentrations were prepared from the main stock and /or intermediate stocks when necessary.

2.2 Cell Lines and Culture Conditions

Two different Ph + ALL (expressing BCR-ABL) cell lines (SD-1 and SUP-B15) were used in the study. These cells were obtained from DSMZ (German Collection of Microorganisms and Cell cultures). Cells were grown in RPMI 1640 (+ L-glutamine, GibcoTM) medium containing 10-20% fetal calf serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2 (carbon dioxide) incubator at 37ºC and medium is changed every 2-3 days.

2.3 MTT Test

MTT cell proliferation test was used to determine the cytotoxic effects of resveratrol, SPT, SK and GCS inhibitors on SD-1 and SUP-B15 cells [189, 190]. In addition, growth suppressive effects of resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor combinations on SD-1 and SUP-B15 cells were determined by MTT test. For this purpose, the cells were seeded into 96-well plates (10.000 SD-1 cells/well and 20.000 SUP-B15 cells/well) after the cell viability was checked with trypan blue staining and the cells were counted using thoma slides. Increasing concentrations of resveratrol

and each inhibtitor were added to the wells and incubated for 48 and 72 hours. The concentrations of resveratrol, SK (SKI II), GCS (PDMP) and SPT (Myriocin) inhibitors used for SD-1 and SUP-B15 cells were as 10-, 20-, 40-, 60-, 80-, 100 uM; 0.25-, 0.5-, 1-, 2.5-, 5-, 10-, 20 uM; 0.25-, 0.5-, 1-, 2.5-, 5-, 10-, 20-, 40 µM and 1-, 5-, 10-, 20-, 40-, 80-, 100 nM, respectively. At the end of the incubation, 20 µl MTT reagent (5mg/ mL, Sigma Aldrich) was added to each well and incubated for 4 hours at 37ºC in 5% CO_2 incubator. The 96-well plates were centrifuged at 1800 rpm for 10 min and the obtained formazan crystals were dissolved in 100 µl DMSO. The absorbance values were read on the spectrophotometer at 570 nm. Cell proliferation graph was drawn based on the spectrophotometric results, and IC_{50} (concentration inhibiting cell growth by 50%) for resveratrol and $IC_{10\text{-}20}$ (concentration inhibiting cell growth by 10-20%) values for PDMP and SK inhibitors were calculated. It has been shown in the literature that $IC_{10\text{-}20}$ concentrations are sufficient to inhibit these enzymes and provide ceramide accumulation [117].

For combination studies (increasing concentrations of resveratrol + $IC_{10\text{-}20}$ concentration of PDMP or SK inhibitors and the highest concentration of SPT inhibitor) were used. 10-, 20-, 40 µM resveratrol were administered to SD-1 cells together with 2.5 µM SKI II, 10 µM PDMP and 100 nM myriocin. In SUP-B15 cells, resveratrol (5- and 10 µM) were applied in combination with 1.0 µM SKI II, 1.0 µM PDMP and 100 nM myriocin.

2.4 Determination of Apoptosis by Annexin-V / Propidium Iodide Dual Staining with Flow Cytometry

The amount and localization of phosphatidyl serine (PS) in resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor treated SD-1 and SUP-B15 cells was determined by flow cytometry using AnnexinV-Propidium Iodide (PI) (BioVision, Inc.) dual staining [189, 190]. $1x10^6$ /2ml cells were incubated for

48 hours with the defined concentrations of resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK-1 inhibitor and resveratrol: GCS inhibitor combinations. At the end of incubation, the cells were centrifuged at 1800 rpm for 10 min and the cells were washed 2 times with cold 1X PBS. Then, 200 µl of annexin binding solution was added and homogenized. 2 µl of Annexin V and 2 µl of propidium iodide were added to the obtained cell suspension and incubated for 15 minutes at room temperature in the dark. Measurements were then performed by flow cytometry (BD). Resveratrol (10-, 20-, 40 µM), SKI II (1-, 2.5- and 5 µM), PDMP (10-, 20-, 40 µM), myriocin (40-, 80-, 100 NM) concentrations and the combination of 10-, 20-, 40 µM resveratrol with 2.5 µM SKI II, 10 µM PDMP and 100 nM myriocin for SD-1 cells were selected as indicated and annexinV-PI staining was performed. In SUP-B15 cells, resveratrol (5-, 10 µM) were administered together with 1 µM SKI II, 1 µM PDMP, 100 nM Myriocin.

2.4.1 Cell Cycle Analysis

Cell cycle analysis was performed using flow cytometry in SD-1 and SUP-B15 cells treated with resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor combinations [190]. $1x10^6$ / 2ml cells were harvested by centrifugation at 260 g for 10 min after incubation with the indicated resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor combinations for 48 hours. Cell pellets were washed twice with 1 ml cold PBS and 4 ml cold ethanol was added samples. Cells were incubated for at least 24 hours in -20 ° C. The cell pellet obtained by centrifugation at 260 g for 10 minutes was homogenized in 5 ml cold PBS and centrifuged. The pellet was dissolved in 1 ml PBS-Triton X100 and 100 µl RNase-A (200 µg / ml, Sigma Aldrich) and incubated at 37 ° C for 30 min. 100 µl propidium iodide (1 mg / ml, Sigma Aldrich) was then added and incubated at room temperature for 10-15 minutes. Cell cycle analysis was performed by flow cytometry. Resveratrol (20 µM), SKI II (2.5 µM), PDMP (10 µM), myriocin

(100 nM) and 20 μM resveratrol together with 2.5 μM SKI II, 10 μM PDMP and 100 nM myriocin for SD-1 cells were used for cell cycle analysis. In SUP-B15 cells, increasing doses of resveratrol (5-, 10 μM) were combined with 1 μM SKI II, 1 μM PDMP and 100 nM Myriocin.

2.5.1 Western Blot Analysis

In resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor treated SD-1 and SUP-B15 cells, the apoptosis molecular markers, caspase-3 and PARP cleavages, were detected by western blot (Baran et al., 2007). $4x10^6$ SD1 cells were treated with 20- and 40 μM resveratrol, 2.5 μM SKI II, 10 μM PDMP, 100 nM myriocin, 20 μM resveratrol + 2.5 μM SKI II, 40 μM resveratrol + 10 μM PDMP, 20 μM resveratrol +100 nM myriocin. $4x10^6$ SUP-B15 cells were treated with 5- and 10 μM resveratrol, 1 μM SKI II, 1 μM PDMP, 100 nM myriocin, 10 μM resveratrol + 1 μM SKI II, 10 μM resveratrol + 1 μM PDMP, 10 μM resveratrol +100 nM myriocin. After 48 h incubation, total protein isolation was performed from the cells using RIPA buffer (Sigma Aldrich) containing proteinase inhibitor and total protein concentrations (mg/ml) were calculated using the RC DCTM Protein Assay (BioRad) kit.

2.5.2 The Protein Isolation Protocol with RIPA Buffer

Cells were treated with resveratrol and resveratrol: inhibitor concentrations at the specified concentrations for 48 hours.

• Cells were collected in 15 ml tubes and centrifuged at 4ºC, 4000 rpm for 5 minutes.

• The supernatant removed after centrifugation. Cells were washed with 1X cold PBS followed by centrifugation at 4ºC, 4000 rpm for 5 min. This procedure was repeated twice.

• After the second wash, 150 μl cold RIPA buffer was added to the pellet. Then, lysis was performed by 1 ml syringes (10-15 times).

• The lysed cells were then left on ice for 25-30 minutes. The cells in RIPA buffer were centrifuged at 4ºC, 1200 rpm for 15 minutes and the isolated proteins were stored at -20 or -80ºC.

2.5.2.1 Preparation of Gamma Globulin Standard

• The RC DCTM Protein Assay (Bio-Rad) kit was used for protein quantification as recommended by the manufacturer.

• Gamma globulin was dissolved in 20 ml of ultra-pure water and a primary stock solution (1.56 mg /ml) was obtained. Our standards are prepared as 0.2 mg / ml - 1.4 mg /ml (Table 2.5.2.1.1)

Globulin (mg/ml)	Main Stock to Add (µl)	RIPA Buffer to Add (µl)	Total Volume (µl)
1,4	89,74	10,26	100
1,2	76,92	23,08	100
1	64,1	35,9	100
0,8	51,28	48,72	100
0,6	38,46	61,54	100
0,4	25,64	74,36	100
0,2	12,82	87,18	100

Table 2.5.2.1.1 Gamma Globulin Standard Preparation

2.5.2.2 Determination of Protein Quantity

• Solution A was obtained by mixing 20 µl of solution S and 1 ml of solution A.

• 5 µl of each of the prepared standards and samples were added to each well and 25 µl of solution A was added to each well.

• Then 200 µl of solution B was added to each well and allowed to incubate for 15 minutes at room temperature.

• At the end of the incubation, absorbance measurement was made at 750 nm and protein concentrations were calculated for each sample.

2.6 Western Blot Protocol

• For each sample, 30 µg /40 µl protein was denatured by boiling at 95˚C for 5 min in 2X laemmli buffer (Santa Cruz Biotechnology) and loaded into the gel wells which were prepared based on the molecular weight of the target proteins. PageRuler ™ Prestained Protein Ladder (Thermo ScientificTM) was used to determine the molecular weight of the proteins.

• The gel was run for 80 minutes at 100 V.

2.6.1 PVDF Membrane Transfer Process

• PVDF membrane was activated in methanol for 10 minutes. The filter papers and gel were soaked in transfer buffer for 10 min.

• The gel is placed between the PVDF membrane and filter papers and placed in the Trans-Turbo (Bio-Rad) device and the transfer is performed at 25V, 1A, 30 min. Ponceau S staining (Sigma Aldrich) was performed in order to determine the success of transfer.

2.6.2 Blocking, Primary and Secondary Antibodies Treatment and Imaging

Following transfer, the membrane was blocked in 1X TBST (Tris-buffered saline, 0.1% Tween 20) containing 5% skimmed milk powder for 1 hour at room temperature.

• After blocking, the membrane was washed 3 times with 1X TBST for 10 minutes.

• Caspase-3 (Cell Signaling), PARP (Cell Signaling), BCR-ABL (Cell Signaling), SK (Cell Signaling) and Beta Actin (Cell Signaling) antibodies were prepared in 1X TBST containing 5% skimmed milk powder at a dilution of 1: 3000. SPT (Novus Biologicals) and GCS (Novus Biologicals) antibodies were diluted as 1:1000. The membrane was shaken overnight at + 4 ° C in these primary antibody solutions.

• After washing three times with 1X TBST for 10 minutes, secondary antibodies (1:10000, Jackson Immuno Research) in 1X TBST containing 5% skimmed milk powder were added onto the membrane and shaken for 1 hour in the dark at room temperature.

• Membrane was washed three times with 1X TBST for 10 min and images taken using the Pierce ™ ECL Western Blotting Substrate kit (Thermo Scientific^{TM}). Densitometric analysis of the immunoreactive bands were analyzed using the imaging software (Bio-Rad, ChemiDoc, Image LabTM 3.0).

2.7 Cytochrome-c Release

The release of cytochrome c, which is localized between the mitochondria inner membrane and outer membrane, is one of the important markers of apoptosis. Cytochrome-c in the cytosol activates caspase-9 by forming complexes with Apaf-1. Activated caspase-9 is responsible for the activation of caspase-3. In order to detect cytochrome-c release, mitochondria were separated from the cytosol after SD-1 and SUP-B15 cells were treated with resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor combinations by using cytochrome-c release apoptosis kit (BioVision, Inc.). Western blot was performed using cytochrome-c specific antibodies included in the kit to determine the amount of cytochrome-c in the cytosol. 10^7/10ml SD-1 and SUP-B15 cells were treated at the specified concentrations for 48 hours and the mitochondria were enriched from the cytosol by following the protocol as recommended (Concentrations for SD1 cell were as 20- and 40 μM resveratrol, 2.5 μM SKI II, 10 μM PDMP, 100 nM myriocin, 20 μM resveratrol + 2.5 μM SKI II, 40 μM resveratrol + 10 μM PDMP, 20 μM resveratrol +100 nM myriocin. Concentrations for SUP-B15 cell were as 5- and 10 μM resveratrol, 1 μM SKI II, 1 μM PDMP, 100 nM myriocin, 10 μM resveratrol + 1 μM SKI II, 10 μM resveratrol + 1 μM PDMP, 10 μM resveratrol +100 nM myriocin). 15μg / 40 μl protein from both cytosolic and mitochondrial fractions were used to detect cytochrome-c release with anti-cytochrome-c (1: 100) antibody by western blot method.

2.8 Statistical Analysis

The experiments were performed in three independent setups and the results are given as mean ± standard error. Statistical analysis were performed using GraphPad Prism 6.0 program. One-way ANOVA (one way analysis of variance) was used for the analysis of MTT and two-way ANOVA was used for cell cycle analysis and anexin-V /PI analysis. $P < 0.05$ was considered as statistically significant.

Chapter 3

3. Results and Discussion

3.1 Resveratrol, Ceramide Metabolism Inhibitors and The Combination of Resveratrol with Inhibitors Have Antiproliferative Effects on Ph + ALL SUP-B15 and SD-1 Cells

To determine the antiproliferative effects of resveratrol, SPT inhibitor (myriocin), SK inhibitor (SKI II) and GCS inhibitor (PDMP), SD1 ($1x10^4$ /well) and SUPB15 ($2x10^4$/well) cells were seeded and incubated with increasing concentrations of resveratrol (10-100 μM), SKI II (0.25-20 μM), PDMP (0.25-40 μM) and myriocin (1-100 nM), respectively for 48 and 72 hours. MTT test was performed at the end of incubation. According to cell proliferation graphs, resveratrol, SKI II and PDMP have cytotoxic effects on the cells in a time and concentration-dependent manner. Using cell proliferation data, IC_{50} concentrations were calculated for SD1 cells as 43- and 37 μM (Resveratrol), 6.6- and 4.5 μM (SKI II), 36- and 25 μM (PDMP) for 48 and 72 hours, respectively. Similarly, IC_{50} concentrations for SUP-B15 cells were calculated as 37- and 20 μM (Resveratrol), 4.5- and 2.3 μM (SKI II), 32- and 18 μM (PDMP) for 48 and 72 hours, respectively. On the other hand, myriocin did not affect cell viability significantly (**Figure 3.1.1 and Figure 3.1.2)**

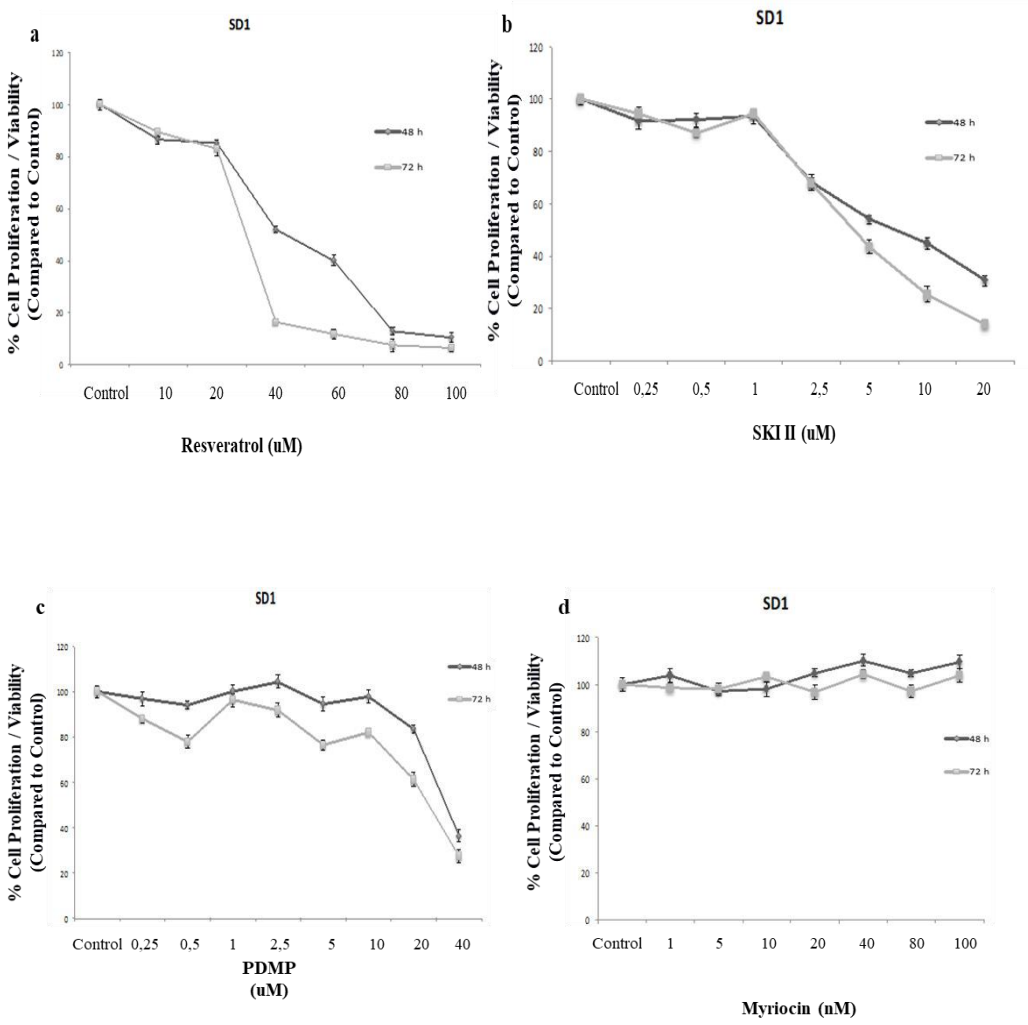


Figure 3.1.1 Cytotoxic effects of resveratrol, SKI II, PDMP and Myriocin on SD1 cells in a time and concentration-dependent manner. The results derived from the means of three independent experiments are represented as mean± SE

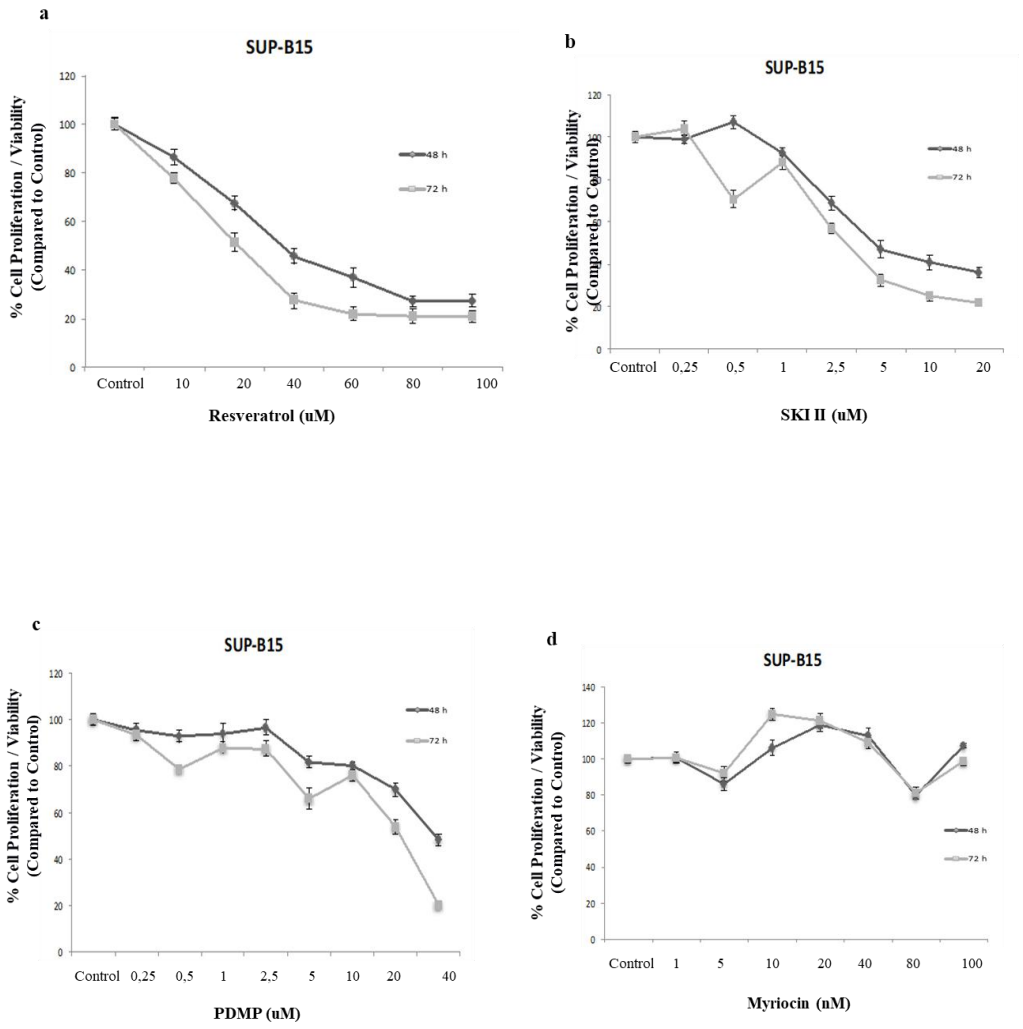


Figure 3.1.2 Cytotoxic effects of resveratrol, SKI II, PDMP and Myriocin on SUP-B15 cells in a time and concentration-dependent manner. The results derived from the means of three independent experiments are represented as mean± SE

It has been known that resveratrol has antiproliferative effects on many solid cancer and leukemia types which occur through different mechanisms depending on the cell type [191]. In many cancer types, *in vitro* effective concentration of resveratrol were different based on the cell type. There are many studies showing resveratrols concentrations below and/ or above 40 µM just as found in our study (IC_{50} values were 43 µM for SD1 cell (Figure 3.1.1.a) and 37 µM for SUP-B15 (Figure 3.1.2.a). For instance, the antiproliferative effect of resveratrol in acute promyelocytic leukemia (APL) was investigated between 12.5-100 µM. IC_{50} was found to be around 30 µM after 48 hours

treatment and 50 μM resveratrol was shown to induce apoptosis [192]. In another study, it was shown that cell proliferation was suppressed in K562 CML cells treated with 50- and 100 μM resveratrol for 24 hours [193]. Therefore, concentrations at which resveratrol inhibited cell proliferation in Ph + ALL cells are consistent with the literature. Similarly, the concentrations of inhibitors targeting the key enzymes of sphingolipid metabolism for SD1 and SUP-B15 cells are consistent with the literature. The effects of SK inhibitor and GCS inhibitor (PDMP) on the proliferation of K562 CML and HL60 APL cells have been studied in the range of 1-100 μM [186] and their cytotoxic effects on cell proliferation has been investigated.

After determining individual effects of each agent, combination studies were performed to figure out whether ceramide, which is known to be apoptotic molecule and expected to increase in the cell especially after inhibiting SK and GCS enzymes, could increase the cytotoxic effect of resveratrol or not. For this purpose, SD1 cells were treated with increasing concentrations of resveratrol (10, 20, 40 μM) in combination with 2.5 μM SKI II, 10 μM PDMP and 100 nM myriocin to determine the synergistic antiproliferative effects. The combination of increasing concentrations of resveratrol (10-, 20-, 40 μM) with 2.5 μM SKI II decreased the cell growth as 73%, 54% and 37%, respectively. The effect of resveratrol on cell proliferation at these concentrations were 88%, 80% and 47%, respectively. Based on these results, the combination of 10-, 20- and 40 μM resveratrol with 2.5 μM SKI II have a synergistic inhibitory effect on cell proliferation. Similarly, increasing concentrations of resveratrol (10-, 20-, 40 μM) combined with 10 μM PDMP showed that cell proliferation decreased in all combinations (cell viability decreased as 80%, 63% and 37% for combinations, respectively). On the other hand, increasing concentrations of resveratrol (10-, 20-, 40 μM) with 100 nM myriocin decreased the cell growth as 70%, 49% and 30%, respectively. (**Figure 3.1.3)**

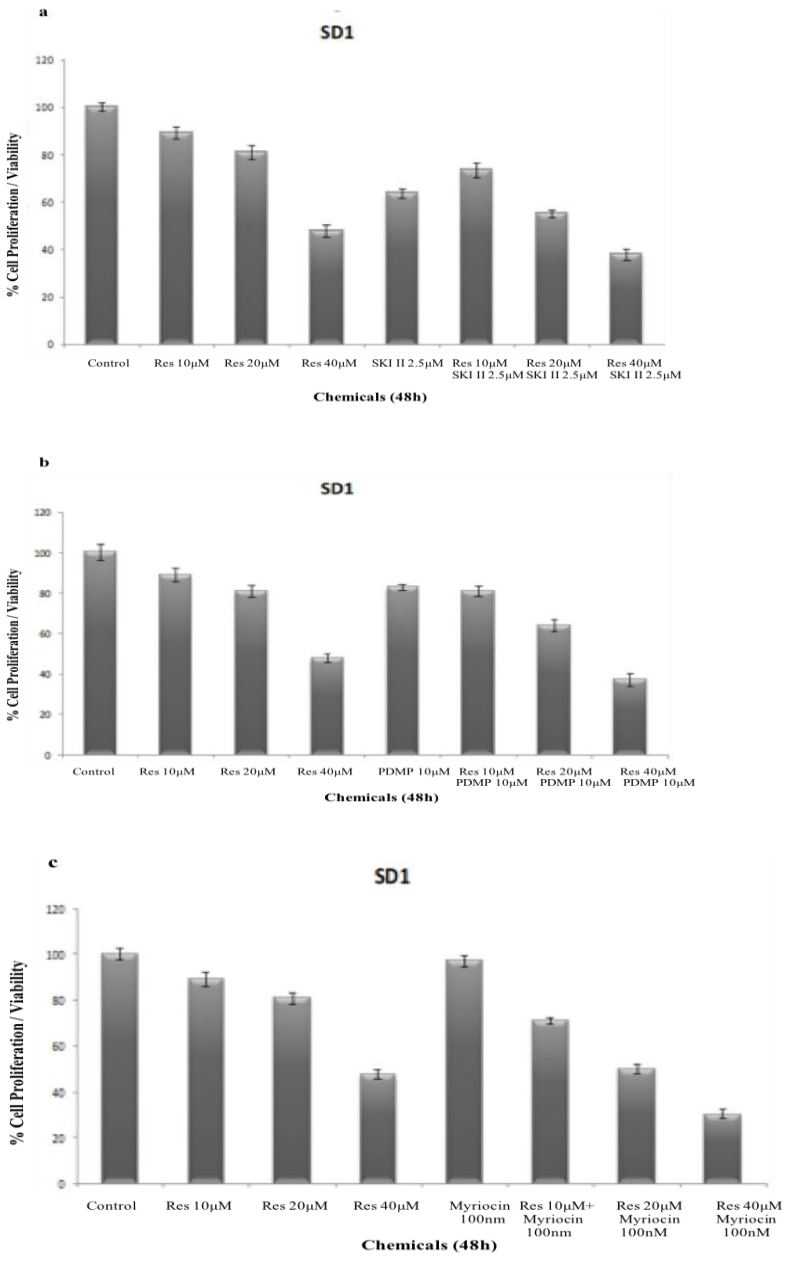


Figure 3.1.3 Synergistic effects of resveratrol in combination with SKI II, PDMP and Myriocin on SD1 cells. The results derived from the means of three independent experiments are represented as mean± SE

Similarly, SUP-B15 cells were treated with increasing concentrations of resveratrol (5 and 10 μM) in combination with 1.0 μM SKI II, 1 μM PDMP and 100 nM Myriocin. The combination of increasing concentrations of resveratrol (5- and 10 μM) with 1.0 μM SKI II decreased the cell growth as 92% and 70%, respectively. The effect of resveratrol on cell proliferation alone at these doses were 99% and 93%, respectively. Proliferation of cells was found to be 81% after treatment with 1.0 μM SKI II. Therefore, the combination of 10 μM resveratrol with 1.0 μM SKI II was the most effective combination on cell proliferation. Similarly, increasing concentrations of resveratrol (5 and 10 μM) combined with 1 μM PDMP showed that cell proliferation decreased effectively only at 10 μM resveratrol + 1 μM PDMP combination. On the other hand, increasing concentrations of resveratrol (5 and 10 μM) with 100 nM myriocin did not affect the cell proliferation as compared to chosen resveratrol concentrations (**Figure 3.1.4)**.

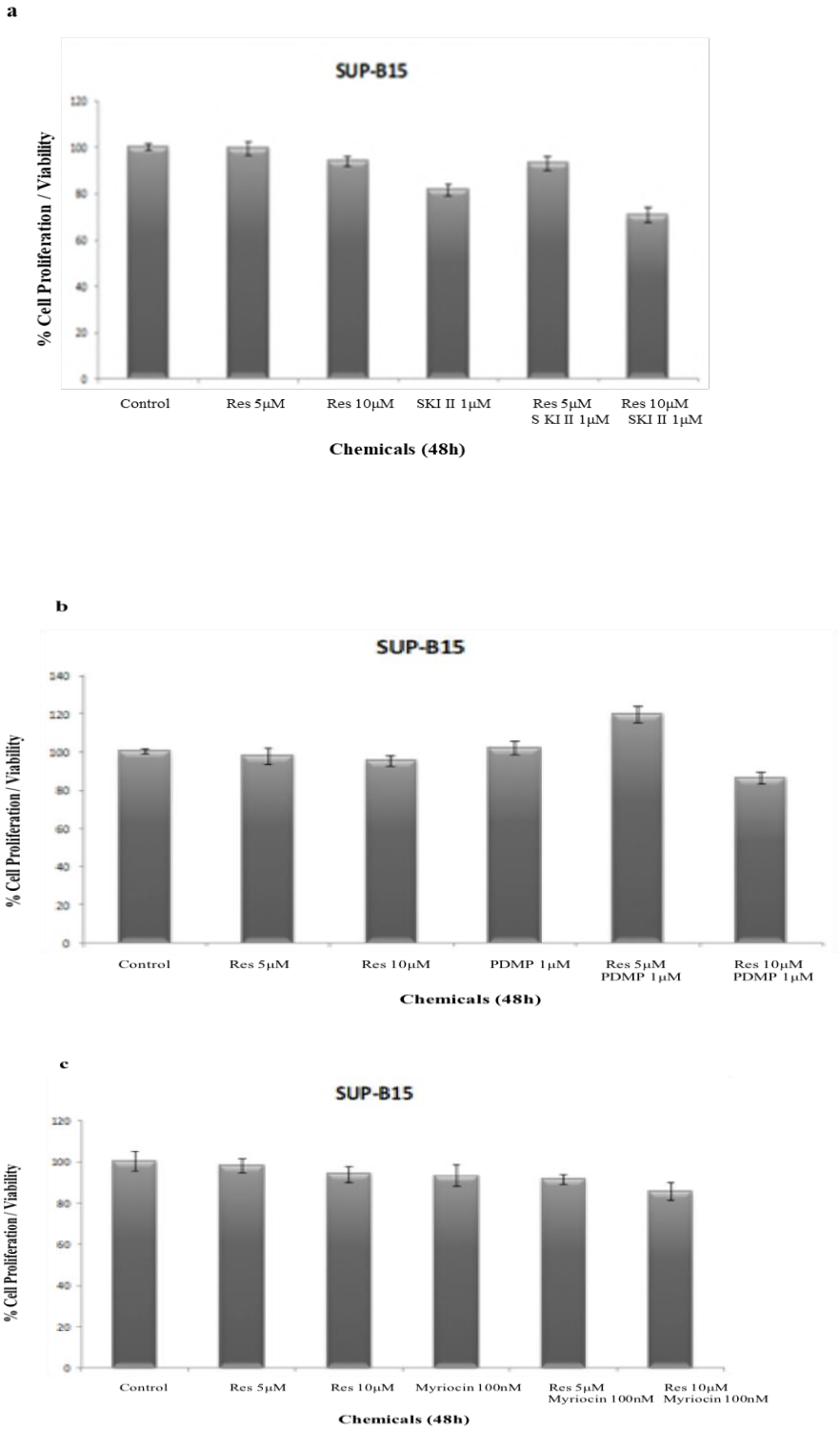


Figure 3.1.4 Synergistic effects of resveratrol in combination with SKI II, PDMP and Myriocin on SUP-B15 cells. The results derived from the means of three independent experiments are represented as mean± SE

In recent years, many studies have shown that one of the mechanisms underlying the therapeutic potential of resveratrol is related to modulation of sphingolipid metabolism. Resveratrol has been shown to induce ceramide-mediated apoptosis by increasing the amount of intracellular ceramide in breast cancer cells [194]. The apoptotic effects of resveratrol on metastatic breast cancer and colon cancer cells have been shown to be mediated by the activation of *de novo* ceramide pathway and this effect is reversed by inhibiting SPT [195, 196]. However, only a few studies have investigated the mechanisms of action of resveratrol in relation to sphingolipid metabolism in leukemia types. In one of these studies, it was found that resveratrol increased the expression of CerS genes while decreased SK-1 and GCS expression in HL60 APL and K562 KML cells [186]. In addition, the mechanisms of action of resveratrol are often investigated in combination strategies in many cancer tyeps including leukemias. For instance, the combinations of resveratrol with PDMP and SK-1 inhibitor have been found to have a synergistic antiproliferative effect in both APL and CML [186]. Similarly, defined concentrations of resveratrol combined with SK and GCS inhibitors have been shown to suppress cell proliferation in SD1 and SUP-B15 cells (Figures 3.1.3.a and b, Figure 3.1.4.a and b). Ceramide has antiproliferative and apoptotic- properties whereas S1P and GCS produced by SK and GCS promote cell proliferation and inhibit apoptosis [197]. Based on the combination results, it is concluded that SK and GCS enzyme inhibitors together with resveratrol inhibited the conversion of ceramide to S1P and GC, thereby increasing intracellular ceramide and suppressing cell proliferation. Myriocin reduces the amount of apoptotic ceramide by inhibiting SPT, which is one of the key enzymes in *de novo* ceramide synthesis [198] Based on the cell proliferation graphs (Figure 3.1.2.d and Figure 3.1.3.d), the proliferation of the cells is not affected significantly or very small effect is observed in the presence of myriocin. However, myriocin given with certain concentrations of resveratrol suppressed cell proliferation especially in SD1 cells (Figure 3.1.3.c). In SUP-B15 cells, cell proliferation is reduced by 10% when 10μM resveratrol is combined with 100 nM myriocin (Figure 3.1.4.c). This result suggests that

myriocin inhibits cell growth by acting through a different mechanism in addition to altering the amount of ceramide in the cell when co-administered with resveratrol. For instance, myriocin has been shown to inhibit cell growth in a dose-dependent manner by activating cell death receptors in lung cancer cells. The combination of myriocin with chemotherapy drugs (cisplatin and docetaxel) synergistically inhibited cell growth [199]. Cell cycle and apoptosis analysis were performed to understand the mechanism of the discussed effects of resveratrol and its combinations on cell proliferation.

3.2 Resveratrol in Combination with SKI II, PDMP and Myriocin Affect the Cell Cycle Progression of SD1 and SUP-B15 Ph + ALL Cells

To understand the potential mechanisms behind the growth suppressive effects of resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor combinations on SD1 and SUP-B15 cells, the distributions of the cells in the cell cycle phases were determined by flow cytometry via Propidium Iodide (PI) staining. 20 μM resveratrol was combined with 2.5 μM SKI II, 10 μM PDMP and 100 nM Myriocin for SD1 cells. Treatment of SD1 cells with 20 μM resveratrol resulted in decreases in cell population at G0 / G1 phase whereas led to increases at S phase as compared to control cells. The percentage of cell population at the G0/ G1 phase was 57.03% and 33.9% while it was 5.7% and 6.5% at the S phase for control and 20 μM resveratrol, respectively. 2.5 μM SKI II treatment reduced the percentage of cells at G0 /G1 phase (36.1%) as compared to the control (57.3%) while increased it at S (7.5%) and very slightly at G2 /M (7.9%) (Control: S: 5.7% and G2 / M: 7.6%). The percentage of cell population at G0 /G1 phase and S phase were as 33.6% and 10% after 20 μM resveratrol and 2.5 μM SKI II treatment and S phase population was found to be increased as compared to 20 μM resveratrol. Thus, the combination treatment led to cell cycle arrest at S phase. This result supported the data shown and described in

Figure 3.1.3.a. Similarly, 20 μM resveratrol + 10 μM PDMP combination arrested the cells at S phase. The percentage of cell population for this combination was 13.7% (Control: 5.7%, 20 μM resveratrol: 6.5%, 10 μM PDMP: 6.8%) (Figure 3.2.1.b). Thus, there is an arrest in the S phase of the cell cycle as a result of the combination treatment. These results support cell proliferation data (Figure 3.1.3.b) and, the distribution of SD1 cells treated with 20 μM resveratrol at G0 / G1, S and G2 / M phases was 33.9%, 6.5%, and 3.1% while it was 57.03%, 5.7% and 7.6% for control cells, respectively. As a result of 100 nM myriocin treatment, the distribution of cells in G0 / G1, S and G2 / M phases was 70%, 6.5% and 3.2%, respectively. 100 nM myriocin treatment resulted in the distribution of cells at G0 / G1, S and G2 / M phases as 70%, 6.5% and 3.2%, respectively. The distribution of cells at G0 / G1, S and G2 / M phases were 46%, 9.3%, and 4.7%, respectively after 20 μM resveratrol + 100 nM myriocin treatment. Based on these results, 20 μM resveratrol + 100 nM myriocin was found to be effective at S phase.

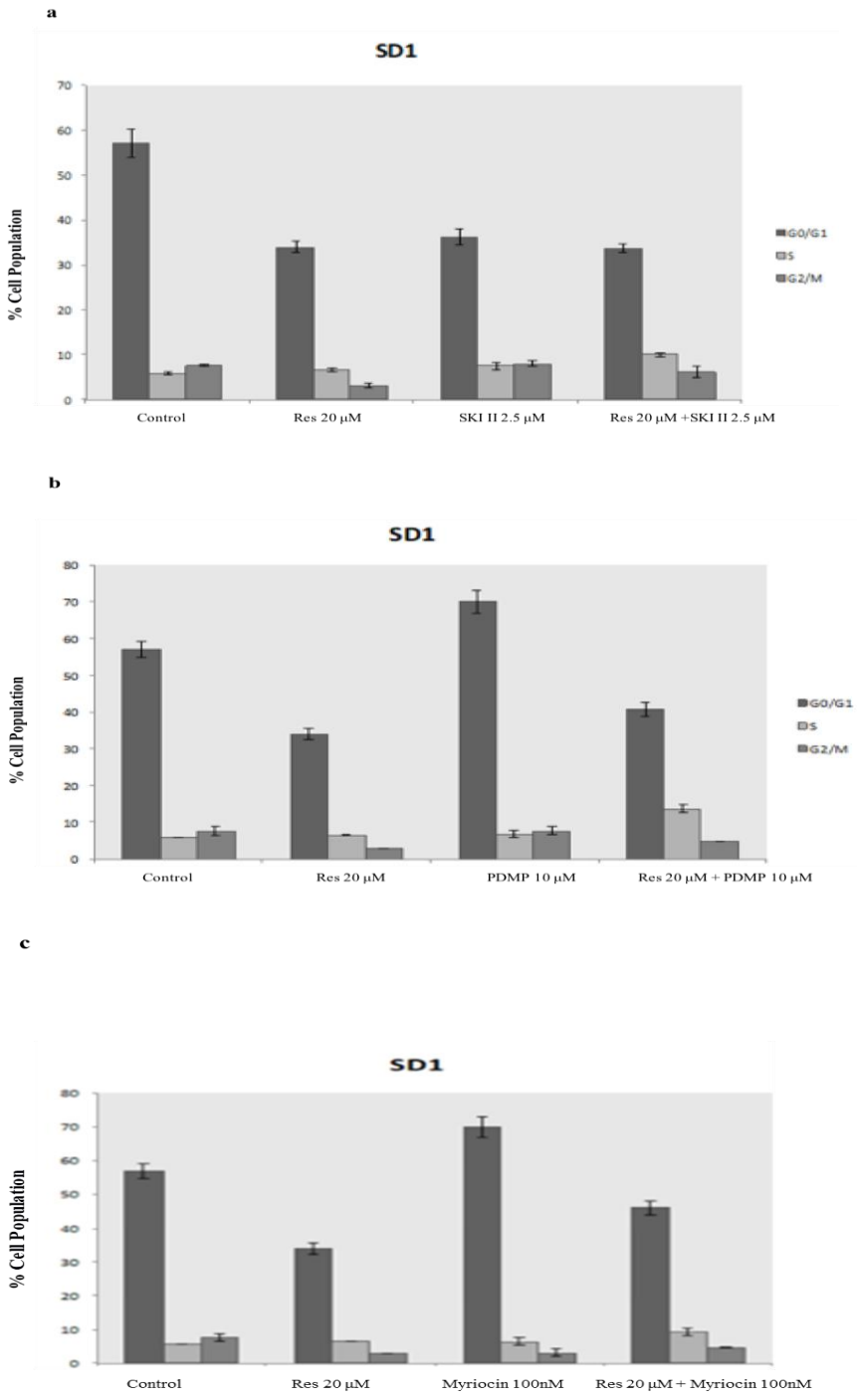


Figure 3.2.1. Cell cycle distrubution of SD1 cells after treatment with Resveratrol, SKI II, PDMP, Myriocin, Resveratrol: SK-1 Inhibitor (a), Resveratrol: GCS inhibitor (b) and Resveratrol: SPT Inhibitor (c) combinations. The results derived from the means of three independent experiments are represented as mean± SE

Treatment of SUP-B15 cells with 5- and 10 μM resveratrol, 1 μM SKI II resulted in decreases in cell population at G0 / G1 phase as compared to control cells, while it increased the cell population at S phase. Distribution of the cells were 53.95%, 45.9% and 42.4% at G0 / G1 phase while S phase cell population was 15.4%, 16.35% and 17.35% for control, 5- and 10 μM resveratrol, respectively (Figure 3.2.2.a). 1 μM SKI II decreased the percentage of cells at G0 / G1 phase (43.8%) while increasing at S (17%) and G2 / M (19.3%) phases compared to the control (G0/G1: 53.95%, S: 15.4% and G2 / M: 17%). 5- and 10 μM resveratrol in combination with 1 μM SKI II synergistically led to decreases at G0 / G1 phase as 41% and 27.3%, respectively (as compared to defined concentrations of resveratrol). Cell populations at G2 / M phase were found to be 17.9% and 3.5% for the combination of 5- and 10 μM resveratrol with 1 μM SKI II, respectively. (G2 / M phase cell population were 19.6% and 17.55% for 5- and 10 μM resveratrol, respectively). On the other hand, 5- and 10 μM resveratrol with 1 μM SKI II arrested the cells at S phase (20.3% and 27%, respectively) compared to defined concentrations of resveratrol. Thus, while combination treatments reduced the percentages of SUP-B15 cells at G0 / G1 and G2 / M phases, the cells were arrested at S phase and the most effective combination was found to be 10 μM resveratrol + 1 μM SKI II (Figure 3.2.2.a). This result was in accordance with the result shown and described in Figure 3.1.4.a. 5- and 10 μM resveratrol + 1 μM PDMP treatment decreased the cell population at G0 / G1 as 44% and 39.7%, respectively (Control: 53.95%, 5 μM resveratrol: 45.9%, 10 μM resveratrol: 42.4%, 1 μM PDMP 49.8%). At S phase, the cell population distribution were 18.5% and 21.3% for 5- and 10 μM resveratrol + 1 μM PDMP combinations, respectively (Control: 15.4%, 5 μM resveratrol: 16.35%, 10 μM resveratrol: 17.35%, PDMP 1 μM: 16.2%) (Figure 3.2.2.b). Thus, the combination treatment resulted in S phase arrest which supported the cell proliferation data (Figure 3.1.4.b). Based on these results, it is proved that combinations of resveratrol with SKI II and PDMP suppress cell proliferation by arresting the cell at S phase and inhibiting DNA synthesis.

The distributions of SUP-B15 cells at G0 /G1, S and G2 / M phases as shown in Figure 3.2.2.a after treatment with 5- and 10 μM resveratrol were 45.9%, 16.35%, 19.6% and 42.4%, 17.35% and %17.55 respectively. For control cells, it was 53.95%, 15.4% and 17%, respectively. The distribution of the cells at G0 / G1, S and G2 / M phases after 100 nM myriocin treatment was 49.5%, 12.7% and 22.4%, respectively. Myriocin caused arrest at G2 / M phase compared to control cells while it decreased the cell population at G0 / G1 and S phases. For the 5- and 10 μM resveratrol + 100 nM myriocin combinations, the distribution of cells at G0 / G1, S and G2 / M phases were 44.1%, 12.2%, 19.1% and 38.3%, 14%, 20.4%, respectively (Figure 3.2.2.c). For both combinations, the cell population was reduced at G0 / G1 phase. Based on these results, the combination of 5- and 10 μM resveratrol with 100 nM myriocin did not appear to be effective on cell cycle progresion, which supported the proliferation data (Figure 3.1.4.c).

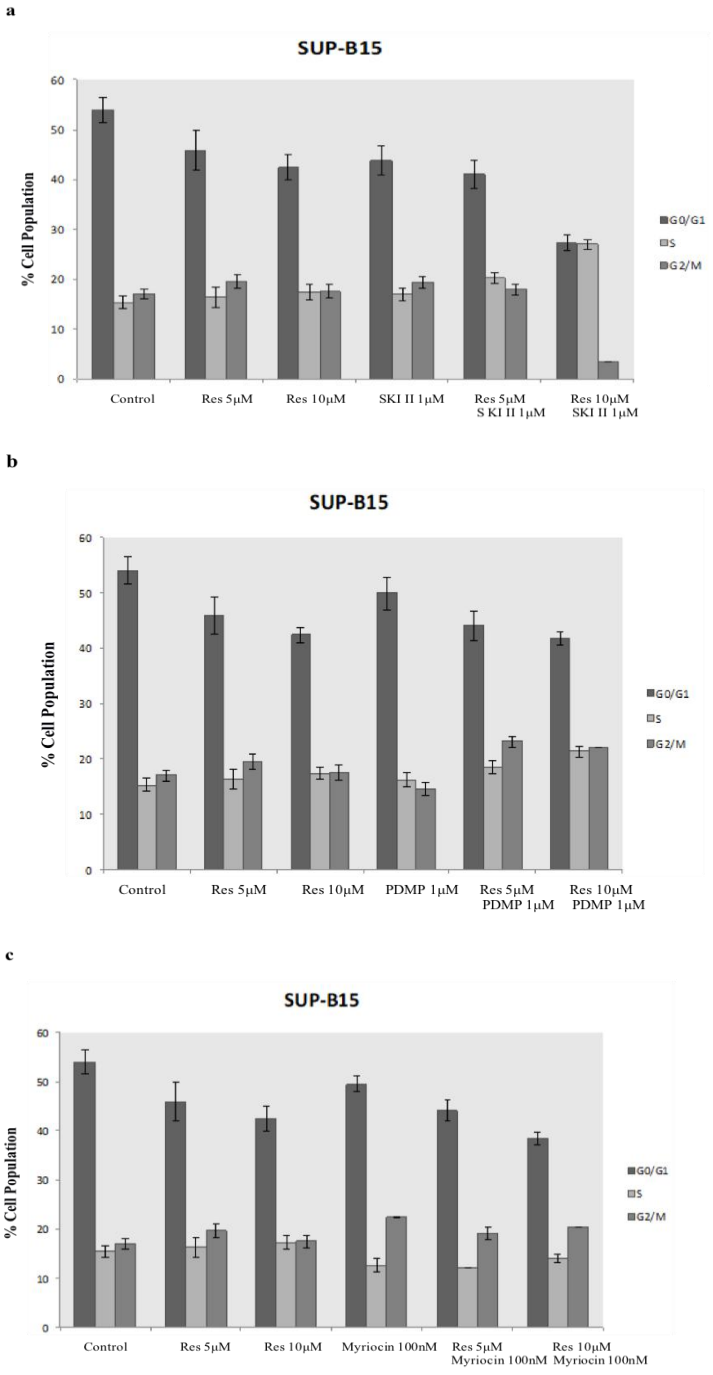


Figure 3.2.2 Cell cycle distrubution of SUP-B15 cells after treatment with Resveratrol, SKI II, PDMP, Myriocin, Resveratrol: SK-1 Inhibitor (a), Resveratrol: GCS inhibitor (b) and Resveratrol: SPT Inhibitor (c) combinations. The results derived from the means of three independent experiments are represented as mean± SE

The reason why inhibitors, resveratrol and resveratrol-inhibitor combinations suppressed the cell proliferation in SD1 and SUP-B15 cells can be explained with the effects of them on cell cycle progression. Based on the cell cycle data for SD1 and SUP-B15 cells, it is thought that combinations of resveratrol with SKI II and PDMP suppress cell proliferation by inhibiting DNA synthesis through causing S phase arrest (Figure 3.2.1.a, b and Figure 3.2.2.a, b). The effects of resveratrol or its combinations with different agents on cell cycle in different types of cancer have been shown in the literature [178]. Determined increased expression of p21 and p27 and decreased expression of cyclin A and D in T-ALL cells treated with resveratrol, which resulted in G0 / G1 phase arrest. In human colorectal cancer cells, resveratrol caused arrest in the S phase and its combination with 5-flurouracil increased S phase population as compared to resveratrol [160]. Resveratrol arrested OCM2 AML cells at S phase [169]. In natural killer cell (NK) lymphoma cells, resveratrol suppressed proliferation by arresting cells at G0 / G1 phase [200]. In B-ALL SUP-B15 cells, 75 μM resveratrol treatment resulted in S phase arrest and proliferation was suppressed [177]. In SUP-B15 cells, myriocin combinations did not have significant effect on the cell cycle (Figure 3.2.2.c) and supported cell proliferation results (Figure 3.1.4.c). The combination of myriocin with resveratrol supported the results of cell proliferation (Figure 3.2.1.c) by arresting SD1 cells at S phase (Figure 3.1.3.c). This study has shown for the first time how resveratrol together with targeting sphingolipid metabolism has an effect on the cell cycle and cell proliferation on the Ph + ALL model.

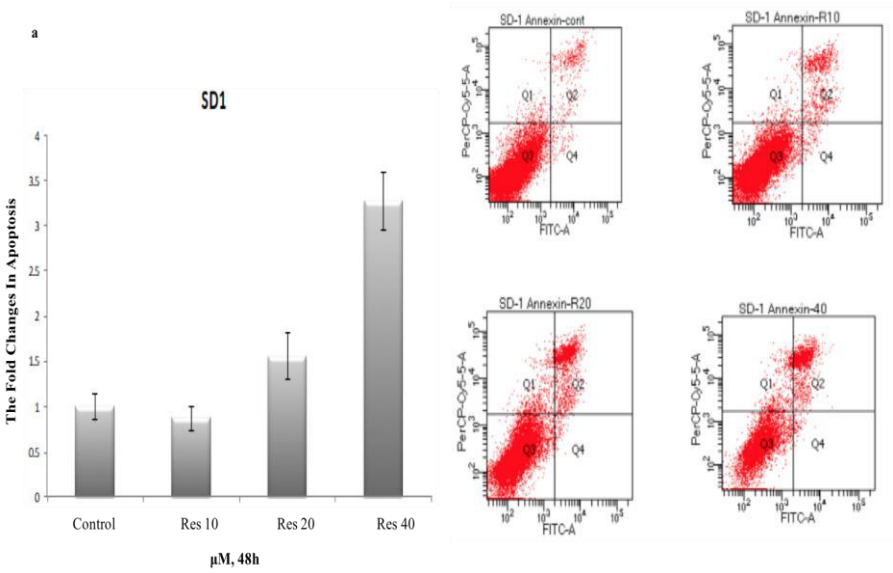
3.3 Combinations of Resveratrol with SKI II, PDMP and Myriocin Regulate Apoptosis by Changing Ceramide/S1P-GC Balance in SD1 and SUP-B15 Ph + ALL Cells

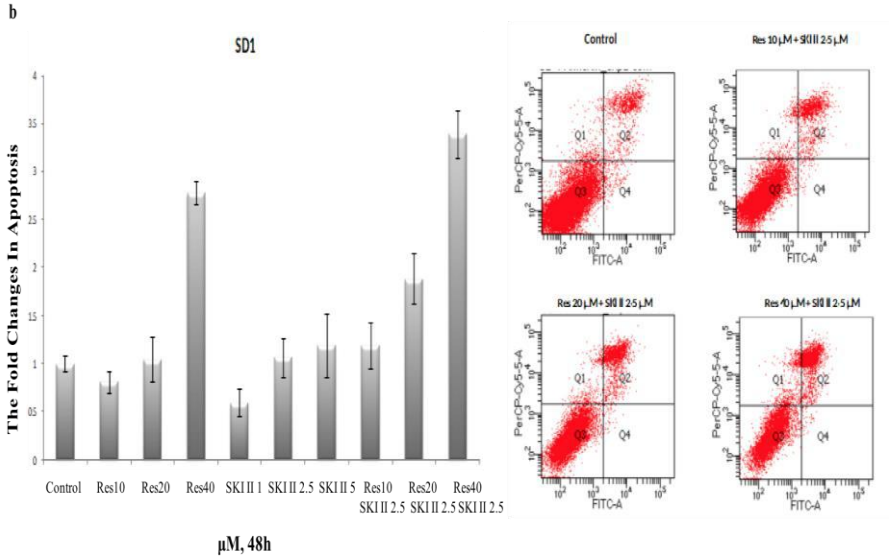
The amount and localization of phosphatidyl serine is one the hallmark of apoptosis, thus the apoptotic effects of resveratrol, SPT, SK1 and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK1 inhibitor and resveratrol: GCS inhibitor combinations on SD1 and SUP-B15 cells were checked by AnnexinV-Propidium Iodide (PI) dual staining method by using flow cytometry.

SD1 cells were treated with 10-, 20- and 40 μM resveratrol and apoptotic cell population was found to be increased by 1.5 and 3.3-fold for 20- and 40 μM resveratrol, respectively as compared to untreated control cells. 10 μM resveratrol did not trigger apoptosis when compared to control (Figure 3.3.1.a).

SD1 cells treated with increasing concentrations of SKI II (1-, 2.5- and 5 μM) had increases in apoptotic cell population especially for 2.5 and 5 μM SKI II as compared to untreated control cells (1.05 and 1.2 folds, respectively). The combination of increased concentrations of resveratrol (10-, 20- and 40 μM) with 2.5 μM SKI II caused increases in apoptotic cell population as 1.19, 1.88 and 3.4 fold, respectively (Figure 3.3.1.b). As a result, the increasing concentrations of resveratrol may induce apoptosis synergistically in the presence of SKI II inhibitor, which is related the inhibition of the SK-1 / SK-2 enzyme, which is involved in the conversion of apoptotic ceramide to antiapoptotic S1P. When SD1 cells were treated with increasing concentrations of myriocin (40-, 80-, and 100 nM), there was a decrease in the apoptotic cell population compared to untreated control cells. Similarly, the combination of increased concentrations of resveratrol (10-, 20- and 40 μM) with 100 nM myriocin resulted in a reduction in apoptosis compared to the respective doses of resveratrol (Figure 3.3.1.c). Since myriocin inhibits *de novo* ceramide formation

by inhibiting the SPT enzyme catalyzing the first step of this pathway, a decrease in apoptosis suggests that resveratrol induces cell death by triggering ceramide formation.





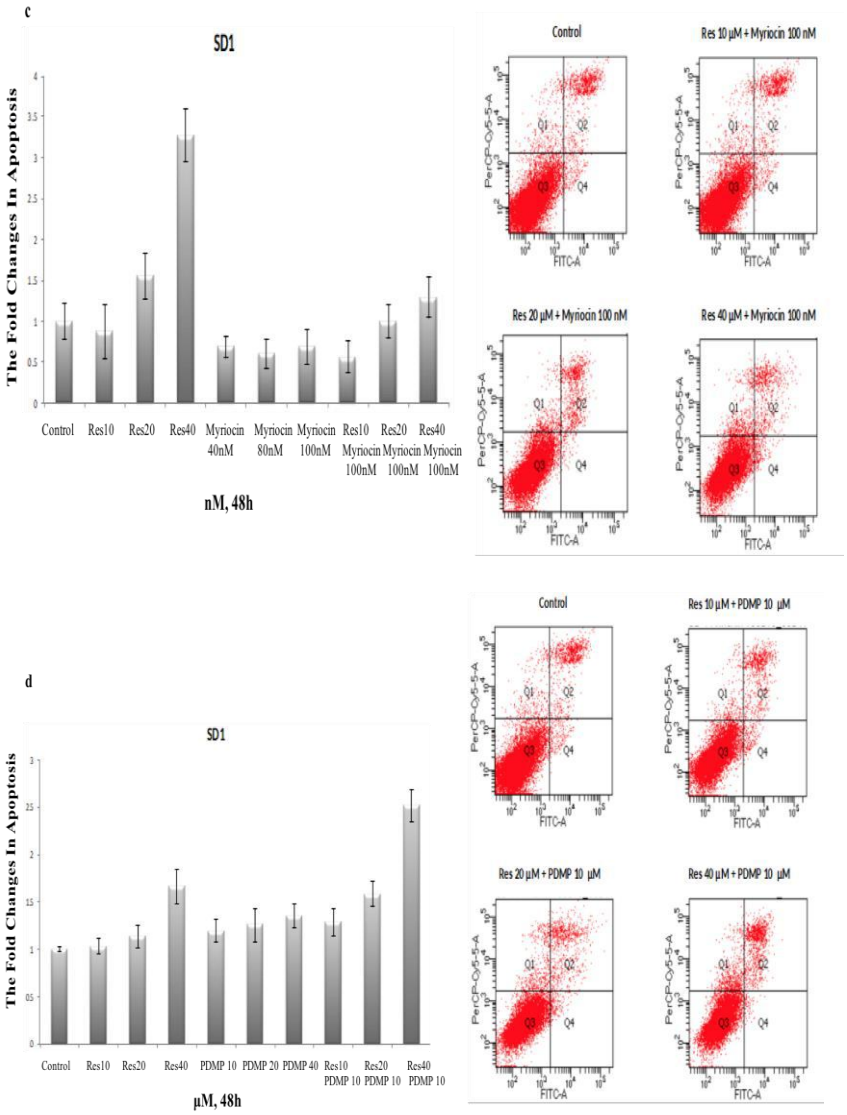


Figure 3.3.1. Apoptotic effects of resveratrol (a), SKI II, PDMP, Myriocin, and Resveratrol: SK-1 inhibitor (b), Resveratrol: SPT inhibitor (c), and Resveratrol: GCS inhibitor combinations (d) on SD1 cells. The results derived from the means of three independent experiments are represented as mean± SE. The cells located in Q4 quadrant show an AnnexinV-positive / PI negative early apoptotic population, and the cells located in Q2 quadrant show an AnnexinV-positive / PI positive late apoptotic population. The given flow cytometer histograms represent one of three independent experiments.

SUP-B15 cells were treated with 5- and 10 μM resveratrol and apoptotic cell population was detected as 1.15 and 2.0-fold more than untreated control cells, respectively (Figure 3.3.2.a).

The increase in apoptotic cell population was 3.6 times more compared to untreated control cells in SUP-B15 cells treated with 1 μM SKI II. 10 μM Resveratrol + 1.0 μM SKI II combination increased the apoptotic cell population by 5.6 fold compared to control cells and compared to 10 μM resveratrol. It was found to be increased approximately 3.0-fold (Figure 3.3.2.b). In conclusion, the potential of inducing apoptosis was increased in the cells after treatment with 10 μM resveratrol in the presence of SKI II and the reason is due to the accumulation of intracellular ceramide by inhibition of SK enzyme which catalyzes the conversion of apoptotic ceramide to antiapoptotic S1P.

There was a 1.7-fold increase in apoptotic cell population in SUP-B15 cells treated with 1 μM PDMP compared to control cells. 10 μM resveratrol together with 1.0 μM PDMP increased apoptotic cell population (2.5 fold compared to control cells and 1.5-fold compared to 10 μM resveratrol) (Figure 3.3.2.c). In conclusion, the potential of inducing apoptosis was increased in the cells after treatment with 10 μM resveratrol in the presence of PDMP and the reason is due to the accumulation of intracellular ceramide by inhibition of GCS enzyme which catalyzes the conversion of apoptotic ceramide to antiapoptotic GS. As explained in the results, the combination of 10 μM resveratrol with SKI II and PDMP appears to suppress cell proliferation (Figure 3.1.4.a and b) and also increase apoptosis (Figures 3.3.2.b and c). Cell proliferation data and apoptosis results support each other.

When SUP-B15 cells were treated with 100 nM myriocin, there was a decrease in the apoptotic cell population as compared to untreated control cells. Similarly, the combination of 5- and 10 μM resveratrol with 100 nM myriocin resulted in reductions in apoptotic cell populations compared to the respective doses of resveratrol (Figure 3.3.2.d). Since myriocin inhibits *de novo* ceramide formation by inhibiting the SPT enzyme, it can be said that there is a decrease in the amount of apoptotic ceramide, which could lead a decrease in the apoptotic

cell population. Therefore, these results can be interpreted as resveratrol induces cell death by triggering ceramide formation.

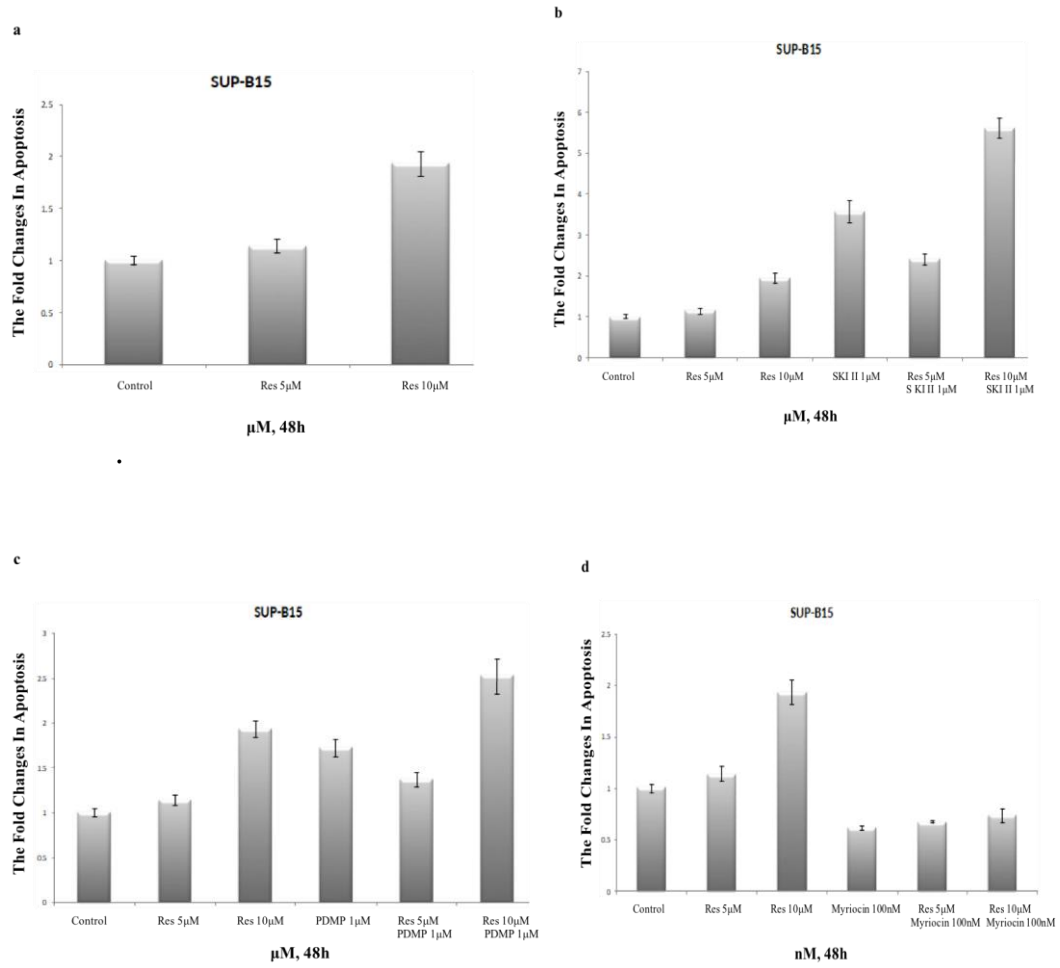


Figure 3.3.2 Apoptotic effects of resveratrol (a), SKI II, PDMP, Myriocin, Resveratrol: SK-1 inhibitor (b), Resveratrol: SPT inhibitor (c), and Resveratrol: GCS inhibitor combinations (d) on SUP-B15 cells. The results derived from the means of three independent experiments are represented as mean± SE. The cells located in Q4 quadrant show an AnnexinV-positive / PI negative early apoptotic population, and the cells located in Q2 quadrant show an AnnexinV-positive / PI positive late apoptotic population. The given flow cytometer histograms represent one of three independent experiments.

Increased concentrations of resveratrol in SD1 and SUP-B15 cells were found to induce apoptosis (Figure 3.3.1.a and Figure 3.3.3.a). It has been shown in the literature that resveratrol triggers apoptosis through very different mechanisms in solid and hematologic cancers. Increasing concentrations of resveratrol triggered apoptosis in K562 CML cells, which was detected by AnnexinV-positive / PI staining [201]. In this study, it was shown that resveratrol increased the mitochondrial membrane potential (MMP), Bax / Bcl-2 ratio, cytochrome c release to cytosol and activated caspase-3 and PARP. In a study published in 2017, 75 μM resveratrol treatment induced apoptosis in T-ALL TALL-104 and B-ALL SUP-B15 cells by triggering caspase-3 activation [177]. This study supported the results obtained in this study. In T-ALL cells, 200 μM resveratrol treatment resulted in decreased expression of antiapoptotic Bcl-2 family members (Mcl-1 and Bcl-2), while it increased the expression of apoptotic members (Bax, Bim, Bad) and cleavaged caspase-3 [178]. Resveratrol triggered apoptosis in natural killer (NK) lymphoma cells by activating caspase-3 and reducing STAT3 acetylation and the expression of STAT3 target proteins, antiapoptotic Mcl-1, Bcl-10 and surviving [200].

In addition to the mechanisms underlying resveratrols prominent therapeutic potential in leukemia and solid cancer types, it is known that resveratrol also regulates various steps of sphingolipid metabolism. As shown in Figure 3.3.1.b and Figure 3.3.2.b, resveratrol-induced apoptosis in SD1 and SUP-B15 cells was increased by inhibiting SK-1 / SK-2, which is responsible for the conversion of apoptotic ceramide to antiapoptotic S1P. Based on this result, it can be concluded that resveratrol can function in Ph + ALL by increasing the amount of intracellular ceramide. Similarly, inhibition of GCS resulted in an increase in the amount of intracellular ceramide and a synergistic effects was detected when it was combined with resveratrol (Figure 3.3.1.d and Figure 3.3.2.c). Although the effect of resveratrol on Ph + ALL SUP-B15 cells has been demonstrated in 2017 [177]. The mechanism of action associated with targeting sphingolipid metabolism has not been previously demonstrated. There are limited number of studies describing the effects of resveratrol on

sphingolipid metabolism in various types of cancer. Cytotoxic and apoptotic effects of resveratrol were increased in gastric cancer cells after combination with dimethylsfingosin (SK inhibitor) [188]. PDMP: resveratrol and SK inhibitor: resveratrol combinations have been found to possess synergistic apoptotic effect by causing changes in MMP and increases in caspase-3 enzyme activity in both APL and CML [186]. As it can be seen in Figure 3.3.1.c and Figure 3.3.2.d, the inhibition of SPT with myriocin, which catalyzes the first step of *de novo* ceramide synthesis, reduced resveratrol-induced apoptosis significantly. This result is consistent with the literature. Co-administration of resveratrol with SPT inhibitors, myriocin and L-cycloserine, reversed apoptotic process induced by resveratrol in metastatic breast cancer cells and reduced or eliminated PARP cleavage [195]. The administration of resveratrol and myriocin and / or L-cycloserine suppressed apoptosis and reversed the effect of resveratrol in nasopharyngeal cancer cells [202].

3.4 Resveratrol Regulates SK-1/SK-2, GCS and SPT Enzymes

In order to understand the role of key enzymes of sphingolipid metabolism in cell growth inhibition and apoptosis induced by resveratrol, changes in protein expression of SK-1, SK-2, GCS and SPT enzymes were investigated. For this purpose, total protein was isolated from SD1 (20- and 40 μM) and SUP-B15 (5- and 10 μM) cells treated with increasing concentrations of resveratrol after 48 hours incubation, as described in Materials and Methods. Changes in the expression of SK-1, SK-2, GCS and SPT enzymes were deteceted.

It was found that increased concentrations of resveratrol did not cause a significant change in SK-1 expression, but caused 0.32 and 0.54-fold decreases in SK-2 as compared to control in SD1 cells (Figure 3.4.1.a). In SUP-B15 cells, 5- and 10 μM resveratrol caused decreases in both SK-1 (0,6 and 0.75-fold) and

SK-2 (0.65 and 0.69-fold) expressions, respectively as compared to control (Figure 3.4.1.a). There are two isoforms of the SK enzyme (SK-1 and SK-2), which are responsible for the conversion of apoptotic sphingosin to antiapoptotic S1P. The role of SK-1 has been investigated in many cancer types as discussed in the literature. In one study, it was shown that while there was no change in total SK-1 expression in K562 CML cells treated with resveratrol in accordance with SD1 results, translocation of SK-1 from membrane to cytoplasm was increased, and its activity was suppressed and the amount of S1P was decreased and cells growth was suppressed [203]. In recent years, it has been shown that the expression of SK-2 in various cancer types is increased, which contributes to neoplastic transformation, tumor formation and development [204]. Pharmacological or genetic inhibition of SK-2 is known to cause growth inhibition through various mechanisms in cancer cells. For example, it has been shown that SK-2 expression is increased in colongiocarcinoma cells and the proliferation of these cells is suppressed by treatment with specific SK-2 inhibitor (ABC294640) and caspase-dependent apoptosis was induced [205]. It is known that SK-2 promotes ALL development by increasing oncogenic Myc expression and genetic/ pharmacological inhibition of SK-2 reduced ALL progression [206]. In this project, resveratrol inhibited the formation of antiapoptotic S1P by inhibiting SK-1/SK-2 in SD1 and SUP-B15 cells, thus inhibiting the growth of cells and triggering apoptosis. The effect of resveratrol on SK-2 has been shown for the first time in this study for Ph + ALL.

20- and 40 μM resveratrol significantly reduced GCS expression in SD1 cells compared to control (0.7 and 0.8-fold, respectively) (Figure 3.3.1.b). In SUP-B15 cells, 10 μM resveratrol reduced GCS expression by 0.32-fold compared to control (Figure 3.4.2.b). This result shows that resveratrol inhibits the growth of cells and triggers apoptosis by inhibiting the enzyme responsible for antiapoptotic GC synthesis. Although there are no studies showing the effect of resveratrol on GCS expression in the literature, the results obtained are consistent with the results of K562 CML and HL60 APL studies [186]. In these studies, the expression of SK-1 and GCS genes decreased as a result of

resveratrol treatment, which was shown by real-time PCR. As explained in cell proliferation and apoptosis results, the combination of resveratrol with SK inhibitor (SKI II) and GCS inhibitor (PDMP) showed a synergistic effect compared to only resveratrol in SD1 and SUP-B15 cells by inhibiting cell proliferation and increasing apoptosis (Figure 3.1.3, 3.1.4, 3.3.1 and 3.3.2). These results show that the underlying cause of the therapeutic potential of resveratrol in Ph + ALL is related to the regulation of the key enzymes of sphingolipid metabolism.

When the effect of increasing concentrations of resveratrol (20- and 40 μM) on SPT expression was examined on SD1 cells, 2.2 and 2.3-fold increases were observed, respectively (Figure 3.4.1.c). In SUP-B15 cells, 10 μM resveratrol caused a 0.38-fold increase (Figure 3.4.2.c). SPT is the enzyme that catalyzes the first step of *de novo* ceramide synthesis (Figure 3.1.1) and its increased expression by resveratrol indicates that resveratrol triggers cell death by increasing the amount of intracellular ceramide. The apoptotic effects of resveratrol on metastatic breast cancer, colon cancer and nasopharynx cancer cells are known to be mediated by the activation of *de novo* ceramide synthesis and this effect was reversed by inhibition of SPT [195, 196, 202]. Similar results have been obtained in SD1 and SUP-B15 cells after treated with resveratrol: SPT inhibitor (myriocin) combination, which suppressed resveratrol-mediated apoptosis (Figure 3.3.1and Figure 3.3.2). The results obtained for the first time in Ph + ALL cells are consistent with the literature.

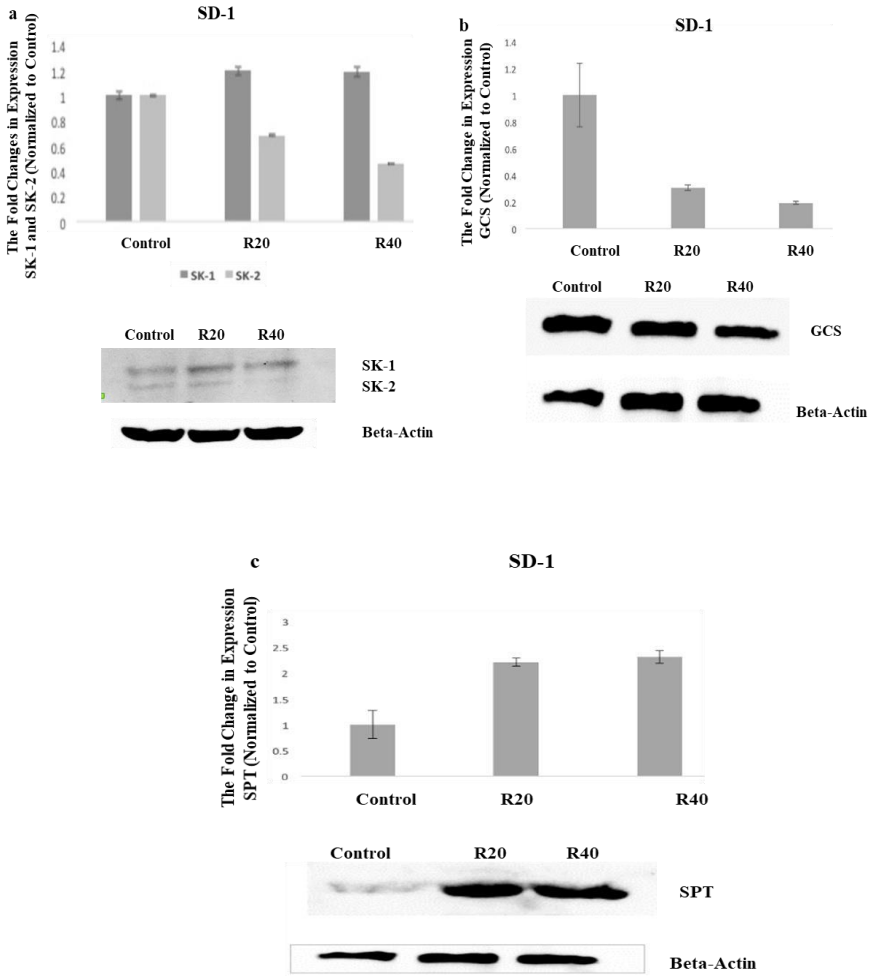


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Figure 3.4.1 Changes in expression of SK-1 / SK-2 (a), GCS (b) and SPT (c) in SD1 cells treated with resveratrol. Beta-Actin was used as loading control. Experimental sets were repeated twice and representative western blot image was used for each set. The results obtained from 2 different experimental sets are given as mean ± standard error. The protein expression of each group was normalized to their Beta-Actins and the graphs were drawn by accepting control intensity as 1.

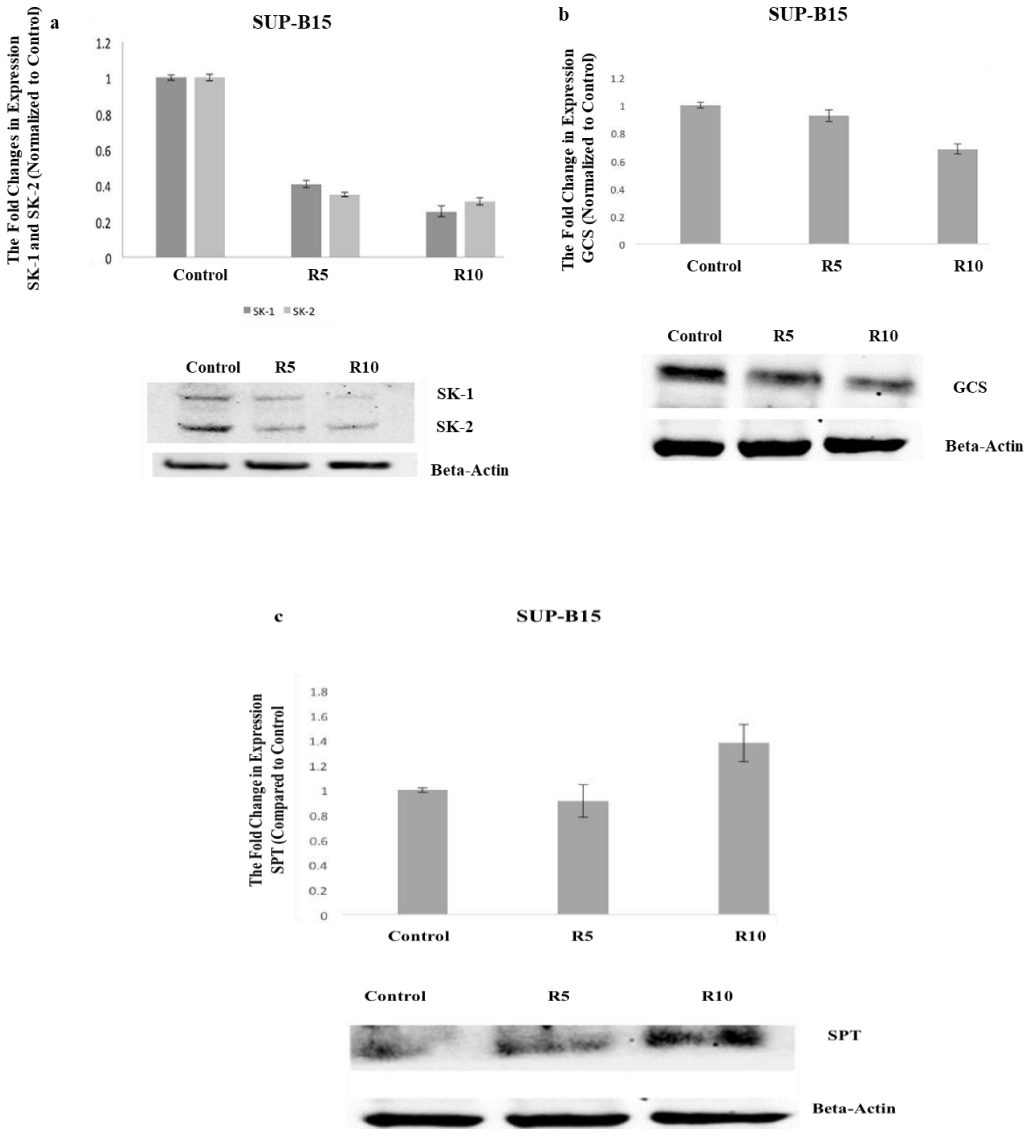


Figure 3.4.2 Changes in expression of SK-1 / SK-2 (a), GCS (b) and SPT (c) in SUP-B15 cells treated with resveratrol. Beta-Actin was used as loading control. Experimental sets were repeated twice and representative western blot image was used for each set. The results obtained from 2 different experimental sets are given as mean ± standard error. The protein expression of each group was normalized to their Beta-Actins and the protein expression of each group was normalized to their Beta-Actins and the graphs were drawn by accepting control intensity as 1.

3.5 Resveratrol and Its Combinations with Sphingolipid Metabolism Inhibitors Regulate Cytochrome c Release, Caspase-3 and PARP Cleavage and BCR-ABL expression

In order to understand how resveratrol and its combinations with sphingolipid metabolism enzyme inhibitors regulate apoptosis at the molecular level, cytochrome c release, caspase-3 and PARP cleavage were checked by western blot in Ph + ALL cells. The release of cytochrome c, which is localized between the mitochondrial inner membrane and outer membrane, is an important marker of intrinsic apoptosis. Cytochrome-c in the cytosol activates caspase-9 by forming complex with Apaf-1. Activated caspase-9 activates caspase-3 which triggers intrinsic apoptosis by cleavage of PARP, a caspase-3 target. Cytosolic and mitochondrial fractions of SD1 and SUP-B15 cells after treatment with resveratrol, inhibitors and resveratrol: inhibitor combinations were collected as described in Materials and Methods and cytochrome-c release into cytosol was examined by western blot analysis. Treatment of SD1 cells with increased concentrations of resveratrol (20- and 40 μM) were found to increase cytoplasmic cytochrome-c release as 0.37 and 0.70-folds, respectively as compared to control. In the nasopharyngeal cancer, retinoblastoma and gastric cancer cells, the amount of cytosolic cytochrome-c increased as a result of resveratrol treatment compared to control [207-209]. 2.5 μM SKI II, 10 μM PDMP and 100 nM myriocin showed 5.2, 2.6 and 6.6-fold increases, respectively. Combinations of 20 μM resveratrol with 2.5 μM SKI II and 100 nM myriocin resulted in 6.0 and 7.5-fold increases. Combination of 40 μM resveratrol with 10 μM PDMP showed a 7.8-fold increase (Figure 3.5.1.a). In SUP-B15 cells, 1 μM SKI II, 1 μM PDMP and 100 nM myriocin resulted in 1.2, 2.7 and 1.5-fold increases in cytochrome-c release, respectively. 5- and 10 μM resveratrol showed no significant change, whereas combinations of 10 μM resveratrol with 1 μM SKI II and 1 μM PDMP showed 4.3 and 3.6-fold

increases, respectively. The combination of 10 μM resveratrol and 100 nM myriocin showed a 1.2-fold increase, but resulted in a 0.2-fold decrease compared to myriocin alone (Figure 3.5.1.b). The results show that combinations of resveratrol with SKI II and PDMP increase the cytochrome c release synergistically and these results support the apoptosis results. The increase in the amount of apoptotic ceramide in the cell as a result of inhibition of SKI II and PDMP in addition to the the inhibitory effect of resveratrol on SK and GCS support the synergy. Myriocin combinations induced cytochrome c release, but can not explain the suppression of apoptosis. There are some studies in which myriocin triggered cytochrome-c release alone or in combinations. For instance, although cytochrome-c release in UV-treated HeLa cells is expected to be inhibited as a result of myriocin treatment (since *de novo* ceramide production is suppressed and the amount of ceramide is reduced), cytochrome-c release is increased and it was also released only in myriocin treatment [210]. However, apoptosis was not prevented in this study. Therefore, the combination of resveratrol with myriocin in Ph + ALL increased apoptosis which can be through cytochrome-c release independent mechanism.

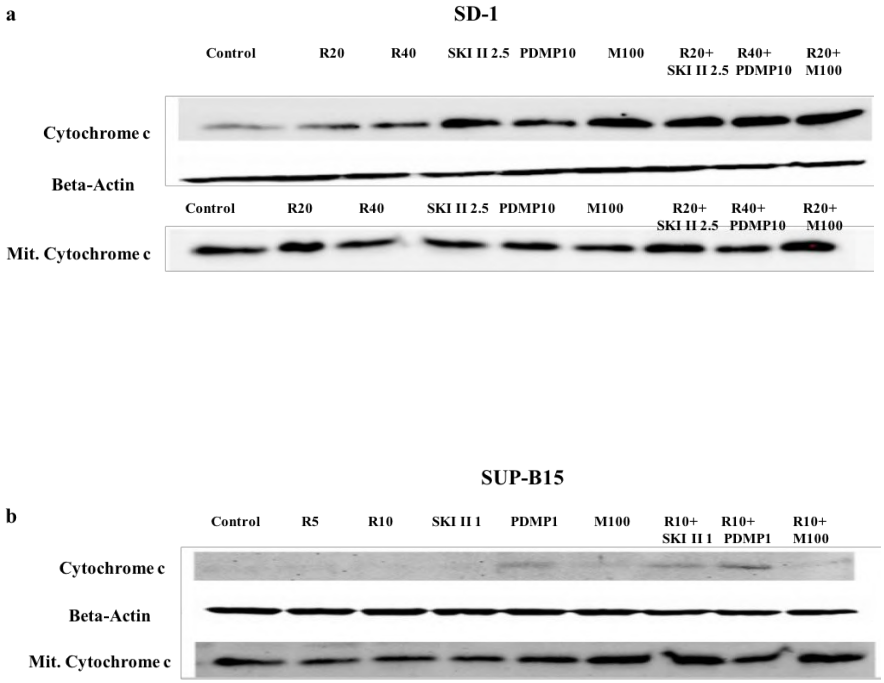


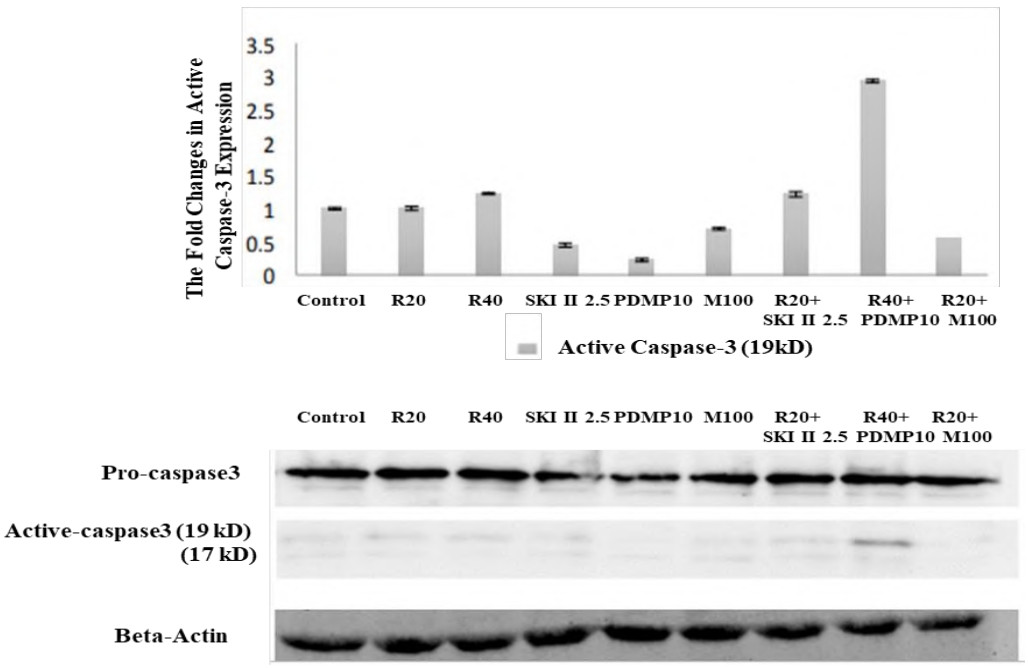
Figure 3.5.1. Cytochrome-c release in SD-1 (a) and SUP-B15 (b) cells treated with resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor. Combinations. Beta-Actin was used as loading control. Experimental sets were repeated twice and representative western blot image was used for each set. The results obtained from 2 different experimental sets are given as mean ± standard error. Cytoplasmic cytochrome-c release of each group was normalized to their Beta-Actins, and graphs were plotted by accepting control value as 1.

The activation of caspase-3, which is involved in the intrinsic apoptosis pathway, and cleavage of caspase-3 substrate PARP in SD1 (Figure 3.5.2.a and b) and SUP-B15 (Figure 3.5.2.c and d) cells were investigated by western blot method. As shown in Figure 3.5.2.a and b, increasing concentrations of resveratrol increased caspase-3 and PARP cleavage in SD1 cells as compared to control. The combination of 20- and 40 μM resveratrol with 2.5 μM SKI II and 10 μM PDMP resulted in 1.22-fold and 2.4 fold increases in active caspase-3 expression, respectively. Cleaved PARP levels were increased as 1.6 fold for 20 μM resveratrol in combination with 2.5 μM SKI II and its expression remained

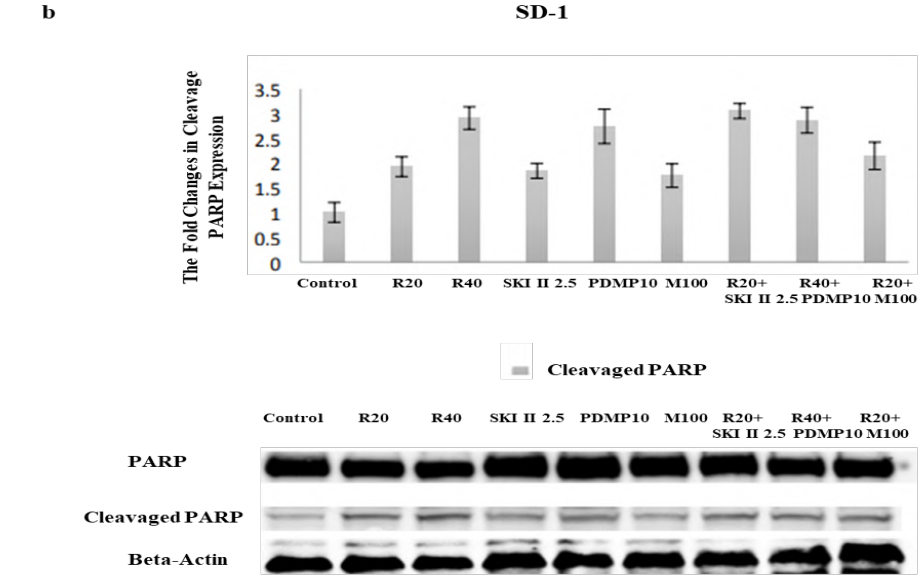
unchanged for 40 μM resveratrol in combination with 10 μM PDMP. 20 μM resveratrol in combination with 100 nM Myriocin resulted in 0.55 fold decrease and 1.23 fold increase in active caspase-3 and PARP levels, respectively.

Increasing concentrations of resveratrol increased caspase-3 and PARP cleavage in SUP-B15 cells as compared to control (Figure 3.5.2.c and d). The combination of 10 μM resveratol with 1 μM SKI II and 1 μM PDMP resulted in 0.23-fold increase and 0.15-fold decrease in active caspase-3 expression while 0.4 and 1.67-fold increases in PARP cleavage, respectively as compared to defined resveratrol comcentration. The combination of 10 μM resveratrol with 100 nM myriocin resulted in a 0.47-fold decrease in active caspase-3 expression and 1.25-fold increase in cleavaged PARP, respectively. Increased concentrations of resveratrol in SD1 and SUP-B15 cells induced apoptosis via intrinsic pathway by increasing caspase-3 and PARP cleavage. This result is consistent with cytochrome-c release for both cell lines (Figure 3.5.1.a and b). As shown in many cancer types, one of the common mechanisms of action of resveratrol is to induce apoptosis via the intrinsic pathway. For instance, resveratrol increased caspase-3 and PARP cleavage in colorectal cancer cells [211]. It was shown that apoptosis was triggered through caspase-3 and PARP cleavage in K562 CML cells after treatment with resveratrol. The combination of resveratrol with SKI II showed a synergistic effect by activating caspase-3 and increasing PARP cleavage. Thus, this combination functions through the mitochondrial pathway. However, combinations of resveratrol with PDMP and myriocin in SUP-B15 cells were found to cause cell death by PARP cleavage and inducing cytochrome-c release, which are caspase-3 independent. Cytochrome-c release is known to induce cell death by triggering the production of free radicals such as mitochondrial superoxide and suppressing ATP production, which can be caspase-3 independent [212]. In addition to caspase-3, there are many cellular proteases such as caspase-1 and -7, calpaines, cathepsins, granzymes, matrix metalloproteinases, which are responsible for PARP cleavage and are involved in the apoptotic process [213].

a


SD-1
The Fold Changes in Active Caspase-3 Expression
3.5
3
2.5
2
1.5
1
0.5
0
Control
R20
R40
SKI II 2.5
PDMP10
M100
R20+ SKI II 2.5
R40+ PDMP10
R20+ M100
Active Caspase-3 (19kD)
Pro-caspase3
Active-caspase3 (19 kD) (17 kD)
Beta-Actin

b


SD-1
The Fold Changes in Cleavage PARP Expression
3.5
3
2.5
2
1.5
1
0.5
0
Control
R20
R40
SKI II 2.5
PDMP10
M100
R20+ SKI II 2.5
R40+ PDMP10
R20+ M100
Cleavaged PARP
PARP
Cleavaged PARP
Beta-Actin

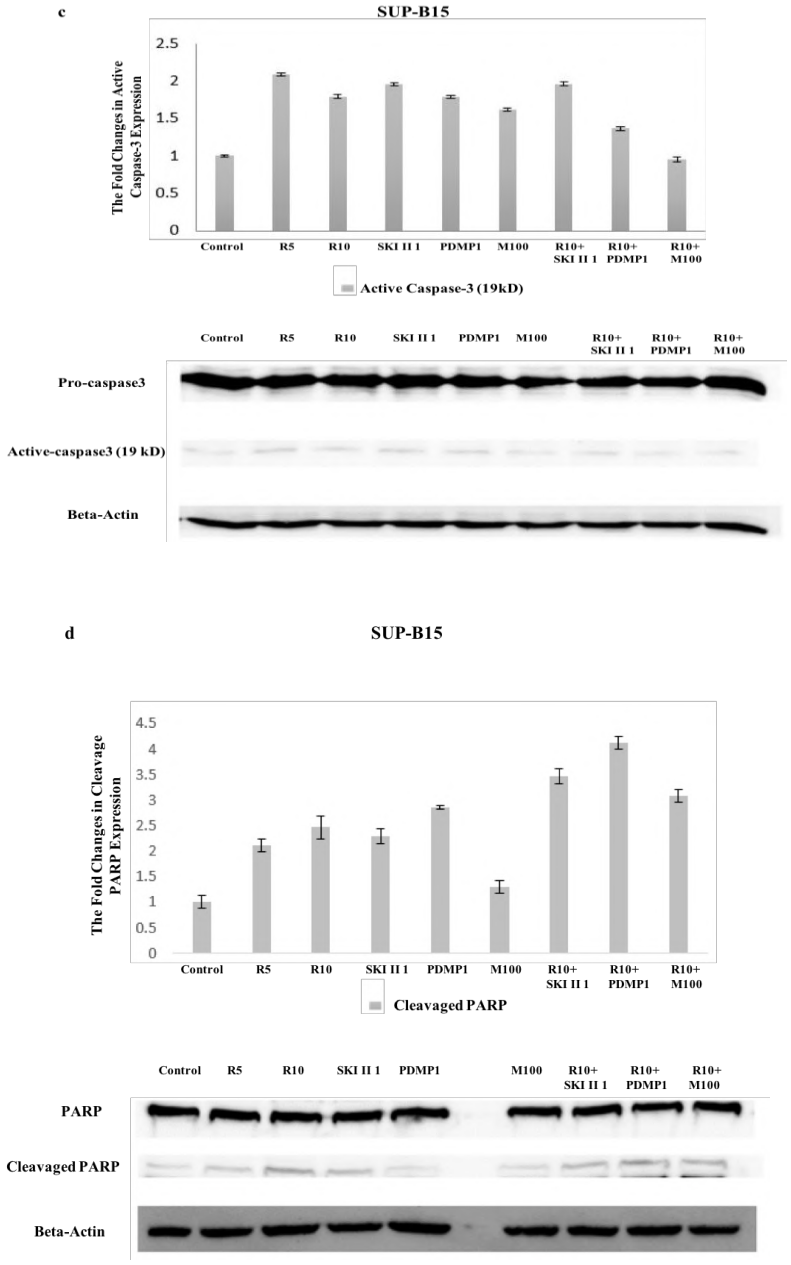


Figure 3.5.2 The changes in active Caspase and PARP expression in SD-1 (a, b) and SUP-B15 (c, d) cells treated with resveratrol, SPT, SK and GCS inhibitors, resveratrol: SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor combinations. Beta-Actin was used as loading control. Experimental sets were repeated twice and representative western blot shape was used for each set. The results obtained from 2 different experimental sets are given as mean ± standard error. The truncated caspase-3 and PARP of each group were normalized to their Beta-Actins, and the graphs were plotted by assuming control value as 1.

In order to understand the changes in expression of BCR-ABL, which is the main oncoprotein in Ph + ALL development, SD1 (Figure 3.5.3.a) and SUP-B15 (Figure 3.5.3.b) cells were treated with resveratrol, sphingolipid metabolism enzyme inhibitors and resveratrol: inhibitor combinations. Western blot was performed following total protein isolation. As shown in Figure 3.5.3.a and b, increasing resveratrol concentrations (SD1: 20- and 40 μM; SUP-B15: 5- and 10 μM) decreased BCR-ABL levels as 0.1 and 0.8-folds for SD1, 0.25 and 0.5 folds for SUP-B15, respectively as compared to the control. 1 μM SKI II and 1 μM PDMP caused 0.48 and 0.37-fold decreases while myriocin led to a 1.5-fold increase in SUP-B15 cells. Similarly, 2.5 μM and 10 μM PDMP resulted in 0.5 and 0.9-fold decreases while myriocin caused 2.4-fold increase in SD1 cells. SKI II and PDMP reduce the amount of anti-apoptotic products S1P and GCS and triggers apoptotic ceramide accumulation, which may be associated with a decrease in BCR-ABL level. On the other hand, myriocin increased BCR-ABL expression. Inhibition of SPT with myriocin may contribute to the growth of cells through an increase in BCR-ABL. Although the roles of sphingolipid metabolism enzymes and products in some BCR-ABL positive hematological cancers are known, its potential effects on Ph + ALL have been explained for the first time in this study. For instance, tyrosine kinase ABL in BCR-ABL positive K562 and LAMA84 CML cells has been shown to inactivate serine palmitoyl transferase long chain 1 (SPTLC1) subunit by phosphorylating tyrosine 164 residue and induced the proliferation of cells [214]. In this study, inhibition of SPT with myriocin suppressed apoptosis as compared to control cells (Figure 3.3.1.c and Figure 3.3.2.d) and increased BCR-ABL expression (Figure 3.5.3.a and b). It has been shown for the first time in Ph + ALL that there is an interaction between BCR-ABL and SPT, supporting the mechanism shown in CML cells. In another study, it was shown that SK-1 / S1P / S1FR2 signaling pathway increased BCR-ABL stability and contributed to the development of resistance to tyrosine kinase inhibitors and cell survival in CML [215]. In CML cells, BCR-ABL has been shown to increase SK-1 expression and activity by regulating pathways such as MAPK, PI3K and JAK2c [216]. In

this study, the reduction of BCR-ABL expression as a result of SK inhibition supported the literature data. The relationship between GCS and BCR-ABL is not known in any cancer type. This study showed that GCS inhibition decreased BCR-ABL and inhibited the growth of cells.

On the other hand, the combination of resveratrol with SKI II and PDMP resulted in increases at BCR-ABL levels in both cell types whereas resveratrol: myriocin combination led to a decrease in BCR-ABL. Resveratrol, SKI II and PDMP inhibited the growth of SD1 and SUP-B15 cells by inhibiting BCR-ABL expression while esveratrol: SKI II and esveratrol: PDMP combinations did not show the expected effect on BCR-ABL. The combination of resveratrol with myriocin was found to have a therapeutic effect by reducing BCR-ABL levels in both cell types. Althogh SPT was activated by resveratrol (Figure 3.4.1.c and Figure 3.4.2.c), the inhibition of SPT with myriocin failed to reverse this effect of resveratrol. Therefore, *de novo* ceramide synthesis pathway or S1P and GCS pathway were not effective in the regulation of BCR-ABL expression in combination studies.

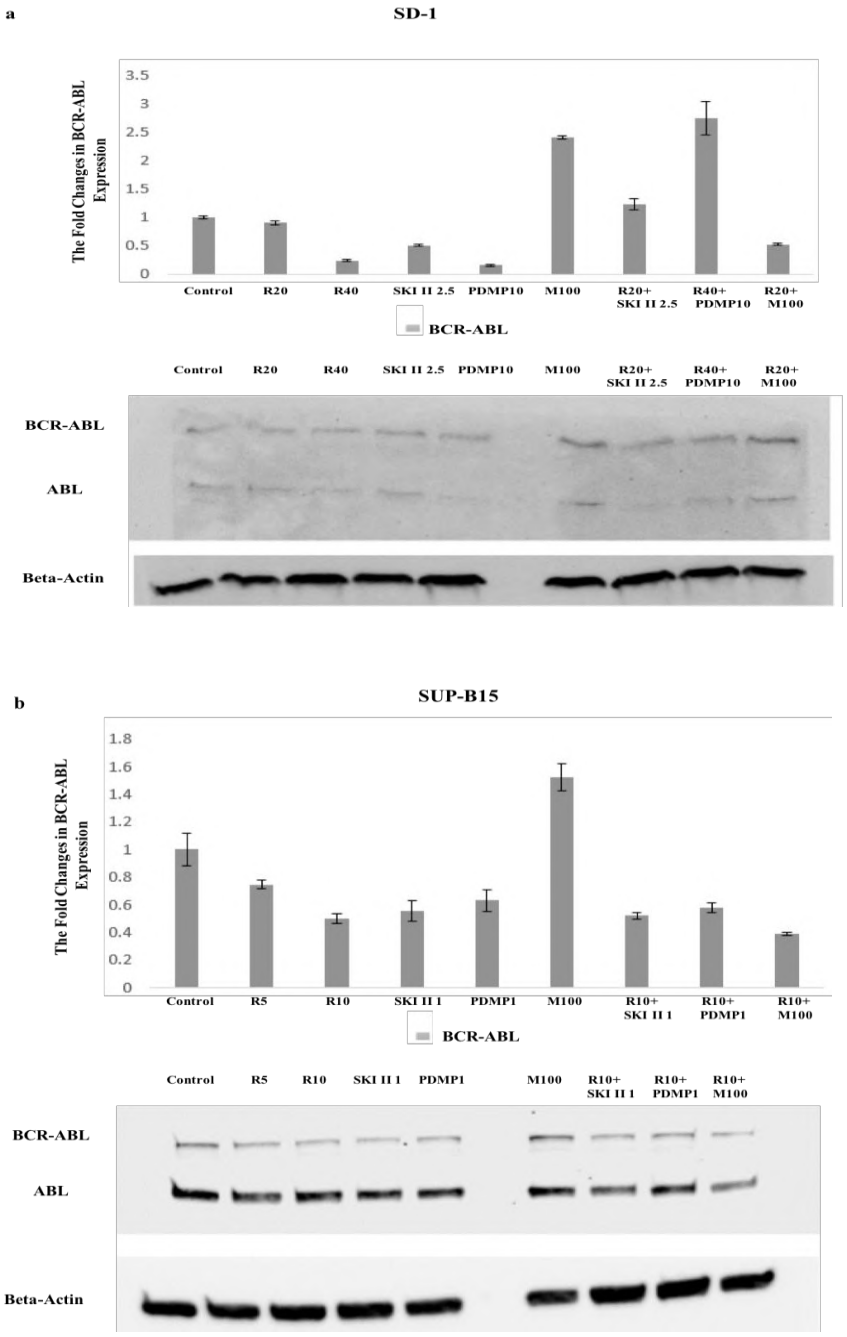


Figure 3.5.3 Changes in BCR-ABL expression in SD-1 (a) and SUP-B15 (B) cells treated with resveratrol, SPT, SK and GCS inhibitors, resveratrol:SPT inhibitor, resveratrol: SK inhibitor and resveratrol: GCS inhibitor combinations. Beta-Actin was used as loading control. Experimental sets were repeated twice and representative western blot image was used for each set. The results obtained from 2 different experimental sets are given as mean ± standard error. The BCR-ABL of each group was normalized to their Beta-Actins and the graphs were plotted by assuming control value as 1.

Chapter 4

4. Conclusion and Future Perspectives

4.1 Conclusion

Molecular signaling pathways behind the antileukemic effect of resveratrol in Ph + SD1 and SUP-B15 ALL cells were investigated by targeting the key enzymes of ceramide metabolism, which are SK, GCS and SPT and by identifying changes in BCR-ABL expression, which is the main oncoprotein for the formation of the disease.

The therapeutic effect of resveratrol was investigated in detail for the first time in Ph + ALL and it was found that resveratrol arrested the cell cycle at S phase and induced apoptosis. The main mechanism behind resveratrol induced apoptosis was related to caspase-3 activation and PARP cleavage together with increased cytochrome-c release. Resveratrol reduced BCR-ABL expression in both cell lines, which was thought to prevent BCR-ABL-mediated cell growth and apoptosis resistance. In addition, resveratrol treatment altered the expression of SPT responsible for the synthesis of apoptotic ceramide through *de novo* pathway and changed the expression of SK and GCS enzymes responsible for the conversion of ceramide to antiapoptotic S1P and GCS in Ph + ALL. Resveratrol decreased SK and GCS expression while increased SPT expression. Thus, resveratrol was also found to suppress cell growth by regulating sphingolipid metabolism. Targeting sphingolipid metabolism with resveratrol and the potential of combination therapy approaches is another target highlighted in this study. Especially, the combination of resveratrol with SKI II induced apoptosis via intrinsic pathway and arrested the cells at S phase. Apoptosis was induced by caspase-3 activation and PARP cleavage together

with cytochrome-c release. Two cell lines responded differently to the combination of resveratrol with PDMP. Caspase-3 independent PARP cleavage and cytochrome-c release were detected in SUP-B15 cells. The resveratrol: myriocin combination was effective through caspase-3 independent PARP cleavage and cytochrome-c release, but did not reverse the apoptotic effect of resveratrol. Resveratrol: SKI II and PDMP combinations did not have significant effects on BCR-ABL expression, but resveratrol: myriocin combination reduced BCR-ABL expression.

This study identified the therapeutic potential of resveratrol in Ph + ALL for the first time. The underlying mechanisms of resveratrol and also the possible effects of combination strategies by targeting sphingolipid metabolism have been defined. Targeting sphingolipid metabolism in Ph + ALL has not been studied extensively. There is only one publication in 2019 [217]. In this study, SK-1 expression increased in ALL and genetic deletion of SK-1 gene resulted in decreases in BCR-ABL induced ALL tumor development in the mouse model. Similarly, BCR-ABL expression was reduced as a result of SK inhibition in Ph + ALL cells. In addition, changes in BCR-ABL expression as a result of inhibition of GCS and SPT were detected for the first time in this study.

4.2 Future Prospects

New projects might be planned by focusing on the sphingolipid metabolism based on the data obtained from this study. The role of triggered signaling pathways (such as SK / S1P / S1PR signaling) might be studied in detail in Ph + ALL. Resveratrol: myriocin combination caused caspase-3 independent PARP cleavage and cytochrome-c release. Therefore, the potential mechanism behind this effect can be investigated by focusing on different cell death mechanisms including ER stress and lysosomal damage.

In addition, the effects of resveratrol, sphingolipid metabolism inhibitors and resveratrol: inhibitor combinations on the cell cycle might be

clarified at the molecular level by investigating the cell cycle regulators such as cyclin and cyclin dependent kinases.

As a result, it was found in the study that resveratrol and targeting sphingolipid metabolism have shown the antileukemic effect on Ph+ ALL through different mechanisms. *In vivo* mouse model studies and / or nanoparticle designs for the targeted therapy are considered as future projects.

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