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THERAPEUTIC POTENTIAL OF RESVERATROL AND THE ROLE OF CERAMIDE METABOLISM IN RESVERATROL-TRIGGERED APOPTOSIS IN FLT3+ ACUTE MYELOID LEUKEMIA

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
Nur Şebnem ERSÖZ
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ABSTRACT

THERAPEUTIC POTENTIAL OF RESVERATROL AND THE ROLE OF CERAMIDE METABOLISM IN RESVERATROL-TRIGGERED APOPTOSIS IN FLT3+ ACUTE MYELOID LEUKEMIA

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MSc in Bioengineering
Advisor: Assist. Prof. Dr. Aysun Adan

September 2021

The mechanisms underlying the growth inhibitory effects of resveratrol on FLT3-ITD + AML cells were investigated by targeting ceramide metabolism. Antiproliferative, apoptotic and cytostatic effects of resveratrol, SK (sphingosine kinase) inhibitor (SKI II), GCS (glucosylceramide synthase) inhibitor (PDMP) alone and on combination on MOLM-13 and MV4-11 cells were investigated by MTT, flow cytometric Annexin-V/PI staining and PI staining respectively. Caspase-3 and PARP cleavages, GCS and SK protein expressions were checked by western blot in response to resveratrol. PARP activation was also checked after co-treatments. Combination indexes were calculated by CompuSyn software. Resveratrol alone and its combinations with SKI II and PDMP suppress cell proliferation with additive or synergistic effects, induced apoptosis and resulted in cell cycle arrest. Resveratrol suppressed GCS and SK-1 expression and induced apoptosis via caspase-3 and PARP cleavage. Co-treatments induced apoptosis through PARP activation mechanistically. Overall, resveratrol’s growth inhibitory effects in FLT3-ITD + AML cells are mediated by inhibiting SK-1 and GCS, which further intensify resveratrol’s activity.

Keywords: FLT3+ AML, resveratrol, glucosyl ceramide synthase, sphingosine kinase

ÖZET

RESVERATROL'ÜN FLT3+ AKUT MİYELOİD LÖSEMİDE TERAPÖTİK POTANSİYELİ VE RESVERATROL TARAFINDAN TETİKLENEN APOPTOZDA SERAMİD METABOLİZMASININ ROLÜ

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Biyomühendislik Anabilim Dalı Yüksek Lisans
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Resveratrol'ün FLT3-ITD+ AML hücreleri üzerindeki büyüme engelleyici etkilerinin altında yatan mekanizmalar, seramid metabolizması hedeflenerek araştırıldı. Resveratrol, SK (sfingosin kinaz) inhibitörü (SKI II), GCS (glukosilseramid sentaz) inhibitörü (PDMP)'nin tek başına ve kombinasyon halinde MOLM-13 ve MV4-11 hücreleri üzerindeki antiproliferatif, apoptotik ve sitostatik etkileri sırasıyla MTT, akış sitometrik Annexin-V/PI boyama ve PI boyama ile araştırıldı. Resveratrol muamelesi sonucu kaspaz-3 ve PARP kesimleri, GCS ve SK-1 protein ifadeleri ve kombinasyon muameleleri sonucu PARP kesimi western blot ile kontrol edildi. Kombinasyon indeksleri CompuSyn yazılımı ile hesaplanmıştır. Resveratrol'ün tek başına ve SKI II ve PDMP ile kombinasyonları, aditif veya sinerjik etkilerle hücre proliferasyonunu baskılamış, apoptozu indüklemesi ve hücre döngüsü ilerlemesini durdurmuştur. Resveratrol, GCS ve SK-1 ifadesini baskılamış ve kaspaz-3 ve PARP kesimi yoluyla apoptozu indüklemiştir. Kombinasyon muameleleri PARP aktivasyonu yoluyla apoptozu indüklemiştir. Sonuç olarak, resveratrolün FLT3-ITD + AML hücrelerinde büyümeyi baskılayıcı etkisi, SK-1 ve GCS'ın inhibe edilmesi aracılığı ile olmuştur ve bu iki enzimin inhibisyonu resveratrolün aktivitesini arttırmıştır.

Anahtar kelimeler: FLT3+, AML, Resveratrol, Glukosil Seramid Sentaz,, Sfingozin Kinase

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LIST OF ABBREVIATIONS

AML	acute myeloid leukemia
SL	sphingolipids
Cer	ceramide
SK	sphingosine kinase
GCS	gulucosyl ceramide synthase
Res	resveratrol
SKI II	sphingosine kinase inhibitor II
FLT3	FMS-like tyrosine kinase-3
TK	receptor tyrosine kinase
ITD	internal tandem duplication
TKIs	tyrosine kinase inhibitors
S1P	sphingosine-1-phosphate
GC	glucosylceramide
SPT	Serine palmitoyltransferase
CerS	ceramidesynthase
GCase	glucoslyceramidase
CDase	ceramidases
GCS	glucosyl ceramide synthase
WHO	World Health Organization
FLT3-ITD	tyrosine kinase mutations
TRM	treatment-related mortality
HSCT	hematopoietic stem cell transplantations
allo-SCT	allogenic stem cell transplantation
auto-SCT	autologous stem cell transplantation
mAbs	monoclonal antibodies
CAR	chimeric antigen receptor
PRMTs	protein arginine methyletransferases
KATs	lysine acetyl transferases
BCL2	B-cell leukemia/lymphoma-2
BH3	BCL-2 homology 3

MCL-1	myeloid cell leukemia-1
RUNX1	Runt-related transcription factor
STAT3	signal transducer and activator of transcription 3
RTK	receptor tyrosine kinase
TKD	tyrosine kinase domain
PDGF	platelet derived growth factor
Ig-like	immunoglobulin like
FLK-2	fetal liver kinase 2
NK	natural killer
DC	dendritic cells
JM	juctamembrane
MOM	mitochondrial outer membrane
C1P	ceramide-1-phosphate
CERT	ceramide transport protein
SPT	serine palmitoyltransferase
DEGS	dihydroceramide desaturase
CerS	ceramide synthase
SKs	sphingosine kinases
GCS	glucosylceramide synthase
C1P	ceramide-1-phosphate
MOMP	mitochondrial outer membrane permeabilization
SM	sphingomyelin
MMP	matrix metalloproteinase
EMT	epithelial to mesenchymal transition
NO	nitric oxide
CML	chronic myeloid leukemia
AKT	serine/thereonine kinase
iNOS	inducible nitric oxide synthase

To My Family…

Chapter 1

Introduction

Acute myeloid leukemia (*AML*) is a heterogeneous disease which is characterized by abnormal accumulation of the myeloid progenitors (blasts) cells in the bone marrow and peripheral blood [1] which is commonly seen in adults. *AML* is classified based on genomic mutations, chromosomal translocations and epigenetic mutations. Those abnormalities cause accumulation of highly proliferated blast cells which have lost the ability of cellular differentiation due to structural chromosomal changes, changes in DNA copy numbers, and sequence mutations. FMS-like tyrosine kinase-3 (*FLT3*), a member of receptor tyrosine kinase (TK) family, plays a key role in the development of the hematopoietic and immune system. *FLT3* receptor is expressed in most pre-B-cell, myeloid and monocytic leukemia cell lines [2]. Activating mutations of *FLT3* are known as the most common molecular abnormality in *AML* and trigger several pathways related to hematological malignancies [3]. *FLT3-ITD* positive *AML* is characterized by the presence of the *FLT3* gene generated by internal tandem duplication (ITD) in juctamembrane (JM) domain which is associated with poor prognosis in *AML* [4]. *FLT3-ITD* deregulates *FLT3* downstream signaling pathways, which resulted in increasing cell proliferation and survival. Even if there are promising treatment strategies including conventional chemotherapy, allogenic stem cell transplantation, CART cell therapy, and tyrosine kinase inhibitors (TKIs), toxicity and the development of drug resistance are the main obstacles faced during the treatment [5]. Hence, the investigation of new signaling pathways to be targeted have been extensively paid attention such as sphingolipid signaling.

Sphingolipids are a major group of lipid family that act as important bioactive signaling molecules to regulate cellular functions such as cell growth, division, senescence, proliferation, cell signaling, immune system regulation, autophagy, migration, apoptosis and drug resistance [6,7]. Sphingolipid metabolism is composed of several important members including ceramide (Cer) as a central molecule, sphingosine-1-phosphate (S1P) and glucosylceramide (GC) [8]. Sphingolipids regulate several

mechanisms which are highly associated with cancer progression, therefore they may be targeted as anticancer treatments [9]. As the central molecule of sphingolipid mechanism, ceramide is synthesized by several pathways such as *de novo* and *salvage pathways*. Serine palmitoyltransferase (SPT) which is the key enzyme in *de novo pathway*, converts serine and palmitoyl CoA to Cer in endoplasmic reticulum. Also, ceramide synthesis is carried out in salvage pathway through metabolizing sphingosine into ceramide by ceramidesynthase (CerS) or the degradation of GC into Cer by glucoslyceramidase (GCase) [10]. All those pathways induce Cer accumulation which triggers apoptosis, autophagy or cell proliferation, whereas S1P or GC triggers cell proliferation or block apoptosis in cancer cells [11]. However, Cer accumulation is reduced by ceramidases (CDase) which convert ceramide to sphingosine than, sphingosine which is converted to S1P by sphingosine kinases (SK). The another enzyme called glucosyl ceramide synthase (GCS) reduces Cer level by converting Cer into GC. Since the Cer metabolism (anabolism/catabolism) determines the cell fate which is called as "sphingolipid rheostat", it is important to elucidate their roles in *FLT3-ITD* positive *AML* pathogenesis and therapy.

As a polyphenol, resveratrol (3,4',5-trihydroxy-trans-stilbene) is a natural phytoalexin which is mainly found in grapes, peanuts and blueberries [12]. Several studies have shown that resveratrol has anti-carcinogenic potential and plays key roles to suppress the proliferation of leukemia and solid cancer cells such as breast cancer, liver cancer, colorectal cancer, pancreatic cancer, prostate cancer [13] by targeting multiple and different cellular signaling pathways such as PI3K, AKT, mTOR, p53, MAPKs, NF-κB, STAT3 [14,15] and molecules based on the cancer type[14]. Nevertheless, the studies on the therapeutic potential of resveratrol and its working mechanisms in *FLT3-ITD AML* still remain unclear.

In this project, it is aimed to investigate the therapeutic potential of resveratrol in *FLT3-ITD positive AML* by targeting Cer metabolism. Besides, new potential therapy approaches are investigated by combining resveratrol with SK-1 inhibitor and GCS inhibitor.

1.1 Acute Myeloid Leukemia

AML is a heterogeneous stem cell disease of malignant immature myeloids and characterized by the accumulation of these myeloid progenitors (blasts) in bone marrow

or peripheral blood [1, 16]. *AML* is commonly seen in adults and approximately 75-60 % of the patients are older than 60 years old [17]. 40% of patients younger than 60 years has a chance to survive for 5 years while 5% of patients older than 60 years could survive less than 5 years [18]. The advances in the *AML* treatment have improved the outcomes in young people derived from off-target effects of chemotherapy adverse biological effects, different genetic background and host factors.

Evidences showed that the disease arises from several hematopoietic stem cell genetic alterations including chromosomal translocations and mutations in genes that control hematopoietic cell proliferation and differentiation (Table 1.1) [19]. These mutations influence the disease phenotype, responses to the therapies, the relapse risk and patient survival by causing differentiation block in the myeloid cells [20].

1.1.1 Classification

Pathological classifications are changed continuously by advanced researches and new findings based on the disease background. The classification of AML provides information about the disease and is used by researchers and physicians. A list of neoplastic diseases of hematopoietic and lymphoid cells has been published by World Health Organization (WHO) which classifies *AML* according to its genetic heterogeneity together with morphology, immunophenotype and clinical features (Table1.2) [21]. In early classification of WHO system subdivided *AML* into four categories: *AML* with recurrent genetic abnormalities, *AML* with myelodysplasia-related changes, therapy-related myeloid neoplasm and *AML* not otherwise specified [22]. In the renewed list, myeloid sarcoma, myeloid proliferation related to Down syndrome and acute leukemia of ambiguous lineage is included [23]. The cytogenetic findings and myelodysplastic changes in *AML* have been contributed to the classification of the disease which provides important prognostic knowledge about disease prognosis.

Table 1.1 WHO Classification of *AML* and related neoplasms [21, 23]

Lineage	Subtype
AML with recurrent genetic abnormalities	AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1 Provisional entity: AML with BCR-ABL1 AML with mutated NPM1 AML with biallelic mutations of CEBPA APL with PML-RARA AML with t(6;9)(p23;q34.1):DEK-NUP214 AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A Provisional entity: AML with mutated RUNX1 AML with t(8;21) (q22;q22.1);RUNX1-RUNX1T1 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
AML not otherwise specified (NOS)	AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Pure erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis (TAM) Myeloid leukemia associated with Down syndrome
Myeloid sarcoma	
Myeloid proliferations related to Down syndrome	Transient abnormal myelopoiesis (TAM) Myeloid leukemia associated with Down syndrome

AML classification has been also published by French American British (FAB) (Table1.3) based on morphological date. This list includes myelogenous, myelogenous and monocytic, monocytic, erythroid/myeloid and megakaryoblastic lineages. M1, M2 and M4 are the largest group among subtypes whereas, M3 and M5 have seen in 10 % of cases and M0, M6 and M7 account for less seen than 5% [24]. The percentage of blast account in the bone marrow or blood is an important parameter for *AML* diagnosing. Therefore, *WHO* classification requires blast threshold higher than 20% in blood whereas, blast amount in bone marrow or peripheral blood must be higher than 30% according to FAB classification to detect cytogenetic abnormalities. [25].

Table 1.2 FAB classification of AML [24, 26]

Lineage	**Abbreviated name**	**Fab subtype**	**Common name**
Myelogenous	AML	M0	Minimally differentiated acute myeloblastic leukemia
		M1	Acute myeloblastic leukemia (t(8;21)(q22,q22))
		M2	Acute myeloblastic leukemia (t(6;9))
	APL	M3	Acute promyelocytic leukemia
Myelogenous and monocytic	AMML	M4	Acute myelomonocytic leukemia
		M4eo	Myelomonocytic leukemia with bone marrow eosinophilia
Monocytic	AMoL	M5a	Acute monoblastic leukemia
		M5b	Acute monocytic leukemia
Erythroid/myeloid	AEL	M6a	Erythroleukemia
		M6b	Very rare pure erythroid leukemia
Megakaryoblastic	AMLL	M7	Acute megakaryoblastic leukemia

1.1.2 Pathophysiology

Although additional hematological disorders and prior therapy (aklylating agents or radiation) can cause *AML* [27], *de novo* malignancy has been observed in most cases. *AML* pathogenesis relies on abnormal proliferation and differentiation of myeloid stem cells due to chromosomal translocation, mutations and epigenetic factors. Even if a large chromosomal abnormality has not been seen, 97% of cases arise from genetic mutations

[28,29]. Animal model studies resulted in the improvement of a two hit model of leukemogenesis, which help to classifying several *AML* related mutations [30,31]. The two hit model is based on pathogenesis and interaction between different somatic alterations and chromosomal rearrangement [32]. In this model, while class I mutations like *FLT3-ITD, TKD, TP53, c-KIT*, cause the pro-proliferative pathway activation, class II mutations like NMP1, disrupt hematopoietic differentiation which is necessary for leukemia development (Table 1.3) [33]. This model shows that class I mutations and class II mutations must occur in together [34]. While class II mutations (*NMP1, CEBPA*) confer a better prognosis, high cytokine secretion such as IL-6 or tyrosine kinase mutations (*FLT3-ITD*) in class I cause tyrosine phosphorylation of STAT3 involved in cellular proliferation and survival [35], which is seen in almost 50% of *AML* cases with worse prognosis [36]. Class III includes mutations which involves DNA methylation related genes such as *DNMT3A, IDH-1, IDH-2* and is observed in 40% of *AML* cases [28]. Class II mutations (*FLT3-ITD*) might also occur together with DNA-methylation related genes in addition to class I mutations.

Table 1.3 Identified important genes involved in AML pathogenesis [37]

Class	**Pathway**	**Genes**
Class I	Signal Transduction	FLT3, KIT, KRas, NRas, JAK2, PTPN11
Class II	Differentiation	RUNX, CBFβ, CEBPA, NPM1, PU1, MLL, RARA
Class III	Epigenetic Regulation	TET2, IDH1, IDH2, ASXL1, EZH2
Other	Tumor Suppressor	TP53, WT1

1.1.3 Prognostic Factors

AML risk stratification is created based on chromosomal abnormalities and genomic mutations for physicians to decide for the right therapy. WHO classifies *AML* according to background of the disease such as clinical factors which includes the risk of treatment resistance, treatment-related mortality (TRM), prognostic factors, cytogenetic changes including chromosomal rearrangements (translocation, insertion) and gene mutations. For example (8;21)(q22:q22) and inv(16)(p13q22)/t(16;16)(p13q22), resulted in the translocation of RUNX1-RUNXT1 and CBFB-MYH11, respectively [38], which are in the cytogenetically risk group and can be treated by combination therapies. However

complex karyotypes such as t(9;11)(p21. 3;q23. 3) involves the translocation of MLLT3-KMT2A with intermediate prognosis and treatments can be harder. The worst, adverse (poor) risk group, t(9;22)(q34. 1;q11. 2) includes the translocation of BCR-ABL1, which is associated with chemo-resistant phenotype and poor prognosis (Figure 1. 1) [39].

Figure 1.1 Risk stratification of AML based on cytogenetic features based on the European Leukemia Net commendation [23]

Identified *AML* mutations significantly have been less than those seen in most solid-tumor, however one or more driver mutation is found 96% of patients with *de novo AML* and 2 or more driver mutations exist in 86% of patients [28,40]. To date, great progress has been made to understand *AML* genomic landscape and alterations that define disease phenotype and prognosis [41]. Frequently mutated genes in *AML* and their prognostic and therapeutic implications are summarized in Figure 1.2.

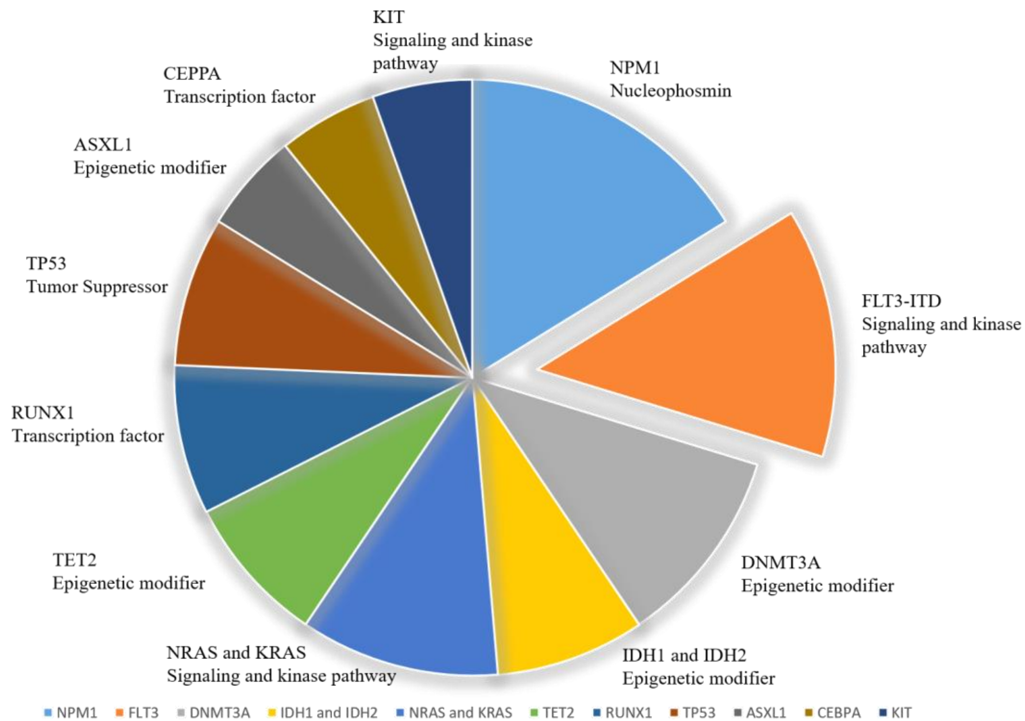


Figure 1.2 The percentage of commonly seen AML mutations [42]

1.2 AML Therapy

AML is the most common form of leukemia in adults with the patients are older than 60 years old. Even if there are improvements in survival during the last decade for *AML* patience among young, prognosis of elderly patience is still remaining poor. Chemotherapy affects elderly patients more than younger patients. Patients who are older than 60 years old, have a lower complete response rate after intensive chemotherapy and a much higher relapse rate [43]. In comparison, multidrug resistance was found in 33% of *AML* patients younger than age 56 (17% favorable cytogenetics) and 57% in patients older than 75 (4% favorable cytogenetics) [44]. The difference in treatment outcome results in different treatment strategy according to disease background, comorbidities and age of patience. It is vital to determine which prognostic subgroups will be benefitted more from treatment. Several new agents have been started to be used in clinical trials and some show promising outputs (Figure 1.3).

Protein kinase inhibitors
- FLT3 inhibitors (midostaurin, quizartinib, gilteritinib, crenolanib)
- KIT inhibitors and PI3K/AKT/mTOR inhibitors

Epigenetic modulators
- New DNA methyltransferase inhibitors (SGI-110)
- HDAC inhibitors and IDH1, IDH2 inhibitors

Chemotherapeutic agents
- Nucleoside analogs

Mitochondrial inhibitors
- Bcl-2, Bcl-xL inhibitors
- Mcl-1 inhibitors

Therapies targeting oncogenic proteins
- Fusion transcrips targeting
- Hedgehog inhibitors

Antibodies and immunotherapies
- Monoclonal antibodies against CD33, CD44,
- CAR T cells or genetically engineered TCR T cells

Figure 1.3 Novel therapies in clinical development in AML [21]

There is a strong relationship between several diseases and sphingolipid metabolism SL metabolic enzymes such as GCS and SKs play a key role in tumorigenesis and resistance to therapy in haematological malignancies [45] (Table 1.4). Stimulus including tumor necrosis factor, fas ligand, stress, chemotherapeutics can cause accumulation of Cer which lead to several molecular mechanisms such as apoptosis [6]. To understand sphingolipid-regulated processes in cancer development and progression is crucial for improve anti-cancer therapeutics.

Table 1.4 The role of SL enzymes in hematological malignancies [45]

Enzyme	**Malignancy**	**Role**
Sphingosine Kinase 1 (SK1)	AML ALL	Overexpressed in patients and increase drug resistance Overexpressed in patients
Sphingosine Kinase 2 (SK2)	ALL	Promote B-ALL disease progression and promote Myc expression.
Glucosyleceramide Synthase (GCS)	AML CLL	Overexpressed in cell resistance to chemotherapy Upregulated in response to B-cell receptor stimulation
Ceramide Synthase	AML	Suppressed by FLT3 signaling and mediated cytotoxicity of FLT3 inhibitors
Acid Cermidase	AML	Increased expression in patient and modulate Mcl-1 expression

1.2.1 Conventional chemotherapy

The standard *AML* treatment is based on two phases as remission induction therapy and post-remission therapy (Figure 1.4). Remission induction therapy is the initial step and aimed to reduce cancer cell amount in the blood by inducing remission. The majority of *AML* patients achieve a complete remission (CR) after induction therapy [46]. Cytarabine and anthracycline (daunorubicin or idarubicin) have been used for forty years as an induction therapy for *AML* [47]. This treatment includes 7 days infusion of standard-dose of a cytarabine with 3 days of an anthracyclines such as daunorobicin which is called as '7+3' regimen [48,49]. Cytarabine inhibits both DNA and RNA synthesis [50]. CPX-351 a liposomal formulation of cytarabine, was evaluated in phase II and phase III trials in *AML* [51–53] CPX-351 has been associated with good prognosis and overall remission rate in 60-75 years old patient diagnosed with secondary *AML* with MDS-related chromosomal abnormalities [47]. The anthracyclines inhibit the progression of topoisomerase II and DNA replication by intercalating with DNA [50].

Remission induction therapy must be followed by post-remission therapy. Post-remission therapy is important to prevent relapse by reducing the number of cancer cells which may remained in the body and to achieve a durable CR. If treatment does not continue with post-remission therapies, it is highly possible that relapse and less survival [54]. Post-remission therapy includes consolidation therapy and hematopoietic stem cell transplantations (HSCT). Consolidation therapy known as intensive chemotherapy, includes high dose of cytarabine which is used alone or combined with anti-tumor antibiotics such as daunorubicin, idarubicin, cladribine, mitoxantrone and thioguanine [53–57]. HSCT can be applied as allogenic stem cell transplantation (allo-SCT) which requires matched blood donor and autologous stem cell transplantation (auto-SCT) which does not require blood donor since patients' own stem cell is used [46]. It is recommended to treat the patients with poor or adverse risk, using allo-SCT if proper donor is available. Patients with favorable-risk group, can be treated with consolidation therapy or auto-SCT [21, 58, 59]. Allo-SCT is the most effective post-remission therapy for the prevention of relapse in *AML* [46, 58,60] . Allo-HSCT has been one of the most successful and widely used stem cell immunotherapy for *AML* for a long time [54, 61, 62]. Allo-HSCT is based on two purpose, replacing an abnormal hematopoietic system with one from a healthy donor and delivering of myeloablative (high-intensity) doses of radiation and

chemotherapy to cure hematologic malignancy [63]. However older patients are not good candidates for allo-HSCT because of its toxicity, complications and high relapse rate [64].

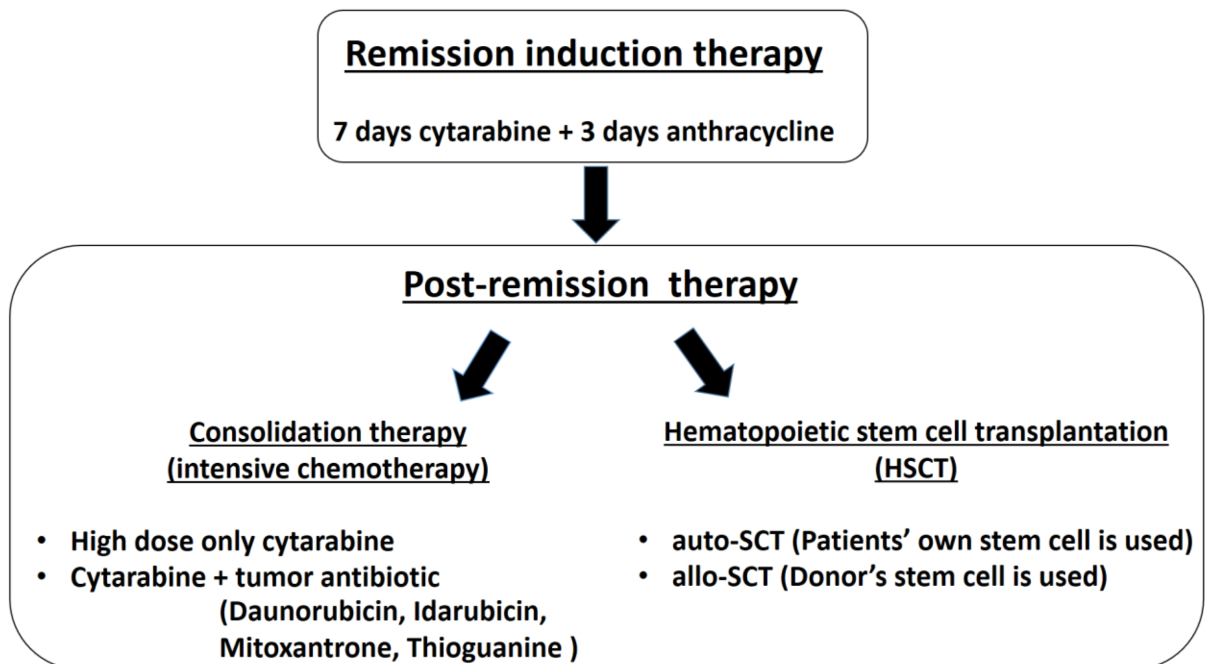


Figure 1.4 AML conventional chemotherapy diagram

1.2.2 Immunotherapy

Evasion from the immune system is a key mechanism of cancer cells to promote malignant progression. Therefore, targeting immune pathways have gained the importance for hematological malignancies especially for *AML* [65]. Immunotherapy has been effective in *AML* treatment and includes allogenic stem cell and donor lymphocyte infusion [66,67].

Immune system identifies foreigns and produces several antibodies in response to antigens. These antibodies recognize specific epitopes on antigens with high specificity. Monoclonal antibodies (mAbs) are made by identical immune cells that are all clones of a parent cell. mAbs are produced in laboratory by using mice spleen which is vaccinated with target antigen to stimulates B-cell which produces antibodies against to target antigen. These synthetic antibodies have anti-tumor activity to act against membrane proteins of cancer cell therefore mAbs have been used as a potential chemotherapy because of their ability to work as a targeted therapy [68]. *AML* cells express several antigens CD45, CD33, CD13 as myeloid markers, and especially CD56 expression in

t(8:21) *AML* is associated with a higher rate of relapse [49, 69]. These markers provide potential therapeutic targets. A humanized mAb which is specific for CD33, gemtuzumab ozogamicin had been used for 10 years [70] and Fc-engineered CD33 mAb have been used in *AML* treatment [71].

Due to some poor outcomes, chimeric antigen receptor (CAR) T-cell was developed to enhance the efficacy of allo-HSCT [72]. CART is a synthetic T-cell receptor which has antibody-like specificity by combining a single- chain variable fragment with a T-cell receptor having a trans-membrane and intracellular domain. CART cell therapy can provide a new insight for the treatment of patients who has relapsed or refractory *AML* [73] . CD33 and CD128 antigens are presents in hematopoietic stem/progenitor cells (HSPC) and two major targets for CART therapy in *AML* [74,75]. It is reported that patients had a transient response to CART33 and demonstrated a clinical response to a combined CD33-CLL CART (C-type lectin molecule-1) [76,77]. Since CD123 is an important antigen due to its expression on hematologic malignancies, CART123 have been used to target *AML* [77].

1.2.3 Epigenetic regulators

AML is genetically heterogeneous disease therefore diverse combination of specific driver mutations make treatment harder and have a huge impact on disease prognosis and response to treatment [78]. Besides, several patients have at least one mutation in an epigenetic modifying protein which plays important role in epigenetic regulator pathway [79]. Therefore, clinic studies also try to target and restore the activity of several epigenetic regulators such as TET2, ASXL1, DNMT31, lysine acetyl transferases (KATs), protein arginine methyletransferases (PRMTs) [80] . For example, ASXL1 leads to myeloid leukemogenesis through aberrant methylation, demethylation and acetylation [47]. Allogenic hematopoietic stem cell transplantation may be therapeutic option for *AML* with ASLXL1 mutation. Also pharmacological inhibition of H3K27 (histone 3 at lysine 27) demethylation can be another potential therapeutic approach in *AML* with ASLX1 mutation [81].

1.2.4 Pro-apoptotic agents

B-cell leukemia/lymphoma-2 (BCL2) is an anti-apoptotic protein responsible for the promotion of leukemic blast survival by regulating mitochondrial apoptotic pathway. BCL-2 homology 3 (BH3) is an antagonist protein of anti-apoptotic proteins and

promotes apoptosis through mitochondrial outer membrane permeabilization [82]. Venotoclax, a BCL2 inhibitor, targets BCL-2 inhibition by BH3 profiling and patient with IDH mutations gives more response to venotoclax [83]. Venotoclax is used in combination with some other drugs such as azacitidine or decitabine in elderly people who has less advantage in standard chemotherapy.

Myeloid cell leukemia-1 (MCL-1) is an another protein which blocks proapopototic proteins such as BAX and BAK. MCL-1 inhibitor agents have still been tested in clinic and its upregulation causes a resistance mechanism to venotoclax. Therefore, venotoclax and some other inhibitors has been planning to be used together [84].

1.2.5 Transcription factor inhibitors

p53 is a transcription factor which maintains genetic stability and cell growth by regulating DNA repair after DNA damage. More than 50% of human cancers possess a TP53 gene missense mutation which leads to protein unfolding and prevents responding of transcription factors to cell cycle arrest and apoptosis under cellular stress [81]. p53 mutations are found in 18% of all *AML* with complex karyotype and causes less survival rate [85–87]. APR-246 which is one of the agents, targets to mutated p53 by covalently binding to the p53 core domain and promote refolding to fulfill the wild type p53 function, therefore triggers cell cycle arrest and apoptosis [88].

Runt-related transcription factor (RUNX1) family consists of RUNX1, RUNX2 and RUNX3 members which form a complex and regulate transcription [89]. RUNX1 regulates the expression of hematopoiesis-associated proteins and cell cycle-associated proteins. Studies suggest that the tumor-promoting transcription factor RUNX1 may be targeted in cancer treatment [89]. New strategy for targeting RUNX uses alkylating agent conjugate pyrrole-imidazole (PI) polymide which inhibits gene transcription by binding to RUNX consensus sequence [89].

Signal transducer and activator of transcription 3 (*STAT3*) which is another transcription factor, regulates expression of anti-apoptotic proteins and cell cycle regulator proteins. JAK-STAT pathway is essential in normal hematopoiesis and hematopoietic malignancies [90]. *STAT3* tyrosine phosphorylation is upregulated in 50% *AML FLT3* receptor ligand result in increase of *STAT3* stimulation and phosphorylation and cause *FLT3 TKI* resistance. C188-9 as one of the *STAT3* inhibitor, decreases *STAT3* phosphorylation and induce apoptosis in *AML* cells [91]. Small molecule FLT3 tyrosine kinase inhibitors used as single agents or in combination with chemotherapy. The

induction of cytotoxicity by *FLT3* inhibition is related with the deactivation of STAT since *FLT3* inhibition is correlated with persistent activation of STAT5 [92] .

1.3 FLT3-ITD Positive Acute Myeloid Leukemia

The regulation of maturation, intracellular communication, proliferation, and differentiation of hematopoietic cells are regulated by membrane bound proteins, growth factors and colony stimulating factors. These hematopoietic regulators mediated their functions via specific receptors which are divided into groups. Ones have intrinsic tyrosine kinase domain (TKD) called as receptor tyrosine kinase (RTK). The growth factor RTK is divided into several classes: class I RTK is represented by erbB2/HER family, class II RTK includes dimeric receptors such as insulin receptors. Class III RTK includes FMS, KIT, platelet derived growth factor (PDGF) [93]. *FLT3* also belong to this group with strong sequence similarities by sharing an extracellular domain comprised of five immunoglobulin like (Ig-like) domains [31]. Class IV RTK includes fibroblast growth factor receptor (FGFR) and FMS-like tyrosine kinase (*FLT*).

Fms-like tyrosine kinase 3 (*FLT3*) is a receptor tyrosine kinase which is expressed by immature hematopoietic cells and plays key roles in the development of stem cell and the immune system. *FLT3* is also known as fetal liver kinase 2 (*FLK-2*) and cluster of differentiation antigen 135 (CD135) [94] (Figure 1.5). *FLT3* localizes on chromosome 13q13 and encodes a 993-amino acid protein in human [95]. It is expressed on 70-100 % of *AML* and >90% of Acute Lymphoblastic Leukemia (ALL) blasts [31]. *FLT3* ligand (*FL*) is expressed by marrow stromal cells and other cells to influence *FLT3* receptor. *FLT3* ligand (*FL)* is a type I trans-membrane protein which is released as a soluble homodimeric protein and act as a growth factor for immature myeloid cells and stem cells. Studies proved that *FL* also plays a key role in immune response by increasing the amount of myeloid progenitor cells as well as dendritic (DC) cells and natural killer (NK) cells. *FLT3* is composed of extracellular domain, transmembrane domain, juctamembrane (JM) domain and TKD (Figure 1. 6).

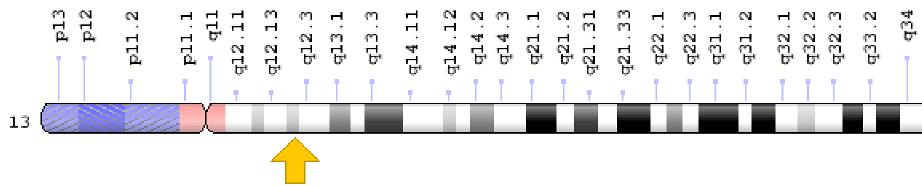


Figure 1.5 Representation FLT3 location on chromosome 13q12[96]

Molecular alterations cause continuing activation of *FLT3*. Two main mutations are detected in which *FLT3* domains have gained sustained kinase activation. One of these mutations is the point mutation in *TKD* which presents in approximately 30% of *AML* cases [97]. The another one is internal tandem duplication (*ITD*) which takes place in between exons 11-12 as the duplication of 3-100 amino acids where JM domain is synthesized [95 ,97]. ITDs represent the most common type of *FLT3* mutations and are associated with poor prognosis in *AML* by altering activation of the enzyme. JM domain is associated with autoregulation of the catalytic activity of *FLT3*. The expression of *ITDs* causes aberrant signaling because of the additional tyrosine residues [98].

After activation of *FLT3* it regulates hematopoiesis through activation of oncogenic pathways including Ras Raf/MEK/ERK, PI3K/Akt/mTOR and phosphorylation of downstream targets such as STAT3/5 [99] (Figure 1.6). HRas, NRas, and KRAs are members of Ras proto-oncogene family and frequently mutated genes in human cancer. Ras small GTPases act as molecular switches to modulate signal transduction and regulates diverse cellular processes such as proliferation, survival, cell cycle regulation, cell migration, and angiogenesis [100, 101]. Mutation in NRas and KRas genes account for more than 30% of pediatric *AML* patients [102]. PI3K/Akt/mTOR pathways is a central pathway for hematopeietic cells and regulates metabolic reprogramming, proliferation differentiation and survival. It is demonstrated that oncogene activating mutations, oncogene amplifications, upstream activation of RTKs, inactivation of tumor suppressor genes cause dysregulation of the PI3K/Akt/mTOR signaling and increase oncogene activity in *AML* [103,104]. PI3K/Akt/mTOR pathway has an oncogenic role in *FLT3*-mutated *AML* cells and decrease overall survival in *AML* patients (figure 1.6) [105].

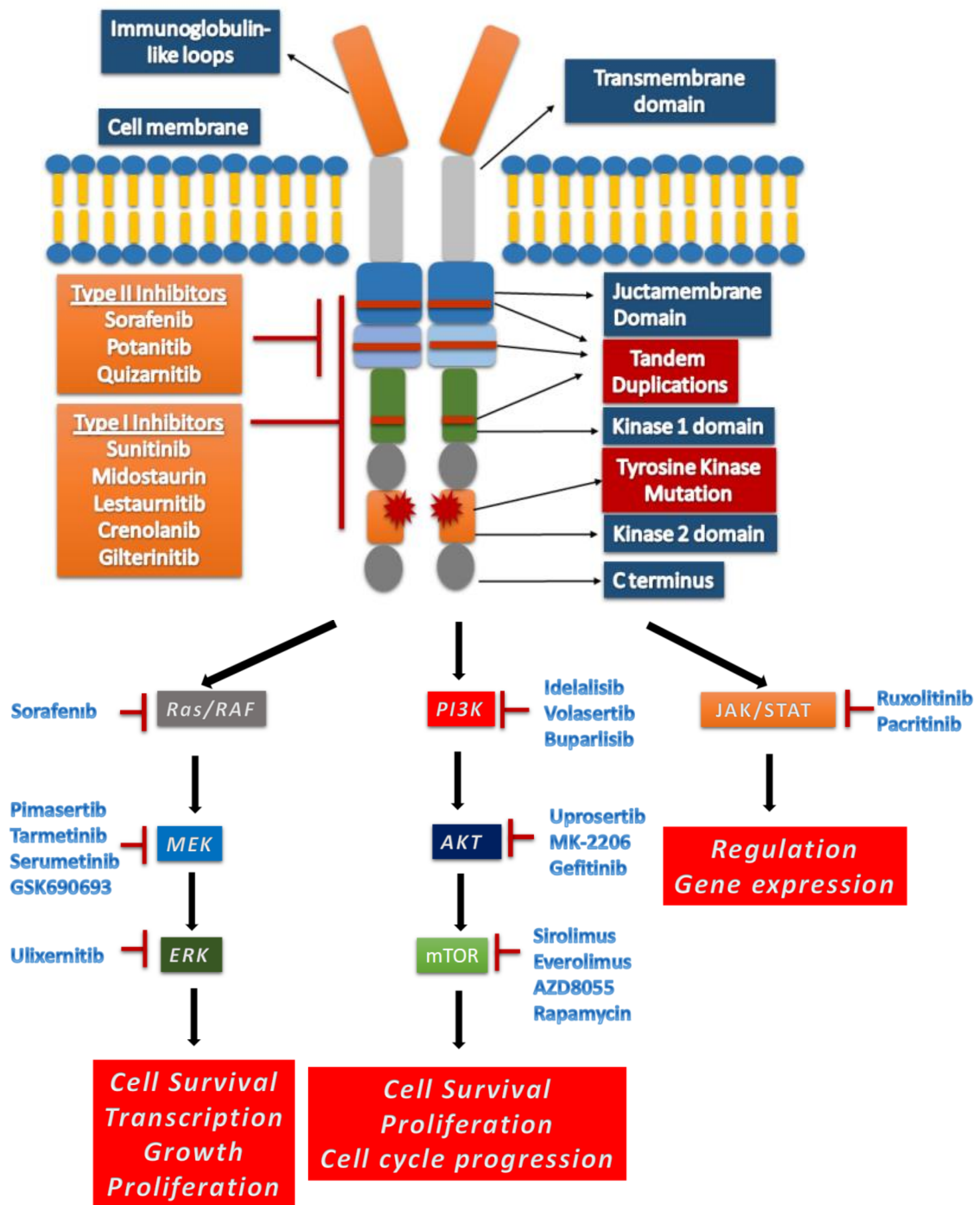


Figure 1.6 The structure of *FLT3* receptor. Localization of Inter Tandem Duplication (ITD) and Tyrosine Kinase (TK) mutations. Type I inhibitors inhibit ITD part of *FLT3* receptor in JM domain and Type II inhibitors inhibit TK part of *FLT3* receptor in kinase domain. *FLT3-ITD* and *FLT3* receptors trigger several pathways which increase cell survival and proliferation [4, 106]

JAK/SAT pathway is a crucial intracellular signaling cascade which controls gene expression by transducing extracellular signals to the nucleus. JAK/SAT pathway works

in collaboration with several cytokines and growth factor to regulate hematopoiesis, fertility, embryogenesis and growth [107–111]. It is reported that STAT3 signaling is upregulated in 20-50% of *AML* patients [112]. Also, constitutive activation of *FLT3* by *FLT3-ITD* mutations leads to aberrant activation of STAT5 downstream pathway [92] (Figure 1.6).

1.3.1 FLT3 AML Therapy

FLT3 is expressed on early myeloid and lymphoid progenitor cells and activated by FL to regulate proliferation and differentiation of hematopoietic cell. *FLT3* mutations occur in around 30% of *AML* patients. *FLT3-ITD* results from head-to-tail duplication of several amino acids within JM.and duplication presents in 20-25% of *AML* patients. *FLT3 TKD* mutations are usually present within the activation segment and found in 5-10% of AML patients [50]. For these reason, *FLT3-ITD* and *FLT3 TKD* inhibition is a potential therapeutic target for *FLT3*-A*ML* treatment. Several different *FLT3* inhibitors have been investigated for clinical use including sunitinib, sorafenib, midostaurin, quizarnitib, crenolanib, gilterinib (Figure1. 7).

Sorafenib as a multikinase inhibitor that is used in clinical trials in combination with induction chometherapy in AML patients [113] or after allo-HSCT [50]. It is approved by FDA for the treatment of hepatocellular, renal cell, differentiated thyroid cancer and AML. In a study group which includes 17% of *FLT3*-mutated AML patients, it was shown that sorafenib combined with stardart 7+3 regimen chemotherapy do not improve event –free survival (EFS) in >60 years old newly diagnosed AML patients, however in younger patients, sorafenib with combined standart induction chemotherapy significantly prolonged EFS [113].

Sunitinib is a small first generation active inhibitor of multiple tyrosine kinase receptors. Sunitib inhibits the signal transduction pathway which is responsible for cell proliferation by blocking of the split-kinase domain family of RTKs such as *FLT3*, PDGFR, VEGFR1/2 and KIT. Sunitib induces G1 phase arrest associated with decreased cyclin D1, cyclin D3 and Cdk2. Besides, sunitinib induces monocytic differentiation and apoptosis by increasing the expression of pro-apoptotic molecules (Bax, Bak, Fas, FasL, DR4, DR5) and decreasing anti-apoptotic molecules' expression (Bcl-2, Mcl-1) in AML cells [114]. It is demonstrated that administration of sutinib in the MV4-11 model of human AML results in sustained inhibition of *FLT3* signaling and prolonged patients'

survival [114]. A study showed that STAT5 phosphorylation was reduced in *FLT3-ITD* patients after sunitinib treatment [115].

Midostaurin is a multitargeted kinase inhibitor with inhibitory activity against *FLT3-ITD* and *FLT3-TKD* mutants. In some studies, *FLT3*-mutated AML patients had been treated with midostaurin and patients' peripheral blood blast were reduced, even if no patient achieved a CR[116]. It may not induce remission but it has biological activity to reduce blast amount in blood and bone marrow. It has been demonstrated that an overall survival benefit has achived in combination with induction chemotherapy for newly diagnosed patients [47 ,115]. Another study showed that midostaurin with intensive chemotherapy can be used in older patients because it significantly increased event-free-survival rate in *FLT3-ITD AML* [117].

Quizarnitib is a next-generation potent and selective *FLT3* inhibitor. It possesses a good bioavailability and its half-life can last until 24 hours. Therefore, it is able to treat more than 40% of AML patients when it is used as single agent [50]. It is shown that quizarnitib yielded 53% overall response rate in *FLT3-ITD* positive patients [118]. Evidences suggest to use quizarnitib monotherapy for the treatment for *FLT3-ITD* mutated relapsed/refractory (R/R) AML [119].

Crenolanib is a potent and selective *FLT3-WT, FLT3-ITD, FLT3-TKD, PDGFR*, KIT inhibitor. When it is compared with quizarnitib, crenolanib is less disruptive to erthroid colony growth which results in less myelosupression. Clinical trials showed that combination of crenolanib with induction chemotherapy (cytarabine and antracycline) inhibits *FLT3*-mutant in newly diagnosed AML[115]. An interesting study showed that CART cell targeting *FLT3* acts synergistically with crenolanib which leads to increased surface expression of *FLT3-ITD AML* cells and enhanced recognition by *FLT3-CAR T*-cells. *FLT3-CAR T*-cells recognize normal HSCs and disrupt normal hematopoiesis as well. Therefore, it is required to apply allo-HSCT to reconstitute the hematopoietic system [120].

Gilternitinib (ASP2215) is a dual *FLT3-ITD* and *FLT3-TKD* inhibitor and reduces the colony-forming capacity of *FLT3-ITD AML* cells by degreasing phosphorylation levels of *FLT3* and its downstream targets without toxicity [115]. Gilternitinib has demonstrated significant single-agent activity in R/R *FLT3-mutated AML* and achieving 40% overall response rate. Food and Drug Administration (FDA) approved gilternitib as

the first *FLT3* inhibitor to be used as monotherapy in *FLT-mutated R/R AML*. Since, it provided significant overall and event-free survival [121].

It is crucial to understand the molecular mechanism of *AML*, since it provides deeper information to design and target specific molecules for defined altered signaling pathways such as *PI3K, AKT, ERK, MEK, RAF* and new promising ones such as sphingolipid signaling.

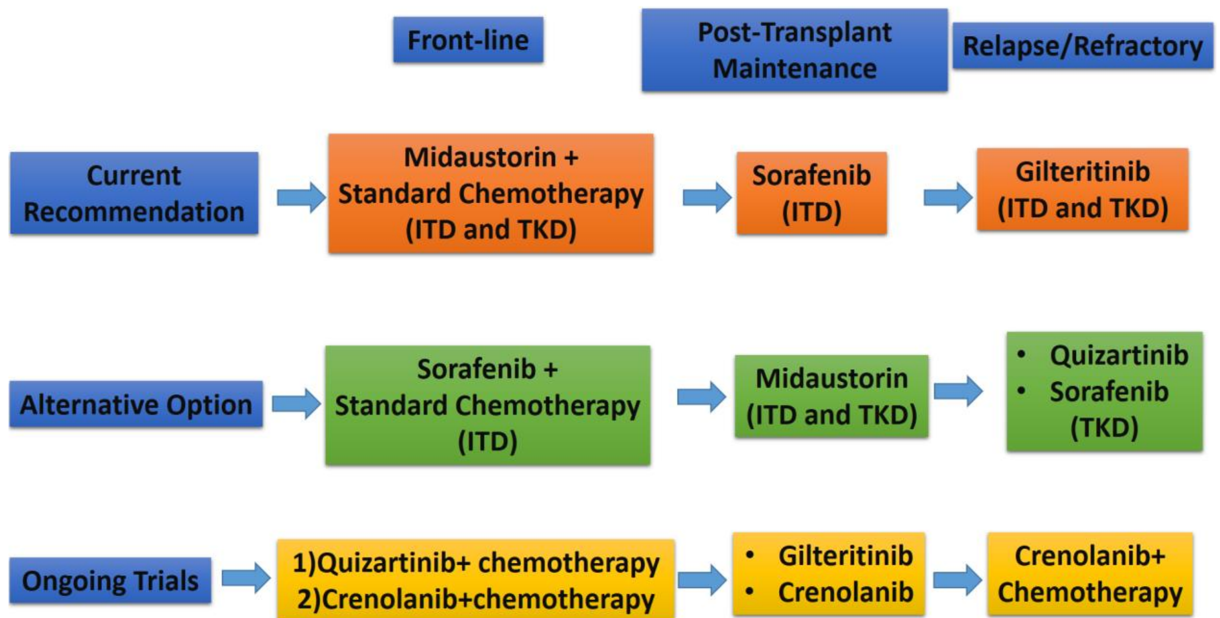


Figure 1.7 FLT3 inhibitors for FLT mutated AML patients [113]

1.4 Sphingolipid Metabolism

Sphingolipids (SLs), the structural components of cell membrane determine cell fate and function. SLs have diverse functions such as cellular signaling, senescence, immune system regulation, cell proliferation, protein sorting and direction, survival and apoptosis. SL regulation in organelles, membranes or cellular compartments contributes to pathological conditions including cancer, obesity, diabetes, oxidative stress, aging, neurodegeneration, and diabetes. Therefore, it could be a big potential target for therapeutic strategies and it has been reported to be biomarker in cancer and other human diseases [122]. SLs can alter mitochondrial morphology via biophysical effects on mitochondrial outer membrane (MOM) and inner membrane. The effects of SLs in mitochondria are quite relevant since alterations of mitochondrial functions have been

associated with cell death and signaling, chemo-resistance, cellular stress, autophagy and mitophagy [122]. SLs recognized as "bioactive lipids" modulate membrane integrity, activity of enzyme and act as ligands of G protein-coupled receptors [123]. The biological complexity of sphingolipids is highly associated with cell type-specific signaling and SL family includes ceramide (Cer), sphingosine-1-phosphate (S1P), sphingosine (Sph), ceramide-1-phosphate (C1P) and glucosylceramide (GC) as bioactive molecules.

Cer is a backbone molecule of sphingolipid metabolism and produced in the endoplasmic reticulum (ER) than transported to the Golgi apparatus by vesicular transport or ceramide transport protein (CERT). Cer has fundamental roles in biological processes such as cancer development, obesity, type2 diabetes mellitus, lipid storage, skin barrier function, senescence and neurological disorders [124]. Cer synthesis is based on two main pathways, *de novo* pathway and salvage pathway. The *de novo* pathway takes places entirely in endoplasmic reticulum (ER) by four step reactions and starts with the condensation of serine and palmitoyl coenzyme A by serine palmitoyltransferase (SPT) to generate 3-keto-dihydrosphingosine. Subsequently, 3-keto-dihydrosphingosine is reduced to form dihydrosphingoanine which is then converted to dihydroceramide by ceramide synthase (CerS). Finally, Cer is formed by dihydroceramide desaturase (DEGS) from dihydroceramide (Figure 1.8). The ER and mitochondria are the central sites for Cer-induced apoptosis since Cer produced through *de novo* pathway in ER may also diffuse to the mitochondria and regulate apoptosis [125,123].

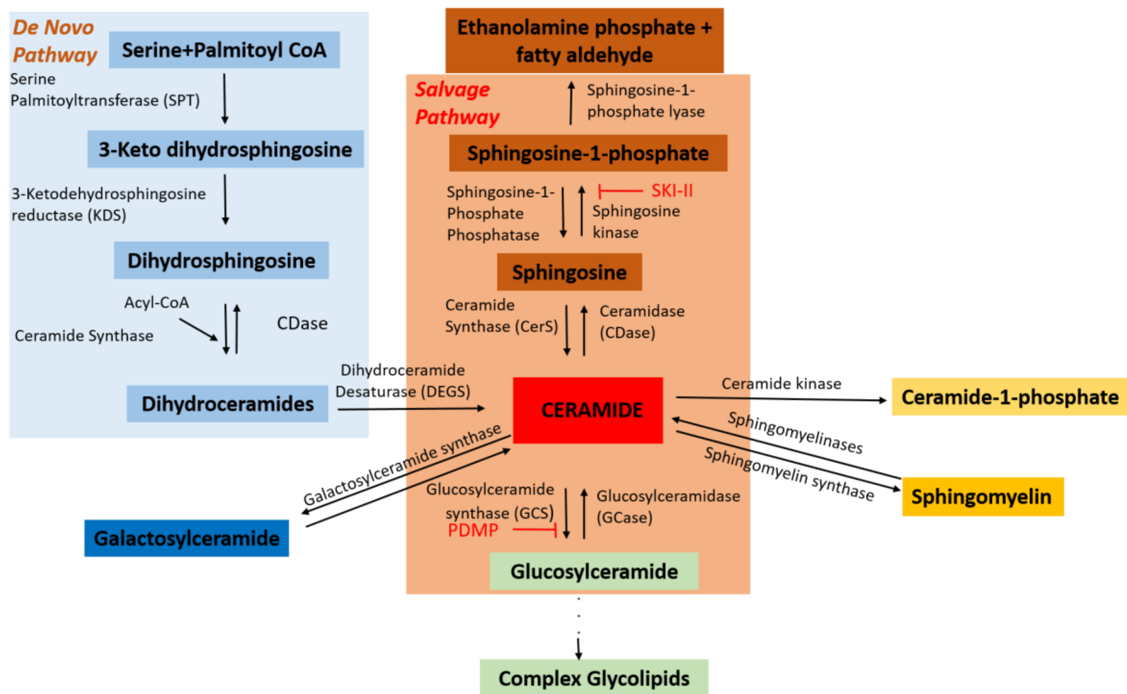


Figure 1.8 The diagram of Sphingolipid metabolism. ***De novo*** **and** ***Salvage pathways*** **are shown** [126]

Cer than can be modified to several SL species to modulate membrane composition and signal transduction. In salvage pathway, Cer is deacetylated by CDase to form sphingosine and subsequently, through the action of sphingosine kinases (SKs), generate S1P. Cer is glucosylated in golgi apparatus by glucosylceramide synthase (GCS) to GC which is intermediary molecule for other complex glicolipids (Figure 1.9). Cer generation made also occur through direct N-acyltion of sphingosine in the ER [123].

Cer can also be synthesized through hydrolysis from sphingomyelin (SM) by sphingomyelinases (SMase) in plasma membrane or lysosome. However, converting of Cer to sphingomyelin and ceramide-1-phosphate (C1P) by sphingomyelin synthase and ceramide kinase, respectively, takes place in golgi apparatus. SM is a major source for the rapid generation of Cer [125](Figure 1.8).

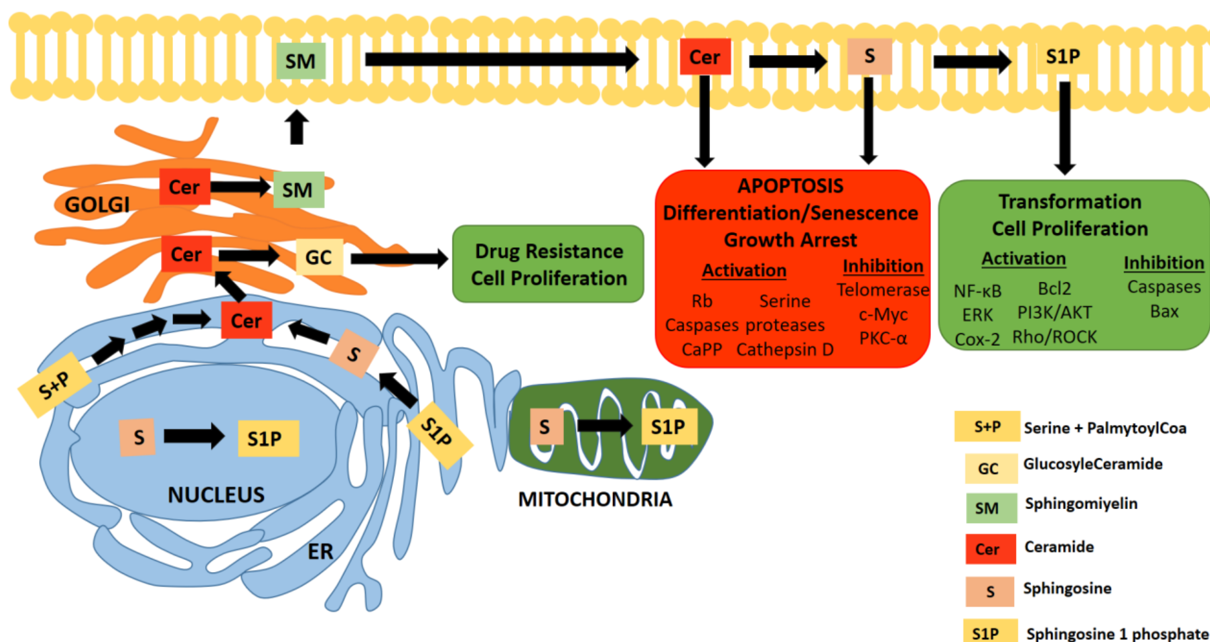


Figure 1.9 The diagram of cellular component where SLs are synthesize [127]

1.4.1 Sphingolipid Metabolism in Cancer

Bioactive SLs have potential roles in the regulation of biological processes which are important for cancer initiation, promotion, progression and response to treatment. Apoptosis, programmed cell death, is a vital process for cell homeostasis. Cancer cells have always been gained the ability to escape from apoptotic signals for tumorigenesis. Studies have been shown that Cer and S1P are cornerstones for lipid metabolism dependent cancerogenesis [128].

Cer acts as a secondary messenger in response to several extracellular stimuli such as growth factors, cytokines, stress (hypoxia, heat stress, irradiation), anticancer drugs and chemical agents. Several extracellular or intracellular stimuli cause increases in Cer level resulting in apoptosis. High Cer in ER could disrupt calcium ion homeostasis and cause ER stress-mediated apoptosis. Cer can induce mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release. It has been shown that Cer metabolites drive mitochondrial cytochrome C release and apoptosis by interaction with the proapoptotic molecules such as BAX and BAK [123]. Cer regulates regulatory pathways by coordinating some protein targets such as AKT, MAPK, PKC, RAS, c-RAF [129].

Cer acts as a tumor suppressor lipid and deficiencies in the generation of Cer cause cancer proliferation and resistance to cancer treatment. While Cer induces

antiproliferative and apoptotic responses in several cancer types, S1P supports cancer progression. Moreover, studies have showed [130] that multidrug resistance in cancer cells result in the increased expression of GCS which converts Cer to GC. Therefore, cell proliferation is increased in cancer cells. The balance between Cer and GCS/S1P determines the cell fate which is called as “SL rheostat”.

In previous studies it is shown that altered level of enzymes involved in sphingolipid metabolism is responsible for cancer pathogenesis [131]. In malignant astrocytoma, reverse correlation between Cer level and disease progression was identified [132]. Similaly, in ovarian tumor, the level of Cer is prominently decreased as compared to normal ovarian tissues. Cer and S1P can be used as diagnostic marker in cancer cells [11]. For instance, high levels of Cer in breast cancer is associated with less aggressive cancer [133] . S1P, Cer and other sphingolipid levels were significantly higher in breast tumor tissues than normal tissues [134]. Epidermal growth factor activation increased SK and therefore S1P levels [135].

It is shown that Cer treatments decreased the accumulation of c-Myc oncogene, increased the activity of PP2A tumor suppressor and enhanced the cytotoxic response to chemotherapy in prostate cancer [136, 137]. In glioblastoma, Cer triggered Fas related extrinsic apoptosis. Cer was responsible for downregulation of FLICE inhibitory protein negative regulator of Fas-FasL signaling [138, 139] and S1P level was higher and Cer level was lower than normal tissues [140,141]. C16 (ceramide 16) and S1P levels have been used as a diagnostic marker in hepatocellular carcinoma [142]. Melatonin increased the amount of Cer by Cer synthesis pathway and SPT inhibition by myriocin inhibited melatonin related autophagy in liver cancer cell and hepatocellular carcinoma [11, 143].

SK-1 is upregulated and SK-1 inhibition reduces proliferation, angiogenesis, metastasis and increases apoptosis in ovarian cancer [144]. Moreover, S1P, GC and SM have mitogenic effect and inhibit apoptosis in ovarian cancer [127]. In melanoma S1P promotes the differentiation of fibroblasts into myofibroblasts and promotes S1P/S1P3, which receptor is important for embryonic angiogenesis[145], dependent metastatic spread of melanoma cells [146]. S1P receptors, S1P1-S1P5 trigger several biological responses, regulate biological processes such as inflammation, angiogenesis, cancer growth, vascular permeability, metastasis [147,148].

Decreased SK1 expression and eliminated NF-κB/IL-6/STAT3 amplification can be useful for treating colon cancer with ulcerative colitis [149]. Cer synthesis has been

linked to apoptotic endothelial cell death and decreased pulmonary barrier function [150]. *De novo* Cer synthesis resulted in apoptotic cell death in lung endothelial cells by both paracellular and TNF-α stimulated intracellular Cer signaling [151,152] . It is revealed that increased SK1 expression and S1P level correlated with cellular migration and invasion in gastric cancer [153,154].

Table 1.5 Sphingolipid enzymes that are known to be differentially expressed or produced in cancer [155]

Sphingolipid enzymes	Inhibitors	Differential expression	Type of cancer
Acid Ceramidase	B13, LCL204, LCL385, NOE, D-MAPP	Increased	Lymphocytic Leukemia, Prostate
Sphingosine Kinase 1	SKI II, Dimethtlsphingosine, Dihydroxyaurone, Doxorubicin, Etoposide	Increased	Lung, Colon, Breast, Ovarian, Stomach, Uterine, Kidney, Glioblastoma, Lymphoma
Glucosyleceramidase Synthase (GCS)	PPMD, PDMP, 4-HPR	Increased	Leukemia, Breast, Melanoma, Neuroblastoma, Ovarian, Colon
S1P Phosphatase	-	Decreased	Colon
Ceramide Kinase	NVP-231	Increased	Liver, Breast, Lung
Serine Palmitoyl-coA Transferase (SPT)	Myriocin	Decreased	Colon
Sphingomyelinase	Desipramine, GW4869	Decreased	Colon, Liver

1.4.2 Effect of Sphingolipid Metabolism in Leukemia

The role of sphingolipid metabolism in cellular growth, apoptosis, differentiation and treatment resistance have been studied by in vitro models of myeloid and lymphoid leukemia [155]. The balance in ceramide metabolism is critical for cancer development [156]. The main product of Cer conversion to sphingomyelin (SM) is made by sphingomyelin synthase enzymes SMS1 and SMS2 (encoded by the SGM1 and SGM2 genes), which found in golgi and plasma membrane [148,157]. Studies show that SMS1 is mainly downregulated in several solid cancers including melanoma [158]. Cer

accumulation could be modulated by several sphingolipid metabolism enzymes such as SPKs, AC, GCS [159]. Therefore, new alternative treatment approaches have been developed to increase Cer mediated apoptosis in many cancer cells and drug resistant [129, 159–162]. One of the well-known and studied enzyme of sphingolipid metabolism in leukemic models is SK1 and SK2, which are isoenzymes. SIP metabolizes in a reaction by the S1P lyase enzyme located on the cytosolic side of ER (Table 1.8) In ALL , it is shown that SK2 has an oncogenic role and regulates MYC which has an vital role in hematological malignancies and promotes survival and proliferation [163]. Another study proved that inhibition of ceramide metabolism combining inhibitors of SK (SKI II) and GCS (PDMP) sensitized leukemic cells to inhibition of BCL2-like proteins which plays important role in regulation of apoptosis [164] .

1.4.3 Targeting Sphingolipid Metabolism

The important roles of anti-apoptotic and pro-apoptotic sphingolipids have been observed in many cancer cells which make them potential targets for therapeutic benefit. Several strategies have been developed to target sphingolipid metabolism including synthesizing long chain synthetic Cer analogs, S1P receptor antagonists and monoclonal antibodies against S1P small molecule enzyme inhibitors of SK, CDase and GCS to increase Cer accumulation and prevent convertion into antiapoptotic sphingolipids. Also other strategies include reactivate genes such as SPL, S1P phosphatase and SMase which are silenced in cancer tissues. These strategies are still in progress in clinical trials in cancer and several diseases [165, 166].

A novel strategy is to remove S1P by sphingomab which is a S1P monoclonal antibody, and preventing pro-angiogenic signaling and tumor- promoting effects of S1P signaling [166].

Recent studies with BML-258 which is a selective SK1 inhibitor, showed that it increased apoptosis in human U937 leukemia cells and decreased proliferation of leukemic blasts [167]. Besides, SK2 inhibition by ABC294640 could induce cell death through autophagy in kidney carcinoma, breast adenocarcinoma and prostate cell lines [168]. A family of naturally sphingolipids called sphingadiene compounds were shown to suppress tumor formation and to induce apoptotic and autophagic cell death trough AKT dependent mechanism in colon cancer[169].

FTY720 is an immune modulator however its antitumor effects on tumor growth, angiogenesis and apoptosis in several cancer types such as prostate, pancreatic, gastric,

hepatocellular and hematopoietic cancers have been shown in literature [170]. FTY720 targets sphingolipid metabolism and inhibits SK-1. In the presence FTY720, in addition to reduction in SK-1 activity, SK1 also undergoes proteosomal degradation which led to apoptosis in myeloma cells [171].

Studies show that sphingosine has promising effect on leukemic cells. In one study, it is shown that low concentrations of sphingosine was highly induced apoptosis in CMK-7, U937 leukemic cell lines [172]. As SK inhibitors, phytosphingosine derivatives promote apoptotic cell death in HL60 leukemic cells [173].

Recent studies have shown promising outcomes such as using CDases to increase Cer levels to induce cytotoxicity and decrease tumor growth [174–176]. Also a ceramide dependent mitophagy in response to *FLT3-ITD* inhibition in *FLT3-ITD AML* has been shown to be a novel cell death mechanism. Therefore, it may provide a novel anticancer treatment strategy to inhibit cancer proliferation and drug resistance [177].

To target sphingolipid metabolism is a growing field in the area of cancer treatment. The strategies that had been studied already created new information and challenging to achieve new insights such as inhibiting S1P enzymes to prevent SP1 signaling in tumor and tumor microenvironment, and to enlighten S1P catabolism and Cer synthesis within tumor. These topics are still remaining unclear and open for new researches for different cancer types.

1.5 Resveratrol and Its Potential in Cancer

Resveratrol ((3,5,4'-trihydroxy- trans-stilbene) is a naturally occuring phytoalexin found in more than 70 plant species including raspberries, grapes, mulberries and peanuts. The word *alexin* is “to protect” in Greek language bacause of its alexin-like protective activity. It is naturally produced by plants in responce to medical injury, UV, radiation and fungal infection. Therefore resveratrol posseses several biological activities such as anti-inflammatory, neuroprotective, antifungal, antiviral and especially anticancer properties. Resveratrol was first purely isolated in 1940, than it attracted more interest in 1990s to use as cardioprotective agent. The anti-leukemia property of resveratrol was reported firstly in 1997 and then several studies have been done to investigate the broad biological and pharmacological activities of resveratrol [178].

Since resveratrol is a naturally occurring compound, it is widely studied for the prevention and treatment of several cancer types including colon cancer, follicular lymphoma, rectal cancer, multiple myeloma, neuroendocrine tumor and liver cancer in combination with conventional chemotherapeutic agents or novel targeted therapy agents [179]. These studies have shown that resveratrol has anticancer effects and it can suppress metastasis by targeting several oncogenic pathways and regulate invasion, migration and chemoresistance of cancer cells. Also, resveratrol works against tumor initiation and cancer progression pathways by promoting cell cycle arrest, which lead to apoptosis in tumor cells. Besides, resveratrol suppresses tumor growth through targeting tumor microenvironment (TME) components.

TME stress factors such as hypoxia, inflammation and reactive oxygen species (ROS) promote cancer progression (figure 1. 10). The rapid proliferation of cancer cell and dysregulated angiogenic processes create a hypoxic area at the core region of a tumor distant from blood vessels. This hypoxic conditions cause epigenetic, transcriptomic and proteomic reprogramming of cancer cells and related to poor prognosis. Hypoxia is an important driver of cancer metastasis. Under hypoxic and low pH conditions in TME, resveratrol can target cancer cells and promotes growth inhibition, internucleosomal DNA fragmentation and apoptosis. HIF-1α is a subunit of a heterodimeric transcription factor and its expression is related with vascularization, angiogenesis, cell survival, drug sensitivity. Treatment with resveratrol trigger pseudo-hypoxic response and regulate HIF-1α, VEGF, STAT3 expression [179]. On the other hand, the excessive production of ROS and dysfunctional anti-oxidation machinery cause in increasing oxidative stress in TM. Cancer cells are able to increase intracellular level of ROS which is correlated with subsequent activation of oncogenic signaling pathways. Resveratrol regulates ROS level in a cell/organ specific manner and ROS-related signaling pathways including hedgehog pathway. As a hallmark of TME, inflammation is a major driver of cancer metastasis and inflammatory cytokines such as IL-1β, IL-6 and TNF-α are upregulated and secreted not only by immune cells but also cancer cells. The segregation of these cytokines cause inflammatory microenvironment of a tumor and promote cancer progression. However, resveratrol modulates the inflammatory cytokine-mediated signaling pathways and inhibits TNF-α induced migration and invasion of cancer cells by downregulating NF-κB expression [179]. Resveratrol can also suppress the expression of extracellular matrix (ECM) remodeling enzymes such as matrix metalloproteinase (MMP)2 and MMP9.

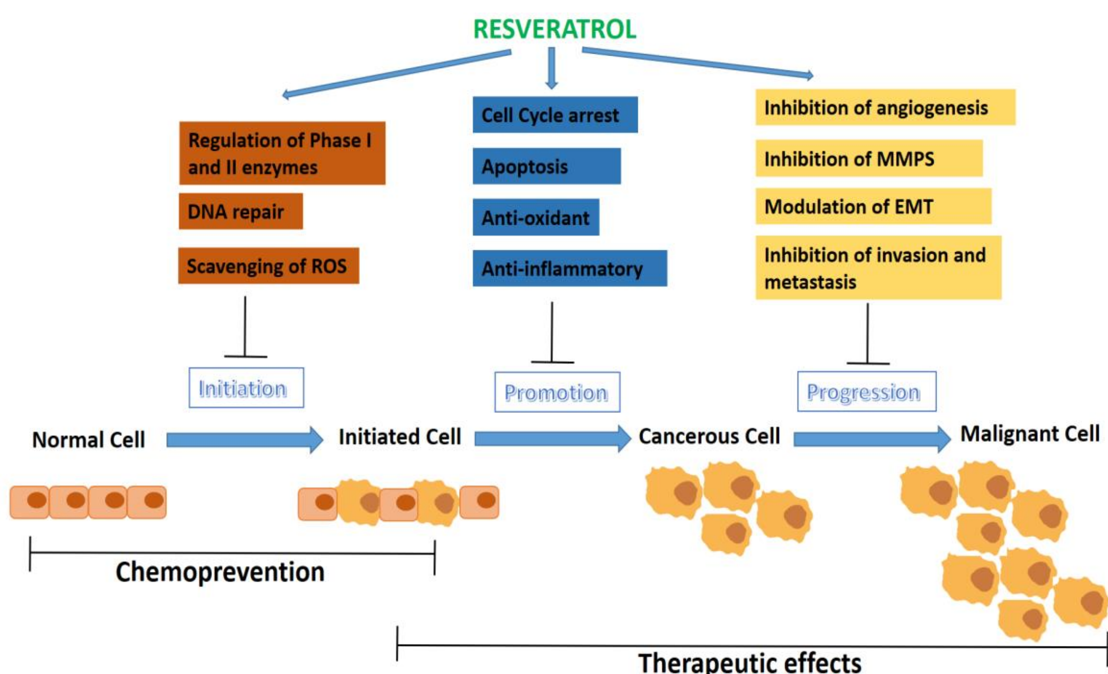


Figure 1.10 Resveratrol as chemo preventive and therapeutic natural compound [180, 181]

Resveratrol induced growth inhibition is related with cell cycle arrest and induction of apoptotic cell death through expression of the anti-apoptotic protein Bcl-2, dysfunction of mitochondria, cytochrome c release and caspase activation.

Resveratrol is also known to act as an antioxidant and block tumor growth by preventing tumor derived nitric oxide (NO) synthase expression and DNA damage which lead tumor formation [180]. Besides, resveratrol decrease DNA binding activity of transcription factors such as PPAR, NRF1, p53, NF-κB , which are upregulated in cancers and drive the transcription of several genes to promote tumor growth [13]. Resveratrol also inhibits epithelial to mesenchymal transition (EMT) processes which are associated with metastasis and chemoresistance[182]. Moreover, resveratrol can degrease cytokines synthesis and heterogeneity of cancer cell population through the inhibition of JAK2/STAT3 signaling [183].

The activation of SIRT1, which deacetylates histones and nonhistone proteins such as transcription factors, by resveratrol is predicted to benefit cancer treatment since SIRT1-regulated pathways affect stress resistance, cellular senescence, metabolism, cell survival, inflammation and endothelial functions[184]

Resveratrol modulates the expression of tumor suppressor small non-coding RNAs such as miRNAs. Resveratrol treatment increased the level of miR-663 targeting growth factor beta (TGFβ)1 transcript and at the same time it decreased the levels of several oncogenic miRNAs which targets genes encoding tumor suppressor and effectors of the TGFβ signaling pathway. In addition to upregulation of many TGFβ signaling pathway components, resveratrol decreases the transcriptional activity of SMADs which are the main signal transducers for TGFβ receptors and play key role in cell development and growth. Due to anti-inflammatory ability of resveratrol, it upregulates miR-663 in THP-1 monocyte, which potentially targets several genes responsible for immune response [185].

1.5.1 The Anti-Leukemic Potential of Resveratrol

Resveratrol takes the attention of researchers and clinicians because of its biological activities including growth inhibition, differentiation induction and apoptosis in leukemic cells (Figure 1.10). In HL-60 (AML) cells, resveratrol-induced cell death is mediated by proteolytic cleavage of caspase substrate poly ADP-ribose polymerase (PARP) in dose dependent manner. Resveratrol also induced FasL-related apoptosis and increased Bax levels, induced the growth arrest and DNA damage-induced gene (GADD45) and caspase-3 expression, while it decrease Bcl-2 expression [178]. AML cell lines OCIM2 and OCI-AML3 produce IL-1β which plays key role in proliferation of AML. It has been showed that resveratrol inhibiteds proliferation of these cell lines and prevent their progression though the cell cycle by arresting the cells at S phase in a dose dependent manner. Besides incubation of cells with resveratrol resulted in apoptotic cell death [186].

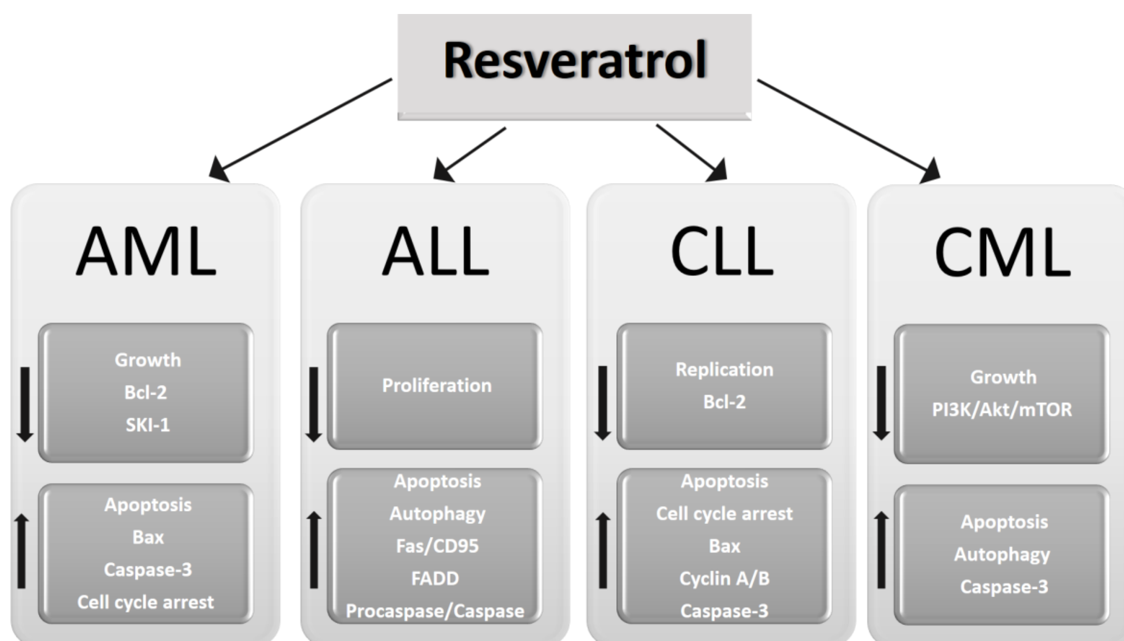


Figure 1. 11 The effect of resveratrol in leukemic cells

The dose related regulatory capacity of resveratrol is also shown in mouse ALL cell lines where resveratrol inhibited cellular proliferation and induced apoptosis and cell cycle arrest at G1 phase [187]. Also in another study, resveratrol increased expression of caspases and induced progressive loss of mitochondrial membrane potential (MMP) in ALL [188].

Resveratrol can also inhibit growth and induce apoptosis in chronic myeloid leukemia (CML) by downregulating STAT5 and up-regulating Bax expression and downregulating Bcl-2, Cyclin D1, Mcl-1. Also resveratrol induced antiproliferation and apoptosis by suppressing the phosphorylation of PI3K, Akt and mTOR, downregulating cyclinD1 and activating caspase-3 in CML (Figure 1.11) [189].

RAS, a protein superfamily of small GTPase activates PI3K/Akt pathway. The serine/thereonine kinase (AKT) regulates several biological processes such as glycogen metabolism, cell survival, proliferation and growth. Resveratrol blocked Akt activity and the reduction in Akt phosphorylation in leukemic cells. Akt prevents the disruption of the mitochondrial inner membrane potential by inhibiting a conformational change in the pro-apoptotic Bax protein and its translocation to mitochondria. However, resveratrol induces apoptosis by reducing activation of Akt and promoting translocation of Bax to mitochondria in leukemic cells (Figure 1.12) [190].

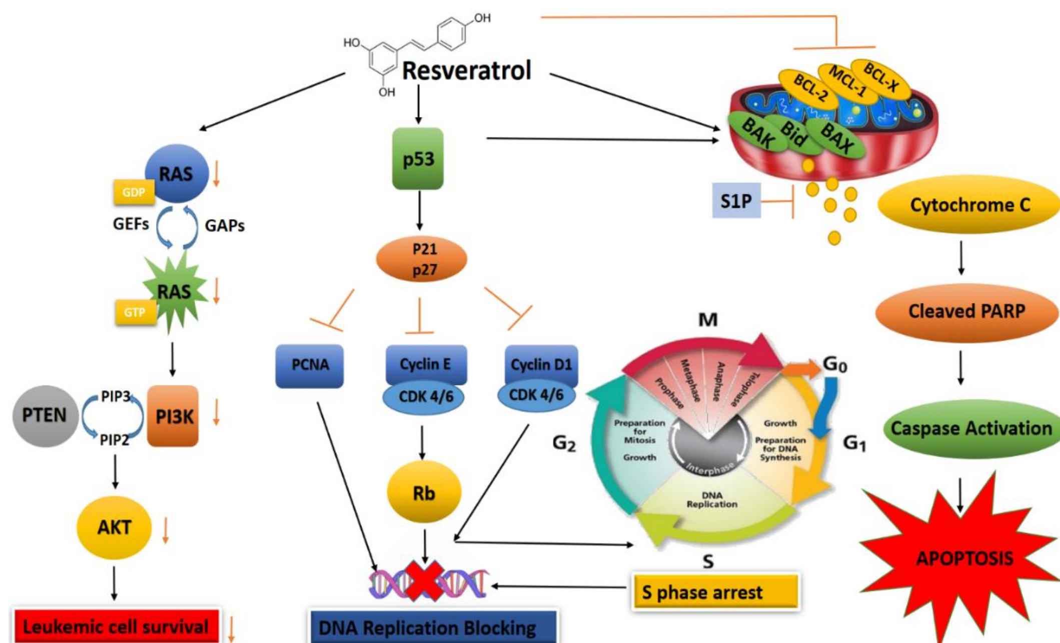


Figure 1.12 Mechanism of Resveratrol induced anti-leukemic effects [190, 191]

Resveratrol inhibited DNA replication and synthesis by arresting cell cycle G2/M phase in CLL cell lines and triggered apoptosis and DNA fragmentation in B-CLL cell lines through the activation of caspase-3, decreasing of MMP, reduction anti-apoptotic protein Bcl-2 and expression of inducible nitric oxide synthase (iNOS) in dose dependent manner [178].

1.5.2 Resveratrol Targets Sphingolipid Metabolism

Studies indicated that resveratrol is an important regulator sphingolipid metabolism where the cell fate is determined according to ceramide level in addition to its multi-targeted effects on cancers. Resveratrol induced alterations in the phospholipid and fatty acid compositions including Cer level in plasma membrane and improved functional activity of membrane lipids in hepatocytes associated with aging [192]. Besides it has been shown that treatment with resveratrol increased the expression of Cer generating proteins and decreased anti-apoptotic SK1 and GCS which resulted in *de novo* Cer generation and accumulation [193]. Sphingolipid metabolism was targeted with resveratrol in BCR-ABL Ph+ ALL cells and it was shown that resveratrol suppressed cell growth and triggered apoptosis by regulating ceramide metabolism [194]. Moreover, the role of Cer metabolizing genes in resveratrol-induced apoptosis have been studied in K562 CML cells and shown that Cer levels increased when resveratrol combined with

sphingolipid enzyme inhibitors PDMP and SKI II [195]. It has been also shown that resveratrol modifieds phospholipids saturated and unsaturated fatty acids in breast cancer cell lines [196]. In human gastric cancer cells, sphingolipid metabolite accumulation could have been used as a novel lipid biomarker of resveratrol-induced cytotoxicity [197]. Human gastric cancer cell that the inhibition of sphingolipid metabolism could increase resveratrol chemotherapy response [197]. The potential cancer prevention and therapy role of resveratrol as a polyphenol has been shown in sphingolipid-mediated mechanisms [198,199]. Resveratrol could induce proliferation inhibition in K562 leukemic cell through modulation of SK-1 pathway [200].

Resveratrol, a plant phytoalexin, possesses pleiotropic anti-carcinogenic activities. In addition to its well identified mechanisms of actions as mentioned, how resveratrol exerts its anti-leukemic actions in relation to modulation of sphingolipid metabolism in *FLT3-ITD* acute myeloid leukemia (AML) remains unclear. In this particular thesis study, we aimed to explore the role of different key rate limiting enzymes of sphingolipid metabolism in resveratrol-induced cytotoxicity in *FLT3-ITD* positive AML cell lines and to suggest a new combinational integrative treatment approach for *FLT3-ITD AML*.

Chapter 2

Material and Method

2.1 Chemicals

Resveratrol (Sigma Aldrich), SK inhibitor (SKI II, Cayman Chemical) and GCS inhibitor (PDMP, Cayman Chemical) stock solutions were prepared in DMSO and stored at -20ºC.

2.2 Cell Lines and Culture Conditions

FLT3 ITD AML cell lines, MOLM-13 and MV4-11 were obtained from DSMZ (German Collection of Microorganisms and Cell cultures). MOLM-13 and MV4-11 cells were grown in RPMI 1640 (+ L-glutamine, GibcoTM) medium containing 10-20% fetal bovine serum (FBS) and 1% penicillin-streptomycin in a 5% CO_2 (carbon dioxide) incubator at 37ºC.

2.3 MTT Cell Proliferation

MTT cell proliferation test was used to determine the cytotoxic effects of resveratrol, SKI II and PDMP on MOLM-13 and MV4-11. Cells were seeded into 96-well plate at density of $1X10^4$ cell/well and treated with increasing concentrations of resveratrol (5-60 µM) and inhibitors SKI II and PDMP (1-100 µM) for 48 and 72 hours. After incubation, 20 µl MTT reagent (5mg/ mL, Sigma Aldrich) was added to each well and incubated for 3 hours at 37ºC in 5% CO_2 incubator. 96-well plates were centrifuged at 1800 rpm for 10 min and 100 µl DMSO was added each well to dissolve formazan crystals. The absorbance values were read on the spectrophotometer at 570 nm and cell proliferation graph was drawn based on the results. IC_{50} (concentration inhibiting cell growth by 50%) concentration for each agent and IC_{20} (concentration inhibiting cell growth by 10-20%) values for the inhibitors were calculated. It has been shown in the

literature that IC_{20} concentrations are sufficient to inhibit these enzymes (Baran et al., 2007).

2.3.1. Calculation of Combination Indexes (CIs)

Increasing concentrations of resveratrol (10-30 µM) were combined with increasing concentrations of SKI II (5-15 µM) and PDMP (5-15 µM for MOLM-13 cells and 20-60 µM for MV4-11 cells) at fixed molar ratio and standard MTT assay was performed after 48 hours incubation to obtain cell proliferation graph.

Combined-effects analyses, based on the method of Chou and Talalay were performed to establish whether combinations of resveratrol SKI II and PDMP result in synergism, additivity or antagonism using CompuSyn software (Biosoft, Cambridge, United Kingdom). A CI of <1, 1.0-1.1, or >1.1 is indicative of synergistic, additive/nearly additive, or antagonistic effects, respectively [201].

2.4 Flow cytometric Detection of Apoptosis by Annexin-V / Propidium Iodide Dual Staining

Flow cytometry was used to determine the amount and localization of phosphatidyl serine (PS) in resveratrol, SK and GCS inhibitors, resveratrol: SK inhibitor and resveratrol: GCS inhibitor treated MOLM-13 and MV4-11 cells by using AnnexinV-Propidium Iodide (PI) (BioVision, Inc.) dual staining (Adan and Baran, 2015; Adan and Baran, 2016). The cells were seeded as $7.5x10^5$ /2ml to six well plate and incubated for 48 hours with the defined concentrations of resveratrol (10-30 µM), SKI II (5-15 µM), PDMP (5-15 µM for MOLM-13 cells and 20-60 µM for MV4-11 cells) alone and their combinations (resveratrol (10-30 µM) with SKI II (5-15 µM) and PDMP (5-15 µM for MOLM-13 cells and 20-60 µM for MV4-11 cells). After 48 hours incubation, the cells were centrifuged at 1800 rpm for 10 min and washed with cold 1X PBS twice. Later on, 200 µl of annexin binding solution was added, homogenized with cells, transferred to flow glass and 2 µl of Annexin V and 2 µl of propidium iodide were added to the each obtained cell suspension. After the 15 min incubation in the dark room, samples were analyzed by flow cytometry (BD FACSCalibur). The histograms were obtained and analyzed using BD FACSDiva™ (BD Biosciences) (Adan and Baran, 2015).

2.5 Cell Cycle Analysis

7.5x10^5 / 2ml MOLM-13 and MV4-11 cells were seeded to six well plate and treated with resveratrol (10-30 µM), SKI II (5-15 µM), PDMP (5-15 µM for MOLM-13 cells and 20-60 µM for MV4-11 cells) alone and their combinations (resveratrol (10-30 µM) with SKI II (5-15 µM) and PDMP (5-15 µM for MOLM-13 cells and 20-60 µM for MV4-11 cells) (Adan and Baran, 2016) and incubated for 48 hours. Then, cells were centrifuged at 260 g for 10 min and cell pellets were washed twice with 1 ml cold PBS. 3 ml cold ethanol was added to each samples and cells were incubated for at least 24 hours in -20 ° C.

Samples were centrifuged at 260 g for 10 minutes and supernatant was removed. Pellets were homogenized in 5 ml cold PBS and centrifuged. 1 ml PBS-Triton X100 and 100 µl RNase-A (200 µg / ml, Sigma Aldrich) were added and mixed with pellet and samples were incubated at 37 ° C for 30 min. Then, samples were dissolved with 100 µl propidium iodide (1 mg / ml, Sigma Aldrich) and incubated at room temperature for 10-15 minutes. Samples were analyzed by flow cytometry (BD FACSCalibur). The histograms were obtained and analyzed using BD FACSDiva^{TM} (BD Biosciences) (Adan and Baran, 2015).

2.6 Western Blot Analysis

5x10^6 cells were treated with resveratrol (10-30 µM) alone for 48 hours and protein levels of SK-1, GCS, caspase-3 and PARP cleavage were checked by western blotting. Moreover, co-treatments (10 µM resveratrol combined with 5 µM SKI II for both cells and with 5 µM PDMP for MOLM-13 cells and 20 µM PDMP for MV4-11 cells) were also subjected to western blotting to check PARP cleavage after 48 hours exposure. Then, cells were collected in 15 ml tubes and centrifuged at 4ºC, 4000 rpm for 5 minutes and supernatant was removed. Cells were washed with 1X cold PBS twice, centifugated at 4ºC, 4000 rpm for 5 minutes and supernatant was removed. Pellet was lysed with 150 µl cold RIPA buffer (Sigma Aldrich) via 1 ml syringes (pull and down 10-15 times) and incubated on ice for 25-30 minutes. Proteins were centrifuged at 4ºC, 12000 rpm for 15 minutes. Protein concentrations were calculated using RC DC^{TM} Protein Assay Kit (Bio-Rad, USA). The isolated proteins were stored at -20 or -80ºC (Baran et al., 2007).

2.6.1 Protein Quality Determination

RC DCTM Protein Assay (Bio-Rad) kit was used to measure protein amounts. To obtain primary stock solution (1.56 mg / ml), gamma globulin was dissolved in 20 ml of ultra-pure water. Standards were prepared as 0.2 mg / ml - 1.4 mg / ml (Table 2. 1)

Table 2. 1 Gamma Globulin Standard Preparation

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

5 µl from each standard (0.2-1.4) and 5 µl from each sample were added to 96 well plates with three replicates. 20 µl of solution S and 1 ml of solution A were homogenized in 1/50 concentration ratio to obtain solution A'. 25 µl from solution A' and 200 µl from solution B were added to each well and incubate for 15 minutes at room temperature. To calculate protein concentrations of each sample, absorbance measurement was performed at 750 nm after the incubation.

2.6.2 SDS Gel Preparation and Running

30 µg / µl protein from each sample homogenized with 2X laemni buffer (Santa Cruz Biotechnology). Samples were denatured by boiling at 95˚C for 5 min and allowed to stay in ice 5 min to make them suitable for separate in gel. SDS gel had been prepared based on the molecular weight of the target proteins. Then, samples were loaded into gel wells. To determine the molecular weight of the proteins, 4 µl PageRuler ™ Prestained Protein Ladder (Thermo ScientificTM) were used. The gel was run at 100V-135V until ladder reach to the bottom of the gel.

2.6.3 Transferring Proteins

The filter papers and gel were soaked in 1X Transfer buffer, which includes 10% 10X Tris-glycine buffer, 10% methanol and 1gr/L SDS, for 10 min. PVDF membrane was activated with methanol for 5 minutes by shaking and then washed with water 10 min. Then, membrane was placed in the Trans-Turbo (Bio-Rad) device and the transfer was carried out at 25V, 1.3A, 7-15 min.

2.6.4 Blocking with BSA, Antibody Treatment and Imaging

After the transfer process, the membrane was blocked for 1-2 hours at room temperature in a 1X TBST (Tris-buffered saline, 0.1% Tween 20) solution which contains 5% skimmed milk powder or BSA (Bovine Serum Albumin). Membrane was washed 3 times with 1X TBST for 5 minutes at room temperature. The primary antibodies Beta Actin (1: 3000, Cell Signaling), Caspase-3 (1: 3000, Cell Signaling), PARP (1: 3000, Cell Signaling), GCS (1: 1000, Novus Biologicals), and SK (1: 3000, Cell Signaling) were prepared in 1X TBST which contains 5% skimmed milk powder or BSA. The membrane was incubated with primary antibody solution overnight at + 4 ° C. Then, membrane was washed 3 times with 1X TBST for 5 minutes at room temperature. The suitable secondary antibodies (1:10000, Jackson Immuno Research) were prepared in 1X TBST solution which contains 5% skimmed milk powder or BSA and the membrane was incubated for 1 hour at room temperature. Then, membrane was washed 3 times with 1X TBST for 5 minutes at room temperature and visualized by the Pierce ™ ECL Western Blotting Substrate kit (Thermo ScientificTM) (Bio-Rad, ChemiDoc). Densitometric analysis of the immunoreactive bands were performed using the imaging software (Bio-Rad, ChemiDoc, Image LabTM 3.0).

2.7 Statistical Analysis

GraphPad software (San Diego, CA) was used to analyze the data and the results were expressed as the mean ± standard error (SEM) from at least two independent experiments. Comparisons among multiple groups were evaluated using one-way ANOVA followed by Dunnett's test. $P<0.05$ was considered as a statistically significant difference.

Chapter 3

Results and Discussion

3.1 Antiproliferative Effect of Resveratrol on FLT3-ITD Positive AML Cells

The growth inhibitory effect of resveratrol on MOLM-13 and MV4-11 cells were evaluated by MTT assay. MOLM-13 and MV4-11 cells were treated with increasing concentration of resveratrol (5-60 μM) for 48-72 hours. The results proved that resveratrol has time and concentration-dependent cytotoxic effects on *FLT3-ITD AML* MOLM-13 cells (Figure 3.1a and Figure 3.1b). IC_{50} (inhibition of cell viability by 50% at a particular concentration) values at 48 and 72 hours were determined for MOLM-13 and MV4-11 cells. IC_{50} values are 22 and 18 μM for MOLM-13 and 30 and 28 μM for MV4-11 cells at 48 and 72 hours, respectively.

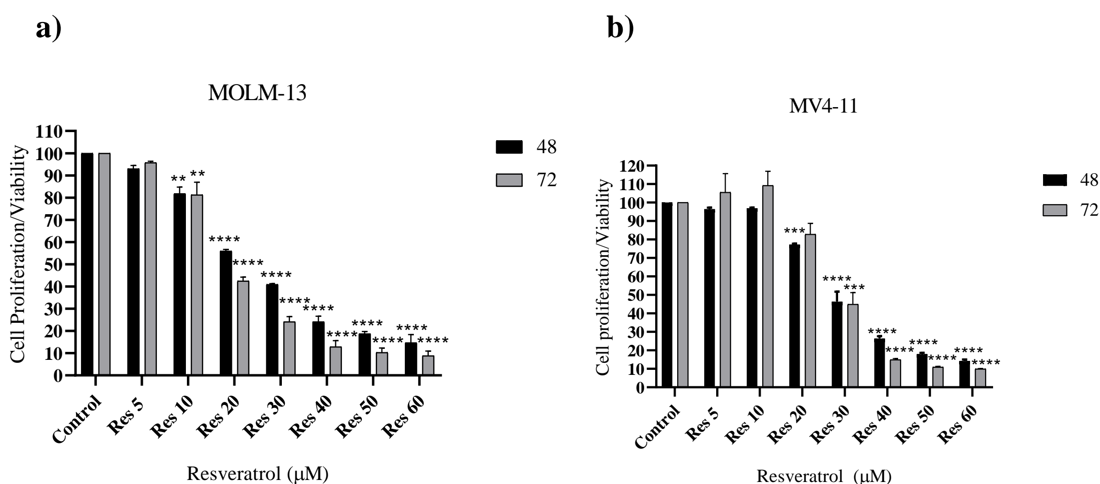


Figure 3.1 Time and concentration-dependent cytotoxic effect of resveratrol on MOLM-13 (a) and MV4-11 (b) cells. Data are presented as the mean ± standard error. **P <0.01, ***P<0.001 and ****P<0.0001 vs controls.

3.2 Sphingolipid Metabolism is Modulated by Resveratrol through inhibition of SK-1 and GCS in FLT3-ITD positive AML Cells

To understand the roles of SK-1 and GCS in resveratrol-mediated growth inhibition in FLT3-ITD positive AML cells, we checked the alterations in SK-1 and GCS protein levels in MOLM-13 and MV4-11 cells in response to increasing concentrations of resveratrol. MOLM-13 and MV4-11 cells are treated with resveratrol (10-30 μM) for 48 hours. The expression of SK-1 and GCS were analyzed by western blot.

Resveratrol significantly reduced GCS expression in both MOLM-13 and MV4-11 cells 0.5 (10 μM), 0.6 (20 μM), and 0.5 (30 μM)-, fold decreases were detected for MOLM-13 (Figure 3.2a) and 0.38 (10 μM), 0.32 (20 μM) and 0.3 (30 μM)- fold decreases for MV4-11 cells compared to control (Figure 3.3a). Data have proved that resveratrol could suppress cell growth by decreasing the levels of GC to prevent the accumulation of apoptotic Cer in both cells.

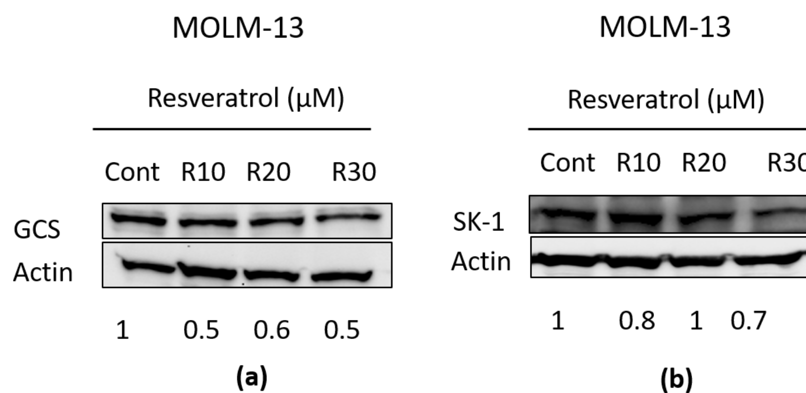


Figure 3.2 Alterations in the expression in GCS (a) and SK-1 (b) in MOLM-13 cells in response to resveratrol. Beta actin was used as a loading control. Experiments were replicated independently and representative western blot image was used. The protein expression of each group was normalized to their Beta-Actins.

Similarly, increasing doses of resveratrol (10-30 μM) downregulated SK-1 in both MOLM-13 and MV4-11 cells especially at higher concentrations. 0.7 (30 μM)- fold decrease for MOLM-13 cells (Figure 3.2b) and 0.4 (30 μM)- fold decrease for MV4-11 cells (Figure 3.3b) were detected as compared to control. These results also suggest that

resveratrol regulated sphingolipid metabolism through SK-1 inhibition which could be the possible mechanism for its antileukemic activity due to decreased amount of antiapoptotic S1P levels. Because SK and GCS promote cell proliferation through the production of S1P and GCS from Cer [129].

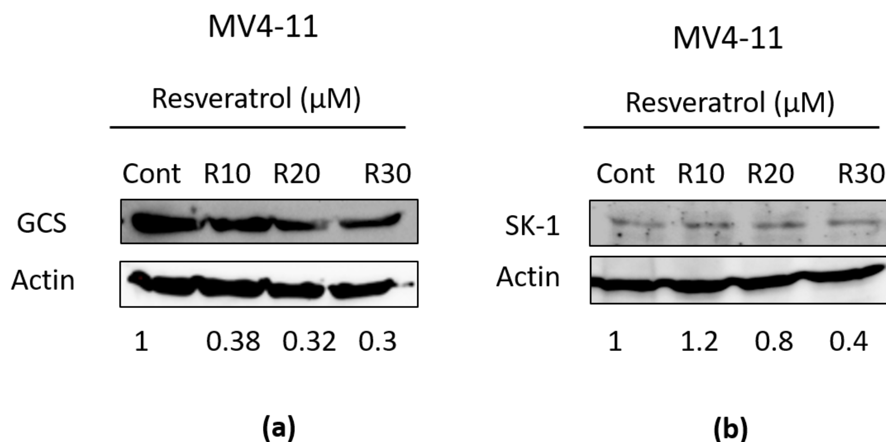


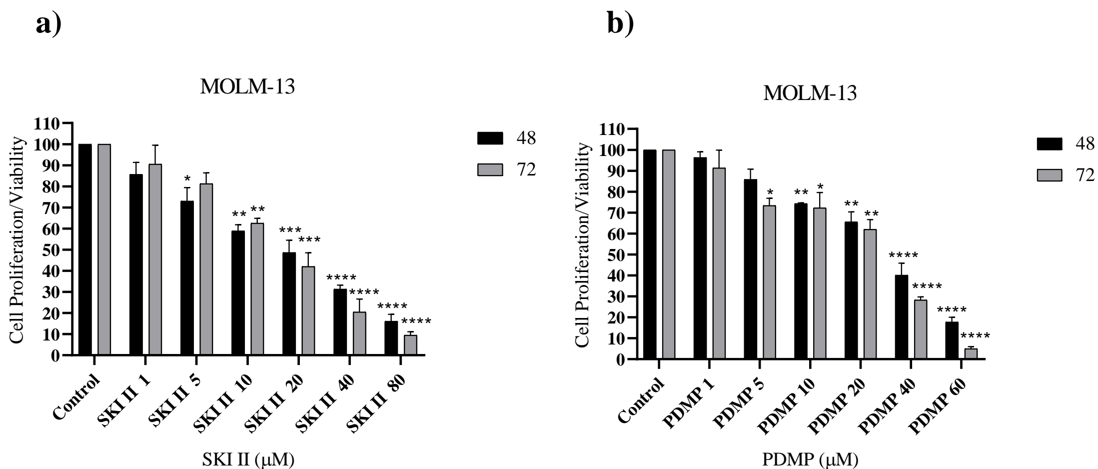
Figure 3.3 Alterations in the expression in GCS (a) and SK-1 (b) in MV4-11 cells in response to resveratrol. Beta actin was used as a loading control. Experiments were replicated independently and representative western blot image was used. The protein expression of each group was normalized to their Beta-Actins.

In the literature, the role of SK has been shown in many cancer types. The cell growth was suppressed in resveratrol treated K562 CML cells through increased level of SK-1 from membrane to cytoplasm while there was no change in the expression SK level [200]. It has been shown that expression of SK-1 and GCS genes were decreased in resveratrol treated K562 CML and HL60 APL cells [195] [193]. In this study, resveratrol could inhibit the formation of antiapoptotic S1P by inhibiting SK-1 and in MOLM-13 and MV4-11 cells and caused the suppression of cell proliferation. It is shown that vatinacol C (VTC), a resveratrol tetramer, regulated sphingolipid rheostat modulation through SK and GCS, suppressed cell growth and increased cytotoxicity in human erythroleukemia cells K562, acute lymphoblastic leukemia cell line NphA2, prostate cancer cells PC3 and their drug resistant subclones [202]. In this study, GCS and SKI protein level is decreased in VTC treated K562 and resistant cells K562 cells. In another study, sorafenib resistant hepatoma cells upregulated GCS expression however, molecular and pharmacological GCS inhibition sentisized hepatoma HepG2 and Hep3B cells to sorafenib treatment [203]. GCS targeting reduces tumor cell proliferation and

vascularization in HepG2 and Hep3B. Therefore, targeting GCS and SK-1 is important to reduce cancer progression and development.

3.3 Inhibition of SK-1 and GCS Enhances Resveratrol's Antiproliferative Effects on FLT3-ITD positive AML Cells

The cytotoxic effect of SKI II and PDMP on MOLM-13 and MV4-11 cells were evaluated by MTT assay. MOLM-13 and MV4-11 cells were treated with increasing concentration of SKI II (1-80 µM) and PDMP (1-100 µM) for 48-72 hours. IC_{20} (inhibition of cell viability by 20% at a particular concentration) and IC_{50} (inhibition of cell viability by 50% at a particular concentration) values at 48 and 72 hours were determined for MOLM-13 and MV4-11 cells. IC_{20} values are 3 and 5 µM (SKI II), 9 and 6 µM (PDMP) and IC_{50} values were 19 and 12 µM (SKI II), 30 and 27 µM (PDMP) for MOLM-13 cells (Figure 3.4a and Figure 3.4b) at 48 and 72 hours, respectively. Similarly, IC_{20} values were calculated as 3 and 1.8 µM (SKI II), 19 and 17 µM (PDMP) and IC_{50} values are 9.5 and 6.6 µM (SKI II), 45 and 38 µM (PDMP) for MV4-11 cells (Figure 3.4c and Figure 3.4d) at 48 and 72 hours, respectively.



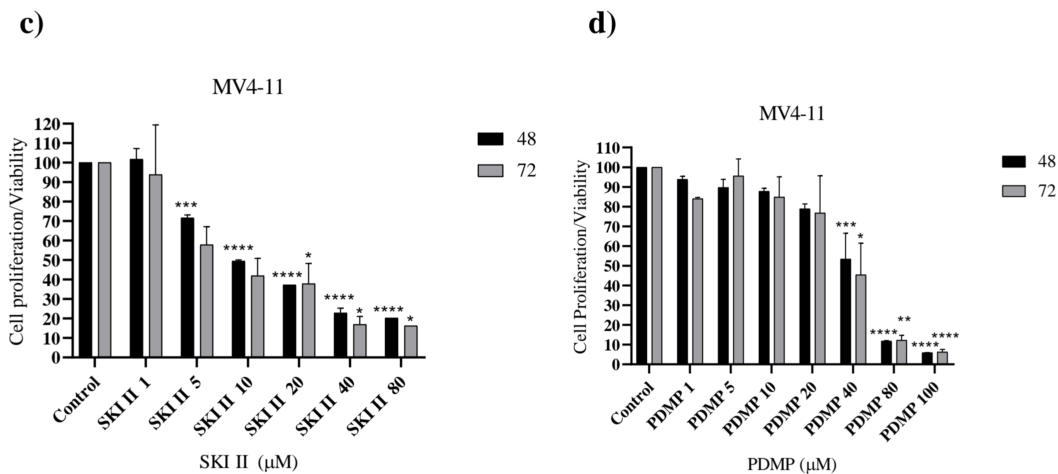


Figure 3.4 Time and concentration dependent cytotoxic effects of SKI II (a,c) and PDMP (b,d) on MOLM-13 and MV4-11 cells, respectively. Data are presented as the mean ± standard error. *P<0.05, **P <0.01, ***P<0.001 and ****P<0.0001 vs controls.

Based on above data, we hypothesized that resveratrol's anti-leukemic activity could be related to inhibition of SK-1 and GCS. Targeting these enzymes together with resveratrol could enhance resveratrol's growth inhibitory effects.

It is expected that Cer accumulation in the cells should be increased after treatments with the inhibitors, which prevent the formation of antiproliferative S1P and GC. Therefore, resveratrol's cytotoxicity should be increased. To understand whether the cytotoxic effects of resveratrol would be enhanced in the presence of SKI II and PDMP, combination studies were performed. MOLM-13 cells were treated with increasing concentration of resveratrol (10-30 μM) in combination with SKI II (5-15 μM) and PDMP (5-15 μM). Co-treatments suppressed the cell viability on MOLM-13 cells as compared to the untreated controls (Figure 3.5a). The results showed that 10-20 μM resveratrol with 5-10 μM SKI II and 10 μM resveratrol with 5 μM PDMP combinations were the most effective ones on cell proliferation as compared to related resveratrol concentrations (Figure 3.5a).

MV4-11 cells also were treated with increasing concentration of resveratrol (10-30 μM) in combination with SKI II (5- 15 μM) and PDMP (20-60 μM). Combinations

inhibited the cell viability of MV4-11 cells as compared to the untreated controls. The results showed that 10 µM resveratrol with 5 µM SKI II and 10-30 µM resveratrol with 20-60 µM PDMP combinations were the most effective ones on cell proliferation as compared to related resveratrol concentrations (Figure 3.5b).

These results might explain that one of main mechanism of resveratrol's action in FLT3-ITD positive AML cells could be related to the regulation of the key enzymes of sphingolipid metabolism.

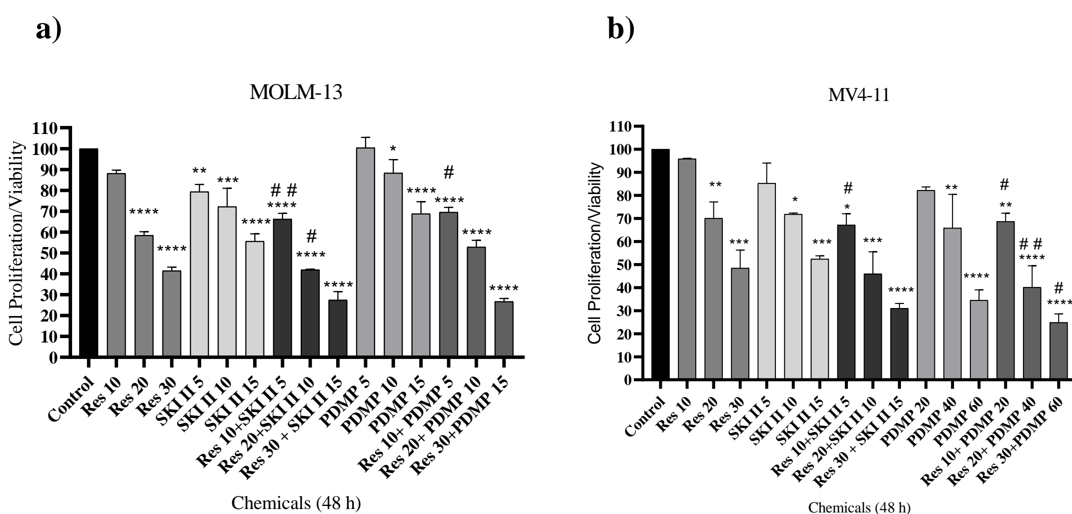
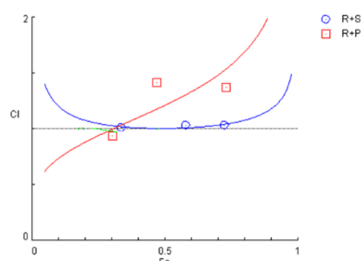


Figure 3.5 Antiproliferative effects of resveratrol together with SKI II and PDMP on MOLM-13 (a) and MV4-11 (b) cells. Data are presented as the mean ± standard error. *P<0.05, **P <0.01, ***P<0.001 and ****P<0.0001 vs control. #P<0.05, ##P <0.01 vs resveratrol alone.

To analyze combined-effects, we performed CompuSyn analysis to detect synergism, additivity or antagonism. A CI of <1, 1.0-1.1, or >1.1 is indicative of synergistic, additive/nearly additive, or antagonistic effects, respectively.

Our results showed that all concentrations of resveratrol with SKI II doses have additive effects, eventhough only 10 µM resveratrol with 5 µM PDMP displayed highest synergistic effect on MOLM-13 cell (Figure 3.6a and Figure 3.6b). In MV4-11 cells, 10 µM resveratrol in combination with 20 µM PDMP has nearly additive effects eventhough 10 µM resveratrol with 5 µM SKI II displayed synergistic effect on MV4-11 cell (Figure 3.6c and Figure 3.6d).

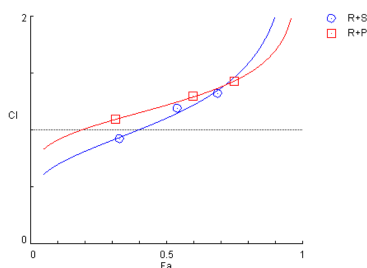
a)



b)

Resveratrol	SKI II	Res+SKI II	PDMP	Res+PDMP
Dose	Dose	CI Value	Dose	CI Value
10	5	1.01303	5	0.93607
20	10	1.02998	10	1.41413
30	15	1.03054	15	1.37417

c)



d)

Resveratrol	SKI II	Res+SKI II	PDMP	Res+PDMP
Dose	Dose	CI Value	Dose	CI Value
10	5	0.92543	20	1.09466
20	10	1.19376	40	1.29736
30	15	1.3218	60	1.43227

Figure 3.6 Combination Index Plots of MOLM-13 (a) and MV4-11(c) cells treated with resveratrol in combination with SKI II and PDMP. CI analysis of resveratrol in combination with SKI II and PDMP for MOLM-13 (b) and MV4-11(d) cells.

It has been shown that resveratrol has an effect on suppressing cell proliferation in many types of leukemia and solid cancer and these effects occur with different mechanisms depending on the cell type [204]. We have calculated that IC_{50} values of resveratrol at 48 hours as 22 μM for MOLM-13 and 30 μM for MV4-11 which are in accordance with the antiproliferative effect of resveratrol on human breast cancer cells [205]. In another study, increasing concentration of resveratrol have antineoplastic effects against AGS gastric cancer cells [206].

It is also known that resveratrol has an anticarcinogenic potential by modulating sphingolipid metabolism. For instance, suppressive effects of SK inhibitor (SKI II), GCS inhibitor (PDMP) and resveratrol alone and in combinations on the proliferation of K562 CML and HL60 APL cells were determined [207] [193].

Resveratrol is able to increase intracellular Cer accumulation in breast cancer cells to induce Cer mediated apoptosis [208]. In another study, increasing concentrations of resveratrol together with dimethylsphingosine (DMS), which is a SK inhibitor, inhibited sphingolipid metabolism and enhanced resveratrol cytotoxicity in human gastric cancer cells [197]. It is shown that resveratrol with SKI II and PDMP treated Ph+ ALL cells inhibited cell proliferation [209]. However, a few studies have been defined the therapeutic potential of resveratrol regarding to sphingolipid metabolism in leukemic cells. Resveratrol has synergistic antiproliferative effect by regulating intracellular generation and accumulation of apoptotic Cer in combination with PDMP and SKI II in APL and CML cells [195]. Based on these results, resveratrol with SK and GCS enzyme inhibitors could inhibit the conversion of Cer to S1P and GCS and suppressed cell proliferation. Furthermore, to be able to understand the main mechanism underlying resveratrol's sphingolipid mediated action on cell proliferation, cell cycle and apoptosis analysis were performed.

3.4 Cytostatic Effects of Resveratrol Alone and in Combination with Ceramide Metabolism Inhibitors on FLT3-ITD positive AML Cells

In order to identify the mechanism behind growth inhibitory effects of resveratrol, SKI II, PDMP alone and in combinations on *FLT3-ITD* AML MOLM-13 MV4-11 cells, flow cytometric propidium iodide (PI) staining was performed to analyses cell cycle phases. Their potential roles in cell cycle were evaluated and the percentages of the cells in G0/G1, S and G2/M phases were calculated.

Resveratrol treated MOLM-13 cells were slightly arrested at S phase. The percentage of the cells accumulating in S phases were 29.1 (10 µM), 31.3 (30 µM) compared to control (26 %). Resveratrol (10-20 µM) treated MOLM-13 cells were slightly accumulated at G2/M phase as 9.4 and 11 % respectively, as compared to control (8.9 %). SKI II (5-15 µM) treated MOLM-13 cells was arrested at G0/G1 phase as 70.35, 73.25, and 77.2 % respectively, as compared to control (65.1 %). In addition, SKI II (5-10 µM) treated cells were slightly accumulated at G2/M phases as 9.5, 9.4 % respectively, as compared to control (8.9 %) (Figure 3.6a). While resveratrol (10 µM) with SKI II (5 µM) treated MOLM-13 cells were arrested at G0/G1 phases (71.6 %) compared to control

(62.6 %), resveratrol (20-30 μM) with SKI II (10-15 μM) treated MOLM-13 cells were slightly arrested at S phases as 27.4 and 28 % respectively, as compared to control (26 %). Besides, 20 μM resveratrol with 10 μM SKI II treated MOLM-13 cells were accumulated at G2/M phase (14.45 %) compared to control (8.9%) (Figure 3.7a). Only PDMP (5-15 μM) treated MOLM-13 cells was arrested at G2/M phases (12.35, 14.95, and 12.9 % respectively) compared to control (8.9 %). Resveratrol (10-30 μM) in combination with PDMP (5-15 μM) treated cells were accumulated in G0/G1 phases (65.8, 67 and 75.9%) compared to control (65.1%). Resveratrol (10-20 μM) with PDMP (5-10 μM) treated MOLM-13 cells arrested at G2/M (10.7 and 13.1% respectively) compared to control (8.9 %), (Figure 3.7b).

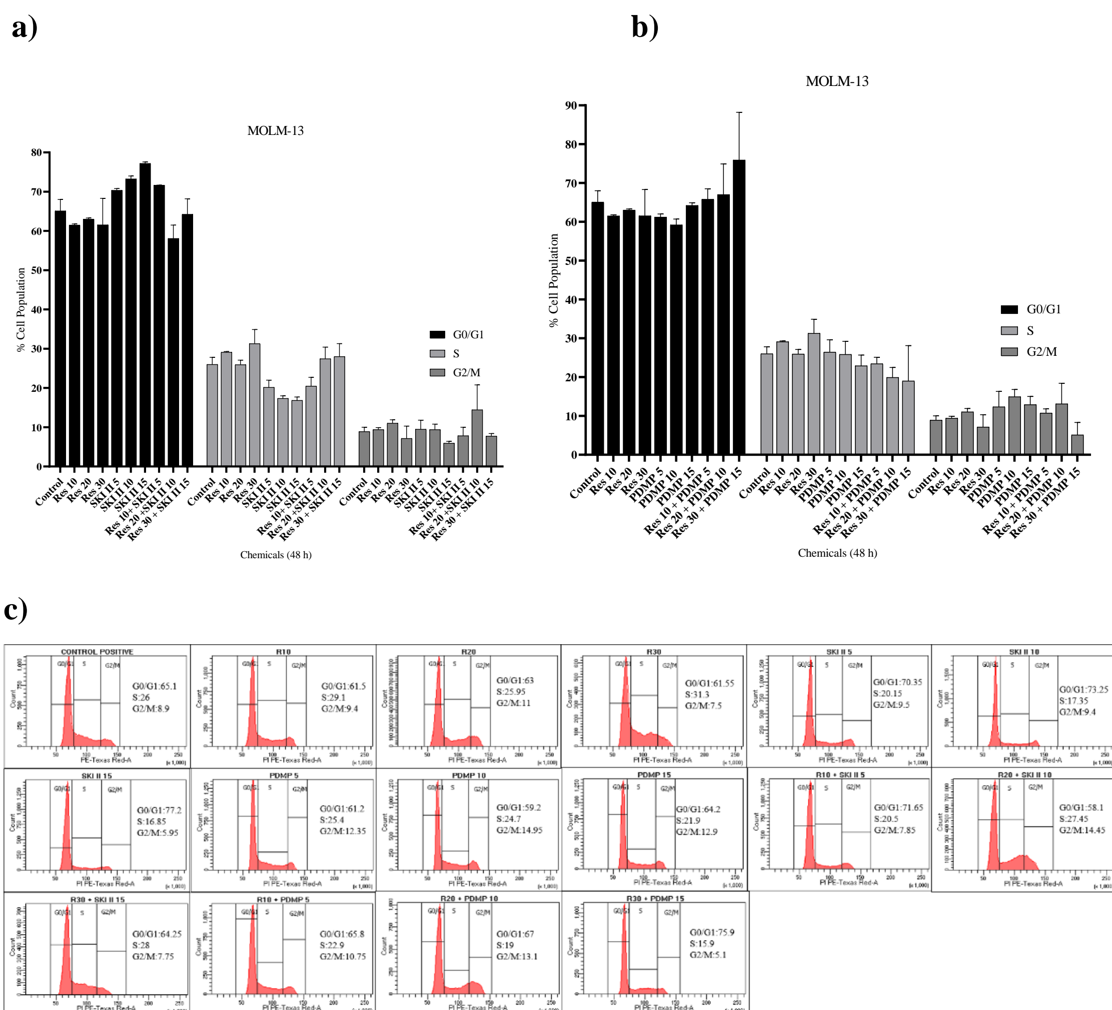
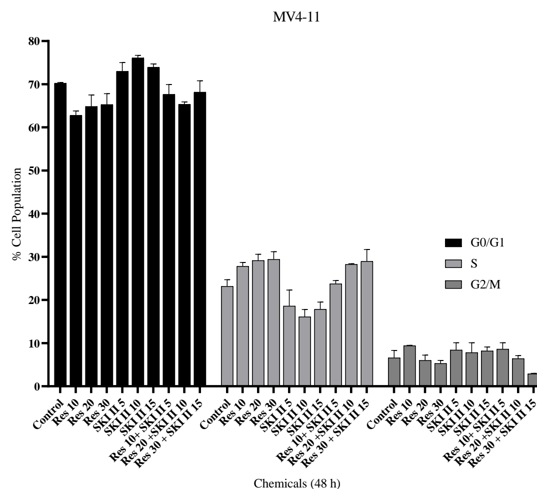


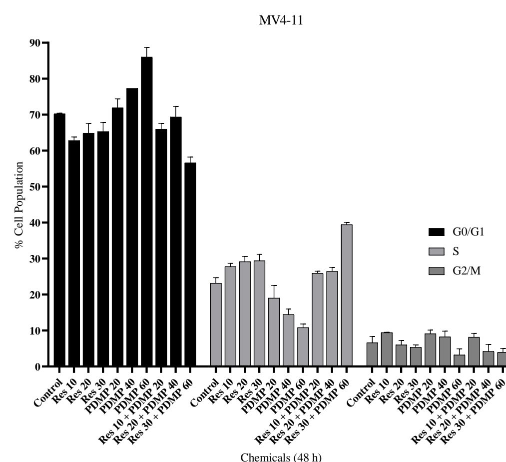
Figure 3.7 Cell cycle distributions of resveratrol, SKI II and PDMP alone and in combinations with SKI II(a) and PDMP (b) in MOLM-13 cells.

The percentage of the resveratrol (10-30 μM) treated MV4-11 cells accumulating in S phases (27.8, 29.15, and 29.4 % respectively) compared to control (23.15%). Resveratrol (10 μM) treatment resulted in a significant increase in cell population at the G1/M phase of the cell cycle as 9.4 % with respect to control (6.6 %) (Figure 3.8). Only SKI II (5-15 μM) treated MV4-11 cells were slightly arrested at G0/G1 (73, 76.1, and 73.9 % respectively) compared to control (70.25%) and accumulated at G2/M phases (8.4, 7.7, and 8.2 % respectively) compared to control (6.6%) (Figure 3.6a). Resveratrol (10-30 μM) with SKI II (5-15 μM) treated MV4-11 cells were arrested at S phases as (23.7, 28.2, 28.9 % respectively) compared to control (23.15 %). Resveratrol (10 μM) with SKI II (5 μM) treated MV4-11 cells resulted in a significant increase in cell population at G2/M phase as 8.6 % respect to control (6.6 %) (Figure 3.6 a). PDMP (20-60) treated MV4-11 cells were arrested at G0/G1 (71.9, 77.3, and 86 % respectively) compared to control (70.25 %). In addition, PDMP (20-40) treated MV4-11 cells were accumulated in G2/M phases were (9.1, 8.2 % respectively) compared to control (6.6%). Resveratrol (10-30) with PDMP (20-60) treated MV4-11 cells were arrested at S phases (25.9, 26.4, and 39.4 %, respectively) compared to control (23.15 %). Resveratrol (10 μM) with PDMP (20 μM) treated MV4-11 cells resulted in a significant increase in cell population at G2/M phase as 8.1 % with respect to control (6.6 %) (Figure 3.8 b).

a)



b)



c)

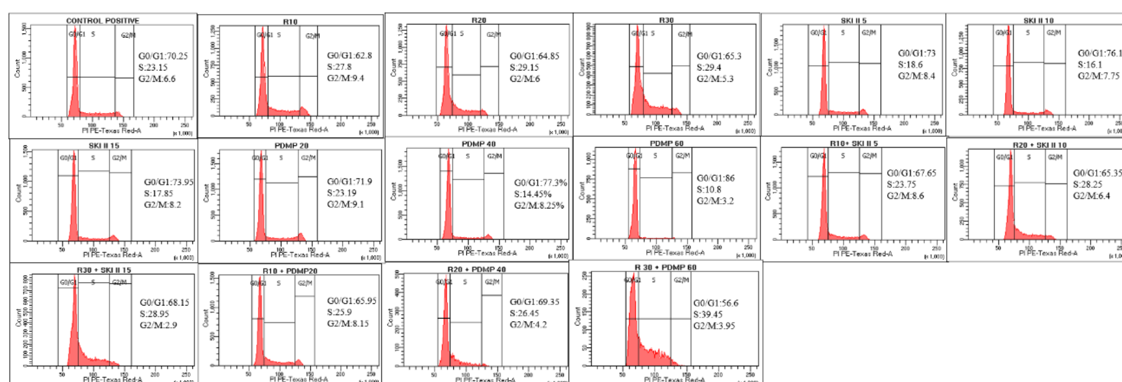


Figure 3.8 Cell cycle distributions of resveratrol, SKI II and PDMP alone and in combinations with SKI II(a) and PDMP (b) in MV4-11cells.

Cell cycle data show how resveratrol with inhibitors suppressed the cell proliferation and regulated cell cycle in MOLM-13 and MV4-11 cells. It can be speculated that resveratrol could suppress cell proliferation through inhibiting DNA synthesis by S phase arrest, which is also supported in different cancer lines where cyclin B1, A, and D1 expression levels were decreased [210]. In T-cell acute lymphoblastic leukemia (T-ALL) cells, resveratrol inhibited cell proliferation and induced G0/G1 cell cycle arrest via upregulating cyclin-dependent kinase (CDK) inhibitors [211]. Resveratrol treatment induced S-phase arrest when combined with 5-fluorouracil (5-FU), which is a conventional therapeutic agent, in human colorectal cancer cells [212]. Resveratrol treated colon cancer cell is determined to be accumulated in S phase by enhancing the levels of cyclin E and cyclin A and decreasing D1 [213]. Resveratrol inhibited proliferation and caused S-phase arrest in AML cell lines OCIM2 and OCI/AML3 [186]. Besides, resveratrol has therapeutic potential against NK cell malignancies such as leukemias and lymphomas through inducing G0/G1 arrest through downregulation of cell-cycle regulator proteins [214]. In gastric cancer cells, resveratrol alone accumulated cells in between G0 and S phases [215]. In prostate cancer cells, resveratrol with docetaxel, which is a chemotherapeutic drug, combination caused G0/G1 to S phase arrest by inhibiting expression of CDK4, cyclin D1, cyclin E1 [191]. In another study, resveratrol targets miR-196b and miR-1290 for its antitumor activity in T-ALL and B-ALL and induces G1 and S cell cycle arrest, respectively [216]. GCS is known to regulate cellular levels of Cer. It is shown that pharmacological inhibition of GCS with PPMP, a GCS inhibitor, resulted in blocking of cell division through arresting of cytokinesis [217].

It was shown that SKI II inhibited cell survival, reduced cell proliferation with accumulation of cells in the G0/G1 phase in human gastric cancer cells [218]. Resveratrol in combination with SKI II and PDMP inhibitors led to cell cycle arrest in S phase in Ph+ ALL cells [194]. Overall, resveratrol alone and in combination with different inhibitors induced cell cycle arrest. In this study, while resveratrol induces cell cycle arrest at S phase, combination with SKI II did not have significant effect upon cell cycle. However, resveratrol combination with PDMP induced apoptosis in G0/G1 and S phases in MOLM-13 and MV4-11 cells.

3.5 Resveratrol Induced Apoptosis through Caspase-3 and PARP cleavage in FLT3-ITD positive Cells

Intrinsic apoptosis pathway is coordinated by the activation of a group of caspases. The initiation of the cleavage of caspase-3 results in degradation of intracellular targets such as PARP in apoptotic cells. Therefore, to understand the main mechanisms of resveratrol mediated apoptosis, western blot experiments were performed to show caspase-3 and PARP cleavage levels in FLT3 ITD + AML cells. Treatment of MOLM-13 cells with increased concentration of resveratrol (10-30 μM) were found to increase caspase-3 cleavage as 2 (10 μM), 8 (20 μM) and 14 (30 μM)- fold as compared to control (Figure 3.9a). Similarly, increased concentration of resveratrol (10-30 μM) also increased PARP cleavage as 1.5 (10 μM), 5.9 (20 μM) and 12.5 (30 μM)- fold, respectively as compared to control (Figure 3.9b)

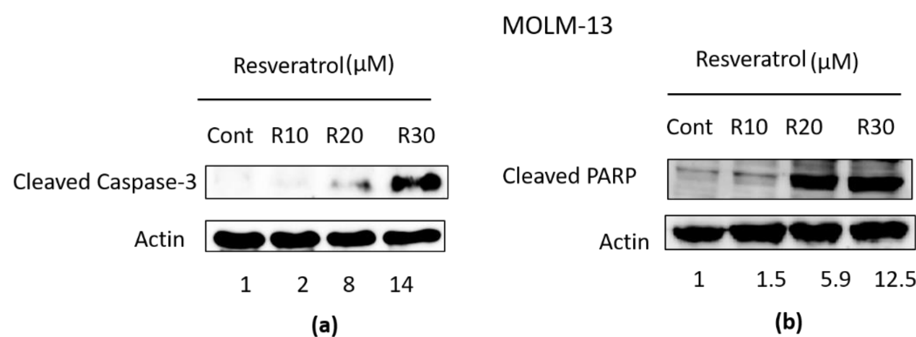


Figure 3.9 Changes in caspase 3 cleavage (a) and PARP cleavage (b) in MOLM-13 cells. Beta actin was used as loading control. Experiments were replicated independently and representative western blot image was used for each set. The protein expression of each group was normalized to their Beta-Actins.

MV4-11 cells treated with increased concentration of resveratrol (10-30 μM) caused increases in both caspase-3 cleavage as 1.6 (10 μM), 4.9 (20 μM) and 14.3 (30 μM)- and PARP cleavage as 4.3 (10 μM), 8.8 (20 μM) and 10.8 (30 μM)- fold, respectively as compared to control (Figure 3.10a and Figure 3.10b).

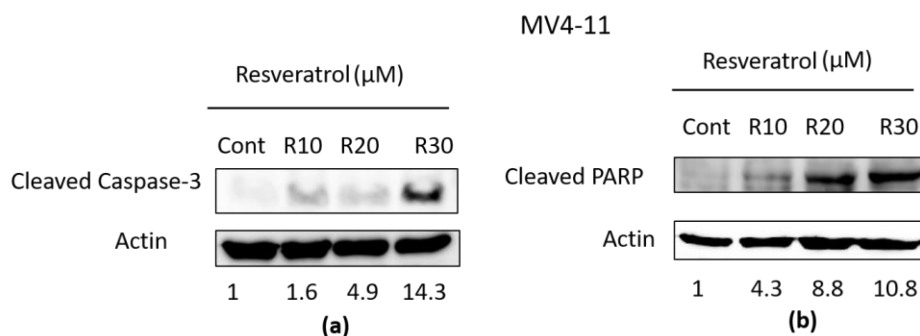


Figure 3.10 Changes in caspase 3 cleavage (a), PARP cleavage (b) in MV4-11 cells. Beta actin was used as loading control. Experiments were replicated independently and representative western blot image was used for each set. The protein expression of each group was normalized to their Beta-Actins.

Overall, the results show that resveratrol induced apoptosis through activation of caspase-3 and PARP cleavage. In the literature, it has been shown that resveratrol induces apoptosis through several mechanisms in many cancer types. In ALL cells, resveratrol induced apoptosis by depolarizing mitochondrial membranes and activating caspase cascade [188]. Resveratrol treated T-ALL cells induced apoptosis and autophagy through inhibiting Akt/mTOR/p70S6K/4E-BP1 and activated p38-MAPK signaling pathways [211]. In NK cells, resveratrol induced apoptotic protein levels such as cleaved caspase-3 and reduced antiapoptotic protein levels such as Survivin, Bcl 10 and MCL1 [214]. In another study, resveratrol (75 μM) treatment significantly increased the apoptosis of both TALL-104 and SUP-B15 cells and elevated the caspase-3 protein expression [216]. It has been shown that resveratrol treated colorectal cancer cells induced caspase-3 and PARP cleavage through p53 mediated apoptosis [219]. Resveratrol induced apoptosis and DNA fragmentation in human nasopharyngeal carcinoma (NPC) cells by upregulating proapoptotic Bax and downregulating antiapoptotic Bcl2 proteins [220]. After increased concentration of resveratrol treatment, U937 (leukemia cell), MOLT-4 (leukemia cell), MCF-7 (breast cancer cell) and HepG2 (liver cancer cell) cells underwent apoptosis and resveratrol-induced DNA fragmentation was observed in leukemic cell lines [190]. In another study, resveratrol induced caspase-3 activation in imitanib resistant

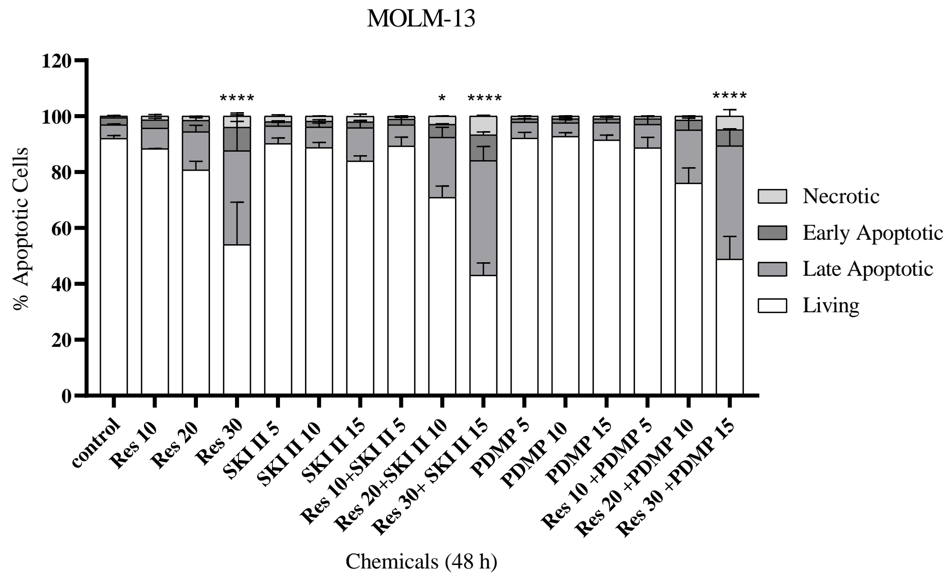
K562 CML [221]. Our findings are consistent with the literature. In this study, increasing doses of resveratrol triggered apoptosis through intrinsic pathway by activating caspase-3 and PARP.

3.6 Synergistic Apoptotic Effects of Resveratrol in Combination with Ceramide Metabolism Inhibitors on FLT3-ITD positive AML

Phosphatidylserine (PS) molecules are pivotal for apoptotic cell recognition, hereby, in order to determine whether the growth inhibitory effect of resveratrol is due to induction of apoptosis, *FLT3-ITD* AML MOLM-13 and MV-11 cells were incubated with increasing doses of resveratrol, SKI II, PDMP and in combinations for 48 hours. The percentage of cells undergoing apoptosis upon treatments were detected by flow cytometry using Annexin V-Propidium Iodide (PI) dual staining.

MOLM-13 cells were treated with resveratrol (10-30 μM) and total apoptotic cell population (early apoptotic cells + late apoptotic cells) was increased as 10.35%, 17.8% and 42 % respectively, compared to control (7.5 %) (Figure 3.11). In SKI II (5-15 μM) treated MOLM-13 cells, apoptotic cell population was increased as 7.95, 9.45 and 13.9 % respectively, compared to control (7.5 %) (Figure 3.11). When MOLM-13 cells were treated resveratrol (10-30 μM) with SKI II (5-15 μM), apoptotic cell population was increased as 9.6, 26.1, and 50.25 % respectively, regarding with control (7.5 %) (Figure 3.11). 20-30 μM resveratrol with 10-15 μM SKI II increased apoptotic MOLM-3 cell population compared to control. PDMP (5-15 μM) treated MOLM-13 cells did not show specific increases in percentages of total apoptotic cell population (7, 5.35 and 7.6 % respectively) compared to control (7.5 %), however, resveratrol (20-30 μM) with PDMP (10-15 μM) increased apoptotic cell population in MOLM-13 cells as 22.6 and 46.35 % respectively, as compared to control (7.5 %) (Figure 3.7.a).

a)



b)

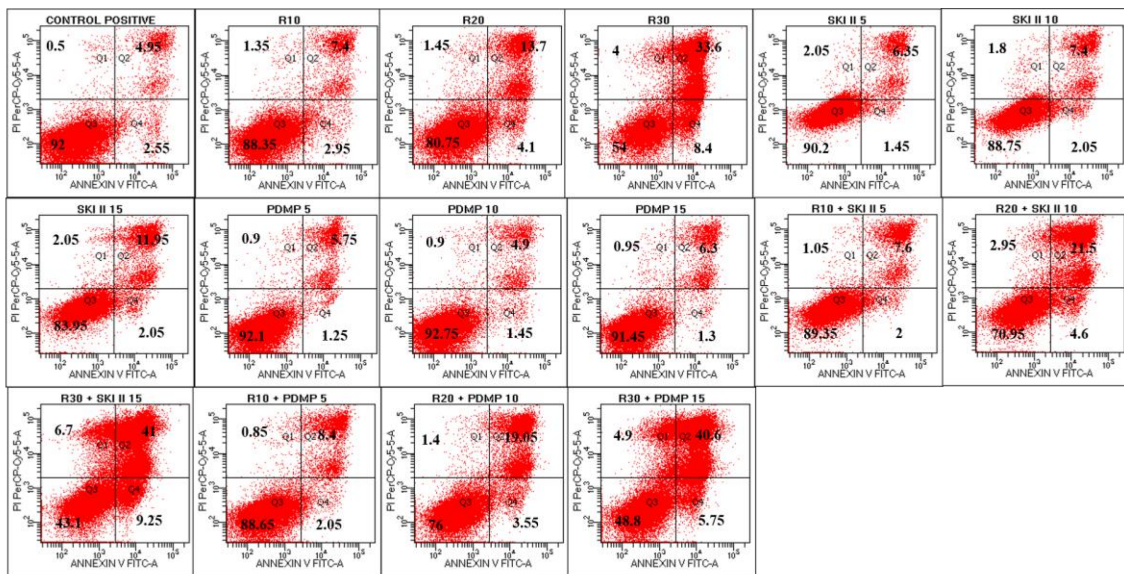
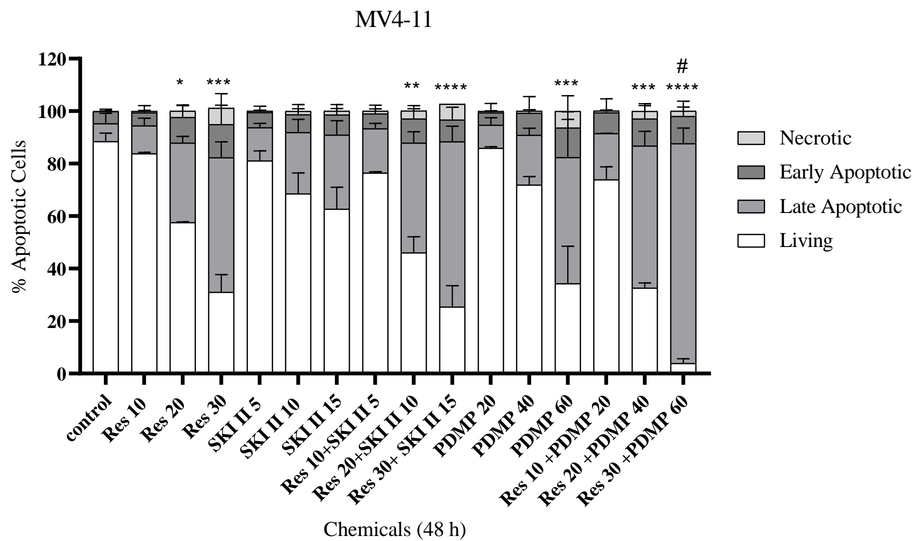


Figure 3. 11 Apoptotic effects of resveratrol with SKI II (a) and PDMP (b) on MOLM-13 cells. Data are presented as the mean ± standard error. *P<0.05 **P <0.005, ***P:0.001 and ****P<0.0001 vs controls.

MV4-11 cells were treated with resveratrol (10-30 µM) and total apoptotic cell population (early apoptotic cells + late apoptotic cells) was increased as 15.9, 40 and 63.9 % respectively, as compared to control (11.4 %) (Figure 3.12). SKI II (5-15 µM) increased levels of apoptotic cell population in MV4-11 cells as 18.4, 30.15 and 35.95 % respectively, compared to control (11.4 %) (Figure 3.12b). In resveratrol (10-30 µM) with

SKI II (5-15 µM) combinations, apoptotic cell population was increased in MV4-11 cells as 22.5, 51.1, and 71.25 % respectively, as compared to control (7.5 %) (Figure 3.12). When MV4-11 cells were treated with PDMP (20-40 µM), apoptotic cell population was increased as 13.5, 27.3, and 60.3 % respectively, as compared to control (7.5 %) (Figure 3.7.b). Resveratrol (10-30 µM) in combination with PDMP (20-60 µM) increased apoptotic cell population in MV4-11 cell as 25.5, 64.5 and 94.2 % as compared to control (7.5 %) (Figure 3.12c).

a)



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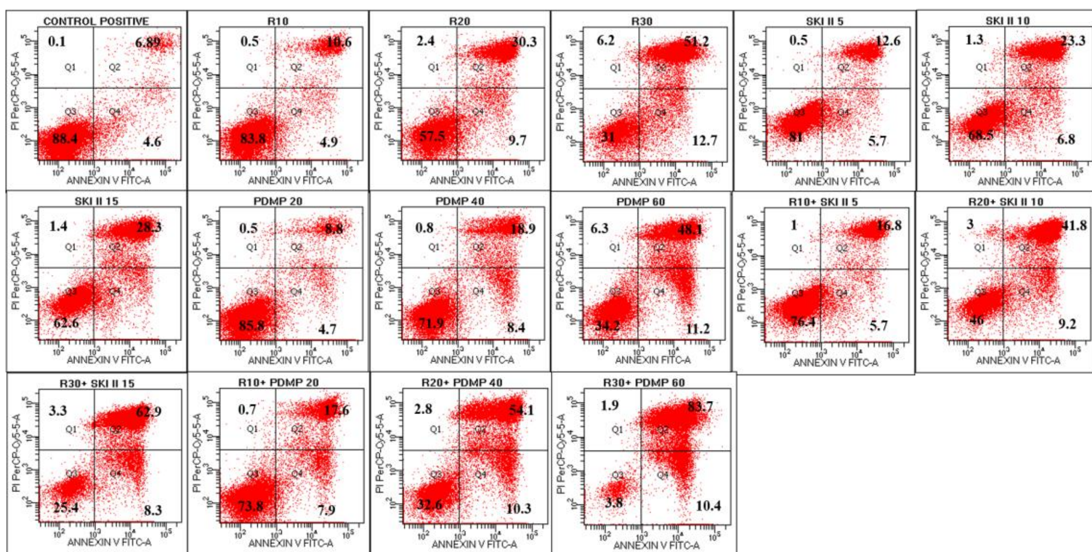

Figure 3.12 Apoptotic effects of resveratrol with SKI II (a) and PDMP (b) on MV4-11 cells. Data are presented as the mean ± standard error. *P<0.05 **P <0.005, ***P:0.001 and ****P<0.0001 vs controls. #P<0.05 vs resveratrol alone.

As discussed earlier, resveratrol triggered apoptosis via caspase-3 and PARP cleavage, which was proven by Annexin V/PI staining. There are limited number of studies which explain the main mechanism underlying resveratrol mediated growth inhibiton in the contex of sphingolipid metabolism. Resveratrol with PDMP and SKI II combinations increased the apoptotic effects of resveratrol through increasing caspase-3 activity and mitochondrial membrane potential K562 CML cells [195]. Resveratrol with dimethylsphingosine (DMS), which is a SK inhibitor, treatment induced apoptosis through exposure of phosphatidylserine to the outher membrne in human gastric cancer cells [197]. These studies support the hypothesis of this study in which co-treatments resulted in increases in apoptotic cell percentages. To understand the main mechanism which resveratrol and its combinations induced apoptosis, PARP cleavage level was checked after treatments in FLT ITD + AML cells. Resveratrol (10 μM) with SKI II (5 μM) combination increased PARP cleavage as 10.7- fold compared to control and resveratrol with PDMP combination increased PARP as 1.8- compared to control (Figure 3.13)

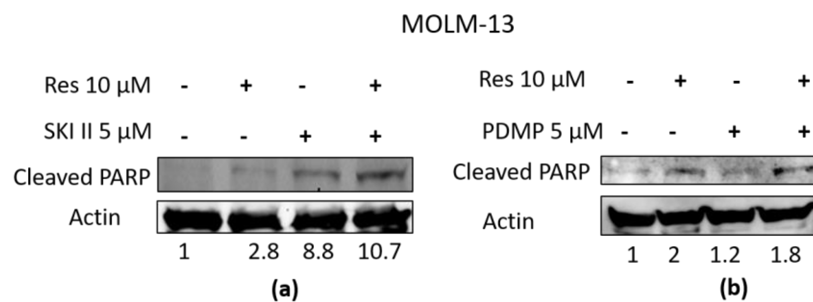

Figure 3.13 The changes in PARP cleavage after resveratrol with SKI II(a) and PDMP (b) treatment in MOLM-13. Beta actin was used as loading control. Experiments were replicated independently and representative western blot image was used for each set. The protein expression of each group was normalized to their Beta-Actins.

Resveratrol with SKI II combination increased PARP cleavage as 4.7- fold compared to control and resveratrol with PDMP combination increases PARP cleavage as 3.9- fold compared to control (Figure 3.14).

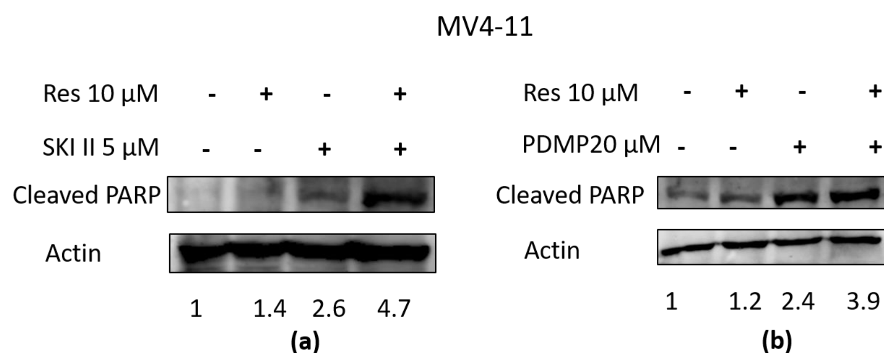

Figure 3.14 The changes in PARP cleavage after resveratrol with SKI II(a) and PDMP (b) treatment in MV4-11 cells. Beta actin was used as loading control. Experiments were replicated independently and representative western blot image was used for each set. The protein expression of each group was normalized to their Beta-Actins.

SK-1 inhibitors induced apoptosis through PARP cleavage in natural killer large granular lymphocyte leukemia [222]. Another study showed that PDMP sensitized pancreatic cancer cells by inducing PARP cleavage [223]. Inhibition of of SK-1 and GCS by SKI II and PDMP caused Cer accumulation which led to apoptosis in cells [224]. In large granular lymphocyte leukemia (LGL) patients, SK and its product S1P are increased therefore, genetic and pharmacological targeting SK resulted in proteosomal degradation of anti-apoptotic Mcl-1 [225]. GCS overexpression is significantly associated with poor prognosis in human cancers and multidrug resistance [226]. In glioblastoma (GBM) cells, GCS was silenced by using siRNA and resulted increasing of chemotherapeutic susceptibility and triggering apoptosis [227]. Therefore, treatment of cancer by targeting Cer metabolism has a big potential [228]. The apoptotic effect of PDMP in ceramide metabolism was shown in HL60 AML and K562 CML cells [193] [207]. In prostate cancer cells, resveratrol inhibited cell growth, induced apoptosis by downregulated SK-1 with *in vivo* and *in vitro* experiments [229]. Since targeting of SK-1 is an important therapeutic approach, resveratrol and its analogous have been designed as anti-cancer agents to target SK-1 and trigger PARP-dependent apoptosis in MCF-7 breast cancer cells [230]. In this study we showed that resveratrol with SKI II and PDMP trigger apoptosis and resulted in increases in the levels of cleaved PARP in AML cells.

Chapter 4

Conclusion and Future Prospects

4.1. Conclusion

The main molecular signaling pathway underlying the antileukemic effects of resveratrol in *FLT3 ITD+ MOLM-13* and *MV4-11 AML* cells were investigated for the first time through the inhibition of SK-1 and GCS which are the key enzymes of ceramide metabolism.

It was shown that resveratrol inhibited cell proliferation and induced S phase arrest. Besides, resveratrol triggered apoptosis through caspase-3 and PARP activation. In addition, resveratrol downregulated SK-1 and GCS enzyme expressions which convert apoptotic Cer to antiapoptotic S1P and GC in *FLT3 ITD+* AML. While resveratrol with SKI inhibitor (SKI II) treatment does not have significant effect on cell cycle arrest, combination with GCS inhibitor (PDMP) induced G0/G1 and S phase arrest. Combination treatments induced apoptosis through PARP cleavage.

In conclusion, it has been determined that resveratrol could have a therapeutic potential by targeting sphingolipid metabolism in *FLT3-ITD+ AML* for the first time. Therefore, after detailed investigation of how resveratrol and SK-1 and GCS regulate *FLT3-ITD+ AML* pathogenesis, it could be suggested that resveratrol would be a complementary agent in *FLT3-ITD+ AML* treatment.

4.2 Societal Impact and Contribution to Global Sustainability

Cancer is a second common disease around the world. Acute Myeloid Leukemia is highly aggressive version of leukemic cancer type. It is not easy to treat *FLT3 ITD* Acute Myeloid Leukemia due to development of resistance, relapse and toxicity even

though clinically approved targeted therapies. AML is commonly seen in adults who are older than 60 years old and the recurrence rate after complete remission is still high. Even if, AML patients who are younger than 60 years old can survive more than 5 years, resistance could be arise and disease relapse. There are several drugs, which targets FLT3 receptors, approved by Food and Drug Administration (FDA). However, drug resistance can develop after chemotherapy. Resistance to chemotherapy in cancer treatment is one of the most important obstacles that limit the response of patients to treatment. Developing resistances may exist in patients before treatment (primary drug resistance), but they can generally develop some time after the treatment is started and are called acquired drug resistance (secondary resistance). The molecular mechanisms of resistance may differ according to the type of cancer. Therefore, it is important to find a novel integrative solution to treat the disease.

Identification of new agents or intracellular signaling pathways for overcoming chemotherapy resistance is especially important for the development of new treatment approaches. Natural products have attracted attention from both clinicians and basic researchers to overcome side-effects of conventional therapies and resveratrol is one of the most important polyphenols with anticarcinogenic properties. Resveratrol as a natural product might be used in combination therapies to target lipid metabolism. With the data which is obtained from this study, the potential of resveratrol can be used as an integrative nutraceutical in FLT3 ITD positive AML treatment. New biomarkers could be defining by determining resveratrol's cytotoxic effect on especially in lipid metabolism.

4.3 Future Prospects

In the future studies, the crosstalk between resveratrol and sphingolipid metabolism would be investigated in more detail by using *in vitro* and *in vivo* models of FLT3+ ITD AML. The apoptotic and cytostatic effects determined in this study could be investigated at molecular level.

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SELECTED PUBLICATIONS AND PRESENTATIONS

J1) N.S. Ersoz, A.Adan, Cytotoxic Effects of Resveratrol and Its Combinations with Ceramide Metabolism Inhibitors on FLT3 Positive Acute Myeloid Leukemia, Erzincan Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 2020

C1) N.S. Ersoz, A.Adan The 3rd international conference on Natural Products for Cancer Prevention and therapy, MDPI, 2019

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