THE ROLES OF CERAMIDE METABOLIZING GENES ON RESVERATROL INDUCED APOPTOSIS IN HUMAN HL60 ACUTE MYELOID LEUKEMIA CELLS

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Zeynep ÇAKIR

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İZMİR

We approve the thesis of **Zeynep ÇAKIR**

Assoc. Prof. Dr. Yusuf BARAN
Supervisor

Assoc. Prof. Dr. Güray SAYDAM
Committee Member

Assist. Prof. Dr. Bünyamin AKGÜL
Committee Member

24 December 2010

Assoc. Prof. Dr. Sami DOĞANLAR
Head of the Department of Molecular Biology and Genetics

Prof. Dr. Durmuş Ali DEMİR
Dean of the Graduate School of Engineering and Sciences

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To my family...

ABSTRACT

THE ROLES OF CERAMIDE METABOLIZING GENES ON RESVERATROL INDUCED APOPTOSIS IN HUMAN HL60 ACUTE MYELOID LEUKEMIA CELLS

Resveratrol (3,5,4'-trihydroxy-trans-stilbene) is naturally occurring phytoalexin, which presents especially in grapes. It is a potential anticancer agent which inhibits tumor initiation, promotion, and progression. Ceramides are the central compounds of sphingolipids metabolism. They are known as second messengers regulating various cellular processes including cell growth, proliferation, differentiation and apoptosis. While ceramide acts as a strong apoptotic molecule, glucosylceramide (GluCer) and sphingosine-1-phosphate (S1P) trigger cell growth and proliferation and inhibit apoptosis. Sphingosine kinase-1 (SK-1) is an enzyme catalyzing the phosphorylation of sphingosine to sphingosine-1-phosphate while glucosylceramide synthase (GCS) converts ceramide to glucosylceramide. Thus, inhibition of GCS by N-(2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl)-decanamide, hydrochloride (PDMP) or SK-1 by sphingosine kinase-1 inhibitor, or exogenous application of ceramide analog (C8:ceramide) results in increased accumulation of ceramides. The aim of the study is to examine the roles of ceramide, glucosylceramide and sphingosine-1-phosphate in resveratrol induced apoptosis in HL60 cells.

In this study, it was demonstrated cytotoxic effects of resveratrol on human acute myeloid leukemia cells in addition to identify a novel mechanism of resveratrol-induced apoptosis by targeting ceramide metabolism. And also it was shown that via targeting ceramide-generating and/or ceramide-clearance genes provided ceramide generation and/or accumulation in response to resveratrol treatment. This study showed for the first time that there were significant induction of apoptosis through increasing intracellular concentrations of ceramides in resveratrol treated HL60 cell. Taking together all these results showed that ceramides may be involved in resveratrol-induced apoptosis.

ÖZET

İNSAN HL60 AKUT MİYELOİD LÖSEMİ HÜCRELERİNDE RESVERATROLÜN TETİKLEDİĞİ PROGRAMLI HÜCRE ÖLÜMLERİ ÜZERİNE SERAMİD METABOLİZMASI GENLERİNİN ROLLERİ

Resveratrol, doğal olarak bitkilerde oluşan anti-oksidan bir fitoaleksindir. En çok kırmızı üzüm ve diğer bazı meyvelerde bulunur. Resveratrolün hücre içindeki fonksiyonları anti-oksidan ve anti-inflamatuar, tümör oluşumunu ve ilerlemesini inhibe etmesidir. Seramidler; hücrede büyüme, farklılaşma ve apoptoz gibi çok önemli metabolik proseste görev alan hücrenin membranlarında bulunan yapısal bir moleküldür. Seramid metabolizmasında rol alan kritik enzimler, sfingozin kinaz-1 ve glukozilseramid sentazdır. Seramidler apoptotik sfingozine seramidazlar ile dönüşür ve bu sfingozinler; sfingozin kinaz-1'ler ile sfingozin-1-fosfata fosfatlanırlar. Sfingozin-1-fosfat, hücrenin yaşamını ve proliferasyonunu sağlar ve apoptozu inhibe eder. Sfingozin kinaz-1 enziminin inhibe edilmesi sonucu seramid birikimi sağlanabilmektedir. Glukozilseramid sentaz (GSS) enzimi, seramidin glukozilseramide glukozillenmesini katalizler. Glukozilseramid ise kanser hücrelerinde ilaç dirençliliğine yol açan bir moleküldür. GSS'nin, PDMP ile inhibisyonu ilaç dirençliliğine neden olan genlerin ekspresyon düzeyinin düşmesine ve aynı zamanda seramid birikmini sağlayarak kanser hücrelerinin apoptoza gitmelerine neden olmaktadır.

Resveratrolün tek başına ya da resveratrol ile kombine edilen seramidlerin, SK-1 inhibitörü ve PDMP'nin HL60 hücreleri üzerine sitotoksik ve apoptotik etkileri gösterilmiştir. Ve ayrıca seramid metabolize eden genlerin expresyon düzeylerindeki değişimler RT-PCR ile gösterilerek CerS 1, 2, 4, 5, 6 genlerinin düzeyindeki artışlar ile SK-1 ve GSS genlerinin düzeylerindeki azalışlar gösterilmiştir. Resveratrol uygulanan HL60 hücrelerinde, seramid biyosentezinde görev alan enzimlerin gen düzeylerindeki artışlar ile seramidi metabolize eden genlerin düzeylerindeki azalışlar sonucu seramidin birikimi sağlanarak hücrelerin apoptoza girmeleri kanser tedavisinde yeni bir tedavi yöntemi olarak düşünülebilir.

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CHAPTER 1

INTRODUCTION

1.1. Acute Myeloid Leukemia (AML)

Acute Myeloid Leukemia is characterized with the rapidly growing of immature blood cells and could be fatal within few weeks if it is untreated (Shipley and Butera, 2009). In AML disease, neoplastic cells are accumulated in the bone marrow, as a consequence hematopoiesis is blocked, in other words leukemic cells replace with normal bone marrow cells. This causes decreases in number of normal platalets, red blood cells and white blood cells. This type of leukemia is resulted from variety type of translocation or inversion at the chromosomal break (Shipley and Butera, 2009). After formation of these variety abnormalities in the chromosome, they encode for proteins with the aberrant functions. These aberrant proteins are also called as 'fusion proteins'. Generally, fusion proteins are made up of a transcription factor and a protein which is related with cell survival and apoptosis in its structure (Alcalay, et al., 2001). Transcription factor, which is found in fusion protein, have effect on myeloid differentiation and hematopoiesis in certain types of AML. The common gene rearrangements are t(15;17) PML-RARα; t(8;21) AML1-ETO; 11q23 MLL rearrangements, and Inv(16), CBFβ-MYH11 in AML (Martens and Stunnenberg, 2010). It is clear that the evaluation of AML pathogenesis is very crucial issue in order to treatment of AML, therefore AML have distinct subtypes in terms of cytogenetics. Therefore, there are two classification is commonly used for classifying AML. One of them is World Health Organization (WHO) and the other one is The French-American-British (FAB) Classification. WHO classification is more prevalent as compared to FAB classification.

1.1.1. The Classification of Acute Myeloid Leukemia

WHO organization classified AML into subtypes according to chromosomal translocations in 2008. The classification of AML contain biological features

(morphology, cytogenetics, immunophenotype) and clinical datas. Table I shows the classification of AML according to 2008 WHO organization.

Table 1.1. WHO Classification of AML

- AML with recurrent genetic abnormalities
    - AML with t(8;21)(q22;q22); (RUNX1-RUNX1T1)
    - AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); (CBFB-MYH11)
    - Acute promyelocytic leukemia (APL) with t(15;17)(q22;q12); (PML-RARA)
    - AML with t(9;11)(p22;q23); (MLLT3-MLL)
    - AML with t(6;9)(p23;q34); (DEK-NUP214)
    - AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); (RPN1-EVI1)
    - AML (megakaryoblastic) with t(1;22)(p13;q13); (RBM15-MKL1)
    - Provisional entity: AML with mutated NPM1
    - Provisional entity: AML with mutated CEBPA
- AML with MDS-related changes
- Therapy-related myeloid neoplasms
- AML-NOS
    - AML with minimal differentiation
    - AML without maturation
    - AML with maturation
    - Acute myelomonocytic leukemia
    - Acute monoblastic/monocytic leukemia
- Acute erythroid leukemias
        - Pure erythroid leukemia
        - Erythroleukemia, erythroid/myeloid
- Acute megakaryoblastic leukemia
- Acute basophilic leukemia
- Acute panmyelosis with myelofibrosis
- Myeloid sarcoma
- Myeloid proliferations related to Down syndrome
    - Transient abnormal myelopoiesis
    - AML associated with Down syndrome

1.1.2. Acute Promyelocytic Leukemia (APL)

Acute Promyelocytic Leukemia (APL) is a subtype of M3-Acute Myeloid Leukemia according to the FAB classification which constitutes of ~10% of AML cases (Tallman, 2008). The frequency of this disease is rare, however it is distinct from the other AMLs with its molecular biology and clinical outcomes. The main clinical outcome of APL disease is the accumulation of progenitor myeloids, promyelocytes, in bone marrow that is based on frustrated differentiation of promyelocytes (Pandolfi, et al., 2001).

APL is characterized with the reciprocal translocation between chromosome 17 and variety of partner chromosomes such as 11, 5, 11, 17 (PLZF, NPM, NuMA, STAT5b, respectively) and generally translocation is observed between chromosomes 17 and 15 (Piazza, et al., 2001). Retinoic Acid Receptor α (RARα) gene located on chromosome 17 and it is involved in all APL cases, fuses generally with PML which are located on choromosome 15. The cells which carry PML/RARα translocation are genetically unstable (Wang, et al., 1998).

Production of PML/RARα fusion protein shows oncogenic activity and blocks myeloid differentiation. Several studies showed that PML/RARα translocation caused accelerated level of leukemogenesis both *in vivo* and *in vitro* (Pandolfi, et al., 2001) (Figure 1.1).

PML/RARα have oncogenic activity at only few type of cells. In fibroblasts, this translocation has no effect on growth however, in non-hematopoietic cells, PML/RARα has growth inhibitory effects, predominantly in hematopoietic cells (Ferrucci, et al., 1997). It is still unclear why PML/RARα translocation cause oncogenic transformation only in specific type of cells. Hematopoietic progenitors which contain PML/RARα fusion protein is resistant to apoptotic stimuli, but still the role of PML/RARα on cell cycle regulation is not clear.

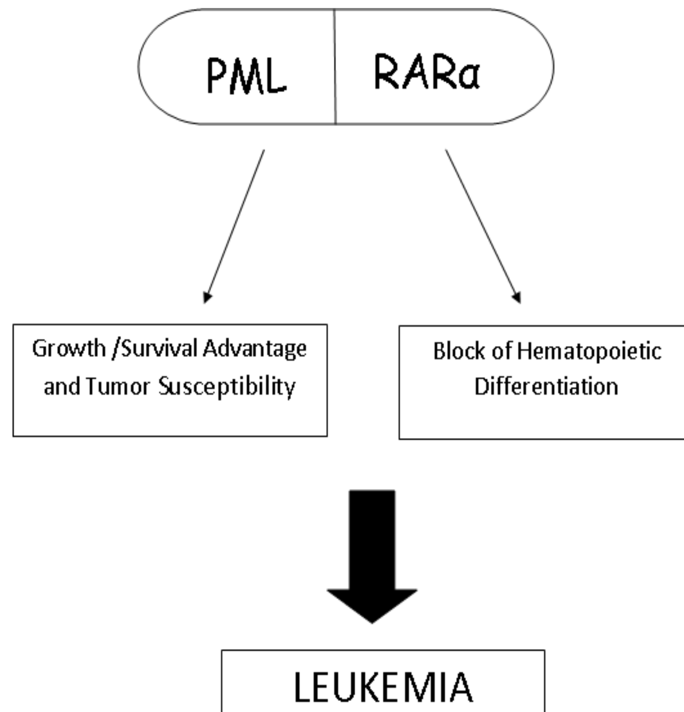


Figure 1.1. PML/ RAR α Fusion Protein Leads to Oncogenic Activation in Hematopoietic Cells (Source: Adapted from Pandolfi, 2001)

The apoptotic stimulis Fas, TNF and interferons are unresponsive in APL cells (Wang, et al., 1998). PML interacts with p53 both *in vivo* and *in vitro*. PML is required for acetylation of p53 in response to γ-irradiation and activates p53 downstream targets such as Bax. PML is not only contributed to p53-dependent pathway, but also contributed to p53-independent pathway (Zhong, et al., 2000) (Figure 1.2).

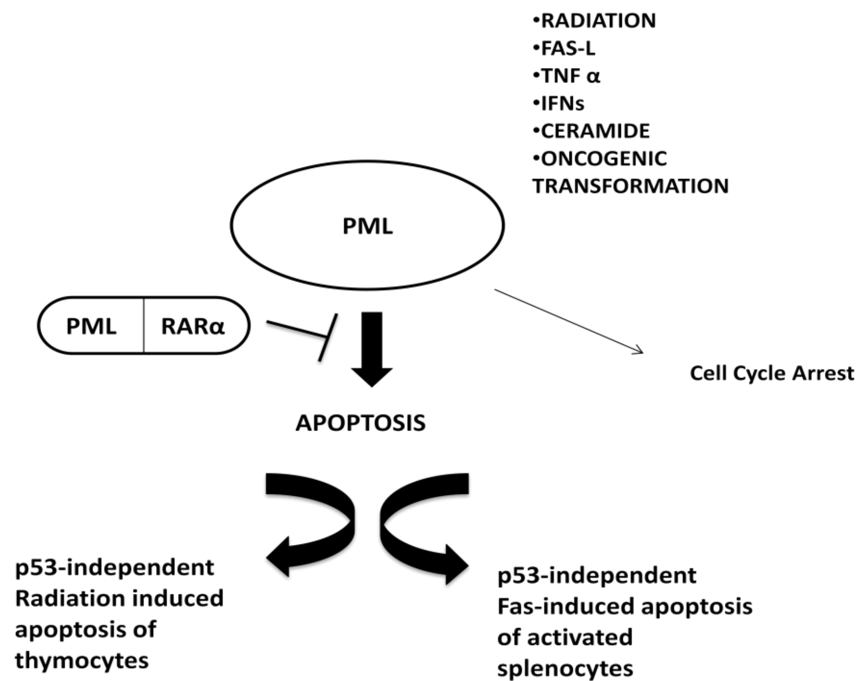


Figure 1.2. Consequence of PML/ RAR α translocation in PML function
(Source:Adapted from Pandolfi,2001)

1.2. Treatment Strategies for Acute Promyelocytic Leukemia

AML comprises %32,6 among all leukemia types according to 2007 statistics (Jemal, et al., 2007). The median age of AML is 70 years old. Generally, younger AML patients give a respond to therapy however, it is difficult to achieve a respond in older AML patients. The standard therapy in AML is administration of cytarabine and anthracyclines such as daunorubicin or idarubicin for the patients of all ages. On the other hand in APL patients, the standard therapy is both administration of all-trans retinoic acid (ATRA) and anthracycline-based chemotherapy. Another treatment approach is administration of Arsenic Trioxide (ATO) (Sanz, 2006). Molecular mechanism of ATRA is induction of differentiation of progenitor neoplastic cells into mature granulocytes. After this irreversible differentiation, leukemic cells enter to apoptotic cell death (Gianni, et al., 2000). ATO is generally used in ATRA resistant patients. ATO reacts with incomplete differentiated cells and induce apoptosis via activation of caspases in leukemic cells (Shen, et al., 1997). It was reported that hematopoietic stem cell transplantation (HSCT) is not suggested in the first line therapy

in APL patients. After ATO or gemtuzumab, HSCT approach can be considered to apply for patients (Tallman, 2007).

Antibodies are also used in APL patients. Gemtuzumab-ozogamicin is one of this conjugated antibodies which determines and binds to CD33 cell surface marker. Gemtuzumab-Ozogamicin is internalized into leukemic cell and conjugated calicheamicin is released and leads to DNA damage and cell death. Calicheamicin belongs to antracycline group which is also effective chemotherapeutic agent in APL treatment (Lo-Coco, et al., 2003).

1.3. Resveratrol

Resveratrol (trans-3,5,4'-trihydroxystilbene, $C_{14}H_{12}O_3$) is a polyphenol that have beneficial effects on body.

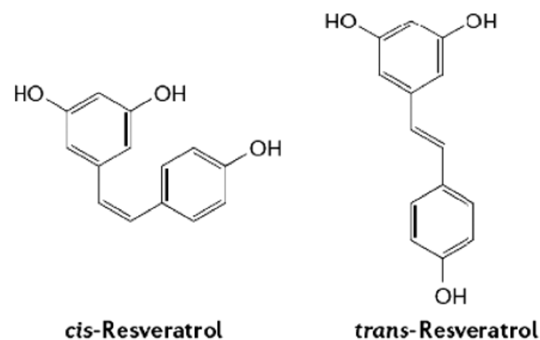


Figure 1.3. The Representation of Resveratrol Structure
(Source: Kraft et al., 2009)

Resveratrol, as a phytoalexin, is derived from plants such as red grape seed and skin, blueberry, peanuts and red wine. It was firstly isolated from the roots of white hellebore and then from *Polygonum cupsidatum* (Baur and Sinclair, 2006). Phytoalexin is a toxic molecule for foreign attacts. Resveratrol is synthesized in plants in response to stress, bacterial or fungal attacks and UV-irradiation. Mostly, it is synthesized in the form of *trans-* configuration because it is thermordynamically stable.

It is known that red wine contains high concentration of resveratrol. Consumption of red wine is correlated with the low risk of cardiovascular diseases in

French population as compared to other industrial countries. This phenomenon is known as French paradox (Richard, 1987).

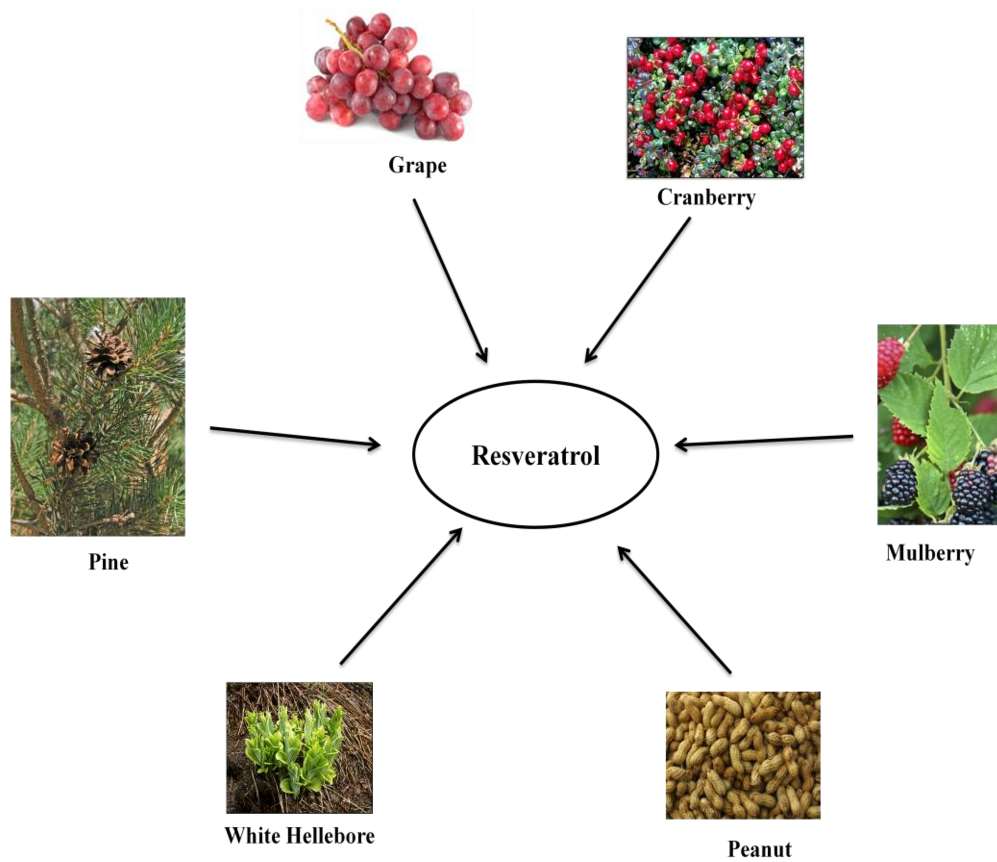


Figure 1.4. The Sources of Resveratrol
(Source: Adapted from Shakibaei et al.,2009)

1.3.1. Biosynthesis of Resveratrol in Plants

Resveratrol is synthesized via phenylpropanoid pathway in plants (Figure 1.5). Phenylalanine is converted to cinnamic acid by phenylalanine ammonia lyase (PAL) and cinnamic acid is transformed into p-coumaric acid (4-coumaric acid) by cinnamic acid 4-hydroxylase (C4H). Then, the enzyme 4-coumarate:CoA ligase (4CL) adds p-coumaric acid to pantetheine group of Coenzyme-A (CoA) and generates 4-coumaroyl-CoA. After that condensation of p-coumaroyl-CoA and malonyl-CoA form resveratrol. Malonyl-CoA is generated from fatty acid biosynthesis (Halls and Yu, 2008).

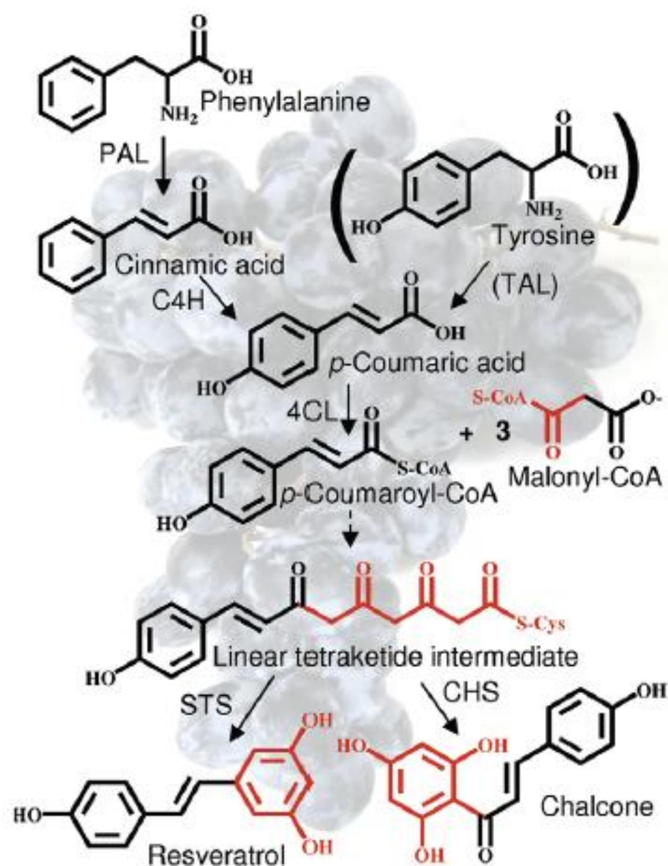


Figure 1.5. Biosynthetic Pathway of Resveratrol
(Source: Halls et al., 2008)

1.3.2. The Roles of Resveratrol in Mammalian Cells and Possible Medical Application

Plant-derived resveratrol targets many intracellular molecules in mammalian cells and activates signaling molecules and enzymes in the cell. The best known target of resveratrol is Sirtuin (SIRT) 1 to 7 enzymes which is involved in DNA repair, cell survival, gluconeogenesis, muscle cell differentiation, cell cycle regulation, lipid metabolism, fat mobilization and insulin sensitivity (Brooks and Gu, 2009). The activation of SIRTs by mediating of resveratrol augments against aging (Brooks and Gu, 2009).

Resveratrol binds to various cell-signaling molecules and modulates cell-cycle regulatory genes. It activates transcription factors, inhibits protein kinases and the expression of antiapoptotic genes, as well as angiogenic and metastatic gene products

and inflammatory biomarkers, induces antioxidant enzymes, and alters the expression of enzymes such as cytochrome P450s that are involved in drug metabolism (Baur and Sinclair, 2006). Resveratrol increases the activity of endothelial nitric oxide synthase enzyme which synthesize vasodilator molecule nitric oxide (Nicholson, et al., 2008). On the other hand resveratrol inhibits platelet aggregation and protects body from cardiovascular diseases (Das and Das, 2010).

Resveratrol is widely known due to its antioxidant activity. With this property, resveratrol scavenges superoxide and peroxynitrite radicals by forming enzymatically or non-enzymatically(de la Lastra and Villegas, 2007). Reactive oxygen species (ROS) are mostly generated as byproducts in metabolism. These molecules are highly reactive and may give damage to DNA. Resveratrol protects towards DNA damage caused by ROS (Jagdeo, et al., 2010).

One of the such roles of resveratrol are inducing apoptosis and inhibiting anti-apoptotic molecules. It was found that resveratrol activates death receptor such as CD95/Fas system in HL60, T47D breast carcinoma cells and colon carcinoma cells (Clement, et al., 1998 and Delmas, et al., 2003). Also, resveratrol may induce apoptosis intrinsically beside that induction of apoptosis via extrinsically. Resveratrol inhibits FIF0 ATP synthase and increases apoptotic molecules such as Bax and p53 is also involved in resveratrol-induced apoptosis and upregulates expression of $p21^{cip1}$ and GADD45 which are downstream targets of p53 (Lu, et al., 2001). Resveratrol interacts with tyrosine kinases which are generally upragulated in many types of cancer and inhibits Src kinases and MAPK pathway (Shen, et al., 2003). Activator protein-1 (AP-1) is normally elevated in cancer and upregulates Fos and Jun families and effects transcription factors. However, it was found that resveratrol suppressed AP-1 stimulation (Manna, et al., 2000).

Szende et al. demonstrated that resveratrol inhibited VEGF activation and prevented angiogenesis in HUVEC (human umbilical vein endothelial cells) cells (Szende, et al., 2000). And also resveratrol induced proteolytic degradation of MMPs which are involved in angiogenesis (Cucciolla, et al., 2007).

1.3.3. Chemotherapeutic Effects of Resveratrol in Cancer Models

Resveratrol has broad spectrum effects from anti-oxidant properties to anti-inflammatory effects on mammary cells. After finding of these properties, Jang et al. demonstrated for the first time resveratrol has anti-carcinogenic effects on cancer cells in 1997 (Jang et al,1997). Then, researchers tend to find molecular mechanisms of resveratrol on cancer cells. And it was reported that resveratrol inhibits initiation, promotion and progression of carcinogenesis, also sensitized chemoresistant or radioresistant cells to apoptosis and metastasis (Garg, et al., 2005). One of the most important point in chemotherapy is regulation of cell proliferation and survival. Some of the chemotherapeutic agents target cell cycle regulatory proteins such as cyclin-dependent kinase (cdk) inhibitors and check point kinases (chk). Resveratrol suppressed cyclin D1, D2 and E and reduced the level of Retinoblastoma protein in A431 epidermoid carcinoma cells (Kim, et al., 2006). Therefore, resveratrol may be considered as a good chemotherapeutic agent.

Resveratrol has antiproliferative roles in many types of cancer such as breast, prostate, lung cancers, melanoma, colorectal and gastric cancer and leukemias (Athar, et al., 2007). In prostate cancer cells, resveratrol downregulated androgen-responsive genes, including prostate-specific antigen (PSA), human glandular kallikrein-2, AR (androgen receptor)-specific coactivator ARA70 (Mitchell, 1999) and also activated apoptotic molecules in LnCap prostate cancer cells (Aziz, 2006) and MAPK signaling and finally activation of p53 in LnCap cells (Lin, et al., 2002).

The molecular structure of resveratrol is similar to synthetic estrogen and can be considered as a phytoestrogen. In breast cancer, resveratrol binds to both α and β estrogen receptors and (17-beta-estradiol) E2 and inhibits the growth of MCF-7 and (Lu and Serrero, 1999), highly invasive MDA-MB-435 cells (Hsieh, et al., 1999).

The environmental pollutants and cigarette smokes contain very harmful carcinogens. Benzo[a]pyrene and PAH (polycyclic aromatic hydrocarbons) are one of these metabolites in human and have crucial roles in lung cancer (Sabapathy, et al., 2004). Resveratrol changed the expression levels of PAH and BaP metabolizing enzymes such as cytocrome P450 (1A1 (CYP1A1) and 1B1 (CYP1B), and glutathione S-transferase P1 (GSTP1) genes (Mollerup, et al., 2001).

Resveratrol induced apoptosis via activation of CD95/CD95 system in HL60 and U937 leukemic cell lines. (Clement, et al., 1998). However, in ALL cells which are resistant to CD95 signaling, induced apoptosis via CD95 signaling independent pathway and activating cell death via mitochondria depolarization and activation of caspase-9 in resveratrol treated cells (Dorrie, et al., 2001).

1.4. Bioactive Sphingolipids

Sphingolipids are a components of membrane lipids which are found in lipid rafts and contribute to many functional roles beside structural roles in the cell. Sphingolipids have roles in differentiation, survival, apoptosis and proliferation. The member of sphingolipid families are ceramide, spingosine, sphingosine-1-phosphate, glucosylceramide and sphingomyelin (Figure 1.6) (Hannun and Obeid, 2008).

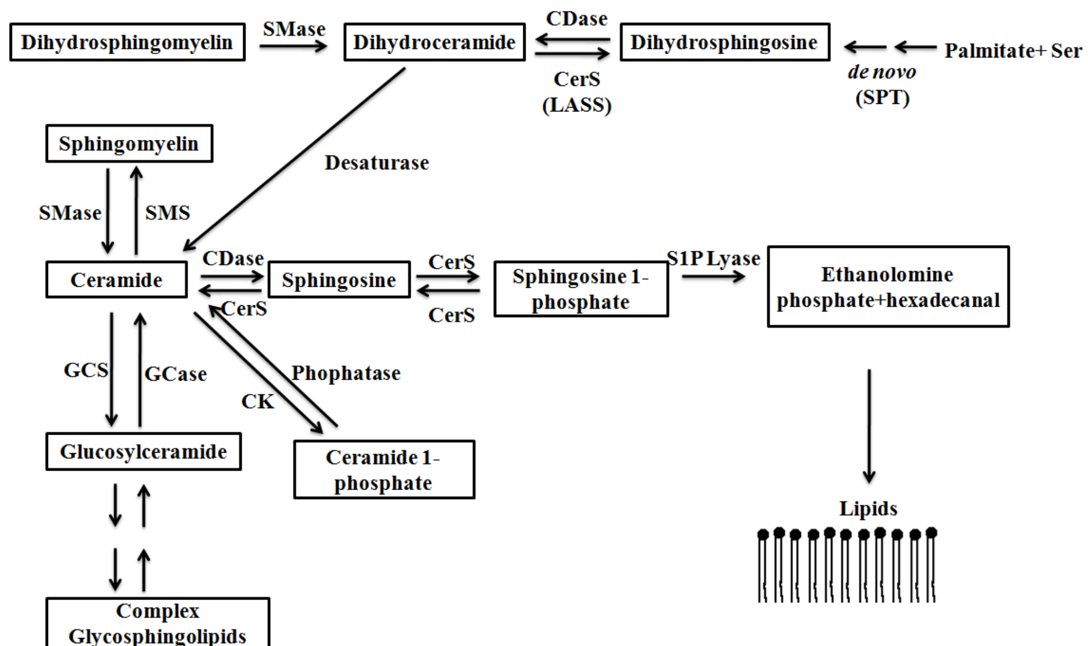


Figure 1.6. Ceramide Metabolism and Interconnection Between Ceramide Metabolites (Source: Adapted from Hannun, 2008)

It was revealed that in last decades sphingolipids have important roles in cancer biology (Ogretmen, 2006). GluCer and S1P play key roles in cell proliferation and drug resistance which means unresponsive to chemotherapeutic agents. On the contrary,

ceramide and shingosine have function in apoptosis, differentiation and growth arrest (Figure 1.7) (Ogretmen and Hannun, 2004).

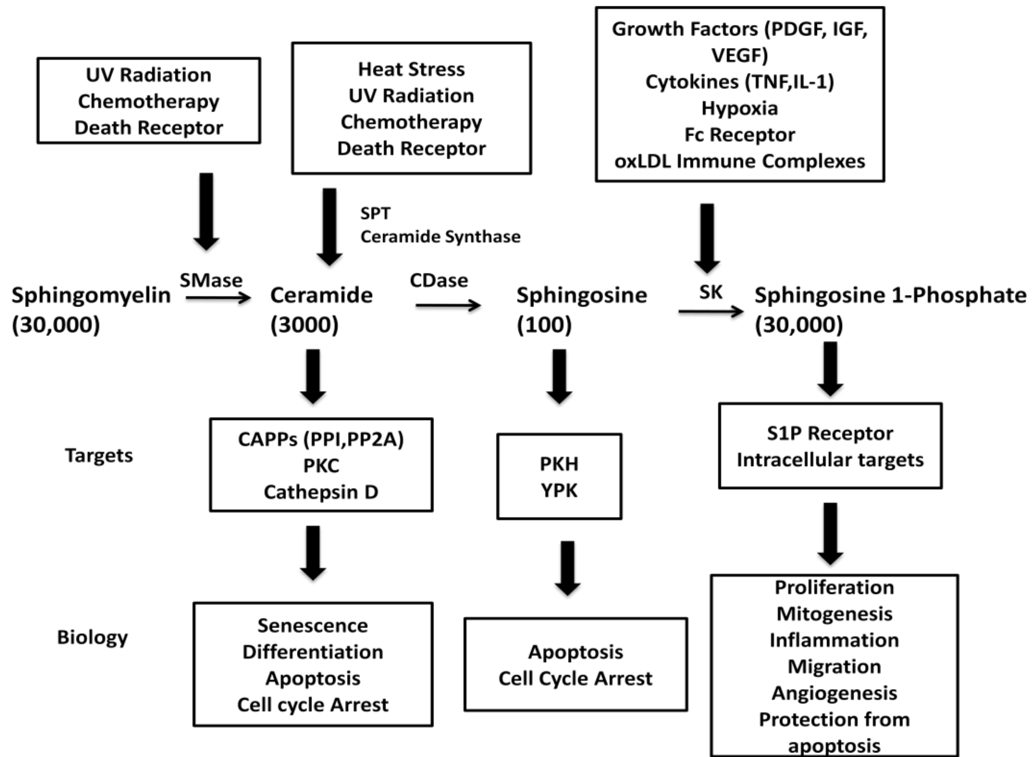
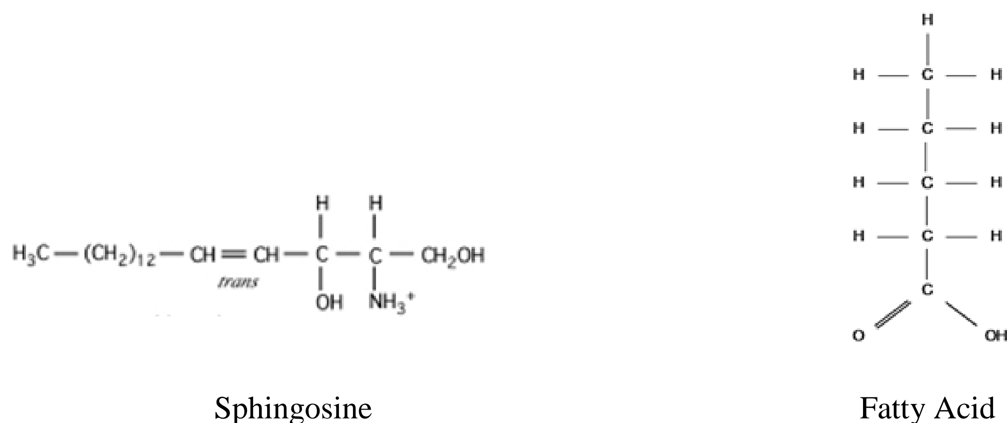


Figure 1.7. Overview of The Roles of Sphingolipids in Cancer Biology
(Source: Adapted from Hannun, 2008)

1.4.1. Ceramides

1.4.1.1. Structure and Metabolism of Ceramides

Ceramides are made up from sphingosine and amide linked to a variety number of fatty acyl chain length (Figure 1.8). These length of fatty acyl chain varied from C_{14} to C_{26}.



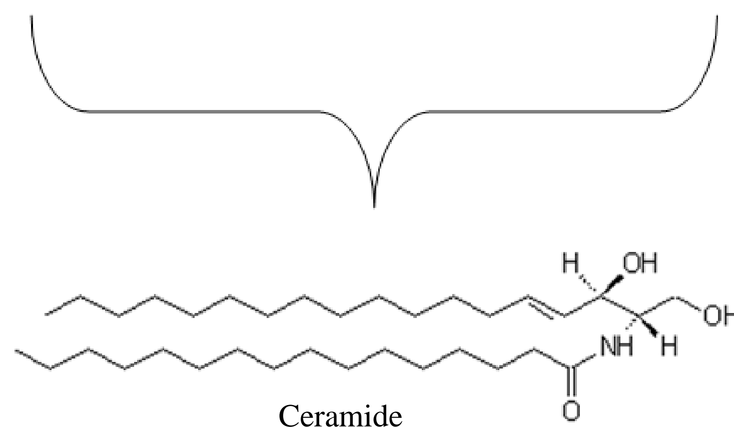


Figure 1.8. The Molecular Structure of Ceramide
(Source: Adapted from Ponnusamy, 2010)

Ceramide acts as a precursor molecule and can be converted into other types of sphingolipids such as sphingomyelin, sphingosine, glucosylceramide and sphingosine-1-phosphate in sphingolipids metabolism (Hannun, 1996). Ceramides are generated through salvage pathway, sphingomyelinase pathway, recycling of exogenous pathway and *de novo* synthesis. In sphingomyelinase pathway, sphingomyelins are hydrolyzed via acid sphingomyelinases or neutral sphingomyelinases at internal membranes or lipid rafts. Neutral sphingomyelinases can be activated via ROS generation in mitochondria and generated ceramides (Kitatani, et al., 2008). The term ‘salvage pathway’ is recycling of sphingolipids in the late endosomes and lysosomes. Recently, it has been found that salvage pathway modulated ceramide formation and metabolism (Jin, et al., 2008). In lysosomes, sphingomyelins are degraded via acid sphingomyelinases into ceramides and further metabolized to sphingosine and exported from lysosomes. However, ceramide seems to not export from the lysosomes and remains in the lysosomes (Kitatani, et al., 2008). After degradation of the long chain

sphingoid bases in lysosomes, short chain sphingoid bases are exported from lysosomes and contribute to sphingolipid metabolism. Furthermore, family of ceramide synthase enzymes synthesize ceramides from long chain sphingosine bases in endoplasmic reticulum after releasing from lysosomes (Smith and Merrill, 1995). Exogenous ceramides also elevate ceramide levels in the cell. Especially, short chain ceramides such as C_2 and C_6 Ceramides are less non-polar and dissolved in water, therefore they could pass from cell membrane easily, contribute to generation of endogenous ceramides and take place in ceramide signaling (Dobrowsky and Hannun, 1992).

1.4.1.2. Ceramide Synthases and *de novo* Generation of Ceramides

De novo synthesis of ceramide starts with condensation of palmitoyl CoA and serine in endoplasmic reticulum (ER). This condensation forms 3-ketosphinganine with Serine-Palmitoyl Transferase. Then, 3-Ketosphiganine is converted to sphinganine via 3-ketosphinganine reductase and finally yields dihydroceramides by ceramide synthases. Dihydroceramides are transformed to ceramide by inserting double bonds into sphingosine backbone. All of these metabolic reactions occur in the ER and the newly synthesized ceramide exports to golgi apparatus by ceramide transfer protein (CERT). Ceramides are synthesized with different acyl chains by ceramide synthases (CerS)1-6 (Figure 1.9). Ceramide synthase family is actually known as Longevity Assurance Gene family (LASS). CerS family show a homology with yeast Longevity Assurance gene-1 (LAG-1). CerS1 and CerS4 synthesize C18 Ceramide, CerS2 and CerS4 synthesize C20, CerS2 and CerS3 synthesize C22, C24 and C26 Ceramide, CerS6 synthesize C14 Ceramide and CerS5 and CerS6 synthesize C16 Ceramide (Levy and Futerman, 2010). CerS has Hox-like domain in its structure. Hox domain plays important roles in development. Different length of ceramides have different roles in the cell such as proliferation, apoptosis, survival and differentiation. CerS1 (LASS1) have different characteristic feature as compared to other CerS because of lacking of Hox-like domains. CerS1 is firstly identified gene and have higher homology to yeast LAG1. CerS1 predominantly find in brain, muscle and skeleatal muscle (Riebeling, et al., 2003). CerS1 is especially have roles in head and neck squamous cell carcinomas (HNSCC), also it was found that the balance between C16 and C18 ceramides were important in HNSCC pathogenesis (Senkal, et al., 2007). CerS2 is also called LASS2

family have Hox-like domain and is found higher levels in kidney and liver (Teufel, et al., 2009). Laviad et al. showed that CerS2 is regulated by S1P. Mutation in S1P receptor-like motif in CerS2 results in preventing the inhibitory effects of S1P on CerS2. The balance between S1P and ceramides are very important in terms of CerS2 activity. Because this balance decides whether the cell survive or not. Furthermore, CerS2 may have roles in unfolded protein response during ER stress (Mullen, et al., 2011). CerS3 is also known as LASS3 synthesizes C24 Ceramide and involved in keratinocyte differentiation (Mizutani, et al., 2008). CerS4 is also called as LASS4 and generated C18-22 Ceramide. The expression of CerS4 increase in neurological disease such as alzheimer's disease. CerS5 is also known as LASS5 and it is implicated in apoptosis by activation of hypoxia. CerS5 have function in cancer and apoptosis. CerS6 is also called LASS6 and generated C16 Ceramide. CerS5 and CerS6 show highly homology between each other. (Levy and Futerman, 2010).

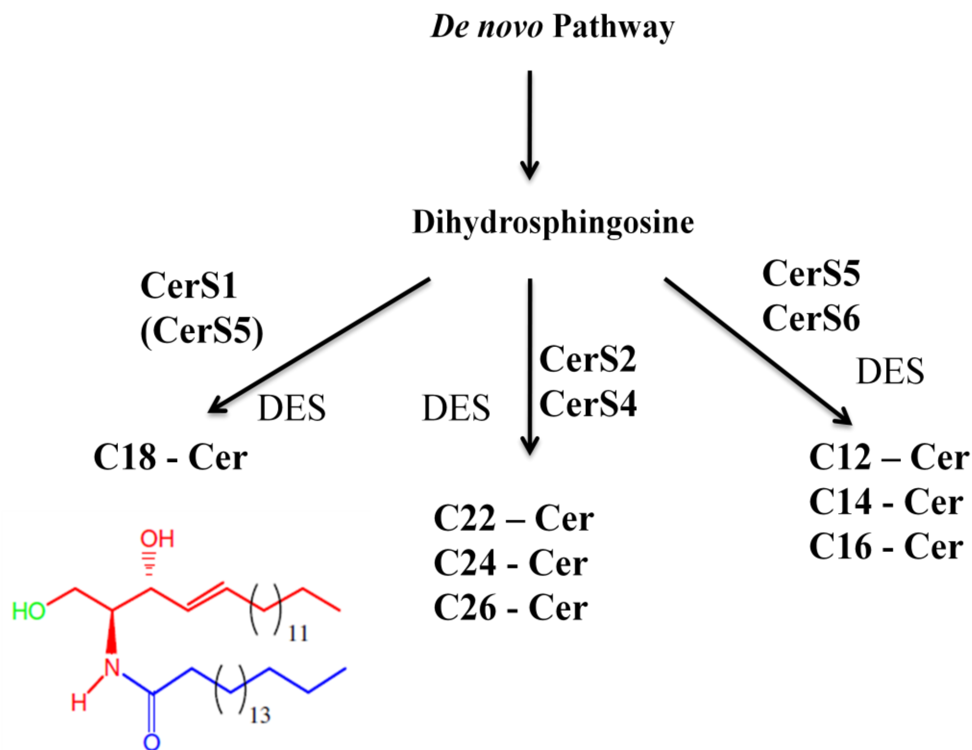


Figure 1.9. Generation of Ceramides with Distinct Chain Lengths
(Source:Adapted from Ogretmen, 2006)

1.4.2. Ceramide and Cancer

Endogenous ceramides have regulatory roles in the cell. They act as a tumor suppressor lipids and induce cell cycle arrest, apoptosis, senescence and differentiation. Ceramide is accumulated in the cell in response to DNA damage, stress, hypoxia and induction of apoptotic molecules (Hannun, 1996). Induction of ceramides activate downstream target molecules such as Cathepsin D, RAF, MEKK, Ceramide activated protein phosphatases (CAPPs) (Figure 1.10) (Ogretmen and Hannun, 2004).

Ceramides are known that reside in lipid rafts and interact with Phospholipase A_2, ceramide-activated protein serine-threonine phosphatases, protein kinase C, c-RAF-1 and kinase suppressor of RAS. In addition to this, ceramides block calcium release by inhibition of activated calcium channels and potassium channels. They contribute to forming of ceramide microdomains (Carpinteiro, et al., 2008). But, still the roles of these ion channels are not clear, but there may be an evidence that involvement in apoptosis via activation of Bax, Bid and Bak (Schneider et al., 2004). Beside these, apoptosis is induced by interaction of death receptors such as CD95 and ceramide translocated onto extracellular plasma membrane (Grassme, et al., 2001). Kim et al. demonstrated that ceramide upregulated p27(kip1) protein levels through induction of protein phosphatase 2A (PP2A) in PC-3 prostate cancer cells (Kim, et al., 2010).

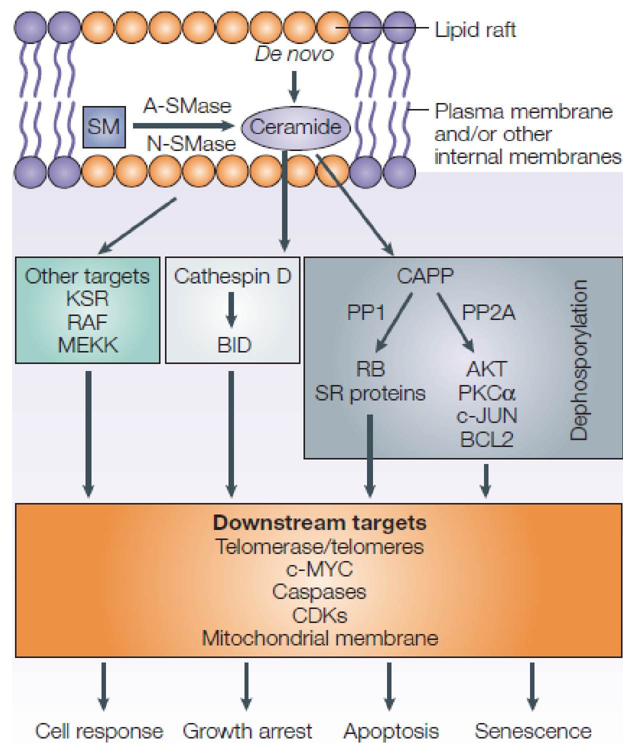


Figure 1.10. Downstream Targets of Ceramide and Activated Pathways.
(Source: Ogretmen, 2004)

Ceramides have also functions in induction of quiescence and senescence. It was demonstrated that ceramide induced CDK inhibitors such as p27(kip1) in HL60 acute myeloid leukemia cells (Kim, et al., 2000). Moreover, ceramides also block the activity of protein kinase C and phospholipase D activities (Venable, et al., 1995).

1.4.3. Sphingosine-1-Phosphate (S1P) and Sphingosine Kinases (SK)

Sphingosine-1 Phosphates are biologically important and active molecules. They promote cell survival, growth and tumorigenesis (Figure 1.11). S1P is synthesized enzymatically via conversion of ceramide to sphingosine and to S1P by ceramidases and SKs. SKs have two isoforms which are SK-1 and SK-2 (Pyne and Pyne, 2010). SK-1 induces cell growth whereas SK-2 inhibits cell growth while overexpression or downregulation occurs in SK2. SK-2 activated cell death is independent from S1P receptors. SK-1 and SK-2 differ in terms of the function and specifity. SK-1 is more dominant than SK-2 to form S1P and activation of cell growth and proliferation. SK-1 is highly expressed in solid tumors (Cuvillier, 2007).

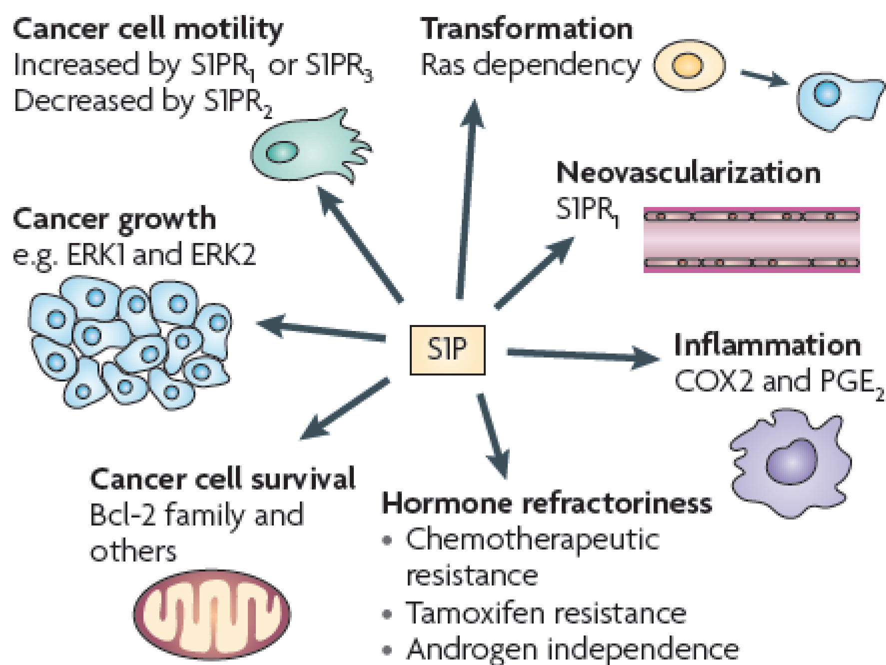


Figure 1.11. The Targets of S1P in Cancer
(Source: Pyne and Pyne, 2010)

S1P can bind to sphingosine-1 phosphate receptor (S1PR). S1PRs are G-protein coupled receptors that are 1 to 5 S1PR. However, SPR2 is distinct from the other with inhibition of migration. And this binding activates signaling cascade and induces tyrosine kinases. S1PR interacts with Rac and activates ERK and hence proliferation. Also, S1PR2 interacts with Rho and inhibits migration. In cancer cells, SK-1 is overexpressed however generally no mutation is encountered in SK-1 and inhibitors of SK-1 induces apoptosis in cancer cells such as hodgin lymphoma, lung and colon cancers (French, et al., 2003 and Bayerl, et al., 2008).S1P contributes to carcinogenesis and interacts with cancer promoting molecules such as VEGF, MAPK and PDGF (Pyne and Pyne, 2010).

1.4.4. Glucosylceramide and Glucosylceramide Synthase

Glucosylceramide synthase is the enzyme that transfers glucose from UDP-glucose to ceramide and produces GlcCer at the cytosolic side of the golgi apparatus (Jeckel, et al., 1992). GlcCer contain hydrophobic lipid group and hydrophilic sugar group. The function of GlcCer are involvement in proliferation, differentiation and tumor metastasis. After formation of GlcCer, it is converted further to gangliosides and

cerebrosides. GCS is a key enzyme in development of multidrug resistance. The activity of GCS is increased in multidrug resistant cells. Multidrug resistant cells are unresponsive to chemotherapeutic agents. It was showed that GCS activity especially increases in breast cancer, leukemia and melanoma (Senchenkov, et al., 2001 and Ogretmen, 2001). GCS activity is related wtih P-gp which is a ABC transporter protein and efflux agents from cytoplasm. This causes development of resistance to chemotherapeutics in the cancer cells.

1.4.5. Targeting of Bioactive Sphingolipids in Cancer Therapy

Sphingolipids as a effector molecules regulate many cellular events such as cell growth, proliferation, differentiation and apoptosis in cancer. This makes sphingolipids considerable targets for cancer treatment and development new chemotherapeutic agents. Ceramide is the crucial molecule for sphingolipid metabolism. The disruption of the balance between ceramides and other molecules may result in drug resistance and/or cell proliferation and growth.

Ceramide activates many apoptotic molecules in cancer cells. Cathepsin D is one of these molecules and involved in induction of apoptosis. Stefanis et al. demonstrated that increasing of ceramide levels in HT-29 colon cancer cells induce apoptosis via activating of Cathepsin D (De Stefanis, et al., 2002). Ceramides are syhtesized in ER and transported to golgi apparatus via CERT protein and processed to SM or GlcCer. Swanton et al. showed that while CERT was downregulated in HCT-116 (HCT) colon carcinoma, MDA-MB-231 (MDA) breast adenocarcinoma, and A549 non-small-cell lung carcinoma cells sensitized to paclitaxel (Swanton, et al., 2007). Pchejetski et al. demonstrated that downregulation of SK-1 enzyme sensitized to docetaxel in prostate adenocarcinoma cells and mouse models (Pchejetski, et al., 2005).

1.5. The Aims of The Study

Ceramides are second messengers in the cell. Normally, they are found in lipid bilayer. Beside the structural roles in the cell, they are also involved in cellular processes such as apoptosis, differentiation and growth. Increases in ceramide levels leads to cell death in cancer cells. But, on the other hand apoptotic ceramides converts

into antiapoptotic sphingosine-1-phosphate via phosphorylation of sphingosines by Sphingosine kinase-1 enzyme. Furthermore, apoptotic ceramides glucosylated to antiapoptotic molecule glucosylceramides by glucosylceramide synthase. Inhibition of GCS by PDMP and inhibition of SK-1 by SK-1 inhibitor may block formation of GlcCer and S1P, also synthesis of ceramides are induced by CerS families. Thus, this pathway may be a good target in chemotherapy (Figure 1.12). Resveratrol is naturally occurring antioxidant in plants and have many important properties such as inhibition of tumor initiation, promotion and progression. It is known that resveratrol induced apoptosis in cancer cells. But still, many unknowns exist in resveratrol induced cell death. Taken together, there may be a link between resveratrol and ceramide induced apoptosis in AML cells. The aim of this project is to examine cytotoxic and apoptotic effects of resveratrol, ceramide analogs and inhibitors of SK-1 and GCS on HL60 cells and determine possible synergistic, cytotoxic, and apoptotic effects. Then, evaluating upregulation or downregulation CerS family genes, GCS and SK-1 gene members in reponse to resveratrol in HL60 cells.

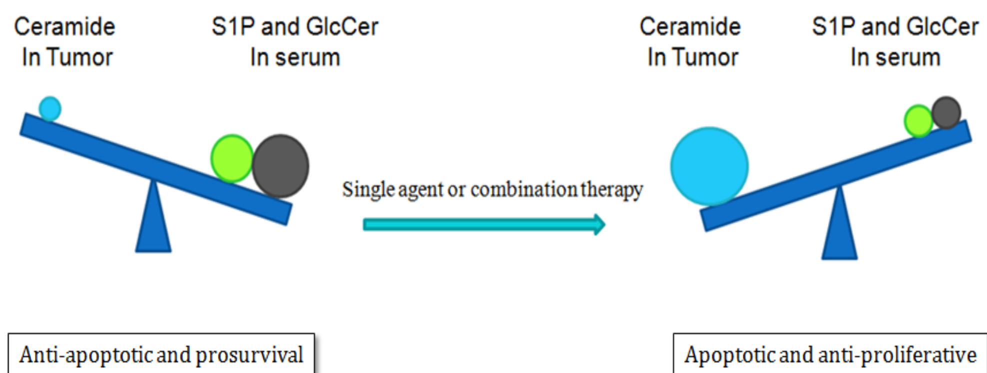


Figure 1.12. Balance Between Ceramide/S1P and Ceramide/GlcCer
(Source: Adapted from Saddoughi, 2008)

CHAPTER 2

MATERIAL AND METHODS

2.1. Chemicals

Resveratrol, bovine serum albumin serum, trypan blue solution, β-mercaptoethanol, dimethyl sulfoxide (DMSO), agarose, coomassie blue were obtained from Sigma, USA. 10 mM stock solution of resveratrol was prepared with DMSO and stored at -20ºC. C8:ceramide, N-(2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl)-decanamide, hydrochloride (PDMP), and SK-1 inhibitor were obtained from Cayman Chemicals (USA), dissolved in DMSO, and stored at -20ºC. The final concentration of DMSO did not exceed more than 0.1% in culture. Heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin were obtained from Biological Industries, Israel. RNA isolation kit was obtained from Macherey Nagel, Germany. Taq DNA Polymerase was obtained from Finnzymes, Finland. dNTP set, DNA ladder, reverse transcriptase reaction kit was obtained from Fermentas, Canada. Caspase-3 colorometric assay kit was obtained from BioVision, USA. JC-1 mitochondrial membrane potential detection kit was obtained from APO LOGICTM JC-1 from BACHEM, USA.

2.2. Cell Lines and Culture Conditions

Human HL60 APL cells were kindly provided by Dr. Ali Ugur Ural from Gulhane Military Medical School, Department of Hematology. They were grown and maintained in RPMI1640 medium containing 10% fetal bovine serum, and 1% penicillin-streptomycin at 37°C in 5% CO_2 incubator. Medium was refreshed every 3 days. The cells were collected from 75cm^2 flask to 50ml falcons and were centrifugated at 800g 10 minutes at room temperature. After centrifugation, supernatant was removed and pellet was resuspended in 4 ml PBS and repeated centrifugation process at 800g 10 minutes. Then, supernatant was removed and pellet was resuspended with 20ml RPMI1640 (%10 FBS, %1 Penicillin-Streptomycin), transferred to 75cm^2 filtered flask and incubated in CO_2 incubator.

2.3. Measurement of Cell Growth by XTT Cell Proliferation Assay

XTT cell proliferation assay is based on the reduction of XTT dye into formazan compound which is measurable via spectrophotometer by active mitochondrial enzyme dehydrogenases.

The IC50 values (drug concentration that inhibits cell growth 50% as compared to control) of resveratrol; a ceramide analogue, C8:ceramide; GCS enzyme inhibitor (PDMP) and IC10 value of SK-1 enzyme inhibitor were determined from cell proliferation plots obtained by XTT assay as described before.(Gucluler and Baran, 2009). $2x10^4$ HL60 cells were seeded into 96-well plates containing 200 µl growth medium in the absence or presence of increasing concentrations of resveratrol, C8:ceramide, PDMP or SK-1 inhibitor. After 72 hr incubation in %5 CO_2 , they were treated with 40 µl of XTT reagent for 4 hours. Then, the absorbances of the samples were measured under 490 nm wavelength of light by Elisa reader (Thermo Electron Corporation Multiscan Spectrum, Finland) and graphed cell proliferation plots. Finally, IC50 values of resveratrol, C8:ceramide or PDMP and IC10 values of SK-1 inhibitor were calculated from cell proliferation plots. In order to determine possible synergistic effects of resveratrol in combination with these ceramide metabolism manipulating chemicals, increasing concentrations of resveratrol were applied together with IC50 value of C8:ceramide or PDMP or IC10 value of SK-1 inhibitor. The same procedure of XTT cell proliferation assay was applied for these set of experiments as mentioned above.

2.4. Isobologram Analysis of Median Dose Effects

It was used the CalcuSyn for Windows computer program (CalcuSyn software, Biosoft, Cambridge, UK) for isobologram analysis (Zhao, et al., 2010). Isobologram analysis was conducted using the computer software and analyzed resveratrol with SK-1 inhibitor, C8:ceramide and PDMP. Experimental data points, represented by dots located below, on, or above the line, indicate synergism, additivity, and antagonism, respectively. The CI is an analysis of the combined effects of 2 drugs, using a median effect plot analysis. A CI value <1 indicates a synergistic effect (0.1-0.5 strong

synergism; <0.1 very strong synergism); a CI value of 1 indicates additive effect; and a CI value >1 an antagonistic effect (3.3-10 strong antagonism, >10 very strong antagonism).

2.5. Measurements of Caspase-3 Enzyme Activity

Changes in caspase-3 enzyme activities were determined using caspase-3 colorimetric assay kit (R&D Systems, USA) as described before (Baran, et al., 2007). $1x10^6$ cells were seeded in 6-well plate in 2 ml growth medium in the absence or presence of increasing concentrations of resveratrol, C8:ceramide, PDMP or SK-1 inhibitor or combination of resveratrol with others. After 72 hr incubation in %5 CO_2, the cells were collected by centrifugation at 1000g for 10 minutes. The pellets were treated with 100 µl of cold lysis buffer (1X) in order to obtain cell lysate. Then, the cell lysates were incubated on ice for 10 minutes and they were centrifuged at 14000g for 1 minute. Following the centrifugation, supernatants were transferred to new eppendorf tubes. In order to measure caspase-3 enzyme activity, reaction mixture include 50 µl of reaction buffer (2X), 50 µl of the samples and 5 µl of caspase-3 colorimetric substrate was added into 96-well plate and incubated for 2 hours at 37˚C in 5% CO_2 incubator. Absorbances of the samples were read under 405 nm wavelength of light via the ELISA reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Then, protein concentrations were measured by Bradford assay. Finally, enzyme activity levels were normalized to protein concentrations that was determined by Bradford assay. In Bradford protein assay, it is used coomassie blue dye which converted into bluer form of dye while binding to proteins. Absorbance was read under 595nm via ELISA reader (Thermo Electron Corporation Multiskan Spectrum, Finland) and while protein concentration was increased, the absorbance was also increased and according to BSA protein concentrations, the concentration of proteins were calculated.

2.6. Detection of the Loss of Mitochondrial Membrane Potential (MMP)

The loss of mitochondrial membrane potential was detected by JC-1 mitochondrial membrane potential (MMP) kit (Cell Technology, USA). $1x10^6$ HL60

cells were seeded in 6-well plate in 2 ml growth medium and treated with different concentrations of resveratrol, C8Ceramide, SK-1 inhibitor or PDMP alone or combination of resveratrol with the others, incubated for 72 hours and after that time, HL60 cells treated with resveratrol, C8:ceramide, SK-1 inhibitor or PDMP alone or combination of resveratrol with the others were collected by centrifugation at 1000g for 10 minutes. Supernatants were removed and the pellets were dissolved in 500 µl of JC-1 dye before their incubation at 37˚C in 5% CO_2 for 15 minutes. Then, the mixtures were centrifuged at 1000g for 5 minutes. Pellets were resuspended in 2 ml of blank media in order to remove excess dye and they were centrifuged at 1000g for 5 minutes. All pellets were resuspended with 500 µl of blank media and 150 µl from each was added into black 96-well plate. The aggregate red form of the dye which is found in inner mitochondria has absorption/emission maxima of 585/590 nm and the monomeric green form of the dye which was released to the cytoplasm due to the loss of the MMP has absorption/emission maxima of 510/527 nm. The plate was read at these wavelengths by fluorescence Elisa reader (Thermo Varioskan Spectrum, Finland). Finally, green/red (510/585) values were calculated to determine the changes in MMP.

2.7. Total RNA Isolation and RT-PCR

The expression levels of ceramide metabolizing genes were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). $3x10^6$ cells were seeded in 6-well plate in 2 ml growth medium and treated with different concentrations of resveratrol for 72 hours in 5% CO_2. Total RNA was extracted using a Nucleospin Total RNA isolation kit (Machery-Nagel, USA) as described by the manufacturer. The amount and quality of RNA were measured by Nanodrop Photospectrometer (NanoDrop 1000 THERMO, USA). 5 µg of total RNA was reverse transcribed into cDNA by reverse transcriptase enzyme (Fermentas, USA). After incubation at 42 °C for 50 min, the reactions were stopped at 95 °C for 5 min.The resulting total cDNA was then used to determine expression levels of CerS1, CerS2, CerS 4, CerS5, CerS6, SK-1, and GCS. Expression levels of β-actin were used as internal positive control. The primer sequences were as follows;

Table 2.1. Forward and Reverse Sequence of Primers

CerS1 Forward	(5’CTATACATGGACACCTGGCGCAA3’)
CerS1 Reverse	(5’TCAGAAGCGCTTGTCCTTCACCA3’)
CerS2 Forward	(5’ GCTGGAGATTCACAT3’)
CerS2 Reverse	(5'GAAGACGATGAAGAT3’)
CerS4 Forward	(5’TGCTGTCCAGTTTCAACGAG3’)
CerS4 Reverse	(5’GAGGAAGTGTTTCTCCAGCG3’)
CerS6 Forward	(5’-CTCCCGCACAATGTCACCTG-3’)
CerS6 Reverse	(5’-TGGCTTCTCCTGATTGCGTC-3’)
SK-1 Forward	(5’CCGACGAGGACTTTGTGCTAAT3’)
SK-1 Reverse	(5’GCCTGTCCCCCCAAAGCATAAC3’)
GCS Forward	(5’ATGACAGAAAAAGTA3’)
GCS Reverse	(5’GGACACCCCTGAGTG3’)
β-Actin Forward	(5’CAGAGCAAGAGAGGCATCCT3’)
β-Actin Reverse	(5’TTGAAGGTCTCAAACATGAT3’)

Products of PCR reactions were visualized under UV after running them on 2% agarose gel electrophoresis including ethidium bromide at 90 V for 1h.

Table 2.2. The Ingredient of Reverse Transcription Reaction

Ingredients	Amount
RNAse Free Water	5 µL
Total RNA (5 µg)	5 µl
10X Buffer	2 µl
Random Primers (0.5 µg/L)	0.7 µL
RNAse Inhibitor (50U/µL)	0,7 µL
MgCl2 (25 mM)	4 µL
dNTP (10 mM)	2 µL
Moloney Murine Reverse Transcriptase Enzyme (200 U/µL)	0,7 µL
Total	20 µL

Table 2.3. Ingredients of PCR Solutions for SK-1 and GCS

SK-1, GCS	
Reaction Mixture	Amount (µL)
PCR grade water	32.3
Reaction buffer (10x)	5
MgCl2 (25 mM)	5
dNTP (10 mM)	4
Primer forward (25 pmol/µL)	0.5
Primer reverse (25 pmol/µL)	0.5
cDNA	2
Taq DNA Polymerase	0.3
Total Mixture	50

Table 2.4. Ingredients of PCR Solutions for CerS1

CerS1	
Reaction Mixture	Amount (µL)
PCR grade water	31.3
Reaction buffer (10x)	5
$MgCl_2$ (25 mM)	5
dNTP (10 mM)	4
Primer forward (25 pmol/µL)	1
Primer reverse (25 pmol/µL)	1
cDNA	2
Taq DNA Polymerase	0.7
Total Mixture	50

Table 2.5. Ingredients of PCR Solutions for CerS2, CerS5 and CerS6

CerS2, CerS5, CerS6	
Reaction Mixture	Amount (µL)
PCR grade water	32.3
Reaction buffer (10x)	5
$MgCl_2$ (25 mM)	5
dNTP (10 mM)	4
Primer forward (25 pmol/µL)	0.5
Primer reverse (25 pmol/µL)	0.5
cDNA	2
Taq DNA Polymerase	0.7
Total Mixture	50

Table 2.6. Ingredients of PCR Solutions for CerS4

CerS4	
Reaction Mixture	Amount (µL)
PCR grade water	31.8
Reaction buffer (10x)	5
MgCl2 (25 mM)	4.5
dNTP (10 mM)	4
Primer forward (25pmol/µL)	0.5
Primer reverse (25 pmol/µL)	0.5
cDNA	2
Taq DNA Polymerase	0.7
Total Mixture	50

Table 2.7. Ingredients of PCR Solutions for β-actin

β-actin	
Reaction Mixture	Amount (µL)
PCR grade water	30.8
Reaction buffer (10x)	5
MgCl2 (25 mM)	5
dNTP (10 mM)	4
Primer forward (25 pmol/µL)	1
Primer reverse (25 pmol/µL)	1
cDNA	2.5
Taq DNA Polymerase	0.7
Total Mixture	50

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cytotoxicity Analysis in HL60 Cells

3.1.1. Cytotoxic Effects Of Resveratrol, C8:Ceramide, Sk-1 Inhibitor and PDMP on HL60 Cells

There were dose dependent cytotoxic effects of resveratrol, C8:Ceramide, PDMP, and SK-1 inhibitor on HL60 cells as measured by XTT assay. The cells treated with increasing concentration of resveratrol which were 1, 5, 10, 20, 50 and 100 µM resveratrol and it was calculated 3, 5, 15, 29, 45 and 55% decreases in cell proliferation of HL60 cells, respectively (Figure 3.1). IC50 values of resveratrol was calculated from cell proliferation plots and found to be 75 µM (Figure 3.1).

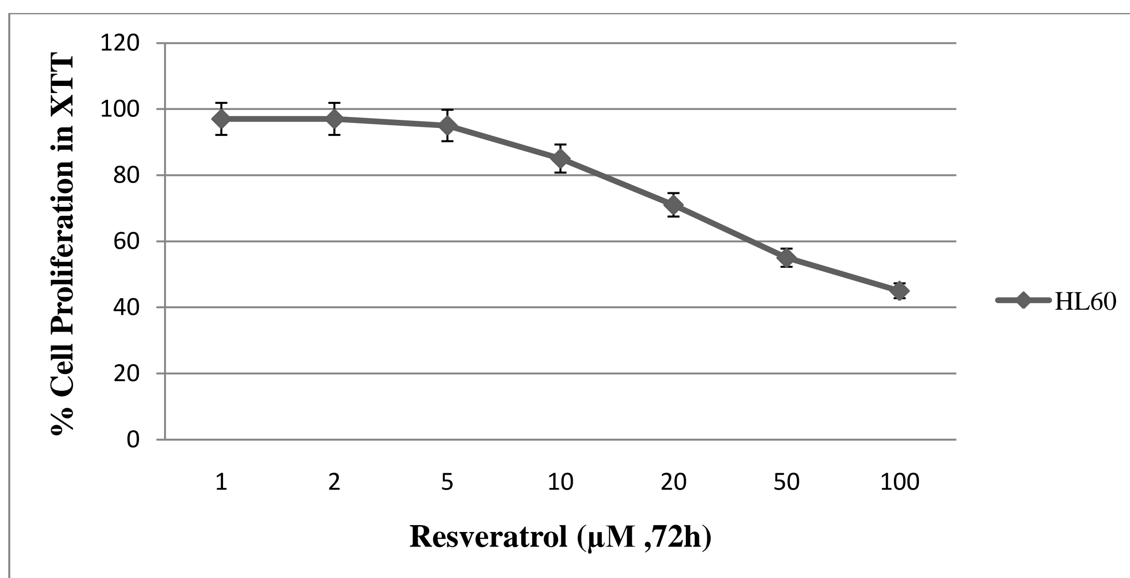


Figure 3.1. Antiproliferative effects of Resveratrol on HL60 cells

HL60 cells were treated with increasing concentrations of exogenous C:8 Ceramide and conducted XTT cell proliferation assay. HL60 cells treated with 1, 10, 20,

50 μM C:8 Ceramide and it was calculated 4, 11, 20, 57 % decrases in cell proliferation, respectively (Figure 3.2.). IC50 value of C8:ceramide is 45 μM (Figure 3.2).

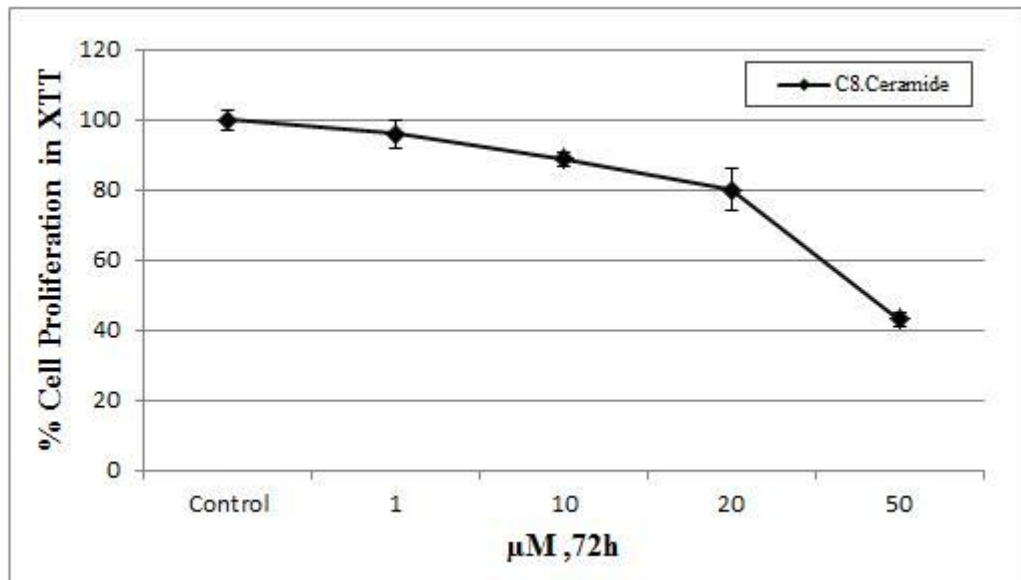


Figure 3.2. Antiproliferative Effects of Ceramide on HL60 cells

Glucosylceramide synthase inhibitor, PDMP, was exposed to HL60 cells with the increasing concentration of 1, 10, 20, 50 μM PDMP and assessed cell proliferation. Then cell proliferation plots were established and it was calculated 0, 9, 21, 41 % decreases in cell proliferation. IC50 value of PDMP was found to be as a 61 μM (Figure 3.3).

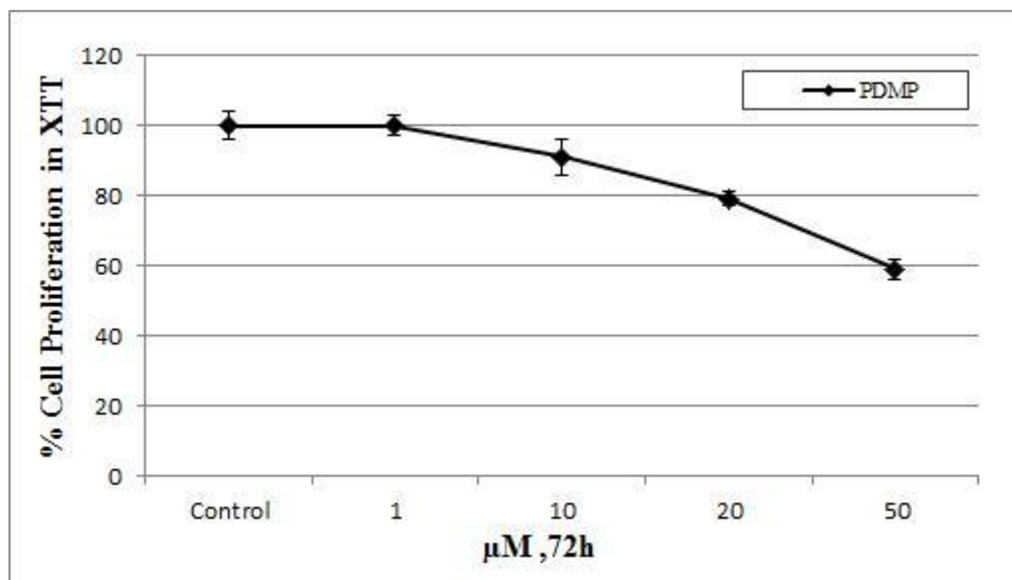


Figure 3.3. Antiproliferative Effects of PDMP on HL60 cells

HL60 cells were exposed to Sphingosine kinase-1 inhibitor in order to assess cytotoxic effects. There were 0, 27, 63, 62, 76 % decreases in response to increasing concentration of 1, 10, 15, 20, 50 µM SK-1 inhibitor (Figure 3.4). IC10 value (Inhibitory concentration of 10%) was calculated according to cell proliferation plots and it was found as 5 µM (Figure 3.4).

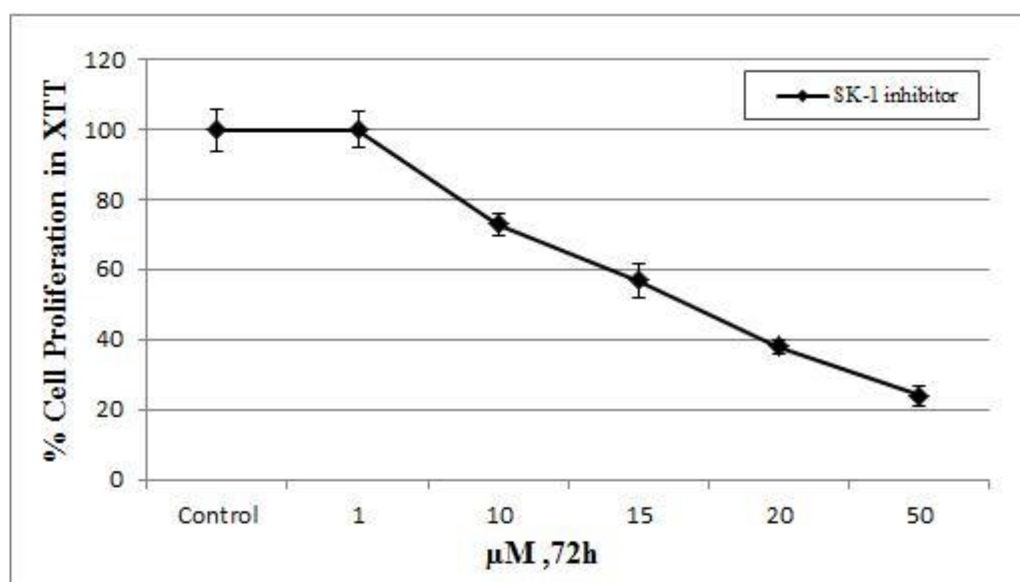


Figure 3.4. Antiproliferative Effects of SK-1 inhibitor on HL60 cells

3.1.2. Resveratrol in Combination with C8:Ceramide, PDMP or SK-1 Inhibitor Showed Significant Synergistic Cytotoxic Effects on HL60 Cells

The possible synergistic antiproliferative effects of combination of resveratrol with the ceramide analogue and inhibitor of ceramide metabolizing enzymes were assessed by XTT cell proliferation assay. Resveratrol combined with C8:ceramide, PDMP and SK-1 inhibitor and determined cytotoxic effects. HL60 cells exposed to IC50 value of 45 μM C8:ceramide, which induces *de novo* generation of ceramides and after 4 hours, increasing concentrations of 1, 2, 5, 10, 20, 50 μM resveratrol were added onto HL60 cells and examined decreases in cell proliferation 75, 76, 80, 81, and 82%, respectively, as compared to untreated controls (Figure 3.5). Combination of the same concentration of resveratrol with IC50 value of 61 μM PDMP which provides accumulation of ceramides, resulted in 66, 71, 76, 88, and 89% decreases in proliferation of HL60 cells, respectively (Figure 3.6). The combination of IC50 value of SK-1 inhibitor and resveratrol did not show any synergistic effect. So it was used IC10 value of SK-1 inhibitor. IC10 value of 5 μM SK-1 inhibitor, in combination with 1, 2, 5, 10, 20, 50 μM resveratrol caused 19, 28, 39, 44, and 71% decreases in cell proliferation, respectively, as compared to untreated controls (Figure 3.7).

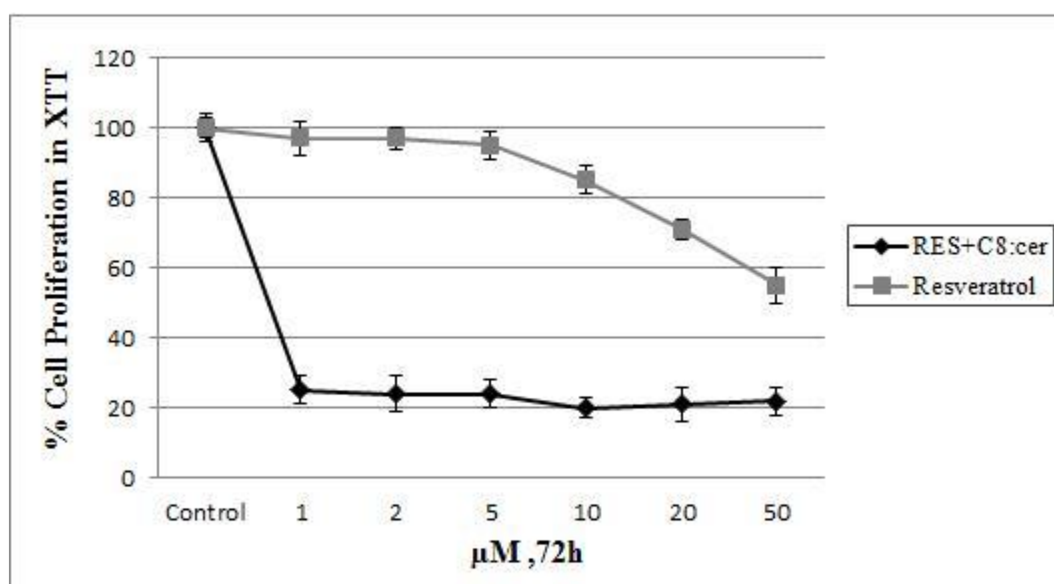


Figure 3.5. Effects of Resveratrol/C8:ceramide combinations on proliferation of HL60 cells

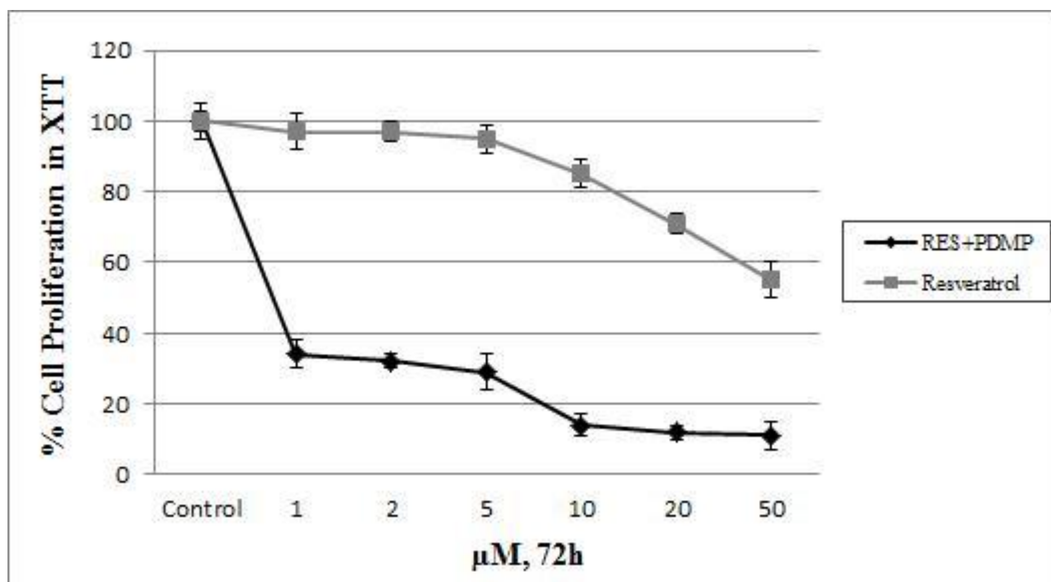


Figure 3.6. Effects of Resveratrol /PDMP combinations on proliferation of HL60 cells

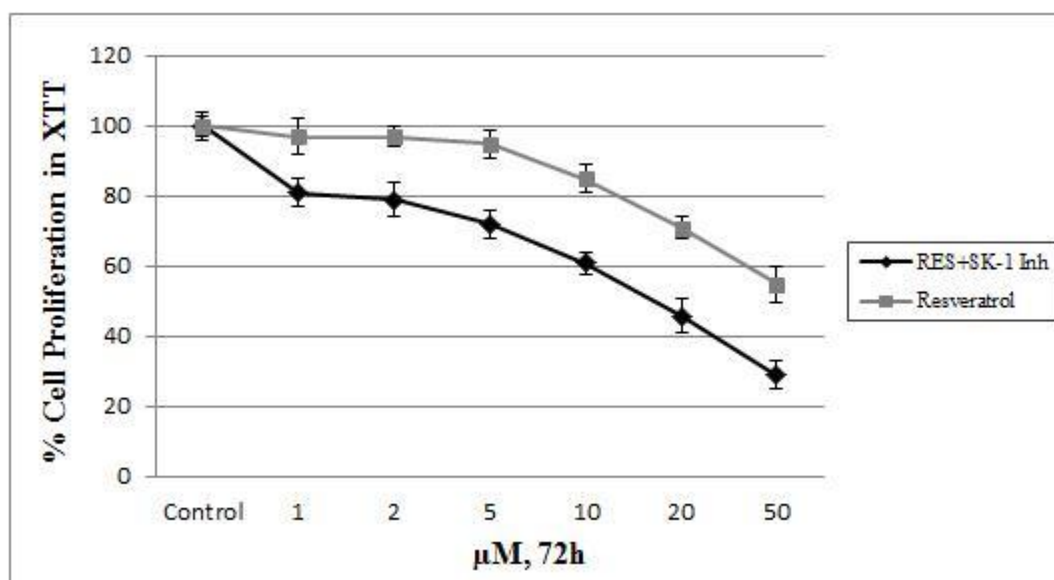


Figure 3.7. Effects of Resveratrol/SK-1 inhibitor combinations on proliferation of HL60 cells

According to isobologram analysis via using CalcuSyn software, synergistic effects of resveratrol in combination with C:8 Ceramide, PDMP and SK-1 inhibitor were determined and calculated CI values which means median doses (Figure 3.8). The CI values of resveratrol in combination with C8:Ceramide, PDMP and SK-1 inhibitor were 0,079615, 0.00716 and 0.74606, respectively, in HL60 cells. CI value which is below than 1 indicates a synergistic effect (0.1-0.5 strong synergism; <0.1 very strong

synergism). The results demonstrated that combination of resveratrol with C:8 Ceramide and PDMP indicated very strong synergism, however combination of resveratrol with SK-1 inhibitor indicated synergism in HL 60 acute myeloid leukemia cells. Because, in this combination, it was used IC10 value of SK-1 inhibitor.

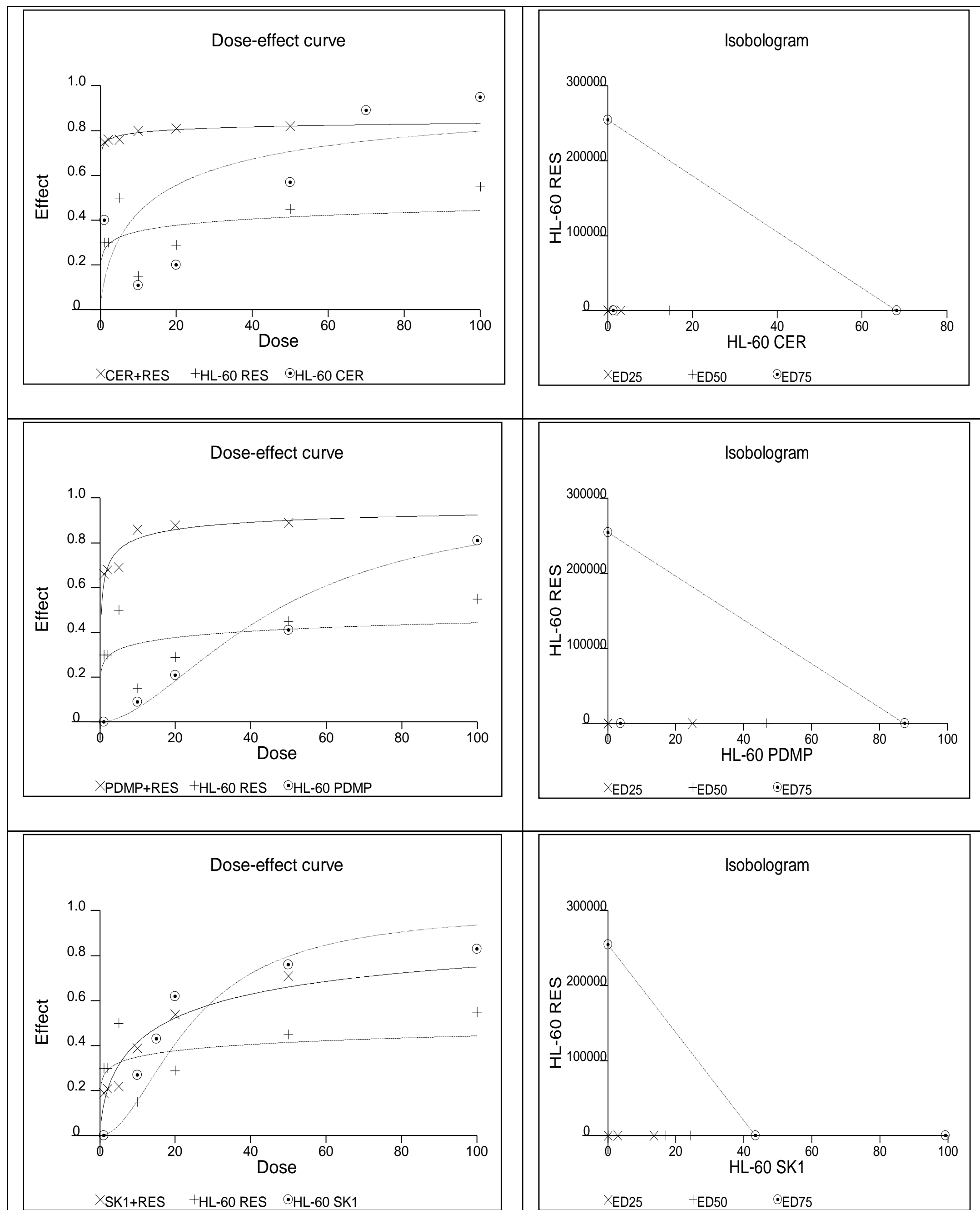


Figure 3.8. Isobologram analysis of resveratrol/C:8 Ceramide, /PDMP and /SK-1 inhibitor on HL60 cells

3.2. Evaluation of Apoptosis in HL60 Cells

3.2.1. Changes in Caspase-3 Enzyme Activity Showed Higher Apoptotic Effects of Resveratrol with C:8 Ceramide, PDMP or SK-1 Inhibitor as Compared to Any Agent Alone

Caspase-3 enzyme is in the center of apoptotic pathway and the activation of this enzyme can be considered as an indicator of apoptosis. Caspase-3 enzyme activity was measured in HL60 cells exposed to any agent alone or combinations of resveratrol/C8:Ceramide, resveratrol/PDMP and resveratrol/SK-1 inhibitor. HL60 cells treated with 5 and 10 µM resveratrol showed 1.11, and 1.14-fold increases in caspase-3 enzyme activity as compared to untreated controls while 45 µM C8:ceramide, 61 µM PDMP, and 5 µM SK-1 inhibitor resulted in 2.17, 1.81, and 1.71-fold increases in caspase-3 enzyme activity (Fig. 3.9). On the other hand, 5 µM Resveratrol in combination with the same concentrations of C8:Ceramide, PDMP, or SK-1 inhibitor resulted in 2.96, 2.62, and 2.45-fold increases in the enzyme activity, respectively (Fig. 3.9). There were 3.77, 2.68, and 2.4-fold increases in caspase-3 enzyme activity in HL60 cells incubated with combination of the same concentrations of C8: Ceramide, PDMP, SK-1 inhibitor and 10 µM resveratrol, respectively (Figure 3.9).

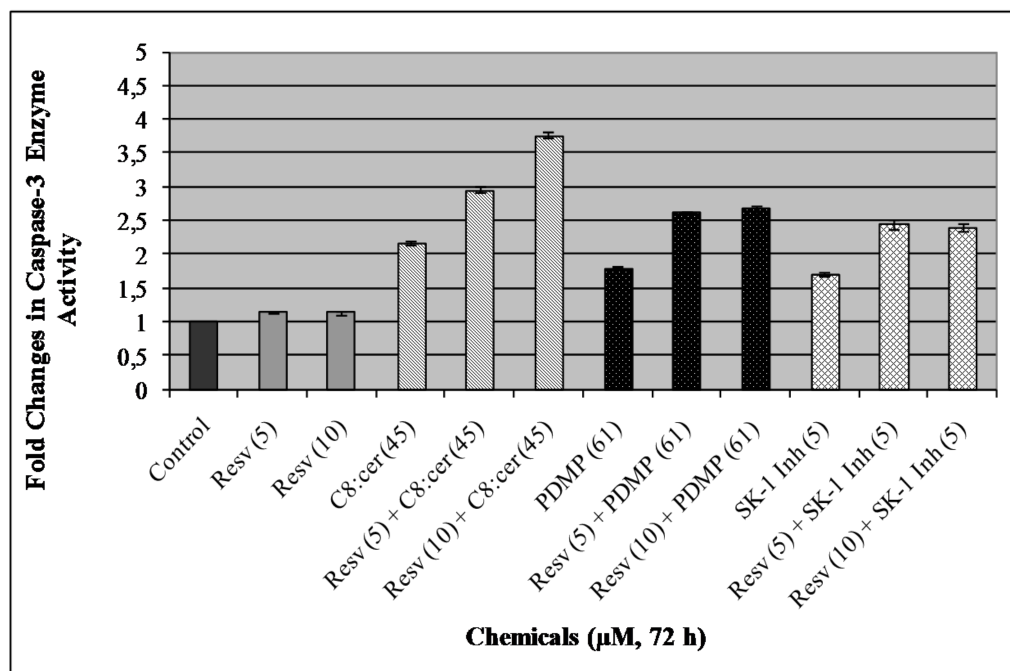


Figure 3.9. Fold changes in caspase-3 enzyme activity in response to resveratrol, C:8 Ceramide, PDMP, SK-1 inhibitor alone and combination of resveratrol, with C8:Ceramide, PDMP and SK-1 inhibitor

3.2.2. Effects of Resveratrol Alone and in Combination with C8:Ceramide, PDMP, or SK-1 Inhibitor on Mitochondrial Membrane Potential

Caspase-3 enzyme activity was significantly increased in HL60 cells in response to combination or alone resveratrol, PDMP, SK-1 and C8:Ceramide. In order to confirm caspase-3 enzyme activity results, it was conducted the same set of experiment to examine the changes in mitochondrial membrane potential. Moreover, mitochondrial membrane potential also changes while apoptosis induced in the cell. The results revealed that there were 1.37 and 1.45-fold increases in cytoplasmic/mitochondrial JC-1 in response to 5 and 10 µM resveratrol, respectively (Fig. 3.10). HL60 cells treated with 45 µM C8:Ceramide or 61 µM PDMP or 5 µM SK-1 inhibitor or showed 25.98, 1.18 or 10.29-fold increases in cytoplasmic/mitochondrial JC-1. We applied combination of 5 and 10 µM resveratrol together with the same doses C8:Ceramide or PDMP or SK-1 inhibitor and observed 32.67 and 79.50-fold or 12.87 and 14.28-fold or 1.75 and 2.41-fold increases in cytoplasmic/mitochondrial JC-1, respectively, as compared to untreated controls (Fig. 3.10).

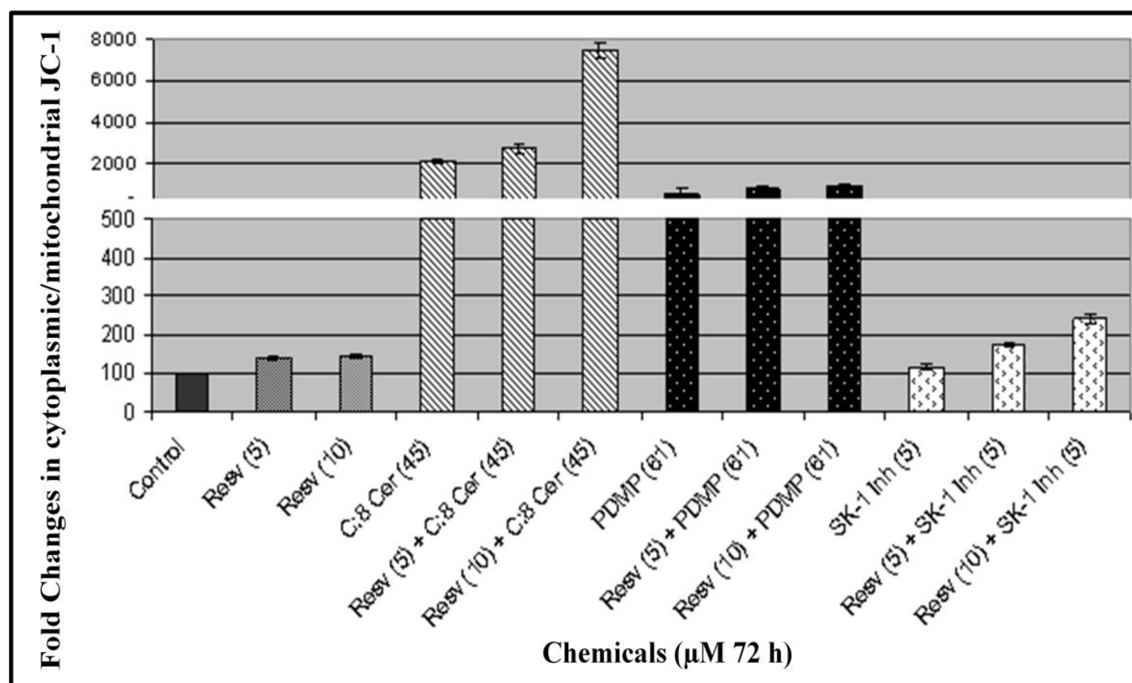


Figure 3.10. Fold changes in cytoplasmic/mitochondrial JC-1 in HL60 cells exposed to alone or combinations of resveratrol with C8:Ceramide, PDMP, or SK-1 inhibitor

3.3. Gene Expression Analysis in HL60 Cells

3.3.1. Resveratrol Induces *de novo* Ceramide Generation and Ceramide Accumulation by Increasing Expression Levels of CerS Genes and Decreasing Expression Levels GCS and SK-1 Genes

Expression levels of ceramide metabolizing genes in HL60 cells treated with increasing concentrations of resveratrol (1, 10, 20, and 50 µM) were determined by RT-PCR. The results revealed that there were significant increases in expression levels of CerS1, CerS2, CerS4, CerS5, and CerS6 genes and decreases in expression levels of GCS and SK-1 in a dose-dependent manner (Fig. 3.11). We quantified expression levels of the genes and detected 1.5, 1.98, 1.79, 1.27 and 1.96-fold increases in expression levels of CerS1, CerS2, CerS4, CerS5, and CerS6 and 0.5 and 0.6-fold decreases in expression levels of GCS and SK-1 genes, respectively, as normalized to Beta Actin levels and compared to untreated control (Table 3.1).

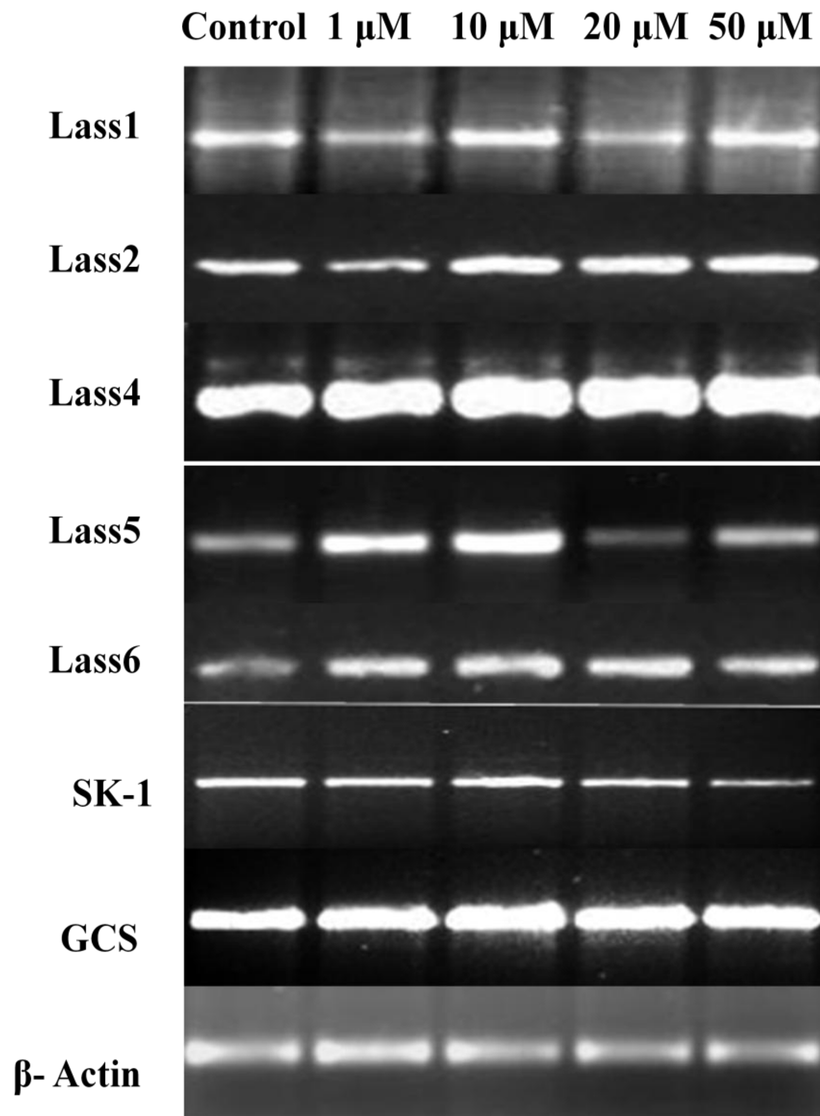


Figure 3.11. mRNA levels of ceramide-metabolizing genes and ceramide synthase genes in response to resveratrol in HL60 cells

Table 3.1. Quantification of gene expression levels in reponse to 50μM resveratrol treatment

Gene	Control	50μM Resveratrol
CerS1	100	150
CerS2	100	198
CerS4	100	179
CerS5	100	127
CerS6	100	196
SK-1	100	50
GCS	100	40

CHAPTER 4

CONCLUSION

There are different strategies for the treatment of acute myeloid leukemia. But, significant problems are observed in patients during treatment with these chemicals. Thus, natural products that may have strong anticancer potential always attract the attention of clinicians and basic science researchers. In this study, it was demonstrated anticancer potentials of resveratrol on human acute myeloid leukemia cells in addition to identifying a novel mechanism of resveratrol-induced apoptosis that involves bioactive sphingolipids. On the other hand, it has increased antiproliferative and apoptotic effects of resveratrol on HL60 cells by targeting ceramide-generating and ceramide clearance genes toward generation and/or accumulation of apoptotic ceramides. It was observed that resveratrol, C8:Ceramide which induce *de novo* generation of apoptotic ceramides, PDMP, which inhibits GCS enzyme, and SK-1 inhibitor showed cytotoxic effects of HL60 cells in a dose-dependent manner. Resveratrol application resulted in increases in caspase-3 enzyme activity and loss of mitochondrial membrane potential. Since the mitochondrial membrane potential and caspases are downstream targets of ceramides, treatment of HL60 cells with C8:ceramide, PDMP, or SK-1 inhibitor also resulted in increases in caspase-3 enzyme activity and loss of mitochondrial membrane potential. As we manipulate the cells to increase intracellular generation or accumulation of ceramides by biochemical approaches, we observed significant synergistic cytotoxic effects of resveratrol on HL60 cells. Combinations of resveratrol with C8:ceramide, PDMP, or SK-1 inhibitor resulted in more apoptotic cells as compared to any agent alone. These findings were in agreement with the literature (Bielawska, et al., 2008, Szulc, et al., 2006 and Baran, et al., 2007). It was shown that application of ceramide analogs or their mimetics induces apoptosis and/or growth arrest in various types of cancer cells (Bielawska, et al., 2008 and Szulc, et al., 2006). It was clearly shown that there was significant inhibition of tumor growth and progression in response to ceramidoids, a synthetic ceramide, in head and neck squamous cancer cells and some other cancer models, *in vivo* (Bielawska, et al., 2008 and Senkal, et al., 2006). Different structural analogs of ceramides have also

been shown to induce apoptosis in breast cancer cells (Struckhoff, et al., 2004) and drug resistant MCF-7/Adr cells (Crawford, et al., 2003). It was shown that increasing expression levels of ceramide synthase gene (CerS1) in imatinib-resistant and sensitive K562 cells triggered apoptosis and increased sensitivity of these cells to imatinib (Baran, et al., 2007). Targeting ceramide clearance enzymes is another important strategy to increase intracellular concentrations of ceramides (Ogretmen, 2006). Inhibition of SK-1 enzyme to block conversion of apoptotic ceramide to antiapoptotic S1P is a novel treatment modality for the treatment of cancers. It was shown in living animal models that inhibition of SK-1 blocked cell growth and proliferation and induced apoptosis (French, et al., 2006). Inhibition of S1P receptors or application of monoclonal antibodies against S1P have also been shown to be effective protocols for the treatment of cancers (Visentin, et al., 2006). Baran et al. showed that partial inhibition of SK-1 by siRNA resulted in apoptosis in imatinib-resistant K562 chronic myeloid leukemia cells (Baran, et al., 2007). Glucosylceramide synthase enzyme has been shown to be involved in drug resistance in cancer. Thus, inhibition of GCS is also a novel treatment option for cancer cells. Adriamycin-resistant MCF-7 cells showed sensitivity and underwent apoptosis in response to GCS inhibitors, while overexpression of GCS in MCF-7 cells resulted in multidrug resistance to several agents (Liu, et al., 1999 and Yuan, et al., 1997). Moreover, combination of GCS inhibitor with some anticancer agents, such as doxorubicin, tamoxifen, vincristine, and taxol, induced apoptosis synergistically in various human cancer cells (Morjani, et al., 2001), (Senchenkov, et al., 2001). Interestingly, our RT-PCR results showed that there were significant increases in the expression levels of CerS1, CerS2, CerS4, CerS5, and CerS6 genes in response to resveratrol in a dose-dependent manner in HL60 cells. In parallel studies, we have also detected significant decreases in expression levels of SK-1 and GCS in a dose-dependent manner in HL60 cells. Increased expression of CerS genes in response to chemotherapeutic stress has also been shown in different cancer cells. There was increased ceramide synthase enzyme activity in response to daunarubicine and etoposide (Bose and Kolesnick, 2000 and Perry, 2000). A camptohotecine derivative CPT-11 and hexadevylephosphocholine agents also induced apoptosis by upregulating ceramide synthase genes (Wieder, et al., 1998). The results of this study showed for the first time that resveratrol induces apoptosis through both increasing *de novo* generation of apoptotic ceramides and decreasing conversion of apoptotic ceramides to antiapoptotic S1P and GluCer. On the other, our data strongly suggest that targeting

ceramide metabolism increased chemosentivity of acute myeloid leukemia cells to resveratrol. Since ceramides are the regulators of vitally important biological processes, targeting ceramide metabolizing genes and their end products may be an open a new way of the treatment of different types of cancers.

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