ACTIVATED SIGNALING PATHWAYS AND APOPTOTIC MECHANISMS IN RESVERATROL APPLIED CHRONIC MYELOID LEUKEMIA CELLS AND THE INVOLVEMENT OF CERAMIDE METABOLIZING GENES ON THESE MECHANISMS

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Melis KARTAL

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We approve the thesis of **Melis KARTAL**

Assoc. Prof. Dr. Yusuf BARAN
Supervisor

Assoc. Prof. Dr. Güray SAYDAM
Committee Member

Assist. Prof. Dr. Bünyamin AKGÜL
Committee Member

05 July 2010

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

Assoc. Prof. Dr. Talat YALÇIN
Dean of the Graduate School of Engineering and Sciences

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ABSTRACT

ACTIVATED SIGNALING PATHWAYS AND APOPTOTIC MECHANISMS IN RESVERATROL APPLIED CHRONIC MYELOID LEUKEMIA CELLS AND THE INVOLVEMENT OF CERAMIDE METABOLIZING GENES ON THESE MECHANISMS

Resveratrol, an important phytoalexin in many plants, has cytotoxic effects on several cancer cells. Ceramide is a significant sphingolipid which affects many signaling pathways regulating cell senescence, migration, and cell cycle arrest. Intracellular ceramide level is balanced by glucosylceramide synthase (GCS), the converter of ceramide to glucosylceramide, and sphingosine kinase-1 (SK-1) that convert ceramide to sphingosine 1-phosphate (S1P). Ceramide functions as an apoptotic molecule whereas glucosylceramide S1P function as anti-apoptotic. An important cell-permeable analogue of natural ceramides, C8:ceramide, increases intracellular ceramide levels significantly, while 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and SK-1 inhibitor increase accumulation of ceramides by inhibiting GCS and SK-1, respectively. Chronic myelogenous leukemia (CML), a hematological disorder, results from the generation of BCR/ABL oncogene.

In this study, we examined the roles of ceramide metabolizing genes in resveratrol-induced apoptosis, and the expression profiles of 84 genes underlying apoptosis, cell cycle control, DNA damage repair, and invasion and metastasis in human K562 CML cells treated with resveratrol. There were synergistic cytotoxic and apoptotic effects of resveratrol with coadministration of C8:ceramide, PDMP and SK-1 inhibitor. We observed significant increases in expression levels of LASS genes, and decreases in expression levels of GCS and SK-1 in K562 cells in response to increasing concentrations of resveratrol. There were also significant increases in the expression levels of SERPINB5, FAS, TNFRSF, MTSS that are related with tumor suppression, and decreases in Myc expression.

Our data, in total, showed for the first time that resveratrol might kill CML cells through increasing intracellular generation and accumulation of apoptotic ceramides.

ÖZET

RESVERATROL UYGULANAN KRONİK MİYELOİD LÖSEMİ HÜCRELERİNDE TETİKLENEN SİNYAL İLETİ YOLAKLARI, ETKİNLEŞTİRİLEN HÜCRE ÖLÜM MEKANİZMALARI VE BU MEKANİZMALAR ÜZERİNE SERAMİD METABOLİZMASI GENLERİNİN ETKİLERİ

Bitki kaynaklı bir antioksidan olan resveratrolün çeşitli kanser hücreleri üzerinde sitotoksik etkilerinin olduğu bilinmektedir. Önemli bir biyoaktif sfingolipid olan seramid, hücre çoğalması, yaşlanması ve apoptoz gibi önemli hücre içi sinyal ileti yolaklarını kontrol etmektedir. Seramidler hücre içinde LASS genleri tarafından sentezlenmektedir. Apoptotik bir molekül olan seramid, glukozilseramid sentaz (GSS) enziminin katalizörlüğünde antiapoptotik glukozilseramide, sfingozin kinaz-1 (SK-1) enziminin katalizörlüğünde ise antiapoptotik sfingozin 1-fosfata dönüşmektedir. Önemli bir seramid analoğu olan C8:seramid, hücre membranından geçebilme özelliğine sahiptir ve hücre içi seramid oluşumunu önemli ölçüde arttırmaktadır. Glukozilseramid sentaz inhibitörü 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) ve SK-1 inhibitörü ise seramidin hücre içinde birikmesine neden olmaktadır. Kronik miyeloid lösemi, resiprokal translokasyon sonucunda oluşan BCR/ABL onkogeni nedeniyle ortaya çıkan bir hematolojik kanser türüdür.

Çalışmamızda, resveratrol uygulanan K562 hücrelerinde seramid metabolize eden genlerin ve hücre yaşlanması, apoptoz, hücre döngüsü kontrolü ve metastaz gibi çeşitli hücresel olayı kontrol eden 84 farklı genin ekspresyon düzeyleri belirlenmiştir. Çalışma sonucunda, resveratrol ile birlikte uygulanan C8:seramid, PDMP veya SK-1 inhibitörünün K562 hücreleri üzerinde sinerjistik sitotoksik ve apoptotik etkilerinin olduğu ortaya konmuştur. Ayrıca, artan dozlarda resveratrol uygulanan K562 hücrelerinde LASS genlerinin ekspresyon düzeyleri doza bağlı olarak artış gösterirken SK-1 ve GSS genlerinin ekspresyon düzeyleri doza bağlı azalma göstermiştir. Öte yandan, tümör baskılanmasında görevli SERPINB5, FAS, TNFRSF, MTSS gibi genlerin ekspresyon düzeylerinde önemli artışlar gözlenirken Myc onkogeninin ekspresyonunda azalma gözlenmiştir.

Sonuç olarak, bu çalışmada elde edilen bulgular, K562 hücrelerinde görülen resveratrol-aracılı apoptozda seramid metabolize eden genlerin de rollerinin

olabileceğini ve ayrıca SERPINB5 gibi önemli tümör baskılayıcı genlerin ekspresyonunun da bu apoptotik yolakta rol aldığını ilk kez ortaya koymuştur.

To indispensible people of my life...

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CHAPTER 1

INTRODUCTION

1.1. Chronic Myeloid Leukemia (CML)

Chronic myeloid leukemia is a hematological disorder arisen from the reciprocal translocation between BCR (Breakpoint cluster region) gene on chromosome 22, and ABL (Abelson murine leukemia virus) gene on chromosome 9, t(9;22)(q34;q11), resulting in the formation of Philadelphia (Ph) chromosome. This Ph chromosome encodes BCR/ABL fusion protein, which has constitutively active tyrosin kinase activity. BCR/ABL consists of more than one different domains. At the N-terminal domain of this protein, there is a cap structure that has two isoforms because of alternative splicing. One another domain, tyrosine kinase domain is split into two subdomains called as Src-homolgy domains, consisting of SH2 and SH3 (Quinta´s-Cardama and Cortes 2009).

Depending on the alternative splicing patterns, molecular weights of BCR/ABL fusion transcripts could be 190-, 210-, or 230 kDa. While 210 kDa-oncoprotein is mainly found in CML and 5 -10% of adults with acute leukemia, 185 kDa-oncoprotein exists in acute lymphocytic leukemia, and also 230 kDa oncoprotein is mainly found in chronic neutrophilic leukemia. In normal conditions, 145-kDa-ABL protein, which localizes in the nucleus, operates in several cellular signaling events such as cell cycle regulation, cellular response to toxic agents, and advising about the environmental conditions (Quinta´s-Cardama and Cortes 2009).

After the fusion of ABL with BCR, ABL no longer translocates into the nucleus, therefore it retains in the cytoplasm. However, it gains limitless tyrosine kinase activity because it is no longer regulated. The resultant BCR/ABL oncoprotein affects many downstream signaling pathways resulting in uncontrolled cell proliferation, growth factor autonomy, and evasion of apoptosis, which are some of the six hallmarks of cancer. BCR/ABL induces several signal transduction pathways which mediate cellular

proliferation and cause the disruption of the genetic maintenance. Such pathways are MAPK, PI3K, and RAS (Jagani, et al. 2008).

CML is characterized by three stages: chronic, accelerated and blast crisis. While chronic phase lasts few years, accelerated phase lasts 4-6 months, and blast crisis phase lasts a few months (Perrotti, et al. 2005).

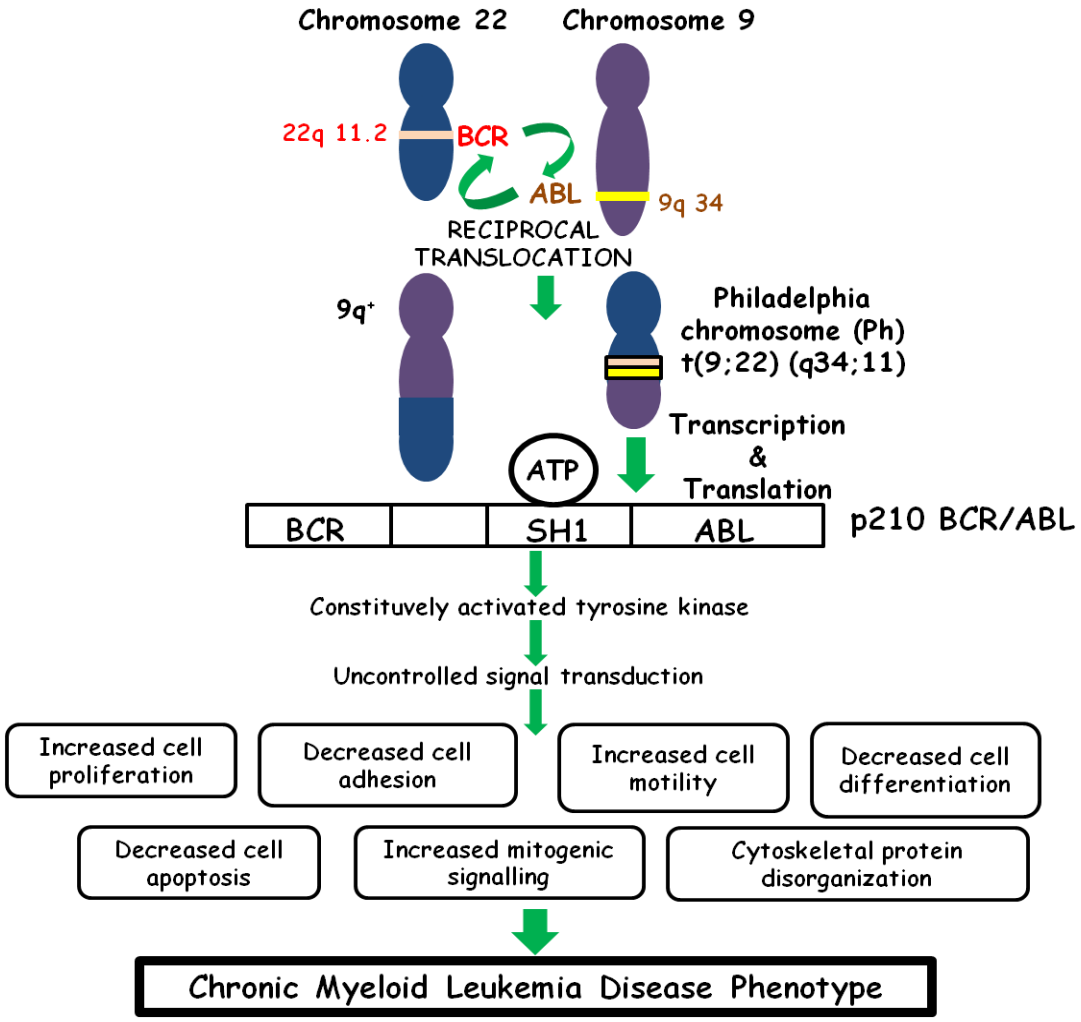

Figure 1.1. Generation of CML phenotype
(Adapted from: Frazer, et al. 2007)

Statistically, the incidence of CML is 1-2 per 100,000 population per year. The median age of presentation is 45 to 55 years, accounting for 20% of leukaemia affecting adults. As in all leukemias, males are affected more than females in CML, with a ratio of 2:1 (Frazer, et al. 2007).

1.1.1. Downstream Signaling Pathways of BCR/ABL

1.1.1.1. BCR/ABL and STAT

The members of signal transduction and activators of transcription (STAT) family occupy in several signaling pathways such as cell growth, inflammation, differentiation, apoptosis, etc. STAT signaling begins with the binding of a ligand to cytokine or growth receptors followed by the phosphorylation, dimerization, and also translocation of STAT (Yu, et al. 2009).

Due to having constitutive tyrosine kinase activity BCR/ABL phosphorylates and activates STAT-1 and -5 constitutively. Active STAT-5 triggers the expressions of anti-apoptotic genes such as Bcl-xL (Jagani, et al. 2008).

1.1.1.2. BCR/ABL and NFκB

NFκB functioning as dimers can be activated by several stimulants such as viruses, cytokines, and oxidative stress. NFκB activity is regulated by the protein IκB (inhibitor of κB), which retains NFκB in the cytoplasm. By the phosphorylation and ubiquitinylation, IκB is degraded, therefore the free and active NFκB can enter the nucleus, and mediates the expression of several genes related with cell survival processes (Li and Sethi 2010).

In many cancer cells, NFκB is constantly activated, and this is generally considered with the development of resistance against apoptosis. Several studies have reported that CML cells with BCR/ABL expression overexpress the p65 (RelA), active subunit of NFκB (Li and Sethi 2010). In addition, for generation of tumors in nude mice by the transformation of hematopoetic cells with BCR/ABL, constant NFκB activation was found to be necessary (Jagani, et al. 2008).

1.1.1.3. BCR/ABL and Ras Signaling

In CML cells expressing BCR/ABL, Ras signaling is constitutively active. In murine bone marrow cells, while Ras is inactivated, it has been observed that the transformation potential of BCR/ ABL decreases (Downward 2003).

The adapter protein Grb2 is the dominant molecule that supplies the connection between BCR/ABL and Ras signaling pathway. This adapter protein binds to tyrosine residue 177 of BCR part of BCR/ABL by its SH2 domain. In vivo studies have been revealed that the pathways Ras and PI3-K/Akt are quite significant for carcinogenesis in CML (Jagani, et al. 2008).

1.1.1.4. BCR/ABL and PP2A Phosphatase

Studies have reported that in CML blast crisis, BCR/ABL causes an increase in the expression levels of SET gene, the inhibitor of PP2A, which is tumor suppressor serine/threonine protein phosphatase. Inhibition of PP2A phosphatase by SET leads to inactivation of pro-apoptotic molecules such as Bad. However, this inhibition of PP2A leads to overexpression of the genes such as Akt and Erk that are related with cell survival and growth (Jagani, et al. 2008).

The studies on CML cell lines that are imatinib-resistant and –sensitive have revealed that reactivation of PP2A causes the inhibition of cell proliferation and survival molecules, the repression of BCR/ABL, and even the degradation of BCR/ABL. Therefore, PP2A should be inhibited in order to provide BCR–ABL-mediated leukemogenesis (Perrotti and Neviani 2006).

1.1.1.5. BCR/ABL and PI3-K/Akt Pathway

Phosphoinositide-3 kinase (PI3-K), a type of lipid kinase, performs critical roles in many cellular processes such as survival, growth, mobility, and proliferation (Carnero 2010). Many studies have been indicated that BCR/ABL expression activates PI3-K (Steelman, et al. 2004, Naughton, et al. 2009). This active PI3-K converts PIP2 to PIP3 via phosphorylation. PIP3 in turn activates Akt. Akt then regulates the functions of many molecules such as Mdm-2, caspase-9, mTOR, Bad, and FoxO (Jagani, et al. 2008). When phosphorylated by Akt, Bad retains in the cytoplasm and cannot promote its apoptotic effects. Despite that, in vitro studies have indicated that Bad sequestration is not fundamental for providing survial that is BCR/ABL-mediated (Neshat, et al. 2000). In addition, one another study has indicated that CML cells treated with PI3-K inhibitor at chronic, acute, and blast crisis phases form less colonies than untreated

ones. Like that, CML cells treated with Akt inhibitor also showed decreased colony formation. These studies also were also confirmed by *in vivo* studies (Jagani, et al. 2008).

When BCR/ABL and inhibitor of Akt-expressing marrow cells were transplanted into SCID mice, leukemia growth was significantly decreased compared to control group (Skorski, et al. 1997). Moreover, PI3-K inhibitor and imatinib have been reported that they act synergistically on triggering apoptosis in CML cells at the phases chronic or blast crisis. Besides Akt inhibitor treatment have sensitized imatinib-resistant CML cells to apoptosis (Klejman, et al. 2002). Furthermore some other studies have reported that PI3-K/Akt signaling is required for CML cells in order to develop resistance against imatinib (Jagani, et al. 2008).

1.1.2. Therapeutic Approaches for CML

1.1.2.1. Allogeneic Stem Cell Transplantation

In allogeneic stem cell transplantation, patient receives stem cells providing blood formation from a donor that is genetically similar. Since 1970, allogeneic stem cell transplantation has been used as an effective therapy for CML. Anyway, this approach could be resulted in deaths in many cases (Goldman and Melo 2003).

Several reports before imatinib era have implicated that bone marrow transplantation provide many patients survival chance. However, emergence of graft versus host disease after transplantation is an important drawback of this therapy (Frazer, et al. 2007).

1.1.2.2. Interpheron Alpha

A biological glycoprotein, interpheron alpha (IFN-α) inhibits proliferation and viral infection. It has been used since 1980s, and many studies have revealed the effects on IFN- α on survival lengths of CML patients.

When combined with other therapeutic agents, its cytotoxic efficacy has increased significantly, however, the disease has not been entirely ameliorated (Frazer, et al. 2007).

1.1.2.3. Imatinib Mesylate (STI571)

Widespread existence of BCR/ABL oncoprotein in patients with CML made this protein an open target for therapeutic applications, therefore tyrosin kinase inhibitors have been developed for therapy. Normally, ATP binds to kinase domain of BCR/ABL, by this way, it phosphorylates tyrosine residues on the substrate. Imatinib mesylate (STI 571, Gleevec, Novartis, Switzerland), the widely used therapeutic agent, blocks binding of ATP to the ATP-binding pocket of BCR/ABL, thus tyrosine kinase activity fails (Fausel 2007). Since 2001, imatinib has been used as "gold standard therapy" due to its strong cytotoxic effects on CML cells (Frazer, et al. 2007).

Comparative studies have revealed that imatinib has more endurable and excellent effects than IFN- α. Presently, doctors are suggesting that patients recently diagnosed with CML should be treated with imatinib. Allogeneic stem cell transplantation should be referred only when imatinib therapy do not respond (Frazer, et al. 2007).

1.1.2.4. Nilotinib (AMN107)

As imatinib does, nilotinib, one of the second generation tyrosine kinase inhibitors, binds to ATP-binding pocket of BCR/ABL resulting in the inhibition of BCR/ABL oncoprotein function. Topographically, nilotinib is more appropriate to the BCR/ABL oncoprotein than imatinib therefore it binds firmlier resulting in more efficient and more susceptible drug action (Frazer, et al. 2007).

Furthermore, many studies have indicated that CML patients with imatinib resistance are more susceptible to nilotinib.

1.1.2.5. Dasatinib (BMS-354825)

In contrast to imatinib and nilotinib, dasatinib functions through binding both active and inactive forms of BCR/ABL. It has a high affinity to BCR/ABL kinase domain since it is quite elastic in binding to several conformational structures of BCR/ABL (Shah, et al. 2004). Despite that, due to having a potential to bind the other

members of Src kinase family, dasatinib is not the most specific inhibitor of BCR/ABL kinase activity (Frazer, et al. 2007).

In the course of time, however, cells could develop resistance against imatinib, and the other therapeutic agents. In addition, these chemotherapeutic agents have many adverse effects such as edema, nausea, rash and musculoskeletal pain. Therefore, new therapeutic agents -especially natural products due to their lower costs and also less side effects- are being investigated for their anticancer potentials.

1.2. Resveratrol

Resveratrol, 3,5,4'-trihydroxystilbene, is a natural product generated by various plants such as red grapes, peanuts, mulberries, blueberries, cranberries, etc., and approximately 70 other plants (Aggarwal, et al. 2004), by the enzyme stilbene synthase, in response to stress conditions such as UV irradiation, ozone exposure, heavy metal exposure, and pathogenic infection (Kundu and Surh 2008). Resveratrol is quite widespread in nature, and also its isomers and derivatives are recognized. UV irradiation and heat can make *trans*- form to turn into *cis*- form. *Trans*- form of resveratrol is more stable than *cis*- form, and it can be stored for months avoiding light (Aggarwal, et al. 2004).

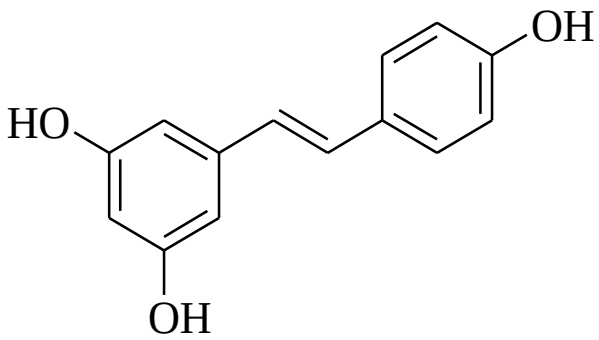

Figure 1.2. Chemical structure of resveratrol

Resveratrol has been reported to have anti-inflammatory (Kang, et al. 2009), anti-angiogenic (Belleri, et al. 2005), anti-metastatic, cardioprotective, neuroprotective, apoptosis- and autophagy-inducer (Signorelli, et al. 2009), spermatogenesis-enhancer,

and also chemosensitizer (Puissant, et al. 2008) and even radiosensitizer (Liao, et al. 2005) effects. The inverse relationship between red wine consumption and protection from cardiovascular diseases is known as “French paradox” (Ulrich, et al. 2005). The anticancer effects of resveratrol were firstly reported by Jang *et al.* in which study showed the inhibition of initiation, promotion, and progression stages of carcinogenesis by resveratrol in 1997. After that, resveratrol attracted increasingly more attention by many cancer researchers due to investigate its molecular targets and functional mechanisms. Many in vivo studies showed that direct application of resveratrol to tumor tissue reduced tumor size, and some of the mechanisms of resveratrol action were clarified (Kundu and Surh 2008). Anticancer effects of resveratrol were mainly reported in prostate (Aziz, et al. 2006), breast (Scarlatti, et al. 2003), and colon (Delmas, et al. 2003) cancers. Despite that, there are still many questionmarks in the mechanistic area of resveratrol. Therefore researchers are still studying with resveratrol to clarify the resveratrol mechanism (Kundu and Surh 2008).

1.2.1. Regulatory Effects of Resveratrol on Cell Cycle Progression

Resveratrol could arrest cell cycle at S phase, G1/S phase, and also G2/M phase transition in many cancer cells (Ulrich, et al. 2005). It induces p21WAF1 and p27KIP1 and downregulates cyclins D1/D2/E, Cdks 2/4/6Cdc2 kinases and pRb which is hyperphosphorylated. Resveratrol also upregulates p21WAF1, p300/CBP, APAF1, and Bak, which are related with the p53 tumor suppressor gene activation, and downregulates Bcl-2 that has anti-apoptotic effects on cancer cells. In addition to p53-dependent ways, resveratrol also induces p21WAF1 and cell cycle arrest by p53-independent ways. In addition, it downregulates ribonucleotide reductase and DNA polymerase thus directly pevents DNA replication (Ulrich, et al. 2005).

C-myc, one of the most expressed oncogene in many cancer cells, is also downregulated by resveratrol, and this is followed by the cell cycle arrest at the S phase (Zhang, et al. 2006). However, resveratrol inhibits Cdk1, the upstream target of myc oncogene, this inhibition in turn downregulates survivin, which is the target of Cdk1 in order to provide survival of cancer cells upregulating myc oncogene. Many in vivo and in vitro studies showed that resveratrol causes cancer cells to undergo apoptosis in myc-

dependent manner. For this reason, Cdk1 inhibition by resveratrol treatment could be a new area for therapy of cancers overexpressing myc oncogene (Ulrich, et al. 2005).

1.2.2. Regulatory Effects of Resveratrol on Apoptosis and Survival

In many studies, resveratrol has been reported to cause cell cycle arrest and and apoptosis by regulating the expressions of the genes related with survival and apoptotic pathways (Kundu and Surh 2004). In many cancer cells, resveratrol induces apoptosis activating p53 tumor supressor gene (Laux, et al. 2004). Resveratrol causes the overexpression of Bim, Bax, Bak, Noxa, and PUMA, which are pro-apoptotic genes, and also the decrease in the expression levels of Bcl2, Bcl-XL, and Mcl-1, that are anti-apoptotic genes, therefore influences the mitochondrial pathway of apoptosis (Athar, et al. 2009). Resveratrol also causes apoptosis through Fas/TRAIL pathway (Ishibashi and Ohtsuki 2008). Moreover, resveratrol sensitizes cancer cells developing resistance against therapeutic agents that cause apoptosis, by inhibiting transcription factors and anti-apoptotic genes, and by increasing the expression levels of apoptotic genes (Puissant, et al. 2008). Resveratrol also leads to the generation of ceramide, the second messenger of sphingomyelin pathway, therefore overcomes drug resistance, and causes apoptosis (Ulrich, et al. 2007). In radio-resistant DU145 prostate cancer cells, resveratrol causes apoptosis sensitizing these cells to radiotherapy due to induction of ceramide generation (Scarlatti, et al. 2007). In addition to prostate cancer cells, resveratrol has also been reported to mediate ceramide generation in breast and colon cancer cells (Scarlatti, et al. 2003, Sala, et al. 2003).

Resveratrol induces not only apoptosis but also autophagocytosis. In 2004, Opipari *et al.* demonstrated that resveratrol had triggered autophagy in human ovarian cancer cells. In addition, Keng-Fu Hsu *et al.*, in 2009, reported that resveratrol had induced autophagy in cervical cancer cells via cathepsin-L, which is a lysosomal endopeptidase playing an important role in protein catabolism. Moreover, resveratrol has been reported recently that it induces autophagy in CML cells through activating the function of AMPK, and causing the expression of p62/SQSTM1 mediated by JNK (Puissant and Auberger 2010). These findings suggest that resveratrol could induce cell death by autophagy rather than apoptosis in some cases, even in the cells that are resistant to chemotherapeutics targeting apoptosis.

1.2.3. Suppression of Invasion and Angiogenesis by Resveratrol

A study reported that in human umbilical endothelial cells (HUVECs), resveratrol prevented cell growth by inhibiting MMP-2, one of the most important type IV collagenase that is an important factor of the basement membrane of whom degradation leads to metastasis, pursued by angiogenesis (Athar, et al. 2009). In these cells resveratrol prevents fibronectin and laminin to be attached by endothelial cells. In addition, many other studies showed that resveratrol decreases the expression levels of MMP-2 and MMP-9 in multiple myeloma and various types of endothelial cells. In the activation of MMP- 9 by DMBA or PMA (phorbol myristate acetate), resveratrol has been reported to inhibit NFκB and AP-1, or JNK, respectively, and also activate protein kinase C for the latter (Athar, et al. 2009). This inhibition of MMPs by resveratrol causes to suppression of invasion and metastasis.

Moreover, resveratrol has been reported to suppress expression levels of VEGF, very important molecule for tumor development and angiogenesis in breast cancer cells (Garvin, et al. 2006). Resveratrol also inhibits ERK 1/2 and Akt, lowering the VEGF levels and the accumulation of hypoxia-induced HIF-1α protein (Wu, et al. 2008). Furthermore, resveratrol inhibits the activation of Src kinase, which is ROS-dependent, and also inhibits VE-cadherin tyrosine phosphorylation, that is important in providing stability to cell-cell junctions (Lin, et al.2003). By this way, it has been reported that resveratrol could successfully prevent angiogenesis induced by VEGF. Studies indicated that resveratrol suppresses the glioma development in rats via inhibiting angiogenesis (Athar, et al. 2009).

1.2.4. Resveratrol Action in Cancer Inflammation

Resveratrol has the ability to suppress the activity of cyclooxygenase-2 (COX-2), the enzyme transforming free arachidonic acid to prostaglandins, resulting in DNA damage. In vivo studies have also indicated the ability of resveratrol in the suppression of COX-2 (Athar, et al. 2009). Resveratrol also regulates the activity of MKP5, which dephosphorylates mitogen-activated protein kinases (MAPKs). By dephosphorylation, MKP5 particularly inhibits protein kinases p38 and JNK that are stress-activated (Theodosiou, et al. 1999).

Inhibition of the activity of p38, which is important in prostate cancer due to its ability in facilitating proinflammatory responses, decreases NFκB activity, the levels of pro-inflammatory cytokines, and also COX-2, IL-6, and IL-8 expression (Nonn, et al. 2007, Athar, et al. 2009).

In MCF-7 and MDA-MB-231, human breast cancer cell lines, resveratrol-mediated apoptosis occurs via the cooperation between COX-2 and ERK1/2 and AP-1. In these cells, ERK1/2 and AP-1 lead to the accumulation of agglomeration of COX-2. In this case, COX-2 is found with the p53, which is phosphorylated, and the co-activator of p53, p 300 (Tang, et al. 2006). With the apoptotic effect of resveratrol, the activity of ERK1/2 decreases resulting in the disintegration of COX-2/p53/p300 complex. Consequently, resveratrol could inhibit the progression of cancer via influencing the activity of inflammatory molecules.

1.2.5. Effects of Resveratrol on Transcription Factors

Inhibition of the activation of NFκB occurs through the inhibition of the phosphorylation of IKK and p65, the degradation of IκBα in many cancer cells (Athar, et al. 2009). In several cancer cells, NFκB inhibition by resveratrol also occurs by different mechanisms such as targeting TNF, H_2O_2, LPS or ceramide (Manna, et al. 2000). Besides NFκB, resveratrol also decreases the expression levels of the genes regulated by NFκB, such as Bcl2, IL6, XIAP, VEGF, MMP-9, and c-IAP (Sun, et al. 2006). In breast cancer cells, resveratrol has been reported as the inhibitor of metastasis, the process that specializes cancer cells mobile ability (Kundu and Surh 2004).

Resveratrol inhibits the binding of AP-1 to DNA via decreasing the expression levels of c-FOS, one of many producers of AP-1. Resveratrol-mediated AP-1 inhibition can also occur via the suppression of c-Jun, and this is also related with the blockade of MEK-ERK1/2 signaling (Kundu, et al. 2006).

Resveratrol-mediated p53 activation involves the serine 15-phosphorylation of this transcription factor, even in other words, “the guardian of the genome”, by ERK and p38 MAPK. It has been reported that resveratrol causes p53-dependent apoptosis by regulating Ras-MAPK signaling in thyroid cancer cells. Resveratrol also activates one of downstream molecules of p53, NAG-1. Resveratrol-mediated increase in NAG-1

expression levels decreases the ability of cancer cells for colony formation (Baek, et al. 2002).

1.2.6. Effects of Resveratrol on Cathepsin-D

An aspartic endoprotease, cathepsin-D is an important mediator of apoptosis, and the translocation of mature cathepsin-D to the cytosol causes the release of cytochrome c from mitochondria and the activation of caspases 9 and 3. This relocalized cathepsin-D can also activates Bax, therefore apoptosis-inducing factor, AIP is released from the mitochondria, following the Bax activation. Resveratrol-induced cathepsin-D activation has been reported as one of the apoptotic mechanisms mediated by resveratrol. Resveratrol triggers cathepsin-D secretion in ER^+ breast cancer cells in a dose-dependent fashion, but not in ER^- cells (Liaudet-Coopman, et al. 2006).

1.2.7. Mitochondrial Effects of Resveratrol and Its Prooxidant Activity

In normal cells, effects of resveratrol are related with the antioxidant activity and the protection of mitochondrial activity whereas in cancer cells, its effects are mostly related with the induction of apoptosis.

Resveratrol inhibits complexI and F0F1-ATPase, which are the important ezymes for mitochondria (Zheng and Ramirez 1999). In addition, resveratrol collapses the redox homeostasis of mitochondria, and triggers apoptosis dependent to mitochondria (Dörrie, et al. 2001). However, resveratrol can also has prooxidant activity producing reactive intermediates. With this activity, resveratrol can causes cellular damage by oxidizing lipids, proteins, and DNA. ROS regulates p53 in a post-translational manner, and triggers the breakdown of membrane permeability of mitochondria, and DNA fragmentation. Resveratrol protects DNA from damages mediated by ROS. In addition, in the presence of metal ions, resveratrol causes DNA degradation. Many studies indicated that in the presence of high levels of copper, which is characteristic for several cancer cells, resveratrol causes the ROS generation resulting in the loss of mitochondrial membrane potential, and finally apoptosis. Morever, treatment of $p53^+$-cancer cells with subapoptotic doses of resveratrol causes to increase in the levels of H_2O_2 and superoxide anion (O_2^-), and also results in cell senescence that

is dependent to ATM. Such resveratrol-mediated cell senescence also includes p53, p38 MAPK, and p21WAF1 activation (Athar, et al. 2009).

Despite of these findings, exact mechanisms underlying resveratrol-induced apoptosis in CML cells have not been clearly identified yet. In order to enlighten this knife edge, bioactive sphingolipids have been studied in CML cells due to their potentials in regulating cellular processes.

1.3. Bioactive Sphingolipids

Sphingolipid family, a member of membrane lipids, have many important regulatory and structural roles in lipid bilayer. The sphingolipids ceramide, ceramide-1-phosphate, glucosylceramide, galactosylceramide, shingosine, sphingosylphospho-choline, and sphingosine-1-phosphate (S1P) are known as effector molecules of sphingolipid metabolism. These molecules have many important roles in several critical processes such as cell proliferation, inflammatory responses, migration, senescence, and also apoptosis (Ogretmen and Hannun 2004).

Ceramide, the key molecule of sphingolipid family, can be generated in response to TNF-α, IL-1, Fas ligand, ionizing radiation, heat stress, oxidative stress, and chemotherapeutics (Ogretmen and Hannun 2004). Increasing intracellular levels of ceramide results in growth inhibition, differentiation, apoptosis, alteration of telomerase activity and telomere length, and senescence whereas increasing S1P levels results in the induction of cell proliferation, transformation, angiogenesis, and mobility, and also increased levels of glucosylceramide synthase (GCS) converting apoptotic ceramide into antiapoptotic glucosylceramide, makes several cancer cells multidrug resistant (Ogretmen and Hannun 2004).

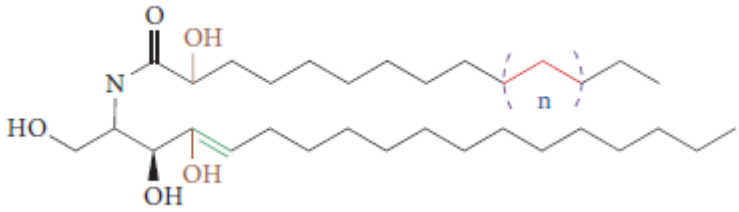

Figure 1.3. Chemical structure of ceramide
(Source: Hannun and Obeid 2008)

1.3.1. Bioactive Sphingolipid Metabolism

Generally, ceramide is produced via two different mechanisms. One mechanism is generation of ceramide by the de novo pathway, and the other one is the generation of ceramide by the lipid hydrolysis, such as sphingomyelin (SM) hydrolysis. In de novo pathway, serine condenses with palmitoyl-CoA by serine palmitoyl transferase to generate 3-ketodihydrosphingosine. 3-keto-dihydrosphingosine is reduced, and dihydrosphingosine (sphinganine) is formed. Then, sphinganine is N-acylated by dihydroceramide (dhCeramide) synthases in order to produce dhCeramide or ceramide. In the hydrolytic pathway, sphingomyelinase hydrolizes sphingomyelin delivering phosphocholine and ceramide. Ceramide kinase phosphorylates ceramide in order to generate ceramide-1-phosphate (Sugiura, et al. 2002).

Ceramide can be converted into sphingosine by ceramidases (El Bawab, et al. 2002), sphingosine is in turn phosphorylated by sphingosine kinases in order to form sphingosine-1-phosphate (S1P). S1P is then converted into sphingosine by phospahatases or by the enzyme lyase, S1P is converted into ethanolamine-1-phosphate and a C16-fatty-aldehyde (Ogretmen and Hannun 2004).

1.3.2. LASS Genes as Ceramide Synthases

LASS genes were firstly investigated in yeasts and called as longevity assurance genes (*lag1*). In mammals, *de novo* ceramide synthesis is conducted by LASS (longevity assurance homologues) genes (LASS 1-6). Each of these six genes generates ceramides in different lengths. While LASS1 generates C18:ceramide, LASS2 and LASS4 generate C22:- and C24:ceramides, respectively. LASS5 and LASS6 generate shorter-chain ceramides such as C14:- and C16:ceramide, respectively. Despite of having lower specificity to fatty acyl-CoAs, LASS3, however, generates C18:-, C22:-, and C24:ceramides (Teufel, et al. 2009). These different-length-ceramides may have different functions. For example, while C16:ceramide is important in the induction of apoptosis in LNCaP cells and hepatocytes, C18:ceramide is important in head and neck squamous cell carcinomas (Teufel, et al. 2009).

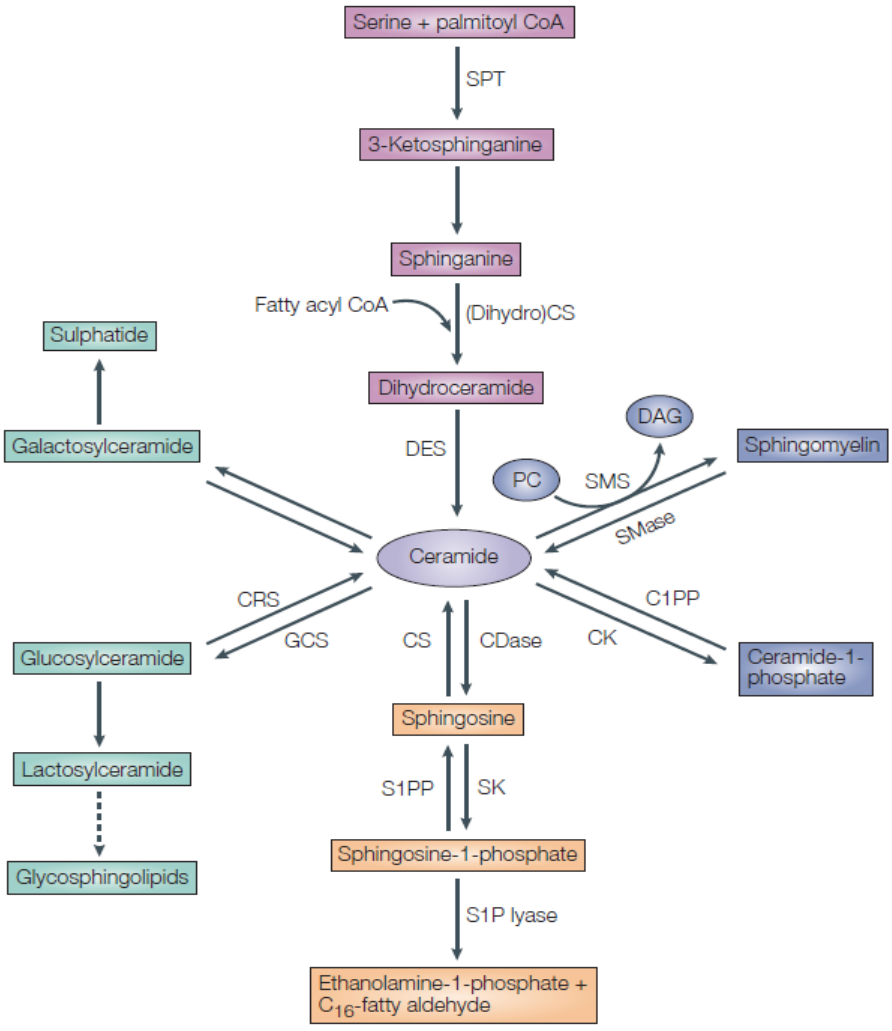

Figure 1.4. Pathways of sphingolipid metabolism
(Source: Ogretmen and Hannun 2004)

1.3.3. Compartmentalization of Sphingolipids

In sphingolipid metabolism, enzymatic reactions are delivered into distinct cellular compartments. *De novo* ceramide synthesis occurs on the cytosolic side of the endoplasmic reticulum (ER) and in ER-associated membranes. Sphingomyelin and glucosylceramide synthesis occurs in the Golgi apparatus. Ceramide can be transported from ER to the Golgi apparatus by two ways. In the first way, ceramide in the form of sphingomyelin, is transported by the activity of the ceramide transfer protein, CERT. In the other way, ceramide in the form of glucosylceramide is transported by vesicules. In

mitochondria, ROS are generated, and these ROS activate neutral sphingomyelinase (N-SMase), which generates ceramide. This ceramide activates PP1 and PP2A, that are ceramide-activated protein phosphatases. PP2A dephosphorylates and inactivates antiapoptotic proteins. Also, PP1 activates Bid. In lysosomes, acid sphingomyelinase generates ceramide. This ceramide activates Cathepsin-D and Bid, and then activates caspases -9 and -3. In addition, ceramide can be converted into sphingolipids by the action of acid ceramidase. In the plasma membrane, ceramide is generated in the lipid rafts, and this generated ceramide affects important signaling pathways (Hannun and Obeid 2008).

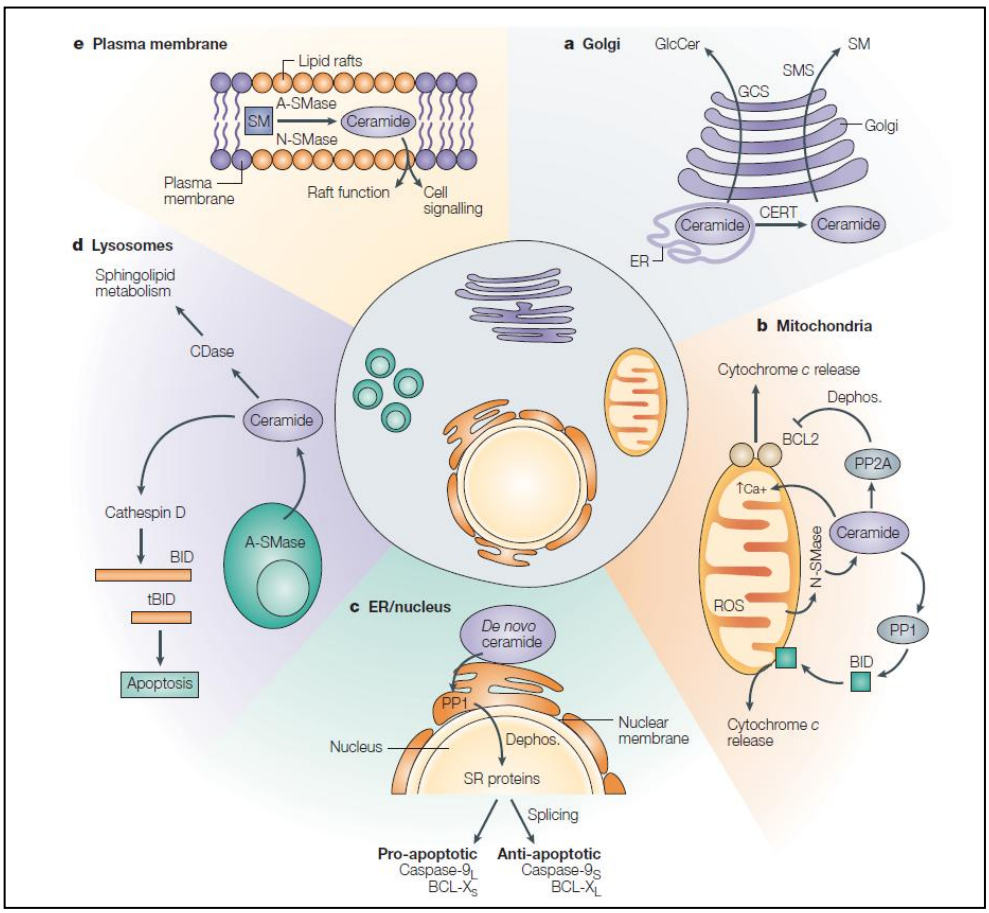

Figure 1.5. Ceramide compartmentalization
(Source: Ogretmen and Hannun 2004)

1.3.4. Roles of Sphingolipid Metabolism in Cancer

Understanding the molecular identifications of the enzymes that play roles in sphingolipid metabolism has provided the observation of molecular pathways occupying in this metabolism. In addition, studies showed that these enzymes occupy different cellular compartments. Such compartmentalized localization of enzymes indicates the complexity of the pathways in sphingolipid metabolism. Moreover, levels of these enzymes show differences in cancer cases, demonstrating that enzymes in sphingolipid metabolism have different functions in carcinogenesis. Many studies showed that ceramide levels and cancer progression are correlated inversely whereas sphingosine kinase-1 levels are related with cancer progression in several cancer cells (Ogretmen and Hannun 2004, Saddoughi, et al. 2008).

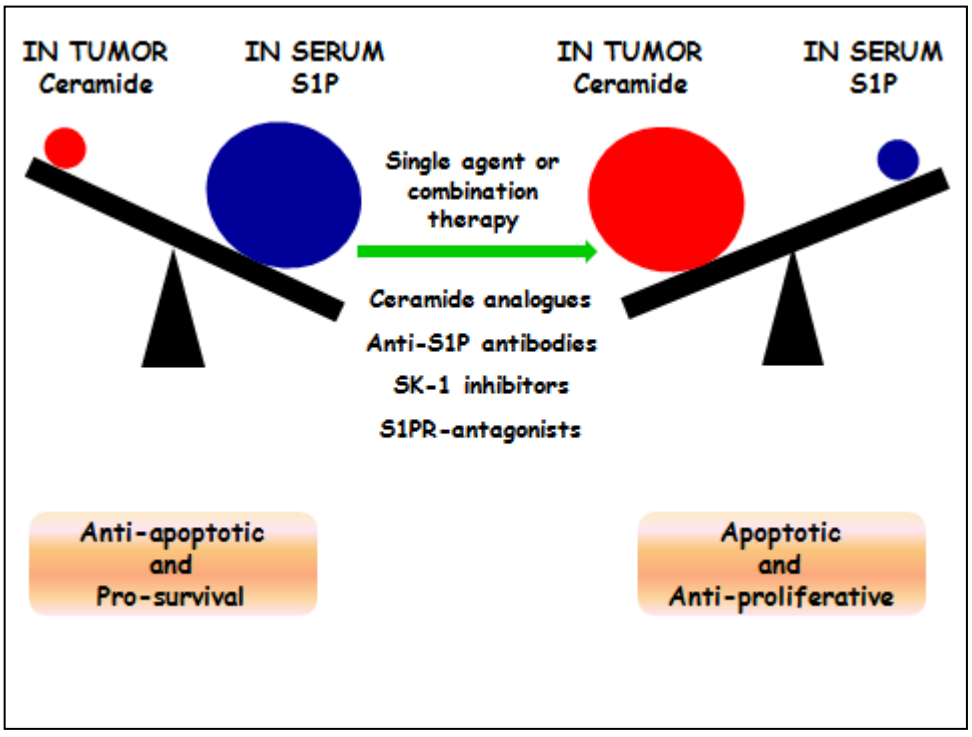

Figure 1.6. Changes in balance between ceramide and S1P in response to therapy (Adapted from: Saddoughi, et al. 2008)

1.3.5. Apoptotic Effects of Ceramide

Several studies showed that ceramide is related with the apoptotic inducers such as TNF-α, FAS/FASL, hypoxia, and DNA damage in many cancer cells (Pettus, et al. 2002). These apoptotic inducers provide ceramide to accumulate in cancer cells via different ways of ceramide metabolism. Studies also showed that the generation of C_{16}-ceramide in response to apoptotic signals occurs via *de novo* pathway. In addition, some chemotherapeutic agents such as daunorubicin, etoposide, gemcitabine, and FAS ligand causes *de novo* generation of C_{16}-ceramide (Ogretmen and Hannun 2004, Saddoughi, et al. 2008).

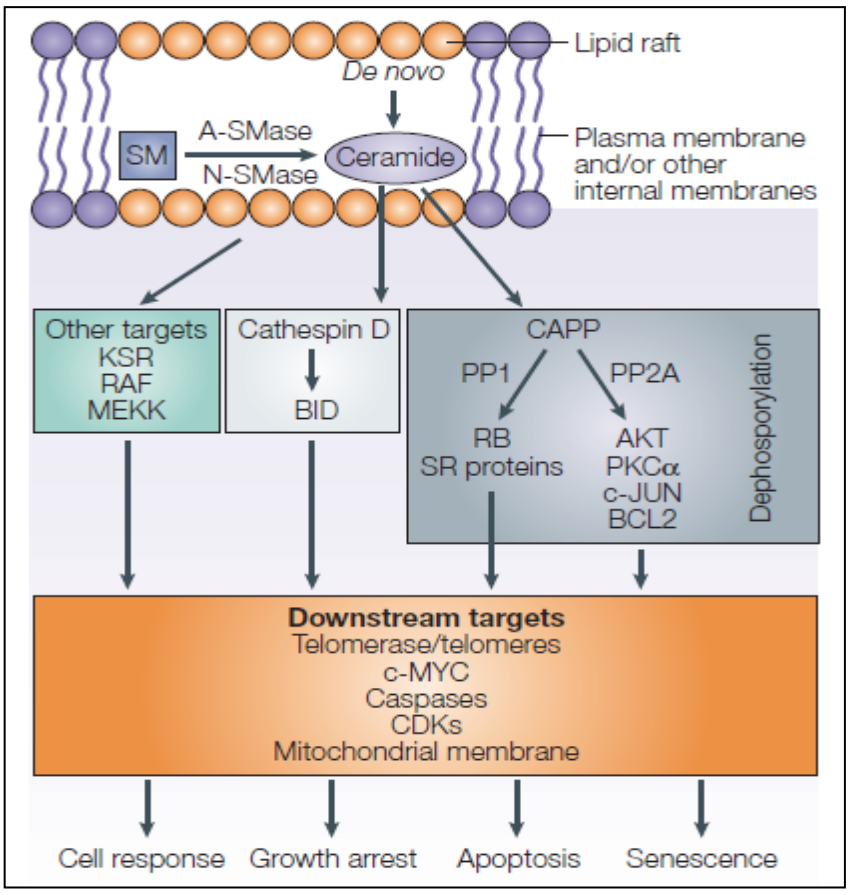

Figure 1.7. Downstream targets of ceramide
(Source: Ogretmen and Hannun 2004)

Generation of ceramide by acid sphingomyelinase (A-SMase) also plays roles in apoptosis. Generally, ionizing radiation and ultraviolet A (UVA) cause an increase in ceramide levels through acid sphingomyelinase activity. Morover, TNF-α is also responsible for generation of ceramide via A-SMase, resulting in cathepsin D activation, which is an endolysosomal aspartate protease. This activation of cathepsin D, in turn, results in the activation of caspase-9 and caspase-3, which is Bid-mediated. Besides A-SMase, neutral sphingomyelinase (N-SMase) also plays roles in ceramide generation and thus ceramide-mediated apoptosis. According to studies, N-SMase-activated apoptotic pathways include TNF in many cancer cells, and also N-SMase requires reactive oxygen species (ROS) for activation, indicating ceramide action in response to ROS-mediated DNA damages (Ogretmen and Hannun 2004).

Furthermore, ceramidases (CDases) also perform in the regulationof ceramide levels. In many cases, nitric oxide (NO) degragades neutral CDases, resulting in the accumulation of ceramide. Subsequently, this ceramide accumulation results in apoptosis (Franzen, et al. 2002).

Some studies indicated that ceramide generation in the same cell type can be controlled by more than one mechanism of ceramide metabolism. For example, in TNF-treated MCF-7 cells, both *de novo* pathway and N-SMase pathway are activated autonomously. Ligand binding to FAS receptor also activates the pathways A-SMase, N-SMase, and *de novo*. Distinct ceramide-induced cell death pathways are activated by every one of these mentioned pathways (Luberto, et al. 2002, Dbaibo, et al. 2001).

1.3.6. Ceramide Action in Quiescence and Senescence

In some cases, ceramide arrests cells at G0/G1 phase. TNF treatment induces dephosphorylation of retinoblastoma (Rb) by ceramide via PP1 activation (Dbaibo, et al. 1995). In addition to Rb, ceramide dephosphorylates and inactivas Cdk2, cyclin-dependent kinase. Ceramide also upregulates p21WAF1 and p27KIP1 in some types of cancer. Human N-SMase 2 regulates cell cycle progression and cell growth by providing the activation of ceramide-mediated growth repression (Ogretmen and Hannun 2004, Lee, et al. 2000).

Ceramide also induces senescence via dephosphorylating Rb, repressing protein kinase C, regulating growth factor signalling, and inhibiting CDKs, diacylglycerol, and

phospholipase D. In addition, ceramide can induce senescence through inhibiting the active expression of telomerase in many cancer cells (Ogretmen and Hannun 2004).

1.3.7. Effects of S1P in Transformation, Proliferation, Apoptotic Inhibition, Angiogenesis, and Inflammation

Many studies showed that, in contrast to ceramide, increased levels of sphingosine 1-phosphate is related with cell proliferation and suppression of apoptosis, which is especially induced by ceramide (Ogretmen and Hannun 2004, Cuvilier, et al. 1996). In several cancer cells, it has been reported that S1P increases invasive properties and carcinogenesis potential (Van Brocklyn, et al. 2003).

S1P can induce the formation of blood vessels in many cancer cells through VEGF signalling, which in turn induceses RAS and MAPK signalling pathways. S1P activates S1P receptors and regulates a family of GTPases, RHO, providing endothelial cells to migrate, and also resulting in the reconstruction of cytoskeleton, and inhibition of apoptosis (Liu, et al. 2001, Wu, et al. 2003).

Some cytokines such as TNF-α and interleukin1 (IL-1) have been reported to activate S1P-SK1 pathway, and cause the development of inflammatory responses. Inflammatory functions of S1P involves the agglomeration of S1P by the induction of SK1, which activates subsequently NFκB. S1P also induces COX-2 and prostaglandin E2 (PGE_2), resulting in the inflammation (Xia, et al. 1998).

1.3.8. Sphingolipid Use as Anticancer Therapy

Summarily, ceramide is a pro-apoptotic molecule whereas S1P converted from ceramide by the enzyme SK1 is anti-apoptotic, and promotes carcinogenesis, and also causes the genes which are responsible for synthesizing ceramide, and by GCS and SK-1 that convert ceramide to glucosylceramide, and sphingosine-1-phosphate (S1P), respectively. Modulation of intracellular levels of these sphingolipids could open a new road in cancer therapy. Moreover, alteration of these levels could circumvent multidrug resistance, increase the efficiency of chemotherapeutic molecules, and also provide defence mechanism to normal cells from toxic substances (Ogretmen and Hannun 2004).

1.4. Aim of the Study

Since the incidence of CML is increasing day by day and emerging of resistance cases of the patients with CML against chemotherapeutic agents is raising, new therapeutic approaches are being investigated nowadays. Resveratrol is the centre of interest due to its potential in repressing the phases cancer initiation, promotion, and progression. Although potential benefits of resveratrol are known, the molecular mechanisms underlying its apoptotic effects is not still completely understood.

Furthermore, ceramides are also the centre of interest due to their strong apoptotic potentials. As known, while ceramide is an apoptotic molecule and leads to many antiproliferative processes, S1P and GCS are highly antiapoptotic and lead to the processes related with cell survival.

Under the light of these informations, we aimed to investigate the molecular mechanisms related with the apoptotic effects of resveratrol, and its possible effects on sphingolipid metabolism in K562 human chronic myeloid leukemia cells. In addition, we intended to determine the possible synergistic effects of resveratrol and ceramide by applying exogenous ceramide, and also by manipulating the intracellular ceramide levels via treating the cells with PDMP, the inhibitor of glucosylceramide synthase that converts apoptotic ceramide into antiapoptotic glucosylceramide, and SK1 inhibitor inhibiting the enzyme sphingosine kinase-1, the converter of apoptotic ceramide to antiapoptotic S1P.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

Resveratrol was obtained from Sigma Aldrich (USA). The stock solution of resveratrol was dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mmol/ml, stored at -20 ^{o}C, and diluted in cell culture medium. C8:ceramide, PDMP (1-phenyl-2-decanoylamino-3- morpholino-1-propanol) that is GCS inhibitor, and SK-1 inhibitor were obtained from Cayman Chemicals (USA), and dissolved in DMSO. Penicillin-streptomycin, RPMI1640, and fetal bovine serum (FBS) were obtained from Invitrogen (Paisley, UK). Total RNA isolation kit was obtained from Macherey-Nagel (Germany). Reverse transcriptase (Moroney Murine Leukemia Virus Reverse Transcriptase), Taq DNA polymerase, and primers were obtained from Fermentas (USA). Bradford dye, and Coomasie blue, bovine serum albumine (BSA), trypan blue solution, β-mercaptoethanol, dimethylsulfoxide (DMSO), and agarose were obtained from Sigma (USA). DNA ladder, gel loading dye, and dNTP set were obtained from Fermentas (USA). Caspase-3 colorimetric assay kit was obtained from BioVision (USA). APO LOGIX JC-1 assay kit was obtained from Cell Technology (USA).

2.2. Cell Line, Culture Conditions, and Maintenance

Human K562 CML cells were obtained from German Collection of Microorganisms and Cell Cultures (Germany). These Ph (+) K562 human CML cells were cultured in RPMI1640 growth medium containing 1% L-glutamine, 10% fetal bovine serum, and 1% penicillin-streptomycin at 37 °C in 5% CO_2. Medium was refreshed in every 2 days. In order to passage the cells, whole cell suspension is taken into a falcon tube, and then centrifuged at 1000 rpm for 10 minutes. After cenrifugation, the supernatant was removed from the tube, and the rest pellet was dissolved in 10 ml of

medium. This cell suspension was then transferred into a sterile 75 cm^2-tissue culture flask containing 10 ml of growth medium.

2.3. Thawing the Frozen Cells

Cells were removed from the frozen storage at -80 ^oC and then immediately thawed in a water bath at 37 ^oC in order to acquire the highest percentage of viable cells. When the ice crystals melted, the content was transferred into 25 cm^2- tissue culture flask containing 5 ml of RPMI1640 medium, and incubated overnight at 37 °C in 5% CO_2. After incubation, cells were passaged as mentioned before.

2.4. Measurement of Cell Viability

In order to measure the viabilities of cells, 30 µl of cells were mixed with 30 µl of trypan blue dye which is an important dye using in distinguishing dead cells from alive cells under microscopy. When cells were treated with trypan blue dye, viable cells would not permeate this dye whereas dead cells would permeate because of their broken cell membranes. Therefore under a microscope, while dead cells would be stained blue, viable cells would not. By this way, cells were counted under microscope by using hemocytometer and then the percentage of unstained cells was calculated. Before each experiment, cell viability assay was conducted.

2.5. Measurement of Cell Growth by XTT Assay

The IC50 values (drug concentration that inhibits cell growth by 50%) of resveratrol and C8:ceramide, and the IC10 values (drug concentration inhibits cell growth by 10%) of PDMP, and SK-1 inhibitor were determined by XTT cell proliferation assay. In short, $2x10^4$ cells/100 µl/well were seeded into 96-well plates containing 100 µl of the growth medium in the absence or presence of increasing concentrations of resveratrol, C8:ceramide, PDMP, or SK-1 inhibitor and then incubated at 37 °C in 5% CO_2. After 72 h incubation period, cells were treated with 50 µl XTT for 4 h. Then, the plates were read under 492 nm wavelengths by Elisa reader

(Thermo Electron Corporation Multiskan Spectrum, Finland). Finally, IC50 values of resveratrol and C8:ceramide, and IC10 values of PDMP and SK-1 inhibitor were calculated according to the cell proliferation plots.

In order to determine the possible synergistic effects of resveratrol in combination with C8:ceramide, PDMP, or SK-1 inhibitor, $2x10^4$ K562 cells were seeded into each well of 96-well plate containing 100 µl of the growth medium. Then combinations of resveratrol with IC50 value of C8:ceramide or IC10 values of PDMP or SK-1 inhibitor were applied onto the cells. After 72 h incubation, the cells were treated with 50 µl of XTT mixture for 4 h and the plates were read under 492 nm wavelengths by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland).

2.6. Evaluation of Apoptosis

2.6.1. Caspase-3 Colorimetric Assay

Changes in caspase-3 enzyme activity of the cells, as an important sign of apoptosis, were examined by caspase-3 colorimetric assay kit (BioVision Research Products, USA). This assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide (*p*NA) after cleavage from the labeled substrate DEVD-*p*NA that can be recognized by caspases.

In short, the cells ($1x10^6$ cells/2 ml/well), induced to undergo apoptosis, were collected by centrifugation at 1000 rpm for 10 min. The cells were lysed by adding 50 µl of chilled Cell Lysis Buffer and incubated on ice for 10 min before centrifugation at 10000 g for 1 min. Supernatants were transferred to new eppendorf tubes and the reaction mixture was prepared in 96-well plates by adding 50 µl of 2X Reaction Buffer (containing 10 mM DTT), 50 µl of sample, and 5 µl of DEVD-pNA substrate, and then incubated for 2 h at 37 °C in carbondioxide incubator. At the end of this period, the plate was read under 405 nm wavelengths by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland).

The absorbance values are normalized to protein concentrations determined by Bradford assay. In this assay, protein samples were diluted by the ratios 1:25, 1:50, and 1:100. Then 90 µl of each sample were added to 96-well plates, containing 10 µl of Bradford reagent. Bradford reagent uses Coomassie blue G-250. Without protein, the

solution is red-brown in its acidic solution. When protein binds, the pKa of the dye shifts causing the dye to become blue. Finally, the dye within the plates was measured at 595 nm by Elisa reader.

2.6.2. JC-1 Mitochondrial Membrane Potential

We have also examined the loss of MMP, another important sign of apoptosis, in response to resveratrol, C8:ceramide, PDMP, and SK-1 inhibitor or combinations of resveratrol with the others in K562 cells by the APO LOGIX JC-1 Mitochondrial Membrane Potential Detection Kit (Cell Technology, USA). This kit uses JC-1, a unique cationic dye, to signal the loss of the MMP. JC-1 accumulates in the mitochondria which stain red in non-apoptotic cells while in apoptotic cells, the MMP collapses, and thus the JC-1 remains in the cytoplasm as a monomer that stains green under fluorescent light.

Briefly, the cells ($1x10^6$ cells/2 ml), induced to undergo apoptosis, were collected by centrifugation at 1000 rpm for 10 min. Supernatants were removed, 500 µl of JC-1 dye was added onto the pellets, and the cells were incubated at 37°C in 5% CO_2 for 15 min. Then, they were centrifuged at 1000 rpm for 5 min and 2 ml of assay buffer was added onto the pellets. After centrifugation at 1000 rpm for 5 min all pellets were resuspended with 500 µl assay buffer and 150 µl from each of them was added into the 96-well plate. The aggregate red form has absorption/emission maxima of 585/590 nm and the monomeric green form has absorption/emission maxima of 510/527 nm. The plate was read in these wavelengths by fluorescence Elisa reader (Thermo Varioskan Spectrum, Finland). At the end, green/red (510/585) values were calculated in order to determine the changes in MMP.

In addition, K562 cells were treated with increasing concentrations of resveratrol in combinations with C8:ceramide, PDMP, or SK-1 inhibitor. For this purpose, K562 cells ($1x10^6$ cells/2 ml) were seeded into 6-well plates and then treated with IC50 value of C8:ceramide or IC10 values of PDMP or SK-1 inhibitor. After incubating the plates for 18 h at 37 °C in 5% CO_2, increasing concentrations of resveratrol were applied into these plates. After 72 h incubation, the assay procedure mentioned above was executed.

2.7. Total RNA Isolation and RT-PCR

Total RNAs of K562 cells, treated with increasing concentrations of resveratrol, were extracted using a Ribolock RNA isolation kit (Macherey-Nagel, Germany) as described by the manufacturer. Concentrations of isolated RNAs were measured by Nanodrop ND-1000 (260/280 and 260/230 ratios). 1 μg of each total RNA was reverse transcribed using reverse transcriptase (Moroney Murine Leukemia Virus Reverse Transcriptase, Fermentas, USA). After 50 min of incubation at 42 °C, the reactions were stopped at 95 °C for 5 min. The resulting total cDNAs were then used in PCR to measure the mRNA levels of LASS2, LASS4, LASS5, LASS6, SK1, GCS and β-actin. The mRNA levels of β-actin were used as internal positive control.

Table 2.1. Primer sequences used in this study

LASS2-F	(5'-GCTGGAGATTCACATTTTAC-3')
LASS2-R	(5'-GAAGACGATGAAGATGTTGT-3')
LASS4-F	(5'-TGCTGTCCAGTTTCAACGAG-3')
LASS4-R	(GAGGAAGTGTTTCTCCAGCG-3')
LASS5-F	(5'-TCCTCAATGGCCTGCTGCTG-3')
LASS5-R	(5'-CCCGGCAATGAAACTCACGC-3')
LASS6-F	(5'-CTCCCGCACAATGTCACCTG-3')
LASS6-R	(5'-TGGCTTCTCCTGATTGCGTC-3')
SK1-F	(5'-CCGACGAGGACTTTGTGCTAAT-3')
SK1-R	(5'-GCCTGTCCCCCCAAAGCATAAC-3')
GCS-F	(5'-ATGACAGAAAAAGTAGGCT-3')
GCS-R	(5'-GGACACCCCTGAGTGGAA-3')
β-actin-F	(5'-CAGAGCAAGAGAGGCATCCT-3')
β-actin-R	(5'-TTGAAGGTCTCAAACATGAT-3')

Table 2.2. Ingredients for cDNA synthesis

Ingredients	**Amount (µl)**
RNase-free water	2.9
Total RNA (5µg)	5
10X Buffer	4
Random Hexamer Primer (0.5 µg/l)	0.7
RNase Inhibitor	0.7
$MgCl_2$ (25mM)	4
dNTP (10mM)	2
Moloney Murine Reverse Transcriptase (200 U/µl)	0.7
Total volume	20

Table 2.3. Ingredients of PCR solutions for LASS2, LASS4, LASS5, GCS, SK1, and β-actin genes

Reaction Mixture	**Amounts for LASS2, LASS4, LASS5, LASS6, GCS, SK1, β-actin (µl)**
PCR-grade water	30.7
Reaction buffer (10X)	5
$MgCl_2$ (25mM)	5
dNTP (2mM)	5
Primer forward (20 pmol/µl)	1
Primer reverse (20 pmol/µl)	1
cDNA	2
Taq DNA Polymerase	0.3
Total volume	50

Table 2.4. Amplification conditions for LASS2

Steps	Temperature (^{o}C)	Duration (min.)
Initial Denaturation	94	5
Denaturation	94	1
Annealing	54	1
Extension	72	1
Final Extension	72	5

Table 2.5. Amplification conditions for LASS4

Steps	Temperature (^{o}C)	Duration
Initial Denaturation	94	5 min
Denaturation	94	30 s
Annealing	57	30 s
Extension	72	45 s
Final Extension	72	5 min

Table 2.6. Amplification conditions for LASS5 and LASS6

Steps	Temperature (^{o}C)	Duration (min)
Initial Denaturation	94	5
Denaturation	94	1
Annealing	65	1
Extension	72	1
Final Extension	72	5

Table 2.7. Amplification conditions for GCS and SK1

Steps	Temperature (^{o}C)	Duration (min.)
Initial Denaturation	94	5
Denaturation	94	2
Annealing	53	2
Extension	72	2
Final Extension	72	5

Table 2.8. Amplification conditions for β-actin

Steps	Temperature (^{o}C)	Duration (min.)
Initial Denaturation	94	5
Denaturation	94	1
Annealing	53	1
Extension	72	1
Final Extension	72	5

After running the PCR products on agarose gel electrophoresis, quantification of expression levels of the genes were performed by Quantity One-1D-Gel-Imaging programme (BIORAD). The results were normalized to β-actin levels.

2.8. PCR Array

In order to investigate the molecular mechanisms underlying resveratrol-induced cell deaths, in this study we also used Human Cancer Pathway Finder RT^2 Profiler PCR Array System (SABiosciences Corporation, USA). With the help of this array, we analyzed the expression profiles of 84 genes which play important roles in cancer initiation, promotion, progression, and resistance.

Total RNAs of K562 cells, treated with 50- and 500 μM of resveratrol, were extracted using High Pure RNA isolation kit (Roche Diagnostics GmbH, Germany) as described by the manufacturer. RNA concentration were measured by Nanodrop ND-

1000 (260/280 and 260/230 ratios). 5 µg of each total RNA was treated with Genomic DNA Elimination Mixture provided by RT^2 First Strand Kit (SABiosciences Corporation, USA). After an incubation period for 5 min at 42 °C, samples were chilled on ice for 1 min. In the course of this incubation period, RT coctail was prepared as described by the manufacturer. Then, after chilling on ice, 10 µl of RT coctail was added to each 10-µl Genomic DNA Elimination Mixture. Samples were incubated at 42 ^oC for 15 min and then the reaction was stopped by heating at 95 ^oC for 5 min, and by this way, cDNAs were acquired. Then, 91 µl of H_2O was added to each 20-µl of cDNA synthesis reaction. Afterwards, expression profiles of the genes which play important roles in cell-cycle control, DNA damage repair, apoptosis, cell senescence, signal transduction and transcription, adhesion, angiogenesis, and also invasion and metastasis were analyzed by RT^2 Profiler PCR Array, which is a reliable and sensitive gene expression profiling technology.

Table 2.9. Ingredients of PCR Array Experimental Coctail

Plate Format	96-well
2X SABiosciences RT^2 qPCR Master Mix	1350 µl
Diluted First Strand cDNA Synthesis Reaction	102 µl
H_2O	1248 µl
Total Volume	2700 µl

After preparing this coctail, 25 µl of experimental coctail was added to each well of the PCR Array, which is a PCR plate preloaded with validated real-time PCR primers for a panel of pathway-focused genes.

Afterwards, PCR Array plates were sealed with the adhesive film. Then, one plate was placed in real-time thermal cycler (Roche LightCycler 480, Germany), and reaction was executed.

Table 2.10. Two-step cycling program executed in Roche LightCycler 480

Cycles	**Duration**	**Temperature**
1	10 min	95 ^{o}C
45	15 s	95 ^{o}C
	1 min	60 ^{o}C

CHAPTER 3

RESULTS AND DISCUSSION

3.1. XTT Cell Proliferation Assay on K562 Cells

3.1.1. Cytotoxic Effects of Resveratrol, C8:Ceramide, PDMP or SK-1 Inhibitor on Human K562 Chronic Myeloid Leukemia Cells

In order to examine antiproliferative effects of resveratrol, C8:ceramide, PDMP, or SK-1 inhibitor on human K562 cells, the cells were incubated with increasing concentrations of the agents for 72 h and XTT cell proliferation assay was conducted. The results showed that there were dose-dependent decreases in cell proliferation as compared to untreated controls. IC50 value of resveratrol (Figure 3.1), and C8:ceramide (Figure 3.2), or IC10 values of PDMP (Figure 3.3) and SK-1 inhibitor (Figure 3.4) were calculated from cell proliferation plots and were found to be 80-, 60-, 20-, and 7 μM, respectively.

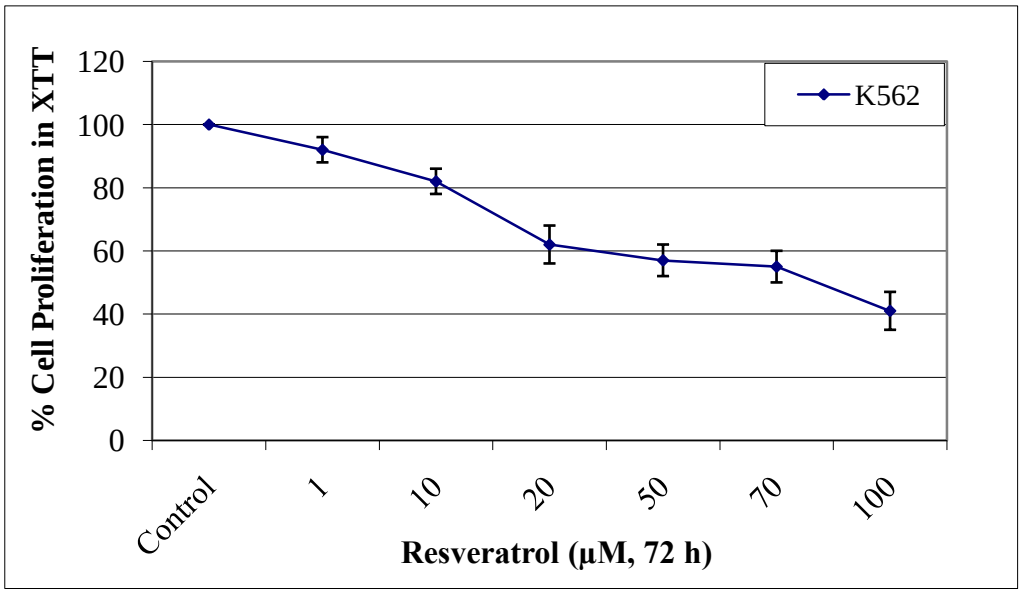

Figure 3.1. Cytotoxic effects of resveratrol on K562 cells

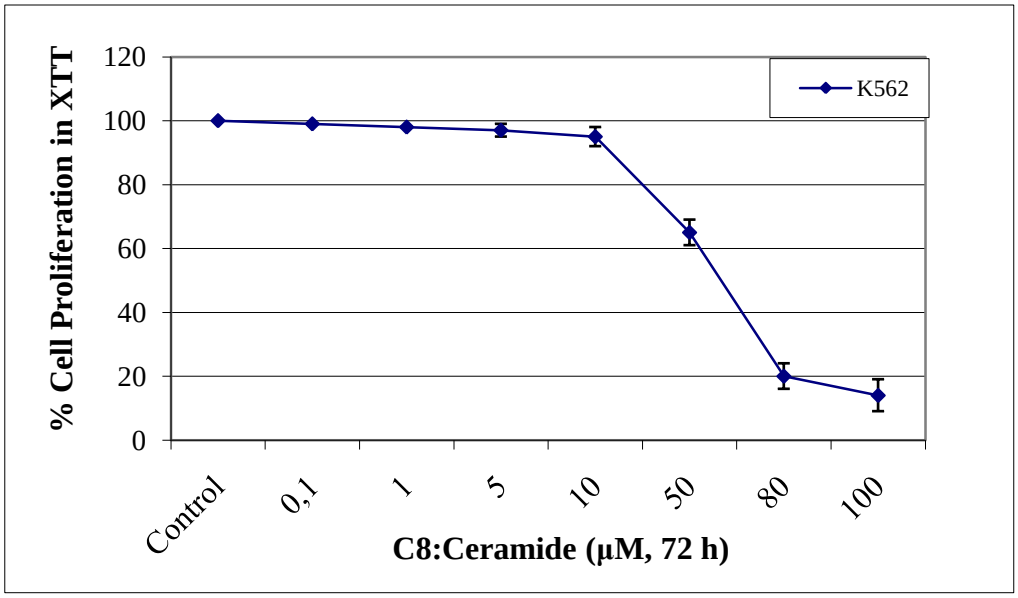

Figure 3.2. Cytotoxic effects of C8:Ceramide on K562 cells

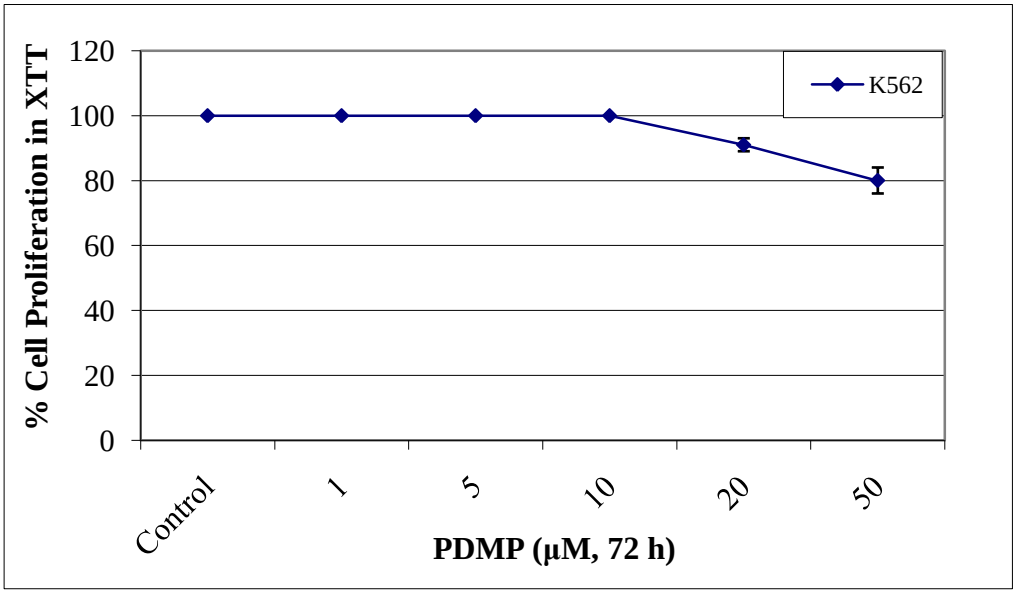

Figure 3.3. Cytotoxic effects of PDMP on K562 cells

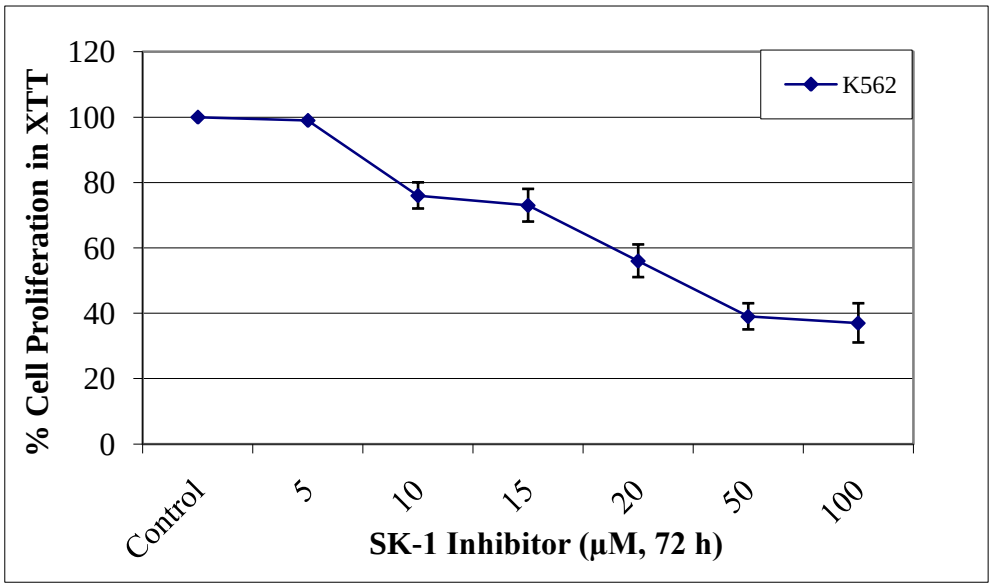

Figure 3.4. Cytotoxic effects of SK-1 inhibitor on K562 cells

3.1.2. Cytotoxic Effects of Resveratrol in Combination with C8:Ceramide, PDMP or SK-1 Inhibitor on K562 Cells

In order to examine the possible synergistic cytotoxicity, the cells were exposed to increasing concentrations of resveratrol from 1- to 100 µM together with 60 µM C8:ceramide, or 20 µM PDMP, or 7 µM SK-1 inhibitor. Combination of 1-, 50-, and 100 µM of resveratrol with 60 µM C8:ceramide decreased proliferation of K562 cells from 87-, 88-, and 89%, while resveratrol application by itself decreased cell proliferation 8-, 43-, and 59%, respectively (Figure 3.5). The same doses of resveratrol in combination with 20 µM PDMP or 7 µM SK1 inhibitor decreased cell proliferation 29-, 55-, and 68% or 23-, 54-, and 73%, respectively (Figure 3.5). This data demonstrate that resveratrol in combination with C8:ceramide, PDMP or SK-1 inhibitor has significant cytotoxic effect on human K562 cells.

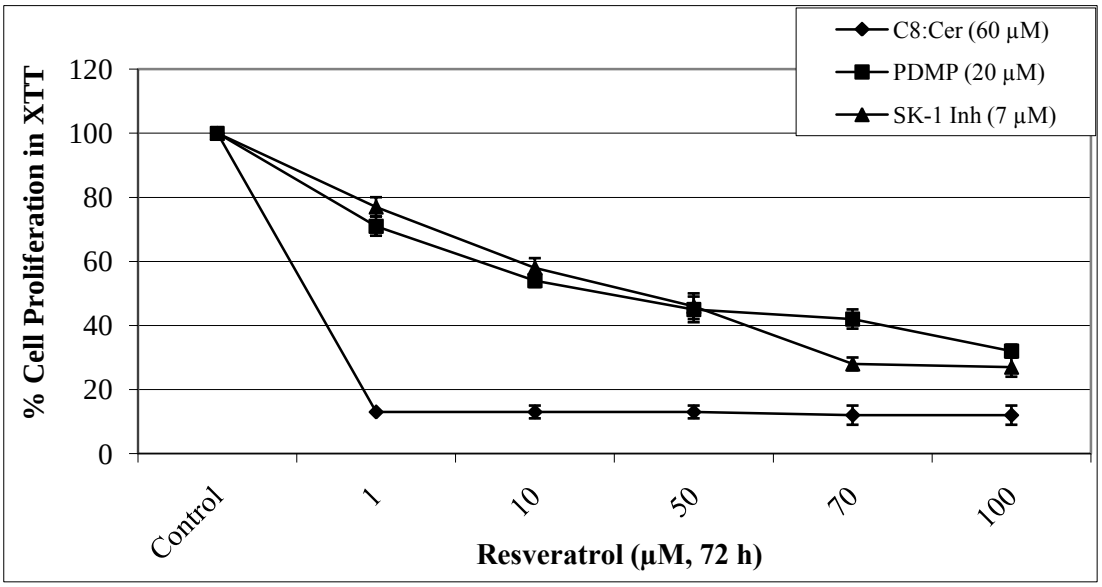

Figure 3.5. Cytotoxic effects of Resveratrol in combination with C8:ceramide, or PDMP, or SK-1 inhibitor on K562 cells

3.2. Evaluation of Apoptosis in K562 Cells

3.2.1. Synergistic Apoptotic Effects of Resveratrol Together with C8:Ceramide, PDMP or SK-1 Inhibitor on K562 Cells

In order to determine the mechanisms of resveratrol in the inhibition of cell proliferation, we examined whether resveratrol, C8:ceramide, PDMP or SK-1 inhibitor induces apoptosis in addition to examining the synergistic apoptotic effects. Apoptosis was evaluated by measuring the changes in caspase-3 enzyme activity and loss of mitochondrial membrane potential. As shown in Figure 3.6, there were 1.12-, and 1.21-fold increases in caspase-3 enzyme activity in response to 1-, and 10 μM resveratrol, respectively. Coadministration of 60 μM C8:ceramide or 20 μM PDMP or 7 μM SK-1 inhibitor with the same doses of resveratrol increased caspase-3 enzyme activity 2.11-, and 3.1- or 1.29-, and 1.87- or 2.36 and 2.49-fold, respectively. The same concentrations of C8:ceramide, PDMP and SK-1 inhibitor caused 1.44-, 1.22-, and 1.83-fold increases in enzyme activity, respectively.

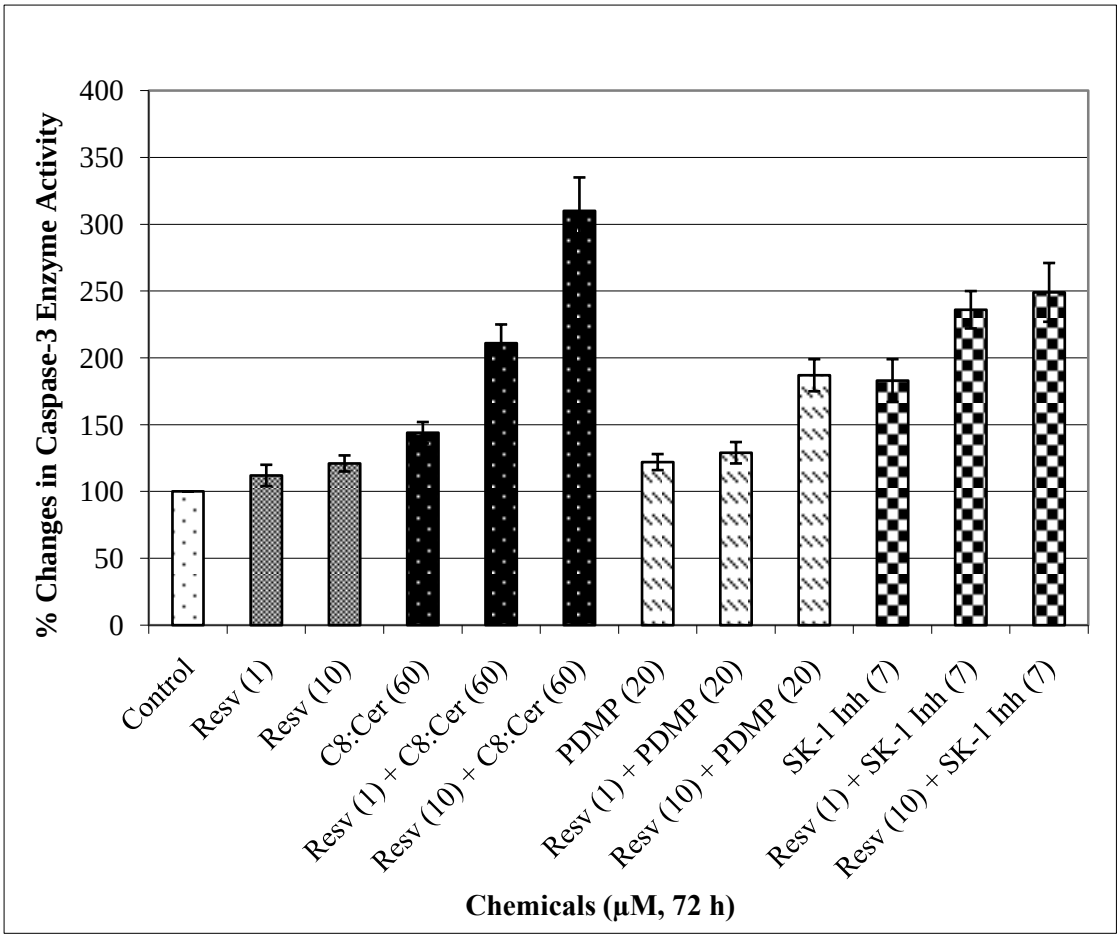

Figure 3.6. Changes in caspase-3 enzyme activity in response to resveratrol, C8:ceramide, PDMP, or SK-1 inhibitor alone, or their combinations

In order to confirm caspase-3 enzyme activity results and examine the roles of mitochondria in resveratrol-induced apoptosis, we also determined the loss of mitochondrial membrane potential. The results were in agreement with our previous data and have shown that all these chemicals induce apoptosis through inducing loss of MMP. Combination of resveratrol with C8:ceramide, PDMP or SK-1 inhibitor caused loss of MMP synergistically as compared to any agent alone and untreated control group (Figure 3.7). As shown in Figure 3.7, there were 1.46-, 1.58- and 88.98- or 1.32-, and 1.32-fold increases in changes of mitochondrial membrane potentials in response to 1-, and 10 μM resveratrol, and 60 μM C8:ceramide or 20 μM PDMP and 7 μM SK-1 inhibitor alone treated K562 cells, respectively, as compared to untreated controls. Coadministration of 60 μM C8:ceramide or 20 μM PDMP or 7 μM SK-1 inhibitor with the same doses of resveratrol increased the changes of mitochondrial membrane

potentials 205.0- and 274.87-, 2.77- and 2.92- or 3.6- and 4.06-fold, respectively, as compared to untreated controls.

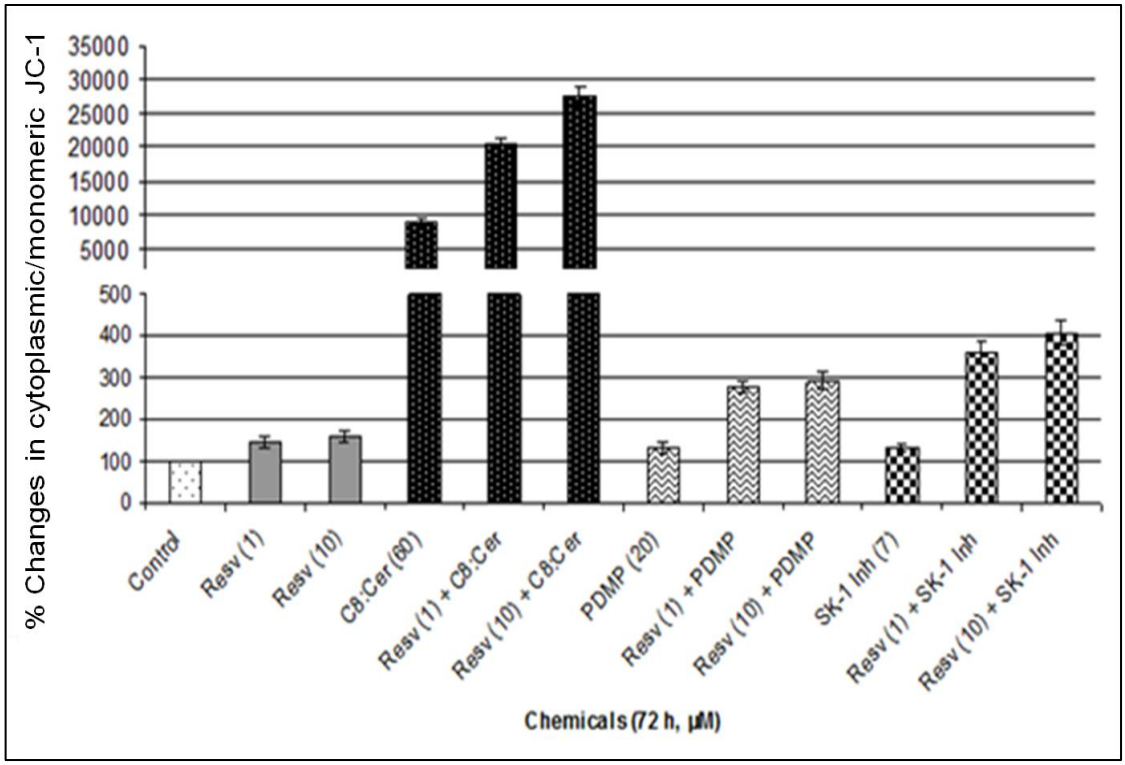

Figure 3.7. Changes in mitochondrial membrane potantial in response to resveratrol, C8:ceramide, PDMP, or SK-1 inhibitor alone, or their combinations

3.3. Estimation of PCR Results

3.3.1. Effects of Resveratrol on Ceramide-Metabolizing Genes in K562 Cells

In order to determine whether ceramide metabolizing genes are involved in resveratrol-induced apoptosis, K562 cells were treated with increasing concentrations of resveratrol (1-, 10-, 20-, and 50 µM) and expression levels of LASS2, LASS4, LASS5, LASS6, GCS, SK-1 and ß-actin were determined by RT-PCR. As shown in Figure 3.8 and table 3.1, resveratrol decreased expression levels of GCS and more significantly of SK-1 genes. On the contrary, expression levels of LASS2, and LASS5, and to a lesser

extent LASS4, and LASS6 were increased in response to resveratrol as compared to untreated controls, and normalized to ß-actin levels (Figure 3.8, Table 3.1).

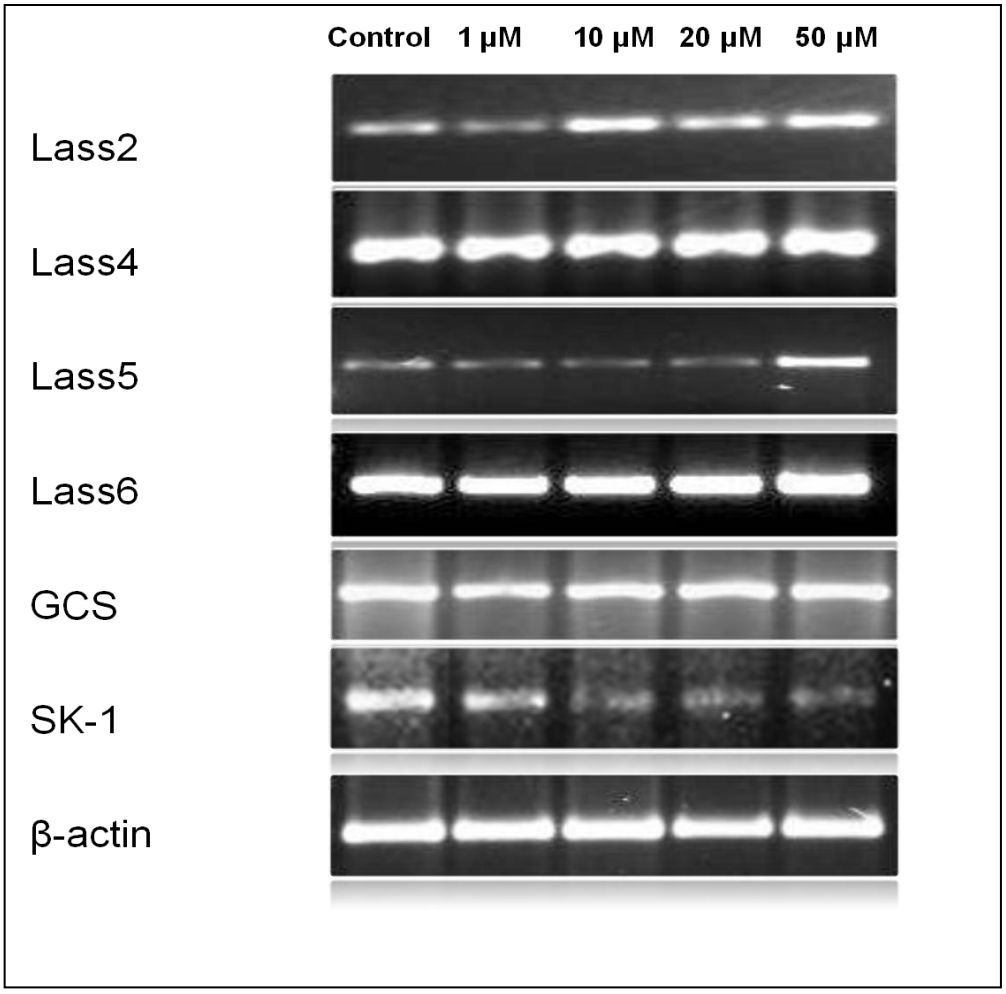

Figure 3.8. Expression levels of LASS2, -4, -5, -6, and GCS, SK-1 genes in response to increasing concentrations of resveratrol

Table 3.1. Quantification of expression levels of the genes in response to increasing concentrations of resveratrol

	GCS	SK-1	LASS2	LASS4	LASS5	LASS6
Control	100	100	100	100	100	100
1	83	69	91	101	73	105
10	82	41	174	102	57	109
20	92	43	182	121	77	129
50	83	35	191	121	188	131

3.4. Assessment of Human Cancer Pathway Finder PCR Array Results

In order to analyze the genes regulated by resveratrol-induced apoptosis, K562 cells were treated with increasing concentrations of resveratrol (10-, and 50 μM) and expression levels of 84 genes involved in cell cycle control and DNA damage repair, apoptosis and cell senescence, signal transduction molecules and transcription factors, adhesion, angiogenesis, and also invasion and metastasis were determined by Human Cancer Pathway Finder PCR Array. As shown in Figure 3.9 and 3.10, there were increases more than 4-fold in expression levels of 25 and 52 genes among 84 genes in response to 10- and 50 μM resveratrol. Moreover, there was a decrease less than 4-fold in only one gene (Myc) in response to 50 μM resveratrol (Figure 3.9 and 3.10). These genes changed significantly were found to be related with tumor suppressing, apoptosis, metastasis, and/or angiogenesis. As compared with untreated control group, 5 housekeeping genes, 3 reverse transcription controls, and 3 positive PCR control groups, expression levels of tumor suppressor SERPINB5, and apoptosis-triggering TNFRSF25, FAS, GZMA genes were found to be the most changed ones among these 84 genes.

Table 3.2. Plate layout of PCR Array

Array Layout											
AKT1 A01	ANGPT1 A02	ANGPT2 A03	APAF1 A04	ATM A05	BAD A06	BAX A07	BCL2 A08	BCL2L1 A09	BRCA1 A10	CASP8 A11	CCNE1 A12
CDC25A B01	CDK2 B02	CDK4 B03	CDKN1A B04	CDKN2A B05	CFLAR B06	CHEK2 B07	COL18A1 B08	E2F1 B09	ERBB2 B10	ETS2 B11	FAS B12
FGFR2 C01	FOS C02	GZMA C03	HTATIP2 C04	IFNA1 C05	IFNB1 C06	IGF1 C07	IL8 C08	ITGA1 C09	ITGA2 C10	ITGA3 C11	ITGA4 C12
ITGAV D01	ITGB1 D02	ITGB3 D03	ITGB5 D04	JUN D05	MAP2K1 D06	MCAM D07	MDM2 D08	MET D09	MMP1 D10	MMP2 D11	MMP9 D12
MTA1 E01	MTA2 E02	MTSS1 E03	MYC E04	NFKB1 E05	NFKBIA E06	NME1 E07	NME4 E08	PDGFA E09	PDGFB E10	PIK3R1 E11	PLAU E12
PLAUR F01	PNN F02	RAF1 F03	RB1 F04	S100A4 F05	SERPINB5 F06	SERPINE1 F07	SNCG F08	SYK F09	TEK F10	TERT F11	TGFB1 F12
TGFBR1 G01	THBS1 G02	TIMP1 G03	TIMP3 G04	TNF G05	TNFRSF10B G06	TNFRSF1A G07	TNFRSF25 G08	TP53 G09	TWIST1 G10	EPDR1 G11	VEGFA G12
B2M H01	HPRT1 H02	RPL13A H03	GAPDH H04	ACTB H05	HGDC H06	RTC H07	RTC H08	RTC H09	PPC H10	PPC H11	PPC H12

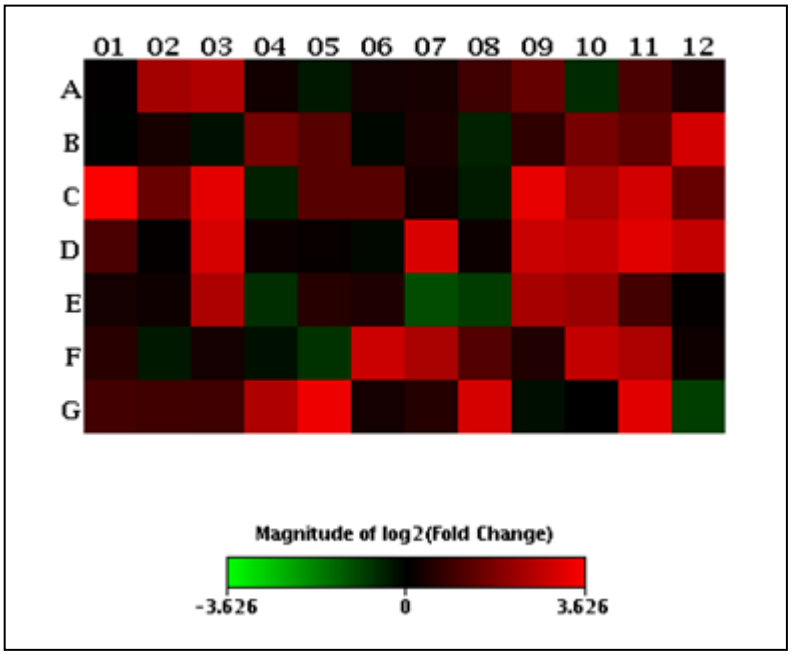

Figure 3.9. Changes in expression levels of 84 genes in response to 10 μM resveratrol

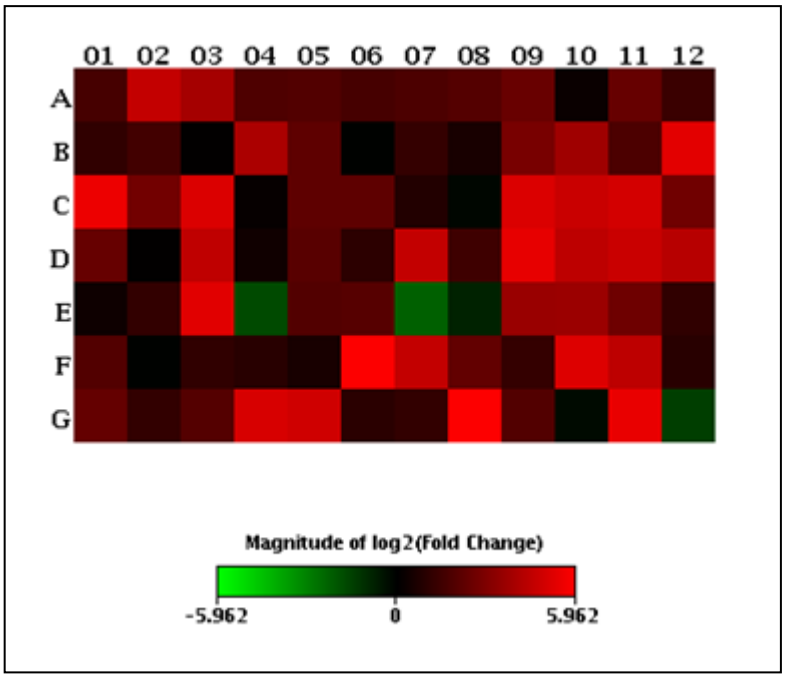

Figure 3.10. Changes in expression levels of 84 genes in response to 50 µM resveratrol

The tumor suppressor SERPINB5 gene expression level was increased 62.33-fold whereas the expression levels of tumor necrosis factor receptor (TNFRSF25), fibroblast growth factor receptor (FGFR), ependymin-related protein (EPDR1), proto-oncogenic mesenchymal epithelial transition (MET), FAS, and also metastasis suppressor gene (MTSS) were increased 61.35-, 47.83-, 44.33-, 43.24-, 40.82-, 39.47-fold, respectively. In addition, TEK endothelial tyrosine kinase, granzyme A, integrin α1 (ITGA1), TIMP metallopeptidase inhibitor 3, and also integrin α3 (ITGA3) genes upregulated by 36.74-, 35.86-, 35.62-, 33-, and 31.29-fold, respectively, as compared with the control groups in response to 50 µM resveratrol. In our study, the expression level of myc gene, an important oncogene in many cancer cells, was decreased 4-fold as compared with the control groups in response to 50 µM resveratrol.

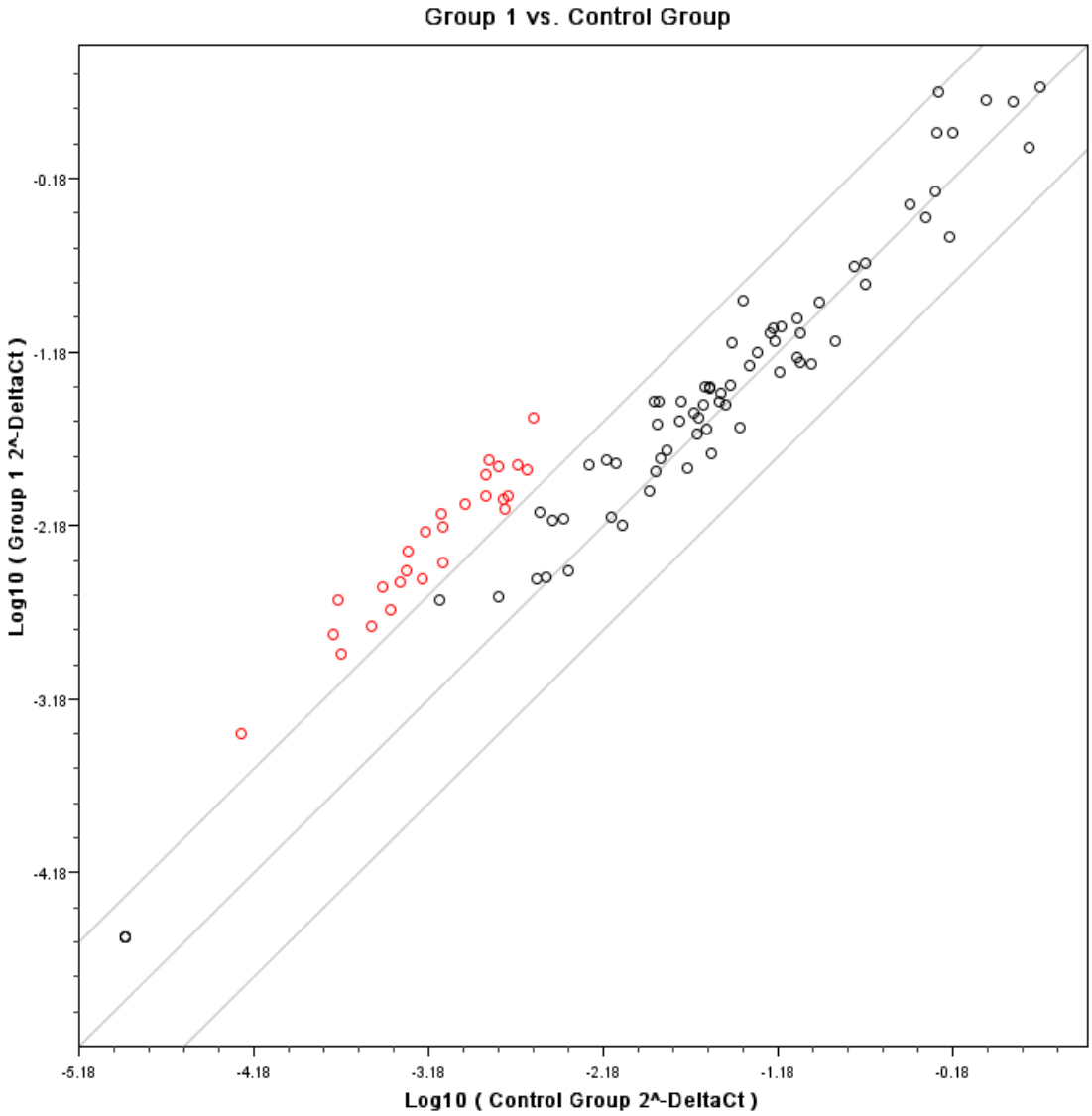

Figure 3.11. Upregulated and downregulated genes in response to 10 µM resveratrol (threshold > 4)

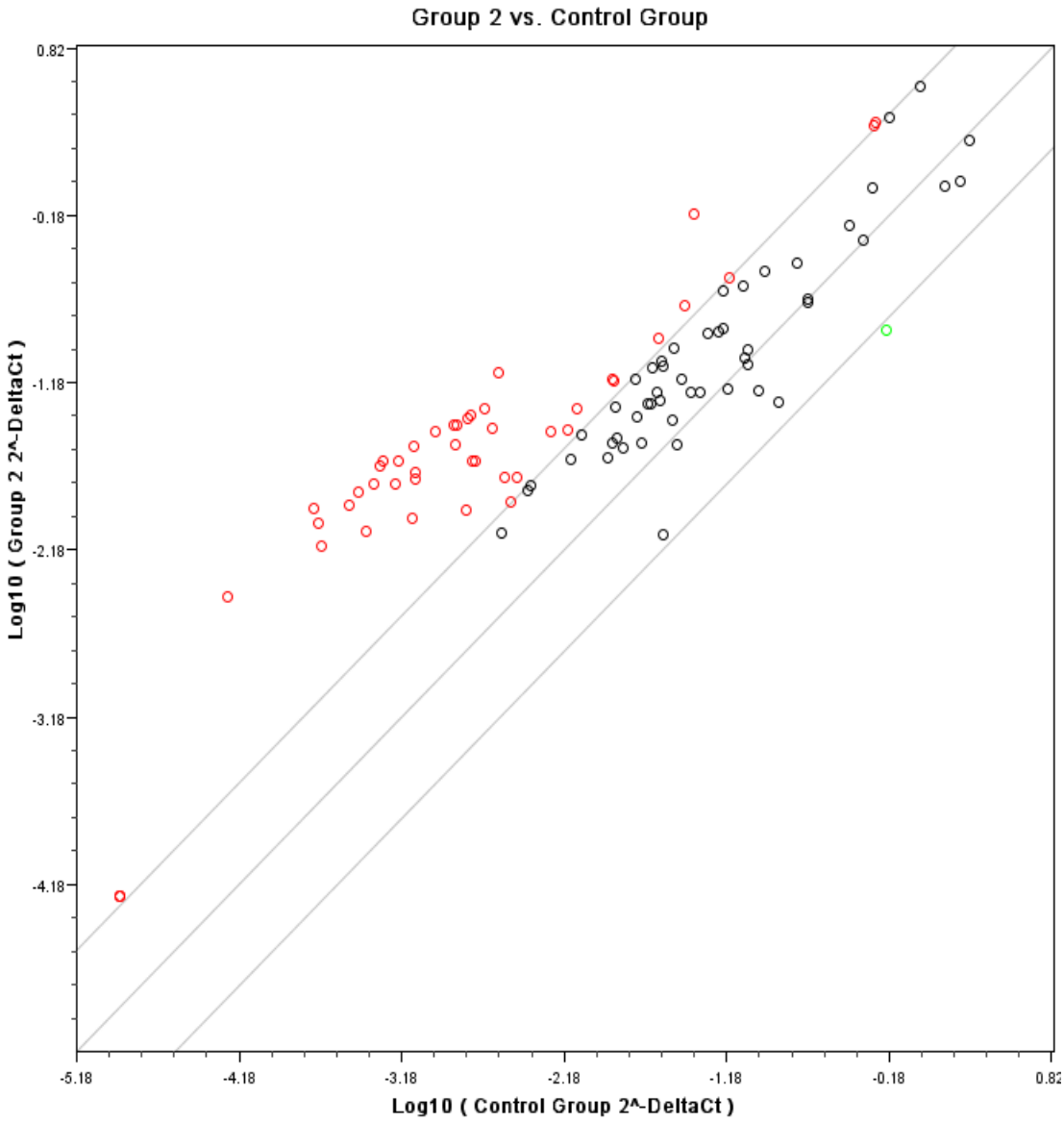

Figure 3.12. Upregulated and downregulated genes in response to 50 µM resveratrol (threshold > 4)

CHAPTER 4

CONCLUSION

Chronic myeloid leukemia incidence is increasing day by day, unfortunately, and new therapeutic approaches are being investigated in order to cure this disease. Although current therapeutic approaches are effective to some extent, resistance cases are developing over time. Biologycally active sphingolipids are thought to be responsible for the maintenance of this resistance balance. Ceramide, the key molecule of sphingolipid family, leads to induction of apoptosis whereas S1P and glucosylceramide lead to induction of cell proliferation, and even emergence of resistance (Baran, et al. 2007, Gouaze-Andersson, et al. 2007). In case of a change in the balance of ceramide/S1P to the direction of S1P pole, cancer cells develop resistance resulting in cell survival. Manipulations of this ceramide/S1P balance by biochemical and/or molecular approaches to the direction of ceramide pole may open a new road in cancer therapy due to the cytotoxic and apoptotic effects of ceramide on cancer cells (Saddoughi, et al. 2008, French, et al. 2006).

Ceramide-mediated apoptosis could be triggered by several different pathways. For instance, drugs used in clinic such as imatinib or docetaxel are known that they induce *de novo* ceramide generation, and also ceramide accumulation by inhibiting sphingomyelinases. Therefore, administration of these drugs results in the increase in intracellular ceramide levels. (Baran, et al. 2007).

In addition to ceramide, resveratrol is also considered to sensitize cancer cells against chemotherapeutic agents in recent years. Resveratrol attracts highly attention because of its potentials such as anticancer, anti-inflammatory, apoptotic, chemosensitizer, etc. (Park, et al. 2009, Scarlatti, et al. 2007, Liao, et al. 2005). Several groups revealed cytotoxic effects of resveratrol on many cancer cells (Sahin, et al. 2007, Harikumar and Aggarwal 2008, Chakraborty, et al. 2008). In addition, chemosensitizer effects of resveratrol in sensitive and even resistant CML cells were reported (Puissant, et al. 2008). Moreover, radiosensitizer effects of resveratrol were reported in prostate, and non-small cell lung cancer cells (Scarlatti, et al. 2007, Liao, et al. 2005). There are also some studies that reveal the effects of resveratrol on ceramide generation (Scarlatti,

et al. 2007). In a study, Paola Signorelli *et al* have reported that resveratrol causes autophagy in gastric cancer cells via increasing intracellular ceramide levels (Signorelli, et al. 2009). In one another study, Ersilia Dolfini *et al* have showed that resveratrol may prevent the growth of MDA-MB-231 breast cancer cells through inducing *de novo* ceramide generation (Dolfini, et al. 2007). Moreover, Filippo Minutolo *et al.* have reported that resveratrol causes the endogenous ceramide accumulation in prostate and breast cancer cells (Minutolo, et al. 2005). Another research group from Germany reported that resveratrol enhances the ceramide synthesis in colorectal carcinoma cells (Ulrich, et al. 2007). However the roles of bioactive sphingolipids in resveratrol induced apoptosis and the effects of resveratrol on ceramide metabolizing genes have never been examined previously in chronic myeloid leukemia. In our study, we tried to show the effects of resveratrol on ceramide metabolism of K562 cells. Since we know C8:ceramide could pass easily through the cellular membrane, and also it has an appropriate fluidity in the cell membrane (Tokudomea, et al. 2009), we treated K562 cells with C8:ceramide.

In this study, we examined the mechanisms of resveratrol-induced apoptosis by focusing on ceramide metabolizing genes in K562 CML cells. We also investigated whether we could increase the sensitivity of K562 cells to resveratrol via increasing intracellular concentrations of ceramides by biochemical approaches. For this purpose, we treated K562 cells by increasing concentrations of resveratrol; C8:ceramide, an important cell-permeable analogue of natural ceramides; PDMP, the key inhibitor of GCS; and SK-1 inhibitor, and determined their cytotoxic effects by XTT cell proliferation assay. Our results showed a dose-dependent decreases in cell proliferation in response to reveratrol and in response to increased intracellular concentrations of ceramides. Apoptosis in K562 cells exposed to increasing concentrations of resveratrol, C8:ceramide, PDMP, and SK-1 inhibitor was also evaluated by examining the changes in caspase-3 enzyme activity and the loss of the mitochondrial membrane potential. The results revealed that resveratrol, C8:ceramide, PDMP, and SK-1 inhibitor induce apoptosis through increasing caspase-3 enzyme activity and inducing loss of mitochondrial membrane potential. Increasing intracellular concentrations of ceramides by C8:ceramide was suggested to be an effective approach to inhibit cancer cell growth (Saddoughi, et al. 2008). In order to determine possible synergistic effects of resveratrol and C8:ceramide or PDMP or SK-1 inhibitor, we assessed combinational treatments of

resveratrol with C8:ceramide, the inducer of *de novo* generation of apoptotic ceramides, PDMP, the inhibitor of GCS, and SK-1 inhibitor. When we applied the IC50 value of C8:ceramide (60 μM), and IC10 values of PDMP (20 μM), or SK-1 inhibitor (7 μM) with increasing concentrations of resveratrol (1- to 100 μM), synergistic antiproliferative effects on K562 cells were observed as compared to any agent alone and untreated controls. 10 μM resveratrol by itself could repress only 18% of cell proliferation while the same concentration of resveratrol together with C8:ceramide, PDMP, or SK-1 inhibitor inhibited 87-, 46-, and 42% of cell proliferation, respectively.

The synergistic apoptotic effects of resveratrol in combination with C8:ceramide, PDMP, or SK-1 inhibitor were also observed by the changes in caspase-3 enzyme activity and loss of mitochondrial membrane potential. As we increased the intracellular concentrations of ceramides by application of exogenous ceramide or by inhibition of the conversion of ceramides to glucosylceramide or sphingosine-1-phosphate, the sensitivity of K562 cells to resveratrol increased synergistically. Our results were in agreement with the literature since Ogretmen *et al* clearly showed that both caspases and mitochondrial membrane potential are well-known downstream targets of ceramides (Ogretmen and Hannun 2004).

Increases in intracellular concentrations of ceramides can be determined by expression levels of LASS gene family, GCS and SK-1 genes, and also by direct detection of ceramides by LC-MS. In this study, we examined the expression levels of ceramide-metabolizing genes by RT-PCR in an effort to determine the increases in intracellular ceramide concentrations. RT-PCR results of this study have shown for the first time that resveratrol treatment may be resulted in the upregulation of LASS2, LASS4, LASS5, and LASS6 genes in a dose-dependent manner in human chronic myeloid leukemia K562 cells. Furthermore, there were significant decreases in the expression levels of GCS and SK-1 genes in response to increasing concentrations of resveratrol in K562 cells. These results suggest that resveratrol increases intracellular concentrations of ceramides via both induction of its *de novo* synthesis, and also inhibition of the conversion of endogenous apoptotic ceramide to anti-apoptotic glucosylceramide and sphingosine-1-phosphate molecules.

In addition, we also examined the expression levels of 84 genes related with apoptosis, cell cycle, angiogenesis, invasion and metastasis, etc. by human cancer pathway finder PCR array, in an effort to determine the effects of resveratrol on K562

cells. The results showed that in cancer cells, there is exactly like a war between the genes inhibitors of cell survival and the inducer ones. The fate of this war depends on the type of the genes upregulated. While we treated K562 cells with 50 μM resveratrol for 72 hours, expression levels of both some apoptotic genes and some cell survival genes were increased. However, the highest increase was determined in the expression level of maspin (SERPINB5) gene, which is a tumor suppressor. Tumor suppressive effects of maspin (MAmmary Serine Protease INhibitor) were reported in many cancer cells such as metastatic breast cancer cells (Zou, et al. 1994, Lele, et al. 2000), prostate cancer cells (Lockett, et al. 2006), and head and neck cancer cells (Marioni, et al. 2009). In cancer cells, maspin is generally inhibited via CpG methylation. However, some chemical agents, for example tamoxifen for breast cancer, and other demethylating agents increase maspin expression in cancer cells (Khalkhali-Ellis 2006). Maspin has been reported as an inhibitor of invasion, metastasis, and angiogenesis (Chen and Yates 2006). PCR array results of our study have shown that expression level of maspin gene increases in response to elevated concentrations (10- and 50 μM) of resveratrol. The second gene among highly upregulated ones in response to 50 μM resveratrol was TNFRSF25, which is known as apoptosis-mediating receptor. This receptor interacts with an adapter molecule, known as TRADD. TNFRSF25 mediates activation of NFκB and induces apoptosis.

However, the third highly upregulated gene in K562 cells in response to 50 μM resveratrol was FGFR2 in the results of the PCR array. Fibroblast growth factors (FGFs) are known as mitogenic signaling molecules playing roles in angiogenesis, wound healing, cell migration, and embryonic development. FGFRs are transmembrane catalytic receptors that have intracellular tyrosine kinase activity. Binding of FGF-FGFR initiates FGFR dimerization. This dimerization allows the cytoplasmic kinase domains to transphosphorylate tyrosine residues and become activated. It is known that FGFR upregulation may lead to cell transformation and cancer. Moreover, the expression level of only one gene, myc oncogene, was decreased in response to 50 μM resveratrol. This downregulation of myc by resveratrol is consistent with the literature (Zhang, et al. 2006).

In conclusion, our overall results showed that there were synergistic apoptotic effects of the combinations of resveratrol with C8:ceramide or PDMP or SK-1 inhibitor on K562 CML cells, and also these results confirmed the cytotoxic effects of resveratrol

on these cells. More importantly, we have shown for the first time that resveratrol triggers apoptosis through increasing expression levels of LASS genes and inhibiting the expression levels of GCS and SK-1 in human K562 CML cell lines. In addition, we also investigated for the first time that resveratrol upregulates the maspin expression, which leads to tumor suppression. Therefore, when we compare the PCR array results with the results of the apoptotic effects of resveratrol, we can suggest that resveratrol contributes some of the anti-survival genes and allows them to be victors of the war. Taking together, all these results may open the way of using resveratrol and ceramides as a novel therapeutic or supportive agents in CML therapy.

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