INCREASING CHEMOSENSITIVITY OF CHRONIC MYELOID LEUKEMIA CELLS TO DASATINIB BY TARGETING BIOACTIVE SPHINGOLIPIDS

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by

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ABSTRACT

INCREASING CHEMOSENSITIVITY OF CHRONIC MYELOID LEUKEMIA CELLS TO DASATINIB BY TARGETING BIOACTIVE SPHINGOLIPIDS

Sphingolipids are bioeffector molecules which control various aspects of cell growth, proliferation, apoptosis, senescence, and drug resistance. Ceramides, the central molecule of sphingolipid metabolism, are inducer of apoptosis and inhibitors of proliferation. Sphingosine-1-phosphate (S1P) and glucosyl ceramide, converted from ceramides by sphingosine kinase-1 (SK1) and glucosyl ceramide synthase (GCS) enzymes respectively, inhibit apoptosis, induce cell proliferation and develop resistance to chemotherapeutic drugs. In this study, we examined the therapeutic potentials of bioactive sphingolipids in chronic myeloid leukemia (CML) by itself and in combination with dasatinib in addition to investigate the roles of ceramide metabolising genes in dasatinib induced apoptosis.

Our results demonstrated that application of ceramide analogs and inhibitors of ceramide clearance enzymes decreased cell proliferation and induced apoptosis. On the other hand, targeting bioactive sphingolipids towards generation/accumulation of ceramides increased apoptotic effects of dasatinib synergistically. It was also shown for the first time by this study that dasatinib induces apoptosis through downregulating expression levels of GCS, and SK-1 genes and upregulating expression levels of LASS1, -2, -4, -5, and -6 in K562 cells. However, in Meg-01 cells, dasatinib downregulates expression levels of apoptotic LASS genes.

Increasing endogenous ceramides through exogenous ceramide analogues or mimetics and decreasing prosurvival lipids, S1P and GC, can open the way of more effective treatment of CML.

ÖZET

BIOAKTIF SFINGOLIPIDLERIN HEDEFLENMESI ILE KRONIK MYELOID LÖSEMI HÜCRELERININ DASATINIB DUYARLILIKLARININ ARTTIRILMASI

Kronik miyeloid lösemi (KML) 9. ve 22. kromozomlar arasında gerçekleşen karşılıklı translokasyon sonucu oluşan hematolojik bir kanserdir. KML tedavisinde kullanılan başlıca ilaçlar BCR/ABL tirozin kinaz inhibitörleridir. Dasatinib, KML tedavisinde kullanılan BCR/ABL ve SFK kinaz inhibitörüdür. Seramidler biyoaktif sfingolipid olup farklılaşma, yaşlanma, proliferasyon, ve apoptoz gibi birçok önemli biyolojik olayda merkezi roller üstlenmektedir. Bu çalışmada hücre içi seramid sentezinin ve birikiminin biyokimyasal yollarla arttırılması ile seramid-dasatinib kombinasyonunun K562 ve MEG–01 KML hücreleri üzerine olası sinerjistik etkileri incelenmiştir.

Dasatinibin yalnız ve C:8 seramid veya apoptotik seramidi antiapoptotik glukozil seramide dönüştüren glukozil seramid sentaz (GSS) enzim inhibitörü PDMP veya apoptotik seramidi antiapoptotik sfingozin-1-fosfata dönüştüren sfingozin kinaz-1 (SK-1) enzim inhibitörü ile kombinasyonunun sitotoksik etkileri, kaspaz-3 enzim aktivitesi ve mitokondri zar potansiyelindeki değişimler gösterilmiştir. Programlı hücre ölümlerini en önemli belirteçlerinden olan mitokondri zar potansiyelindeki azalış ve kaspaz-3 enzim aktivitesindeki artışlar da seramid metabolizmasının hücre içi seramid birikimini arttıracak yönde hedeflenmesinin dasatinibin apoptotik etkisini önemli ölçüde arttırdığını göstermiştir. Dasatinibin artan dozlarına maruz bırakılan K562 ve MEG–01 hücrelerindeki seramid metabolizması genlerinin ekspresyon düzeylerine bakıldığında SK–1 geninin ekspresyonu her iki hücre hattında da baskılanırken GSS geni K562 hücrelerinde artmakta MEG–01 hücrelerinde ise baskılanmaktadır. Öte yandan seramidin sentezlenmesinden sorumlu olan LASS 1, -2, -4, -5 ve -6 genlerinin ekspresyonunun K562 hücrelerinde artış, Meg01 hücrelerinde is bir azalma gözlenmiştir. Sonuç olarak, bu çalışma ile ilk defa K562 ve MEG-01 hücrelerinde seramid metabolize eden genlerin hücre içi seramid miktarlarını arttıracak şekilde hedeflenmesi ile dasatinibin sitotoksik ve apoptotik etkilerinin önemli ölçüde arttırdığı gösterilmiştir.

To my family...

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CHAPTER 1

INTRODUCTION

1.1. Chronic Myeloid Leukemia

Chronic Myeloid leukemia is a hematopoietic stem cell disorder that causes uncontrolled cell proliferation of white blood cells (Druker, et al. 2001). According to the statistics of 2009 CML stands for approximately 10-15% of all leukemias. The average risk of getting CML in a person's life time is about 1 in 625 with an average diagnosis age of 60-65. This disease is more commonly seen in men when we compare to women with percentage of 56- 44%, respectively. CML is composed of three stages which are chronic phase, accelerated phase and blast crisis. Chronic phase is very early stages of CML and is very hard to diagnose except routine blood tests. Immature white blood cells are very low (5% or fewer) in the blood and bone marrow. The second phase is accelerated phase in which the levels of immature white blood cells are higher than chronic phase at about 5-30 %. In this phase some symptoms are seen such as weigth loss, poor apetite. The last and the most serious phase of CML is blast crisis. Many of the patients are diagnosed in this phase. There are mostly immature white blood cells in the blood and bone marrow (more than 30%). Some serious symptoms are observed such as such as anemia, tiredness, fever, an enlarged spleen and recurring infections are typical (Aguilera, et al. 2009).

Chronic myeloid leukemia is a very important disease for the investigation of cancer with a molecular perspective. It is seen as a model for other cancer types in many ways. CML is explained as a multistep developed disease with chronic phase, accelerated phase and blast crisis. Its cytogenetic definition is explained as a translocation and to overcome this mutation targeted therapies are come up in CML. Drug development is gained much more importance with the targeted therapy perspective.

CML results from a reciprocal translocation between the chromosome 9 which contains Abelson Kinase domain and the chromosome 22 which contains Breakpoint Cluster Region t(9;22) (Shah 2007). As a result of this translocation the regenerated

chromosome is called Philedelphia Chromosome (Ph). Approximately % 95 of CML patients has this Ph chromosome. This Ph chromosome encodes for an oncoprotein called BCR/ABL. This oncoprotein has a tyrosine kinase activity which phosohorylates a number of substrates and causes increased mitogenic activation, uncontrolled cell proliferation and decreased apoptosis (Deininger, et al. 2000). This oncoprotein has two binding sites on it, an ATP binding site and a substrate binding site. The oncoprotein has an inactive state when ATP is not bound but with the binding of ATP, oncoprotein switches into an active state (Peng, et al. 2004). Since the protein has a tyrosine kinase activity, it transfers phosphate groups from ATP to a number of subtrates. When the substrates are phosphorylated they become active and binds to effector proteins.

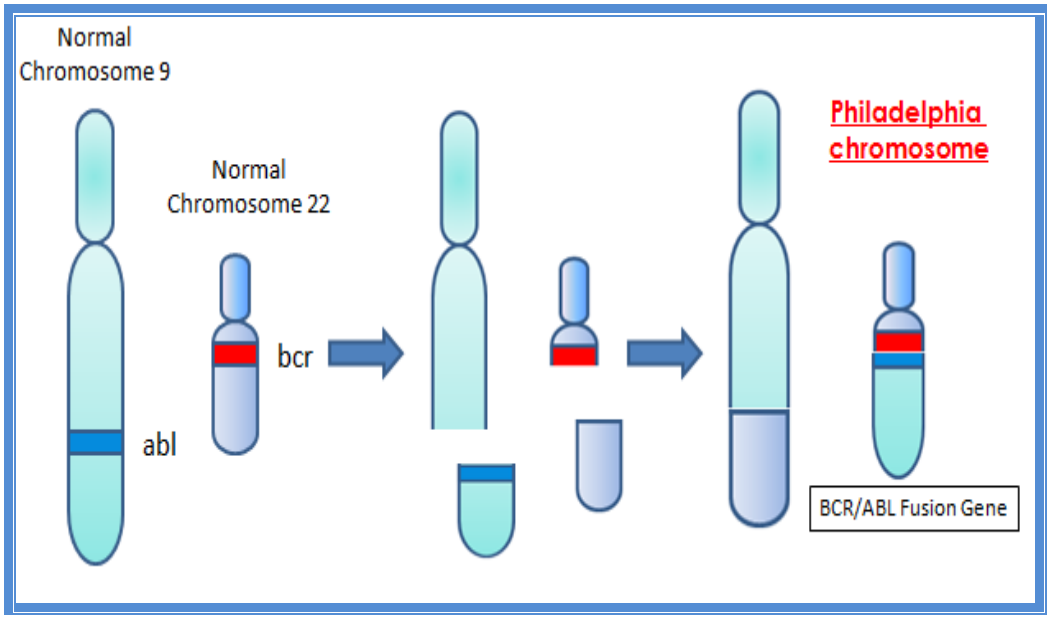


Figure 1.1. The illustration of the translocation between chromosome 9 and 22 which in turn generates BCR/ABL oncogene (Adapted from Kalidas, et al. 2001)

There are many mechanisms that the CML transforms into blast crisis. Although these mechanisms are partly understood, we can classify them as follows (Shtivelman, et al. 1985).

-BCR-ABL1 expression
-Arrest of differentiation
-Genomic instability and DNA repair
-Additional chromosomal abnormalities

-Inactivation of tumor suppressor genes

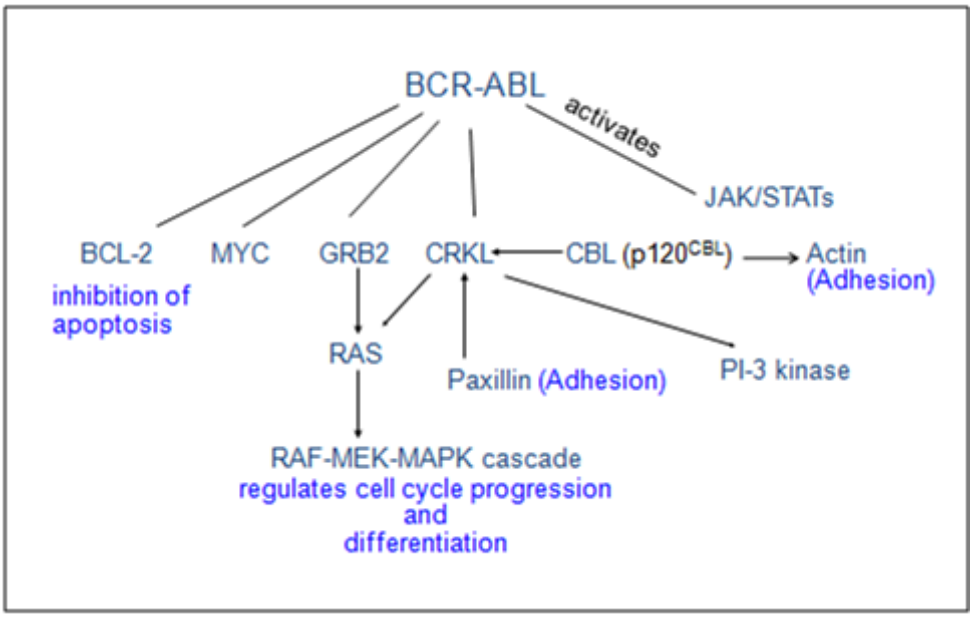


Figure 1.2. Molecular pathways that BCR/ABL effects
(Adapted from Pasternak, et al. 1998)

1.2. Molecular Pathways of CML

1.2.1. c-myc

c-myc is a proto-oncogene that encodes for c-myc protein which is a transcription factor. The vital importance of this protein is to promote cell cycle progression. However this protein has key roles for not only cell cycle but also differentiation, cell death and angiogenesis. During development the expression of c-myc is present almost in every growing tissue (Cole and McMahon 1999). In adult body c-myc expression is observed in highly dividing and rapid growing tissues such as epithelium of skin. Elevated and deregulated expression pattern of c-myc is observed in various types of cancer and sometimes c-myc can be a diagnostic marker for some cancer types. The cancer types that are related to c-myc are breast, lung, colon, cervical, osteocarcinoma, melanoma and myeloid leukemias (Dang 1999). Sawyers et al showed that c-myc activity is demanded for Bcr/Abl transformation but the exact mechanism is not known (Sawyers 1992). Fang et al revealed that the Bcr/Abl caused survival is regulated at transcriptional level via JAK2/PI3K signaling pathway that is activated by

c-myc transcription factor. In this study shRNA mediated silencing of c-myc completely eliminated the colony formation of K562 cells on semi-solid culture system (Fang, 2009). Okuda et al showed that the expression c-myc reduces when the C-terminal of Abl is deleted. This supports the finding that C-terminal Abl is required for the Jak2 binding.In another study they clarify the downstream signaling pathways of p210 Bcr/Abl that is regulated by c-myc mRNA in hematopoietic cells. They studied in murine myeloid cell lines which have a stable p210 Bcr/Abl expression. In these cells an active expression pattern of c-myc is observed and they observed that promoter in p210 BCR-ABL transformed cells transcription is initiated from P2 promoter. In p210 Bcr/Abl transformed cell the expression of E2F1 protein is required for the E2F binding to the P2 promoter site of c-myc. As a result of these relationships, E2F components of the retinoblastoma-cyclin pathway are directed to the transformation into p210 Bcr/Abl to c-myc transcription, which is crucial for the transformation (Okuda, et al. 1998).

1.2.2. Phosphatidyl Inositol 3 Kinase (PI-3 Kinase)

Phosphoinositide 3-kinases are a family of enzymes that have important roles in cell growth, proliferation, differentiation, motility, survival. It has also very important and vital role in cancer (Hickey and Cotter 2006). These enzymes are characterized by the lipid kinase family. They are activated through the G-coupled protein and they phosphorylate the inositol ring of phosphotydyl inositol-3,4-diphosphate (PIP2) that in turn generates phosphotydyl inositol-3,4,5-triphosphate. PIP3 is important in inducing survival effects by regulating downstream pathways that include AKT and PKB. In many cancer cell types deregulated PI3K is critical by either downregulation of tumor suppressor PTEN or upregulation of oncogenic AKT pathways and also mTOR (Kim, et al. 2005). Bcr/Abl tyrosine kinase activates this pathway by the binding of Bcr/Abl to p85 that is the regulatory subunit of PI3K. It has been showed that PI3K/Akt pathway is crucial for Bcr/Abl leukemogenesis that is proved by the specific inhibitor of PI3K. This inhibitor decreased the BCR/ABL dependent colony formation of murine marrow cells at very low concentrations. Skorsky et al demonstrated that the mouse marrow cells that are lack of SH2 domain of Bcr/Abl are unable to upregulate the expression of c-myc and Bcl-2 when they infect the mouse retrovirally. In conclusion their findings

prove that SH2 domain of Bcr/Abl is very vital in PI3K activation and this can be a target to inhibit the Bcr/Abl leukemogenesis (Skorski, et al. 1997).

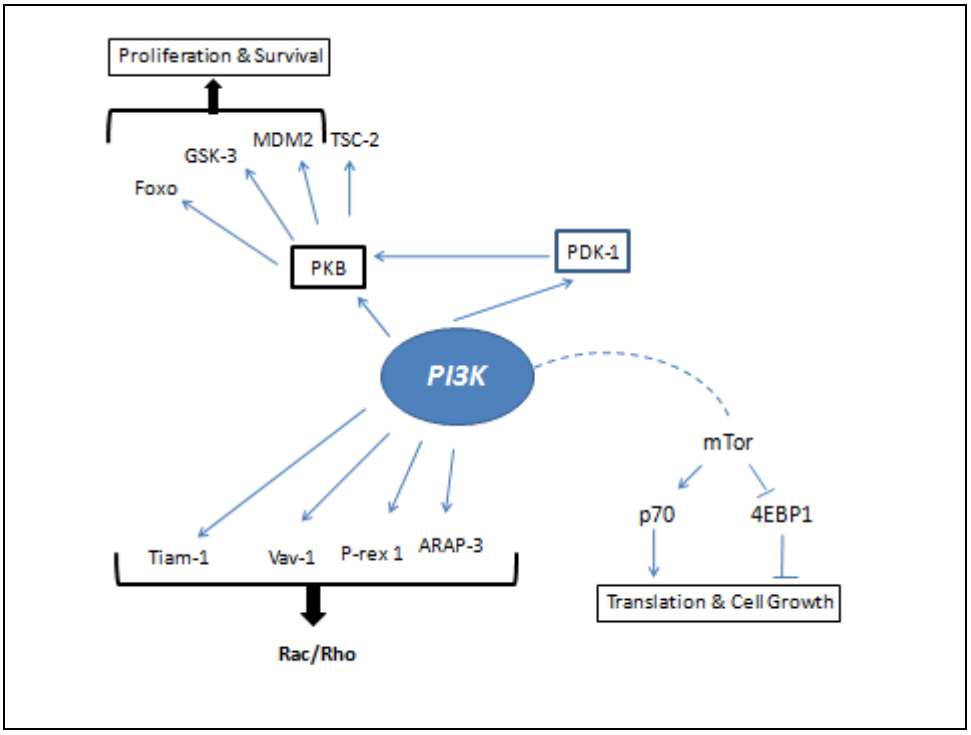


Figure 1.3. Signalling pathways of phosphotidyl inositole-3 kinase
(Adapted from: Workman, et al. 2006)

1.2.3. Bcl-2

Bcl-2 is a proto-oncogene that is located on chromosome 18. The product of this oncogene is an integral membrane protein that can be located in endoplasmic reticulum membrane, nuclear envelope and in the outer membrane of the mitochondria. This gene was first discovered in B-cell leukemia (Bcl) which takes its name from. The formation of this oncogene is generated from a reciprocal translocation between chromosome 18 and 14 t(14;18). This protein has a very vital importance in regulation of cell death because it is crucial for cytochrome c release (Pelengaris and Khan 2009).

Bcl-2 subfamily is known to be pro-survival and this class of protein includes Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1 while Bax subfamily is pro-apoptotic and includes Bax, Bak and Bok. The third class of proteins is again pro-apoptotic and they are BH3 subfamily which includes Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL.

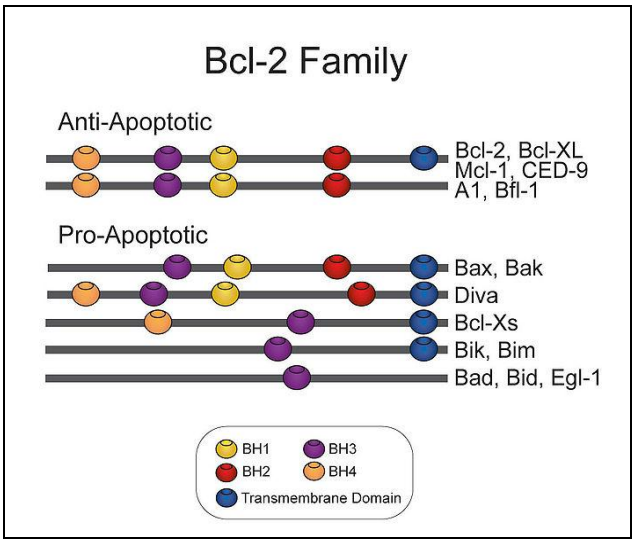


Figure 1.4. Pro-apoptotic and anti-apoptotic subfamilies of BCL2 family proteins (Source: Chao and Korsmeyer 1998)

Under stress conditions apoptosis can be stopped by antiapoptotic Bcl-2 family proteins via mitochondrial membrane by blocking the release of some apoptotic factors such as cytochrome c, SMAC/Diablo and AIF since apoptosis is initiated by the formation of apoptosome complex which is composed of APAF-1, ATP, cytochrome c and pro-caspase-9 molecules (Cirinna`, et al. 2000).

Jaiswal et al demonstrated that in half of the mice in their study showed an increased expression of Bcr/Abl in myelomonocytic cells but not in hematopoietic stem cells which synergizes with enhanced expression levels of Bcl-2 which causes the induction of blast crisis in those mice. They suppose that the cooperative action between increased Bcl-2 expression and decreased Bcr/Abl expression may be the key event of transformation into blast crisis (Jaiswal, et al. 2003). In another study the generated Bcr/Abl oncogene inhibit apoptosis through inducing the expression levels of Bcl-2. Moreover, the Bcr/Abl expressing cells return back to condition in which they show non-tumorigenicity when they suppress Bcl-2 expression. These results help to explain the ability of BCR-ABL oncogenes to synergize with c-myc in cell transformation (Gracia and Grütz 1995).

1.2.4 JAK/ STAT

The JAK/STAT pathway consist of three families of proteins, Janus Kinases (JAKs), signal transducers and activators of transcription (STATs) and their endogenous inhibitors which are SOCS family. This pathway includes the signals from membrane via cytokines to nucleus (Danial, et al. 1995). Activation of the JAK kinases leads to the activation of STAT transcription factors. JAKs are large tyrosine kinases with the molecular weight between 120-140 kDa. In mammals there are four JAKs identified; JAK1, JAK2, JAK3 and TYK2. However the STAT proteins consist of seven proteins; STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, STAT6. These proteins are relatively have lower molecular weights from 75-95 kDa (Pelengaris and Khan 2009).

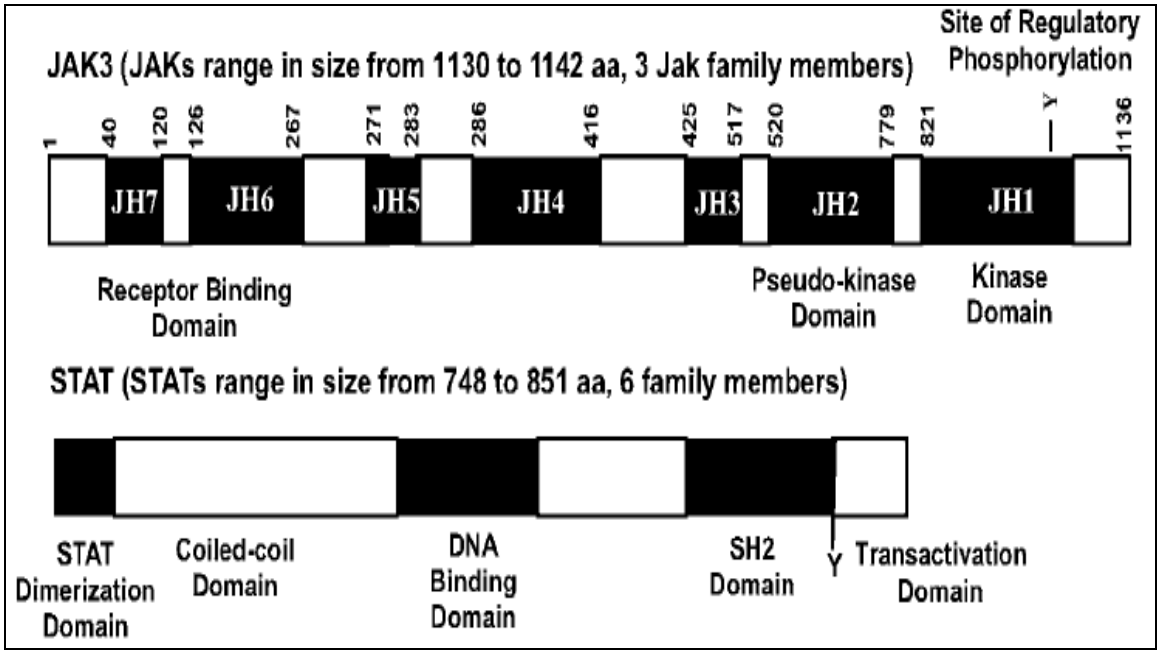


Figure 1.5 Molecular structures of JAK and STAT proteins
(Source: Steelman, et al. 2004)

With the binding of cytokines to their receptors on cell membrane generates the homodimer or heterodimer formation of JAK proteins. This receptor aggregation mediates coming closer of them and transphosphorilation of the receptors occur through JAK kinase activity (Rawlings, et al. 2004). One of the studies that prove transphosphorilation is that IL-2 cannot activate JAK1 in the absence of JAK3. These studies showed that this event is very important for the activation of the receptors.

STAT overexpression is frequently seen in many cancer types. STAT3 is accepted as an oncogene and this make us think that other STAT proteins may be oncogenic. Groot et al observed that STAT5 is an important player in transformation of Bcr/Abl oncogene. They examined the phosphorilation levels of STAT5 proteins (STAT5A and STAT5B) in K562 CML cell line and they showed that they are constantly phosphorilated from tyrosine residues and they become active transcriptionally (Groot, et al. 1999).

1.3. Transformation into Blast Crisis

The cytogenetic marker of chronic myeloid leukemia is the existence of Ph chromosome. This chromosome is appear as a translocation between chromosome 9 and 22. The c-abl gene which is a protooncogene is located on the terminal part of the chromosome 9 wtih a 145 kD molecular weight. The breakpoint on chromosome 9 can take place within a relatively large region. In contrast to this large breakpoint region on chromosome 9, the bcr gene on chromosome 22 intensified into 6 kb region. The translocation takes the oncogene c-abl to its new location that is on Ph chromosome. As a result of this event, a new fusion gene consist of c-abl on the 3'end and bcr gene on the 5' end is generated. The transcription of this gene generates a 8.5 kb mRNA. The mass of the protein product of this specific mRNA can be variable. They can be 190 kD, 210 kD and 230 kD in weight. Although p190 and p230 BCR/ABL oncoproteins can be present in CML, the most abundant one is p210. This p210 protein is necessary and also adequate for CML disease progression. (Calabretta and Perrotti 2004)

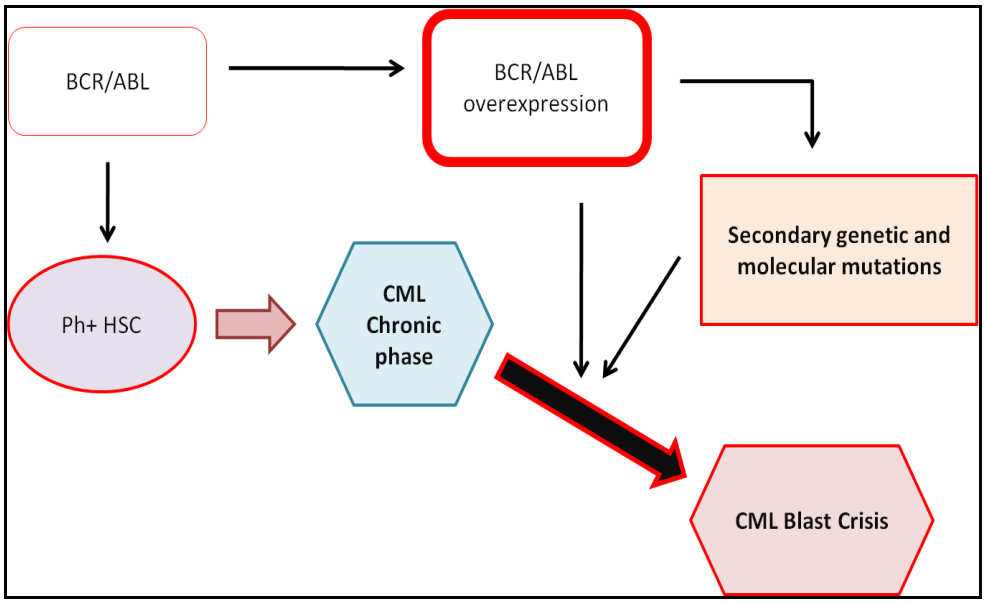


Figure 1.6. Transformation into blast crisis
(Source: Calabretta and Perrotti 2004)

Blast crisis is an inescapable end of CML if no treatment is executed. Progression into blast crisis is also seen in different lineages. While in myeloid lineages %60 of cases transform into blast crisis, in lymphoid and erythroid lineages %16-30, %10 of cases turn into blast crisis respectively. Progression of the disease is divided into three phases. The chronic phase progress slowly while balst crisis is not. Blast crisis is characterized as increased aggressive proliferation of immature hematopoietic cells that are arrested at an early state of differentiation (Pullarkat, et al. 2008). Besides the translocation of chromosome 9 and 22 causes genomic rearrangements and recruites secondary chromosomal abnormalities. Most of these genetic abnormalities result from mutations in p53 tumor suppressor gene and vice versa, which means that the genetic alterations have a direct or indirect effect on p53 (Calabretta and Perrotti 2004).

1.3.1. Secondary Mutations in CML

1.3.1.1. Chromosome 8 Trisomy

Myc gene is located on 8q24 and because of the position of this gene on chromosome 8, an aberration on this chromosome would cause an overexpression of this gene. Blick et al showed that however, a c-myc overexpression is observed in CML

blast crisis there is no definite relationship between chromosome 8 trisomy and c-myc overexpression (Blick, et al. 1987). On the other hand there are strong evidences that there might be such a correlation between these two happenings. Sawyer and his colleagues showed that expression of dominant-negative c-Myc molecules suppressed BCR/ABL-dependent transformation, and Skorski et al observed that inhibition of colony formation in vitro and in vivo leukemiagenesis is observed when they treat the CML-BC cells with c-Myc antisense oligodeoxynucleotides (Sawyer, et al. 1992, Skorski, et al. 1996).

1.3.1.2. Isochromosome i (17q)

Isochromosome i(17q) is associated with the loss of the short arm (17p) and duplication of the long arm (17q) which causes the abnormality (Schutte, et al. 1993). Since p53 gene is located on the short arm of chromosome 17, isochromosome 17q is known to be the most common precedent in cancer. This rearrangement leads to tumor progression and initiation. However, p53 mutations were not found in CML cases with the i(17q), raising the possibility that the relevant pathogenetic mechanism in CML-BC patients with the i(17q) abnormality is loss of function of yet unidentified genes (Fioretos, et al. 1999).

1.4. Targeted Therapies

1.4.1. Imatinib

Targeted therapies for chronic myeloid leukemia is a very important and vital step for the development of molecular medicine. CML is the most extensively studied hematologic malignancy and its mechanism of action is highlighted completely. Imatinib (Glivec, Novartis, Basel, Switzerland) is the first tyrosine kinase inhibitor that is developed and approved for CML and it is the gold standart for treatment (Michor, et al. 2005). Imatinib is an analogue of ATP and acts as a competitive inhibitor at the ATP binding site. When imatinib binds to ATP binding site just like ATP, it prevents the

phosphorilation of substrate molecule and by this way, inhibits the downstream signalling of BCR/ABL (Skobridis, et al. 2010).

The mutations that effects the activity of imatinib can occur in more than one site. They can be ATP bindind site which is named as P-loop, imatinib binding site, activation loop that controls the kinase activity and the catalytic domain. The mutations that exist in P-loop are reported to be the worse prognosis. Detection of these mutations in early stage of disease provides a great advantage for patient because this makes a well-timed treatment for prevention of disease progression (Ramirez and DiParsio 2008).

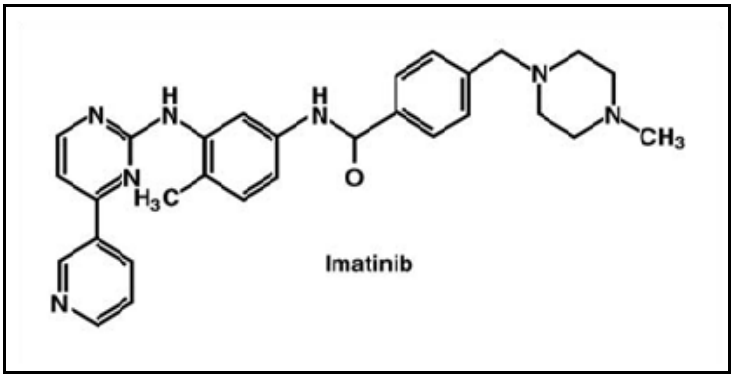


Figure 1.7 Chemical structure of Imatinib
(Source: Kantarjian, et al. 2007)

1.4.1.1. Imatinib Resistance

Patients can give different responses to imatinib therapy. They can either respond or not respond to imatinib. This resistance is observed as lack of hematologic, cytogenic or molecular responses to therapy. Besides the lack of respond to imatinib intolerance can rise up in patients which cause toxicity. Many resistance cases occur while using imatinib most often in accelerated and blast crisis phases (Gambacorti-Passerini, et al. 2002). There are two kinds of resistance that can be developed to imatinib. The first one is intrinsic resistance which is primary resistance while the other one is acquired resitance during treatment which is secondary treatment. The major resistance mechanism is the mutations that occur in the kinase domain which causes a conformational change in the BCR/ABL oncoprotein that prevents imatinib binding. In addition to mutations there are other resistance mechanisms against imatinib. These

mechanisms can be categorized into two groups. While mutations in the kinase domain and increased expression of BCR/ABL kinase through gene amplification is known as the BCR/ABL dependent mechanisms, decreased intracellular imatinib concentrations caused by transporters, imatinib binding by plasma proteins and other kinases such as Src family kinases are categorized as BCR/ABL independent mechanisms (Ramirez and DiParsio 2008).

1.4.1.1.1. BCR/ABL Dependent Mechanism

BCR/ABL dependent mechanisms result from the mutations in the tyrosine kinase domain and overexpression of the oncoprotein that as a result of BCR/ABL gene amplification. As it is mentioned above there are four sections in BCR/ABL oncoprotein; kinase domain, catalytic domain, activation domain and ATP binding domain. The mutations can be classified into four groups. The first one is the mutations that directly disrupts the imatinib binding, second one is those that target ATP binding site, thirdly the activation loop can be interrupted that leads the blocking the conformational change that is required for imatinib binding and finally we can categorize the mutations that are present within the catalytic domain (Deininger, et al. 2005).

Among these mutations the point mutations that take place in ABL kinase domain are the most important and more commonly acquired in primary resistance. T315I mutation is the most important mutation that is too difficult to overcome. In this mutation the aminoacid threonine at position 315 is switched into isoleucine. It is the first mutation that is confirmed in resistant patients against imatinib (Weisberg, et al. 2007). It is believed that this mutation causes the resistance by two main ways. The first one is caused by the absence of oxygen atom that is provided by threonine. The switch into isoleucine prevents the formation of hydrogen bond between imatinib and the protein because of the lack of oxygen atom. Secondly, isoleucine has a hydrocarbon group and this group limits the binding of imatinib sterically (Deininger, et al. 2005).

Besides the mutations that take place in kinase domain, the ones that cluster in ATP binding domain are also very important for the formation of the resistance. This domain is rich in glycine and this glycine rich sequence spans 248-256 aminoacids. This concensus sequence interacts with imatinib via hydrogen bonds and van der waals

bonds. These mutations cause a conformational change that is not suitable for imatinib to bind and by this way these changes causes insensitivity to imatinib.

In addition, the activation loop of the Abl kinase domain can exposed to the mutations. This loop starts from the 318th aminoacid with a conserved aminoacid sequence of aspartete-phenylalanine-glycine. This region is the determinant of the active and inactive conformation of the oncoprotein. Imatinib has the ability to bind to inactive (closed) conformation of BCR/ABL. However, the mutation that changes the conformation into on open conformation cause the imatinib no longer bind to the region (Tokarski, et al. 2005).

BCR/ABL oncoprotein over expression is the second resistance mechanism that is dependent to BCR/ABL. This overexpression is caused as a result of amplification of the oncogene. The increased levels of the target protein cause the requirement of the high amounts of therapeutic agent. In a study, it is observed that 3 patients form 11 in blast crisis shown to have multiple copies of BCR/ABL which is proved by FISH. In another study 7 patients out of 55 showed an approximately 10 times more transcript levels and 2 out of 32 patients were shown to have a genomic amplification of BCR/ABL. Another interesting finding about the resistance to imatinib is the transient overexpression of BCR/ABL (Hochhaus, et al. 2002).

1.4.1.1.2. BCR/ABL Independent Mechanisms

Besides BCR/ABL dependent mechanisms independent mechanisms also have a great importance in resistance. Multidrug resistance is a cross resistance to a variety of drugs such as anticancer drugs in mammalian cells. This resistance is mediated through transport proteins, either influx or efflux proteins, that are present on cell membrabe (Schinkel, et al. 2003). One of these proteins is P-glycoprotein (P-gp) which is an ATP dependent efflux pump that leads to a reduction of the concentrations of the drug and reduces the insufficient reach of drug to its target (Mimeault, et al. 2008). Imatinib is a substrate of P-gp and this protein reduces the levels of imatinib before it binds to BCR/ABL (Gorre, et al. 2001). In a study, the imatinib resistant CML cell line that is exposed to increasing doses of imatinib showed an upregulation of P-gp expression and also MDR1, another multidrug resistnce protein, is shown to be overexpressed in CML cell lines that are resistant to imatinib. However, in resistant CML patients P-gp

overexpression is not observed but when the imatinib treated cells from resistant patients are exposed to an inhibitor of P-gp pump, PSC833, a significant decrease in colony formation is observed (Mahon, et al. 2003). In addition to P-gp there are two other drug transporters that are thought to play a role in imatinib resistance. Breast cancer resistance protein (BCRP) and human organic cation transporter1 (hOCT1) have been involved in imatinib resistance. Imatinib has been showed to be a substrate for BCRP drug efflux protein which is also overexpressed in CML stem cells. On the other hand hOCT1 protein mediates the active transport of imatinib into the cell. In contrast to overexpression of drug efflux proteins, the inhibition of the hOCT1 protein causes the resistance to imatinib because of the limited enterance into the cell (Chuah and Melo 2009).

Other than multidrug resistance proteins, alpha-1 acid glycoprotein (AGP) is another factor that reduces the levels of imatinib inside the cell. There is a strong a relationship between AGP and imatinib. When this protein binds to imatinib, it prevents the activity of the drug (Hamada, et al. 2003). In an interesting study, AGP is isolated from healthy donors and applied with imatinib to K562 CML cells in vitro. They observed that growth inhibitory effects of imatinib are affected when tey applied via this combination against cells. They also isolated AGP from CML patients and they observed that they have significant higher levels of this protein when compared to healthy donors. Besides the same combination applied to CML cells in vitro and they showed that AGP from CML patients showed an inhibitory effects on imatinib activity (Jorgensen, et al. 2002). While STI571 is known to be bound in human serum by both AGP and albumin, the role of increased AGP levels in STI571 resistance in CML patients remains unclear (Shah and Sawyers 2003).

Secondary tyrosine kinases such as SRC family kinases are activated in CML. The Src family kinases, including Lyn and Hck, are activated in BCR-ABL-expressing cell lines (Jabbour, et al. 2009). Lyn is overexpressed and activated in an imatinib-resistant CML cells both in vivo and in vitro. Lyn suppression by a Src kinase inhibitor resulted in reduced proliferation and survival of the imatinib-resistant but not the sensitive cell line (Donato, et al. 2003).

1.4.2. Dasatinib

Resistance cases that rise up after imatinib therapy, some other agents are needed to be discovered. For this purpose different second generation tyrosine kinases are rationally designed. Dasatinib (BMS-354825) is one of them and is a novel, oral, potent, multitargeted kinase inhibitor of both Bcr-Abl, Src family kinases (SFK), c-KIT, ephrin A and PDGF-R and is 325 times more effective than imatinib (Aguilera, et al. 2009). Dasatinib is originally designed as SFK inhibitor. It acts as a competitive inhibitor at the adenosine triphosphate (ATP) binding site of BCR/ABL, preventing tyrosine phosphorylation of the substrate molecule and downstream signaling, causing to growth arrest and apoptosis (Tokarski, et al. 2006). Src proteins show a high sequence homology with ABL protein. Active conformation of ABL is very similar to Src structurally. Dasatinib is a very potent inhibitor of CML cell proliferation when we take into consideration that Src and ABL have very strong similarities functually and structurally. Dasatinib has many superiorities against imatinib. Firstly dasatinib is not a substrate for drug efflux protein that pumps imatinib out. This efflux causes a decrease in imatinib concentrations and resistance is observed. Since dasatinib is not pumped out of the cell, it effects its target more efficiently. Oncogenic activation requires ATP binding to BCR/ABL and conformational change that switches the protein into an active state. Imatinib can only bind to inactive form of BCR/ABL in which no ATP binding takes place. The unique property of dasatinib is that it can bind both active and inactive conformations of BCR/ABL which provides the ability to inhibit oncogenic tyrosine kinase activity progression (Tokarski, et al. 2006). Moreover this second generation TKI is active against most of the mutations except T315I point mutation and dowregulates BCR/ABL (Olivieri, et al. 2007).

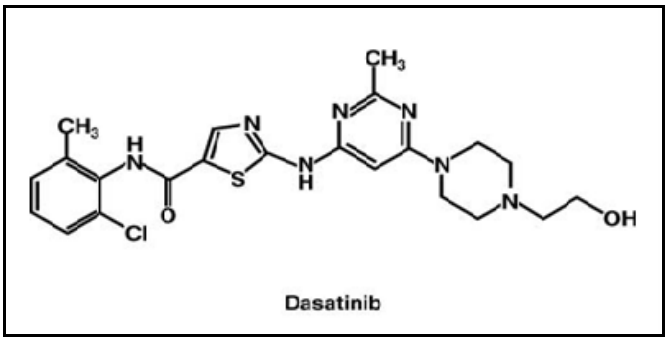


Figure 1.8. Chemical structure of Dasatinib
(Source: Shah, et al. 2004)

In the treatment of lymphoid malignancies, the levels of glucocorticoids are very important because increased levels of glucocorticoids are a part of cure since they induce apoptosis. In this study they used chronic lymphoid leukemia as a model for lymphoid malignancies. Since Lck has a strong relationship with resistance to dexamethasone, they found that dasatinib, a strong SRC family kinase inhibitor, induces apoptosis through enhancing glucocorticoid levels by inhibiting Lck levels (Harr, et al. 2010). In prostate cancer, the connection between osteoblasts and prostate cancer cells causes metastasis to bone which is an unwanted situation. In this study, Lee et al. observed that dasatinib inhibited osteoblast proliferation which is induced by fibroblast growth factor-2. Besides this dasatinib induces the differentiation of osteoblasts by inhibiting SRC and ABL (Lee, et al. 2010). Metastasis and invasion is a hallmark of cancer and this property of cancer cells shows a significant problem for therapy. In this study its is shown that Lyn is an intermediary factor of invasion in breast cancer model and this could be a determinant for epithelial-mesenchymal transition (EMT) which is a very important parameter in metastasis and invasion. In these models, dasatinib showed an inhibitory effect against invasion by inhibiting Lyn kinase activity at nanomolar concentrations (Choi, et al. 2010). This study revealed the signaling pathways that are active in lung cancer and also targeted by dasatinib. They examined approximately 40 kinase targets of Dasatinib and they found out that especially Src family kinases(SFKs) and EGFR is targeted by Dasatinib in lung cancer (Li, et al. 2010). Dasatinib also causes growth inhibition and apoptosis in molecularly heterogenous acute myeloid leukemia. They used both growt factor dependent and independent leukemia cell lines and they observed inhibiton of Src family kinases for all lines (Guerrouahen, et al. 2010). In another study besides the tyrosine kinase inhibitory effect, dasatinib also showed antitumor immunity. They analyzed the effect of dasatinib on NK cells and they used K562 and Meg-01 CML cell lines. Dasatinib showed an activity of reduced NK cell cytotoxicity and IFN-gamma production. Also, when they examined the signaling patterns of NK cells, they observed that dasatinib blocks the signalling events that caused reduced PI3K and ERK phosphorilation that are very active on NK cell activation (Salih, et al. 2010). Haura et al. combined dasatinib, an SFK inhibitor, and erlotinib which are an EGFR inhibitor in non-small lung cancer patients. They showed that combinations of these two agents endurable for treatment with an inhibitory effect on angiogenesis (Haura, et al. 2010) Together with the importance that SFK gained as a target for cancer therapy, dasatinib also acquire a great function on many cancer types.

Liang et al. carried out both in vivo and in vitro studies of dasatinib on the effect on tumor microenvironment. They isolated tumor cell, endothelial cell, and myeloid cell compartments of tumor microenvironment from mice and treat these cells with dasatinib. Dasatinib did not showed any cytotoxic effects in cell culture but it showed tumor suppressive effect through enhancing apoptosis. Dasatinib blocked motility and also other functions of endothelial and myeloid cells. Matrix metalloprotease-9 activity which has a role in cell proliferation, migration, differentiation, angiogenesis is also inhibited by dasatinib (Liang, et al. 2010). In another study dasatinib showed on inhibitory effect on B-cell receptor signaling which provides a new therapy option for chronic lymphoid leukemia (CLL). Apoptosis is induced by dasatinib in CLL cells through downregulation of SYK and phospholipase C gamma2. It is suggested that these molecules can be goog biomarkers for the effectiveness of dasatinib (Song, et al. 2010) Dasatinib inhibits the proliferation of breast cancer cells which have an overexpression of EGFR, HER-2 and HER-3. Besides the inhibition of proliferation, dasatinib also inhibit angiogenesis, migration, invasion, colony formation through targeting c-Src, Akt and Erk **(**Nautiyal, et al. 2010**).**

The response to dasatinib does not appear to be compromised among patients with F359C/V, which is a mutation in the catalytically important P-loop region of BCR-ABL. However, patients with T315A/I, F317I/L, and V299L are unlikely to respond to dasatinib therapy. Nilotinib resistant mutations have been identified as T315I, Y253H, and E255V. It is noteworthy that both dasatinib and nilotinib are ineffective against T315I BCR-ABL. Indeed, this mutation is resistant to all available TKIs, and survival outcomes of patients with T315I remain stage dependent.

Although good cytotogeneic and hematologic responses are obtained, resistance cases against dasatinib still remain a problem. This resistance is a major obstacle to overcome chronic myeloid leukemia. So combinational treatment strategies are needed to be discovered and tried. Sphingolipids are important players in cancer and their importance is proved in resistance cases. With the knowledge of the importance of sphingolipids in cancer arena, we purposed to enhance the cytotoxicity of dasatinib against CML cells.

1.5. Sphingolipid Metabolism

1.5.1. Sphingolipid Biosynthesis

Bioactive sphingolipids such as ceramide, sphingosine 1-phophate (S1P), sphingosine, and glucosylceramide (GlcCer), function as bioeffector molecules, which are involved in the regulation of various aspects of cancer pathogenesis and therapy, including apoptosis, cell proliferation, cell migration, senescence, or inflammation (Futerman and Hannun 2004, Kok and Sietsma 2004, Ogretmen and Hannun 2004, Reynolds, et al. 2004, Fox, et al. 2006, Modrak, et al. 2006). Formation of ceramide is induced by a variety of signals including TNF-α, Fas ligand, heat stress, oxidative stress, ionizing radiation and chemotherapoetics (Segui, et al. 2000). The sphingolipid metabolism is composed of integrated pathways and various enzymes. The de novo synthesis of ceramide occurs via the condensation of serin and fatty acyl CoA with the action of serin palmitoyl transferase (SPT) as a first step in endoplasmic reticulum. The formation of 3-ketosphinganine from serine and palmitoyl CoA via SPT is followed by the reduction of the product to sphinganine by ketosphinganine reductase. Then fatty acyl CoA is added to sphinganine by ceramide synthase to form dihydroceramide. This acylation process is carried out in microsomes. The desaturation of 4-5 C of dihydroceramide into double bond generates cermaide with the action of dihydroceradmide desaturase enzyme. Ceramide gives rise to different sphingolipids which are sphingosine, galactocerebrosidase, glycosphingolipids, ceramide-1-phosphate and sphingomyelins by ceramidase, ceramide galactosyltransferase, glcosylceramide synthase, ceramide kinase and sphingomyelinase, respectively (Perry, et al. 1998).

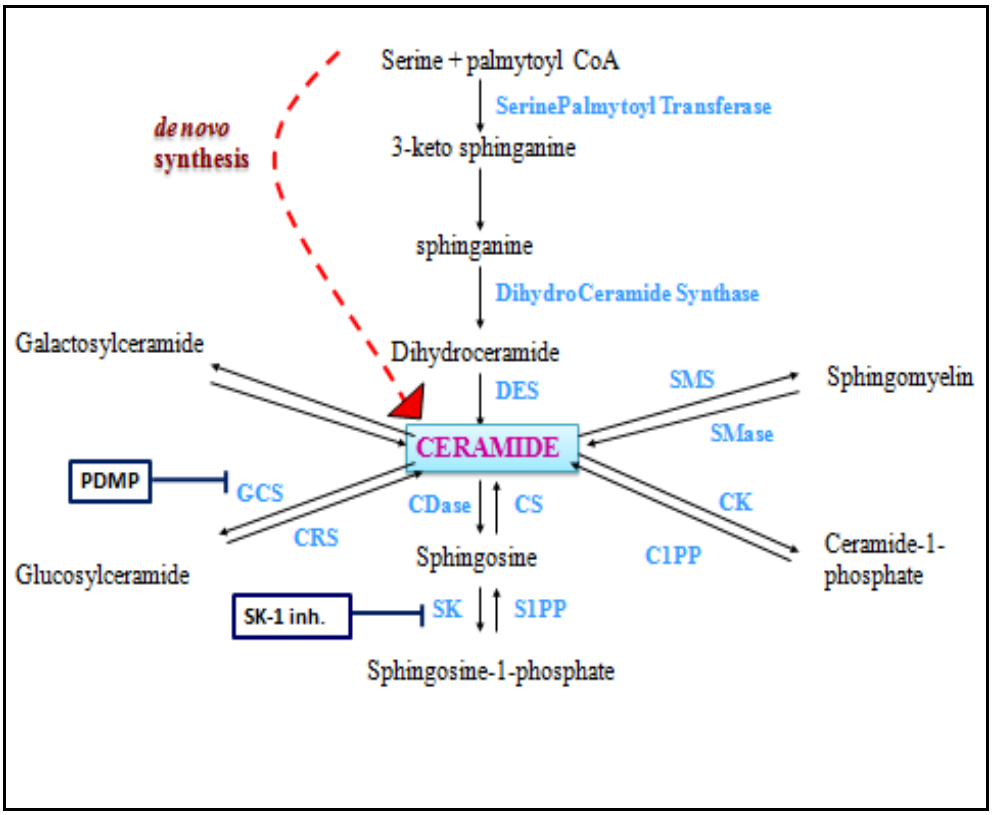


Figure 1.9. Sphingolipid biosynthesis pathways
(Source: Ogretmen 2006)

1.5.2. Downstream Targets of Ceramide

Ceramide regulates many pathways by effecting some kinases or phosphatases such as AKT, protein kinase C, mitogen activated protein kinases, phospholipase D. Ceramide targets directly ceramide activated ptoein phosphatases (CAPP) which is a serine/threonine protein phosphatases PP1 and PP2, cathepsin D and some other kinases such as RAF, MEKK and KSR. The phosphatases PP1 and PP2 act on many substrates such as retinoblastoma (RB), BCL2, c-Jun, protein kinase C (PKC), AKT and SR proteins. SR proteins have important roles in constitutive and alternative splicing. These pathways are activated by ceramides in different compartments. For example, cathepsin D is activated by ceramide that is generated in lysosomal memebranes. Activation of cathepsin D causes the activation of proapoptotic BID. Ceramide also activates AKT, PKC, c-Jun and BCL2 through PP2 which in turn activates many apoptotic stimuli. All these proteins that are somehow activated by ceramide effects telomeres and telomerases (Ogretmen and Hannun 2001). This effect explains the relationship of

ceramides with senescence because shortening of telomeres causes senescence. Also c-myc is regulated by these activated proteins. C-myc is known to play very important and pivotal roles as a oncogene and its gene product, c-myc, is a transcription factor that regulates cycle regulation, metabolism, apoptosis, differentiation, cell adhesion, and tumorigenesis. Caspases, the key point of all aoptotic signals, are also regulated through cermaide mediated activation of mediator proteins. Mitochondrial membrane potential decrease, pore formation on mitochondrial outer membrane and cytochrome c release from mitochondria is a known intrinsic pathway of apoptosis. Cakir et al showed that ceramide decreases the MMP in breast cancer, chronic myeloid leukemia and acute promyelocytic cell lines (Cakir, et al. 2010). Cyclin dependent kinases are also activated as a downstream target of ceramide.

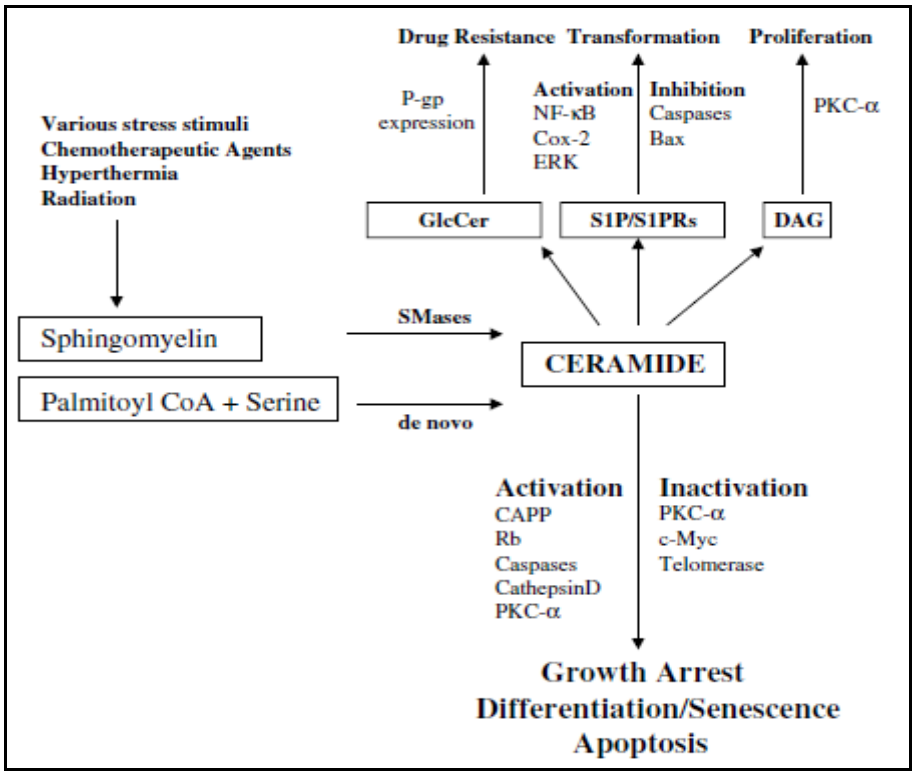


Figure1.10 Downstream targets of ceramide
(Source: Ogretmen 2006)

1.5.3. Biological Functions of Ceramide

1.5.3.1. Growth Arrest

Ceramide have very vital roles in many important cellular processes. It is found that exogenous ceramide has the aility to induce G0/G1 cell cyle arrest. This result is due to the dephosphorilation of retinoblastoma gene product (Dbaibo, et al. 1995). Also another study showed that when NIH 3T3 cell line is treated with GCS enzyme inhibitor which causes the accumulation of ceramide causes cell cycle arrest through the inhibition of cyclin dependent p34cdc2 and cdk2 kinases (Rani, et al. 1995). In another study it has been observed that ceramide inactivartes specifically cdk2 but not cdk4 (Lee, et al. 2000).

1.5.3.2. Differentiation

In acute myeloid leukemia cell lines HL60 and U037 treatment with vitamin D3 causes an increase in sphingomylein hydrolysis by neutral sphingomyeliase which causes increased cermide levels and resulting in monocytic and not neurophilic or macrophage type differentiation of these cells (Okazaki, et al. 1989). In neuronal cell line, ceramide act like a nerve growth factor function and then causes the differentiaon of these cells into T9 glioma cells, Purkinje cells and hippocampal neurons (Dobrowski, et al. 1994).

1.5.3.3 Senescence

Senescence has been proposed as a mechanism to block immortalization and tumorigenesis. Moreover, in addition to apoptosis, premature or inducible senescence was identified as an effective response to chemotherapy. A nearly irreversible stage of permanent G1cell-cycle arrest, which is linked to morphological changes, is metabolic changes and changes in gene expression. The induction of senescence depends on p53 and cell-cycle inhibitors such as p21 and p16. A very strong evidence that shows ceramide is related to senescence is ceramide levels are increased significantly while

human fibroblasts entered senescent phase and also the addition of ceramide to fibroblasts induced many changes that are related to senescence such as activation of RB, regulation of cdks.ceramide causes this induction of senescence by inhibiting phospholipase D that causes diacylglycerol formationand inhibition of translocation and activation of protein kinase C to the membrane (Venable, et al. 1995). Hannun et al also showed that ceramide inhibits the telomerase activity of A549 lung cancer cell line.there are also evidences that ceramide has roles in senescence in yeast. In yeast there are lac1 and lag1 aging genes (Ogretmen, et al. 2001). These genes are analogues of LASS genes in human. This finding shows us that ceramide is not an important molecule not only for senescence but also in aging (Ogretmen and Hannun 2001).

1.5.3.4. Apoptosis

Ceramide is known as a strong apoptotic molecule. Intracellular accumulation or exogenous ceramides causes anti-proliferative responses. As a result of apoptotic stimuli a cascade metabolism of caspases is induced. Our results showed us that ceramide increases caspase-3 activity when we compare to untreated control. In many studies addition of exogenous ceramide causes cell death (Jarvis, et al. 1994, Cifone, et al. 1994, Obeid, et al. 1993). Although ceramide is a very strong apoptotic molecule, molecules that are very similar to ceramide are not always showed this effect. For example, dihydroceramide is very similar in metabolism and uptake with ceramide, but this molecule does not show apoptotic response (Bielawska, et al. 1992). Also diacylglycerol reverses the apoptotic effects of ceramide. Inhibitors that cause the accumulation of ceramide such as PDMP, a strong inhibitor of glucosylceramide, and sphingosine 1 kinase inhibitor causes increases in ceramide levels and this leads to enhanced apoptosis and growth repression (Ogretmen and Hannun 2001). A study showed that ionizing radiation causes ceramide formation through action of an acid sphingomyelinase and mice that is lack of this acid sphingomyelinase become unable to response irradiation and become resistant to high doses of radition (Santana, et al. 1993).

1.5.4. Structure and Metabolism of Ceramide

The length of ceramide varies form C14 to C26. Ceramide is composed of sphingosine and an amide bond linked fatty acyl chain (Ogretmen 2006).

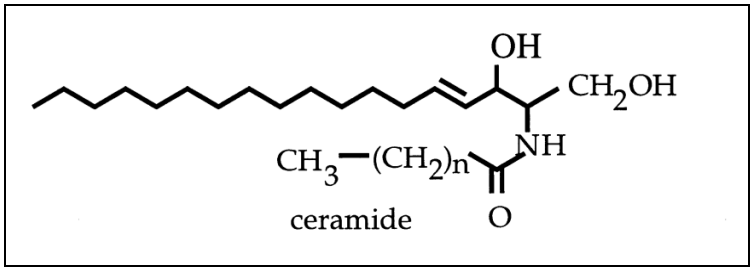


Figure1.11. Chemical structure of Ceramide
(Source: Perry and Hannun 1998)

There are six genes responsible for the sythesis of ceramide in mammals named as LASS1-6 (longetivity assurance homologues) and nowadays renamed as CerS1-6 (ceramide synthase) (Reynolds, et al. 2004). These genes are analogues of LAG1 genes in yeast which is responsible for the regulation of life span and longetivity of *Saccaromyces cerevisiaea* (D'Mello, et al. 1994). Each LASS enzyme is responsible for the synthesis of a specific ceramide which are differ in fatty acyl chain length. While LASS1 is responsible for synthesis of C18-ceramide (Venktamaran, et al. 2002), LASS2 and LASS4 increases the levels of C24-ceramides and C22-ceramides, respectively (Mizutani, et al. 2005, Riebeling, et al, 2003). C14- and C16-ceramides were sythesised by LASS5 and LASS6 genes (Weinmann, et al. 2005, Xu, et al. 2005).

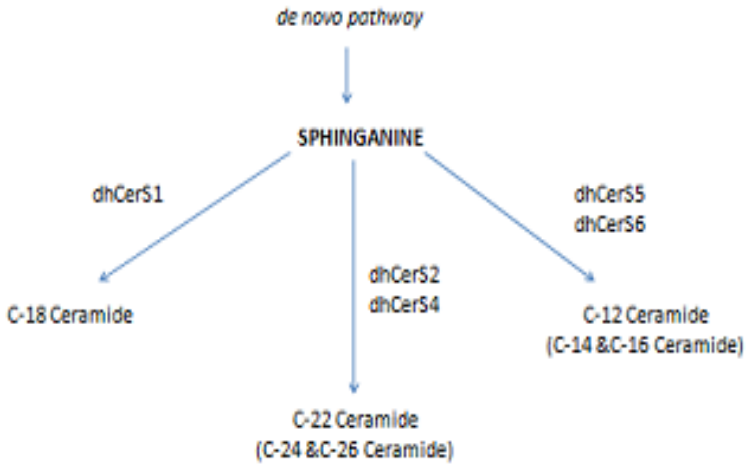


Figure 1.12. The Ceramide Synthase (CerS) enzymes that are responsible for the synthesis of different length ceramides (Source: Ogretmen 2006)

Ceramide is synthesized in ER membrane, and the following processes are carried out in Golgi apparatus.the transport of ceramide to Golgi is done by ceramide transport protein (CERT) via nonvesicular transport. The addition of glucose to ceramide, thus formation of glucosylceramide occurs in Golgi apparatuswhich is CERT independent. The nonvesicular transport of GlcCer from its synthesis site to distal Golgi is done by FAPP2 (four phosphate adaptor protein). Sphingosine 1 phosphate is produced by the phosphorilation of sphingosine by sphingosine kinase. Sphingomylein is formed by addition of phosphocholine to ceramide by sphingomyeline synthase and sphingomyelinase converts it into ceramide and phosphocholine (Hannun and Obeid 2008).

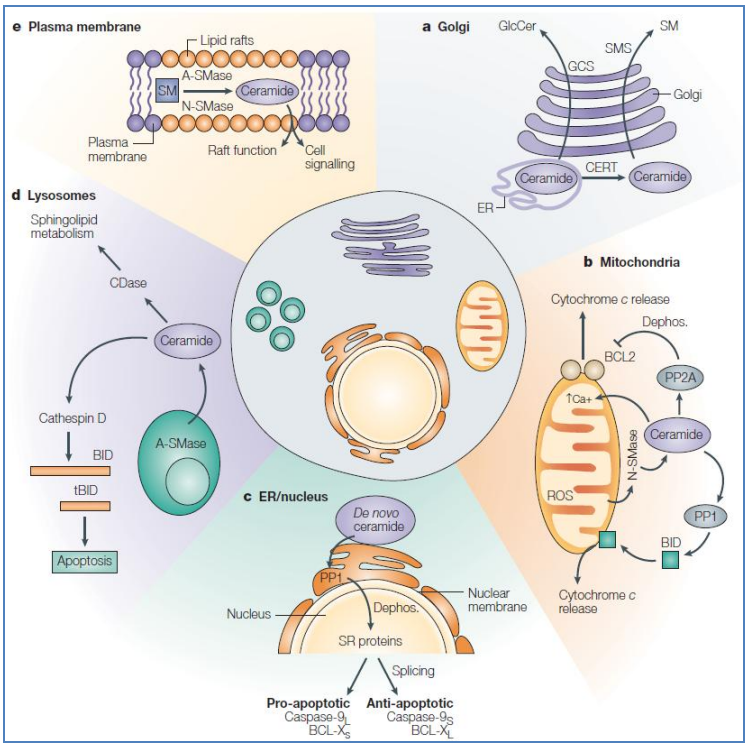


Figure 1.13. Compartmentalization of boiactive sphingolipid synthesis (Source: Ogretmen and Hannun 2004)

1.5.5. Ceramides and Drug Resistance

In cancer chemmotherapy development of resistance to anticancer drugs and the relapse of disease is the major obstacle to achieve success. Ceramides act as strong antitumoral molecules supressing cell growth and proliferation and inducing apoptosis and differentiation (Pettus, et al. 2002). On the other hand, GlcCer and S1P molecules converted from ceramide by glucosylceramide synthase (GCS) and sphingosine kinase-1 (SK-1) enzymes act as strong antiapoptotic molecules inducing cell growth and proliferation and inhibiting apoptosis and differentiation. GCS enzyme regulate the balance between ceramide and glucosylceramide which also means that the levels of sensitivity to anticancer drugs (Cabot, et al. 2002). There are very strong evidences that shows the correlation between GCS and drug resistance (Senchenkov, et al. 2001). In another study GCS gene has introduced into sensitive MCF-7 breast cancer cell line and they observed a an apparently increase in the GCS expression levels and as a result the cells became adriamycin and exogenious ceramide resistant. This study shows that GCS activity adjust drug resistance (Liu, et al. 1999). Liu et al downregulated GCS gene with

antisense RNA in adriamycin resistant MCF7 breats adenocarcinoma cells and they observed a decrease in resistance to anticancer agents which shows that GCS is very closely related to drug resistance in breast cancer (Liu, et al. 2001). There are many studies that shows us that using inhibitors of GCS anzyme can open a door evading from resistance that is caused by GCS enzyme acitivty. These inhibitors sentesizes cancer cells to chemotherapoetic agents. Morjani et al showed that in multidrug resistant MCF7 cells there is an accumulation of GlcCer which is visualized with the help of fluorescent GlcCer bodipy. These droplets are seen in cytoplasm. Treatment of these cells with PPMP, an inhibitor of GCS, reduces the amount of droplets whisc leads to decrease in resistance of cells (Morjani, et al. 2001). P-gp is a specific transporter of GlcCer and in cells that have an overexpression of P-gp have an accumulation of GlcCer. In consistent with this informaiton, Gouaze et al showed a relationship between P-gp and GCS. They observed an inhibition of MDR1 gene when they silenced the GCS gene with siRNA and reported the reversal of the resistance of adriamycin resistant MCF7 cells (Gouazé, et al. 2005).

Sphingosine kinase-1 (SK1) converts sphingosine to sphingosine-1-phosphate (S-1-P), which means that this enzyme reduces the levels of apoptotic ceramide. In a study that is carried out with A375 melanoma cells, it is showed that these cells gain resistance to Fas and ceramide mediated apoptosis when they increased the expression levels of SK1 and they reduced the sensitivity. This result gives the idea that the resistance can be reversed with the inhibition of SK1 activity (Bektas, et al. 2005). In prostate cancer cell line, PC3, high levels of SK1 expression is observed in response to camptothecin. Besides, inhibition of the SK1 expression in this cell line also inhibited cell growth and camptothecin treatment induces the SK1 pathway (Akao, et al. 2006). These results shows us that SK1/S1PR signalling prevents the cells from chemotherapy inguced apoptosis. It was shown that SK-1 is a prosurvival, antiapoptotic and a migratory factor for breast cancer cells by Sarkar et al. in 2005 (Sarkar, et al. 2005). Besides, high levels of SK-1 are observed in tumors from lung cancer patients (Johnsonn, et al. 2005). French and his collegues observed that using inhibitors of SK-1 inhibits the cell proliferation without any toxic effects in animal models in vivo (French, et al. 2006).

1.5.6. Targetting Ceramide Metabolism to Overcome Drug Resistance

As it is mentioned above, many studies showed the importance of sphingolipid metabolism in cancer. Ceramide shows apoptotic, antiproliferative responses, while glucosylceramide and sphingosine 1 phosphate enhances growth and proliferation, strategies that provides increases in ceramide levels or decreases GlcCer and S1P levels or inhibiting the conversion of ceramide into these anti apoptotic molecules should be developed. These strategies will provide efficient cancer therapy, increase the action of drugs, provide the reversal of resistance and prevent the adverse effects of chemotherapy to normal cells.

With the help of all information, sensitization of cancer cells to anti cancer drugs is possible when we target the sphingolipid metabolism. Molecules that target the conversion of ceramide into GlcCer or to sphingomyelin by sphingomyelin synthase, to S1P by sphingosine kinase 1 could be helpful to sensitize cancer cells. Moreover, combinational therapies that mimic or antagonizes sphingolipid molecules or synthesis of new inhibitors that prevent the conversion of ceramide to GlcCer or S1P, also providing enhancement of endogenous ceramide levels provides the cells undergo apoptosis and increases the cytotoxicity of cancer cells. In our laboratory there are many studies that reports combination of C:8 ceramide with anticancer drugs showed a significant decrease in cell proliferation (Gucluler and Baran 2009, Ekiz, et al. 2009). In a study it is observed that combination of C:6 ceramide and paclitaxel combination significantly inhibits cell proliferation in human head and neck squamous cells in the S and G2/M phase of cell cycle (Mehta, et al. 2000).

It should also be thought that relapse of the disease may be caused by the alterations in sphingolipid metabolism and dysfunctional ceramide metabolism. However, manipulations on this dysfunctional sphingolipid metabolism can reverse this effect and enhance the sensitivity of cells to anticancer drugs.

In vitro studies approved the effects of ceramide metabolism however; in vivo studies should be done to observe the effects in an organism. Modrak et al observed that intravenous administration of sphingomyelin as 10 mg/day for seven days potentiated 5-FU chemotherapy in human colonic xenograft bearing nude mice (Modrak, el al. 2000). In another interesting study, in two human colon cancer cell lines the main digestion product of sphingolipids is detected as sphingosine and treatment of these cell lines with

sphingosine caused apoptosis. These reports show us the digestion products and the activity of sphingolipids in cancer types (Schmelz, et al. 2001).

Day by day, it is becoming more and more clear that ceramide metabolism, the balance of ceramide/GlcCer and ceramide/sphingosine-1-phosphate decides the fate of the cell, to undergo apoptosis or to survive.

CHAPTER 2

MATERIAL AND METHODS

2.1. Reagents

Dasatinib was kindly provided by BMS, USA. It was dissolved in DMSO and 10mM stock solution was prepared and stored at -20ºC. C:8ceramide, 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 -20 ºC. The final concentration of DMSO did not exceed more than 0.1% in culture. Primers were obtained from Eurofins, Germany. RPMI1640, heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillinstreptomycin and trypsin EDTA were obtained from Biological Industries, Israel. RNeasy RNA isolation kit, QIAquick gel extraction kit, Taq DNA Polymerase was obtained from Finnzymes, FI. Bradford dye, 4-5 % SDS polyacrylamide gel, coomassie blue, tween-20, 10X trisglycine- EDTA were obtained from Sigma, USA. dNTP set, DNA ladder was obtained from AMRESCO, USA. Caspase-3 colorometric assay kit was obtained from BioVision, USA. JC-1 mitiochondrial membrane potential detection kit was obtained from APO LOGICTM JC-1 from BACHEM, USA. Reverse-Transcription system and bovine serum albumine (BSA), trypan blue solution, β-mercaptoethanol, dimethyl sulfoxide (DMSO), agarose were obtained from Sigma, USA.

2.2. Cell lines and Culture Conditions

K562 and Meg-01 human CML cells were obtained from the German Collection of Microorganisms and Cell Cultures (Germany). The cells were cultured in RPMI-1640 growth medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) at 37˚C in 5% CO_2. Medium was refreshed in every 3 days. The cell suspension was taken from tissue culture flask into a sterile falcon tube and then the cells were centrifuged for 10 minutes at 1000 rpm. After centrifugation the

supernatant was removed and the pellet washed with 3 mL PBS. After washing the cells were centrifuged again at 1000 rpm for 10 minutes. After centrifugation, supernatant was removed and the pellet was resuspended in 20 mL of RPMI1640 and transferred into a sterile 75 cm^2 tissue culture flask.

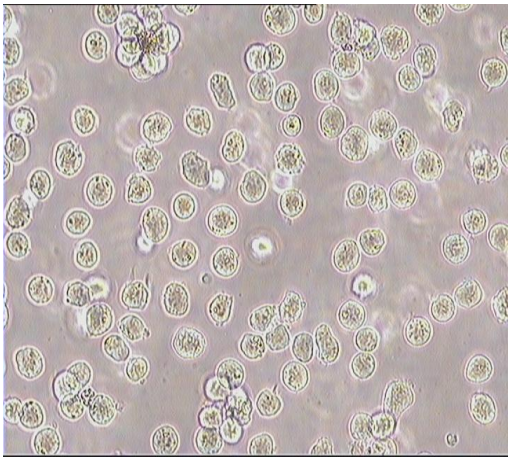
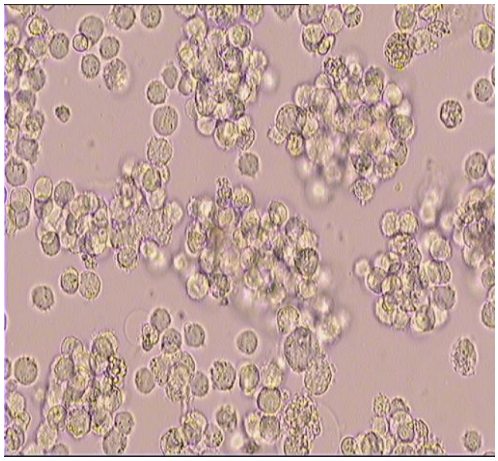
a  b 

Figure 2.1. Chronic myeloid leukemia cell lines a) K562 b) Meg-01

2.3. Cell Proliferation Assay

Living cells have enzymes on the mitochondria membrane that catalyzes the formation of tetrazolium salts into formazan. These formazan molecules give an absorbance at 492 nm. Anti-proliferative effects of dasatinib, C8:ceramide, SK-1 inhibitor, and PDMP were determined by XTT cell proliferation assay as described previously (Ekiz, et al. 2009). Briefly, 96-well plates were seeded as $2x10^4$ cells/well containing 200 µl of the growth medium in the absence or presence of increasing concentrations of these agents. The cells were incubated at 37˚C in 5% CO_2 for 72 hours. Then, they were treated with 40 µl XTT for 4 hours at CO_2 incubator.

After that, the plates were read under 490 nm wavelengths by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland). Finally, IC50 (the concentration of the chemical that inhibit 50% of cell proliferation as compared to untreated control) and IC10 values were calculated from cell proliferation plots.

2.4. Isobologram Analysis for Median Dose Effect

We used the CalcuSyn for Windows computer program (CalcuSyn software, Biosoft, Cambridge, UK) for isobologram analysis (Zhao, et al. 2010). We studied the isobologram analysis of dasatinib with C8:ceramide, PDMP, and SK-1 inhibitor using the computer software. 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. Measurement of Changes in Caspase-3 Enzyme Activity

Caspase-3 enzyme is the key enzyme that stands in the center of apoptotic pathways. This enzyme is activated by the signals that are induced either by extrinsic or intrinsic pathways. The activation is dependent of the release of cytochrome-c molecules form mitochondria. The cytochorome-c molecules are then binds to APAF-1 and porcaspase-9 in order to form apoptosome complex. Finally caspase-3 enzyme is activated by the activated caspase-9 via cytochrome-c. Changes in caspase-3 enzyme activity in response to applied anyone of these chemical agents were assessed by caspase-3 colorimetric assay kit (R&D Systems, USA) as described previously (Gucluler and Baran 2009).

The cells were treated with C8:ceramide, PDMP or SK-1 inhibitor itself and in combination with dasatinib for 72 hours. Untreated cells were used as control group. The cells were collected by centrifugation at 1000 rpm for 10 minutes, and treated with 100 µl of cold lysis buffer (1X) to obtain cell lysate. After incubating the cell lysates on ice for 10 minutes, they were centrifuged at 14000 rpm for 1 minute. Then, the supernatants were transferred to new microcentrifuge tubes. In order to measure caspase-3 enzyme activity, reaction mixture including 20 µl of assay buffer (5X), 25 µl of sample, 50 µl of sterilized water, and 5 µl of caspase-3 colorimetric substrate was prepared in 96-well plates, and incubated for 2 hours at 37˚C. The samples were read

under 405 nm wavelengths by Elisa reader (Thermo Electron Corporation Multiskan Spectrum, Finland).

2.6. Bradford Protein Assay

In Bradford Protein assay, we used a dye called Coomassie Brilliant Blue to detect the levels of protein levels. The form of the dye that is bound to proteins has an absorption spectrum maximum at 595 nm. The cationic form, that is unbound form of the dye, is brown while binding of the dye to protein stabilizes it turns into a blue color that is anionic form. The measured absorbance rises with the increasing concentrations of protein. We prepared a series of standard protein solutions by using BSA (Bovine Serum Albumine) diluted with PBS (Phosphate Buffered Saline).

After measuring protein concentrations by Bradford assay, enzyme activity levels were normalized to protein concentrations.

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

Mitochondrion is a very important molecule which mediates the release of cytochrome-c molecules. The decrease of mitochondrial membrane potential creates pores on the outer mitochondrial membrane and cytochrome-c escapes from these pores into the cytoplasm. This decrease of the potential is the first step to induce an apoptotic response. APO LOGIX JC-1 Assay Kit (Cell Technology, USA) was used to measure the changes in mitochondrial membrane potential in K562 and Meg-01 cells as described previously (Gucluler and Baran 2009).

Shortly, the cells that had been induced to apoptosis were collected by centrifugation at 1000 rpm for 10 minutes. Supernatants were removed, and 500 µl of JC-1 dye (1%) was added onto the pellets. After incubation of cells for 15 minutes at 37˚C in 5% CO_2, they were centrifuged at 1000 rpm for 5 minutes. Then, 2 ml of assay buffer was added onto the pellets, and they were centrifuged for 5 minutes at 1000 rpm. All the pellets were resuspended with 500 µl assay buffer, and 150 µl from each of them was added into black 96-well plate for a triplet measurement. The aggregate red form has absorption/emission maxima of 585/590 nm, and the green monomeric form has

absorption/emission maxima of 510/527 nm. The plate was read in these wavelengths by fluorescence Elisa reader (Thermo Varioskan Spectrum, Finland). Finally, green/red (510/585) values were calculated to determine the changes in MMP.

2.8. Phosphatidylserin Exposure

Apoptotic cell death was evaluated by using annexin V/propidium iodide double staining method based on apoptosis-related cell membrane modifications. First of all, the cells that had been induced to apoptosis were washed with cold 1X PBS, and then resuspended in 1X binding buffer. 100 µl from each solution was transferred to microcentrifuge tubes. 5 µl Annexin V-FITC, and 5 µl PI was added onto the cells. After gentle vortexing, they were incubated for 15 minutes at room temperature in the dark. 400 µl of binding buffer (1X) was added to each tube, and they were analyzed by flow cytometry (BD Facscanto Flowcytometry, Belgium) within 1 hour.

2.9. Total RNA Isolation and Reverse Transcriptase-PCR

The involvement of ceramide synthase genes (LASS1-6), and ceramide clearance genes (SK-1 and GCS) in dasatinib-induced apoptosis was investigated by examining the expression levels of these genes in response to dasatinib in K562 and Meg-01 cells. To achive this aim, the cells were incubated in the absence and presence of increasing concentrations of dasatinib, and total cellular RNAs were isolated by using RNA Isolation Kit (Macherey-Nagel, USA). mRNAs from total RNA population were reverse transcribed into cDNA by using reverse transcriptase enzyme (Moroney Murine Leukemia Virus Reverse Transcriptase, Fermentas, USA). After 50 min incubation at 42 ˚C, the reactions were stopped at 95 C˚ for 5 minutes. The resulting total cDNA was used in PCR to measure the mRNA levels of LASS1-6, SK-1, GCS, apoptosis genes and transport genes. mRNA levels of β actin were used internal positive control. PCR mixture was prepared in the sterile 0.5 mL eppendorf tubes.

Table 2.1. Forward and reverse primer sequences of LASS1-6, GCS, SK-1, BCR/ABL and β-actin genes

β-actin-Forward	(5’-CAGAGCAAGAGAGGCATCCT-3’)
β-actin-Reverse	(5’-TTGAAGGTCTCAAACATGAT-3’)
GCS-Forward	(5’-ATGACAGAAAAAGTA-3’)
GCS-Reverse	(5’-GGACACCCCTGAGTG-3’)
SK-1-Forward	(5’-CCGACGAGGACTTTGTGCTAAT-3’),
SK-1-Reverse	(5’-GCCTGTCCCCCCAAAGCATAAC-3’)
LASS1-Forward	(5’-CTATACATGGACACCTGGCGCAA-3’)
LASS1-Reverse	(5’-TCAGAAGCGCTTGTCCTTCACCA-3’)
LASS2-Forward	(5’-GCTGGAGATTCACAT-3’,)
LASS2-Reverse	(5'-GAAGACGATGAAGAT-3’)
LASS4-Forward	(5’-TGCTGTCCAGTTTCAACGAG-3’)
LASS4-Reverse	(5’-GAGGAAGTGTTTCTCCAGCG-3’)
LASS5-Forward	(5’-TCCTCAATGGCCTGCTGCTG-3’)
LASS5-Reverse	(5’-CCCGGCAATGAAACTCACGC-3’)
LASS6-Forward	(5’-CTCCCGCACAATGTCACCTG-3’)
LASS6-Reverse	(5’-TGGCTTCTCCTGATTGCGTC-3’)

The expression levels were quantified using the imaging system after running PCR products in agarose gels, followed by ethidium bromide staining.

Table 2.2. Ingredients 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, 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 LASS1

LASS1	
Reaction Mixture	Amount (µL)
PCR grade water	31.3
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
Taq DNA Polymerase	0.7
Total Mixture	50

Table 2.5. Ingredients of PCR solutions for LASS2, LASS5 and LASS6

LASS 2, LASS 5, LASS 6	
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	0.7
Total Mixture	50

Table 2.6. Ingredients of PCR solutions for LASS4

LASS 4	
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 BCR/ABL

BCR/ABL	
Reaction Mixture	Amount (µL)
PCR grade water	29.7
Reaction buffer (10x)	5
MgCl2 (25 mM)	5
dNTP (10 mM)	4
BCR-C	1
B2B	1
C5E	1
CA3	1
cDNA	2
Taq DNA Polymerase	0.7
Total Mixture	50

Table 2.8. 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. Cell Proliferation Analysis on K562 and Meg-01 Cells

3.1.1. Cytotoxic Effects of Dasatinib on CML Cells

We have shown anti-proliferative effects of dasatinib on both K562 and Meg-01 cell lines by XTT cell proliferation assay. The cells exposed to increasing concentrations of dasatinib from 0.01 to 100 nM for K562 cells and from 1 to 500 nM for Meg-01 cells and they showed a dose-dependent decrease in proliferation as compared to untreated controls. There were 15, 26, 31, 87, and 88% decrease of cell proliferation when cells are exposed to 0.01, 0.1, 1, 10, 100 nM dasatinib for K562 cells. However in Meg-01 cell line when cells are exposed to 1, 5, 10, 20, 50, 100, 500 nM dasatinib the proliferation is decreased as 0, 8, 17, 24, 46, 83 and 84% as compared to untreated controls. As a result the IC50 of dasatinib is 4 nM and 55 nM for K562 and Meg-01 cells, respectively.

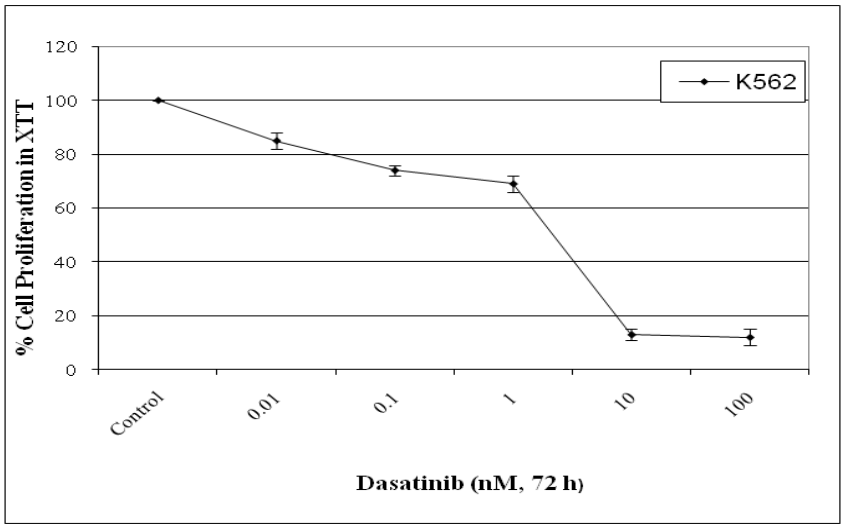


Figure 3.1. Antiproliferative effects of dasatinib on K562 cells.

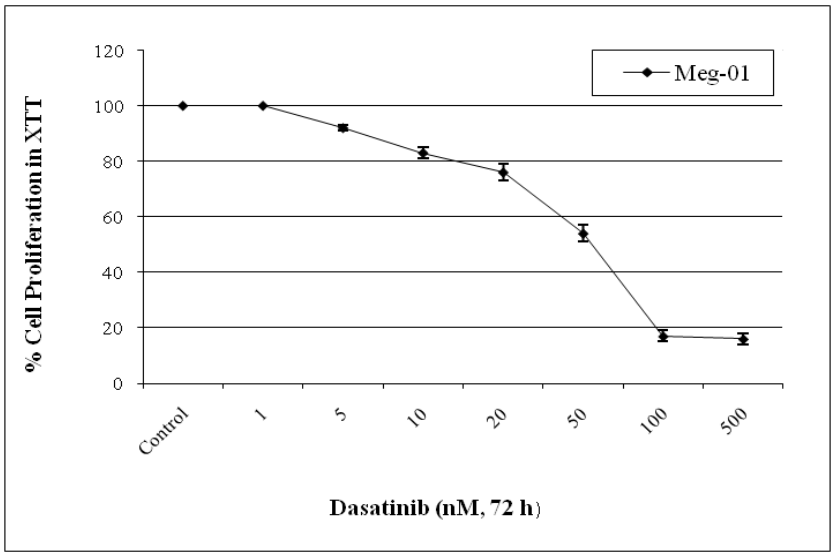


Figure 3.2. Antiproliferative effects of dasatinib on Meg-01 cells.

3.1.2. The Cytotoxic Effects of PDMP, SK-1 Inhibitor or C-8:ceramide Significantly Decreases the Proliferation of Cells

We used ceramide analogues and ceramide metabolizing enzyme inhibitors in combination with dasatinib. C:8 ceramide, an analogue of ceramide, is used in order to increase the intracellular concentrations of ceramide. We supposed to observe an increase in apoptosis when we compare to dasatinib alone. PDMP and SK-1 inhibitors are inhibitory agent of glucosyl ceramide synthase and sphingosine kinase-1 enzyme, respectively. These enzymes decrease the ceramide levels and cause depletion in apoptosis. For this purpose we inhibited the activities of these enzymes in order to provide the accumulation of ceramide in the cell. The cytotoxicity analyses of PDMP, a strong inhibitor for glucosyl ceramide synthase, sphingosine kinase-1 inhibitor and ceramide were carried out with XTT proliferation assay. When we treat K562 cells with increasing concentrations of PDMP from 1 to 50 µM, the IC10 value is found as 20 µM. For SK-1 inhibitor concentrations from 5 to 50 µM is applied and IC10 value is found as 7 µM. Finally, for C8:ceramide from 0.1 to 100 µM concentrations were used and IC50 value is determined as 60 µM. Then we do the same XTT assay procedure for Meg-01 cells and we calculated the IC50 values for each agent. When the Meg-01 cells exposed to increasing concentrations of PDMP from 10 to 100 µM, IC10 value is calculated as 50 µM. The concentrations for SK-1 inhibitor are as 1, 5, 10, 50, 100 µM

and the IC10 value is determined as 5 µM. The IC50 value for C:8 ceramide is found as 70 µM when we treat Meg-01 cells with increasing ceramide concentrations.

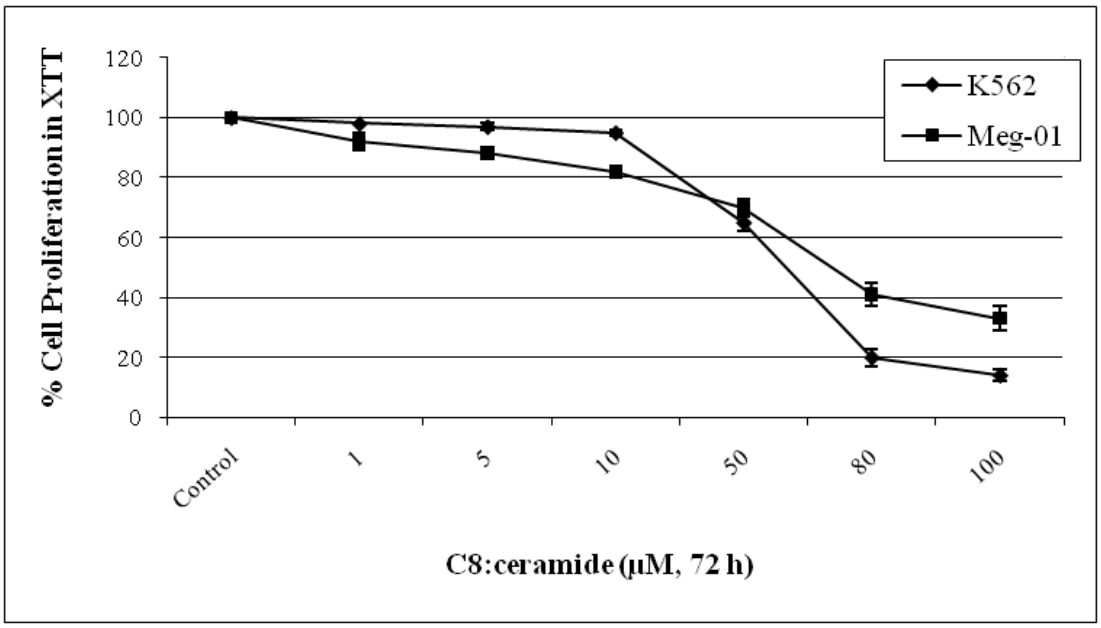


Figure 3.3. Effects of ceramide analog, C:8ceramide on proliferation of K562 and Meg-01 cells.

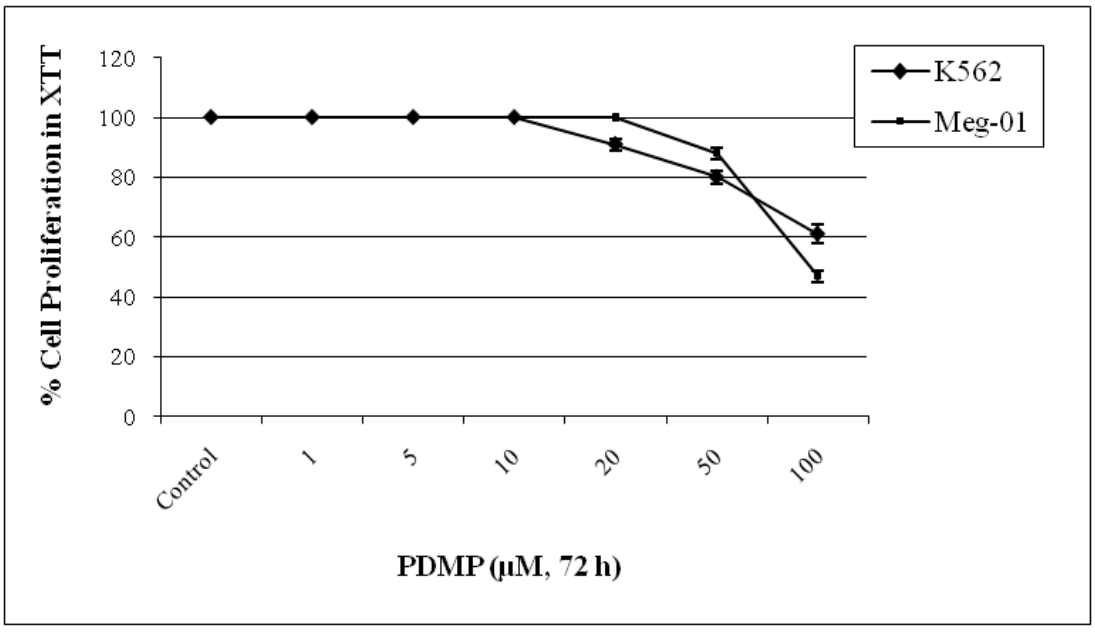


Figure 3.4. Effects of GCS inhibitor, PDMP, on proliferation of K562 and Meg-01 Cells.

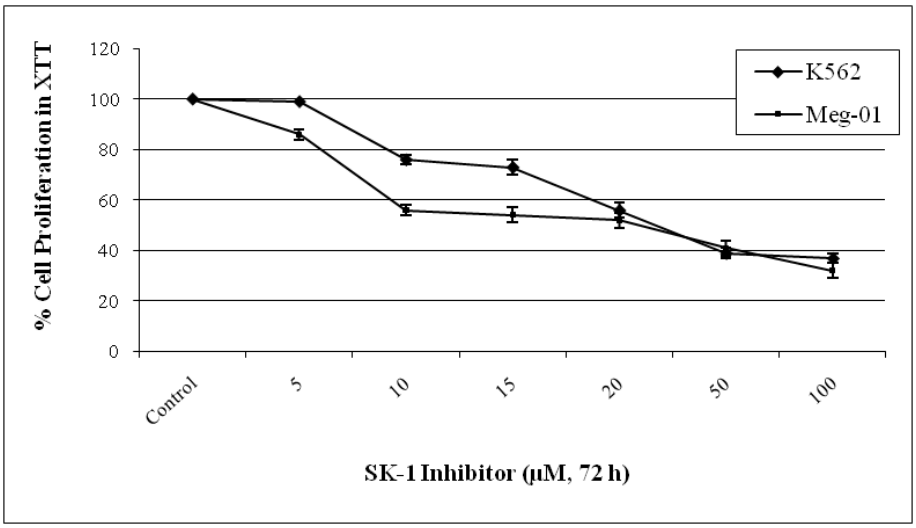


Figure 3.5. Effects of SK-1 inhibitor on proliferation of K562 and Meg-01 Cells.

3.1.3. Antiproliferative Effects of Dasatinib/C8:ceramide, /PDMP, and /SK-1 Inhibitor Combinations on CML Cells

The possible synergistic antiproliferative effects of combination of dasatinib with the ceramide analogue and inhibitor of ceramide clearance enzymes were determined by XTT assay. IC50 concentration of C8:ceramide, and IC10 concentrations of PDMP and SK-1 inhibitor were applied to K562 and Meg-01 cells in combination with increasing concentrations of dasatinib for 72 hours. IC50 concentrations of C8:ceramide was chosen based on its inhibitory effects on proliferation while IC10 values of PDMP and SK-1 inhibitor were preferred because at these concentrations they only inhibit enzyme activity but has no effect on proliferation.

The combinatorial studies for dasatinib and these chemicals are also carried out. K562 cells are exposed to PDMP or SK-1 inhibitor or ceramide and dasatinib. The combination of dasatinib with PDMP decreases the IC50 value of dasatinib significantly. When we treat the cells with 0.1, 1, 10, 100, 500 nM dasatinib with the 20 µM PDMP, IC10 value, the proliferation decreases 42, 49, 91, 91, 92% and the IC50 value of dasatinib is decreases to 1.2 nM from 4 nM. SK-1 inhibitor also decreases the cell proliferation in a dose-dependent manner. When the concentrations 0.1, 1, 10, 100, 500 nM of dasatinib are exposed to K562 cells with 7 µM, there were 40, 45, 85, 88 and 96% decreases of proliferation is observed. Finally for ceramide, the combinatorial studies respond very effectively. The increasing concentrations of dasatinib is applied as

0.01, 0.1, 1, 10, 100 nM with 60 µM C:8 ceramide and the decreases of proliferation are 72, 76, 80, 90 and 92% which is very significant.

When we do the same combinational studies for Meg-01 cells it is observed that combinational therapies are effective. When we treat Meg-01 cells with PDMP in a dose-dependent manner, 1, 5, 10, 20, 50, 100, 500 nM dasatinib is used with 50 µM PDMP and the cell proliferation is decreased by 67, 76, 77, 78, 80, 88 and 93% while combination with 5 µM SK-1 inhibitor resulted in 38, 51, 62, 75, 76, 92 and 100% decreases in cell proliferation, respectively. When we combine 1, 5, 10, 20, 50, 100 and 500 nM dasatinib with 70 µM C8:ceramide the decreases in proliferation are observed as 72, 76, 78, 81, 82, 88 and 94%.

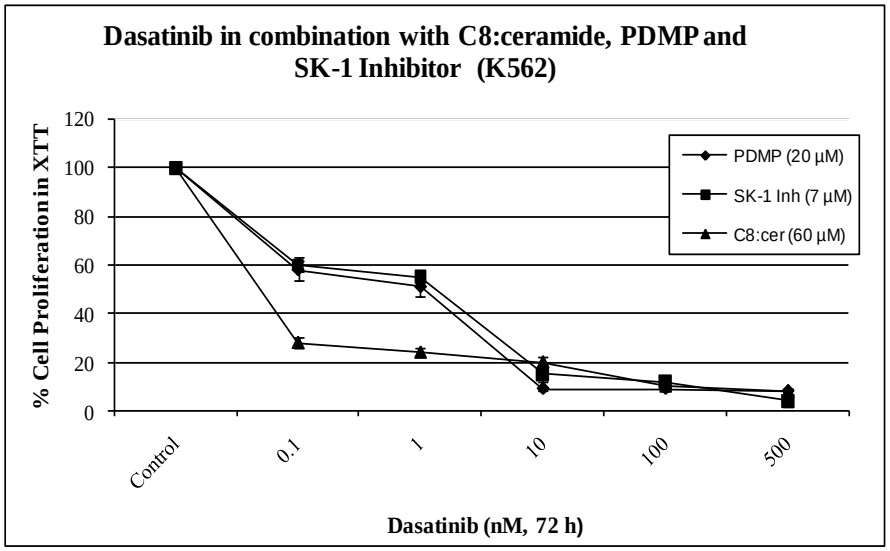


Figure 3.6. Effects of dasatinib/C8:ceramide, /PDMP, and /SK-1 inhibitor combinations on proliferation of K562 cells.

Similar set of experiment were performed for Meg-01 cells. The results also demonstrated that there were synergistic antiproliferative effects of increasing concentrations of dasatinib (1-500 nM) with C8:ceramide, PDMP, and SK-1 inhibitor as compared to any agent alone and to untreated control group.

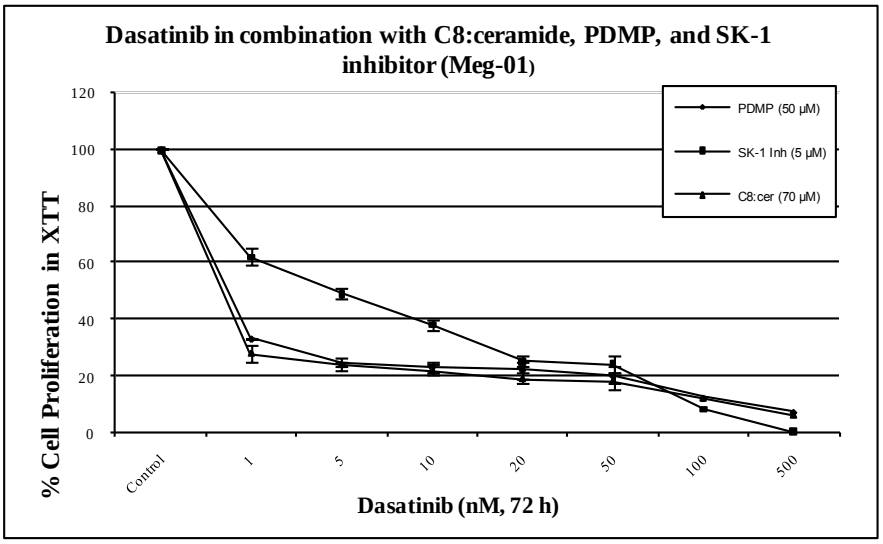


Figure 3.7. Effects of dasatinib/C8:ceramide, /PDMP, and /SK-1 inhibitor combinations on proliferation of Meg-01 cells.

As shown in Figure 3.7., the CI values of dasatinib in combination with C8:ceramide, PDMP and SK-1 inhibitor were 0.00160, 0.26330 and 0.26330, respectively, in K562 cells. Combinations of dasatinib with C8:ceramide, PDMP and SK-1 inhibitor gave the CI values of 0.00258, 0.00253, and 0.23522 respectively, in Meg-01 cells (Figure 3.8). A CI value <1 indicates a synergistic effect (0.1-0.5 strong synergism; <0.1 very strong synergism). The results demonstrated that increasing intracellular concentrations of ceramide in dasatinib applied CML cells resulted in strong or very strong synergism.

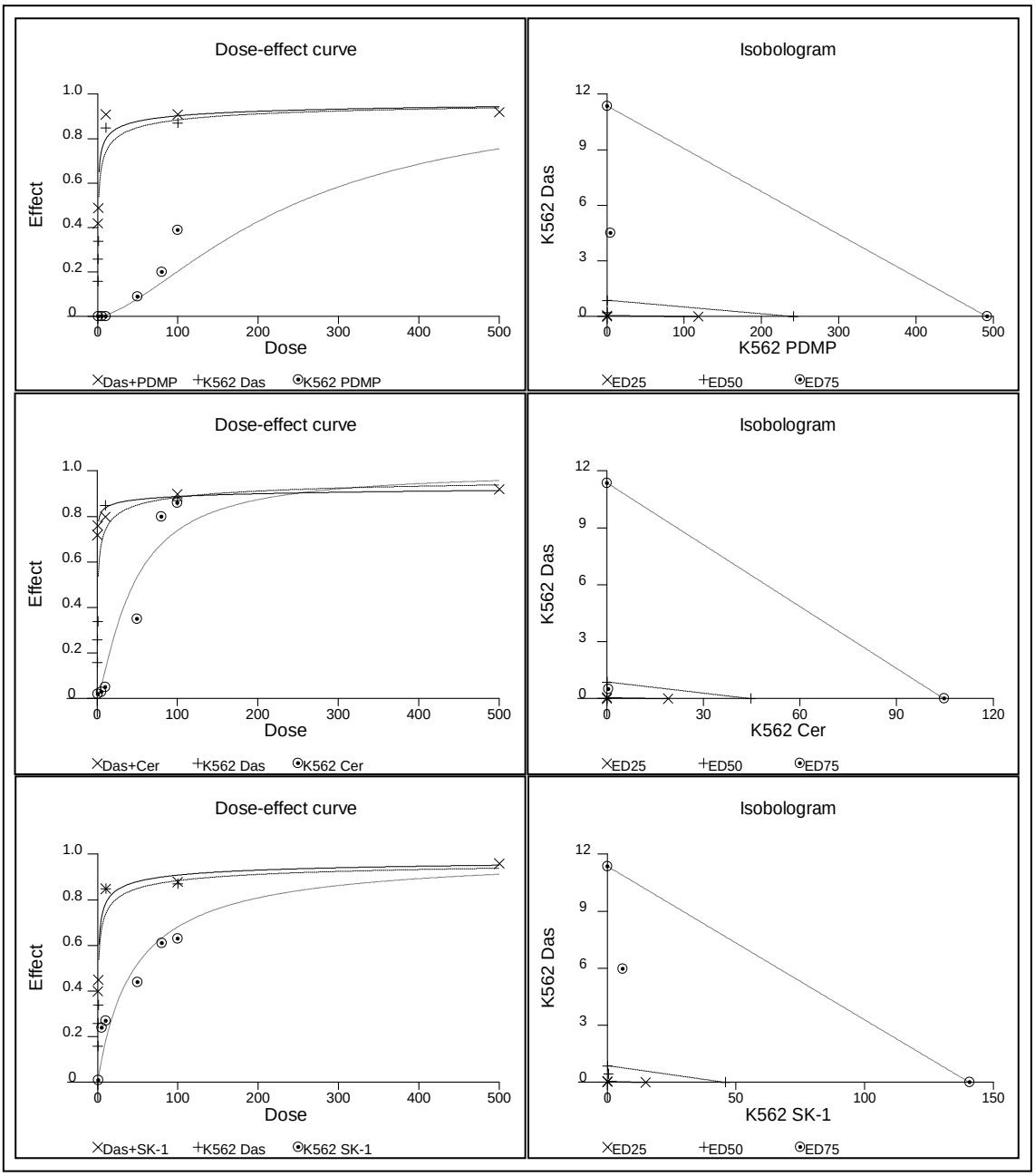


Figure 3.8. Isobalogram Analysis of combination of dasatinib with C8:ceramide, PDMP and SK-1 inhibitor in K562 cells.

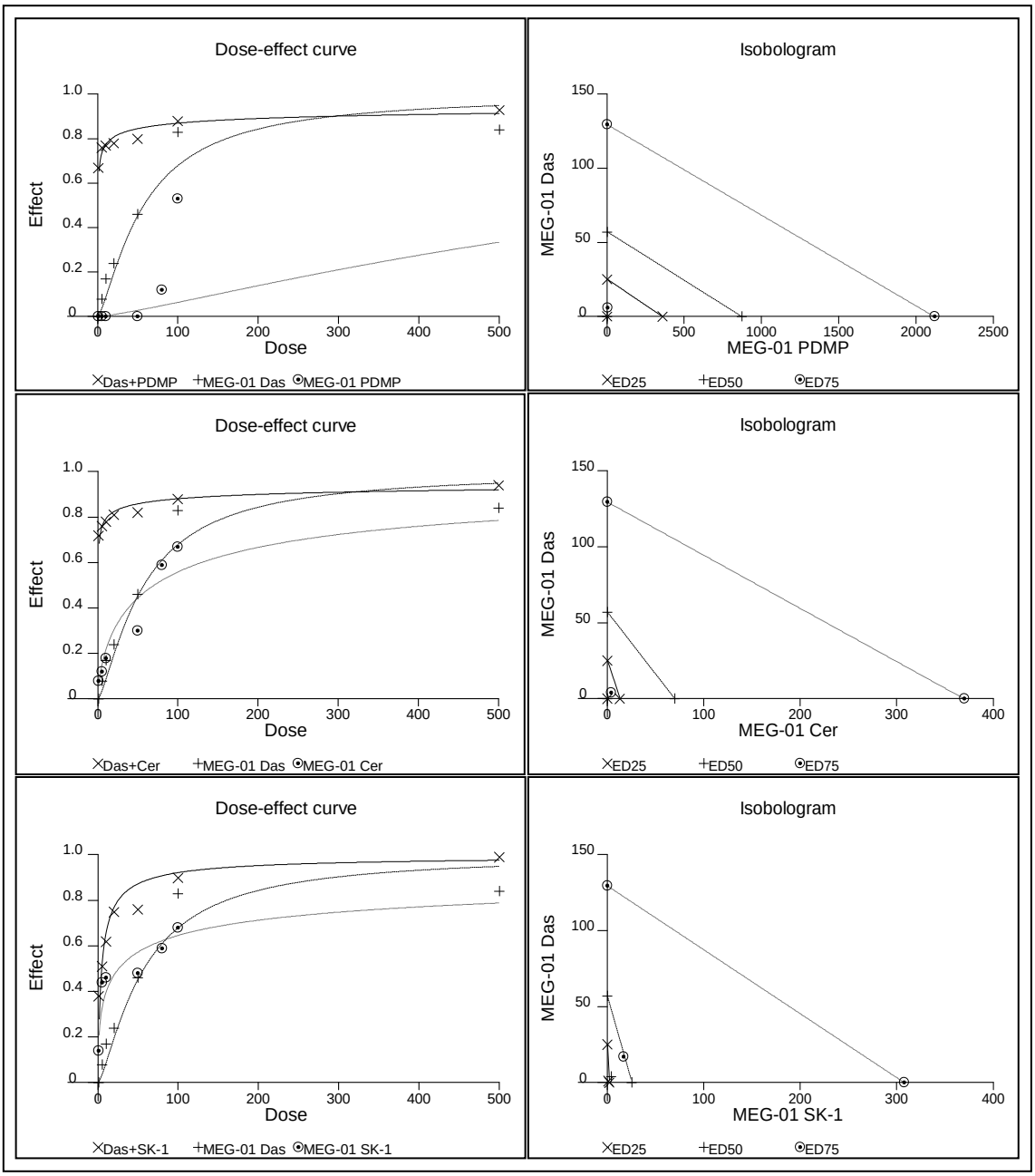


Figure 3.9. Isobalogram Analysis of combination of dasatinib with C8:ceramide, PDMP and SK-1 inhibitor in Meg-01 cells

3.2. Synergistic Effects of Dasatinib and C8:ceramide, PDMP or SK-1 Inhibitor Significantly Decreases Mitochondrial Membrane Potential of K562 and Meg-01 Cells and Induces Apoptosis

The combinational effects of dasatinib with PDMP, SK-1 inhibitor and C8:ceramide were observed with JC1 MMP kit. K562 cells were treated with 0.1-, 1 nM dasatinib and 20 µM PDMP, 7 µM SK-1 inhibitor or 60 µM ceramide alone and the combinations of these chemicals with the same concentrations of dasatinib. We treated K562 cells with 0.1, 1 nM dasatinib and 20 µM PDMP, 7 µM SK-1 inhibitor or 60 µM ceramide. We observed 1.7-, 1.71-, 2.0-, 2,81-, 4.4- fold increases in cytoplasmic/monomeric JC-1 in 20 µM PDMP alone, 0.1-, 1 nM dasatinib and the combinations of PDMP with the same concentrations of dasatinib, respectively, as compared to untreated controls. When we treat K562 cells with 7 µM SK-1 inhibitor and the same concentrations of dasatinib we determined 1.25-, 1.7-, 2.0-, 2.11-, 2.6- fold increases in cytoplasmic/monomeric JC-1 in 7 µM SK-1 inhibitor alone and 0.1-, 1- nM dasatinib and the combination of dasatinib and SK-1 inhibitor, respectively when we compared to untreated controls. Finally we determined 1.57-, 1.70-, 2.0-, 31.3- and 36.1- fold increases in cytoplasmic/monomeric JC-1 in ceramide alone, 0.1-, 1 nM dasatinib and the combinations of ceramide with the same concentrations of dasatinib, respectively.

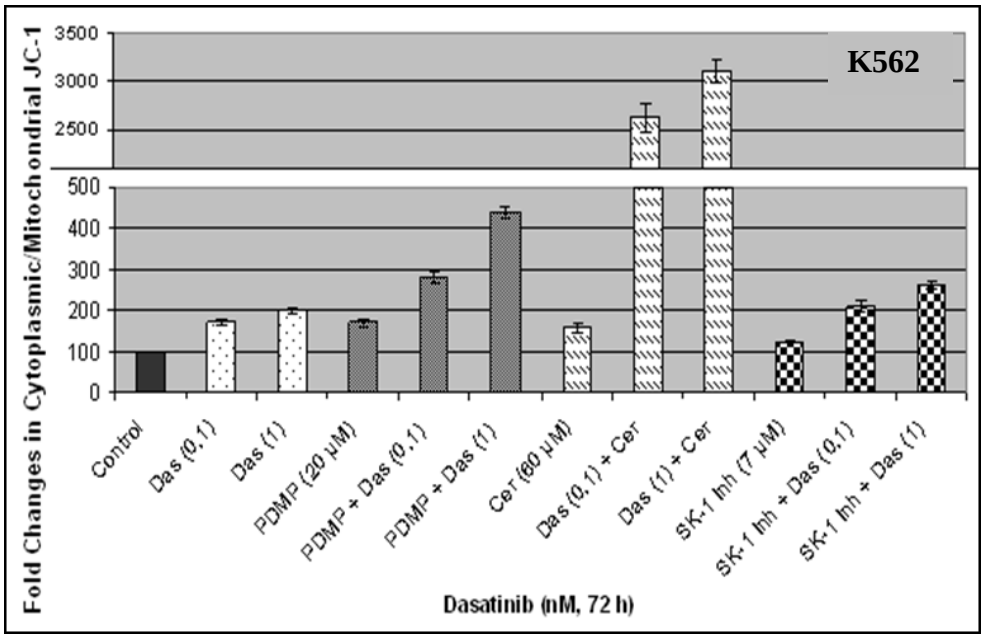


Figure 3.10. Fold changes in cytoplasmic/mitochondrial JC-1 in CML cells treated with combinations of dasatinib with C8:ceramide, PDMP, and SK-1 inhibitor on K562 cells.

We determined 1.13-, 1.18- and 1.55- or 1.25- or 1.22- fold increases in the cytoplasmic/monomeric JC-1 in 1-, 10 nM dasatinib and 50 µM PDMP, 5 µM SK-1 inhibitor or 70 µM ceramide alone treated Meg-01 cells, respectively, as compared to untreated controls. Combinations of the 50 µM PDMP, 7 µM SK-1 inhibitor and 70 µM ceramide, or with the same concentrations of dasatinib increased the cytoplasmic/monomeric JC-1 for 24.5- and 26.6-, 1.28- and 1.55- or 1.27- and 1.48 - fold, respectively, as compared to untreated controls.

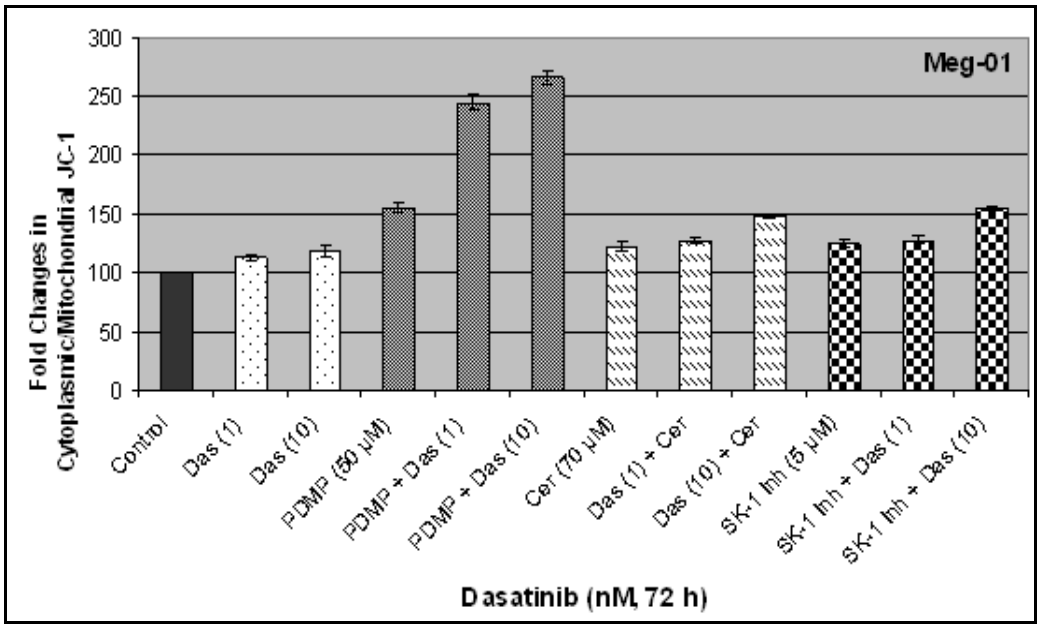


Figure 3.11. Fold changes in cytoplasmic/mitochondrial JC-1 in CML cells treated with combinations of dasatinib with C8:ceramide, PDMP, and SK-1 inhibitor on Meg-01 cells.

3.3. Combinations of Dasatinib with PDMP, SK-1 Inhibitor and C8:ceramide Significantly Increases Caspase-3 Enzyme Activity

The caspase-3 enzyme activity analyses of K562 cells treated with 0.1-, 1 nM dasatinib, and 20 µM PDMP, 7 µM SK-1 inhibitor, 60 µM C8:ceramide alone showed that there were 1.18-, 1.36- and 1.36- or 1.17- and 1.36 -fold increases, respectively, as compared to untreated controls. Besides, we observed that the combinations of 20 µM PDMP µM, 7 µM SK-1 inhibitor or 60 µM C8:ceramide with 1- and 10 µM dasatinib show in 1.64- and 2.41-, 1.53- and 1.6- or 1.84- and 2.41 -fold increases, respectively, in caspase-3 enzyme activities of K562 cells, when compared to untreated controls.

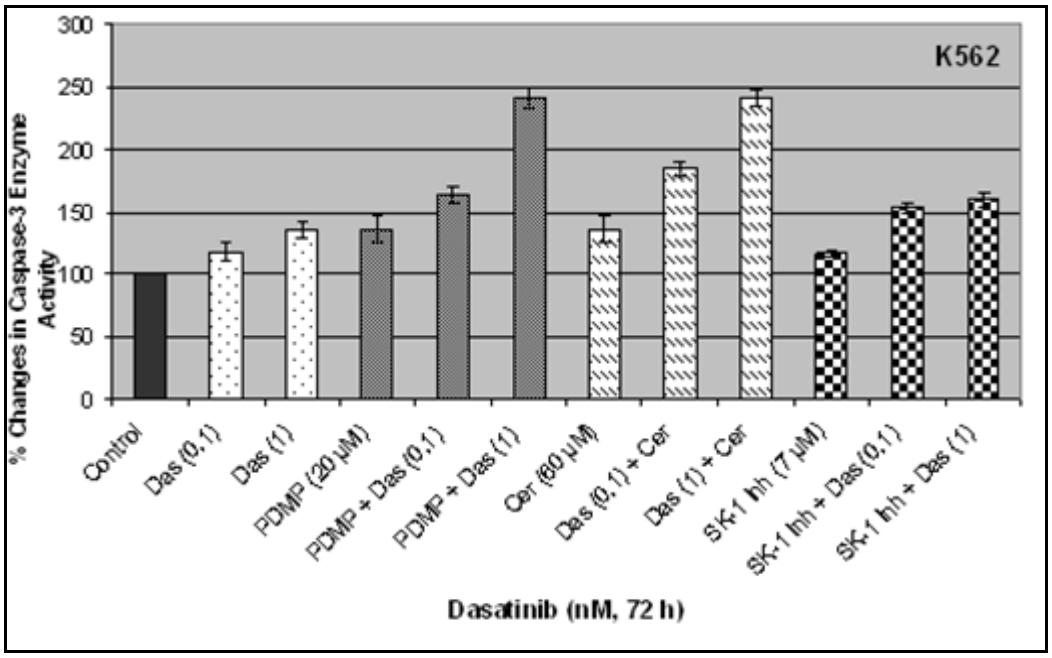


Figure 3.12. Fold changes in caspase-3 enzyme activity in response to coadministration of dasatinib with C8:ceramide, PDMP, and SK-1 inhibitor on K562 cells.

When the caspase-3 enzyme activity is analyzed for Meg-01 cell line we also showed great synergistic effects. When Meg-01 cells treated with 1- and 10 nM dasatinib and 50 µM PDMP, 5 µM SK-1 inhibitor and 70 µM C8:ceramide we observed 1.3-, 1.52-, 1.40-, 1.20- and 1.24- fold increases, respectively when compared to untreated controls. When the combinational treatment of Meg-01 cells with 50 µM PDMP, 5 µM SK-1 inhibitor and 70 µM ceramide with 1- and 10- nM dasatinib resulted in 1.81- and 4.38-, 1.60- and 1.95- or 1.94- and 3.58- fold increases in caspase-3 enzyme activity, respectively, as compared to untreated controls.

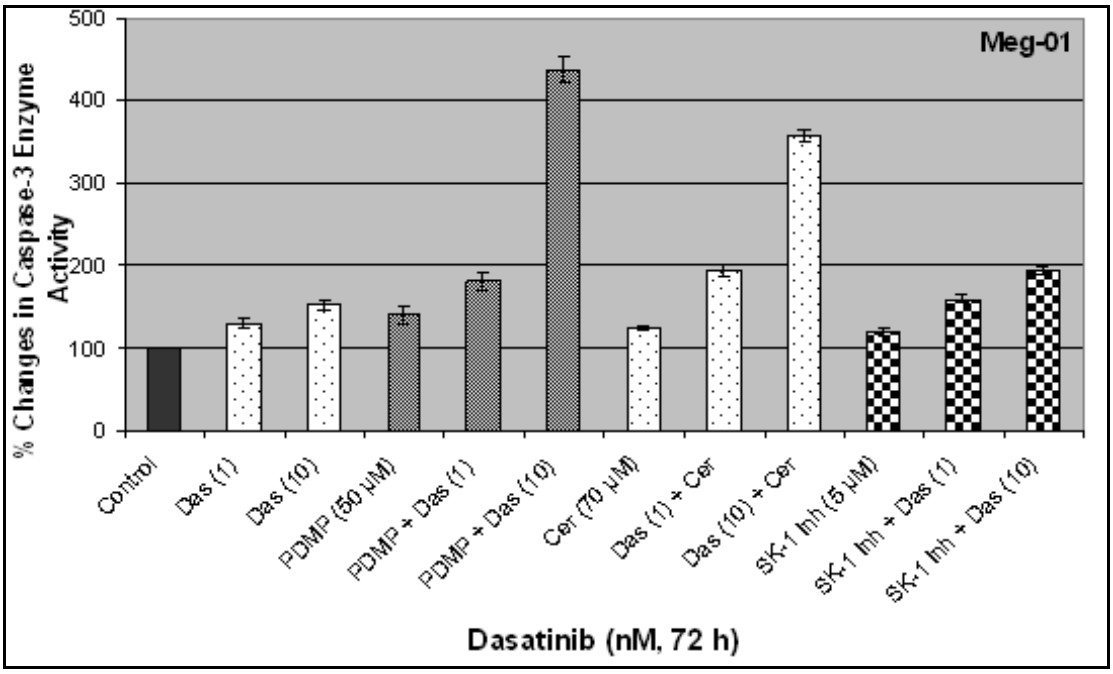


Figure 3.13. Fold changes in caspase-3 enzyme activity in response to coadministration of dasatinib with C8:ceramide, PDMP, and SK-1 inhibitor on Meg-01.

3.4. Gene Expression Levels of Ceramide Metabolism Related Genes by RT-PCR with Dasatinib Treated K562 and Meg-01 Cells

K562 cells were treated with increasing concentrations of dasatinib. The concentrations that are applied to cells were 0.01, 0.1, 1 nM. The cells are incubated with dasatinib for 72 hours and then RNA isolation is carried out with Nucleospin RNA isolation kit. The RNA isolated from cells then converted into cDNA and the cDNAs were amplified by RT-PCR. The amplified products are loaded on a %2 agarose gel. There were slight decreases in xpression levels of SK-1, which is responsible for ceramide degredation, are downregulated in K562 which causes the accumulation of ceramide intracellularly while no change was observed for expression levels of GCS in response to dasatinib. The LASS genes that are responsible for de novo synthesis of ceramide were also examined. The expression levels of LASS1, -2, -4, 5, and -6 are increased with the increasing concentrations of dasatinib as expected.

Meg-01 cells are also exposed to increasing concentrations of dasatinib and the same procedures are done for Meg-01 cells. GCS and SK-1 genes are also dowregulated for in Meg-01 cells exposed to increased doses of dasatinib. For Meg-01 cells the LASS1, -2, 5, and -6 genes are also downregulated in a dose dependent manner.

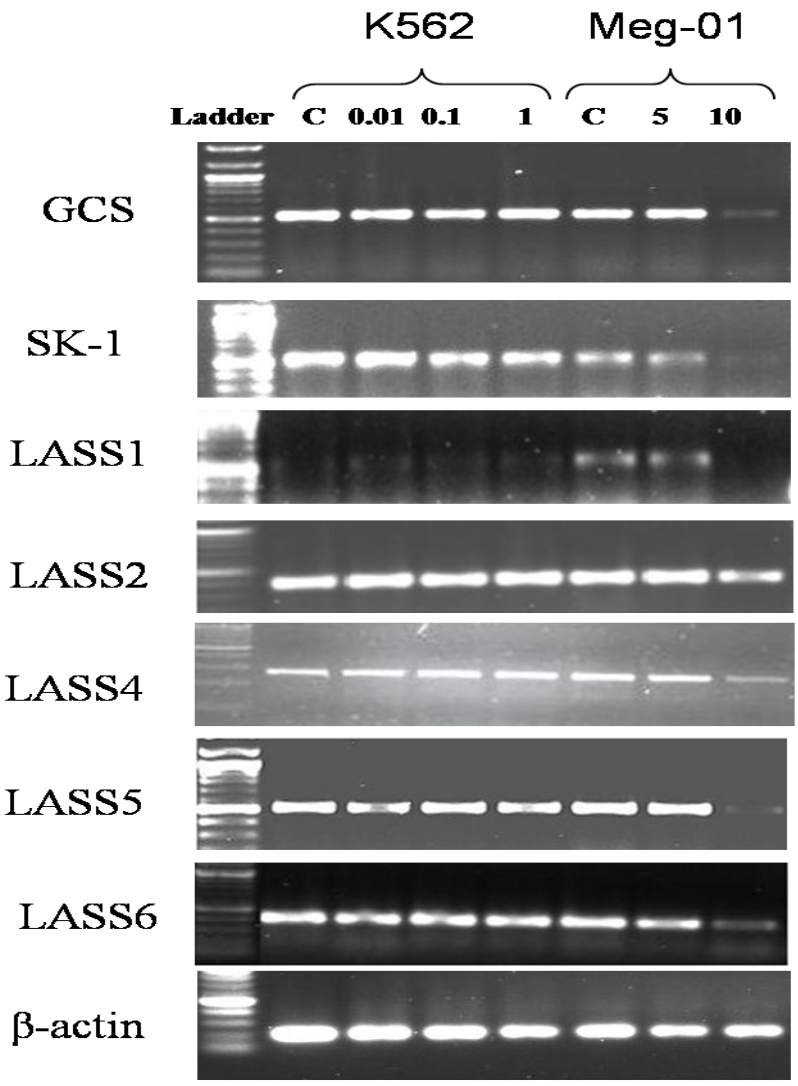


Figure 3.14. Expression levels ceramide generating and ceramide clearance genes in response to dasatinib K562 and Meg-01 cells.

CHAPTER 4

CONCLUSION

Chronic myeloid leukemia is the most important type of disease that opens a gate for molecular medicine. CML is known to be a model for oncology according to its multi step evolution as chronic phase, accelerated phase and blast crisis. CML was a model disease from its discovery.

CML was the first neoplasm associated with a chromosomal aberration, known as the Philadelphia chromosome (Hehlmann, et al. 2007). The explanation of the molecular pathogenesis of this disease led to the development of a therapy for the cause of disease. It is clarified that leukemia is a stem cell disorder. In recent studies it is indicated that leukemic stem cells are progenitor cells that give rise to leukemia. Chronic myeloid leukemia is characterized by the clonal expansion of hematopoietic stem cells which are result from the gene translocations of BCR gene on chromosome 9 and ABL on chromosome 22. This reciprocal translocation causes the production of oncoprotein BCR/ABL. Resultant hybrid protein is the main cause of CML and it is the target of chemotherapeutic agents. As an anti-cancer agent many tyrosine kinases are used in clinic. Imatinib mesylate is the "gold standard" for CML therapy and it is the first choice of chemotherapeutic agent which binds to the ATP binding site of BCR/ABL and causes minimum side effect. Imatinib has the ability to bind inactive form of BCR/ABL protein. The low side effects that make it the first choice for chemotherapy and unfortunately many resistance cases occur during therapy. This resistance stem from the mutations that came out in BCR/ABL kinase domain, catalytic domain, activation domain and ATP binding domain. Resistance occur more than one reason. First one is the mutations that occur in imatinib binding sites but not in active kinase sites generate high rates of resistance. The other resistance mechanism is that imatinib substrate for the multidrug resistance protein P-glycoprotein. Since, imatinib is a substrate for this transport protein and transported out of the cell, decreased levels of imatinib causes problems. The third mechanism is alpha-1-acidglycoprotein binding to imatinib. This interaction between the drug and the protein decreases the intracellular levels of drug. The gene amplification of BCR/ABL is another cause of resistance

because the amplification is related with the increased levels of Ph chromosome and again decreased levels of imatinib effect. The last mechanism is BCR/ABL independent. CML cases are approximately %95 Ph+ but other kinases can also a reason for CML progression, such as SRC family kinases. Mutations that came out alters the oncoprotein in an active form, in a form that imatinib can no longer bind. Because of all these problematic reasons secondary tyrosine kinase inhibitors are needed to be discovered. Dasatinib (BMS-354289) is a second generation tyrosine kinase inhibitor that not only BCR/ABL but also SFK, c-KIT, PDGF. Dasatinib can bind both inactive and active form of BCR/ABL which is an advantage against imatinib. This second generation TKI is active against most of the mutations except T315I point mutation and downregulates BCR/ABL. Dasatinib is effective against not only hematological malignancies but also on solid tumors. There are many studies that are showing that dasatinib effectively inhibits the progression of breast, lung, prostate, head and neck cancers. Unlike imatinib dasatinib is not substrate of P-glycoprotein and also dasatinib can bind to 33 amino acids at the ATP binding site of BCR/ABL whereas imatinib can bind to only 21. Despite the fact that dasatinib is much more efficient than imatinib resistance to dasatinib cases are also present. Dasatinib has no inhibitory effect against the mutation T351I which is the result of the change of tyrosine residue at the position of 315 with isoleucine. Also the mutations at phenylalanine 317 changing into isoluecine or valine or leucine or cysteine (F317I-I-L-C) causes dasatinib resistance.

Lipid metabolism is intimately related to each other, generating an interconnected network. One of the subclass of lipids is sphingolipids which have very important roles in a vast numbers of cellular processes. Sphingolipids function as bioactive mediators of different cellular processes, mostly proliferation, survival, differentiation and apoptosis, besides being structural components of cellular membranes. Involvement of sphingolipid metabolism in cancerogenesis was demonstrated in solid tumors as well as in hematological malignancies (Ricci, et al. 2006).

Bioactive lipids are ceramide, sphingosine, sphingosine-1‑phosphate (S1P), ceramide-1‑phosphate and lyso-sphingomyelin. They have roles in the regulation of cell growth, death, senescence, adhesion, migration, inflammation, angiogenesis and intracellular trafficking. Their synthesis of sphingolipids starts with the condensation of serine and palmitate to form 3-keto-dihydrosphingosine through the action of serine

palmytoil transferase that is called *de novo* synthesis. Ceramide is a very important molecule which has roles in proliferation, cell cycle arrest, differentiation and senescence. Ceramide is known as a very strong apoptotic molecule. The importance of ceramide is increasing day by day because of the vital importance of this molecule. While ceramide is a strong apoptotic molecule, the conversion of ceramide into antiapoptotic sphingosine-1-phosphate and glucosylceramide by sphingosine kinase-1 (SK-1) and glucosyl ceramide synthase (GCS), respectively, causes resistance in cancer cells. The involvement of S-1-P in cancer resistance is reported in many cases in literature. It was shown that SK-1 is a prosurvival, antiapoptotic and a migratory factor for breast cancer cells. (Sarkar, et al. 2005) Besides, high levels of SK-1 are observed in tumors from lung cancer patients. (Johnson, et al. 2005) In vivo studies showed that using inhibitors of SK-1 inhibits the cell proliferation in animal models. (French, et al. 2006) Glucosyl ceramide synthase is the enzyme that transfers glucose to ceramide (Bleicher and Cabot 2002). There are very strong evidences that show the correlation of GCS and drug resistance. (Senchenkov, et al. 2001) Another study shows the obvious relationship between GCS and drug resistance. They introduce GCS gene into sensitive MCF-7 breast cancer cell line and they observed an apparently increase in the GCS expression levels and as a result the cells became adriamycin and exogenous ceramide resistant. This study shows that GCS activity adjusts drug resistance (Lui, et al. 1999).

According to our results we determine the IC50 values from proliferation plots for K562 and Meg-01 cell lines as 4 nM and 55 nM, respectively. Thus, we can say that dasatinib has a more inhibitory effect on K562 cells. When we compare the inhibitory effects of exogenous C-8 ceramide, PDMP and SK-1 inhibitor on K562 and Meg-01 cells we observe different effect on both cell lines which is seen in Figure 2. Although both cell lines are belong to the same disease, the effects are different. This result supports the idea that the effect of same drug could be different on different patients.

On the other hand our study demonstrated that the inhibition of SK-1 and GCS with SK-1 inhibitor and PDMP, respectively, to overcome resistance in K562 and Meg-01 CML cells. Besides, to enhance apoptosis, we treat the cells with C:8 ceramide to provide the accumulation of intracellular ceramide. For both cell lines we observed very strong synergistic cell proliferation inhibition, decreases in mitochondrial membrane potential and increases in caspase-3 enzyme activity when we combined dasatinib with these agents. Also we proved that dasatinib induces apoptosis of K562 and Meg-01 cell through ceramide metabolism which is proved not only with biochemical assays but

also by gene expression levels for the first time. This is a new data about dasatinib that it induces apoptosis through ceramide metabolism and when we manipulate ceramide metabolism we observed increased levels of apoptosis.

In order to understand the mechanisms of dasatinib-induced apoptosis we examined the expression patterns of ceramide metabolizing genes in CML cells exposed to dasatinib. RT-PCR results revealed that there were significant increases in expression levels of LASS1, LASS2, LASS4, LASS5, and LASS6 (ceramide synthase) genes in response to dasatinib in K562 cells in a dose dependent manner while SK-1 is downregulated and no change is obwerved in expression pattern of GCS as compared to untreated controls. However, in contrast to our expectations, dasatinib application downregulated expression levels of LASS genes and downregulated antiapoptotic and prosurvival GCS and SK-1 genes in Meg-01 cells. The results of RT-PCR analyses may explain the sensitivity of K562 cells to dasatinib as compared to Meg-01 cells. While IC50 value of dasatinib in K562 cells was 4 nM, it was 55 nM for Meg-01 cells. While dasatinib downregulates expression levels of SK-1 slightly, it shows no effect on GCS and increases expression levels of LASS genes that result in increased generation and accumulation of apoptotic ceramides in K562 cells. However, in Meg-01 cells, since dasatinib downregulates both ceramide generating genes, LASS genes, and ceramide clearence genes, SK-1 and GCS, there may be no incrase in intracellular concentrations of apoptotic ceramide. On the other hand, there may be some other unknown mechanisms of dasatinib-induced apoptosis regulated differently in K562 and Meg-01 cells that results in differences in their sensitivity to dasatinib.

It is obvious that development of drug resistance is the major obstacle to fight against cancer. Due to the fact that single drug therapy is not efficient, the treatment protocols are changing in a way that combinational therapy gains importance. For this purpose increasing the effectiveness of the drug is the main purpose of our study. In cancer cells the ceramide balance is disturbed, but a strategy that adjusts this balance would be very powerful. We treat the cells with C:8 ceramide in addition to dasatinib in order to enhance apoptosis and as a result we have observed significant induction of apoptosis. Also, inhibition of conversion of apoptotic ceramide to anti-apoptotic glucosyle ceramide or sphingosine-1-phophate increased cytotoxic effects of dasatinib as compared to any agent alone. These results are proved by decreases in cell proliferation, decreases in MMP and increases in caspase-3 enzyme activities.

Taking all together our data here presents new mechanistic information about ceramide metabolism in dasatinib induced apoptosis and the involvement in mitochondrial membrane potential and caspase-3 enzyme activity on K562 and Meg-01 CML cells. Manipulation of sphingolipid metabolism for CML treatment can open a new gate for combinational chemotherapy.

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