THE MECHANISMS RESPONSIBLE FOR NILOTINIB RESISTANCE IN HUMAN CHRONIC MYELOID LEUKEMIA CELLS

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ABSTRACT

THE MECHANISMS RESPONSIBLE FOR NILOTINIB RESISTANCE IN HUMAN CHRONIC MYELOID LEUKEMIA CELLS

Multidrug resistance remains a significant obstacle to successful chemotherapy. The ability to determine the possible resistance mechanisms and surmount the resistance is likely to improve chemotherapy. Nilotinib is a very effective drug in the treatment of sensitive or Imatinib resistant patients. Although very successful hematologic and cytogenetics responses have been obtained in Nilotinib-treated patients, in recent years resistance cases were observed.

The main objective of the project is to understand the mechanisms underlying multidrug resistance to Nilotinib to provide new targets for the treatment of chronic myeloid leukemia (CML). In this study, continuous exposure of cells to step-wise increasing concentrations of Nilotinib resulted in the selection of cells resistant to 50 nM Nilotinib and referred to as K562/NIL-50. Expression analyses of BCR-ABL gene demonstrated BCR-ABL was upregulated in resistant cells as compared to parental sensitive cells. However, nucleotide sequence analyses of ABL kinase gene revealed that there was no mutation in Nilotinib binding region of the gene in resistant cells. There was also an increase in expression levels of MRP1 gene in resistant cells, which transports the toxic substances outside of cells. Besides, Bax, which is one of the apoptosis inducing genes, was dowregulated in resistant cells. In addition to this, in resistant cells, while GCS and SK-1 genes were overexpressed, decrease in expression levels of LASS1 gene was observed.

In conclusion, we determined mechanisms involved in Nilotinib resistance in CML *in vitro*. Targeting this mechanisms, besides inhibition of BCR-ABL may be a good way of treatment of CML.

ÖZET

İNSAN KRONİK MİYELOİD LÖSEMİ HÜCRELERİNDE NİLOTİNİB DİRENCİNDEN SORUMLU MEKANİZMALAR

Çoklu ilaç direnci,başarılı kemoterapinin önünde önemli bir engel olmaya devam etmektedir. Olası direnç mekanizmalarını belirleyebilme ve direncin üstesinden gelebilme yetisi kemoterapinin etkinleştirilmesi ile mümkün hale gelmektedir. Nilotinib duyarlı veya Imatinib dirençli hastaların tedavisinde çok etkili bir ilaçtır. Nilotinib ile tedavi edilmiş hastalarda çok başarılı hematolojik ve sitogenetik yanıtlar elde edilmesine rağmen, son dönemlerde direnç vakaları gözlemlenmiştir.

Projenin temel hedefi, kronik miyeloid lösemide (KML) yeni hedefler bulmak için Nilotinib çoklu direncinin altında yatan gerçek mekanizmaları anlamaktır. Bu çalışmada, K56 insan kronik myeloid lösemi hücrelerinde Nilotinibin yol açtığı apoptoza karşı geliştirilen direnç mekanizmaları incelenmiştir. Hücrelerin sürekli artan dozlarda Nilotinibe maruz bırakılmaları ile 50 nM Nilotinibe dirençli hücreler elde edilmiştir ve K562/NIL-50 olarak adlandırılmıştır. Atasal duyarlı hücrelerle kıyaslandığında dirençli hücrelerde, BCR-ABL gen ifade analizleri BCR-ABL nin upregüle olduğunu gösterdi. Ancak, ABL kinaz geni nükleotid dizisi analizleri dirençli hücrelerde ABL geninin Nilotinib bağlanma bölgesinde hiçbir mutasyon olmadığını göstermiştir. Ayrıca, dirençli hücrelerde, toksik maddeleri hücrenin dışına taşıyan MRP1 gen ifade düzeyinde artış vardı. Yanı sıra, apoptotozla ilgili genlerden biri olan Bax' ın, dirençli hücrelerde ifadesi baskılanmıştır. Buna ek olarak, dirençli hücrelerde GSS and SK-1 genleri aşırı ifade edilirken, LASS1 geninin ifadesinde azalma görülmüştür.

Sonuç itibariyle, biz Nilotinib dirençliliğiyle ile ilgili mekanizmaları KML de *in vitro* olarak belirledik. BCR-ABL nin inhibisyonunun yanısıra bu mekanizmaların hedeflenmesi KML tedavisinde iyi bir yol olabilir.

To my family...

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CHAPTER 1

INTRODUCTION

1.1. Chronic Myeloid Leukemia

Chronic Myeloid Leukemia (CML) is a hematopoietic stem cell disorder characterized by immature white blood cell count (Druker 2002). CML was the first haematological malignancy to be associated with a specific genetic lesion (Goldman and Melo 2003) and so it became a model disease for neoplastic diseases (Brien, et al. 2004). CML accounts for 20% of adult leukemias and its incidence is approximately 1-2 per 100,000 population per year. As with all leukemias, CML is more commonly seen in men than in women with a 2:1 ratio. CML is generally a disease of the older people with a median age at diagnosis of around 65 years (Faderl, et al. 1999).

The progression of CML has three stages which are chronic phase, accelerated phase and blast crisis. Chronic phase is the very early stage and approximately 85% of patients are diagnosed in this phase. The second phase is accelerated phase in which the levels of immature white blood cells are higher than chronic phase at about 5-30 %. The final and the most serious phase of CML is blast crisis. There are mostly immature white blood cells in the blood and bone marrow (Aguilera, et al. 2009, Cortes 2004). As the disease progresses through the accelerated phase and into the blast crisis, there are many changes that are not known exactly. However, they can be classified as follows BCR-ABL expression, arrest of differentiation, genomic instability, DNA repair (Shtivelman, et al. 1985) and additional chromosomal abnormalities (Druker 2002).

CML is driven by a specific chromosomal abnormality called the Philadelphia (Ph) chromosome. The Ph chromosome is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22. This exchange brings together two genes: the BCR (breakpoint cluster region) gene on chromosome 22 and the proto-oncogene ABL (Ableson leukemia virus oncogene) on chromosome 9 (Wong, et al. 2004). The resulting fusion gene BCR-ABL codes for a protein with constitutive

tyrosine kinase activity which activates signal transduction pathways, leading to uncontrolled cell growth and reduced apoptosis.

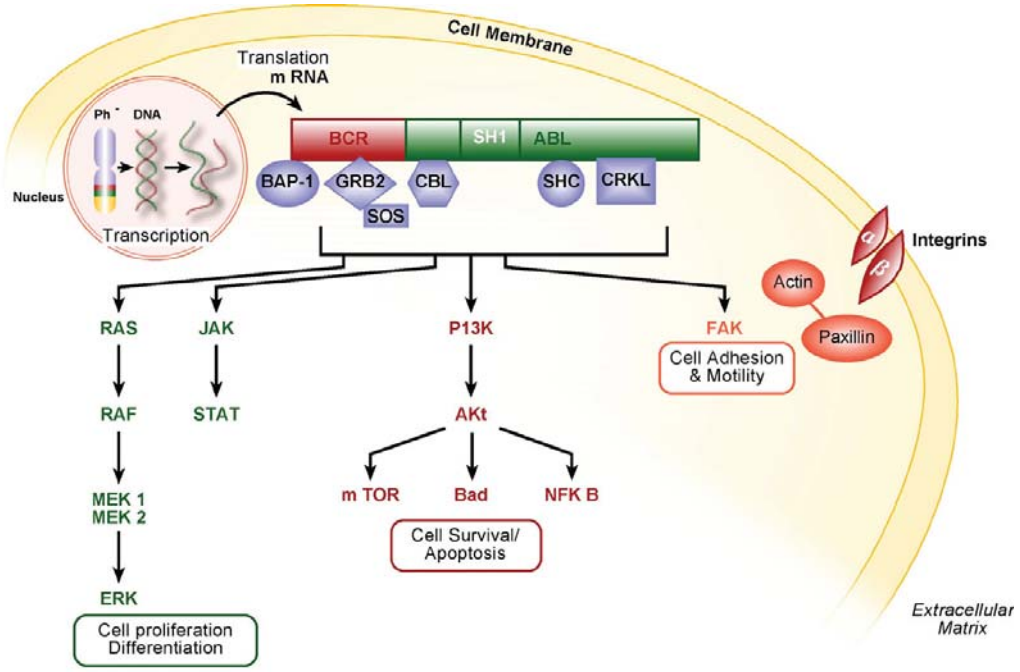

Figure 1.1. Molecular signaling pathways that BCR-ABL affects
(Source: Frazer, et al. 2007)

1.2. Molecular Biology of CML

CML is characterized by a reciprocal translocation between chromosomes 9 and 22. However, 5% of CML patients don't have the reciprocal translocation which results in juxtaposition of 3' sequences from the Abl-proto-oncogene on chromosome 9, with the 5' sequences of the truncated Bcr (breakpoint cluster region) on chromosome 22. Fusion mRNA molecules of different lengths are produced and subsequently transcribed into chimeric protein products. Although these oncogenic proteins can be 190, 210 or 230 kDA depending on the breakpoint on the BCR gene (Lugo, et al. 1990), the most common form is 210 kDA (Eren, et al. 2000). The SH1 domain of ABL encodes a non-receptor tyrosine kinase. Protein kinases are enzymes that transfer phosphate groups from ATP to substrate proteins, thereby governing cellular processes such as growth and differentiation. In normal conditions, the native ABL kinase is located mainly in the

nucleus, while the BCR-ABL fusion protein is located in the cytoplasm (Sawyers 1999). The regulation of tyrosine kinase activity is controlled tightly, and if not maintained, deregulated kinase activity can lead to transformation and malignancy (Goldman and Melo 2003). The portion of ABL responsible for governing regulation of the SH1 domain is lost during the reciprocal translocation. The addition of the *BCR* sequence constitutively activates the tyrosine kinase activity of the SH1 domain that is the most crucial for oncogenic transformation. Its activity disturbs the normal physiological functions of the ABL enzyme, as it interacts with a number of effector proteins (Vigneri, et al. 2001). Thus, BCR-ABL is the most ideal and attractive target for molecular-targeted therapy.

There are also possible secondary translocations that are occurred in CML. The first one is chromosome 8 trisomy which causes an overexpression of Myc gene located on 8q24. 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, Sawyer and his colleagues showed that expression of dominant-negative c-Myc molecules suppressed BCR-ABL dependent transformation (Sawyer, et al. 1992). The other one is Isochromosome i(17q) associated with the loss of the short arm (17p) and duplication of the long arm (17q) that causes the abnormality (Schutte, et al. 1993). Since p53 gene is located on the short arm of chromosome 17. That's why isochromosome 17q is known to be the most common precedent in cancer. This rearrangement results in 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).

1.3. Activated Signaling Pathways

There are three major pathways that have been activated in the malignant transformation by BCR-ABL, namely constitutively active mitogenic signaling (Puil, et al. 1994), altered adhesion to extracellular matrix (Gordon,et al. 1987) and inhibited apoptosis (Bedi, et al. 1994).

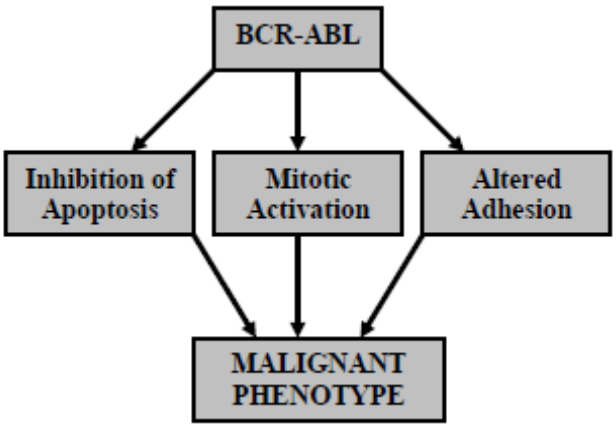

Figure 1.2. Mechanisms implicated in the pathogenesis of CML
(Adapted from Deininger, et al. 2000)

1.3.1. Constitutively Active Mitogenic Signaling

1.3.1.1. Ras and the MAP Kinase Pathways

Several links between BCR-ABL and Ras have been determined. Autophosphorylation of tyrosine 177 provides a docking site for the adapter molecule Grb-2 (Pendergast, et al. 1993) which binds to the Sos protein stabilizing Ras in its active GTP-bound form. Two other adapter molecules, Shc and Crkl can also activate Ras. They are substrates of BCR-ABL and bind it by their SH2 (Shc) or SH3 (Crkl) domains. The Ras pathway is important because no further activating mutations are required, even in the blast crisis phase (Watzinger, et al. 1994).

Stimulation of cytokine receptors such as IL- 3 leads to the activation of Ras and to the subsequent recruitment of the serine threonine kinase Raf to the cell membrane (Marais, et al. 1995). Raf initiates a signaling cascade through the serine threonine kinases Mek1/Mek2 and Erk, which ultimately leads to the activation of gene transcription (Cahill, et al. 1996). The activation of individual paths depends on the cell type, but the MAP kinase system appears to play a central role.

1.3.1.2. Jak-STAT Pathway

Activation of the JAK kinases leads to the activation of STAT transcription factors. For the first time, the Jak-STAT pathway as a activated signaling pathway by BCR-ABL was shown in a study with v-ABL-transformed B cells (Danial, et al. 1995). Constitutive phosphorylation of STAT transcription factors (STAT1 and STAT5) has since been reported in several BCR-ABL-positive cell lines (Ilaria, et al. 1996) and in primary CML cells (Chai, et al. 1997). In addition to this, increased activation of STAT3 has been observed in numerous leukemias (Coffer, et al. 2000). STAT5 activation appears to contribute to malignant transformation (De Groot, et al. 1999). Effect of STAT5 in BCR-ABL transformed cells appears to be primarily anti-apoptotic and involves transcriptional activation of Bcl-XL (Horita, et al. 2000). In contrast to the activation of the Jak-STAT pathway by physiologic stimuli, BCR-ABL may directly activate STAT1 and STAT5 without prior phosphorylation of Jak proteins.

1.3.1.3. Myc Pathway

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. Overexpression of Myc has been demonstrated in many human malignancies and that's why, c-myc can be a diagnostic marker for some cancer types. It is thought to act as a transcription factor. Activation of Myc by BCR-ABL is dependent on the SH2 domain, and the overexpression of Myc partially rescues transformation-defective SH2 deletion mutants whereas the overexpression of a dominant-negative mutant suppresses transformation (Sawyers, et al. 1992). The results obtained in v-ABL-transformed cells suggest that the signal is transduced through Ras/Raf, cyclin-dependent kinases (cdks), and E2F transcription factors that ultimately activate the Myc promoter. Another study from the same group that showed similar results for BCR/ABL transformed murine myeloid cells (Stewart, et al. 1995). It can be said that the effects of Myc in Ph-positive cells are probably not different from those in other tumors. Depending on the cellular context, Myc may constitute a proliferative or an apoptotic signal (Bissonnette, et al.

1992). It is therefore likely that the apoptotic arm of its dual function is counterbalanced in CML cells by different mechanisms, such as the PI3 kinase pathway.

1.3.1.4. Phosphatidyl Inositol-3 Kinase Pathway

Phosphatidyl Inositol-3 (PI3) 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), such as requirement for the proliferation of BCR-ABL positive cells (Skorski, et al. 1995). BCR-ABL forms multimeric complexes with PI3 kinase, Cbl, and the adapter molecules Crk and Crkl, where PI3 kinase is activated. The next relevant substrate in this cascade appears to be the serine threonine kinase Akt (Skorski, et al. 1997). In another report identified the pro-apoptotic protein Bad as a key substrate of Akt through the downstream cascade of the IL-3 receptor (Del Peso, et al. 1997). Phosphorylated Bad is inactive because it is no longer able to bind anti-apoptotic proteins such as Bcl-XL and it is kept back by cytoplasmic 14-3-3 proteins. Altogether this indicates that BCR-ABL might be able to mimic the physiological IL-3 survival signal in a PI3 kinase dependent manner.

1.3.2. Altered Adhesion Properties

CML progenitor cells exhibit decreased adhesion to bone marrow stroma cells and extracellular matrix (Verfaillie, et al. 1997). Adhesion to stroma negatively regulates cell proliferation, and CML cells escape this regulation by virtue of their perturbed adhesion properties. Interferon-a (IFN-a), an old therapeutic agent used in CML, seems to reverse the adhesion defect (Bhatia, et al. 1994). Another data about adhesion molecules, CML cells express an adhesion-inhibitory variant of b1 integrin that is not found in normal progenitors (Zhao, et al. 1997). The valuable property of integrins is capable of initiating normal signal transduction from outside to inside on binding to their receptors (Lewis, et al. 1996). Thus, it can be thought that the transfer of signals that normally inhibit proliferation is impaired in CML cells. Moreover, Crkl is involved in the regulation of cellular motility and in integrin mediated cell adhesion

by association with other focal adhesion proteins such as paxillin, the focal adhesion kinase Fak, p130Cas and Hef1 (Deininger, et al. 2000).

1.3.3. Inhibition of Apoptosis

In response to cellular stress such as DNA damage induced by chemotherapeutic drugs, the cell's mitochondria are triggered to release cytochrome c (a component of the electron transport chain) into the cytosol. Once released, cytochrome c plays a critical role in the formation of a proteolytic cell death machine known as the apoptosome. The formation of the apoptosome results in the activation of a group of zymogenic cysteine proteases (caspases), which carry out the cell death program (Olson *et al*, 2001). Cytosolic cytochrome c initiates apoptosome formation by binding to the adaptor protein Apaf-1 and promoting its oligomerization into a higher-ordered structure. Oligomerization of Apaf-1 then allows binding of the initiator caspase 9, which results in dimerization-induced self-activation (Srinivasula SM, 1998). Once activated, caspase-9 can cleave and activate effectors, caspases 3 and -7, which subsequently cleave a number of cellular substrates. This results in orderly dismantling of the cell and in the hallmark features of apoptosis (Wang *et al*, 2000).

The release of cytochrome c from the mitochondria is tightly regulated by Bcl-2 proteins, a family comprising both pro-apoptotic Bax and Bak and antiapoptotic Bcl-2 and Bcl-XL family members (Danial, et al. 2004). These proteins act as mitochondrial gatekeepers and regulate apoptosis by governing the release of cytochrome c. While pro-apoptotic Bak and Bax promote mitochondrial cytochrome c release, the anti-apoptotic Bcl-2 and Bcl-XL proteins maintain the integrity of the mitochondria to prevent the release of cytochrome c.

Alterations of apoptotic signaling pathways at a number of loci allow malignant cells to evade cell death which is one of the hall marks of cancer (Hanahan, et al. 2000). Before releasing of cytochrome c, BCR-ABL can inhibit apoptosis through regulation of Bcl-2 family members. Specifically, BCR-ABL increases expression of antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL through activation of the transcription factor STAT5 (Sanz, et al. 2002). Despite of all these, BCR-ABL has recently been

reported to be a more effective inhibitor of apoptosis than either Bcl-2 or Bcl-XL (Baran 2007).

1.4. Treatment Strategies for Chronic Myeloid Leukemia

Until interferon was introduced and was found to be better than known cytotoxic chemotherapy in CML, the treatment of choice for chronic phase CML was orally administered chemotherapy with either hydroxyurea or busulphan for a long time (Melo, et al. 2003). Also, allogenic stem cell transplant has been used since the 1970s in the treatment of CML and is the only curative therapy for CML, however, it bears a significant mortality risk (Frazer, et al. 2007). When CML progresses to the accelerated and blastic phase, the available treatments including bone marrow transplantation become less effective.

A major advance in the treatment of CML has the advent of Imatinib, as the first targeted therapy for CML which has shown striking activity in the chronic phase, in the accelerated phase, but less so in the blast phase. This drug will be the first of a new series of small organic compounds designed to inhibit specific molecular sites in the cascades of cellular activation pathways (Buchdunger, et al. 1996). Despite of success of Imatinib, there have been developed resistant to Imatinib in use of long term. In effort to improve on the long term outcomes and overcome the developing resistance, several second generation tyrosine kinase inhibitors are designed and are used widely in CML treatment.

1.5. Targeted Therapies

1.5.1. Imatinib

The SH1 domain responsible for oncogenic transformation is an extremely attractive target in struggling CML. Imatinib (Glivec, Novartis, Basel, Switzerland) is the first tyrosine kinase inhibitor that targets SH1 domain, approved for the first line treatment of adult patients with Ph+ CML at all disease stages (Guilhot 2004). The

introduction of this drug has dramatically changed the management of CML due to very successful hematologic and cytogenetic response (Deininger, et al. 2004).

Imatinib binds to the amino acids of the BCR-ABL tyrosine kinase through ATP binding domain and stabilizes the inactive, non-ATP-binding form of BCR-ABL, thereby preventing tyrosine autophosphorylation, and in turn, phosphorylation of its substrates (Schindler, et al. 2000). At the end of this process, the downstream signaling pathways that promote leukemogenesis are closed. A further characteristic of Imatinib is its striking degree of specificity for the ATP binding pocket, so that its effect on other cellular tyrosine kinases is negligible (Savage and Antman 2002). According to the IRIS study (International Randomised Study of Interferon and STI571), a Phase III clinical trial showed that the haematologic and cytogenetic responses in terms of tolerability and likelihood of progression to accelerated or blast phase CML, provided superior results with Imatinib (O'Brien, et al. 2003, Merx, et al. 2002).

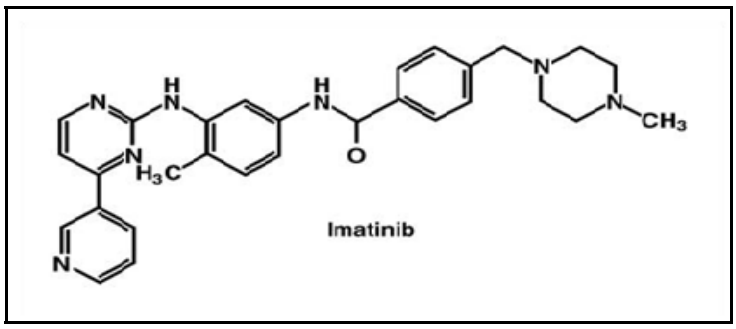

Figure 1.3. Chemical structure of Imatinib
(Source: Kantarjian, et al. 2007)

Despite high rates of hematologic and cytogenetic responses to Imatinib therapy, after exposure of drugs, resistance to Imatinib has been recognized as a major obstacle in the treatment of Ph-positive leukemia (Gambacorti-Passerini 2003, Hochhaus and La Rosee 2004). Several resistance mechanisms are responsible for the overall poor efficacy of cancer chemotherapy (Shah, et al. 2002, Hochhaus, et al. 2002).

1.5.1.1. Multidrug Resistance

Multidrug resistance describes a phenomenon whereby resistance to one anticancer drug is accompanied by resistance to drugs whose structures and mechanisms

of action may be totally different. Multidrug resistance can be classified into two main types. The first one is acquired resistance which can gain during treatment by patients and the second one is primary resistance which is pre-existing at the time of diagnosis. Somehow, there have been seen different responses to Imatinib therapy in patients. Despite high rates of hematologic and cytogenetic responses, resistance cases have been observed in a proportion of patients. This resistance is observed as a lack of hematologic, cytogenetic or molecular responses to therapy. Many resistance cases occur while using Imatinib generally in accelerated and blast crisis phases (Gambacorti-Passerini, et al. 2002). Although the major and also the best characterized Imatinib resistance mechanism is the mutations that occur in the kinase domain (Wang, et al. 2007), to summarize all the other resistance mechanisms against Imatinib, they 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).

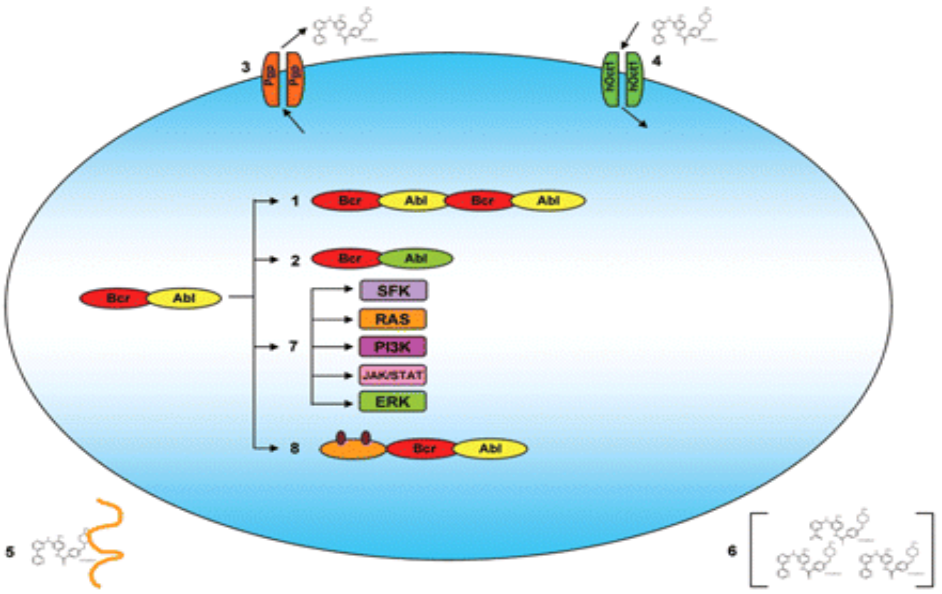

Figure 1.4.The possible mechanisms of Imatinib resistance. 1. Denotes duplication or amplification of the BCR-ABL sequence; 2. Shows mutations in the BCR-ABL sequence; 3. denotes imatinib export by the P-glycoprotein export protein; 4. Depictes import of Imatinib by the hOCT1 protein; 5. Denotes binding of imatinib in the plasma by α1-acid glycoprotein (AGP); 6. Denotes variability in the plasma level of imatinib; 7. Shows activation of alternative signaling cascades leading to BCR-ABL independent growth; and 8. Depicts alterations in the epigenetic regulation of the expression of the BCR-ABL sequence (Source: Bixby and Talpaz 2009)

1.5.1.1.1. BCR-ABL Dependent Mechanisms

1.5.1.1.1.1. Mutations

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 (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 consensus 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).

1.5.1.1.1.2. Overexpression/Amplification of BCR-ABL

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).

1.5.1.1.2. BCR-ABL Independent Mechanisms

1.5.1.1.2.1. ATP Cassette Transporters

BCR-ABL 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 membrane (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 resistance 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 entrance into the cell (Chuah and Melo 2009).

1.5.1.1.2.2. Bioactive Sphingolipids in CML

In cancer chemotherapy 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 GCS and SK-1 enzymes act as strong antiapoptotic molecules inducing cell growth and proliferation and inhibiting apoptosis and differentiation. GCS enzyme regulates the balance between ceramide and glucosylceramide which also means that the levels of sensitivity to anticancer drugs (Gouazé, et al. 2002). There are very strong evidences that show the correlation between GCS and drug resistance (Senchenkov, et al. 2001). In another study GCS gene was introduced 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 exogenious ceramide resistant. This study shows that GCS activity adjusts 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 show us that using inhibitors of GCS enzyme can open a door evading from resistance that is caused by GCS enzyme activity. These inhibitors synthesize cancer cells to chemotherapeutic 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 which leads to decrease in resistance of cells (Morjani, et al. 2001). P-gp is 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).

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 show us that SK1/S1PR signaling prevents the cells from chemotherapy induced 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 (Johnson, 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. 2003).

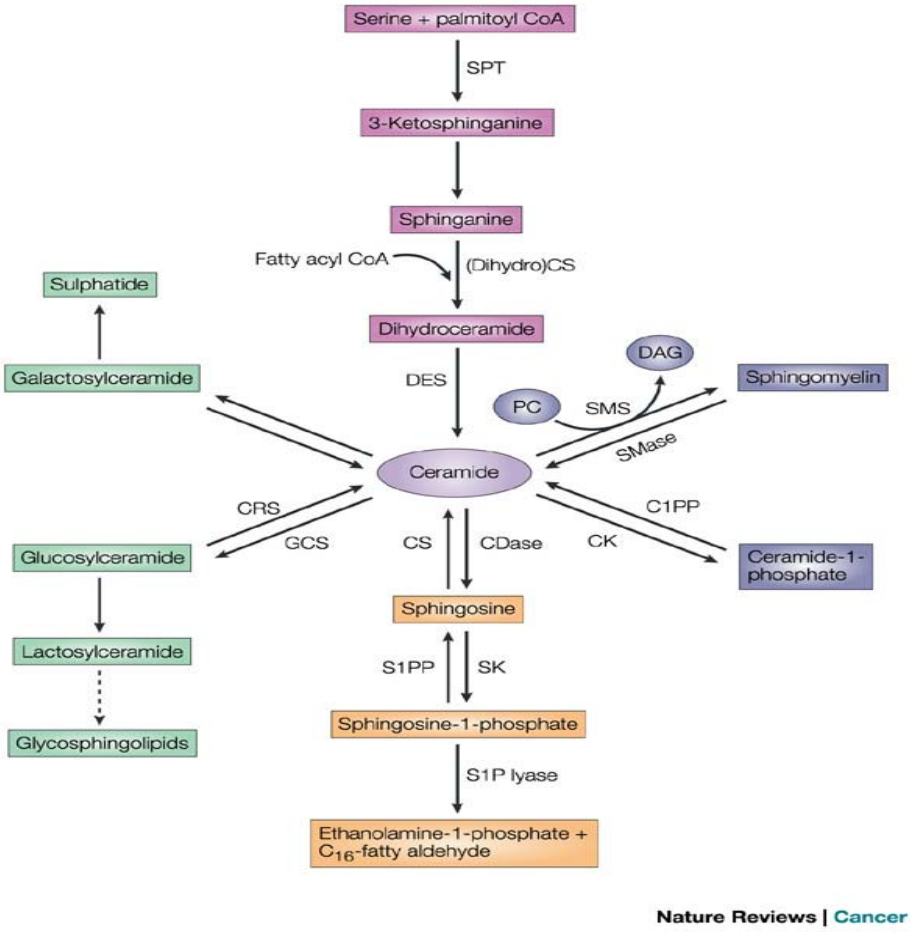

Figure 1.5. Major synthetic and metabolic pathways for ceramide metabolism (Source: Ogretmen and Hannun 2004)

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 mimic or antagonize 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.

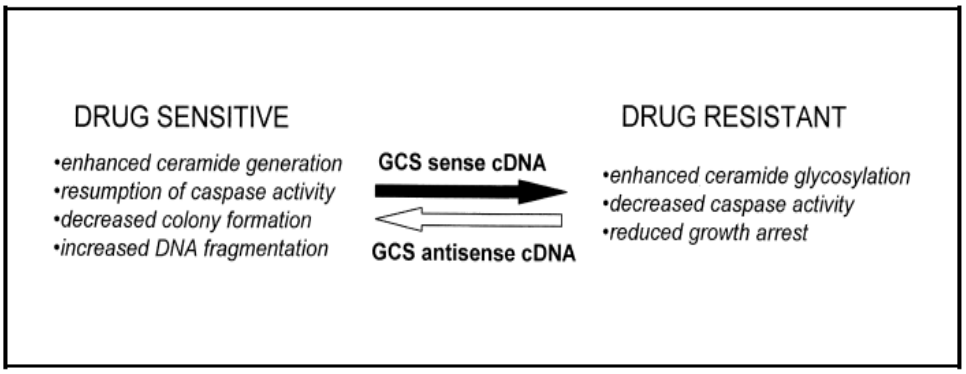

Figure 1.6. Influence of sense and antisense GCS transfection on response to chemotherapy (Source: Senchenkov, et al. 2001)

In a study it is observed that combination of C:8 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.

1.5.1.1.2.3. Alpha-1 Acid Glycoprotein

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 they 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).

1.5.1.1.2.4. Activation of Secondary Tyrosine Kinases

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). Cancer stem cells have been identified in leukemias and some solid tumors (Donnenberg 2005). Many researchers now suspect that all cancers are composed of a mixture of stem cells and proliferative cells. These cancer stem cells make up as few as 1% of the total tumor cells, making them difficult to detect and study. Therefore, the existence of cancer stem cells provides a tumor reservoir that is the source of disease recurrence and metastasis. ABCB1 and ABCG2 genes are expressed in both normal stem cells and most tumor stem cells (Lou and Dean 2007). Thus, the major barrier to therapy may be the quiescent tumor stem cell with constitutive MDR (Liu 2009).

1.5.2. Nilotinib

To overcome Imatinib resistance, more selective second-generation ABL kinase inhibitors were developed and have been used in clinical practice (Kantarjian, et al. 2006, Talpaz, et al. 2006).

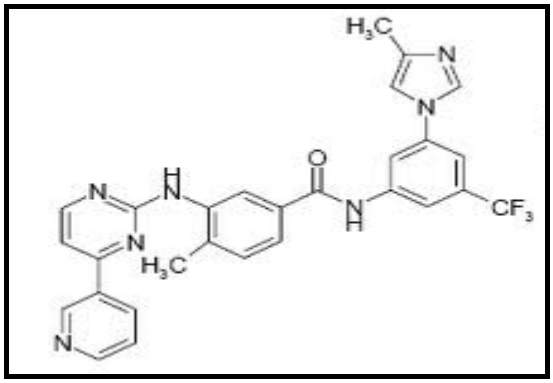

Figure 1.7. Chemical structure of Nilotinib
(Source: Weisberg, et al. 2006)

Nilotnib (Tasigna) is one of the most commonly used second generation kinase inhibitors. Nilotinib is an orally administered kinase inhibitor made by the Novartis Pharmaceuticals Corporation. Nilotinib was methodically and rationally designed to create a better topological fit in the ABL kinase domain of BCR-ABL resulting in enhanced BCR-ABL inhibition. It is an aminopyrimidine derivative of Imatinib, structurally changed to eliminate two energetically unfavorable hydrogen bonds with the replacement of the N-methylpiperazine ring of Imatnib by a trifluoromethyl-substituted phenyl group (Weisberg, et al. 2005 , O'Hare, et al. 2005). Nilotinib does not inhibit only BCR-ABL kinase activity, but also inhibits c-KIT and platelet derived growth factor (PDGFR). Like Ima, it only binds the inactive conformation of ABL and it does not inhibit Src kinases (Weisberg, et al. 2005).

Nilotinib uptake does not involve OCT (White, et al. 2006) which is a properity of Nilotinib keeping in mind while designing. Nilotinib's various characteristics makes it 20-fold more potent against BCR-ABL expressing cells with well-documented activity against 32 of 33 Imatinib resistant BCR-ABL mutants. However, like Imatinib Nilotinib is unable to overcome the resistance of the T351 I mutation (Weisberg, et al. 2006).

Nilotinib is orally bioavailable and has a half life of approximately 15 hours. It should be taken 2-hours before or 1 hour after meals as food consumption increases its drug bioavailability. It is metabolized by hepatic oxidation reactions, mainly involving the CYP3A4 pathway, therefore has potential to interact with CYP3A4 inhibitors and inducers (Weisberg, et al. 2006).

Nilotinib has been shown to be effective in patients with Imatinib-resistant and Imatinib-intolerant CML-BC. In 136 patients with CML-BC treated in a phase II study, overall survival was 42% at 12 months and 27% at 24 months. At 24 months, nilotinib induced MCyR and CCyR in 38% and 30% respectively of patients with myeloid blast crises and 52% and 32% respectively of patients with lymphoid blast crises (Giles, et al. 2010, Giles, et al. 2008). In addition, Nilotinib is effective in patients with CML following Imatinib and Dasatinib (a second generation kinase inhibitor) failure. A phase II study, evaluating the safety of Nilotinib 400 mg BID in CML-CP, -AP, and –BC who either failed or were intolerant of both Imatinib and Dasatinib, demonstrated clinical activity in these patients (Giles, et al. 2007). Nilotinib is generally well tolerated. The most common hematologic adverse events are neutropenia and thrombocytopenia.

In patients with baseline kinase domain point mutations, the *in vitro* sensitivity of the mutant colone correlates with response. Baseline mutation database has been evaluated in 288 patients with CML-CP from the phase II nilotinib registration trial. After 12 months of therapy, those with highly *in vitro* nilotinib sensitive mutations (cellular IC50 $\leq$ 150 nM) showed the best responses with MCyR,CCyR and MMR of 60%,40% and 29% respectively, which were equivalent to those without baseline mutations. These include M244V, L248V, G250E, Q252H, E275K, D276G, F317L, M351T, E355A, E355G, L387F and F486S. The patients who had less sensitive mutations (cellular IC50 $\leq$ 201-800 nM) had less favorable responses to nilotinib and none achieved CCyR. These mutations include Y253H, E255K/V, and F359C/V Patients with the T315I mutation, which has IC50 >10,000 nM (Weisberg et al. 2006) remain highly resistant to nilotinib (Hughes, et al. 2009, Saglio, et al. 2010).

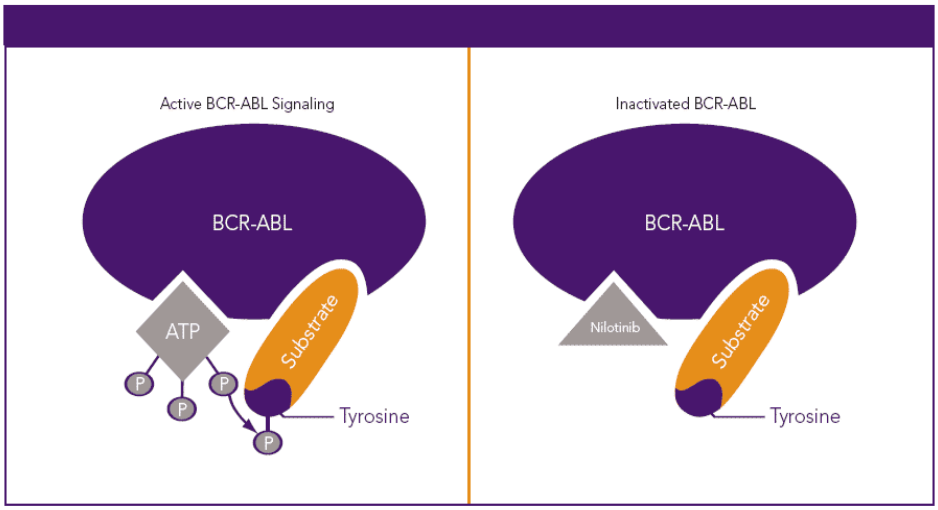

Figure 1.8. The action of Nilotinib binding
(Source: Novartis 2010)

1.6. Aims of the Study

Multidrug resistance remains a significant impediment to successful chemotherapy. The ability to determine the possible resistance mechanisms and circumvent the resistance is likely to improve treatment with Nilotinib. Nilotinib is a very effective drug in the treatment of Imatinib-resistant patients and even first diagnosed chronic myeloid leukemia patients.

Although very high hematologic and cytogenetics responses have been obtained in Nilotinib-treated patients, resistance cases were observed recently. The main objectives of the project are firstly to develop Nilotinib resistance and secondly to understand the mechanisms underlying multidrug resistance to Nilotinib in order to define new targets for the treatment of CML.

CHAPTER 2

MATERIAL AND METHODS

2.1. Reagents

Nilotinib was a gift from Novartis, (Switzerland). It was dissolved in DMSO and 10mM stock solution was prepared and stored at -20ºC. 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, penicillin-streptomycin 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). Long PCR Enzyme Mix was obtained from Fermentas, (USA). Bradford dye and coomassie blue were obtained from Sigma, (USA). dNTP set, DNA ladder was obtained from AMRESCO, (USA). Caspase-3 colorimetric assay kit was obtained from BioVision, (USA). JC-1 mitochondrial 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 also obtained from Sigma, (USA).

2.2. Cell Lines and Culture Conditions

K562 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 10-20% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) at 37˚C in 5% CO_2. Medium was refreshed 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 (min) at 1000 rpm. After centrifugation the supernatant was removed and the pellet washed with 2 mL PBS. After washing the cells were centrifuged again at 1000 rpm for 10 min. 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.

2.3.Thawing the Frozen Cells

Cells were removed from frozen storage and quickly thawed in a 37 C° water bath to obtain the highest percentage of viable cells. When the ice crystals melted, the content was quickly taken into a sterile falcon tube and washed with PBS or medium. Then the cells were cultured in 25 cm^2 tissue culture flask in RPMI1640 medium.

2.4. Cell Viability Assay with Trypan Blue

Viable cells are normally impermeable to trypan blue dye. Breakdown in membrane integrity causes the uptake of the trypan blue into cell. Cells can be observed as unstained cells that are live or blue stained cells that are dead. For trypan blue dye exclusion analysis, cells were counted using a hematocytometer in the presence of trypan blue solution at a 1:1 ratio (volume/volume), before each experiment.

2.5. Generation of Nilotinib Resistant K562 Cells

Generation of resistant sub-lines was carried out as described previously (Baran, et al. 2007). K562 human CML parental cells maintained in liquid cultures were exposed to stepwise increasing concentrations of Nilotinib, starting with a concentration of 1 nM. After the cells acquired the ability to grow in the presence of a specific concentration of the drug, cells were grown at the next highest drug level, again. The level of resistance was defined by the Nilotinib concentration at which the growth rate of cells was comparable to that of untreated parental cells. As a final dose, 50 nM Nilotinib was applied to cells and subpopulations of the cells that were able to grow in the presence of 50 nM Nilotinib, were referred to as K562/NIL-50.

2.6. Cell Proliferation Assay

Anti-proliferative effects of Nilotinib were determined by XTT cell proliferation assay. The principle of this assay based on the formation of tetrazolium salts into formazan by the mitochondrial enzyme and these formazan molecules give an absorbance at 490 nm. Briefly, 96-well plates were seeded as $2x10^4$ cells/well containing 200µl of the growth medium in the absence or presence of increasing concentrations of Nilotinib. The cells were incubated at 37˚C in 5% CO_2 for 72 hours (h). Then, they were treated with 40 µl XTT for 4 h 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 drug that inhibits 50% of cell proliferation as compared to untreated control) was calculated from cell proliferation plots.

2.7. Measurement of Changes in Caspase-3 Enzyme Activity

Caspase-3 Fluorometric Assay Kit (R & D Systems, USA) was used for the detection of caspase-3 activity. The activity assay was performed as described by the manufacturer. Briefly, 1 X 10^6 cells were seeded in six-well plate in 2 ml growth medium in the absence or presence of increasing concentrations of Nilotinib for 72 h. Then, they were collected by centrifugation in a Falcon tube at 1000 rpm for 10 min. The supernatant was gently removed and discarded while the cell pellet was lysed by the addition of the Lysis Buffer. Cold Lysis Buffer (100 µL) was added for each sample. The cell lysate was incubated on ice for 10 min before centrifugation at 14000 rpm for 1 min. Then, the supernatants were transferred to new microcentrifuge tubes. The enzymatic reaction for caspase activity was carried out in a 96-well flat bottom microplate that can be read with a microplate reader equipped with fluorescence detection capabilities. For each reaction, 20 µl of assay buffer (5X), 25 µl of sample, 50 µl of sterilized water, and 5 µl of caspase-3 colorimetric substrate (DEVD-pNA) was prepared 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).

2.8. Determination of Protein Concentration by Bradford Assay

Caspase-3 activity levels were normalized to total protein amounts for each sample determined by Bradford Assay. For this purpose, a dye called Coomassie Brilliant Blue was used 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 colour 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 1X PBS (Phosphate Buffered Saline). After measuring protein concentrations by Bradford assay, enzyme activity levels were normalized to protein concentrations.

2.9. Detection of the Loss of Mitochondrial Membrane Potential

Mitochondrion has a crucial role in the induction of intrinsic apoptosis via the loss of mitochondrial membrane potential (MMP). During this process, the electrochemical gradient across the mitochondrial membrane collapses. The reasons of this collapse is thought that the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bak, or Bad proteins. Activation of these pro-apoptotic proteins causes the release of cytochrome c into the cytoplasm. APO LOGIX JC-1 Assay Kit (Cell Technology, USA) was used to measure the loos of mitochondrial membrane potential in both K562 and K562/NIL-50 cells as described by the manufacturer.

In short, 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 as triplicate. 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.

2.10. Nucleotide Sequence Analyses of ATP Binding Site of ABL Kinase Domain in K562 and K562/NIL-50 Cells

To see whether a point mutation in the BCR-ABL ATP-binding domain was responsible for the resistance in K562/NIL-50 cells against to Nilotinib, sequencing of the cDNA portion corresponding the entire ABL kinase domain was performed. Total RNAs, isolated from K562 and K562/NIL-50 cell lines, were converted to cDNA by Reverse Transcriptase enzyme (Table 2.1). Then, to amplify the ABL kinase domain of the BCR-ABL allele with forward primer BCRF (5'-TGACCAACTCGTGTGTGAAACTC-3') and reverse primer ABLKinaseR (5'-TCCACTTCGTCTGAGATACTGGATT-3'), a long PCR methods was used as described previously (Brandford, et al. 2000). ABLkinaseF (5'-CGCAACAAGCCCACTGTCT-3') as a forward primer and ABLkinaseR as a reverse primer were used for a second-stage PCR. After that, the final PCR products were run on a 1% agarose gel at 90 V for 1 h and the ABL band was isolated from gel by DNA Gel Extraction Kit as described by the manufacturer (Fermentas, USA). The entire kinase domain was sequenced in the forward and reverse directions (Applied Biosystems 3130xl). Finally, the 863 bases including area compared to the c-ABL known sequence (Gene Bank accession number: M14752).

Table 2.1. Ingredients of reverse transcription reaction

Ingredients	**Amount (μL)**
RNAse Free Water	6,5
Total RNA (1,5 μg)	3
5X Buffer	4
Random Primers (0.5 μg/μL)	1
RNAse Inhibitor (50U/μL)	0,5
dNTP (10 mM)	4
Moloney Murine Reverse Transcriptase enzyme (200 U/μL)	1
Total	20

2.11. Total RNA Isolation and Reverse Transcriptase-PCR

The expression levels of ceramide synthase genes (LASS1-6), and ceramide clearance genes (SK-1 and GCS) were investigated by RT-PCR in response to Nilotinib in K562 cells. Firstly, the cells were incubated in the absence and presence of increasing concentrations of Nilotinib, and total cellular RNAs were isolated by using RNA Isolation Kit (Macherey-Nagel, USA). Recovered RNA concentration was measured by Nanodrop ND-1000 (260/280 and 260/230 ratios). 1 μ*g* of total RNA was reverse transcribed into cDNA by using reverse transcriptase enzyme (Moroney Murine Leukemia Virus Reverse Transcriptase, Fermentas, USA). After 60 min incubation at 42 ˚C, the reactions were stopped at 70 C˚ for 10 min. The resulting total cDNA was used in PCR to measure the mRNA levels of LASS1-6, SK-1, GCS, apoptosis genes, transport genes and BCR-ABL with β actin as an internal positive control. Primer sequences were given in Table 2.2.

Table 2.2. Forward and reverse primers used in this study

Houskeeping Gene	
β-actin-Forward	(5'-CAGAGCAAGAGAGGCATCCT-3')
β-actin-Reverse	(5'-TTGAAGGTCTCAAACATGAT-3')
Ceramide Metabolising Genes	
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')
Apoptosis Genes	
Bax- Forward	(5'-ACCAAGAAGCTGAGCGAGTGT-3')
Bax- Reverse	(5'-ACAAACATGGTCACGGTCTGC-3')
Bcl-xL Forward	(5'-GGAGCTGGTGGTTGACTTTCT-3')
Bcl-xL Reverse	(5'-CCGGAAGGTTCATTCACTACT-3')
CASPASE-3 Reverse	(5'-GGTTAACCCGGGTAAGAATGTGCA-3')
CASPASE-3 Forward	(5'-CTCGGTCTGGTACAGATGTCGATG-3')
BcL-2 Forward	(5'-AGATGTCCAGCCAGCTGCACCTGAC-3')
BcL-2 Reverse	(5'-AGATAGGCACCCAGGGTGATGCAAGCTT-3')
Transporter Genes	
LRP Forward	(5'-CGCTGCTTGATTTTGAGGAT-3')
LRP Reverse	(5'-CGAGAATCACGCAGTAGTTG- 3')
MRP1 Forward	(5'-TAGAGGACTTCGTGTCAGCC-3')
MRP1 Reverse	(5'-GTCCATGATGGTGTTGAGCC-3')
MDR1 Forward	(5'-TACAGTGGAATTGGTGCTGGG-3')
MDR1 Reverse	(5'-CCCAGTGAAAAAATGTTGCCA-3')
BCRP Forward	(5'-TACAGTTCTCAGCAGCTCTTCG-3')
BCRP Reverse	(5'-CAACTTGAAGATGGAATATCGAG-3')
BCR-ABL	
B2B	(5'-ACAGAATTCGCTGACCATCAATAAG-3')
CA3	(5'-TGTTGACTGGCGTGATGTAGTTGCTTGG-3')

PCR mixture was prepared in the sterile 0.5 mL eppendorf tubes (Table 2.3). Amplification conditions were maintained in 35 cycles. The expression levels were quantified by Quantitive1 program (BioRad, USA) using the imaging system after running PCR products in agarose gels, followed by ethidium bromide staining.

Table 2.3. Ingredients of PCR solutions for all genes

Reaction Mixture	**Amount (μL)**
PCR grade water	32.5
Reaction buffer (10x)	5
MgCl2 (25 mM)	5
dNTP (2 mM)	4
Primer forward (20 pmol/μL)	0.5
Primer reverse (20 pmol/μL)	0.5
cDNA	2
Taq DNA Polymerase	0.5
Total Mixture	50

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cytotoxic Effects of Nilotinib on K562 and K562/NIL-50 Cells

To explore the mechanisms responsible for Nilotinib resistance, human K562 CML cells were exposed to step-wise increasing concentrations of the drug (1-50 nM) for several months, and the sub-clones that expressed resistance were selected. Firstly, the degree of resistance was determined by measuring the IC50 values of Nilotinib at 72 h using XTT cell proliferation assay.

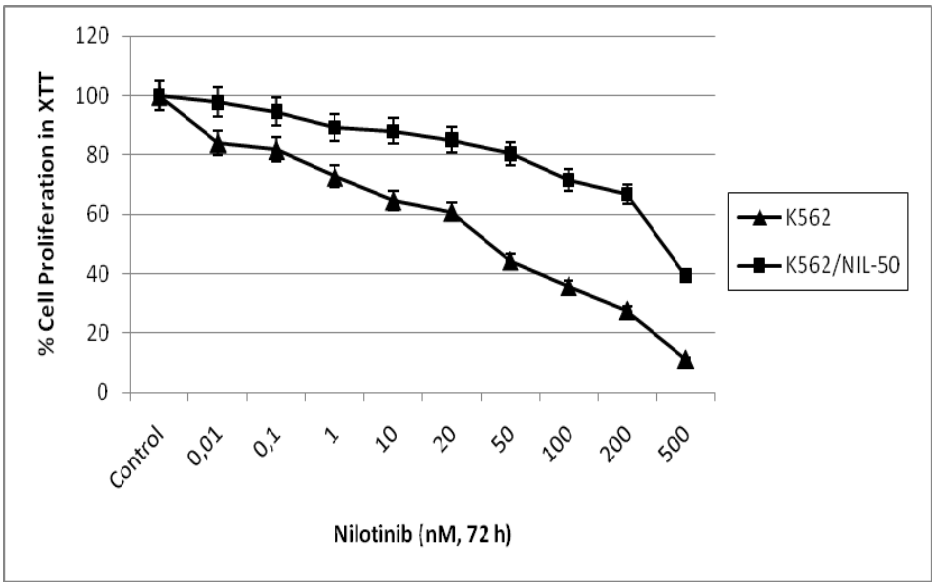

Figure 3.1. Antiproliferative effects of Nilotinib on K562 and K562/NIL-50 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

As shown in Figure 3.1, K562 cells that survived upon chronic exposure to 50 nM Nilotinib, which were referred to as K562/NIL-50 and, showed more than 10-fold resistance, as compared to its parental sensitive counterparts. The inhibitory

concentration (IC50) values of Nilotinib that inhibits growth by 50% in these cells were 35 nM and 386,5 nM for K562 and K562/NIL-50 cells, respectively (Figure 3.1).

3.2. Apoptotic Effects of Nilotinib on K562 and K562/NIL-50 Cells

To make sure whether the resistant cells (K562/NIL-50) are resistant to Nilotinib or not, we performed apoptotic assays including mitochondrial membrane potential and Caspase-3 enzyme activity, as two important apoptotic indicators.

Firstly, changes in MMP was measured in K562 and K562/NIL-50 cells using the JC-1 mitochondrial membrane potential detection kit by varioscan. Treatment with 10 nM Nilotinib for 72 h caused a significant loss of MMP (about 5-fold), as measured by increased accumulation of cytoplasmic monomeric form of JC-1, in parental K562, but not in resistant K562/NIL-50 cells (about 1,15 fold). Moreover, when 500 nM Nilotinib such a very high dose was even applied to resistant cells, decrease in MMP (1,65- fold) was still below the decrease in MMP in 10 nM Nilotinib treated cells (Figure 3.2 and Figure 3.3). Thus, these data confirmed that K562/NIL-50 cells exert significant resistance to Nilotinib-induced loss of MMP.

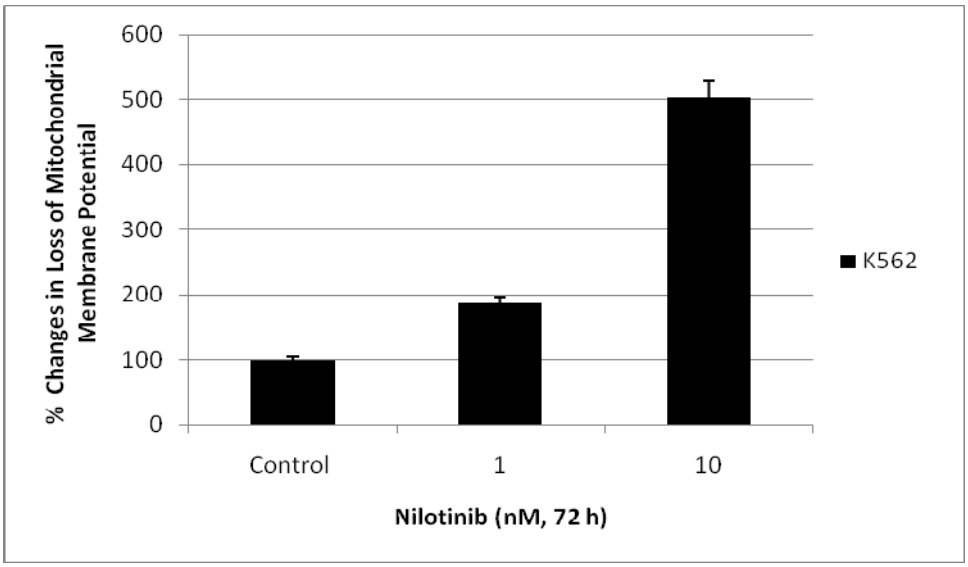

Figure 3.2. Percent changes in mitochondrial membrane potential in K562 cells treated with Nilotinib. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

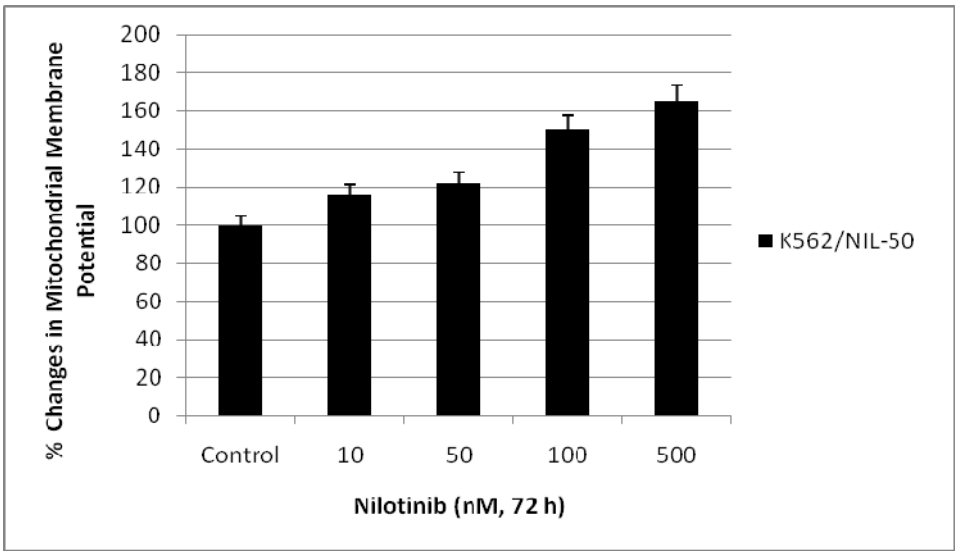

Figure 3.3. Percent changes in mitochondrial membrane potential in K562/NIL-50 cells treated with Nilotinib. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

Secondly, the activation of pro-caspase-3 of parental and resistant K562 cells was measured using the caspase-3 activity assay by fluoresecent spectrophotometer. Treatment of parental K562 cells for 72 h with 10 nM Nilotinib resulted in 1,24-fold increase in caspase-3 activity (Figure 3.4) , whereas treatment of K562/NIL-50 cells at the same concentration only have partial effects, as 0,6-fold increase, on the activation of caspase-3 (Figure 3.5). Thus, these data confirmed that K562/NIL-50 cells exert significant resistance to Nilotinib-induced caspase activation.

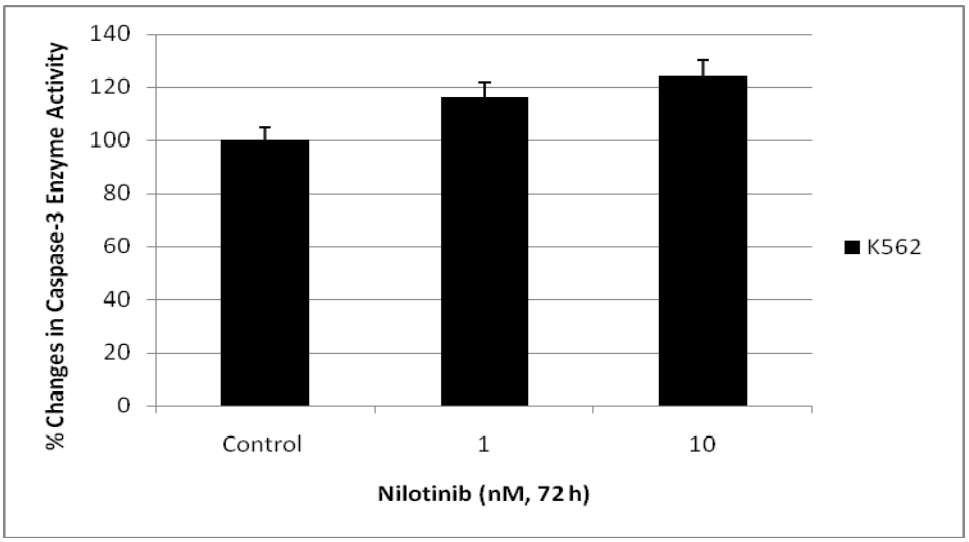

Figure 3.4. Fold changes in caspase-3 enzyme activity in response to Nilotinib in K562 parental cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

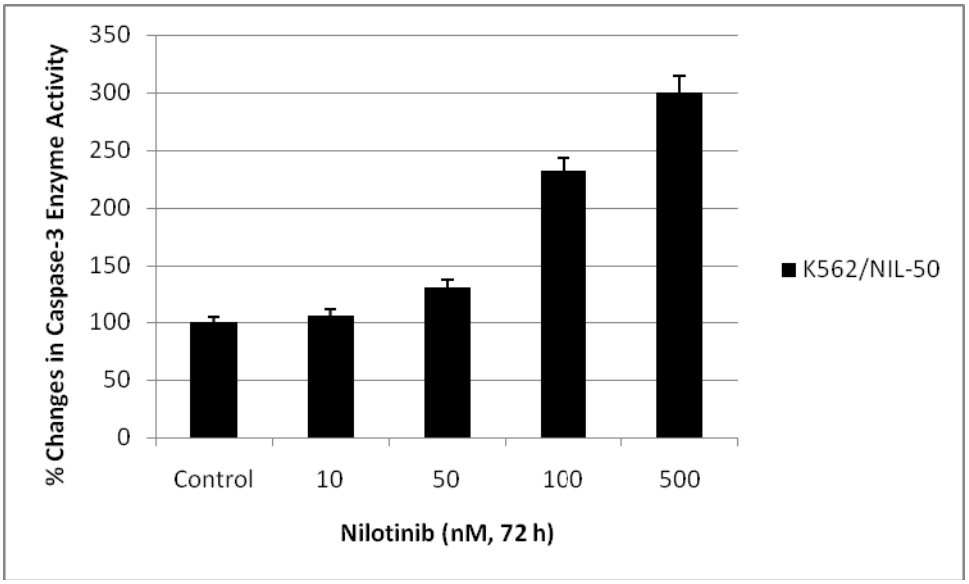

Figure 3.5. Fold changes in caspase-3 enzyme activity in response to Nilotinib in K562/NIL-50 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

3.3. Mechanisms of Nilotinib Resistance in CML

3.3.1. Expression Levels of BCR-ABL Gene in K562 and K562/NIL-50 Cells

Expression levels of BCR-ABL were analyzed in K562 and K562/NIL-50 cells by RT-PCR. Indeed, the data showed that K562/NIL-50 cells overexpress BCR-ABL when compared to their parental sensitive counterparts (Figure 3.6). Quantification analyses of BCR-ABL gene expression was conducted by Quantitive1 programme and the results showed that there was 51% increase in expression of BCR-ABL gene in K562/NIL-50 cells, as compared to parental K562 cells.

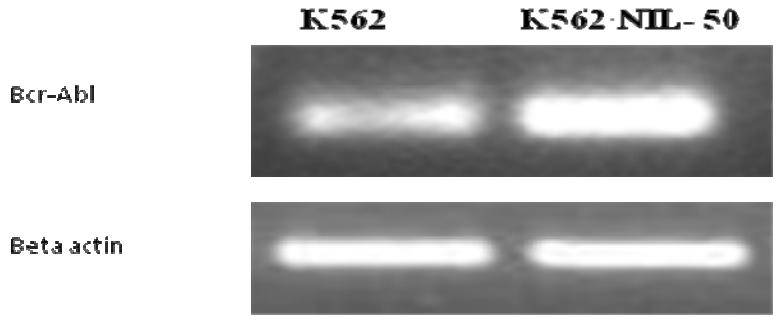

Figure 3.6. Expression analyses of BCR-ABL in parental and resistant CML cells.

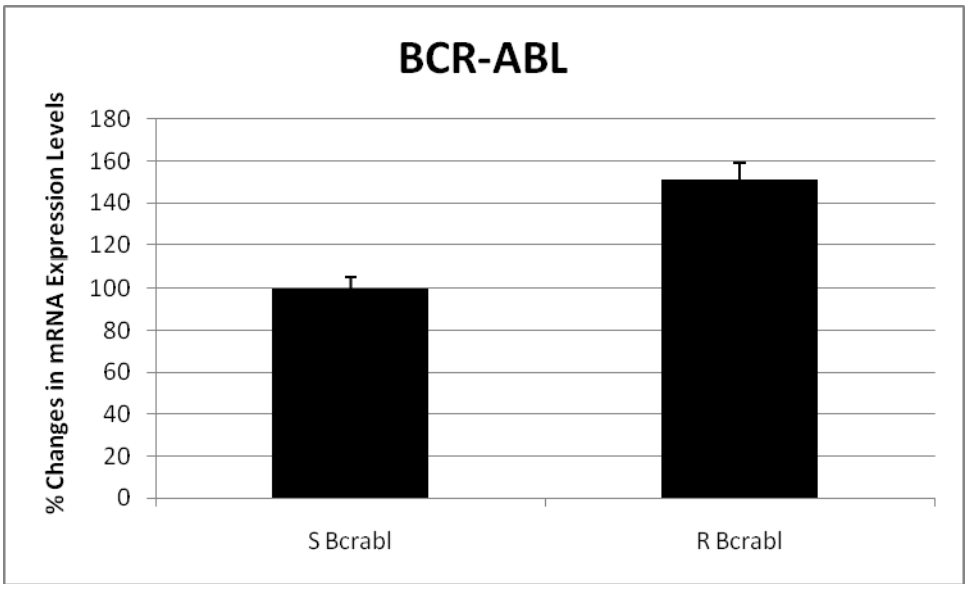

Figure 3.7. Quantification of expression analysis of BCR-ABLgene in K562 and K562/NIL-50 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

3.3.2. Sequence Analysis in Nilotinib Binding Site of ABL Kinase Region in Sensitive and Resistant Cells

To evaluate whether mutations on the ABL kinase domain effect the binding efficiency of Nilotinib or not, sequence analyses of ABL kinase region were examined in parental and resistant K562 cells. The data revealed that there were no detectable mutations on this site of BCR-ABL in any of the K562/NIL-50 (Figure 3.8) comparing to parental K562 (Figure 3.9).

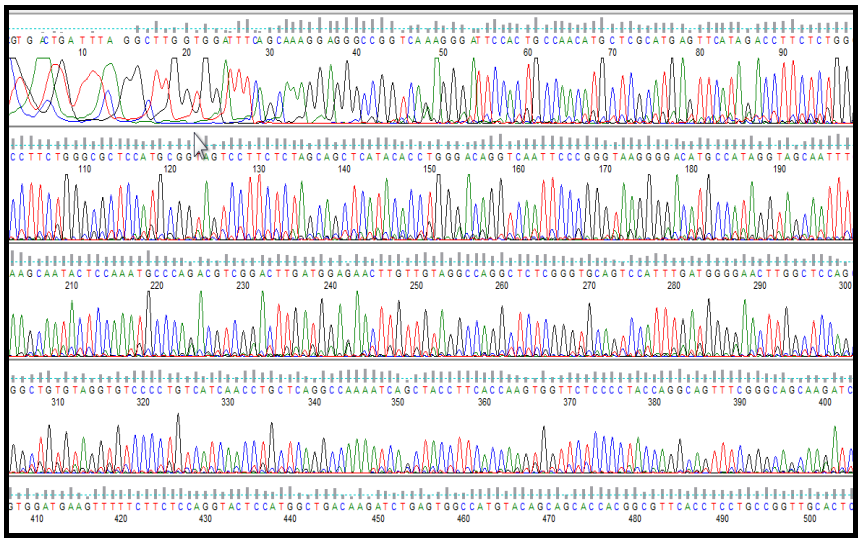

Figure 3.8. Nucleotide sequence analyses of ABL kinase region of resistant K562/NIL-50 cells.

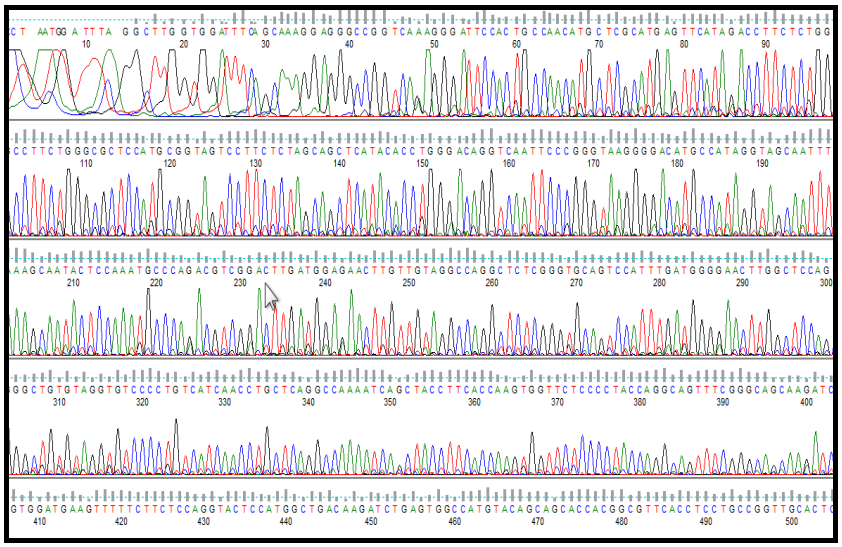

Figure 3.9. Nucleotide sequence analyses of ABL kinase region of parental K562 cells.

3.3.3. Expression Levels of Apoptosis Related Genes in K562 and K562/NIL-50 Cells

Expression levels of apoptosis related genes including Bcl-2, Bcl-xL, Cas3 and Bax were analyzed in K562 and K562/NIL-50 cells by RT-PCR (Figure 3.10). The data showed that K562/NIL-50 cells significantly inhibit expression of Bax gene when compared to its parental sensitive counterparts. However, there were not significant changes in expression levels of the other genes (Bcl-2, Bcl-xL, Cas3). Quantification analyses of the genes expression was conducted by Quantitive1 programme and the

results showed that there were around 2%, 3%, 0% and 25% decrease in K562/NIL-50 cells in expression of Bcl-2, Bcl-xL, Cas3 and Bax genes , respectively, as compared to parental counterpart cells (Figure 3.11).

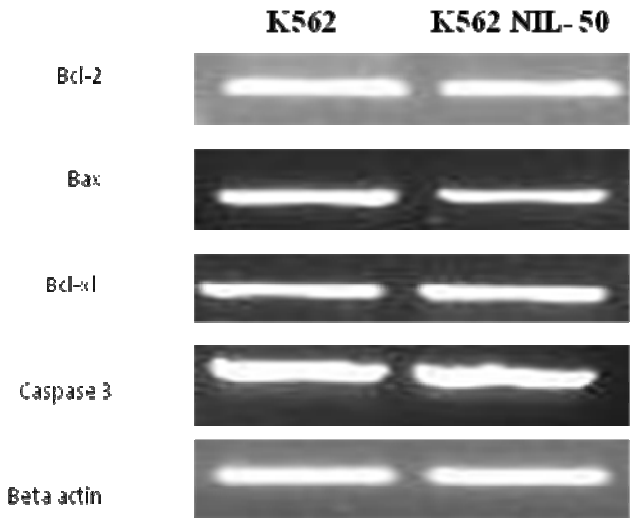

Figure 3.10. Expression analysis of apoptosis related genes in K562 and K562/NIL-50 cells.

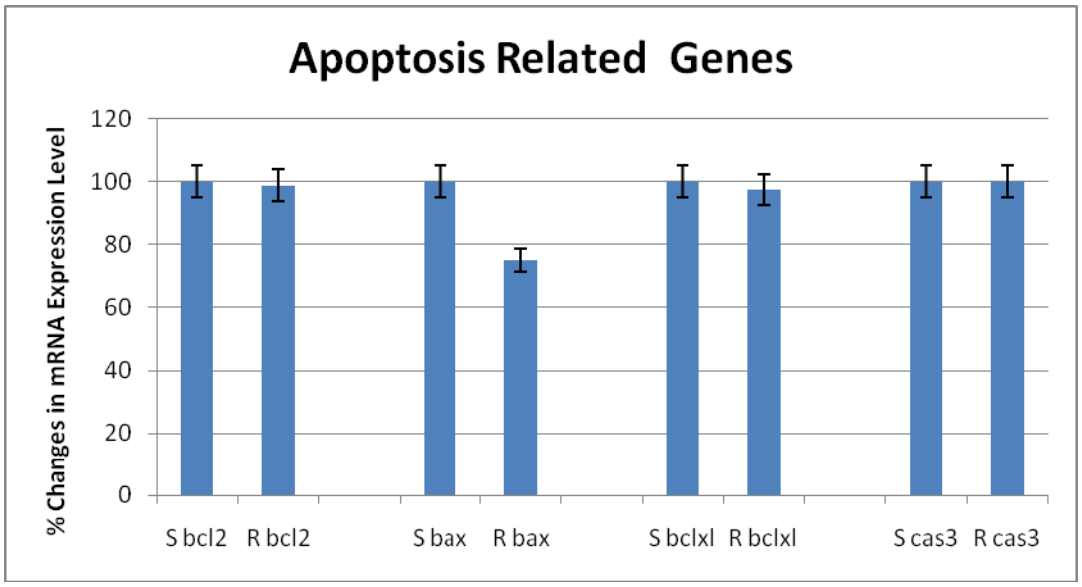

Figure 3.11. Quantification of expression analysis of apoptosis related genes in K562 and K562/NIL-50 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

3.3.4. Expression Levels of Ceramide Metabolising Genes in K562 and K562/NIL-50 Cells

To determine that whether bioactive sphingolipids are involved in Nilotinib resistance, expression levels of GCS, SK-1 and Lass family genes were analyzed. The data showed that K562/NIL-50 cells overexpress significantly GCS and SK-1 genes when compared to its parental sensitive counterparts. Besides, a decrease in expression levels of Lass1 gene was observed, while the other lass family genes including Lass2, Lass4, Lass5 and Lass6 were unexpectedly upregulated. Quantification analyses of the genes expression was conducted by Quantitive1 programme and the results showed that there were 55% and 70% significant increase in K562/NIL-50 cells in the expression levels of GCS and SK-1 genes , respectively, as compared to parental counterpart cells (Figure 3.13).

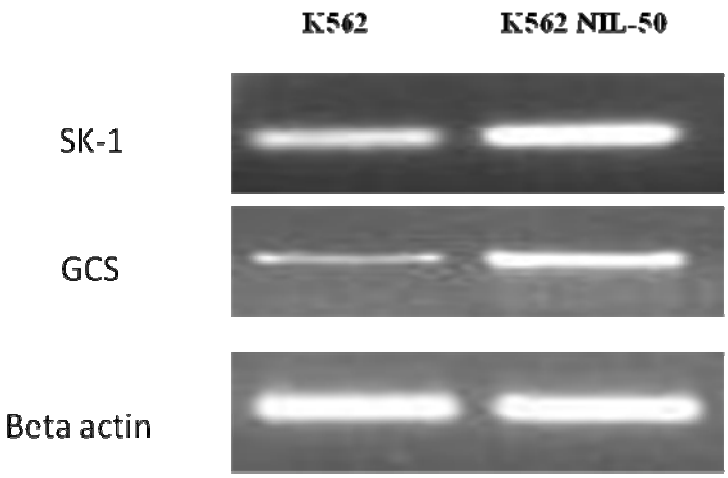

Figure 3.12. Expression analysis of GCS and SK-1 genes in K562 and K562/NIL-50 cells.

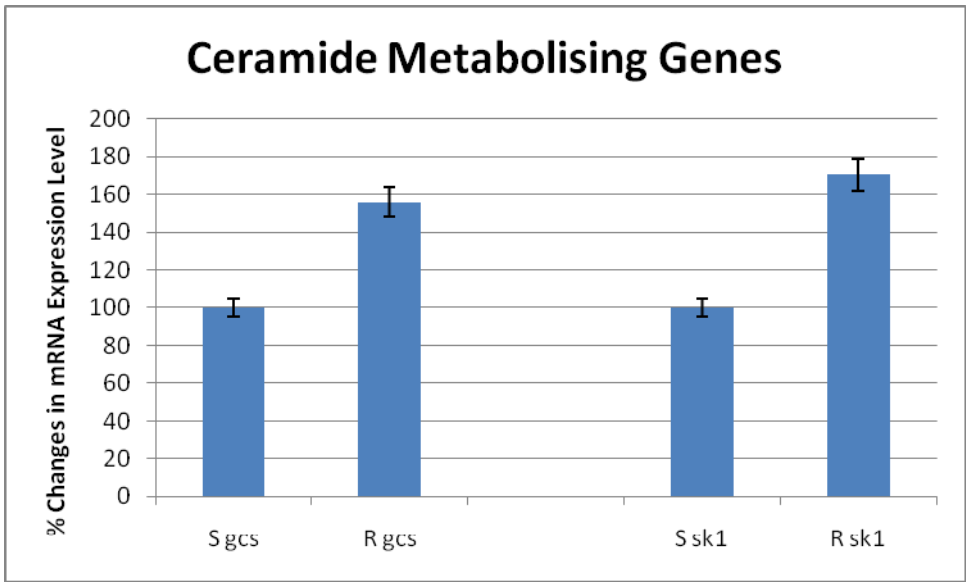

Figure 3.13. Quantification of expression analysis of GCS and SK-1 genes in K562 and K562/NIL-50 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

Moreover, there was around 9% decrease in the gene expression level of Lass1. But there were unexpectedly 10%, 13%, 2% and 12% increase in the expression levels of Lass2, Lass4, Lass5 and Lass6, respectively (Figure 3.15).

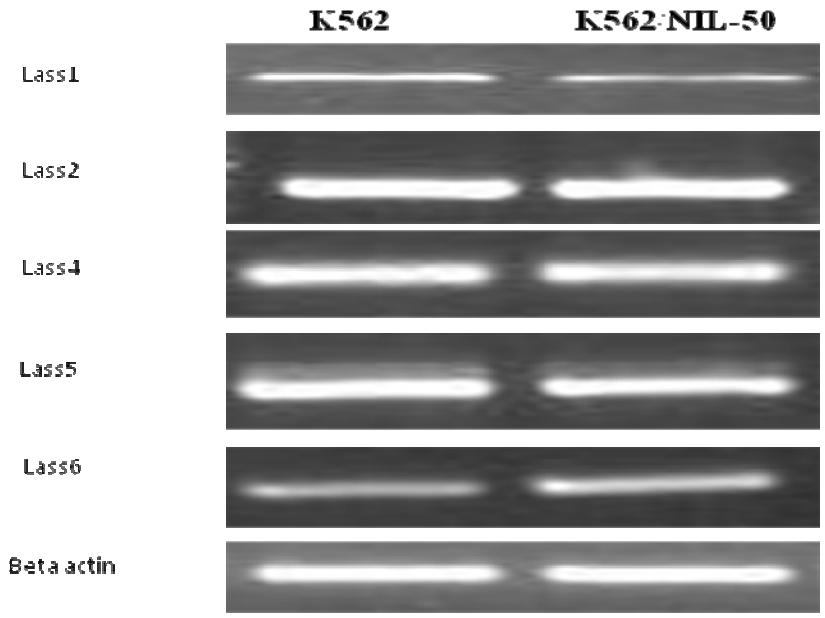

Figure 3.14. Expression analysis of Lass family genes in K562 and K562/NIL-50 cells.

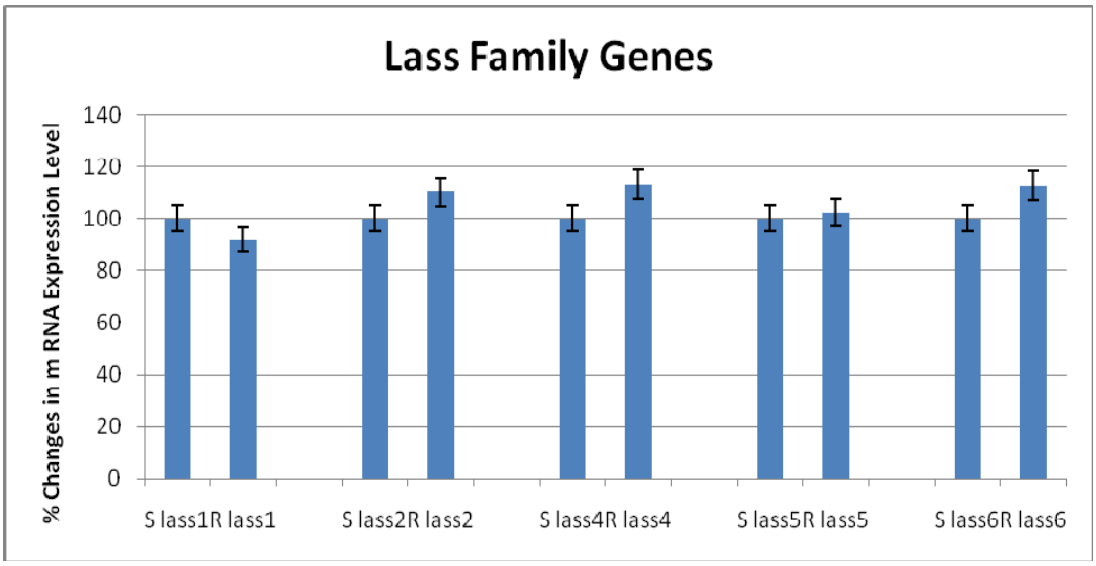

Figure 3.15. Quantification of expression analysis of Lass family genes in K562 and K562/NIL-50 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

3.3.5. Expression Levels of Transport Family Genes in K562 and K562/NIL-50 Cells

To see the role of transport genes on Nilotinib resistance , expression levels of transport family genes including MDR1, MRP1, BCRP and LRP were analyzed in K562 and K562/NIL-50 cells by RT-PCR. The data showed that K562/NIL-50 cells inhibit expression of MDR1, BCRP and LRP genes when compared to its parental sensitive counterparts, while these cells overexpress MRP1 gene. Quantification analyses of the genes expression was conducted by Quantitive1 programme and the results showed that there were around 30%, 6%, 19% decrease and 14% increase in K562/NIL-50 cells in expression of MDR1, BCRP, LRP and MRP1 genes , respectively, as compared to parental counterpart cells (Figure 3.17).

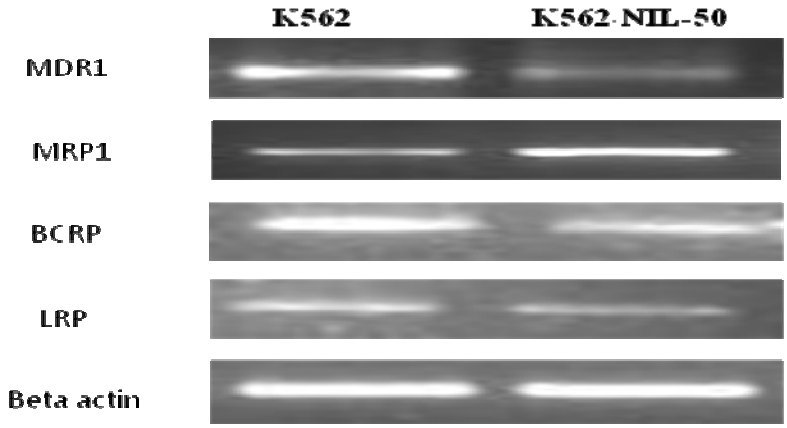

Figure 3.16. Expression analysis of transport family genes in K562 and K562/NIL-50 cells.

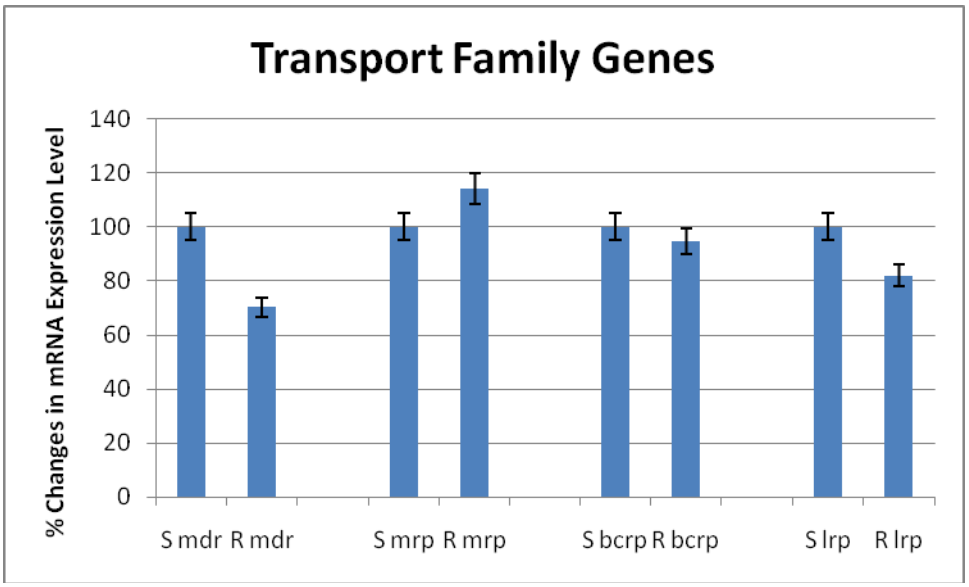

Figure 3.17. Quantification of expression analysis of transport family genes in K562 and K562/NIL-50 cells. The error bars represent the standard deviations, and when not seen, they are smaller than the thickness of the lines on the graphs. Statistical significance was determined using two-way analysis of variance, and P,0.05 was considered significant.

CHAPTER 4

CONCLUSION

CML is a model disease since its discovery. Because CML was the first neoplasm associated with a chromosomal translocation, known as the Philadelphia chromosome. This feature has opened the door that provides an adequate and effective treatment for CML. After a while, the availability of a molecular targeted therapy has profoundly changed the management of CML and defied general ideas about cancer treatment (Hehlmann, et al. 2007).

Imatinib is the first tyrosine kinase inhibitor showing strong activity in chronic and accelerated phase while it is less effective in the blast phase of CML (Savage and Antham 2002). Unfortunately, the emergence of resistance was observed which is the major problem in CML treatment (Walz and Sattler 2006, Deininger 2005). Then, the second generation tyrosine kinase inhibitors were designed to overhelm the resistance, such as Nilotinib. Nilotinib now is used as a first line treatment with approval by FDA since July 2010. However, there have been recently observed Nilotinib resistance cases in CML patients.

In order to produce Nilotinib resistant sub-lines of human CML cell line, K562 cells were cultured in the presence of gradually increasing concentrations (1 nM to 50 nM) of Nilotinib over a period of 15 months. However, rare Ph positive K562 cells were observed which were unaffected by concentrations of Nilotinib that suppress the proliferation of most CML cells. These sub-lines with differential sensitivity to Nilotinib were generated from Nilotinib sensitive BCR-ABL positive human CML cells and the possible molecular mechanisms of resistance to Nilotinib induced apoptosis were investigated.

The overall difficulty through this study was to generate resistant sublines from the parental sensitive cells. Obtaining rare survivors from all cells, even when subjected to a gradual exposure to Nilotinib in liquid culture, suggested that resistant cells arose in response to selective pressure of the inhibitor. This point is important because it is emphasized the specificity and high efficacy of this drug for the control of proliferation of BCR-ABL positive cells. Also, it was observed that although the tyrosine kinase

inhibitor did not kill these resistant sublines, in the beginning their proliferation was slowed in comparison with the parental cultures, suggesting that a delaying effect over the cell cycle was still elicited by the drug.

Although similar approach was used in various studies to derive Imatinib resistance starting with Ph-positive cell lines previously, including AR230, LAMA84 and K562 (Mahon, et al. 2000, Baran, et al. 2007), there are not much studies using similar methods about Nilotinib resistance. By the same group, it was also developed Nilotinib resistant AR230, LAMA84 and K562 cell lines (Mahon, et al. 2008). However, the degree of resistance was just 20 nM Nilotinib. We could develope 50 nM Nilotinib resistant K562 cell line, to our current knowledge, the highest concentration in the literature.

It has been well documented that the degree of BCR-ABL expression appears to be directly proportional to the levels of drug resistance (Mahon, et al. 2000, Weisberg, et al. 2000). In this study, in mRNA levels it was observed that BCR-ABL was overexpressed in K562/NIL-50 cells as compared to parental sensitive counterparts. Besides, Mahon et al. showed that resistance to Nilotinib may be mediated by upregulated expression of BCR-ABL (Mahon, et al. 2008).

Another mechanism for the development of resistance, which is today best-characterized, was due to selection of cells with mutated BCR-ABL in the Imatinib binding domain in various CML cell lines (Wang, et al. 2007). An extensive search for the presence of all mutations in parental and resistant K562/NIL-50 cells using RT-PCR followed by direct sequencing failed to identify any of these mutations. These findings may show that resistance to Nilotinib in these resistant human CML cells did not result from any mutation in Nilotinib binding site of ABL kinase domain.

On the other hand, there has been BCR-ABL independent mechanisms reported for leading resistance in various CML cells. Nilotinib resistance can be due to a failure to induce apoptosis. The signals inducing apoptosis could be blocked and/or anti-apoptotic genes' overexpression can be observed in resistant cells. The Bcl-2 protein can block apoptosis induced by most chemotherapeutic agents (Reed, et al. 1996). Upregulation of anti-apoptotic Bcl-2 by Lyn-kinase-dependent mechanism (Dai, et al. 2004) and by BCR-ABL gene in hematopoietic cells (Salomoni, et al. 2000) has been shown to be involved in resistance to Imatinib. In our results, it was observed that only Bax was dowregulated. While expecting upregulation in expression levels of Bcl-2 and

Bcl-xL, there were only moderate changes. Bax is a pro-apoptotic molacule which forms pore resulting in a loss of mitochondrial membrane potential which activates proteases (caspase-3, -7 and -9) and nucleases by cytochrome-c release from mitochondria (Zamzami, et al. 1998). These data are in agreement with this study which showed that in Nilotinib treated parental human CML cells, there were significant losses in mitochondrial membrane potential and an increase in caspase-3 enzyme activity resulting in higher number of cells in apoptosis. Thus, the pro-apoptotic effects of Nilotinib may be explained by a rapid and sustained inhibition of BCR-ABL, leading to induction of mitochondria dependent apoptosis in parental cells. In steady state levels, decreases were detected in loss of mitochondrial membrane potential and in caspase-3 activity in resistant cells as compared to parental sensitive counterparts. Mitochondrial membrane potential and caspase-3 enzyme activity analyses revealed that although higher concentrations of Nilotinib were applied, there were almost no loss of mitochondrial membrane potential and no increase in caspase-3 enzyme activity in resistant cells.

In addition, transporter mediated tyrosine kinase inhibitor efflux has been implicated as a possible mechanism for resistance to Imatinib (Thomas, et al. 2004, Jiang, et al. 2007). However, there has been significant controversy in the field regarding the potential of ABC transporters to confer drug resistance. There are several studuies showing that MDR1 and BCRP transporters are involved in Nilotinib resistance as a substrate or an inhibitor (Dohse, et al. 2010, Brendel, et al. 2007). The report that has been published as describing the interaction of Nilotinib with BCRP, suggesting Nilotinib is a BCRP substrate (Brendel, et al. 2007), while there is another study showed that in higher concentrations, Nilotinib reduced both MDR1 and BCRP activities (Hegedus, et al. 2009). Like these results, we showed that 50 nM Nilotinib concentration might inhibit expression levels of MDR1 and BCRP significantly in K562 CML cells. Besides this, in this study, we showed that MRP1 overexpression may be responsible for Nilotinib resistance. Although there are only a few reports about the role of MRP1 in drug resistance in the literature, Shen et al. showed that Nilotinib reverses MRP7 mediated paclitaxel resistance at very high concentration (5 μM), another member of MRP family transporter. Concentration may be responsible for determining whether Nilotinib assumes the phenotype of substrate or inhibitor. Our results also showed that there was a inhibition in expression levels of LRP, but there is not sufficent

data in the literature about the role of LRP in Nilotinib resistance. After confirmation with different perspectives that show LRP is really dowregulated, it can be said Nilotinib may be inhibitor of LRP.

Furthermore, we examined the roles of bioactive sphingolipids in the regulation of Nilotinib resistance in human K562 cells, as another BCR-ABL independent mechanism. Bioactive sphingolipid ceramide is involved in mediating anti-proliferative responses via various different mechanisms in human cancer cells (Ogretmen and Hannun, 2004). It has been well documented that treatment with some chemotherapeutic agents results in increased generation and/or accumulation of endogenous ceramide either via the activation of the *de novo* pathway (LASS1-6 genes), or by increased activity of SMases (Ogretmen and Hannun, 2004). However, any role for Nilotinib in inducing the generation of ceramide in human CML cells has not been examined previously. Here, the data showed that there were significant increase in expression levels of GCS and SK-1 genes which are responsible enzymes for increasing levels of antiapoptotic forms of ceramide. There are several reports showing SK-1 is overexpressed in distinct cancer types (Sobue, et al. 2006, French, et al. 2003, Li, et al. 2007). Besides, GCS overexpression has been observed in Imatinib resistant human CML cells (Unpublished data from our lab). Another support for the involvement of GCS in drug resistance came from transfection experiments, which showed that overexpression of the enzyme resulted in increased resistance to adriamycin in drug sensitive MCF-7 tumor cells (Liu, et al. 1999). Decrease in expression levels of LASS1 that selectively regulates the synthesis of C_{18}-ceramide (Venkataraman, et al. 2002), was observed, while LASS2, LASS4, LASS5 and LASS6 were still upregulated. These data are in agreement with the study which showed that when LASS1 was overexpressed, Imatinib resistant cells increased the sensitivity to Imatinib, suggesting that upregulation of ceramide generation might help for improving response to Imatinib. However, the overexpression of LASS2, LASS5 and LASS6 did not cause any change in Imatinib resistant cells (Baran, et al. 2007). According to these results, decrease in expression of LASS1 may also be responsible for Nilotinib resistance.

What is more, the data presented here have important implication for designing novel therapies for the treatment of CML. For example, in addition to the inhibition of BCR-ABL, targeting ceramide metabolism by increasing its synthesis and/or modulating its metabolism may provide improved strategies for the treatment of CML.

As a conclusion, in this study, Nilotinib resistant cell line is developed at very high concentration, such as 50 nM. More importantly, these results show that ceramide metabolising genes including GCS, SK-1 and LASS1 may be involved in the regulation of Nilotinib resistance in K562 cells.

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