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# STAT3 Contributes to Resistance Towards BCR-ABL Inhibitors in a Bone Marrow Microenvironment Model of Drug Resistance in Chronic Myeloid Leukemia Cells

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by

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| | |
|---|---|
| ABL | Abelson kinase |
| AKT | Protein kinase B |
| ALL | Acute lymphoblastic leukemia |
| AP | Accelerated phase |
| Bcr | Breakpoint cluster region |
| BME | Bone marrow microenvironment |
| BP | Blast crisis phase |
| CaLB | Calcium-dependent lipid binding |
| CD | Cluster of differentiation |
| CML | Chronic myeloid leukemia |
| CNL | Chronic neutrophilic leukemia |
| CP | Chronic phase |
| CRP | Complement-regulatory proteins |
| ECM | Extracellular matrix |
| GAB2 | GRB-2-associated binding protein |
| GAP | GTPase activating protein |
| GEF | Guanine-nucleotide exchange factor |
| GM-CSF | Granulocyte macrophage-colony stimulating factor |

| | |
|---|---|
| GRB-2 | Growth factor-binding protein 2 |
| HSC | Hematopoietic stem cell |
| $IC_{50}$ | Half-maximal inhibitory concentration |
| IkB | Inhibitor of kappa B |
| IL | Interleukin |
| IM | Imatinib mesylate |
| JAK | Janus kinase |
| kb | Kilobases |
| kD | Kilodalton |
| MAPK | Mitogen activated protein kinase |
| MDR1 | Multidrug resistance-1 drug transporter |
| MRD | Minimal residual disease |
| NF-kB | Nuclear factor kappa B |
| NK cells | Natural killer cells |
| PDK1 | Phosphoinositide-dependent protein kinase 1 |
| PH | Pleckstrin homology |
| PI | Phosphotidylinositides |
| PI3K | Phosphoinositide 3-kinase |
| PMN or PML | Polymorphonuclear leukocytes |
| qRT-PCR | Quantitative real time-polymerase chain reaction |
| SFK | SRC family kinase |
| SCID | Severe combined immunodeficient |

| | |
|---|---|
| STAT | Signal transducer and activator of transcription |
| VEGF | Vascular endothelial growth factor |
| WBC | White blood cell |

STAT3 Contributes to Resistance Towards BCR-ABL Inhibitors in a Bone Marrow Microenvironment Model of Drug Resistance

Nadine N. Bewry

## ABSTRACT

Imatinib mesylate (imatinib) represents a potent molecularly targeted therapy against the oncogenic tyrosine kinase, BCR-ABL. Although imatinib has shown considerable efficacy against chronic myeloid leukemia (CML), displaying high rates of complete hematological and complete cytogenetic responses, treatment with imatinib is not curative and overtime advanced-stage CML patients often become refractory to further treatment. Acquired resistance to imatinib has been associated with mutations within the kinase domain of BCR-ABL, *BCR-ABL* gene amplification, leukemic stem cell quiescence as well as over-expression of the multidrug resistance (MDR1) gene. However, *in vitro* resistance models often fail to consider the role of the tumor microenvironment in the emergence of the imatinib-resistant phenotype. The bone marrow is the predominant microenvironment of CML and is a rich source of both soluble factors and extracellular matrixes, which may influence drug response. To address the influence of the bone marrow microenvironment on imatinib

sensitivity, we utilized an *in vitro* co-culture bone marrow stroma model. Using a transwell system, we demonstrated that soluble factors secreted by the human bone marrow stroma cell line, HS-5, were sufficient to cause resistance to apoptosis induced by imatinib in CML cell lines. We subsequently determined that culturing CML cells in HS-5-derived conditioned media (CM) inhibits apoptosis induced by imatinib and other second generation BCR-ABL inhibitors. These data suggest that more potent BCR-ABL inhibitors will not overcome resistance associated with the bone marrow microenvironment. Additionally, we determined that CM increases the clonogenic survival of CML cells following treatment with imatinib. HS-5 cells are reported to express several cytokines and growth factors known to activate signal transducer and activator of transcription 3 (STAT3). Given its crucial role in the survival of hematopoietic cells, we asked whether, 1) CM derived from HS-5 cells can activate STAT3 in CML cells and 2) does activation of STAT3 confer resistance to BCR-ABL inhibitors. We demonstrated that exposure of the CML cell lines, K562 and KU812, to CM caused an increase in phospho-Tyr STAT3, while no increases in phospho-Tyr STAT5 were noted. Moreover, resistance was associated with increased levels of the STAT3 target genes, Bcl-xl, Mcl-1 and survivin. Furthermore, reducing STAT3 levels with siRNA sensitized K562 cells cultured in CM to imatinib-induced cell death ($p<0.05$, Student's t-test). Importantly, STAT3 dependency was specific for cells grown in CM, as reducing STAT3 levels in regular growth conditions had no effect on imatinib sensitivity. Together, these data support a

novel mechanism of BCR-ABL-independent imatinib resistance and provide preclinical rationale for using STAT3 inhibitors to increase the efficacy of imatinib within the context of the bone marrow microenvironment.

# CHAPTER I

# INTRODUCTION

## Hematopoiesis

Although an estimated 1 x $10^{10}$ red blood cells and 1 x $10^{9}$ white blood cells are produced per hour, mature blood cells have a limited lifespan of only a few hours to a few days and, therefore, require continuous production. Hematopoiesis is the highly regulated process of blood cell production from hematopoietic stem cells (HSCs) within the bone marrow (Figure 1). This process, which continues throughout adulthood, first begins in the embryonic yolk sack during the first weeks of embryonic development, proceeds in the liver and then the spleen. In adults, the bone marrow becomes the major site of hematopoiesis and ceases in the liver and spleen. The primary locations of HSC production are femurs, hip, ribs, sternum, as well as other bones.

HSCs are small, non-adherent, rounded cells that possess a rounded nucleus and can be identified by their low cytoplasm-to-nucleus ratio. Characterized by their multipotency and high replicative and differentiation capacity, HSCs are capable of giving rise to all types of blood cells, which are divided into three distinct lineages: lymphoid, myeloid and erythroid. The lymphoid lineage of HSCs is composed predominantly of T-cells and B-cells, a

category of leukocytes or white blood cells (WBCs) called lymphocytes. Although they are the smallest WBCs, they are the cornerstones of the immune system, playing vital roles in the cell-mediated and humoral components of adaptive immunity, respectively. This lineage also consists of natural-killer (NK) cells, which plays a central role in defending the body from both tumors and virally infected cells. The myeloid lineage produces another category of leukocytes that include granulocytes, megakaryocytes and macrophages. Granulocytes, also known as polymorphonuclear leukocytes (PMN or PML) because of the varying shapes of the nucleus, consist of: 1) basophils, the least common of the granulocytes that play a role in regulating the immune inflammatory response to allergens and drugs; 2) eosinophils, which also respond to allergens and are involved in defending against infection and parasites; and 3) neutrophils, the most abundant WBCs in the body that contain granules of bacteria-killing enzymes in their cytoplasm and function as phagocytes, responding primarily to acute inflammation due to bacterial infection or fungi. Megakaryocytes produce blood platelets, which are small cells necessary for normal blood clotting. Macrophages are phagocytes involved in both innate and adaptive immunity. The third lineage of HSCs, the erythroid lineage, gives rise to oxygen-carrying red blood cells (RBC), which make up 45% of blood volume.

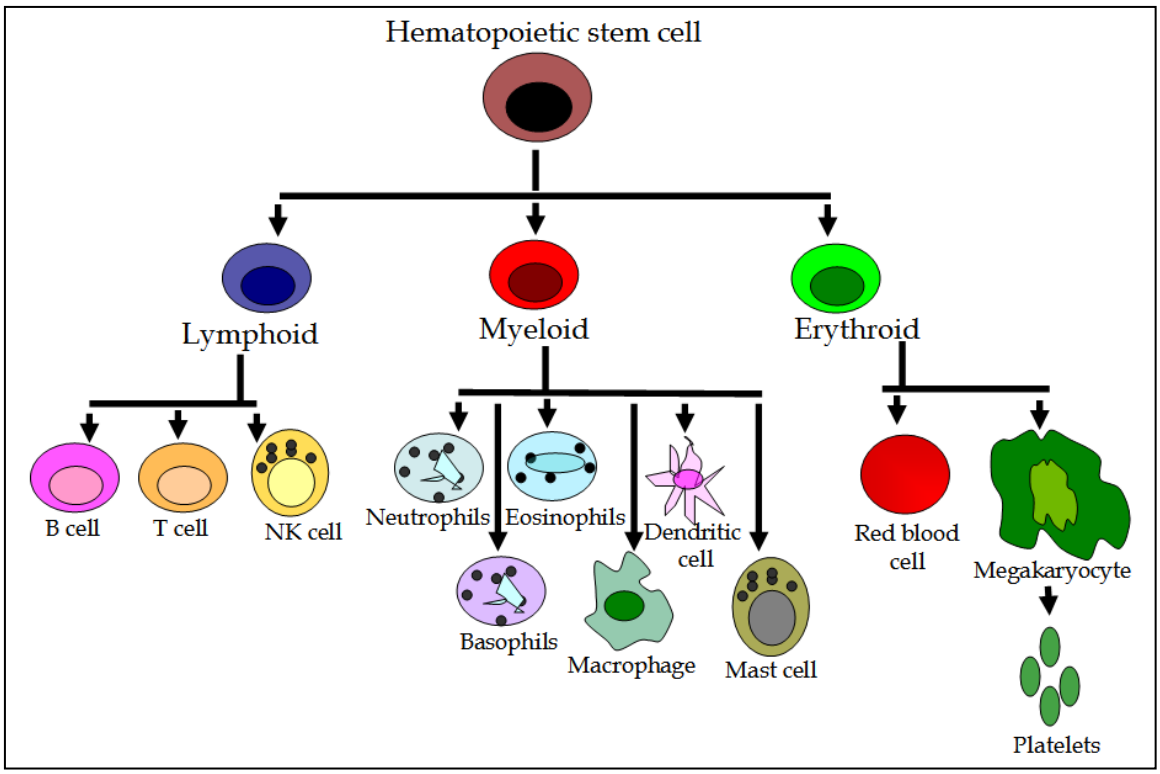



**Figure 1**. **The Process of Hematopoiesis**. All of the cellular components of the blood are derived from hematopoietic stem cells. Blood cells are divided into three lineages: lymphoid, myeloid and erythroid.

**Types of Leukemia**

Hematopoiesis is highly regulated by a balance of growth and death signals that determine the fate of each cell in the hematopoietic system. Consequently, mutations of stem cells can disrupt the hematopoietic system to confer a growth advantage. This deregulated hematopoiesis creates an imbalance between cell production, destruction and differentiation and can lead to a number of hematologic diseases. Leukemia, the abnormal proliferation of

blood cells in the bone marrow, results in deregulated blood cell production and cell differentiation.

Leukemia is classified as acute or chronic based on pathological and clinical characteristics, such as the speed with which the disease progresses and the phenotype of the affected cells. While acute leukemia is distinguished by the rapid increase of WBCs, an increase in mature, yet abnormal blood cells typifies chronic leukemia. Leukemia can be further sub-divided as lymphoid or myeloid based on the lineage of the blood cells that are affected (Table 1).

**Table 1: Types of Leukemia**

| | Chronic | Acute |
|---|---|---|
| | Characterized by the excessive build up of relatively mature, but still abnormal, blood cells | Characterized by the rapid proliferation of immature blood cells. |
| **Lymphocystic Leukemia** | Chronic lymphocytic leukemia (CLL) most often affects adults over the age of 55. CLL is an abnormal, cancerous proliferation of B-cells, which is a lymphocyte that fights infections. | Acute lymphocytic leukemia (also known as Acute Lymphoblastic Leukemia, or ALL) is most common in childhood and young adulthood, with a peak incidence at 4-5 years of age. refers to the undifferentiated, immature state of the circulating lymphocytes ("blasts"), and to the rapid progression of disease. |
| **Myelogenous Leukemia** | Chronic myelogenous leukemia (CML) can occur in all age groups, but is most commonly seen in the middle-aged and elderly. It is characterized by the increase and unregulated growth of myeloid progenator cells. | Acute myelogenous leukemia (also known as Acute Myeloid Leukemia, or AML) occurs more commonly in adults than in children. AML is the most common acute leukemia affecting adults, and its incidence increases with age. |

**Normal vs. Leukemic Hematopoietic Stem Cells**

Normal HSCs are a rare, quiescent homogeneous cell population whose production is very tightly regulated. Additional characteristics of stem cells include their multipotent differentiation capacity and their ability to self-renew [1].

Based on the latter characteristic, HSCs can be subdivided by their stem cell hierarchy, or the degree to which they are able to self-renew, as short- or long-term repopulating cells [2].

Despite major advances in our understanding of stem cells, little is known about their role of stem cells in human malignancies. There is a growing body of evidence that lends support to the existence of cancer stem cells (CSCs), a small, primitive subpopulation of cancer cells that escape normal control and are capable of initiating, propagating and maintaining the cancer cell population [3]. Studies show that CSCs have been implicated in a number of human malignancies, including breast and brain tumors [4, 5].

Among the CSC subpopulation, studies have identified and characterized cancer-causing leukemic stem cells (LSCs). These cells share many of the canonical properties of normal HSCs, such as multipotency, the ability to self-renew, and extensive proliferative capacity [6, 7]. However, unlike normal HSCs, these LSCs display a reduced or absent exogenous growth factor-dependence [8]. Instead, these cancer-causing leukemic cells are capable of generating their own growth signals, most notably via the autocrine production of cytokines such as interleukein-3 (IL-3) and/or granulocyte colony-stimulating factor (GM-CSF) [9].

Early studies using immunodeficient mice as xenotransplantation models demonstrated the existence of leukemia-initiating stem cells in CML patients [10-13]. Like normal stem cells, these cells lack lineage markers (Lin-) and are

distinguished as follows: CD34+CD38- Thy-1+Lin- (Baum C, 1992), where CD34 is a single-pass transmembrane glycoprotein that functions as a cell-cell adhesion factor and is expressed early in hematopoietic tissues [14]; CD38 is an early cell surface marker for white blood cells [15] and CD90 or Thy-1 is the thymocyte (T-cell precursor) marker [16]. Research done in these early CML models confirmed that while chronic phase CD34+ CML patient cells contained both normal and LSC fractions, patients in blast crisis phase CML have a greater preponderance of LSCs [17]. CML LSCs are not composed of a functionally homogeneous cell population as previously believed, but like normal HSCs, are comprised of a heterogeneous pool of short-term and long-term repopulating cells [18-20].

Addressing the failures of cell cycle-active chemotherapeutic agents to eradicate CSCs *in vivo* and *in vitro* poses a challenge for CML patient care, as primitive quiescent cells are resistant to imatinib. This resistant, quiescent LSC phenotype coupled with an augmented stem cell population, an enhanced self-renewal capacity and decreased growth-factor dependence/increased autocrine secretion of growth factors may contribute to chemo-resistance in CML patients and subsequent relapse.

## CHAPTER II

## CHRONIC MYELOID LEUKEMIA AND THE 'PHILADELPHIA' CHROMOSOME

Currently within the United States every 1 in 4 deaths is due to cancer [21]. Among cancers, chronic myeloid leukemia (CML) is possibly the most well-studied. CML is a myeloproliferative disorder that is characterized by the neoplastic transformation and malignant expansion of pluripotent hematopoietic stem cells (HSCs) within the bone marrow resulting in cancer of the white blood cells. It accounts for 7% to 20% of all cases of leukemia, and according to the America Cancer Society estimates, in 2008 there will be 4,830 newly diagnosed cases of CML with 450 of those resulting in deaths. While it affects all age groups, it is predominantly affects middle-aged to elderly individuals at a rate of 1-2 per 100,000. CML is the first disease to be linked to a clear, consistent genetic abnormality, the Philadelphia (Ph') chromosome, which was first described in 1960 by Peter Nowell of the University of Pennsylvania and David Hungerford of the Fox Chase Cancer Center, two scientists from Philadelphia, Pennsylvania [22]. The team described a tiny acrocentric chromosome in cells cultured from the blood of seven patients harboring the disease. This chromosome later became the unique genetic signature and diagnostic clinical marker of CML patients [23]

The Ph' chromosome is formed by the reciprocal translocation and fusion between the long arms of chromosomes 9 and 22 [t(9,22)(q34;q11)] [24-26] (see Figures 2A and B). Chromosome 9 encodes the proto-oncogene *ABL,* while chromosome 22 encodes the breakpoint cluster region gene (*bcr*), a serine/threonine kinase named after the site where it is located. This translocation event results in the formation of the chimeric oncogene, *BCR-ABL*, which gives rise to an aberrant and deregulated, constitutively active tyrosine kinase capable of activating numerous downstream targets.

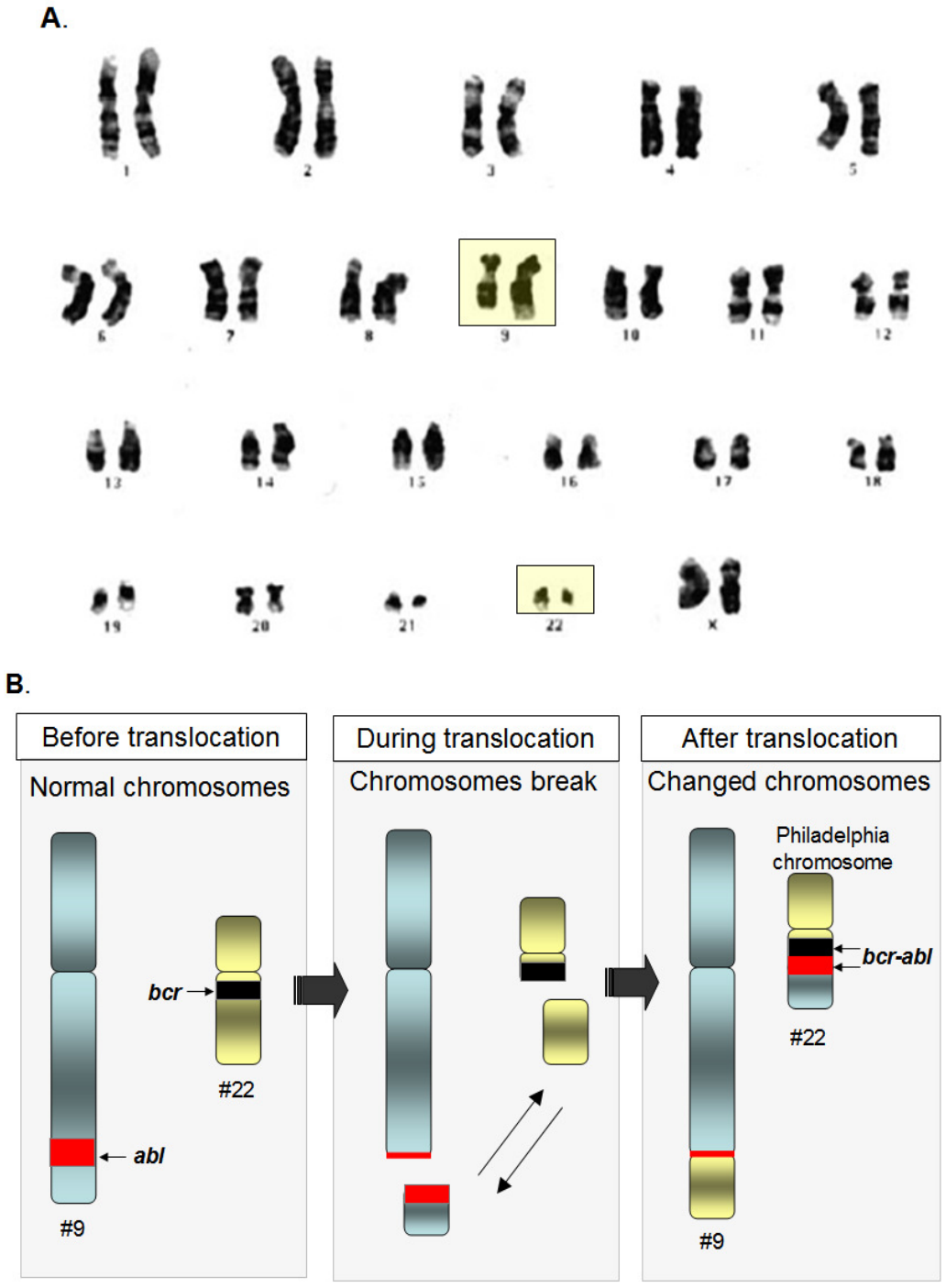
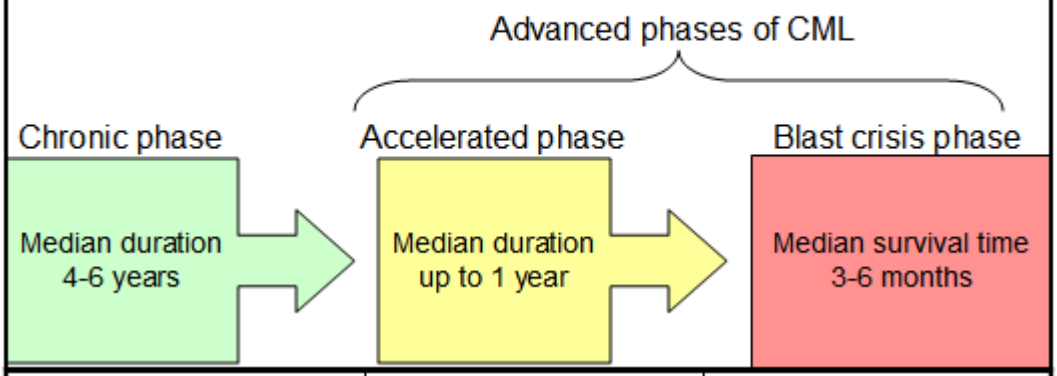



**Figure 2: The Philadelphia (Ph') chromosome**. CML is the first disease to be linked to a clear and consistent genetic abnormality, the Philadelphia chromosome. (**A**) Human karyotype, with chromosomes 9 and 22 highlighted; (**B**)

Formation of the Philadelphia chromosome. The Philadelphia chromosome is formed by the head-to-tail reciprocal translocation between the long arms of chromosome 9, which contains the proto-oncogenic *ABL* non-receptor tyrosine kinase, and chromosome 22, which contains the *bcr* serine/threonine kinase [t(9:22)(q34:11)].

CML is divided into three phases based on clinical characteristics that such as the amount of blast cells (immature, abnormal white blood cells) in the blood and bone marrow and the severity of symptoms presented (see Table 2). The initial chronic phase (CP), diagnosed in approximately 95% of patients, has an average duration of 4 to 6 years and is often asymptomatic, or patients may experience mild symptoms of fatigue, anemia, splenomelgaly, upper abdominal pain or mass. Less common presentations may include fever, weight loss, anorexia or gout. Chronic phase CML is characterized by an overproduction of immature myeloid and mature granulocytes in the spleen, peripheral blood and bone marrow; however, the cells retain the ability to differentiate and function normally. The lack of therapeutic intervention and the presence of additional genetic and/or epigenetic defects cause the disease to progress to an accelerated phase (AP), characterized by the presence of 10–20% primitive blast cells in the peripheral blood and bone marrow. When the disease advances to the 'blast-crisis' phase it resembles acute leukemias and is characterized by the presence of >20% undifferentiated blasts in the peripheral blood and bone marrow. At this grave stage patients have a life expectancy of 3-6 months.

**Table 2: Three Phases of Chronic Myeloid Leukemia**




| | | |
|---|---|---|
| •Approximately 85% of patients with CML are in the chronic phase at the time of clinical diagnosis.<br>•Patients may be asymptomatic or may have mild symptoms of exhaustion.<br>•This phase is characterized by overproduction of immature myeloid and mature granulocytes in the spleen, peripheral blood and bone marrow. | Accelerated phase is characterized by the presence of primitive blast cells in the peripheral blood and bone marrow | Blastic or blast crisis phase is characterized by the presence of 30% undifferentiated blasts in the peripheral blood and bone |

While the cause of the initial translocation event which gives rise to CML remains unclear, studies have shown that translocation can be induced by ionizing radiation (IR) [27]. Additionally, the physical distance between the *BCR* gene and the *ABL* gene in human hematopoietic progenitor cells is shorter than might be expected by chance and could favor translocation between the two genes [28, 29].

## The BCR-ABL Protein

BCR-ABL's oncogenic capacity is mediated by its multiple modular domains inherited from BCR and ABL that facilitate diverse protein-protein interactions (some of BCR-ABL's protein targets are described in Table 3).

**Table 3: Some of BCR-ABL Substrates**

| Substrate | Function | Associated Pathway |
|---|---|---|
| **Grb-2** | Adaptor molecule | Ras/MEK survival pathway |
| **Shc** | Adaptor molecule | Ras/MEK survival pathway |
| **SOS** | Adaptor molecule; Ras guanine-nucleotide-exchange factor | Ras/MEK survival pathway |
| **P62$^{DOK}$** | Adaptor molecule; phosphoprotein | Ras/MEK survival pathway |
| **CrkI** | Adaptor molecule | Ras/MEK survival pathway; PI3/Akt anti-apoptotic pathway |
| **Crk** | Adaptor molecule | Ras/MEK survival pathway; PI3/Akt anti-apoptotic pathway |
| **Talin** | Focal adhesion protein; cytoskeletal/cell membrane rearrangement | Integrin-mediated signaling |
| **Paxillin** | Focal adhesion adaptor protein; cytoskeletal/cell membrane rearrangement | Integrin-mediated signaling |
| **Fak** | Focal adhesion protein; cytoskeletal/cell membrane rearrangement | Integrin-mediated signaling |
| **Fes** | Tyrosine kinase | Myeloid differentiation |
| **Raf-1** | Serine/threonine kinase | Ras/MEK survival pathway |
| **Ras** | signaling switch, cellular oncogene | Ras/MEK survival pathway |
| **Ras-GAP** | Ras-GTPase activation | Ras/MEK survival pathway |
| **PI3 kinase (p85)** | Phospholipid serine kinase; pro-survival signaling | PI3/Akt anti-apoptotic pathway |
| **Akt** | Protein kinase | PI3/Akt anti-apoptotic pathway |
| **Bap-1** | 14-3-3 protein | Ras/MEK survival pathway; PI3/Akt anti-apoptotic pathway |
| **Cbl** | Unspecified | PI3/Akt anti-apoptotic pathway? |
| **Vav** | Adaptor molecule; guanine-nucleotide-exchange factor | STAT-mediated cell survival pathway |
| **Bad** | Apoptotic regulator | PI3/Akt anti-apoptotic pathway |
| **Bcl-xL** | Apoptotic regulator | PI3/Akt anti-apoptotic pathway |
| **STAT1** | Transcriptional activator | JAK/STAT cell survival pathway |
| **STAT5** | Transcriptional activator | JAK/STAT cell survival pathway |

**Structure of c-BCR Protein**

The 160-kd (p160) breakpoint cluster region protein, BCR, is a large multi-domain, ubiquitously expressed serine/threonine kinase comprised of 1,271 amino acids (Figure 3). At its $NH_2$-terminus, BCR has a coiled-coil/oligomeric domain which enables the protein to dimerize *in vivo* [30]. The serine/threonine kinase domain defines the protein's function by phosphorylating substrate on either serine or threonine residues. Known substrates of BCR include Bap-1, a member of the 14-3-3 family of proteins [30, 31] and BCR itself [32]. Within the center of the protein is the Rho/guanine nucleotide exchange factor (Rho/GEF) domain that is specific for the Rho-subfamily of small GTPases, RhoA and CDC42. Both small GTPases regulate actin cytoskeleton rearrangement and control cell functions such as cell migration, morphology and cell cycle progression. The Rho/GEF domain also interacts with and activates transcription factors such as NF-kB [33]. This domain is followed by the Pleckstrin homology (PH)/Calcium-dependent lipid binding (CaLB) domain that binds phospholipids, such as phosphotidylinositides (PI). This interaction plays an important role in signal transduction by localizing the protein to cellular membranes and activating second messenger. The carboxyl-terminus of the protein contains a Rac-GTPase activating protein (Rac-GAP) domain [34], a small GTPase belonging to the Ras super-family that plays a role in regulating actin polymerization. Additionally, BCR's tyrosine 177 (Y177) residue is an autophosphorylation site that is essential for binding to the adaptor molecule, *GRB-2*, and activating the mitogen

activated protein kinase (MAPK) signal transduction pathway. BCR-ABL autophosphorylation also occurs on Y283, Y328 and Y360 [35-37].

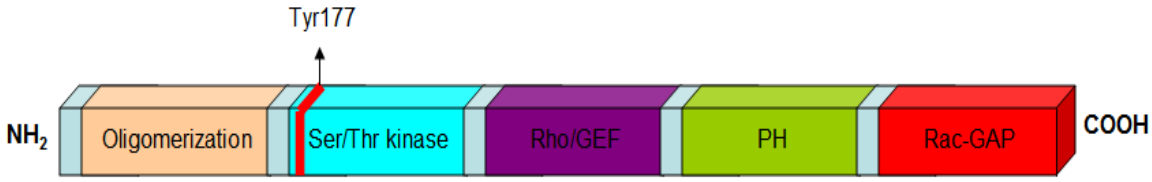



**Figure 3: Structural Domains of The c-BCR Protein**. There are several functional domains found within BCR: At its N-terminus lies an oligomerization domain; this is followed by a serine/threonine kinase domain; a Rho/Guanine nucleotide exchange factor (Rho/GEF) domain; a Pleckstrin homology (PH) domain and a Rac-GTPase activating protein (Rac-GAP) domain. Key residues include Y177 which enables mitogenic activation of the MAPK pathway via binding of the adaptor molecule *GRB-2*.

**Structure of c-ABL Protein**

The *c-ABL* gene was initially identified as the human homologue of the Abelson murine leukemia virus (A-MuLV), which is the acute transforming retrovirus encoding the *v-ABL* oncogene [38]. It is located on chromosome 9q34, spans 230 kilobases (kb) and possesses 11 exons that encodes a 145 kilodalton (kD) (1,097 amino acids) non-receptor tyrosine kinase. In addition to c-ABL, the ABL family of non-receptor tyrosine kinases is also comprised of ARG (ABL-related gene, designated ABL2).

Like BCR, ABL is expressed ubiquitously within the cell, but predominates within the nucleus and cytoplasm. Its diverse localization within the cell reflects its diverse functions, which are mediated by its protein-protein interactions.

These interactions facilitates ABL's involvement in numerous cellular processes, including cellular response to DNA damage and genotoxic stress [32, 39-41], cell cycle regulation [28, 42] and rearrangement and cell migration [43]. There are two isoforms, designated 1b and 1a, which are generated by alternative splicing of the first exon (Figure 4) and are driven by their own promoters. Differing only in their $NH_2$-terminus sequences, the 1b isoform encodes a 6.5 kb mRNA, while the 1a isoform encodes a slightly shorter 5 kb transcript that is 19 amino acids shorter than 1b.

Within the amino-terminus of the 1b isoform lies a myristoylation site which enables plasma membrane- attachment. This site is missing in the 1a isoform. Further within the $NH_2$-terminus are three SRC-homology domains, SH1-SH3. The SH1 tyrosine kinase domain defines protein's function. It contains two key residues: Y412, the site of phosphorylation in transformed cells, and Y393, the major site of autophosphorylation within the proto-oncogene's kinase domain. Both the SH2 and SH3 domains are involved in mediating protein-protein interactions: the SH2 domain binds to the phosphotyrosine regions of target proteins, while the SH3 domain recognizes proline residues. Within the center of the protein is a proline-rich region (P) which, conversely, binds the SH3 domains of other protein substrates [44]. Towards the carboxyl-terminus there are three (NLS) [42, 45], a DNA binding domain (DNA BD) [45], a nuclear exporting signal (NES), and an actin-binding domain which mediates binding to globular/ monomeric (G) and filamentous (F) actin.

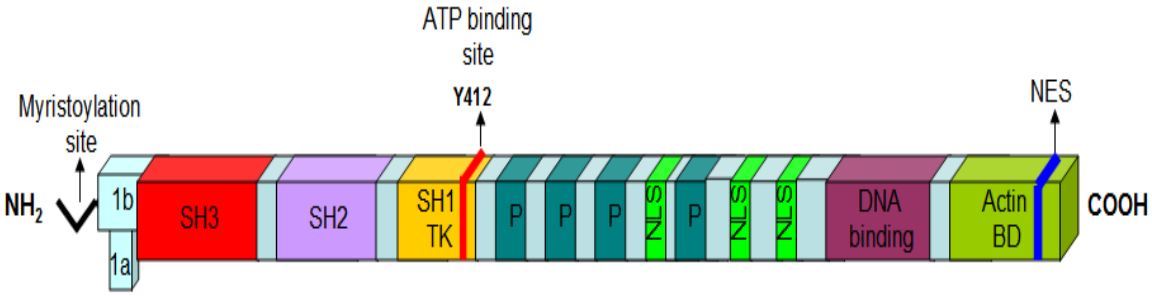



**Figure 4: Structural Domains of The c-ABL Protein**. There are several functional domains found within ABL: a myristoylation site; SRC-homology (SH) domains SH1-3; four proline-rich regions (P); three nuclear localization signal (NLS) regions; a DNA binding domain (DNA BD; an actin binding domain and a nuclear export signal (NES).

### One *BCR-ABL* Oncogene, 3 BCR-ABL Proteins Isoforms

Breakpoints within the *BCR* gene results in primary fusion *BCR-ABL* transcripts with the same portion of *ABL* sequence in the carboxyl-terminus, but include different amounts of *bcr* sequence at the NH2-terminus (see Figure 5A). Unlike *ABL,* which has only one breakpoint within a region greater than 300 kb at its 5' end, *BCR's* breakpoints are localized within three 'breakpoint cluster regions' (bcr) resulting in three BCR-ABL fusion proteins varying in size from 190 to 230 kD (Figure 5B). Each isoform is associated with a different type of leukemia.

In 95% of CML patients and 1% Ph-positive acute lymphoblastic leukemia (ALL) patients, the breakpoint occurs within the major breakpoint cluster region of *BCR* (M-bcr), a 5.8 kb area stretching *across* exons b12-b16. These patients

possess the p210 BCR-ABL isoform, which is formed when either exon b12 or b13 of BCR fuses with exon a2 of ABL.

Most ALL patients possess the breakpoint within the minor bcr region (m-bcr), which spans a 54.5 kb area between exons b1 and b2. The resulting p190 BCR-ABL fusion protein is characteristic of ALL.

The p230 BCR-ABL isoform is seen in patients with chronic neutrophilic leukemia (CNL), a rare disease in which too many stem cells develop into neutrophils. CNL may stay the same or progress quickly into acute leukemia. The recently characterized mu-bcr (μ-bcr), is associated with this form of leukemia and is located downstream of exon b19.

The research presented here is focused on the role of p210 BCR-ABL in CML pathogenesis.

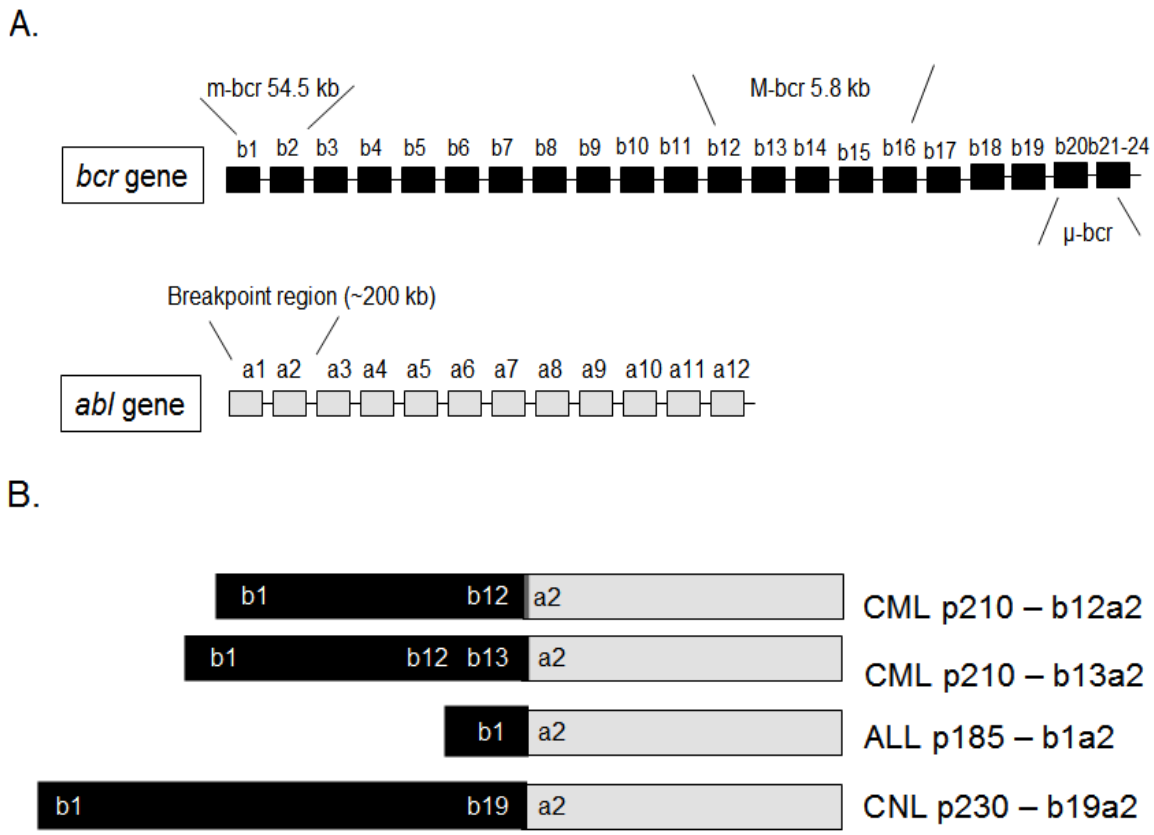



**Figure 5: 1 BCR-ABL Oncogene, 3 BCR-ABL Proteins Isoforms**. Fusion transcripts contain the same portion of the *ABL* within the carboxyl-terminus but different amounts of *BCR* sequence at the amino-terminus depending on where the breakpoints occur within *BCR*. (**A**), Breakpoint locations within the *ABL* and *BCR* genes. 3 breakpoint cluster regions (bcr) are found within the *BCR* gene: the major bcr (M-bcr), the minor bcr (m-bcr), and the mu bcr (µ-bcr). Breakpoints within each of these regions give rise to a different form of leukemia. *ABL* has a >300 kb breakpoint region between exons a1 and a2. (**B**), Structure of the chimeric *BCR-ABL* transcripts. The p190 BCR-ABL isoform is found in Ph-positive ALL, the p210 isoform is found in CML, and the p230 isoform is found in CNL (Clinical Cancer Research Vol. 8, 2177-2187, July 2002).

## CHAPTER III

## BCR-ABL PATHOGENESIS

### The Role of Modular Domains

Features of key domains and motifs contribute to p210 BCR-ABL-mediated malignant transformation in CML patients (Figure 6A). The oligomerization domain of BCR performs several crucial roles: 1) it enables the chimeric BCR-ABL proteins to form dimers (or tetramers) and to trans-autophosphorylate, which abrogates the need for regulatory external kinases (see Figure 6B); 2) it is an essential activator of ABL kinase activity [30]; and, 3) it promotes the association of BCR–ABL with F-actin which facilitates cytoskeletal rearrangement, cell spreading and cell migration [43]. The *GRB-2* binding site at tyrosine 177 activates both the MAPK survival pathway [46, 47], and the phosphatidylinositol 3-kinase (PI3K) anti-apoptotic pathway [47].

Purified ABL is kinase-active, therefore regulatory cis- and trans-acting elements are believed to be involved in its constitutive inhibition. Intermolecular interactions within the first three tandem domains of ABL facilitate their assembly into an auto-inhibitory structure that negatively regulates the protein. The SH3 domain interacts with Pro1124 within the SH2-linker region and results in a 'clamp' structure that confines the kinase in an inactive confirmation [44, 48].

Furthermore, the myristoylation site binds to the tyrosine-kinase domain of ABL, operating as a 'latch' that reinforces the SH3–SH2 'clamp' [48, 49]. Studies show that the SH3 domain is intrinsically capable of suppressing the transforming ability of ABL and its deletion or mutation fully activates the kinase [39]. Additionally, a number of proteins also bind to the SH3 domain of ABL and serve as negative regulators of the proto-oncoprotein. ABL interactor proteins 1 and 2 (Abi-1 and Abi-2) bind to ABL's SH3 domain and activate the inhibitory function of this domain [50-52]. Additionally Pag/Msp23, another ABL SH3-binding protein, dissociates from ABL under oxidative stress and facilitates its tyrosine kinase activity [53].

Fusion of BCR with ABL breaks the intermolecular bonds within ABL's SH domains and disrupts its constitutively inhibited regulatory configuration. Furthermore, activated ABL tyrosine kinase triggers the ubiquitin-proteasome-mediated destruction of the Abi proteins that normally antagonize its oncogenic potential [54].

Collectively, deregulation of BCR and ABL's multiple domains is a major contributing factor in CML pathogenesis.

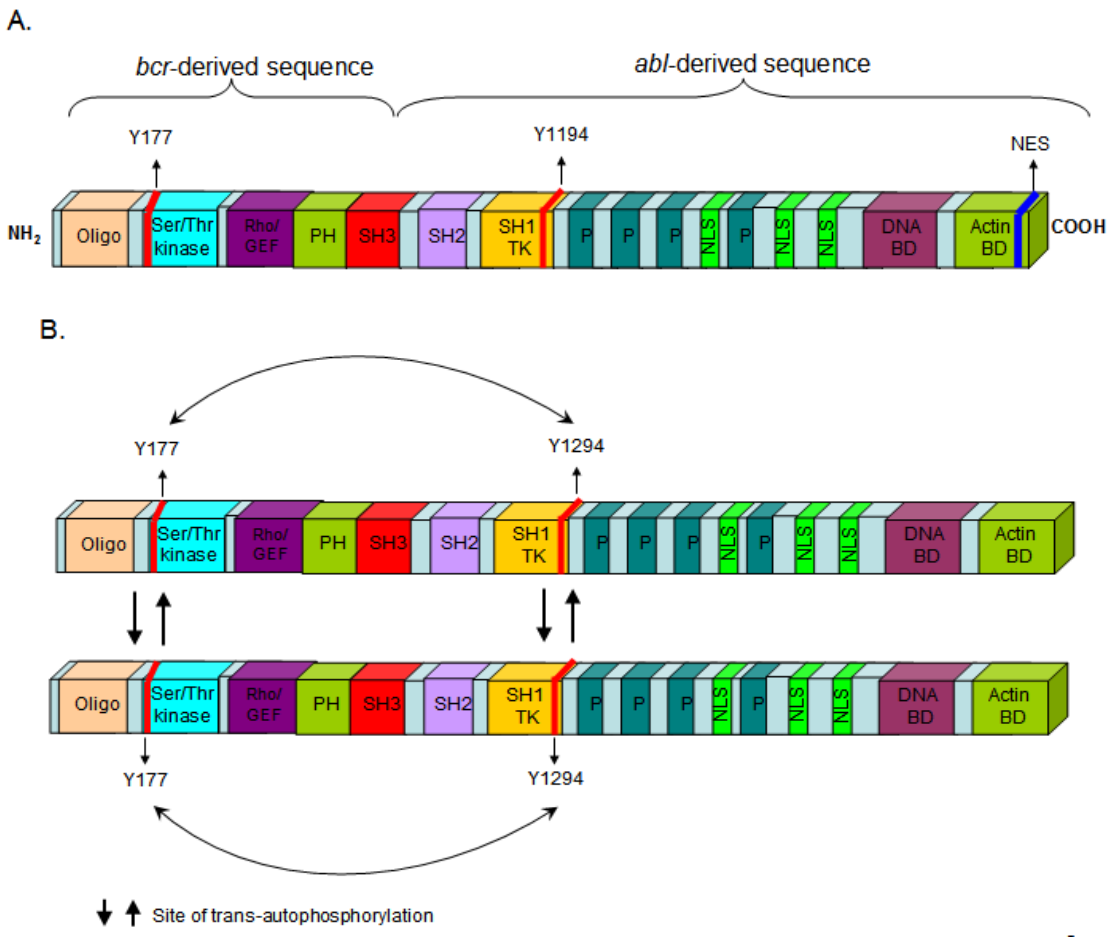



**Figure 6: Structure of p210 BCR-ABL Protein**. (**A**), BCR-derived sequences and ABL-derived sequences. Fusion of BCR to ABL is central to the pathogenesis of CML as it not only disrupts ABL's regulatory configuration but also disrupts BCR-ABL's localization within the cell. (**B**), Location of trans-autophosphorylation sties within BCR-ABL. Proteins are able to dimerize via their oligomerization domains and trans-self-phosphorylate on both Y177, a key binding site for *GRB-2*, and Y1124 within the activation loop of ABL's kinase domain.

### Activation of Multiple Signaling Pathways

BCR-ABL activates numerous pathways commonly used in hematopoietic growth factor receptor signaling. These activated pathways contribute to CML

pathogenesis by causing 1) constitutive mitogenic activation, inhibition of apoptosis and decreased adhesion to bone marrow stroma extracellular matrices (Figure 7).

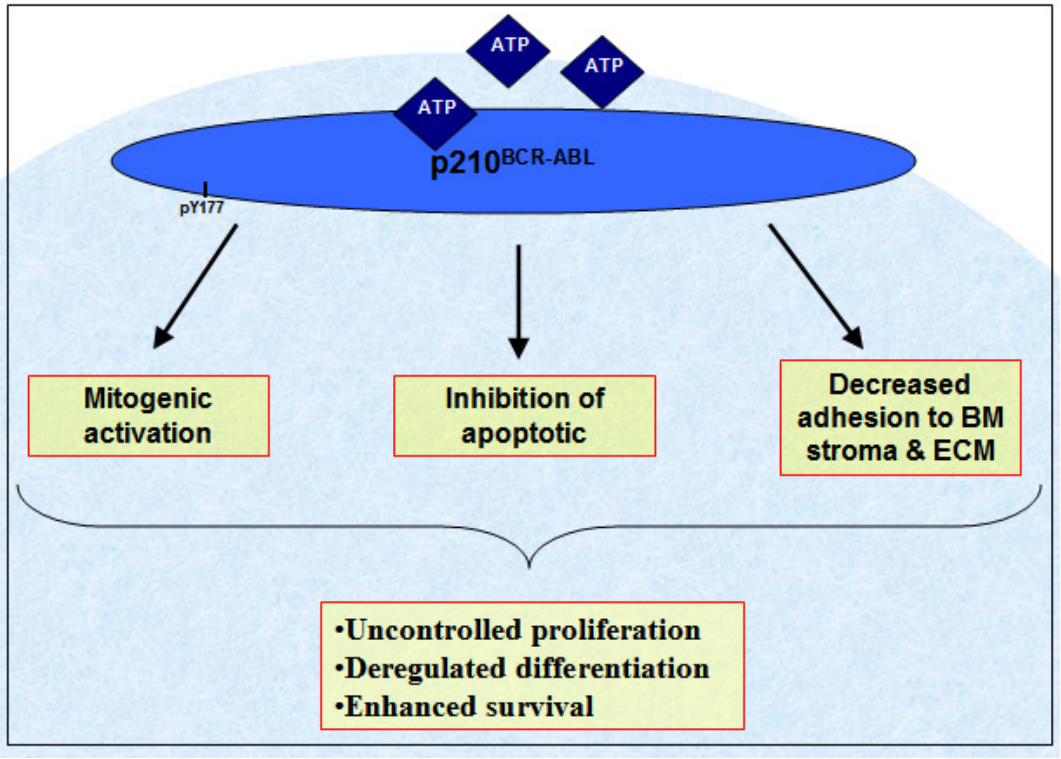



**Figure 7: Mechanisms of Malignant Cell Transformation by BCR-ABL**. BCR-ABL-mediated malignant transformation in CML pathogenesis occurs via three major mechanisms: constitutive mitogenic activation, inhibition of apoptosis and decreased adhesion to bone marrow stroma extracellular matrices.

**Constitutive Mitogenic Activation: The Ras/MEK Pathway**

The Ras/extracellular-signal-regulated kinase (ERK) (Ras/ERK) transmits extracellular signals into the nucleus and results in gene transcription [55-57]. The autophosphorylated Y177 residue within BCR-ABL's first exon serves as a docking site for growth factor-binding protein 2 (GRB-2)'s SH2 domain and

facilitates signal transduction (Figure 8). GRB-2 forms a complex with the guanine nucleotide exchange factor, son of sevenless (SOS) protein, which stabilizes RAS in its active GTP-bound conformation and induces its activation. RAS activation can also be mediated by three other adaptor molecules and BCR-ABL substrates, SH2-containing protein (Shc), Crk-like protein (Crkl) and phospho-protein $p62^{DOK}$ [58-60].

RAS activation initiates a cascade of signaling events involved in CML pathogenesis. Activated Raf-1 (MAP KKK) phosphorylates MEK1/2 (MAP KK) on both serine and threonine residues; MEK1/2 then phosphorylates and activates the extracellular-signal regulated kinases, ERK1/2 (MAPK), which results in the phosphorylation and activation of the transcription factor Elk1. Upon activation, Elk-1 translocates to the nucleus and binds the serum response factor (SRF) transcription factor. This ternary complex then binds the serum response element (SRE) of the immediate early genes and proto-oncogenes, c-Fos and c-Jun, to direct their transcriptional activation and regulation.

Together, c-Fos and c-Jun DNA-binding proteins heterodimerize to form the AP-1 transcription factor which upregulates the transcription of genes involved in cell proliferation, cell differentiation and mediating growth-factor independence. These genes include the G1-cyclin dependent kinases (CDKs) [61]; CDK regulator, cyclin D1 [62]; c-Jun [63]; the anti-apoptotic protein Bcl-xL [64]; granulocyte-macrophage colony-stimulating factor (GM-CSF) hematopoietic cytokine [65]; the second messenger, cyclic adenosine monophosphate (cAMP)

[66]; and cAMP response element binding protein (CREB) [67]. The upregulation of these genes is characteristic of CML pathogenesis.

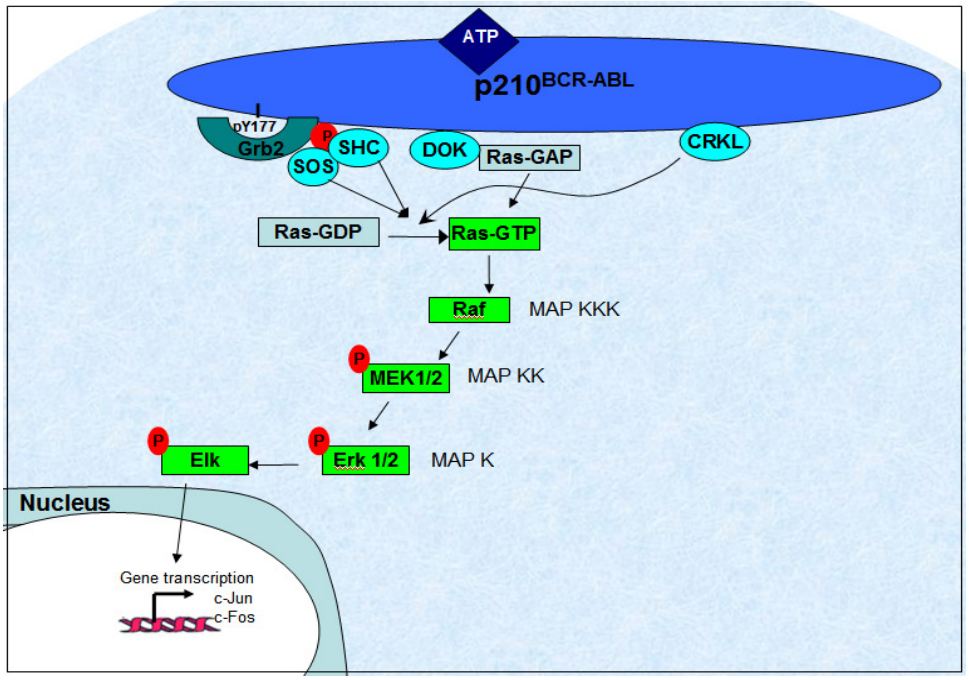



**Figure 8: BCR-ABL Activates the Ras/MEK Mitogenic Signal Transduction Pathway.** Autophosphorylation of BCR-ABL on Y177 results in its interaction with the adaptor molecule, *GRB-2*. *GRB-2* interacts with the guanine nucleotide-exchange factor, SOS, which in turn mediates RAS activation. The adaptor molecules Shc and Crkl can also mediate RAS activation. RAS is coupled to the mitogen activated protein kinase (MAPK) pathway by the serine/threonine kinase, Raf. Raf in turn phosphorylates mitogen activated and extracellular-signal regulated kinase kinases 1 and 2 (MEK1/2). This lead to a phosphorylation and activation of and extracellular-signal regulated kinase kinases 1 and 2 (Erk1/2) and the subsequent activation of the transcription factor Elk.

## Inhibition of Apoptosis

*The PI3/AKT Pathway*

The lipid kinase phosphoinositide 3-kinase (PI3K) represents a family of cytosolic, intracellular signaling proteins whose deregulation is associated with malignant cell transformation [68]. Class I PI3Ks, which are commonly activated in CML, are heterodimeric molecules that are composed of a regulatory and a catalytic subunit and can be divided into two subclasses, class 1A and B. While class1A consists of an 85 kD regulatory subunit (p85) and a 110 kD catalytic subunit (p110), class1B consists of only a 110 kD catalytic subunit (p110γ/PI3Kγ). The class 1A PI3K pathway is activated either in one of two ways: 1) the p85 regulatory subunit binding to an activated tyrosine residue on the activated interleukin (IL)-3 receptor, or 2) by the p110 subunit binding to activated Ras. Once activated, PI3K translocates to the cell plasma membrane where it phosphorylates the membrane phospholipid PI(4,5)P2 to produce PI(3,4,5)P3. This in turn activates phosphoinositide-dependent protein kinase 1 (PDK1) and facilitates its membrane localization where it phosphorylates and activates protein kinase B (AKT), PI3K primary downstream effector protein.

Activation of PI3K/AKT survival pathway is essential to BCR-ABL-mediated leukemogenesis [69] (Figure 9). BCR-ABL interacts indirectly with the p85 subunit of PI3K through the adaptor molecules GRB-2 and SHC to stimulate the constitutive activation of the p110 catalytic subunit [70]. This interaction is mediated by BCR-ABL's recruitment of GRB-2-associated binding protein

(GAB2) through GRB-2. GAB2 then activates the p85 regulatory subunit of PI3K, leading to constitutive AKT activation, which can also be achieved by SHC's binding to the SH2 domain within BCR-ABL's carboxyl-terminus.

Mutations to the p85 subunit of PI3K resulted in the inhibition of BCR-ABL-dependent growth in hematopoietic CML cells, which highlights the role of PI3K in BCR-ABL-mediated pathogenesis [71]. Additionally, BCR-ABL upregulates the transcription of the p110γ subunit of PI3K in several CML cell lines.

Constitutive activation of the PI3K/AKT pathway suppresses apoptosis in several ways. AKT phosphorylates the pro-apoptotic protein, Bad, which is scavenged by the cytosolic protein 14-3-3 and neutralized. This decreases apoptosis by preventing Bad from binding and inhibiting the anti-apoptotic protein Bcl-xL [72]. AKT promotes cell survival by phosphorylating and suppressing the activity of FKHRL1/FoxO3, a member of the 14-3-3 Fork-head family of transcription factors. Phosphorylation by AKT sequesters FoxO3 in the cytoplasm, preventing it from translocating to the nucleus where it activates genes necessary for cell death, such as Bad and Bim [73, 74]. AKT-induced phosphorylation of the "initiator" death caspase, caspase-9, also decreases apoptosis by inhibiting its proteolytic activities directly [75]. The ubiquitously expressed serine/threonine kinase glycogen synthase kinase-3 (GSK-3), another key downstream target of AKT, also induces apoptosis in tumor cells [76]. However, induction of apoptosis by GSK-3 is inhibited when it is phosphorylated by AKT. Lastly, AKT activates the nuclear factor-kB (NF-kB) transcription-factor,

which is involved in the transcription of anti-apoptotic genes. Normally NF-kB is held in the cytoplasm in an inactive complex with its inhibitor, IkB. However, phosphorylation of NF-kB by AKT releases the transcription factor and enables it to perform its anti-apoptotic gene transcription [77].

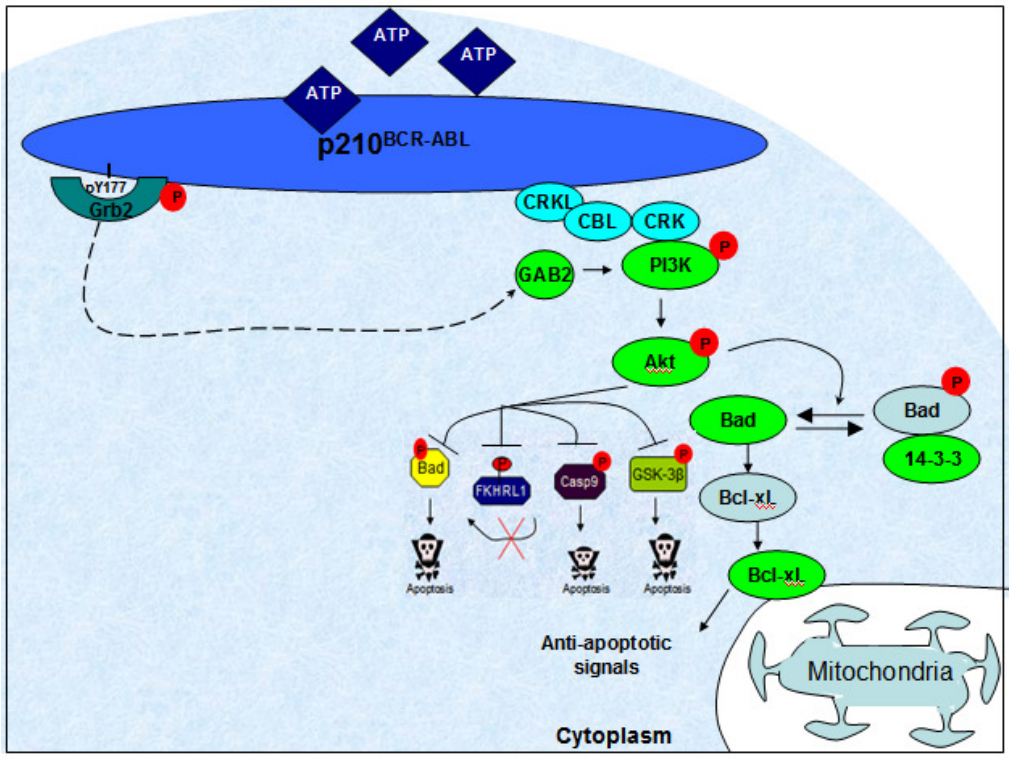



**Figure 9: BCR-ABL Activates the PI3K/AKT Anti-Apoptotic Signal Transduction Pathway.** By recruiting *GRB-2*-associated binding protein (GAB2) or the adaptor molecule Shc, BCR-ABL activates the PI3K/AKT pro-survival pathway; a process which can also be mediated by the signaling adaptor protein Crk. This interaction facilitates PI3K/AKT –mediated anti-apoptotic signaling in by phosphorylation and inhibition of: 1) the pro-apoptotic protein Bad, making it incapable of suppressing the activity of Bcl-xL; 2) FKHRL1/FoxO3 , making it incapable of translocating to the nucleus to activate pro-apoptotic proteins; 3) the cysteine protease, caspase-9; 4) the serine/threonine kinase glycogen synthase kinase-3 (GSK-3).

*The JAK/STAT Pathway*

Signal transducer and activators of transcription (STATs) were originally characterized as latent cytoplasmic transcription factors. They are members of a family transcription factors that are involved in normal cellular response to cytokines, such as cell differentiation. There are seven STAT proteins: STAT1, STAT2, STAT3, STAT4, STAT5 (which consists of two closely related proteins, STAT5A and STAT5B) and STAT6. They have diverse biological roles and control critical cellular functions including cycle progression, cell proliferation, survival, differentiation, apoptosis and oncogenesis.

STAT-mediated signal transduction occurs in several steps. Extracellular binding of cytokines to their cognate receptors, followed by receptor dimerization, leads to the engagement of associated Janus kinases (JAKs), a family of four cytoplasmic non-receptor tyrosine kinases (JAK1, JAK2, JAK3 and Tyk2), to the receptors' cytoplasmic tail. JAKs then become activated by auto-phosphorylation, causing them to phosphorylate tyrosine residues within the receptor 's cytoplasmic tails. These receptor-associated phosphotyrosine residues serve as docking sites that recruit latent cytoplasmic STAT monomers through its SH2 domain. Further tyrosine phosphorylation by JAKs leads to activation of STAT molecules, which in turn induces the formation of STAT homo- or heterodimers through interaction of the phosphotyrosine of one molecule with the SH2 domain of the other molecule. STATs can also be activated directly by growth factor receptors, such as epidermal growth factor receptor (EGFR), platelet derived

growth factor receptor (PDGFR) and GM-CSF receptor (or CD116), as well as by cytoplasmic kinases and oncoproteins, such as SRC and ABL. Activated STAT dimers are translocated into the nucleus via importin α/β as well as Ran-GDP. Once inside the nucleus activated STAT molecules bind a unique cytokine inducible sequence named GAS (γ-interferon activated sequences) within the promoter of target genes, thereby activating gene transcription. The activation of STATs is required for repopulation of the stem cell pool, as well as cell proliferation and differentiation in response to external stimuli.

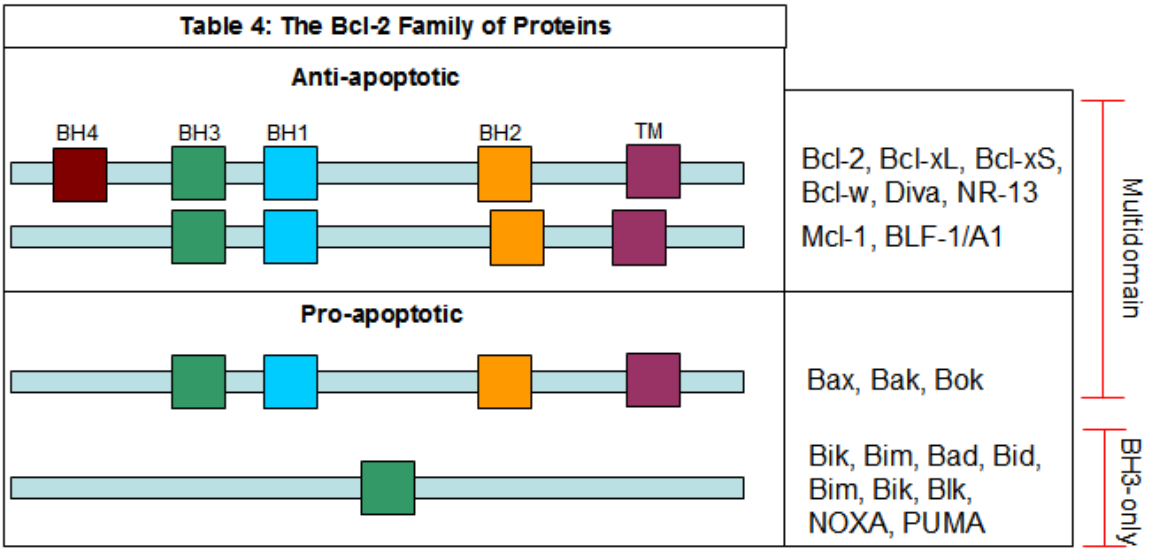
Several STATs, particularly STATs 1, 3 and 5, are constitutively activated in several types of cancers. Typically STAT1 is involved in cell growth arrest [78, 79], in promoting apoptosis [80] and is implicated as a tumor suppressor [81]. STAT3 and STAT5 are involved in cellular transformation, cell cycle progression and preventing apoptosis [82-84].

Aberrant activation of cytokine-mediated signal transduction pathways involving STATs are capable of conferring cytokine-independent growth to leukemia cells [85]. In CML, BCR-ABL can induce phosphorylation and activation of STAT5 via its SH2 and SH3 domains, hijacking normal cellular functions by circumventing the need for cytokine-mediated STAT5 activation. BCR-ABL kinase-dependent STAT5 activation in CML cells has been shown to be an essential component of CML's pathogenesis [86, 87] (see Figure 10).

Cells with constitutive STAT activation promote malignant cell proliferation by upregulating the gene expression cyclin D1, a cell cycle regulator that controls

cell cycle progression from G1 to S phase, and c-Myc, a key transcription factor that promotes cell cycle progression by inhibiting the expression of p21, a cyclin-dependent kinase inhibitor [88, 89]. c-Myc is also capable of upregulating the expression cyclins. Additionally, constitutively activated STATs inhibits apoptosis by upregulating the expression of members of the Bcl-2 family of anti-apoptotic proteins Bcl-xL, Bcl-2 and Mcl-1 (see Table 4 for list of pro- and anti-apoptotic Bcl-2 family of proteins).

The combined effects of 1) deregulated cell cycle progression and, 2) reduced apoptosis, results in uncontrolled cell proliferation and malignant cell transformation characteristic in CML pathogenesis.




**The BCL-2 Family of Proteins.** The Bcl-2 (B-cell lymphoma-2) family of proteins governs mitochondrial outer membrane permeability (MOMP). They are categorized functionally by their ability to promote or inhibit cell death (pro- or anti-apoptotic) and structurally by their Bcl-2 homology (BH) domains. Each protein possesses at least one of four of the characteristic BH domains, named

BH1-4, and can therefore be subdivided further into those that have multidomain and those that have BH3-only domain. With the exception of Mcl-1 and BLF/A1, which have three conserved BH domains, the anti-apoptotic family of proteins possesses all four domains. Conversely, with the exception of Bax, Bak and Bok which are multidomain proteins, all the pro-apoptotic family of proteins are BH3-only domain-containing proteins. TM, transmembrane.

**Decreased Adhesion to Bone Marrow Stroma and Extracellular Matrices**

During hematopoiesis, cell differentiation and proliferation is tightly regulated through specific interactions between HSCs and bone marrow stroma. Transformed CML progenitor cells escape normal negative regulatory signals and exhibit enhanced motility and perturbed adhesion properties. Rho GTP activating proteins (GTPases) are important regulators of cell motility, cell adhesion, actin assembly and cell migration [90]. They play important roles within the cell by transducing extracellular regulatory signals to effector molecules (reviewed in [91]. Rac GTPases, Rac 1-3, are members of the Rho GTPase family and cycle between GDP- and GTP-bound states. BCR-ABL activates RhoA and Rac1 GTPases via BCR's Rho/GEF domain and ABL's Rac GAP domain respectively. Additionally, Rac1 and Rac2 are significantly upregulated in CD34+ chronic phase CML cells [92].

BCR-ABL recruits and activates Rac1 through its interaction with its hematopoietic-specific GEF, Vav, which leads to alternations in actin assembly. Deregulation of actin assembly enhances the motility of CML cells and facilitates their diminished adhesion to bone marrow stroma [93]. CML cells also display

increased expression of focal adhesion proteins, such as focal adhesion kinase (FAK), talin and paxillin, when compared to normal cells. These focal adhesion proteins play important roles in cell motility and survival, and their increased expression enable CML cells to escape the normal negative regulatory signals provided by component of the BME [94]. Additionally, CML cells express an adhesion-inhibitory variant of β1 integrin that is not found in normal progenitor cells [95].

Taken together, BCR-ABL-mediated cytoskeletal rearrangement contributes to CML pathogenesis.

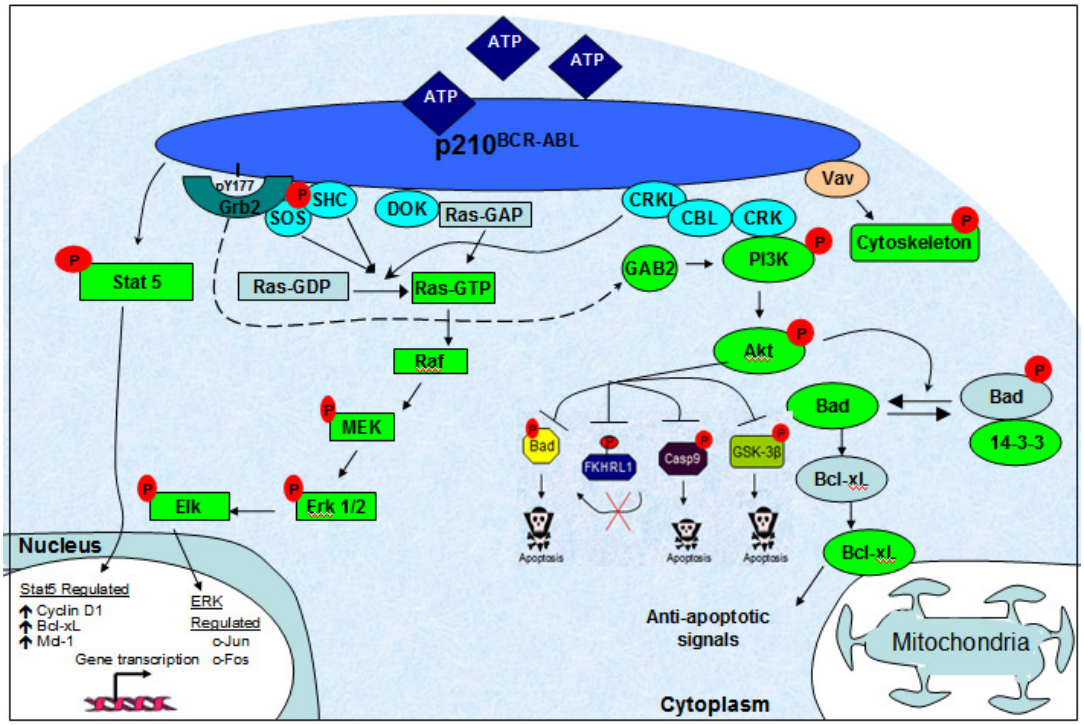



**Figure 10: Summary of Signal Transduction Pathways Activated in BCR-ABL-Mediated Leukemogenesis.** BCR-ABL is capable of phosphorylating numerous substrates to activate signal transduction pathways shared by cytokine

receptors and are involved in the growth and differentiation of hematopoietic cells. These activated signal transduction pathways include: Ras/MEK, which results in promitotic transcriptional gene regulation; PI3/AKT; which results in suppression of apoptosis; JAK/STAT5, which results in pro-survival gene transcription; Rac-GTPase activation, which results in cytoskeletal reorganization and anchorage-independent growth.

## CHAPTER IV

## TREATMENT FOR CHRONIC MYLOID LEUKEMIA

### Before Signal Transduction Inhibitors

Prior to the development of targeted therapy against CML, therapeutic options included cytotoxic drugs such as busufan, hydroxyurea, recombinant interferon α (rIFNα), bone marrow transplant (BMT) and allogeneic stem cell transplantation (allo-SCT).

### Busulfan

Busulfan is an inexpensive, oral alkylating agent that produces DNA-crosslinks resulting in the inhibition of DNA and protein synthesis. It produces periods of hematologic response in patients and is often restricted to those who are intolerant or resistant to hydroxyurea (see Table 5 for a list of definitions of the types of response to CML treatment). While it lowers white blood cell (WBC) count, its myelosuppressive effects were often delayed and persistent, which complicated therapy. Additional draw-backs to using busulfan include hyperpigmentation and interstitial lung disease [96, 97].

**Hydroxyurea**

Hydroxyurea is also a DNA-synthesis inhibitor that achieved hematologic remission in patients within 1-2 months of use and is normally well tolerated in CML patients. Although it has been shown to be better than busulfan, both drugs rarely induced cytogenic responses. Neither busulfan nor hydroxyurea prevented disease progression from chronic phase to acute phase in CML patients.

**Recombinant Interferon-Alpha**

Recombinant interferon-alpha (IFNα) treatment produces cytogenetic response in 70% of patients, with 20% of those patients experiencing complete cytogenic response. Studies show that rIFNα works well against CML alone or in combination with cytarabine, another DNA-damaging agent. It increased patients' survival rates and is the treatment of choice for patients who do not have a HLA-matched bone marrow donor or are too old for BMT [98, 99]. Under rIFNα treatment patient survival at 5 years was 57%, compared to 42% in the other previously discussed treatment groups (CML Trailists' Coorperative Group, J Nat Cancer Inst. 1997; 89:1616-1620). Unfortunately, rIFNa treatment is limited by its toxicity profile, with 15-25% of patients discontinuing treatment due to intolerable side effects and another 35-50% requiring dose reduction due to poor drug tolerance. Side effects include anorexia, fever, chills and postnasal drip.

**Allogeneic Stem Cell Transplantation**

At present, only allogeneic stem cell transplantation (allo-SCT) is an effective and curative treatment option for CML patients, providing long-term disease eradication [100]. Stem cells are derived from the bone marrow or peripheral blood of donors. Results are best for patients in chronic phase when compared to those in accelerated or blast crisis phase. The long term survival rate using this treatment method is 50-60% for CML chronic phase patients, 15-20 for accelerated phase patients, and <10% for blast crisis phase patients [101].

Treatment-related morbidity and mortality rates in allo-SCT patients are high and often associated with organ toxicity, complications from infection and chronic graft-versus-host disease (GVHD) [102]. Due to the difficulty in finding a healthy, young HLA-matched sibling or donor, allo-SCT is often reserved for patients under the age of 55 years old who are unresponsive to secondary BCR-ABL inhibitors.

| Table 5: Definition of The Types of Response to CML Treatment | | |
|---|---|---|
| **Field** | **Types of response** | **Definition** |
| Hematology | | The study of blood, blood-forming organs and blood diseases |
| | Hematologic response | The evaluation of white blood cell (leukocyte) counts |
| | Partial | The reduction and normalization of leukocyte count in the peripheral blood |
| | Complete | Normal leukocyte count in peripheral blood |
| Cytogenetics | | The study and evaluation of chromosomes and cell division |
| | Conventional | Microscopic examination of cells in metaphase of cell division |
| | Molecular | The use of DNA-binding probes to label and visualize one or more specific regions of the genome e.g. fluorescence *in situ* hybridization (FISH) |
| | Cytogenetic response | Cytogenetic monitoring of response to treatment in CML cells within the peripheral blood and bone marrow |
| | Major/Partial | Less than 35% (1/3) of cells is Ph+; at least 2/3 of blood cells are normal (Ph-) |
| | Complete | There is no detectable Ph+ cells from >20 bone marrow cells in metaphase |

## Rationally Designed BCR-ABL Signal Transduction Inhibitors

### Imatinib Mesylate

Constitutive BCR-ABL kinase activity in HSCs is an essential component of CML pathogenesis. The identification of BCR-ABL as the hallmark oncogenic transforming protein in CML made it an ideal target for drug development. This gave rise to rationally designed, small bioavailable signal transduction inhibitors (STI) specific for the tyrosine kinase domain of BCR-ABL. Imatinib mesylate (imatinib, also known as Gleevec; formerly STI571 and CGP 57158; Novartis Pharmaceuticals Corporation, Basel, Switzerland) was identified as a lead

compound in a high throughput *in vitro* screening for tyrosine kinase inhibitors (TKIs). The 2-phenylaminopyrimidine-derivative was designed to bind the ATP binding site of ABL kinases (see Figure 11) While imatinib is also capable of binding to other kinases, such as the stem cell factor (SCF) cytokine receptor, c-kit, and platelet-derived growth factor receptor (PDGFR), it is specific for ABL oncoproteins, including c-ABL and ETV1-ABL.

Functionally, imatinib easily transverses the cell membrane and selectively binds the tyrosine kinase domain of BCR-ABL. This domain consists of a bi-lobed structure in which Mg-ATP is located in a deep cleft between the amino- and carboxyl-terminal lobes. BCR-ABL cycles between two distinct active and inactive states, acting as a "molecular switch" depending on the conformation of its activation loop which contains the highly conserved asparagine-phenylalanine-glycine (DFG) motif.

In an active kinase conformation, Y1294 in the activation loop becomes phosphorylated and takes on a 'DFG-out' configuration, in which these conserved residues are flipped out of their usual position. This open conformation provides a platform for substrate binding and activation.

However, in the inactive kinase conformation, this DFG motif is folded into the ATP-binding site and makes a channel opening outside the threonine 315 gatekeeper residue [103]. This residue is essential as it is located near the ABL catalytic domain in the middle of the imatinib binding site and controls access to the hydrophobic region of the enzymatic active site. The inactive conformation of

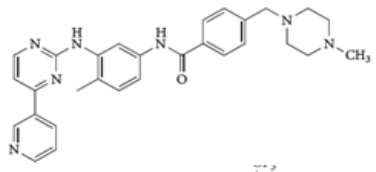
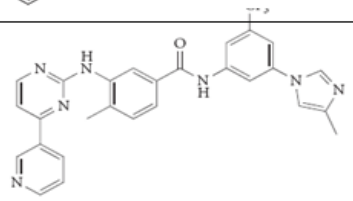
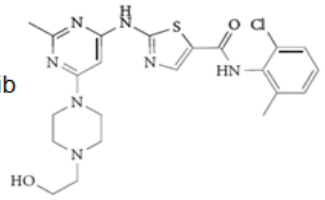
BCR-ABL causes an auxiliary binding site within the ATP-binding pocket to open, which facilitates imatinib binding by the formation of hydrogen bonds. Interaction with imatinib traps and stabilizes BCR-ABL tyrosine kinase in an inactive, "closed" conformation, which terminates its kinase activity and inhibits its auto- and substrate phosphorylation [104].

With has an $IC_{50}$ value of 0.5 µM, imatinib has been shown to induce apoptosis in BCR-ABL+ cell lines as well as in primary leukemia cells from CML patients [105] (see Table 6 for a profile of BCR-ABL inhibitors). Cells undergo apoptosis within 48 to 72 hours following imatinib treatment.

Chronic phase CML patients who had failed rIFNα-therapy and were treated with 400 mg/day of imatinib did not progress to accelerated or blast crisis phase. Additionally, 95% of achieved complete hematologic response and 60% had a major cytogenic response (MCR). A long-term follow-up of these patients showed a 13% increase in the MCR rate to 73%, with 63% of patients having a CCR. Studies done in accelerated-phase CML patients were encouraging, though less dramatic. Eighty two percent of these patients showed hematologic responses, with MCR seen at 24% and CCR at 17%.

Imatinib's efficacy has revolutionized CML patient care ([104, 106]. By obstructing BCR-ABL's tyrosine kinase domain and its ability to commandeer signal transduction pathways crucial for CML pathogenesis, imatinib inhibits BCR-ABL's role in cell transformation at micromolar concentrations.

Yet despite the efficacy of imatinib, CML patients in advanced stages of the disease (whose in accelerated or blast crisis phase) eventually display inadequate responses to treatment and often experience relapse within a year [107].

| Table 6: Profile of BCR-ABL STIs | | | CML Response Rates | | |
|---|---|---|---|---|---|
| Structural formula | $IC_{50}$ | P | HR | CCR | |
| | | | | CP | AP |
| Imatinib  | 500 nM | 1X | 95% | 63% | 17% |
| Nilotinib  | 30 nM | 30X | 90% | 70% | 29% |
| Dasatinib  | 1nM | 300X | 100% | 95% | 82% |

Key: STIs = signal transduction inhibitors; P = Potency; HR = hematological response; CCR = complete cytogenic response; CP= chronic phase CML; AP = accelerated phase CML

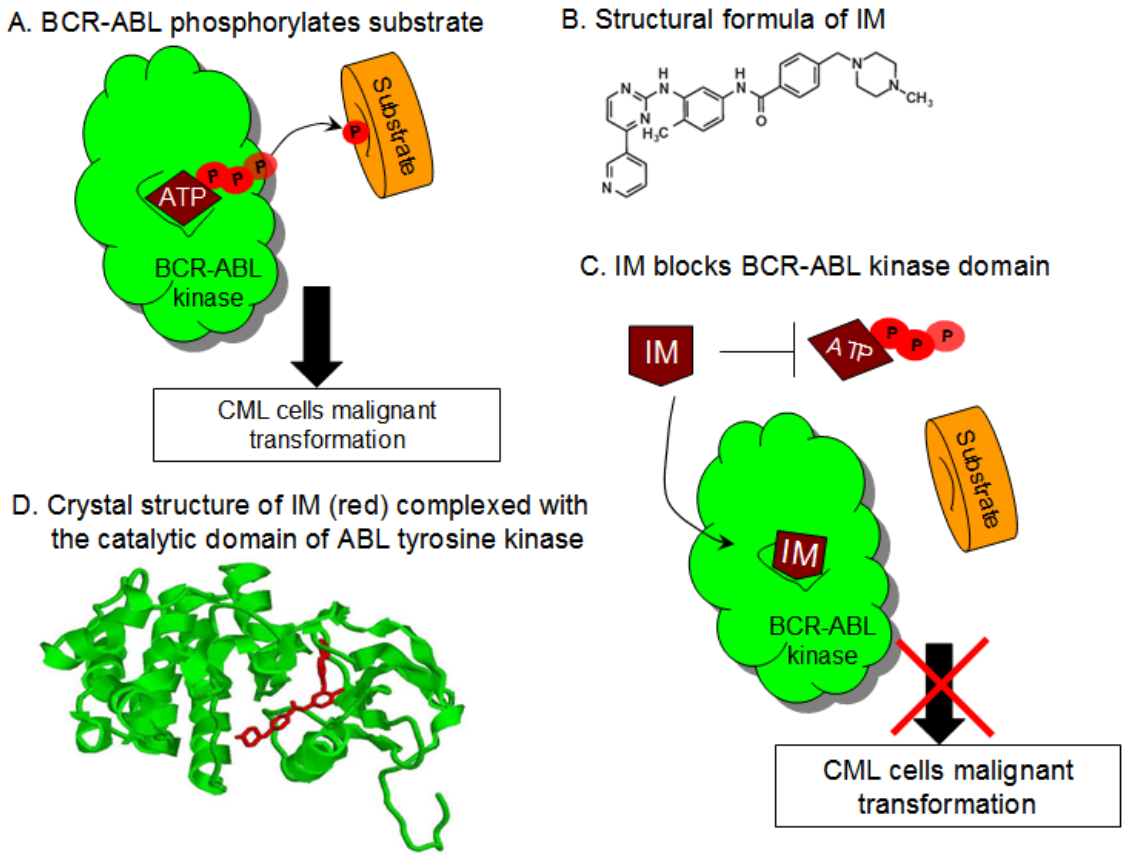



**Figure 11: Mechanism of Imatinib Action in Chronic Myeloid Leukemia Cells**. (**A**), CML pathogenesis is mediated by the constitutive activation of the BCR-ABL oncoprotein, which binds ATP to phosphorylate and activate numerous substrates involved signal transduction and malignant cell transformation. (**B**), Chemical formula of imatinib (STI-571). (**C**), Imatinib works as a competitive inhibitor and ATP-mimic that selectively competes for and binds to the ATP binding site/tyrosine kinase (TK) domain of BCR-ABL. (**D**), Ribbon representation of the three dimensional crystal structure of the catalytic domain of the ABL tyrosine kinase in complex with imatinib (figure taken from Wikimedia Commons).

**Imatinib Resistant Chronic Myeloid Leukemia**

*In vitro* resistance models have played a critical role in identifying various mechanisms of primary (acquired) resistance that thwart the effects of imatinib

treatment. Patient relapse is associated to three main mechanisms of imatinib resistance: 1) point mutations within BCR-ABL's tyrosine kinase domain that (a) directly interfere with the ability of imatinib to bind to BCR-ABL kinase domain, or (b) impair the ability of BCR-ABL to achieve the inactive conformation required for imatinib binding [108-111]; 2) *bcr-ABL* gene amplification, and 3) overexpression of drug transporters.

*Point Mutations within the BCR-ABL Kinase Domain*

Gorre *et al.* was the first to demonstrate the relationship between a point mutation at ABL kinase domain and imatinib resistance [108]. The most resistant kinase mutation occurs when threonine 315 gatekeeper residue changes to isoleucine (T315I). This change directly blocks the ability of imatinib to bind the kinase domain while still retaining BCR-ABL's capacity to bind ATP and catalyze substrate phosphorylation. The T315I point mutation causes a shift in the equilibrium between BCR-ABL's inactive and active states, restoring BCR-ABL kinase activity. This can be attributed to T315I-mutation-specific changes in phosphorylation pattern within the kinase domain. This mutation results in a shift in the phosphorylation of two key tyrosine residues within the ATP binding loop that confers an oncogenic fitness advantage to cells possessing this mutation [112].

Imatinib mesylate resistance is commonly associated with mutations that alter the flexibility of the BCR-ABL kinase domain to adopt the inactive conformation needed for imatinib binding. A summary of some of these well-

characterized mutations is provided in Table 7. Like T315I, these mutations result from amino acid substitutions and consist of approximately 25 amino acids residues distributed throughout the ABL kinase domain that provide varying degrees of imatinib resistance. In some patients with stable chronic phase CML a number of these mutations are associated with disease progression to advanced stages [113]. The most commonly characterized mutations that account for 60–70% of all kinase mutations affect residues Gly250, Tyr253, Glu255, Met351 and Phe359.

**Table 7: Some Imatinib-Resistant BCR-ABL Kinase Mutations**

| Mutations | Description |
|---|---|
| Leu248Val | destabilization of the inactive state |
| Gly250Glu | stabilization of the active or other conformational state to which imatinib does not bind |
| Gln252His | destabilization of the inactive state |
| Gln252Arg | destabilization of the inactive state |
| Tyr253His | loss of interaction with imatinib; destabilization of the inactive state |
| Tyr253Phe | destabilization of the inactive state |
| Glu255Lys | destabilization of the inactive state |
| Glu255Val | destabilization of the inactive state |
| Phe311Ile | destabilization of the inactive state |
| Phe311Leu | destabilization of the inactive state |
| His396Arg | destabilization of the inactive state |
| His396Pro | destabilization of the inactive state |

Reviewed by Weisberg *et al.*, 2007

*BCR-ABL Gene Amplification*

Gene amplification of target proteins is often characteristic of malignant cell transformation and is a frequently used mechanism to generate drug resistant neoplastic cells. Studies show that resistance to imatinib also occurs through *BCR-ABL* gene amplification. Imatinib-resistant CML cells have displayed higher levels of BCR-ABL protein expression and phosphorylation when compared to parental, imatinib-sensitive cells due to robust overexpression of *BCR-ABL* mRNA transcripts [114]. Studies also show *BCR-ABL* gene amplification linked to the presence of additional copies of the gene in some CML patients [115]. In other patients, unusual karyotyping using banding cytogenetics coupled with the overexpression of *BCR-ABL* led to the discovery of multiple double minutes , which are small fragments of extrachromosomal DNA encoding the *BCR-ABL* gene [116] (Figure 12).

Disease progression from chronic to accelerated or blast crisis phase in CML patients is associated with an increase in *BCR-ABL* transcripts during imatinib treatment [108, 117]. This increase in transcripts correlates with enhanced CML cell survival determined by their ability to overcome the apoptotic effects of imatinib. These results underscore the fact that *BCR-ABL* gene amplification is an important mechanism of acquired resistance to imatinib.

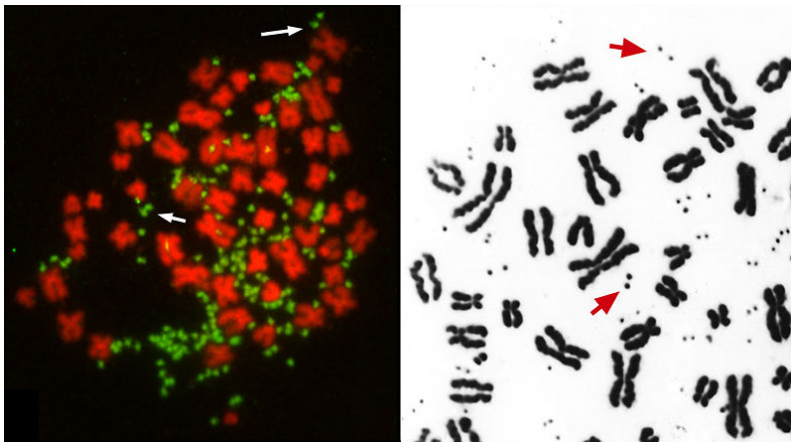


**Figure 12: Gene Amplification Due to the Presence of Double Minutes**. FISH showing cells in metaphase. Chromosomes stained in red and double minutes stained in green (left) and all nuclear DNA stained black (right). In both panels, the double minutes are indicated by arrow. *Image taken from Wikipedia. This work is in the public domain in the United States because it is a work of the United States Federal Government.*

*Overexpression of Drug Transporters*

Cancer cells exposed to chemotherapeutic agents for an extended period of time often develop a multidrug resistance (MDR) phenotype that is characterized by overexpression of the multidrug resistance1(MDR1; also called P-glycoprotein (P-gy), ABCB1 and CD243) efflux pump. The broad substrate specificity of MDR1 enables it to regulate the distribution and bioavailability of numerous drugs. In CML patients this results in an excessive efflux of imatinib, rendering the chemotherapeutic agent ineffective.

The importance of MDR1 in imatinib resistance is reflected in one recent study that demonstrated that polymorphisms of the MDR1 gene are indicative of the type of molecular response CML patients may have to imatinib treatment

[118]. MDR1. Retroviral-mediated transfection of BCR-ABL+ cells with the MDR1 gene greatly decreased its sensitivity to the apoptotic effects of imatinib, a phenotype that was could be reversed with the addition of MDR1 pump modulators [119]. Additionally, cell lines resistant to imatinib were capable of growing continuously in its presence due to increased expression of the MDR1 gene. Furthermore, Dulucq *et. al.* also revealed that the presence of MDR-1 polymorphisms correlated to suboptimal patient response to imatinib treatment [118]. Similarly, patients with CML and treated with high dose imatinib displayed drug clearance and drug resistance in a manner consistent with MDR1-mediated elimination [120].

**Nilotinib**

The need to address imatinib-resistant CML ushered in an active area of research focused on the development of second generation inhibitors. The observation that imatinib-resistance is predominantly associated with mutations within BCR-ABL kinase domain led to the development of other small-molecule ABL kinase inhibitors with less stringent binding requirements and that are more effective in combating resistance.

One such inhibitor is nilotinib (AMN107, Novartis Pharmaceuticals Corporation, Basel, Switzerland) (AMN107; Novartis), another phenylaminopyrimidine derivative that followed a rational drug design based on the crystal structures of imatinib in complexes with ABL. It was developed by modifying the *N*-methylpiperazine ring within the imatinib molecule. This

modification facilitates greater hydrogen-bond interactions with wild-type and many imatinib-resistant kinase mutants by providing a better topological fit to the kinase domain. Clinical studies show that nilotinib is active against 32 of the 33 mutant forms of BCR-ABL [121, 122]. It is approximately 30-fold more potent than imatinib as an ABL inhibitor, with an $IC_{50}$ value of 30 nM. However, like imatinib, it possesses no significant activity against the T315I mutant protein.

Nilotinib is effective in patients who are unresponsive to treatment with imatinib. When CML patients in accelerated-phase were treated with nilotinib they experienced a hematologic response rate of 47% and a MCR rate of 29%, with a 12-month survival rate of 79% [123]. Clinical studies show that nilotinib is also well-tolerated at doses of up to 600 mg daily [124].

**Dasatinib**

The need to address imatinib-resistant CML also resulted in the development of dasatinib (BMS-354825; Bristol-Myers Squibb), a dual BCR-ABL/SRC family kinase inhibitor (SFK: Fyn, Yes, SRC, Lyn, Lck, Fgr, Blk, Hck). It also has additional activity against c-Kit and PDGFR. Dasatinib is clinically more active than imatinib and nilotinib against both wild-type and mutant BCR-ABL proteins responsible for drug resistance [121, 125].

Dasatinib has less stringent conformational requirements for interaction with BCR-ABL kinase domain than nilotinib. Consequently, it binds both the active and inactive conformations. Dasatinib induces complete hematologic remissions and major cytogenetic responses in imatinib-resistant CML patients in

all three phases, chronic, accelerated, and blast phases. Response rates show that 95% of patients with chronic-phase CML and 82% of patients in accelerated-phase CML respond to dasatinib treatment during a >12 months median follow-up timeframe [126]. However, like imatinib and nilotinib, dasatinib has no effect against the T315I mutation and nearly all blast crisis phase patients experience relapse in less than a year of treatment

## CHAPTER V

## MINIMAL RESIDUAL DISEASE CHRONIC MYELOID LEUKEMIA

Despite the efficacy of imatinib, nilotinib and dasatinib in achieving high rates of cytogenetic and hematological responses to varying degrees, they are unable to override all forms of drug-resistant kinase mutations. Overtime some CML patients, particularly those in advanced stages of the disease, become refractory to further treatment. In almost all patients the *BCR-ABL* transcript persists below the level of cytogenetic detection while undergoing treatment, indicating the presence of minimal residual disease (MRD) CML. This suggests that treatment with imatinib and more potent BCR-ABL inhibitors is not sufficient to eradicate this disease.

Several factors have been implicated in contributing to MDR CML: 1) the failure to kill leukemic stem cells, 2) *BCR-ABL* gene amplification, 3) the presence of pre-existing BCR-ABL kinase mutations, 4) epigenetic changes, and 5) the tumor microenvironment.

### Failure to Kill Leukemic Stem Cells

The identification of a rare population of primitive quiescent BCR-ABL+ cells with an intrinsic insensitivity to imatinib treatment lends support this model [127-129]. *In vitro* studies of quiescent CD34+ leukemic cells revealed that they

were resilient against high-dose imatinib treatment of up to 10-folds higher than the normal dosage. Additionally, while imatinib suppressed the proliferation of leukemic colony-forming cells (CFCs) and long-term culture-initiating cells (LTC-ICs) it did not induce apoptosis in these primitive cells.

Furthermore, residual BCR-ABL+ stem cells persisted in the bone marrow of CML patients who had achieved CCR with imatinib. This indicates that primitive BCR-ABL+ CML cells that are capable of entering the cell cycle could repopulate the bone marrow compartment to facilitate patient relapse when imatinib is withdrawn.

The insensitivity of leukemic stem cells to the apoptotic effects of imatinib, coupled with their self-renewing capacity are important contributing factors to MRD CML.

**Pre-existing Mutations within the BCR-ABL Tyrosine Kinase Domain**

In one study 12% of CML patients who had no cytogenetic response to imatinib therapy and who did not display *bcr-ABL* gene amplification possessed rare cells that had point mutations within the kinase domain at the time of diagnosis. This included the highly resistant Thr315Ile mutation [130]. Additionally, BCR-ABL+ imatinib-naive cells from CML patients possessed the Glu255Lys point mutation, which is involved in preventing the BCR-ABL kinase from adopting the inactive conformation needed for imatinib interaction [131].

Pre-existing mutations in BCR-ABL+ cells could facilitate their outgrowth during therapy owing to the selective pressure of imatinib. The ‘fitness’

advantage conferred to these mutated cells would enable a clonal selection of the minor population of cells carrying the mutation.

**Epigenetic Contributions**

While the t(9;22) translocation that produces the BCR-ABL oncogenic tyrosine kinase is the major transforming even in CML, additional molecular and/or epigenetic changes have been implicated in CML oncogenesis. These changes are also associated with the transition from chronic to blast crisis phase that accompanies the emergence of MRD CML and patient relapse.

Disease progression in CML patients has been linked to increase *de novo* DNA-methylation of the *ABL* promoter contained within a CpG island, which is associated with *ABL* gene silencing [132, 133]. This suggests that the course of the disease may be documented by the extent of CpG methylation.

During chronic phase CML the expanded granulocytic lineage still retains its ability to differentiate. However, the accelerated and blast crisis phases are characterized by decreased cell differentiation and the subsequent accumulation of immature hematopoietic cells. Molecular changes resulting in differentiation arrest are associated with this transition. Gain-of-function mutations in GATA-2, a gene that serves as a negative regulator of hematopoietic stem progenitor cell differentiation, resulted in its enhanced transactivation capacity and augmented inhibition of hematopoietic cell differentiation [134].

**Tumor Microenvironment**

The bone marrow microenvironment (BME) provides CML cells direct interaction with stromal cells and extracellular matrices (ECM), which may modulate drug response. We reported previously that adhesion to fibronectin was sufficient to protect K562 CML cells from imatinib-induced cell death (Damiano [135, 136]. Similar studies reveal that direct contact of leukemic cells with the human stromal cell line, HS-5, significantly increased cell proliferation, viability and colony formation [137]. Additionally, leukemic-stromal cell interactions enhanced *in vitro* leukemic cell survival while attenuating chemotherapy-induced cell killing. Using a co-culture model consisting of leukemic cells and bone marrow-derived stromal cells, studies show that this interaction resulted in increased activation of the several signal transduction pathways involved in cell survival, including PI3K/AKT, Ras/ERK and STAT3 [138].

The BME is also a rich source of cytokines and growth factors that could also influence drug response. While the retroviral transduction of BCR-ABL into severe combined immunodeficient (SCID) bone marrow progenitor cells grown healthy mice resulted in rapid, fatal, imatinib-resistant leukemia, its transduction into similar cells lacking the cytokine receptor common γ-chain resulted in imatinib-sensitivity. This suggests that cytokines within the microenvironment are responsible for conferring imatinib resistance [139]. Studies to identify cytokines involved in modulating imatinib sensitivity revealed that autocrine secretion of GM-CSF was involved. Its secretion activates the JAK/STAT5 pathway and

protects imatinib-naive and CML progenitor cells from death induce by BCR-ABL inhibitors [140]. Additionally, mutated CML cells were able to spread resistance to non-mutated cells through overexpression of IL-3, which resulted in the activation of the Ras/MEK and JAK2/STAT5 signal transduction pathways [141].

This highlights the importance of the bone marrow microenvironment in modulating imatinib response. This resistance can be conferred by either direct adhesion to bone marrow stroma or by the secretion of bone-marrow derived soluble factors.

## CHAPTER VI

## THE ROLE SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION (STATS) IN CHRONIC MYELOID LEUKEMIA ONCOGENESIS AND DRUG RESISTANCE

Receptor engagement by hematopoietic cytokines results in their phosphorylation and the activation of JAKs. These receptors consist of a unique ligand-binding subunit and a signal transducing subunit [142] (see Figure 13 for cytokine receptor families). STATs are activated transiently by numerous cytokines via JAKs [143], as well as SFKs [144].

Various types of hematologic malignancies are associated with the aberrant, constitutive, cytokine-mediated activation of STAT proteins. In CML, BCR-ABL activates signal transduction pathways shared by many hematopoietic cytokines, including the STAT3 and STAT5 signaling pathways.

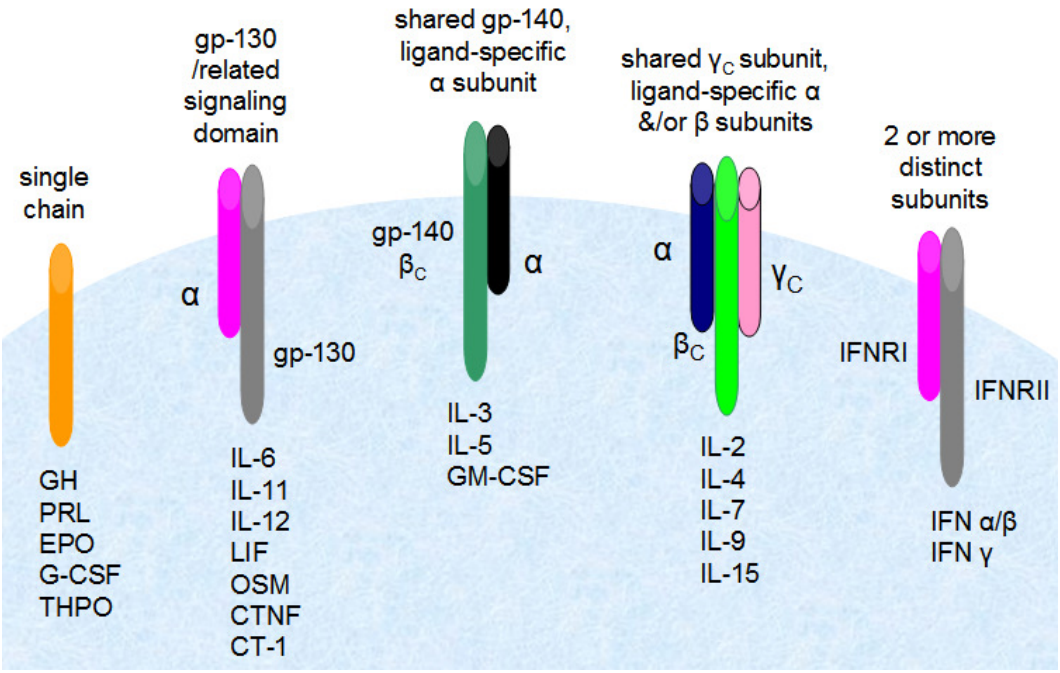



**Figure 13: Cytokine-Receptor Families**. Cytokine-receptors consist of a ligand-binding subunit and a signal-transducing subunit. Hematopoietic cytokine receptors can be categorized based on their signaling subunit as follows: 1) those that signal through a single subunit, which includes growth hormone (GH), prolactin (PRL), erythropoietin (EPO); granulocyte colony-stimulating factor (G-CSF), and thrombopoietin )THPO); 2) those that signal through the gp130 or gp 130-related subunit, which includes interleukins (IL)-6, 11 and 12, leukemia inhibitory factor (LIF), oncostatin M, ciliary neurotrophic factor (CTNF), and cardiotropin-1 (CT-1); 3). those that signal through the gp140β-subunit or a ligand-binding α subunit, which includes IL-3 and 5, and granulocyte-macrophage colony-stimulating factor (GM-CSF); 4) those that signal through a common γ-chain ($\gamma_C$) and a α or β ligand-binding subunits, which includes IL-2,4,7,9, and 15; 5) those that signal through two more subunits, such as the interferon-γ (IFN-γ) subfamily of receptors, which includes IFNRI and II.

**The Role of STAT3**

STAT3 governs signal transduction in growth factor-mediated control of hematopoiesis and myeloid cell differentiation. Its activation up-regulates the expression of genes associated with cell survival and proliferation including Bcl-x, Mcl-1, and cyclin D1 [89, 145, 146]. Persistent STAT3 activation has been detected in a variety of hematopoietic malignancies and solid tumors [147-149]. The enforced expression of BCR-ABL in primitive embryonic stem cells (ESCs) resulted in their persistent STAT3 activation and caused the cells to retain their primitive morphology by inhibiting their differentiation [150]. The increased activation of STAT3 in primary leukemia blast cells lends support to its involvement in disease progression of CML patients and their' transition from chronic phase to blast crisis phase [151].

Taken together, these observations highlight the role of amplified STAT3 activation in the pathogenesis of CML and underscore its involvement in imatinib resistance.

**The Role of STAT5**

STAT5 has gained prominence because it is often constitutively activated in a wide variety of human cancers, ranging from solid tumors seen prostate, lung, breast and colon cancers, to hematologic malignancies, such as myeloproliferative disorders [152-155]. It is activated by non-receptor tyrosine kinases, such as BCR-ABL and SFKs. Like STAT3, STAT5 activation up-regulates the expression of Bcl-x, Mcl-1, and cyclin D1.

BCR-ABL-dependent STAT5 activation was observed in several leukemic cell lines possessing the Ph+ clone, but not untransformed cells or BCR-ABL-negative leukemia cells [154, 156]. Furthermore, the expression of BCR-ABL in IL-3/GM-CSF-dependent leukemic cells resulted in aberrant STAT5 activation and growth factor independence [157]. Similarly, constitutive activation of STAT5 by the SFK, Hyk, resulted in cytokine-independent cell growth in cytokine-dependent leukemic cells [158]. STAT5 activation is also associated with chronic-to-blast crisis phase transition in leukemia patients. As indicated previously, in up to 95% of AML blast cells constitutive activation of STAT3 and STAT5 could be detected [151].

Interestingly, increased STAT5 activation in BCR-ABL-positive leukemic cells and STAT5's ability to confer resistance to dasatinib has been shown to be directly correlated with leukemic cell density [159]. This recent study revealed that an increase in the density of leukemic cells resulted in a decrease in dasatinib's efficacy against STAT5-mediated signaling. Results indicate that this observation may attribute to the upregulation of STAT5-regulated anti-apoptotic proteins.

Taken together, this demonstrates that constitutive STAT5 activation promotes CML pathogenesis and imatinib resistance by up-regulating the expression of anti-apoptotic proteins, Bcl-x, Mcl-1 and cyclin D1. Additionally, enhanced STAT5 activation is capable of mediating cytokine-independent growth in leukemic cells, leading to increased cell survival.

## CHAPTER VII

## OBJECTIVES

The *BCR-ABL* fusion gene is critical for the development of CML. Treatment with imatinib is highly effective against CML; however treatment response in patients is compromised by the emergence of imatinib resistance. *In vitro* resistance models often fail to consider the role of the tumor microenvironment in the emergence of imatinib resistance. The predominant microenvironment of CML is the bone marrow, a rich source of both soluble factors and extracellular matrixes which may influence drug response. This study was developed to address the influence of the bone marrow microenvironment on imatinib sensitivity. We utilized an *in vitro* co-culture bone marrow stroma model to demonstrate that stable soluble factors secreted by the HS-5 human bone marrow stroma cell line were sufficient to protect K562 CML cells against imatinib-induced cell death. Subsequently, we observed that HS-5-derived conditioned media (CM) also provided protection against cell death. Previous studies showed that HS-5 stromal cells secrete cytokines and growth factors involved in the growth and differentiation of hematopoietic stem cells [160]. Some of these cytokines are capable of activating signal transduction pathways involved in myeloid differentiation. Investigations into the activation of survival

pathways that reconstitute BCR-ABL signaling in CM may provide novel insights into the mechanism of environment-mediated drug resistance (EMDR). One such pathway being investigated is the signal transducer and activator of transcription 3 (STAT3) survival pathway. A review of the current literature led to the following hypothesis:

**Bone marrow-derived soluble factors in conditioned media contribute to the failure of BCR-ABL inhibitors to eradicate minimal residual disease CML.**

In order to refute or support this hypothesis, the following objectives were formulated:

1. To determine if HS-5-derived CM protects CML cell lines from death induced by second generation BCR-ABL inhibitors
2. To determine whether HS-5-derived CM increased the proliferation and clonogenic survival of CML cells
3. To determine whether HS-5-derived CM mediate the activation of STAT3 and STAT5 cell survival pathways in CML cells

# CHAPTER VIII

# MATERIALS AND METHODS

## Cell Cultures

Human CML K562 and KU812 cells (obtained from the American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% L-glutamine, and penicillin/streptomycin [regular medium (RM); Life Technologies] at 37°C in 5% $CO_2$ in a humidified incubator. The human stromal cell line HS-5 (also obtained from the American Type Culture Collection) was maintained under the same conditions.

## Generation of Conditioned Media (CM)

CM was generated by culturing 5 x $10^5$ HS-5 cells/mL in RM overnight in a humidified atmosphere at 37°C with 5% $CO_2$ to achieve 75% to 80% confluency. Subsequently, the medium was removed and the cells were incubated in fresh medium for 3 hours. The supernatant was then collected and cleared of contaminating cells by centrifuging at 2,000 rpm for 5 minutes. Supernatant was aliquoted and stored at -80°C for future use.

## Drugs and Reagents

Imatinib mesylate and nilotinib (AMN107), supplied by Novartis Pharma, were dissolved in DMSO as a 10 mM stock solution and stored in aliquots at -20°C. Dasatinib, supplied by Bristol-Myers Squibb, was treated similarly.

Treatment with Tyrosine Kinase Inhibitors

## Preparation of Lysates and Western Blotting

K562 or KU812 cells were cultured at a density of 4 x $10^5$/mL in either RM or CM for various time points in the presence or absence of either a matched vehicle control (DMSO) or drug (imatinib or dasatinib). Subsequently, cells were pelleted and lysed in radioimmunoprecipitation assay lysis buffer supplemented with phosphatase and protease inhibitors (1 mM $Na_3VO_4$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL leupeptin, and 10 µg/mL aprotinin). Insoluble materials were removed by centrifugation at 4°C for 20 minutes at 15,000 rcf . Antibodies used for Western blotting were anti-STAT3, anti-STAT3 (Tyr705), anti-Bcl-xl, anti-Mcl-1, anti-Survivin, and p-SRC (all from Cell Signaling Technologies).

## Apoptosis Assay

Apoptosis was measured using the Annexin V-FITC apoptosis detection kit (Alexis Biochemicals) according to the manufacturer's recommendations. Data were acquired using CellQuest Pro software version 4.0.2 (BD Biosciences) and analyzed using FlowJo software version 7.2.2 (Tree Star).

## siRNA Transfection

K562 cells cultured in RM were transiently transfected with either STAT3 siRNA (siGENOME ON-TARGETplus SMARTpool reagent; Dharmacon) or a control siRNA (ONTARGETplus siCONTROL Nontargeting reagent; Dharmacon) using Amaxa Nucleofector methodology (Amaxa). Briefly, 1 x $10^6$ K562 cells were transfected per manufacturer's instructions. Thirty-six hours post-transfection, the process was repeated to ensure continued suppression of STAT3 levels. Transfected K562 cells (2.0 x $10^5$/mL) were then cultured in RM or CM for 3 hours then treated with 250 nM imatinib or a vehicle control (DMSO). Cell death was measured using Annexin V apoptosis assay as described previously.

## Bromodeoxyuridine (BrdU) Antibody Staining

2.5 x $10^5$ K562 cells/mL were cultured in RM or CM for 24 hours then pulsed with 30 µg/mL 5-bromo-2-deoxyuridine (BrdU) for 30 minutes. Cells were fixed with ethanol and BrdU incorporation was detected using FITC-conjugated anti-BrdU antibody (Calbiochem, Gibbstown, NJ). Data acquisition and analysis were done using fluorescence-activated cell sorting (FACS).

## Clonogenic Assay

K562 cells were diluted to a concentration of 10,000 cells/mL in RM or CM for 6 hours in the absence or presence of imatinib and incubated at 37°C with 5% $CO_2$. Cells are transferred to 0.3% final agar solution reconstituted in RM or CM. Once the agar solidifies, cells were allowed to incubate for 8-10 additional days. Cell colonies (>50 cells) were then counted on 2 mm grid culture dish (Corning).

## Preparation of Nuclear Extracts

Nuclear extracts were prepared by resuspending 5 x 106 K562 cells in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM PMSF, 2 µg/mL leupeptin, 0.15 µg/mL aprotinin) followed by incubation on ice 5 minutes. Cells were centrifuged for 10 seconds at 14000 rpm and washed once with hypotonic buffer. The nuclei were collected by centrifugation for 5 minutes at 14000 rpm and washed once with hypotonic buffer. The nuclear pellet was resuspended in 50 µL of hypertonic buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 450 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 2 µg/mL leupeptin, 0.15 µg/mL aprotinin), incubated for 30 minutes at 4ºC followed by centrifugation for 2 minutes at 14000 rpm.

## Electrophoretic Mobility Shift Assay (EMSA)

A total of 2 µL nuclear extract was incubated with a double-stranded 32P-labeled oligonucleotide (1 ng) sis-inducible element (SIE), (5'-TCGAGTATTTCCCAGAAAAGGAACAGCT-3' and its complement) in 10 µL binding buffer (25 mM HEPES, pH 7.9, 100 µM ethylene glycol tetraacetic acid (EGTA), 200 µM MgCl2, 500 µM dithiothreitol, 1 µg/mL BSA, 0.2 µg/mL poly dI:dC) for 15 minutes at room temperature. Protein-DNA complexes were detected by autoradiography and quantified using a phosphoimager.

## MTT Assay.

Incubate 50,000 K562 cells/ mL for 3 hours in either regular media or conditioned media. Add varying concentrations of imatinib. Incubate drug-treated cells for 72

hours at 37℃, 5% $CO_2$. Add MTT dye and incubate for 3 hours at 37℃, 5% $CO_2$. Read absorbance at 540 nm on a spectrophotometer.

### Statistical Analysis

A preliminary examination of the data was done using descriptive summary analysis and the Anderson-Darling statistic. Significance testing of the dose-response apoptosis assays was done using analysis of covariance. The Student's t test was used for the siRNA experiments to compare the log-transformed fold change values, with the null hypothesis being a log (fold change) = 0 (or conversely the fold-change = 1). A significance level of 0.05 was considered statistically significant for all tests. The geometric means of the four ratios for siRNA experiments and the 95% confidence intervals were also calculated

# RESULTS

## Co-culture Bone Marrow Stromal Model Protects K562 CML Cells from Imatinib-Induced Apoptosis

Previous work done by Torok-Storb *et. al.* revealed that HS-5 human stromal cells are capable of producing cytokines and growth factors that are involved in the growth and differentiation of HSC and are also capable of supporting the *ex vivo* expansion of both immature and mature progenitor cells [160]. To test our hypothesis that the bone marrow microenvironment contributes to the failure of BCR-ABL inhibitors to eradicate minimal residual disease CML, we utilized a co-culture bone marrow microenvironment transwell model system and took advantage of HS-5 stroma cells' ability to support HSC and their progenitors. We wanted to determine whether the bone marrow microenvironment protects the K562 CML cell line, which originated from the pleural effusion of a CML female patient in terminal blast crises phase, from death induced by imatinib.

Using this co-culture model, we first wanted to delineate the contributions of (1) HS-5-derived soluble factors alone, and (2) direct adhesion to HS-5 cells on imatinib resistance in K562 CML cells treated with 1 µM of imatinib (Figure 14). Our control samples consisted of K562 cells cultured in regular media (RM).

Here we saw approximately 35% cell death. Next, we determined the effects of transiently secreted soluble factors on K562 cells cultured in the upper chamber of the transwell (TSF), and here we saw protection from cell death at approximately 10%. We then examined the effects that direct adhesion to HS-5 stroma cells as well as secreted soluble factors on imatinib-induced K562 cell death (SF+A). Here we observed an even greater protection from cell death at 5%. Lastly, we examined the effects of HS-5-derived stable soluble factors alone on apoptosis by using HS-5 conditioned media (CM). Here we saw 15% cell death. Taken together, these data revealed that the co-culture model was not required for the production of the protective soluble factors produced by HS-5. It also revealed that HS-5 conditioned media contained stable soluble factors that provided protection against imatinib-induced cell death. Therefore, to simplify our bone marrow stroma model, we utilized HS-5-derived conditioned media in our subsequent experiments.

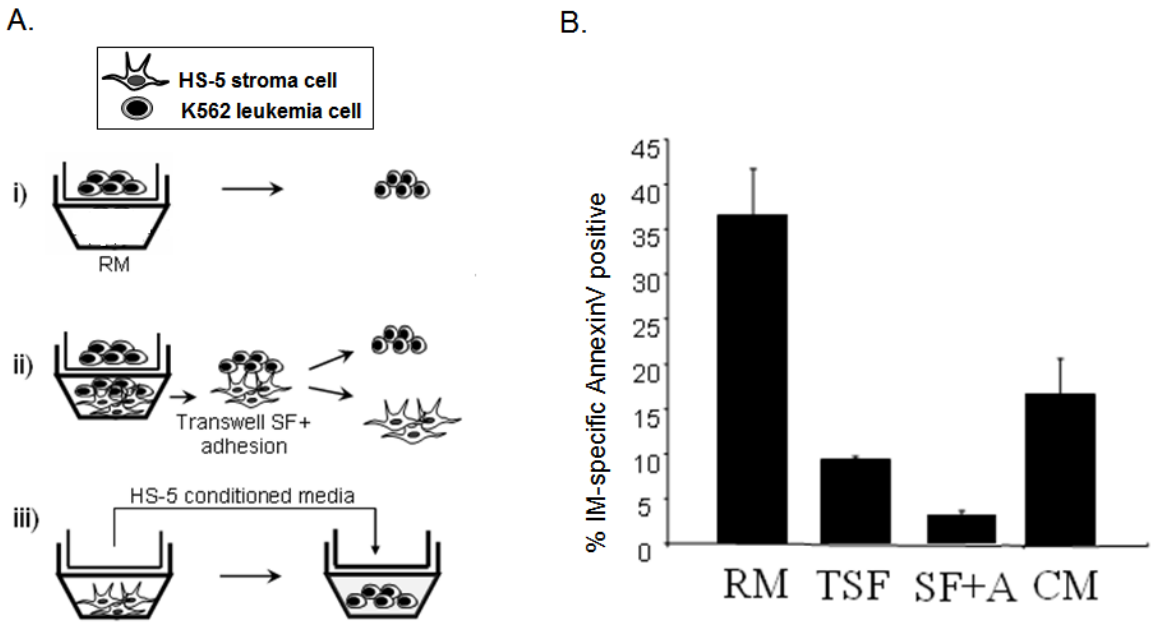



**Figure 14: Co-culture Bone Marrow Transwell Stromal Model**. (**A**) Co-culture transwell model system of K562 chronic myeloid leukemia (CML) cultured in: i) regular media (RM: RPMI + 10% FBS, 1% Penicillin /Streptomycin); ii) the upper chamber of the transwell system with HS-5-secreting transient soluble factors (TSF) or with direct adhesion to HS-5 stroma cells (SF+A); iii) .HS-5-derived conditioned media (CM: RM collected after 3-hour incubation with HS-5 cells). (**B**) Annexin V apoptosis assay of conditions described in A.

## Characterizing Conditioned Media

### Collecting Conditioned Media beyond 3 Hours does not Provide Greater Protection against Imatinib-Induced Cell Death

To verify whether 3-hour HS-5-derived conditioned media provided the best protection against imatinib-induced cell death, we generated the conditioned media as described before, however, instead of only collecting at 3 hours it was collected at varying time-points: 1, 3, 6 and 24 hours.

As seen in Figure 15, our data revealed that conditioned media generated beyond 3 hours did not provide greater protection from death induced by 500 nM of imatinib.

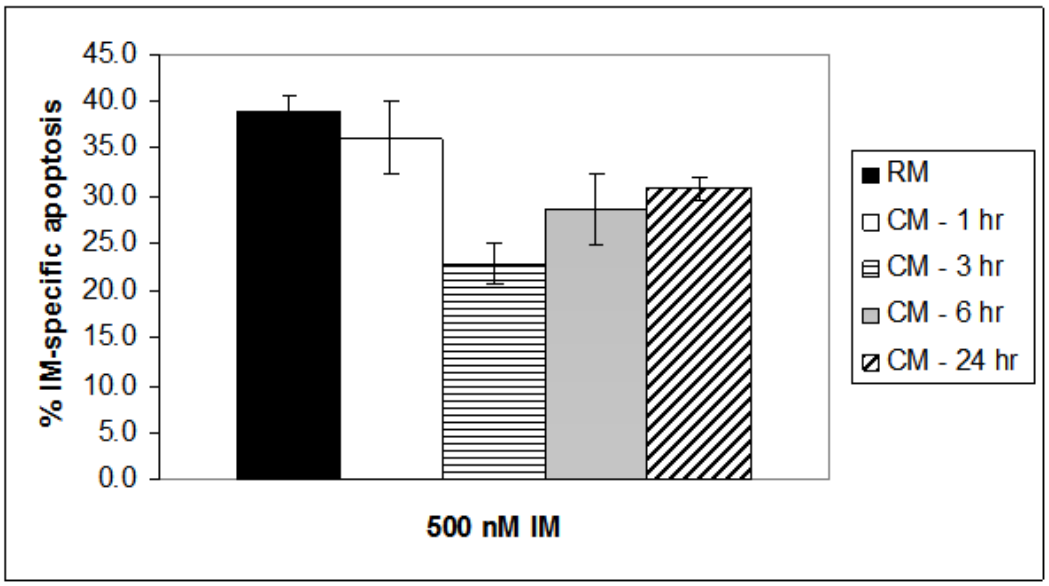



**Figure 15: The Effects of Conditioned Media Collected at Various Times on Imatinib-Induced Cell Death in K562 CML Cells.** HS-5 stromal cells cultured to 75-80% confluency in regular media were incubated for 1, 3, 6, 24 hours in fresh media. Cell death was measured using the Annexin V apoptosis assay following treatment with 500 nM of imatinib for 48 hours. Results were analyzed using flow cytometry.

**Conditioned Media Stored for up to One Week Still Provided Protection against Imatinib-Induced Cell Death**

To determine the effects of storage on the protective characteristic of 3 hour-derived conditioned media, we cultured K562 cells in regular media and

conditioned media that was freshly collected, one day and one week old. One-day and one-week old conditioned media were stored at -80℃.

After treating K452 cells with 500 nM of imatinib, our results were analyzed with Annexin V apoptosis assay. Figure 16 reveals that 3-hour-derived conditioned media that was stored at -80℃ for up to one week could still protect K562 CML cells from death induced by imatinib. This confirmed that HS-5-derived soluble factor(s) in the conditioned media is stable and does not have a short half-life. To ensure consistency and preserve the integrity of our data, we did not store conditioned media for longer than one week.

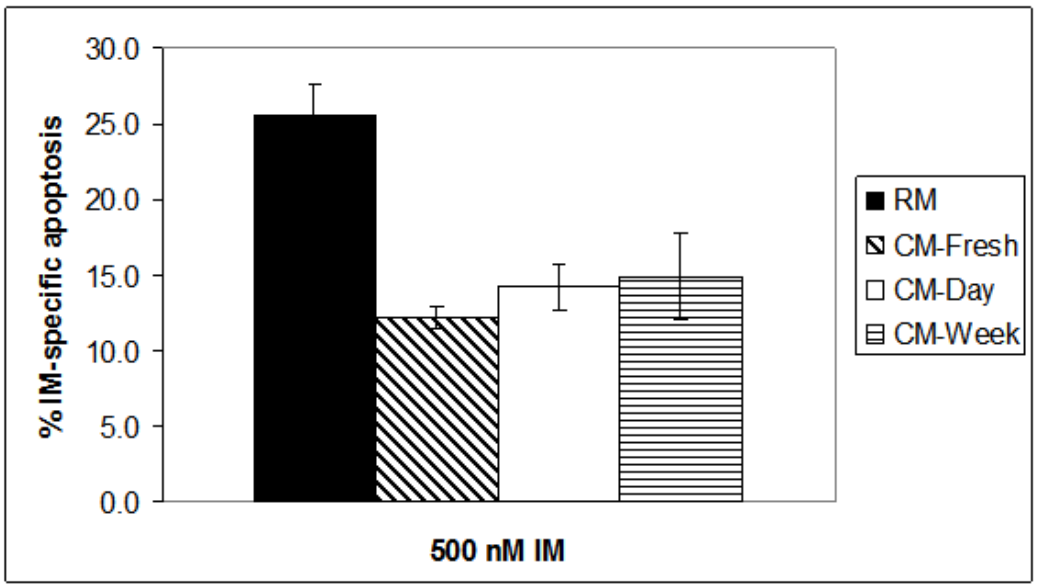



**Figure 16: The Effects of Storage on 3-Hour-Derived Conditioned Media.** To make the time-dependent conditioned media, HS-5 stromal cells were grown to 75-80% confluency in regular media (RM) for 3 hours. Media was then collected and used fresh, after one day of storage at -80℃ or after one week of storage. Cell death was measured using the Annexin V apoptosis assay following

treatment with 500 nM of imatinib for 48 hours. Results were again analyzed using flow cytometry.

**Serum is not Required for Production of the Protective Soluble Factor(s) Found in HS-5-Derived Conditioned Media**

We next wanted to determine if HS-5 human stromal cells require serum to produce the activate component in conditioned media. To answer this question we examined whether serum-free conditioned media (SF-CM) can still protect K562 cells from apoptosis induced by imatinib. HS-5 stromal cells were allowed to incubate for 3 hours in regular media void of serum. This SF-CM was then collected and supplemented with 10% FBS then utilized in our experiments. As before, K562 cells were cultured in regular media (RM), conditioned media (CM) and SF-CM. Our results were analyzed using the Annexin V apoptosis assay followed by flow cytometry analysis.

As seen in Figure 17, HS-5 stromal cells were capable of producing their protective soluble factor(s) in the absence of FBS. Additionally, since media containing serum is more difficult to fractionate, these data demonstrates that future studies to identify the protective component of conditioned media using fractionation can be readily accomplished with this SF-CM.

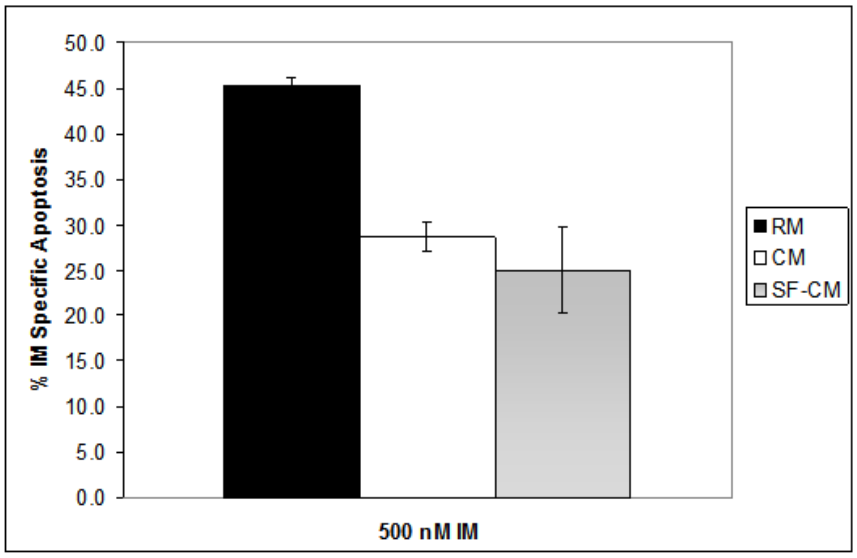



**Figure 17: The Effects of Serum-Free Conditioned Media on Imatinib-Induced K562 Cell Death.** To make the time-dependent serum-free (SF) conditioned media, HS-5 stromal cells were grown to 75-80% confluency in regular media (RM) for 3 hours. Media was then collected and 10% fetal calf serum (FBS) was added. SF-CM was used fresh, after one day of storage at -80℃ or after one week of storage. Cell death was measured using the Annexin V apoptosis assay following treatment with 500 nM of imatinib for 48 hours. Results were again analyzed using FACS.

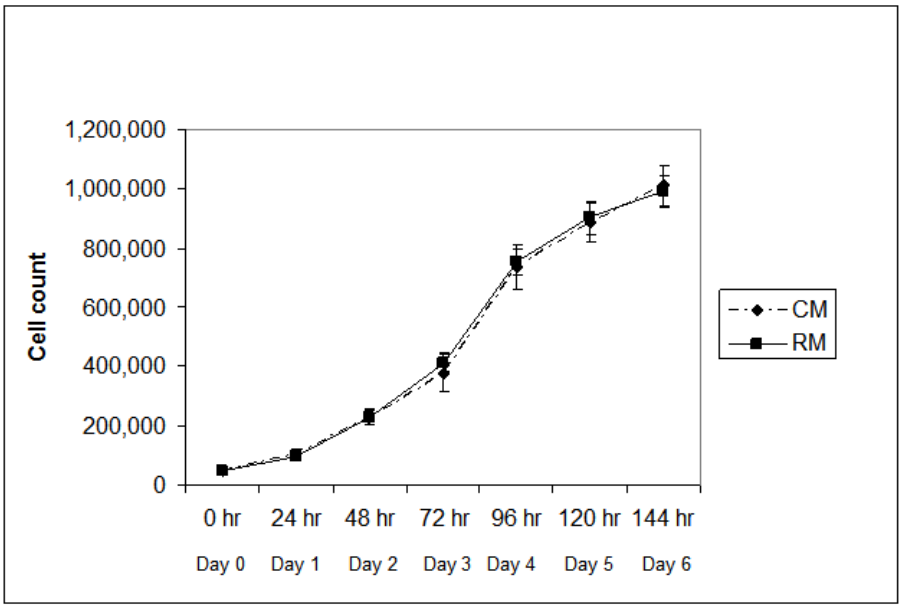
**Conditioned Media does not Convey a Growth Advantage to K562 CML Cells**

Given our previous results which show that conditioned media protects K562 cells from death induced by imatinib within our bone marrow stromal model, we decided to investigate whether conditioned is capable of increasing the rate of K562 cell proliferation and DNA synthesis.

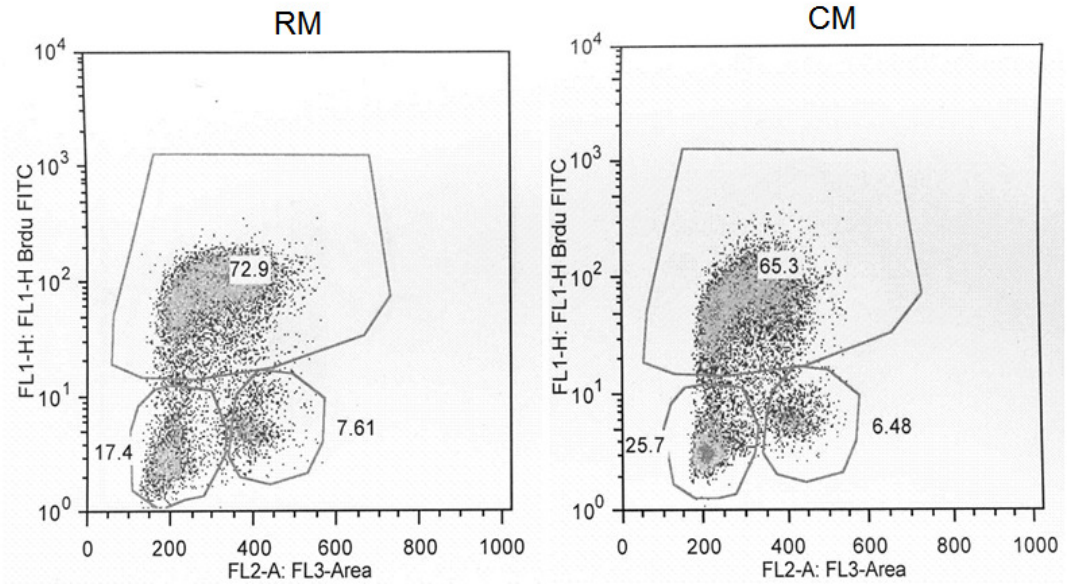
Using trypan blue exclusion staining method, Figure 18A shows that when cells were counted over the course of six days, there was no increase in cell proliferation in K562 cells regardless of culture conditions.

To verify these results, the rate of DNA synthesis, which is indicative of cell proliferation, was also measured via BrdU incorporation staining and detection with a FITC-conjugated anti-BrdU antibody. Figure 18B shows that DNA synthesis is also not increased in conditioned media. Figure 18C is a representative of three independent BrdU incorporation staining assays.

**A.**




**B.**



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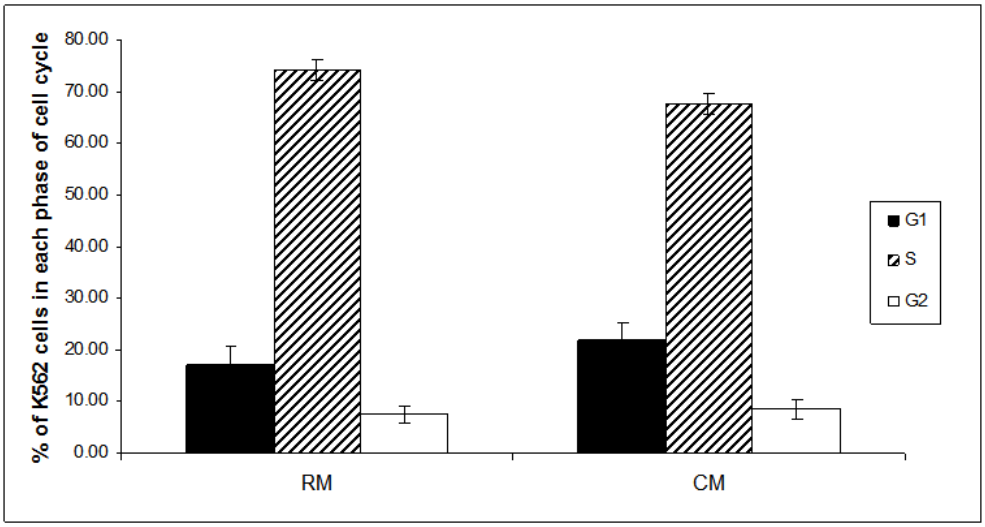



**Figure 18: The Effects of Conditioned Media on K562 Cell Proliferation and DNA Synthesis.** (**A**) 50,000 K562 cells were cultured in regular media or conditioned media and total cell count was determined every 24 hours using trypan blue exclusion staining. Results and standard deviation represents the average of 4 independent experiments. (**B**) The rate of DNA synthesis was determined by 5-bromo-2-deoxyuridine (BrdU) antibody staining. 2.5 x $10^5$ K562 cells/mL were cultured in regular media or conditioned media for 24 hours then pulsed with 30 µg/mL of BrdU for 30 minutes. Cells were then fixed with ethanol and BrdU incorporation was detected using FITC-conjugated anti-BrdU antibody. Data acquisition and analysis were done using FACS. (**C**) Representative figure of BrdU incorporation staining (n=3).

### Heat-Inactivated Conditioned Media does not Protect against Imatinib Mesylate-Induced Cell Death

Cytokines are proteins that initiate cellular communication. To determine if the HS-5-derived protective soluble factor(s) found in conditioned media is a cytokine we cultivated K562 CML cells in regular media and heat-inactivated

conditioned media, treated them with 500 nM imatinib for 48 hours then performed an Annexin V apoptosis to determine cell death. Results were analyzed via FACS.

Figure 19 shows that when K562 CML cells were cultured in heat-inactivated conditioned media there was no protection from death induced by imatinib. This demonstrates that the protective soluble factor in HS-5 conditioned media is a protein.

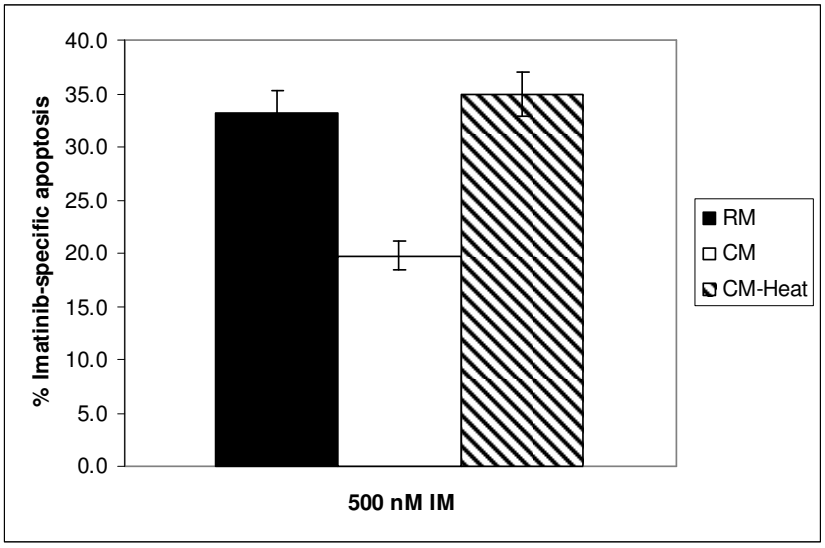



**Figure 19: Heat-Inactivated Conditioned Media does not Protect K562 Cells Against Imatinib-Induced Cell Death.** K562 cells were cultured for 3 hours in either regular media or conditioned media that was heat-inactivated at 95°C for 10 minutes. Cells were treated with 500 nM imatinib or 0.1% DMSO for 48 hours. Cell death was measured using Annexin V apoptosis assay followed by fluorescence-activated cell sorting analysis.

**HS-5-Derived Conditioned Media Protects K562 and KU812 CML Cell Lines from Death Induced by Imatinib Mesylate**

Results from our co-culture bone marrow stromal model had revealed that HS-5 conditioned media alone can also protect K562 CML cells from imatinib-induced cell death. We wanted to determine whether the effects we saw was cell line specific by doing a dose response profile. To address this question, we performed an imatinib-dose response profile using the CML cell lines, K562 cells and KU812, which is a pre-basophilic cell line that originated from the peripheral blood of a CML patient in blast crisis phase. Cells were cultured in regular media or conditioned and treated with increasing doses of imatinib (0, 125, 250, 500 nM imatinib) for 48 hours.

The results of the Annexin V apoptosis revealed that exposing either K562 or KU182 cells to conditioned media for 3 hours before drug treatment was sufficient to inhibit apoptosis induced by imatinib (Figures 20A and B) and suggested that the protective effects of conditioned media is not cell-line specific.

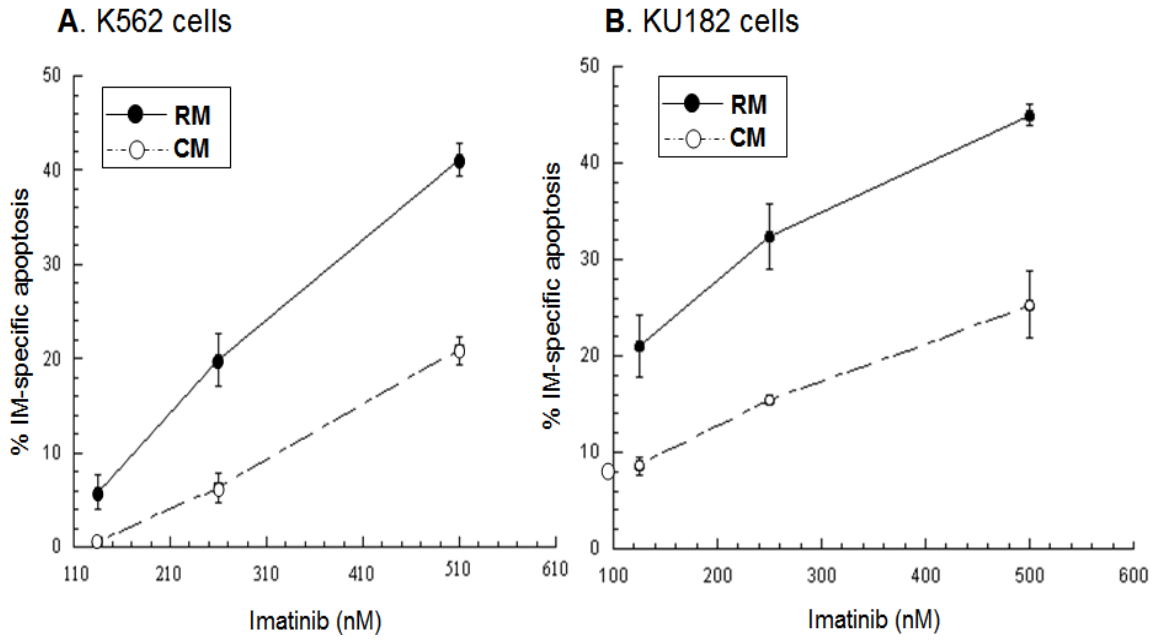
Additionally, we performed a clonogenic assay, or colony formation assay, to determine whether conditioned media increased the ability of K562 cells to divide and form colonies. The clonogenic assay is an *in vitro* cell survival assay based on the ability of a single cell to grow into a colony. It is a measure of cancer cells' ability to repopulate and form colonies in semisolid media. The colony is defined to consist of at least 50 cells.

First, we wanted to determine the optimal K562 cell concentration to use for the clonogenic assay. To determine this, we performed clonogenic assays using varying concentrations of K562 cells to obtain a cell-density profile. As shown in Figure 20C, in the absence of imatinib, conditioned media did not increase the clonogenic survival of K562 cells across the differing cell concentrations indicated. Additionally, in the absence of imatinib, the initial cell concentrations of 2,500 and 5,000 cells provided too few colonies at the end of 10 days to justify the use of these concentrations. Therefore, we utilized the initial cell concentration of 10,000 cells in our subsequent clonogenic assays to ensure that there would be enough viable colonies to count, even after high-dose imatinib treatment.

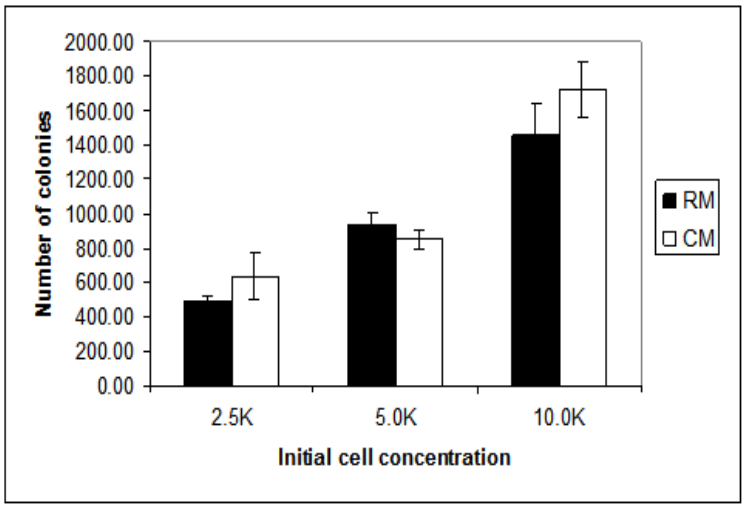
Figure 20D shows that when K562 cells are treated with varying concentrations of imatinib, the clonogenic survival of those cells cultured in conditioned media was increased across all imatinib concentrations when compared to those control cells cultured in regular media. Taken together, these data indicate that not only does conditioned media protect from imatinib-induced apoptosis or cell death but also results in a higher percentage of cells capable of dividing and repopulating.

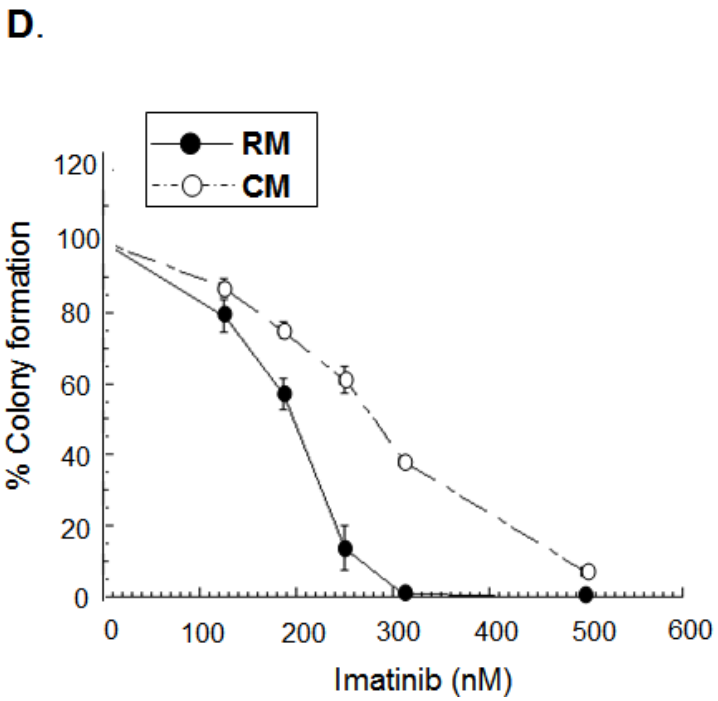
Finally, as shown in Figure 20E, removal of K562 cells from conditioned media into regular media partially reverses resistance to imatinib-induced cell death. This suggests that imatinib-resistance in CML cells occurs in the presence

of HS-derived soluble factors and in the absence of the soluble factors CML cells become re-sensitized to the apoptotic effects of imatinib.




C.





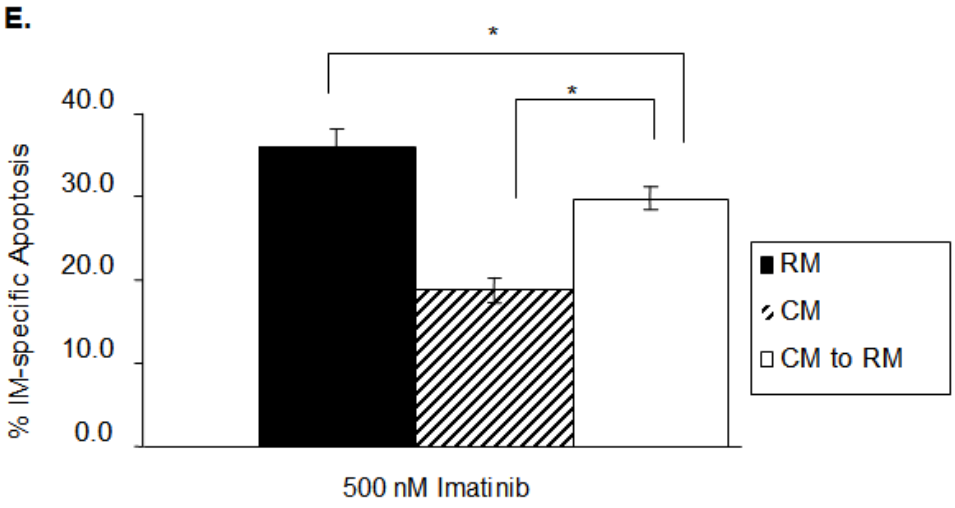




**Figure 20: HS-5-Derived Conditioned Media Protects K562 and KU182 CML Cells from Death Induced by Imatinib.** (**A**) K562 or (**B**) KU182 cells were cultured in either regular media or conditioned media for 3 h and before the addition of various concentrations of imatinib or 0.1% DMSO for 36 hours. Cell

death was measured using Annexin V apoptosis assay followed by fluorescence-activated cell sorting analysis. % Drug-specific apoptosis was calculated by subtracting the background cell death in control DMSO-treated cells from drug treated cell death. **A**, Conditioned media significantly protects K562 cells from imatinib-mediated cell death ($P < 0.05$, analysis of covariance). **B**, Conditioned media significantly protects KU182 cells from imatinib-mediated cell death ($P < 0.05$, analysis of covariance). **C**, Cell-density profile of K562 cells shows similar clonogenic survival in regular media and conditioned media. K562 cells were cultured in regular media or conditioned media without imatinib. After 6 hours incubation at 37°C, 5% $CO_2$, cells were cultured in 0.3% agar made of regular media or conditioned media and containing their respective imatinib concentrations. Cells were allowed to incubate at 37°C, 5% $CO_2$ for 10 days. Cell colonies (>50 cells) were counted. Representative figure in triplicate (n = 3 independent experiments). The starting concentration of 10,000 cells was used in subsequent clonogenic assays. **D**, Conditioned media increases the clonogenic survival of K562 CML cells treated with imatinib. K562 cells were cultured in regular media or conditioned media and treated with increasing concentrations of imatinib as depicted. After 6 hours incubation at 37°C, 5% $CO_2$, cells were cultured in 0.3% agar made of regular media or conditioned media and containing their respective imatinib concentrations. Cells were allowed to incubate at 37°C, 5% $CO_2$ for 10 days. Cell colonies (>50 cells) were counted. Representative figure in triplicate (n = 3 independent experiments). **E**, Removal of conditioned media partially reverses resistance to imatinib ($P < 0.05$, Student *t*-test). K562 cells were grown in either conditioned media or regular media for 3 hours. Following 3 hours, appropriate samples were removed from conditioned media and placed in regular media for an additional 3 hours. Cells were treated with 500 nmol/L imatinib for 36 hours and apoptosis was measured by Annexin V positivity. Representative figure of experiments done in triplicates.

**Conditioned Media from Non-Stromal Cell Lines do not Protect K562 CML Cells from Death Induced by Imatinib Mesylate**

Some cells are capable of secreting their own soluble factors via an autocrine loop and condition their own media. We investigated whether the protection against cell death that we observed using HS-5-derived conditioned media was specific to this human stromal cell type. To address this, we generated conditioned media from several other non-stromal cells. K562 CML cells, U937 lymphoma cells and 8226 myeloma cells were incubated in regular media for three hours, after which their conditioned media was collected. To perform the experiment, K562 cells were cultured for three hours in regular media and the conditioned media of the different cell lines previously described. The cells were then treated with 500 nM of imatinib for 48 hours. An Annexin V apoptosis assay was performed followed by flow cytometry analysis.

As you can see in Figure 21, there was no protection from imatinib-induced cell death in K562 cells cultured in its own conditioned media, as well as in conditioned media from U937 and 8226 cell lines. This suggests that the secreted, protective soluble factor(s) responsible for conferring the imatinib-resistant phenotype are specific to bone marrow stromal cells.

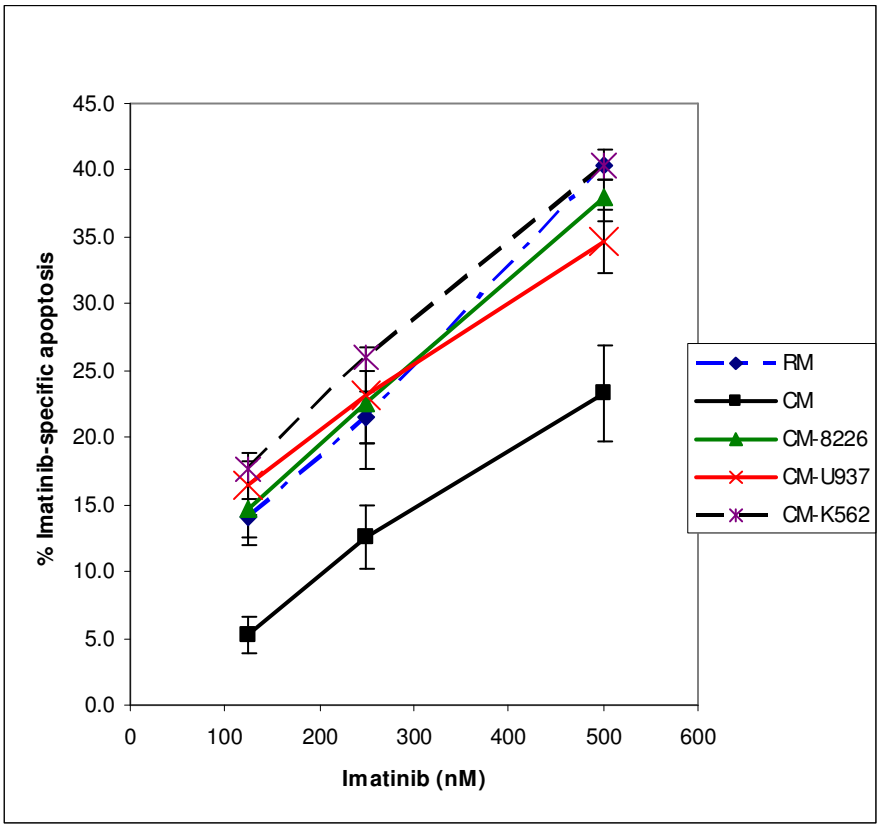



**Figure 21: Conditioned Media from Non-Stroma Cell Lines does not Protect K562 CML Cells from Death Induced by Imatinib.** K562 cells were cultured for 3 hours in either regular media or conditioned media generated from K562, U937 and 8226 cell lines. Cells were treated with 500 nM imatinib or 0.1% DMSO for 48 hours. Cell death was measured using Annexin V apoptosis assay followed by fluorescence-activated cell sorting analysis.

### Conditioned Media Protects K562 CML Cells from Death Induced by 2nd Generation BCR-ABL Inhibitors, Nilotinib and Dasatinib

One of the shortcomings of imatinib therapy stems from the fact that, over the course of treatment, many patients develop resistance which results in relapse and disease progression. Studies have shown that imatinib resistance is

often caused by point mutations in the BCR-ABL tyrosine kinase domain [113, 161]. This discovery has led to the design and development of more potent, second generation kinase inhibitors that target imatinib-resistant BCR-ABL mutants. Dasatinib (Sprycel, BMS-354825, Bristol-Myers Squibb) and nilotinib (AMN107) represent two potent second-generation BCR-ABL inhibitors, both of which are more potent than imatinib and show considerable efficacy against most of the well-characterized BCR-ABL mutants. Both imatinib and nilotinib bind to the inactive conformation of BCR-ABL, although nilotinib was designed to provide a better topological fit to the enzyme's tyrosine kinase domain and binds with approximately thirty times higher affinity than imatinib. In contrast, dasatinib binds both the active and inactive conformations of BCR-ABL kinase due to its less stringent conformational requirements for binding and it is a less selective kinase inhibitor, targeting SRC kinase family members as well. It is approximately 300 times potent than imatinib against wild-type BCR-ABL expressing cells and, therefore, inhibits BCR-ABL kinase activity at low-nanomolar concentration (between 0.75 to 1.0 nM).

As shown in Figures 22A and B, conditioned media protects from both nilotinib- and dasatinib-induced cell death. These data indicate that the development of more potent BCR-ABL inhibitors will not circumvent resistance associated with exposure of CML cells to soluble factors produced by the bone marrow microenvironment.

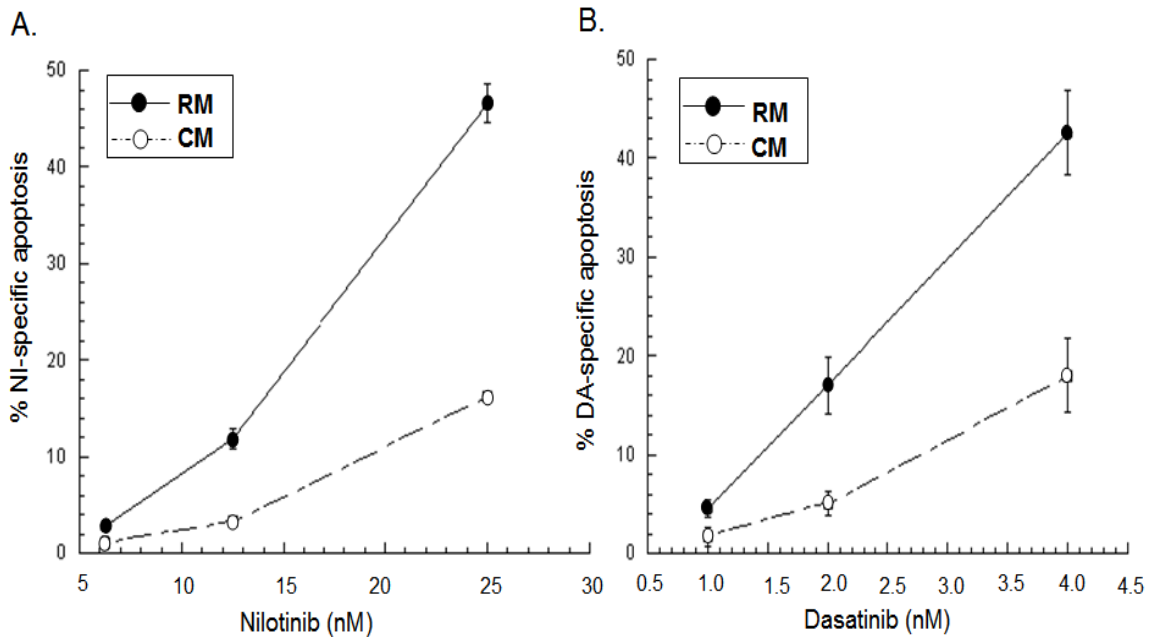



**Figure 22**: **HS-5-Derived Conditioned Media Protects K562 CML Cells from Death Induced by 2nd Generation BCR-ABL Inhibitors, Nilotinib And Dasatinib**. K562 cells were allowed to incubate for either 48 hours (nilotinib) or 24 hours (dasatinib). Conditioned media significantly protects K562 cells from (A) nilotinib-mediated cell death ($P < 0.05$, analysis of covariance) and (B) dasatinib-mediated cell death ($P < 0.05$, analysis of covariance). Representative graph in triplicates (n = 2 independent experiments)

### Conditioned Media Activates STAT3 in K562 and KU812 CML Cell Lines

Signal transducers and activators of transcription (STATs) are members of a family of transcription factors that were originally characterized as mediators of cytokine- and growth factor-induced signaling. Many of the growth factors and cytokines reported to be expressed in the HS-5 cell line are known activators of STAT3 and STAT5, including granulocyte-macrophage colony-stimulating factor, G-CSF, interleukin-6, and vascular endothelial growth factor [162-167]. Thus, to

determine the mechanism(s) of drug resistance observed in conditioned media, we decided to exam the roles of both STAT3 and STAT5 within our bone marrow stromal model. We first examined the effects of conditioned media on STAT3 activation. We wanted to determine whether culturing K562 cells in conditioned media caused an increase in the phosphorylation of STAT3.

To address this question, we performed Western blotting of K562 and KU812 CML cell lines cultured in regular media or conditioned media across various time points and used antibodies to probe for pTyr705-STAT3. Total STAT3 and β-actin serve as loading controls. As shown in Figures 23A and B, a rapid and sustained increase in pTyr705-STAT3 levels is observed across the indicated time points in both K562 and KU182 cells cultured in conditioned media as compared to those cells cultured in regular media. This suggests that our drug-resistant phenotype may be due to the anti-apoptotic affects of increased STAT3 activation and also demonstrates that our results were not cell-line specific.

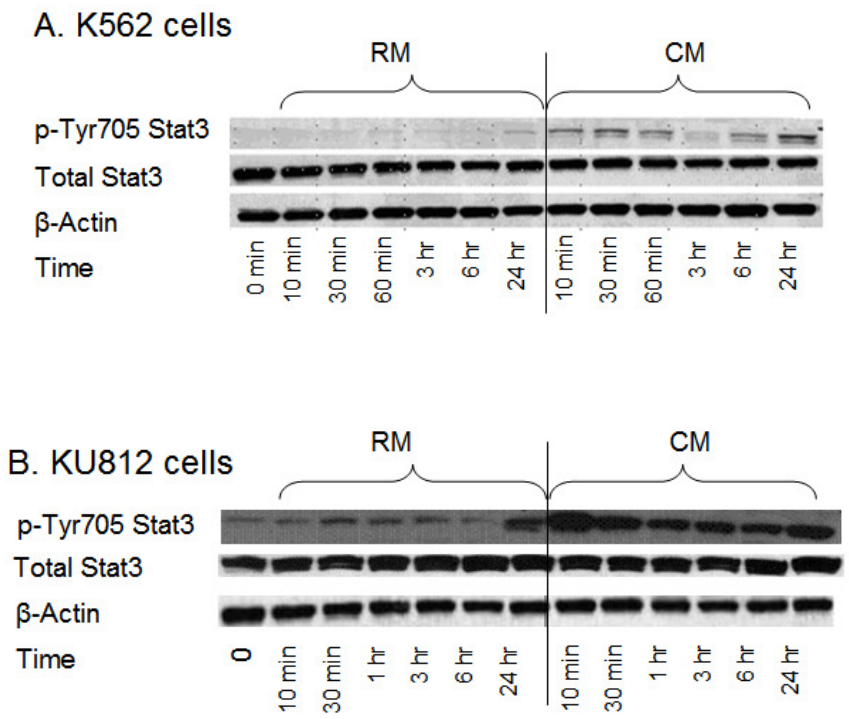



**Figure 23: STAT3 Phospho-Y705 is Increased in K562 and KU812 CML Cells Cultured in Conditioned Media.** (**A**) K652 cells were cultured in regular media or conditioned media for the time points indicated. Cells were collected, lysed, and analyzed for either pTyr705 or total STAT3 via Western blotting. β-actin was used as a loading control. (**B**) KU812 cells were cultured in RM or CM for the time points indicated. Cells were collected, lysed, and analyzed for either pTyr705 or total STAT3 via Western blotting. β-actin was used as a loading control. Representative figure (n = 3 independent experiments).

### STAT3 Activation in CML Cells is BCR-ABL-Independent

Previous studies have shown that BCR-ABL activates STAT3 by phosphorylating Ser727 via the MEK pathway [168]. To determine whether conditioned media-mediated STAT3 activation in CML cells is BCR-ABL dependent, we cultured K562 cells in regular media or conditioned media for 3

hours, then inhibited BCR-ABL signaling by treating cells with varying doses of imatinib for 48 hours. Cells were subsequently lysed followed by Western blotting for p-Y705 STAT3 and total STAT3 as a loading control.

We see in Figure 24 that treating K562 cells cultured in conditioned media with increasing concentrations of imatinib did not reduce STAT activation. In fact, there is an increase in the basal levels of p-Y705 STAT3 in cells grown in conditioned media when compared to those cultured in regular media and there is also sustained STAT3 activation in the presence of imatinib. This data lends greater support to our previous findings that our drug-resistant phenotype may be due to the anti-apoptotic affects of increased, aberrant STAT3 activation in CML cells cultured in conditioned media.

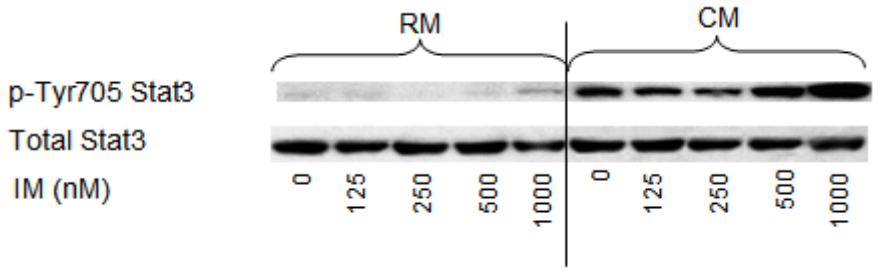



**Figure 24: Basal Phospho-Y705 STAT3 is Increased in K562 CML Cells Cultured in Conditioned Media and is Sustained in the Presence of Imatinib**. K562 cells were cultured in regular media or conditioned media for 3 hours prior to treating cells with varying doses of imatinib for 48 hours and followed by Western blotting for pSTAT3 and total STAT3.

**STAT5 activation is BCR-ABL-dependent**

Previous work done by de Groot revealed that BCR-ABL-mediated STAT5 activation can contribute to K562 leukemic cells' transformation [169]. To investigate the potential contribution of STAT5 activation in our drug resistant phenotype seen in conditioned media, we decided to also examine whether STAT5 activation in our model is BCR-ABL dependent. As before, we cultured K562 cells in regular media or conditioned media for 3 hours, inhibited BCR-ABL signaling with varying doses of imatinib for 48 hours and performed Western blotting for p-Y694 STAT5, with total STAT5 used as a loading control.

In contrast to our observations with STAT3, culturing K562 cells in conditioned media did not increase the basal levels of pSTAT5 (Figure 25). Furthermore, pSTAT5 levels were equally inhibited regardless of the culture condition. This indicates that STAT5 activation is BCR-ABL-dependent and eliminates STAT5 as a mediator in the drug-resistant phenotype conveyed by conditioned media.

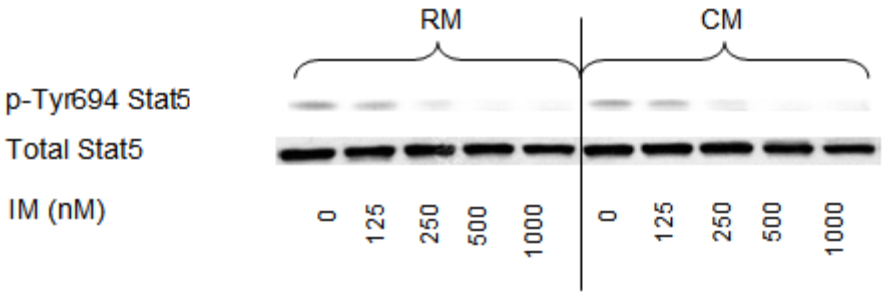



**Figure 25: K562 Cells Cultured in Conditioned Media Show Equal Levels of Phospho-Tyr STAT5 and Equal Inhibition of Phospho-Tyr STAT5 after Imatinib Treatment.** K562 cells were cultured in regular media or conditioned media for 3 hours; cells were treated with varying doses of imatinib for 48 hours and probed for pSTAT5 and total STAT5 via Western blotting.

### STAT3 Activation is not SRC-Dependent

Previous studies have shown that STAT3 activation can occur via activation of c-SRC, a member of the proto-oncogenic tyrosine kinases [170]. To examine whether STAT3 activation within our model is also being mediated by SRC, we cultured K562 cells in regular media or HS-5-derived conditioned media, then treated the cells with increasing concentrations of the dual BCR-ABL/SRC-kinase inhibitor, dasatinib, for 24 hour, followed by Western blotting for pY705 STAT3 and p-SRC. Total STAT3 and GAPDH were used as loading controls.

Work done in our lab revealed that while dasatinib inhibited p-SRC activity, it did not have any inhibit p-Y705 STAT3 activity (Figure 26). This suggests that STAT3 activation in conditioned media is also independent of SRC activity.

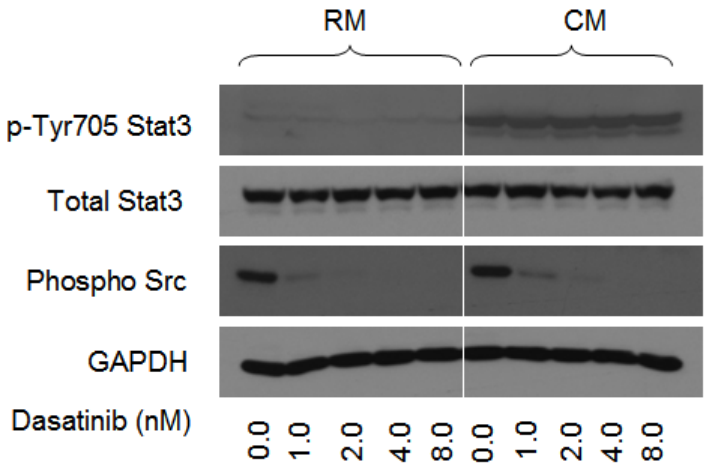



**Figure 26: Dasatinib Inhibited Phospho-SRC Activity but not Phospho-Y705 STAT3 Activity.** Treating K562 CML cells with dasatinib inhibited levels of p-SRC family members but did not attenuate the levels of pSTAT3 when K562 cells were cultured in CM. K562 cells were grown in regular media or conditioned media, and then treated with increasing doses of the dual BCR-ABL/SRC-kinase inhibitor, dasatinib, for 24 hours. This was followed by Western blotting for p-Y705 STAT3 and p-SRC. Total STAT3 and GAPDH were used as loading controls. Representative figure (n = 3 independent experiments).

## Protein Expression Levels of STAT3 Downstream Targets are Increased in K562 Cells Cultured in Conditioned Media

We next examined whether increased activation of STAT3 corresponded with an increased expression of its downstream targets, Bcl-xL, Mcl-1 and survivin.

Previous reports indicated that inhibition of BCR-ABL results in decreased expression of STAT5-regulated genes including Bcl-xL (24). As shown in Figure 27, while basal levels of Bcl-xL, Mcl-1 and survivin were not increased in K562 cells cultured in conditioned media compared to those cultured in regular media

(0 nM imatinib), when those cells were treated with imatinib in the context of conditioned media these protein expression levels were sustained. Specifically, we observed that, in the presence of 500 nmol/L imatinib, Bcl-xl, Mcl-1, and survivin were increased by 4.19 ± 1.82, 3.48 ± 1.84, and 7.0 ± 3.41, respectively (n = 3 independent experiments) when cells were cultured in conditioned media compared with cells cultured in regular media. These data suggest that activation of STAT3 may be responsible for persistent expression of STAT-regulated genes despite inhibition of STAT5 following imatinib treatment.

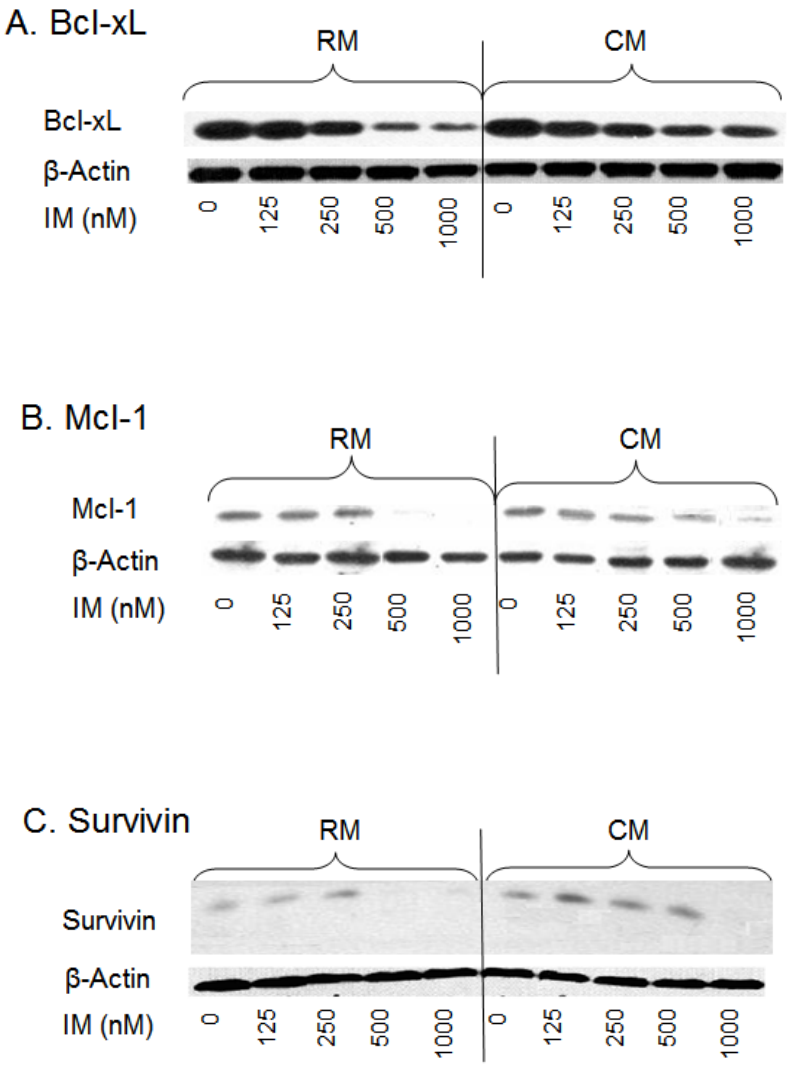



**Figure 27: The Effects of Conditioned Media on STAT3 Downstream Targets, Bcl-Xl, Mcl-1 and Survivin.** K562 CML cells were cultured in regular media or conditioned media for 3 hours then treated with imatinib in a dose-dependent manner for 48 hours. Western blot analysis was done with specific antibodies to Bcl-xl, Mcl-1, and survivin as described in Materials and Methods. Western blots showing: (**A**) Bcl-xl; (**B**), Mcl-1; and (**C**), survivin. β-actin was used as a loading control. Representative figure (n = 3 independent experiments).

**Reducing STAT3 Levels with siRNA Increases Sensitivity to Imatinib Mesylate in Conditioned Media**

SiRNA technology was used to determine the causative role for activation of STAT3 with respect to mediating resistance to imatinib when K562 cells were cultured in conditioned media. Figure 27A depicts the timeline used in performing these time-course experiments.

As shown in Figure 28B, STAT3 was reduced at 84 hours and remained reduced for 120 hours. Based on these time-course experiments, apoptotic assays were designed as follows: K562 CML cells were transfected with either a control siRNA or STAT3 siRNA and this process was repeated 36 hours later to ensure that STAT3 gene expression remained silenced; 84 hours following imatinib treatment, control siRNA and STAT3 siRNA-transfected cells were treated with either 250 nmol/L imatinib or vehicle control for 36 hours (120 h after initial transfection with siRNA) and apoptosis was detected by Annexin V binding. Because some tumor types require STAT3 for survival, we first analyzed whether reducing STAT3 levels with siRNA was sufficient to induce apoptosis when cells were cultured in either regular media or conditioned media. As shown in Figure 28C, reducing STAT3 levels (120 hours time point) with siRNA did not cause apoptosis in either growth condition. However, reducing STAT3 levels sensitized K562 to imatinib-induced cell death when K562 cells were grown in conditioned media but not in regular media (see Figure 28D for a representative figure).

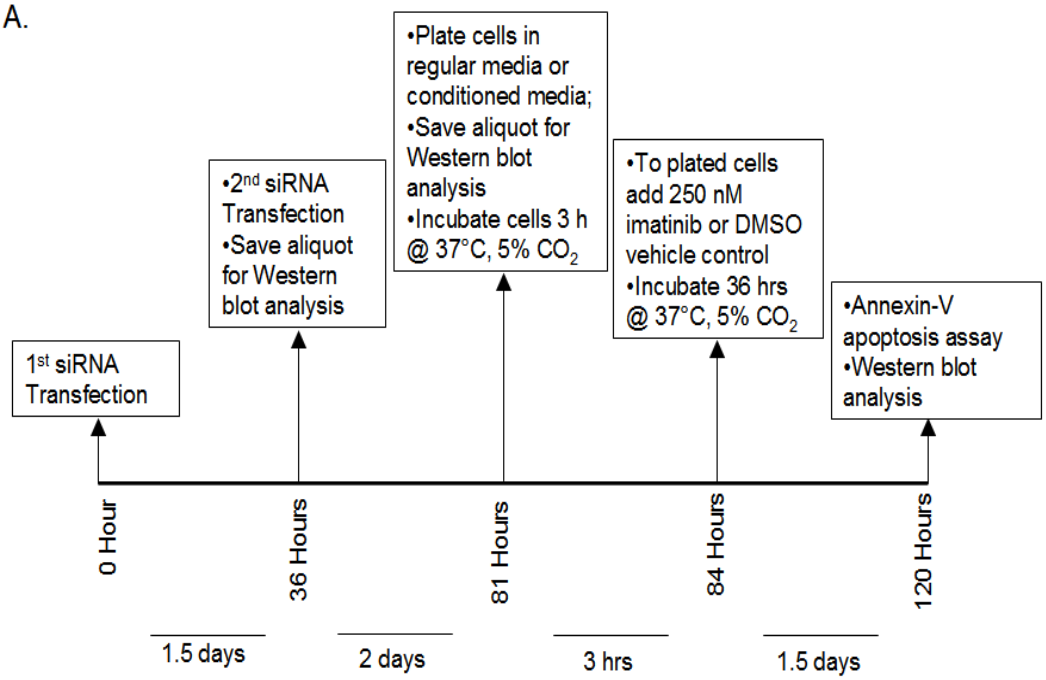
In Figure 28E we graphed the geometric mean of the ratios of each group depicted, showing the 95% confidence intervals and the P-values. We used the geometric mean instead of the arithmetic mean because with the arithmetic mean values below 1 are limited to numbers between 1 and 0. But values above 1 are limited to 1 and infinity. Therefore, using the geometric mean enabled us to address this asymmetry. The first group we analyzed was those cells transfected with the control siRNA and treated with imatinib regardless of culture conditions.

Our results in Figure 28E show that in the control siRNA-transfected cells, there was a significant difference in imatinib-specific cell death seen in K562 cells cultured in regular when compared to those cultured in conditioned media. We next analyzed the STAT3 siRNA-transfected cells that were treated with imatinib in either regular media or conditioned media. Our results show that when STAT3 is knocked-down using siRNA there was no significant difference in imatinib-induced apoptosis in cells cultured in regular media compared to those cultured in conditioned media. In essence, we were able to reverse the drug-resistant phenotype seen in conditioned media by knocking-down STAT3. We then analyzed those cells cultured in regular media, regardless of their transfection state. As seen in Figure 28E, our results show that when cultured in regular media, whether or not STAT3 is knocked-down, there was no significant difference in apoptosis induced by imatinib. Lastly, we analyzed those cells cultured in conditioned media, regardless of their transfection state. Our results

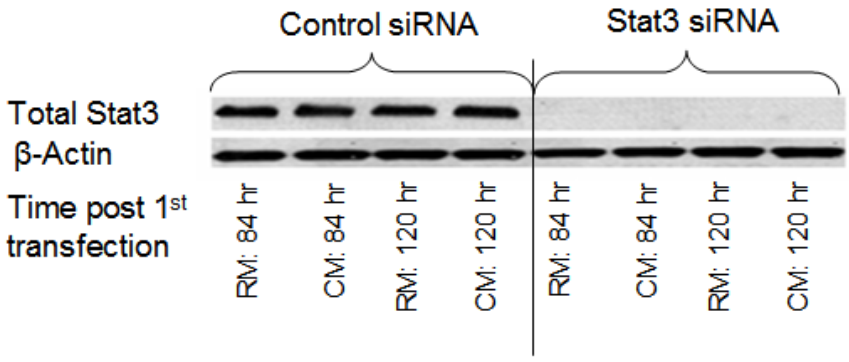
show that when cultured in conditioned media and STAT3 is knocked-down, there was a significant difference in apoptosis induced by imatinib.

Taken together, these data indicate that STAT3 contributes to imatinib resistance only when cells are cultured within the context of bone marrow stroma-derived conditioned media. Furthermore, these data suggest that STAT3 can compensate for BCR-ABL survival signals and thus represents a potential BCR-ABL-independent mechanism of drug resistance.

A.




B.



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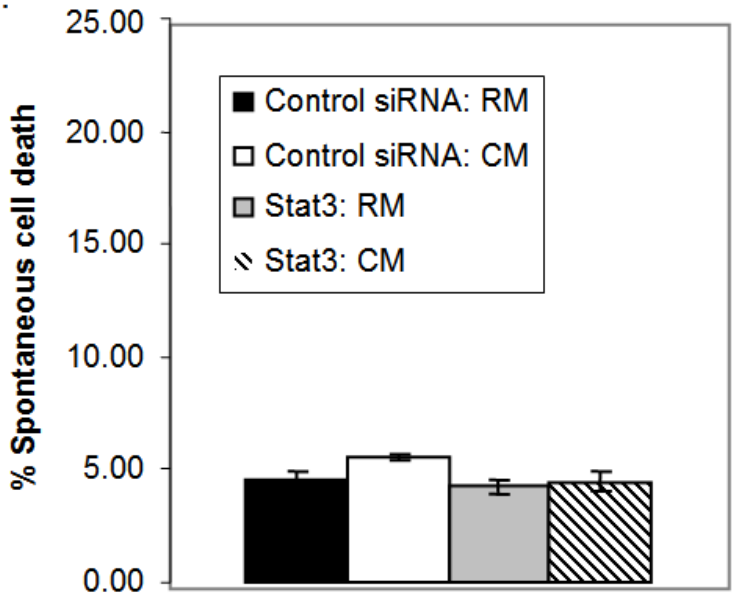


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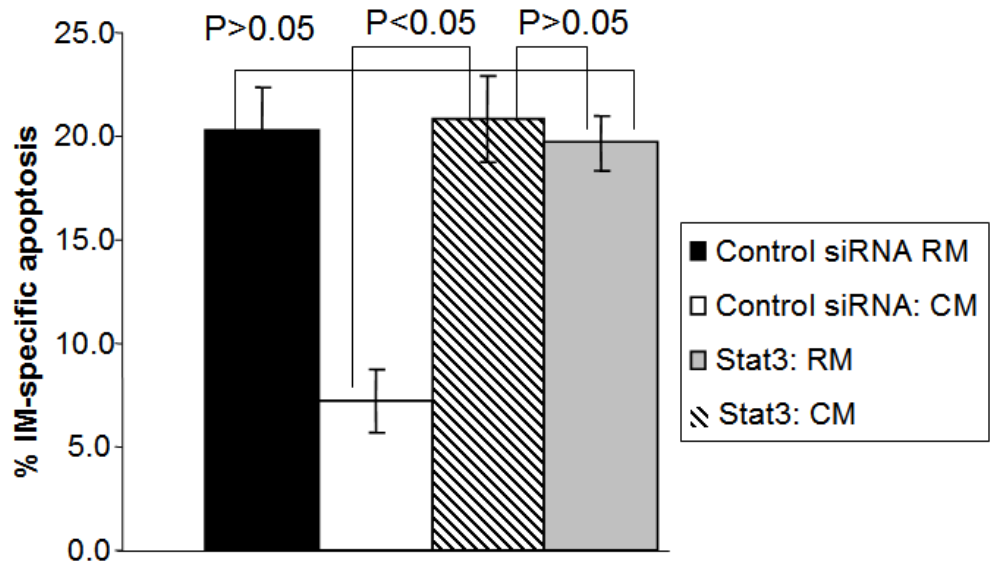

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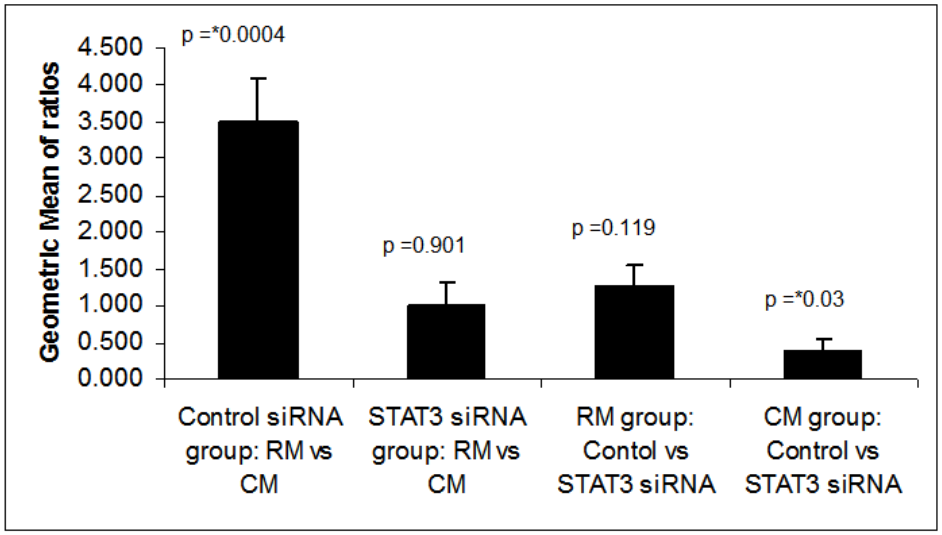



**Figure 28: Reducing STAT3 Levels with siRNA Reverses Imatinib Resistance in K562 Cells Cultured in Conditioned Media.** K562 cells were cultured in either regular media or conditioned media and treated with either siRNA to STAT3 or control siRNA. (**A**), Time line for conducting siRNA experiments. (**B**), STAT3 knockdown was confirmed using Western blotting and STAT3 was noted to be maximally decreased at 84 hours and remained reduced for at least 120 hours (n = 5 independent experiments). (**C**), reducing STAT3 levels was not sufficient to cause cell death in cells cultured in regular media or conditioned media. Mean ± SD of 5 independent experiments. (**D**, reducing STAT3 levels enhances sensitivity to imatinib when K562 cells are cultured in conditioned media. Apoptosis of K562 cells cultured in regular media or conditioned media, treated with either STAT3 siRNA or control siRNA, in the presence or absence of 250 nmol/L imatinib was done using the Annexin V detection of apoptotic cells and fluorescence-activated cell sorting analysis. % Specific apoptosis was calculated by subtracting background cell death from imatinib-mediated cell death. (**E**), Geometric mean of the 5 combined independent experiments showing the 95% confidence intervals and the corresponding P-values. The geometric mean was calculated as the 5th root of

the product of all the members of the data set in all 5 experiments. The formula is depicted as follows: $\sqrt[5]{a1 * a2 * a3 * a4 * a5}$, where 'a' represents the data of each experiments (1-5). We decided *a priori* that a p-value <0.05 is statistically significant. Representative figure in triplicates (n = 5 independent experiments).

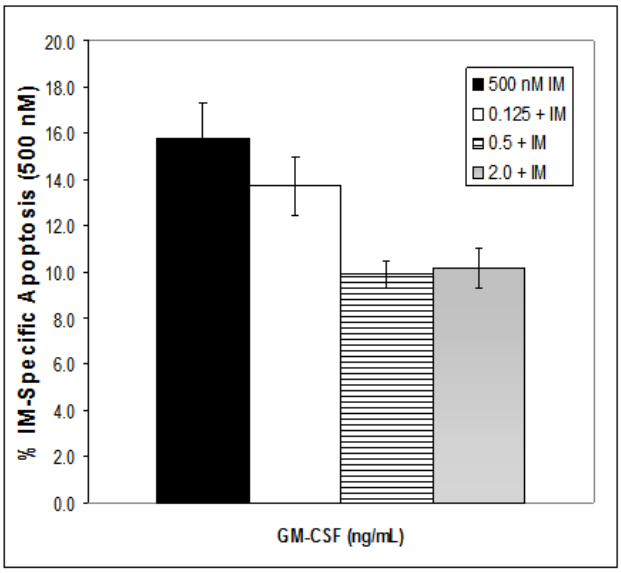
**Addition of GM-CSF, IL-6 and VEGF to Regular Media Induces the Imatinib-Resistant Phenotype Associated with Conditioned Media**

Previous studies highlighted the involvement of multiple cytokines in mediating drug-resistance in CML cells and demonstrate the involvement of more than one cytokine in stroma-mediated protection of leukemic cells [171]. Therefore, we decided to investigate the role of key cytokines, GM-CSF, IL-6 and VEGF, on our conditioned media-induced imatinib-resistant phenotype within our model system. We wanted to determine whether the addition of these STAT3-activating cytokines to regular media induce imatinib-resistance in K562 CML cells. To answer this question we cultured K562 cells for 3 hours in regular media containing varying concentrations of GM-CSF (0.125 - 2.0 ng/mL), IL-6 (0.25 – 4.0 ng/mL) or VEGF (3.0 – 48.0 ng/mL), treated the cells with vehicle control or 500 nmol/L imatinib for 48 hours and analyzed our results via Annexin V apoptosis assay followed by FACS analysis.

Our preliminary results indicate that the addition of these cytokines to regular media induced the imatinib-resistant phenotype that has been observed in K562 CML cells cultured in conditioned media. Figures 29A and B show that 2.0 ng/mL GM-CSF and 4.0 ng/mL IL-6, respectively, induced the conditioned

media- associated imatinib-resistant phenotype to K562 cells cultured in regular media. At 48.0 ng/mL VEGF partially restores this phenotype to K562 cells cultured in regular media (Figure 28C). Together, these results suggest that multiple cytokines, including GM-CSF, IL-6 and VEGF, may be involved in mediating imatinib resistance to CML cells within the context of the bone marrow stroma.

A.

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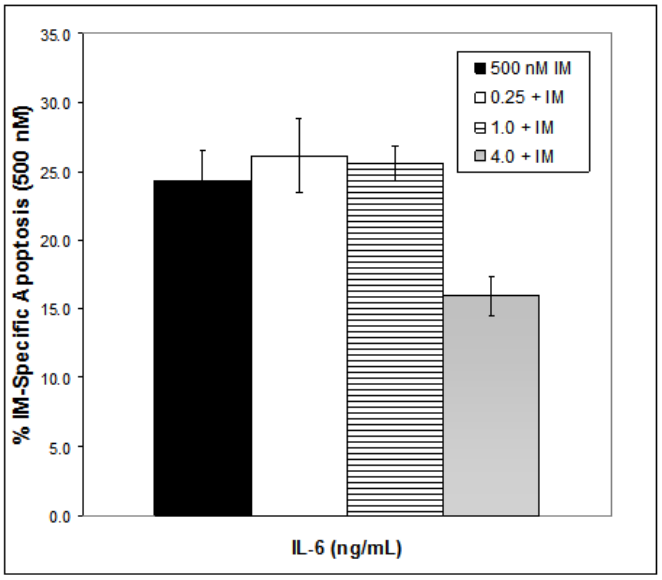

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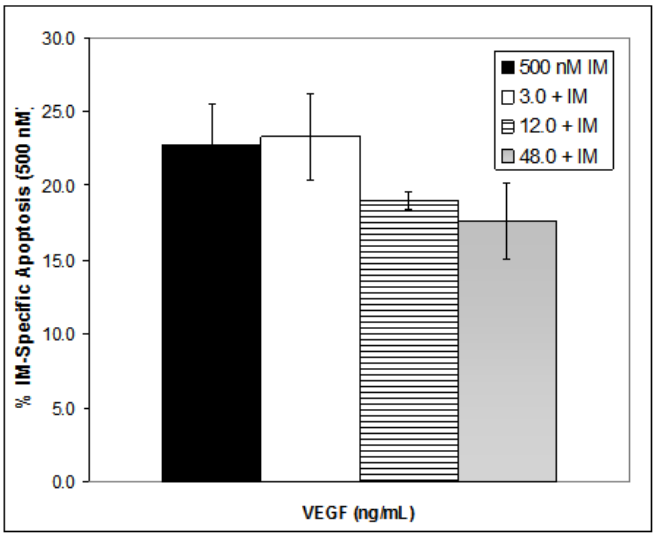


**Figure 29: The Effects of GM-CSF-, IL-6-, or VEGF-Supplemented Regular Media on Imatinib-Sensitivity in K562 CML Cells.** K562 CML cells were cultured for 3 hours in regular media supplemented with varying concentrations of GM-CSF, IL-6 and VEGF as indicated added. After treatment with DMSO vehicle control or 500 nmol/L imatinib, cells were allowed to incubate 48 hours at 37℃, 5% $CO_2$. Cell death was measure using AnnexinV apoptosis assay and data was analyzed using FACS. Representative figure (n = 2 independent experiments).

## CHAPTER X

## DISCUSSION AND FUTURE DIRECTION

Over 40 years ago ground-breaking research led to the identification of the BCR-ABL chimeric oncoprotein as the initial transforming event in chronic myeloid leukemia (CML). This discovery revolutionized the treatment of CML and made BCR-ABL an ideal target for drug development. This gave rise to rationally designed, small molecule signal transduction inhibitors (STI) specific for the tyrosine kinase domain of BCR-ABL, whose constitutive activation is the hallmark of BCR-ABL-mediated cell transformation.

The novel BCR-ABL tyrosine kinase (TK) inhibitor, imatinib mesylate (imatinib; IM), represents the first molecularly targeted therapy in patient care and has become the gold standard in the treatment for CML patients. By working as an ATP-mimic that selectively competes for and binds to the TK domain of BCR-ABL, imatinib essentially stabilizes the oncoprotein in an inactive conformation that inhibits its enzymatic and transforming activities. The remarkable efficacy of this targeted-therapy approach to CML treatment is evidenced by the fact that imatinib produces a complete hematological response rate, which described the amount of normal leukocyte count in the peripheral blood, of 95% in newly diagnosed patients. Furthermore, it produces complete

cytogenetic response, which is the absence of detectable Ph-positive cells from more than 20 bone marrow cells in metaphase, in 90% of patients with minimal toxicity.

However, despite imatinib's efficacy and the efficacy of more potent BCR-ABL inhibitors such as nilotinib and dasatinib, the emergence drug-resistant CML challenges us to re-visit our understanding of the mechanisms involved in CML disease occurrence, progression and drug-resistance. Clinical data arising from studies targeting BCR-ABL inhibitors indicate that although BCR-ABL inhibitors are very effective, targeted therapy using a single agent does not eliminate minimal residual disease (MRD). This is due to the observation that BCR-ABL-positive clones are still detectable by quantitative real time-PCR in patients undergoing treatment with these inhibitors. Furthermore, these findings are consistent with clinical data indicating that patients that discontinue imatinib therapy rapidly relapse to a tumor burden which is as great or greater then before treatment, suggesting that imatinib does not induce cell death in the stem cell population or the population capable of self-renewal [172, 173].

Imatinib was specifically designed to bind to and block the ATP-binding pocket of the BCR-ABL kinase domain while in an inactive conformation. Mutations within the kinase domain disrupt that conformation and thwart the ability of imatinib to bind. Therefore, the predominant and most characterized mechanism of imatinib-resistance is the emergence of clones possessing point mutations within the kinase domain of BCR-ABL. These observations led to the

development of second generation BCR-ABL inhibitors, nilotinib and dasatinib, which are 30 and 300 times more potent than imatinib, respectively. However, despite the efficacy of these inhibitors, their use has not remedied the problem of all chemo-resistant CML associated with kinase mutations. Some patients present with indomitable pre-existing mutations, such as T315I, which these inhibitors cannot eradicate, while other patients acquire this mutation and the resistant phenotype during the course of treatment with these inhibitors. Furthermore, recent evidence reveals that mutations of the BCR-ABL kinase domain occur in approximately 40% to 60% of imatinib-resistant patients [174]. Therefore, approximately 50% of these imatinib-resistant CML patients do not have kinase domain mutations but are resistant to imatinib by alternative means. This suggests that BCR-ABL-independent mechanisms could contribute to imatinib-resistance and underscore the need to identify and target BCR-ABL-independent pathways.

Models used to investigate the mechanisms of drug resistance seen in CML often highlight the cell-autonomous modes of resistance: mutations within the tyrosine kinase domain of BCR-ABL itself that either block imatinib binding or impair the ability of the kinase to assume the correct conformation required for imatinib to bind. But these models often fail to consider the role of the surrounding cells and tissues, the “microenvironment”, on the cancer itself and the role of this microenvironment on the emergence of the tumor drug-resistance phenotype. However, recent studies have draw attention to the involvement of

the tumor microenvironment in providing cancer cells an escape from chemotherapy-induced cell death. While studying drug resistance in Ph-positive acute lymphoblastic leukemia (ALL), William *et al*. reported on a non-autonomous mechanism of drug resistance involving cytokine signaling within the hematopoietic microenvironment [139]. This study revealed that the contribution of BCR-ABL-independent mechanisms was capable of protecting CML cells from imatinib-induced cell death. Therefore, the use of an *in vitro* non-autonomous model of drug resistance may provide further insights into the role of host-derived stimuli on CML cells in conferring resistance to BCR-ABL inhibitors and may provide a greater comprehension of how to combat this mechanism of resistance.

To investigate the role of the bone marrow microenvironment on resistance to BCR-ABL inhibitors, we utilized an *in vitro* bone marrow stromal model that exposes CML cells to multiple soluble factors produced by bone marrow stroma cells. The human bone marrow stromal cell line, HS-5, served as the model of the bone marrow microenvironment as it closely approximates the bone marrow-associated cytoprotection observed in drug-treated leukemia patients. HS-5 is fibroblastic and has been shown to secrete numerous cytokines that are capable of supporting the *ex vivo* expansion of mature and immature hematopoietic cells and their progenitors ([175]. Two hematopoietic CML cell lines were used to model the disease: K562 cells originated in the bone marrow, are highly undifferentiated and were derived from the pleural effusion of a CML

patient in blast crisis phase; KU812 cells are pre-basophilic and originated from the peripheral blood of a blast-crisis-phase CML patient.

Previous work done in our laboratory revealed that adhesion of K562 CML cells to fibronectin could provide cell adhesion mediated-drug resistance (CAM-DR) against imatinib-induced cell death through anti-apoptotic integrin-mediated signaling [135]. Similarly, our initial set of experiments using a co-culture transwell model system revealed that direct adhesion of K562 cells to HS-5 stoma cells conferred protection against cell death induced by imatinib, possibly through a similar mechanism. Interestingly, however, was the observation that HS-5-derived soluble factors were also capable of modulating imatinib response in CML cells and confer resistance. This data indicates that both direct contact with the bone marrow stroma and soluble factors produced by supporting stroma cells play a role in the emergence of the CML drug-resistant phenotype. This data also lends support to previous observations that, in addition to targeted therapy against BCR-ABL, the tumor-microenvironmental interactions may play a crucial role in protecting CML cells from the anti-apoptotic and anti-proliferative effects of imatinib. To delineate the contributions of bone marrow stroma-derived soluble factors on resistance to BCR-ABL inhibitors and to simplify our model the data presented in this dissertation were obtained from the use of HS-5-derived conditioned media.

It has been demonstrated here that imatinib induces a high rate of cell death in CML cells cultured in regular media (RM: RPMI + 10% fetal bovine

serum). However, using the same media conditioned for three hours by the HS-5 human stroma cells (CM) is sufficient to cause resistance to imatinib-induced cell death in K562 and KU182 CML cells. Since HS-5 stromal cells produce cytokines that support the growth and differentiation of HSCs, this data suggests that HS-5-derived soluble factor(s) may be involved in mediating this imatinib-resistant phenotype in CML cells. Furthermore, HS-5 conditioned media could be stored at -80℃ and retain its ability to produce imatinib resistance in these CMLS cells, while heat-inactivated conditioned media lost this ability. These data indicate that the soluble factor(s) conferring resistance to imatinib is quite stable and capable of preserving its function during the freezing and thawing process. Additionally, heat-inactivation of conditioned media would destroy its protein components, which includes growth factors and cytokines. The observation that heat-inactivated conditioned media restores imatinib-sensitivity to CML cells demonstrates that the protective component in conditioned media is a protein. Based on findings of similar studies, the hematopoietic cytokine granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin 6 (IL-6) are two potential cytokines that may be involved in mediating the BCR-ABL-independent imatinib-resistant phenotype seen in our model.

While HS-5-derived conditioned media increased the clonogenic survival of K562 cells, exposure of CML cells to the soluble factors in conditioned media did not lead to an increase in cell proliferation. This indicates that the soluble factor(s) mediate imatinib-resistance through cell survival pathways. There are

several well-characterized cytokine-receptor-activated cell survival pathways that may be involved in promoting clonogenic survival of these cells. The Janus activated kinase (JAK)/STAT signal transduction pathway is important in converting cytokine-receptor signals into downstream survival signals and is often constitutively activated in hematopoietic malignancies. Studies show that in BCR-ABL-expressing cells, constitutive activation of JAKs is associated with constitutive STAT3 Try-705 phosphorylation. Studies show that STAT5 is also constitutively activated in CML cells. Other survival pathways that may enhance the clonogenic survival of CML cells are the anti-apoptotic phosphatidylinositol 3-kinase (PI3K)/AKT signaling pathway, whose activation has been shown to not only be involved in BCR-ABL-mediated cell transformation but also resistance to BCR-ABL inhibitors. Additionally, BCR-ABL also signals through the extracellular signal regulated kinase 1/2 (ERK-1/2) pathway and has been implicated in the anti-apoptotic activities of BCR-ABL.

Data presented here revealed that when CML cells were no longer exposed to the protective soluble factor(s) associated with HS-5 conditioned media and were re-cultured in regular media, the imatinib-resistant phenotype diminished. This demonstrates that withdrawal of CML cells from bone marrow stroma-secreted soluble factor(s) was sufficient to re-sensitize these cells to the apoptotic effects of imatinib. It is possible that this observation is attributable to the termination of cytokine-receptor-mediated signaling. Furthermore, this data reveals a direct correlation between the presence of HS-5-derived soluble factors

and the emergence of imatinib resistance, an observation that has also been highlighted in other studies.

The hypothesis within this dissertation is based on the importance of soluble factors specific to the bone marrow microenvironment in promoting chemoresistance in CML cells. To accurately test this hypothesis it was important to investigate whether K562 CML cells could condition its own media via autocrine secretion of its own soluble factors and induce chemoresistance. Additionally, we investigated whether other non-stromal cell lines were capable of conveying protection against cell death induced by imatinib. In either case, if there is protection against cell death induced by BCR-ABL inhibitors then this would demonstrate that the bone marrow microenvironment may not be responsible for providing chemo-resistance to CML cells and would discredit its role in modulating drug response within our model. Conditioned media from K562 CML cells and two hematopoietic cell lines, U937 and 8226, were generated in the same manner as HS-5-derived conditioned media: these cells were cultured in regular media for 3 hours, enabling the media to become conditioned by the different cell lines; the conditioned media was then collected by centrifugation to remove debris and utilized in subsequent experiments. K562 CML cells were cultured in regular media and several types of conditioned media, as previously described. The cells were treated with imatinib for 48 hours to induce cell death, which was measured using the Annexin V apoptosis assay. Our data revealed that of the different conditioned media generated, only HS-5-derived conditioned

media was capable of conveying imatinib-resistance in K562 CML cells. This data validated the unique role and importance of soluble factors specific to the bone marrow stroma in protecting CML cells from death induced by imatinib.

There are numerous bone marrow stroma-derived soluble factors and cytokines involved in the growth and differentiation hematopoietic cells that may also be crucial in conferring resistance to BCR-ABL inhibitors. According to Torok-Storb (1999), the human HS-5 stroma cell produces a cocktail of cytokines that include IL-6, GM-CSF, G-CSF and VEGF. It is conceivable that these cytokines could confer protection against imatinib-induced cell death to varying degrees either individually on in combination with each other. In fact, while studies by Wang *et al*. focused on the role of the autocrine secretion of GM-CSF in mediating resistance to BCR-ABL inhibitors in CML cells [140], Weisberg *et al.* emphasized the involvement of the bone marrow stroma as a whole in mediating protection against CML cell death by using a cocktail of stroma-derived cytokines [171].

Our data from the bone marrow stromal model lends credence to the involvement of survival signaling mediated by stroma-derived soluble factors in the emergence of chemo-resistant CML. This is also evidenced by our observations that neither nilotinib nor dasatinib are capable of overriding the protective effects of HS-5-secreted soluble factors on K562 CML cells. This demonstrates that even though one inhibitor was designed to bind more stringently to the tyrosine kinase domain of BCR-ABL and the other was

designed to recognize and bind to both its active and inactive conformations, the development and use of these more potent, rationally-designed inhibitors was not sufficient to address this mechanism of resistance involving the bone marrow microenvironment. There is growing interest in research to target components of the hematopoietic tumor microenvironment as a way to enhance the efficacy of targeted therapy against CML. While research that addresses BCR-ABL-independent mechanisms of imatinib resistance have gained momentum, there are also concerns about the effects that targeting the bone marrow microenvironment may have on normal hematopoiesis and surrounding cells. Studies suggest that the best initial approach is to target individual signal transduction pathways that are constitutively activated in malignant hematopoietic cells.

BCR-ABL's modular domains make it capable of activating several signal transduction pathways that can contribute to enhanced CML cell survival, including Ras/ERK, PI3K/AKT, and STAT5 signal transduction pathways. These pathways can also be activated by external signals including growth factors, cytokines, and interactions with extracellular matrices. Furthermore, studies suggest that these pathways can be reconstituted in a BCR-ABL-independent manner by these external signals. Our data supports this observation and reveals that HS-5-derived soluble factors are capable of BCR-ABL-independent constitutive STAT3 activation via Tyr-705 phosphorylation. Studies show that constitutive STAT3 Tyr-705 phosphorylation and activation is mediated by

activated JAKs. Activated STAT3 homo- or heterodimerizes and translocates to the nucleus where it leads to the transcriptional activation of STAT3 target anti-apoptotic genes.

Studies show that cytokines that are capable of STAT3 activation include IL-6, vascular endothelial growth factor (VEGF), and GM-CSF. Here we demonstrate that not only does the exposure of either K562 or KU812 CML cells to conditioned media derived from HS-5 cells resulted in the rapid and sustained increase in phospho-Y705 STAT3 levels, but we also show that this exposure to HS-5-derived soluble factors also led to an increase in the protein expression levels of STAT3 downstream targets, Bcl-xL, Mcl-1 and survivin. This suggests that HS-5-derived soluble factors may be involved in the activation of STAT3, which in turn resulted in STAT3-mediated expression of these anti-apoptotic proteins. Furthermore, the addition of imatinib does not reduce phospho-Y705 STAT3 levels or the levels of its downstream targets. This demonstrates that activation of STAT3 is indeed independent of BCR-ABL activity and highlights the importance of BCR-ABL-independent mechanisms in facilitating the imatinib-resistant phenotype. This data also contradicts an earlier observation made by Coppo *et. al.* which showed that the constitutive phosphorylation of STAT3 on Tyr-705 was dependent on BCR–ABL [168]. This difference may be attributable to the fact that they utilized a hematopoietic cell line that was retrovirally transduced with a p210-BCR-ABL expressing vector as oppose to utilizing a cell line obtained from CML patients.

Since studies show that STAT3 activation can also be accomplished through the activities of SRC family kinases (SFKs), it was important to determine whether the increase in BCR-ABL-independent STAT3 activity was attributable to SFKs. Our data showed that when K562 CML cells were treated with the dual BCR-ABL/SFK inhibitor, dasatinib, sustained phosphorylation of STAT3 was still observed in those cells cultured in HS-5-derived conditioned media, while phospho-SRC levels were attenuated. This observation eliminates the involvement of SFKs in the increased activation of STAT3 that we observed in our model and lends greater support to the involvement of HS-5 derived soluble factors in mediating STAT3 activation, possible through cytokine receptor-engagement and JAKs activation. While investigating the role of STAT3 activation in K562 CML cells exposed to HS-5 soluble factors and assessing the mechanism(s) involved in its activation, one interesting and consistent observation was that the use of increasing concentrations of imatinib resulted in increased STAT3 activation. In examining this further, other studies have reported that BCR-ABL can activate STAT5 and STAT1 independently of the activation of JAKs [154]. Furthermore, a dominant-negative STAT5 was shown to inhibit colony formation of K562 cells and blocking BCR-ABL was shown to inhibit STAT5-dependent DNA binding as well as STAT5 target genes, Bcl-xL and Mcl-1 [169, 176-180]. Bcl-xl, Mcl-1, and cyclin D1 represent genes that can be regulated by either STAT5 or STAT3 activation. Therefore, based on these observations, we propose that within the bone marrow microenvironment STAT3

can compensate for BCR-ABL-dependent activation of STAT5-dependent genes, which are critical for CML cell survival, when STAT5 activity is blocked by BCR-ABL inhibitors. In support of this hypothesis, our data also indicate that when K562 cells were treated with imatinib, the levels of Bcl-xL, survivin, and Mcl-1 were increased when cells were cultured in conditioned media compared with cells cultured in regular media. Together, these data suggest that, depending on culture conditions, K562 CML cells may have plasticity with respect to STAT-dependency.

To determine the causal role of STAT3 activation in our drug-resistant phenotype associated with conditioned media, we reduced STAT3 levels using siRNA technology. Here we show that this reduction in STAT3 levels led to the enhanced sensitivity of K562 CML cells to imatinib-induced cell death. Importantly, this increased imatinib-sensitivity in CML cells was observed only within the context of the bone marrow stroma model, as reducing STAT3 levels in regular media did not result in increased cell death. These data highlight the contribution and significance of BCR-ABL-independent STAT3 activation in reconstituting BCR-ABL-mediated survival signaling events that promote CML progression. Furthermore, our data indicate that while the initial BCR-ABL insult that initiated the disease within the primitive hematopoietic stem cell population is crucial to CML pathogenesis, clearly soluble factors unique to the hematopoietic microenvironment are capable of protecting CML cells from the apoptotic effects of BCR-ABL inhibitors. To address the question recently posed by Charles

Sawyers in his news brief entitled 'Where lies the blame for resistance - tumor or host?' [181], the synthesis of data presented here, along with data presented in previous studies, suggest that both the tumor and the host are responsible. Consequently, the use of BCR-ABL inhibitors alone is not sufficient to address this type of resistance, which is associated with BCR-ABL-independent STAT3 activation. While BCR-ABL inhibitors target the fusion oncoprotein, it does not target STAT3. Therefore, these data also provide pre-clinical rationale for targeting STAT3 in order to increase the efficacy of BCR-ABL inhibitors in treating drug-resistant CML cells. To that end, a multi-targeted approach to CML patient care that involves the combined use of BCR-ABL inhibitors and STAT3 inhibitors may prove more effective in addressing this type of drug-resistant CML. *In vitro* studies to identify compounds that are highly selective for STAT3 have highlighted the effectiveness of cisplatin/IS3 295 [182] as well as cucurbitacin Q [183] in inhibiting STAT3 activation.

Bone marrow stroma-derived conditioned media consists of a complex mixture of cytokines and other soluble factors that can be source of survival signals affecting cell differentiation, expansion and survival. The functional redundancy of many of these soluble factors and their signaling receptors may converge to activate STAT3 and circumvent cell death induced by BCR-ABL inhibitors. Since several studies have highlighted the involvement of GM-CSF, IL-6 and VEGF in the emergence and progression of hematological malignancies, we examined the contribution of these cytokines in our drug-resistant phenotype.

We wanted to determine whether exposure of CML cells to these cytokines can modulate their response BCR-ABL inhibitors. Here, our data revealed that when these cytokines were added to regular media in varying concentrations a dose-dependent imatinib resistant phenotype emerged. This is evidenced by the steady reduction in imatinib-induced CML cell deaths, which correlated with increased cytokine concentrations. The addition of 2.0 ng/mL of GM-CSF, 4.0 ng/mL of IL-6 or 48.0 ng/mL of VEGF to regular media initiated the return of the imatinib-resistant phenotype associated with HS-5 conditioned media. These results, which are consistent with observations made in similar studies, indicate that these cytokines may be working individually or cooperatively to protect CML cells from death induced by BCR-ABL inhibitors.

Furthermore, data from other studies revealed that conditioned media from malignant cell lines expressing high levels of phospho-STAT3 also contained cytokines that are capable of stimulating STAT3 activation, such as IL-6. To determine the involvement of cytokines such as IL-6, GM-CSF and VEGF within our model, their activity in conditioned media could also be impeded using blocking antibodies. For example, IL-6 signal transduction, which activates STAT3 through the glycoprotein (gp) 130/JAK pathway and is notable for its pleiotropic tumor-promoting activities, could be blocked using a gp130-blocking antibody or an IL-6-blocking antibody (such as BR-3 and 522, respectively; provided by Cell Sciences, Inc.). Alternatively, to validate the results obtained from the use of cytokine-blocking antibodies, the gene expression of these

cytokines in HS-5 cells could also be sequentially silenced using siRNA technology.

To identify the specific soluble factor(s) causative for imatinib resistance and STAT3 activation within our model, one global approach is to utilize multiplexed bead-based assays that efficiently detect and quantify several cytokines simultaneously (Invitrogen). These assays consist of capture-antibodies conjugated to biologically inert beads and are designed to identify various soluble factors, including cytokines, with enhanced speed and accuracy. Furthermore, since our data revealed that HS-5 cells do not require serum to produce the protective soluble factors, we could identify the active soluble factor(s) in HS-5 conditioned media that confers imatinib resistance by fractionating the SF-CM into its protein components and performing the Annexin V apoptosis assay on each fraction. Fractionation could be accomplished in several ways. Centricon spin columns (Miliipore) can separate the components of conditioned media by molecular weight. Additionally, fractionation can also be accomplished using liquid chromatography or mass spectrometry.

Constitutive STAT3 activation is associated with several human malignancies, including solid tumors and hematological diseases. Interestingly, we observed that reducing STAT3 levels sensitized imatinib-resistant CML cells to its apoptotic effects. It is possible that while BCR-ABL-mediated oncogenic signaling events are being impeded by imatinib, the non-autonomous activation of STAT3 by soluble factors within the bone marrow microenvironment facilitates

these signal transduction events (see Figure 30 for proposed model). Additionally, our data provides preclinical rationale to support the use of STAT3 inhibitors as BCR-ABL-inhibitor sensitizers and for conduction validating our observations in studies using primary patient specimens. One set of experiments would be to confirm data presented in other studies that show increased STAT3 activation in primary CML patient samples when compared to healthy bone marrow donors. Furthermore, cells from CML patient can be used to delineate whether small molecule inhibitors targeting STAT3, such as phosphotyrosyl peptides [184], are capable of enhancing the efficacy of BCR-ABL inhibitors using *in vivo* models.

Furthermore, patient's samples could be used to examine whether STAT3 activation enables CML cells to escape complement-dependent cytotoxicity via CD46 expression. It has been reported that STAT3 activation is capable of inducing the expression of CD46, one of the complement-regulatory proteins (CRPs) expressed on the surface of normal and transformed cells. While complement proteins target bacteria and cancerous cells for lysis [185], cancer cells often escape elimination by complement proteins. This escape mechanism is due in part to the expression of this membrane-bound CRP, which inactivates complement components. It would be of interest to e whether increased STAT3 activation in patient samples also resulted in this escape mechanism.

Studies show that BCR-ABL-positive MRD in some imatinib-resistant CML cells are characterized by increased activation of the PI3K/AKT and Ras/MEK

signal transduction pathways. These pathways are capable of mediating cell survival by transmitting signals from multiple cell surface receptors to transcription factors within the nucleus. However, the mechanisms responsible for the enhanced activation of these survival pathways remain poorly understood. Future studies may include investigating whether the PI3K/AKT and Ras/MEK signal transduction remains activated in imatinib-treated CML cells cultured in conditioned media. Furthermore, a SCID-hu *in vivo* model could be implemented to validate the involvement of these pathways in modulating imatinib response in CML cells.

In conclusion, while imatinib and other BCR-ABL inhibitors are highly effective in treating CML patients and addressing mechanisms of acquired resistance, the high rates of patient relapse and the persistence of MRD CML challenges us to continuously expand our understanding of CML pathogenesis and seek innovative solutions to combat this disease. Data presented here demonstrate that targeted therapy using a single agent does not eradicate drug resistance associated with the bone marrow microenvironment. However, the use of combinational therapy that targets BCR-ABL as well as stroma-derived soluble factors promises to increase the efficacy of BCR-ABL inhibitors by sensitizing cells to their apoptotic effects. This accomplishment would translate into increased remission rates among CML patients and further revolutionize oncology therapeutics. Additionally, these data demonstrate the importance of bone marrow stroma models that consider the tumor microenvironment in

identifying novel targets that enhance the efficiency of existing targeted drug therapies.

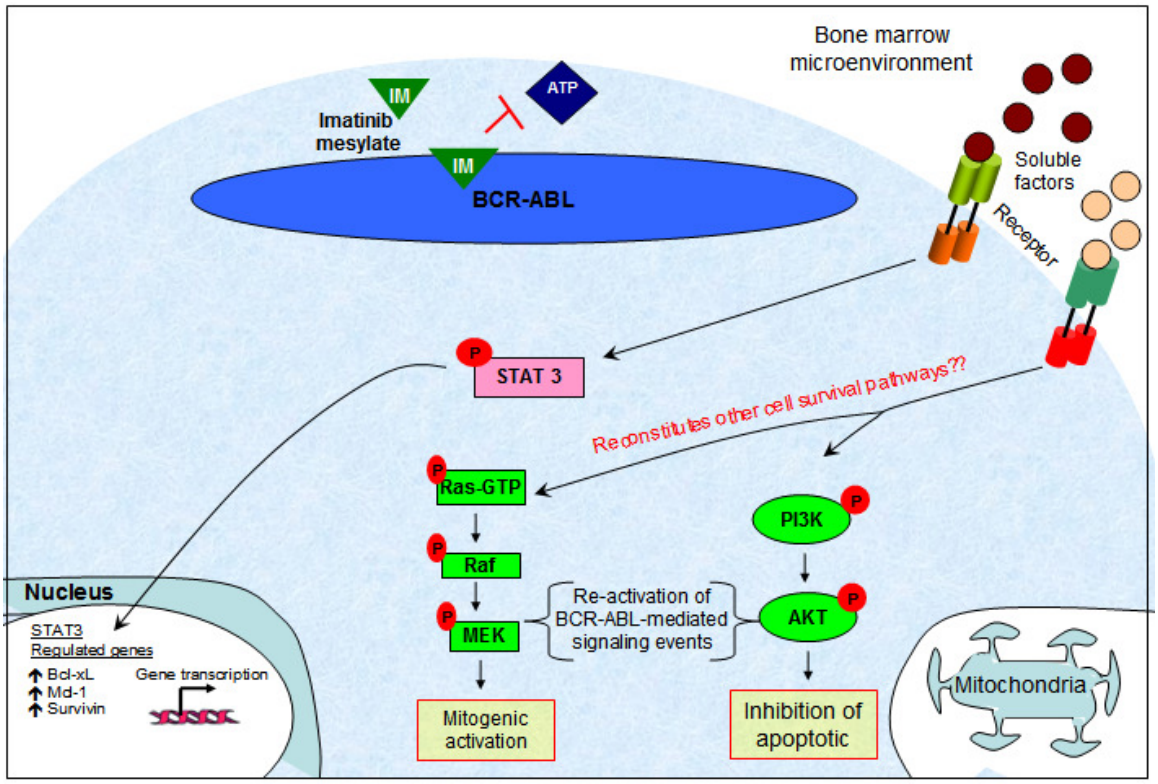


**Figure 30: Proposed Mechanisms of Resistance to BCR-ABL Inhibitors in Chronic Myeloid Leukemia Cells**. Data from our bone marrow stroma model show increased and sustained STAT3 activation in CML cells. Bone marrow stroma cells secrete numerous soluble factors that are capable of STAT3 activation. Additionally, soluble factors within the bone marrow microenvironment may be capable of reconstituting BCR-ABL downstream signaling events, such as the Ras/MEK and PI3K/AKT cell survival signal transduction pathways.

## LITERATURE CITED


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## PRESENTATION OF STUDIES

Publications resulting from these studies include one first-authored paper in press, and one manuscript in press. These results were also presented as an oral presentation at the American Association of Cancer Research 2008 Annual Meeting. These presentations and papers are listed below.

Bewry NN, Rajesh R. Nair, Michael F. Emmons, David Boulware, Lori A. Hazlehurst. 2008. STAT3 contributes to resistance towards BCR-ABL inhibitors in a bone marrow microenvironment model of drug resistance *(Manuscript in press)*

Bewry NN, Rajesh R. Nair, Michael F. Emmons, Lori A. Hazlehurst. 2008. Bone marrow stromal cells activate STAT3 and confer resistance to BCR-ABL inhibitors in K562 CML cells. American Association of Cancer Research Annual Meeting; Minisymposia: Molecular Mechanisms of Drug Resistance, San Diego, CA

Bewry NN, Rajesh R. Nair, Michael F. Emmons, Lori A. Hazlehurst. 2007. Role of the Bone Marrow Microenvironment and STAT3 Activation in the Imatinib-

resistant Phenotype of K562 Chronic Myeloid Leukemia (CML) Cells. Seminar, Department of Molecular Medicine, USF, Tampa, FL

Bewry NN, Rajesh R. Nair, Michael F. Emmons, Lori A. Hazlehurst. 2007. Role of the Bone Marrow Microenvironment in Mediating Drug Resistance in Chronic Myeloid Leukemia (CML). Department of Molecular Medicine Scientific Retreat, Brookville, FL

Bewry NN, Rajesh R. Nair, Michael F. Emmons, Lori A. Hazlehurst. 2007. Imatinib Resistance in Chronic Myeloid Leukemia (CML). McKnight Doctoral Fellowship Mid-Year Research & Writing Conference, Tampa, FL

Bewry NN, Emmons M., Dalton W., Hazlehurst L. 2007. Contribution of the bone marrow microenvironment in mediating resistance to Bcr-Abl inhibitors in Chronic Myeloid Leukemia (CML). Moffitt Scientific Retreat, Tampa, FL.

## ABOUT THE AUTHOR

Nadine N. Bewry completed her undergraduate studies at Tennessee State University with a Bachelors of Science degree in Biology. In 2004 she entered the University of South Florida as a doctorate student in the Medical Sciences Program at the College of Medicine. During her matriculation, Nadine was a recipient of the Diversity Student Success Fellowship and the Genshaft Family Doctoral Fellowship. Additionally, Nadine was the recipient of the American Association for Cancer Research's Brigid G. Leventhal Scholar-in-Training Award. She was also invited to give an oral presentation at AACR's 2008 Annual Meeting at the Minisymposia on the Molecular Mechanisms of Drug Resistance.

Nadine has published two manuscripts, one in the *Journal of Biological Chemistry* (2007) and the other in *Molecular Cancer Therapeutics* (2008), which is based on her doctoral research.

Nadine's two wonderful children, Yishmael and Tovliyah, are her blessing, as they keep her happy, motivated and energized.