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The effects of Gleevec upon K-562 cell differentiation and adherence properties

Scott Frederick Schimmel
University of Tennessee

To the Graduate Council:

I am submitting herewith a thesis written by Scott Frederick Schimmel entitled "The effects of Gleevec upon K-562 cell differentiation and adherence properties." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Comparative and Experimental Medicine.

Albert T. Ichiki, Major Professor

We have read this thesis and recommend its acceptance:

Carmen B. Lozzio, Robert L. Donnell

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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The Effects of Gleevec Upon K-562 Cell Differentiation and Adherence Properties

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Scott Frederick Schimmel
May 2002

DEDICATION

To my Family,
for their inspiration
and encouragement.

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I sincerely appreciate the support, knowledge and patience of my mentor, Dr. Albert Ichiki. His efforts have greatly advanced my scientific education and I welcome his continuing influence.

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ABSTRACT

Chronic myelogenous leukemia (CML) is a hematological stem cell malignancy characterized by excessive myeloid proliferation. K-562 cells, developed from a CML patient in blast crisis, provide an excellent cell model with which to study multi-lineage leukemia cell differentiation and pathology. Virtually all patients with CML express the BCR-ABL protein. This chimeric protein is the result of a reciprocal (9;22) chromosomal translocation. The fusion gene generates a constitutively active tyrosine kinase, which plays a fundamental role in the regulation of cell proliferation, growth, and function. Gleevec, a small molecule that selectively inhibits the BCR-ABL tyrosine kinase by competitively binding to the ATP binding site, was recently approved by the FDA. This thesis provides evidence that Gleevec induces *de novo* morphological changes and adherence characteristics in K-562 cells. We characterize some of the features of initial treatment with Gleevec and the progression towards resistance.

K-562 cells co-cultured with Gleevec showed morphologic changes along the dendritic pathway. These induced changes were consistent for three K-562 sublines as examined by sequential temporal observation. Gleevec-treated cells displayed altered adhesive characteristics, which included homotypic aggregation and adherence to plastic. Brighter fluorescence at points of cell-cell contact suggests that f-actin was involved at these adhesive junctions. Additionally, induced morphological alterations,

including dendritic hairs, pseudopodia and filopodia, were all directly associated with f-actin staining.

Resistance to Gleevec has been a clinical occurrence, especially with CML patients in blast crisis. We demonstrated that K-562 cells are capable of becoming resistant to concentrations of Gleevec as high as 3.0μM. Resistant cell lines were shown to have increased BCR-ABL mRNA and BCR-ABL protein expression as compared to the controls. We also determined that the over-amplification of the BCR-ABL gene in K-562 cells makes it difficult to conclude quantifiable results from FISH analysis.

This study provides further insight regarding the complexities of altered adhesion in BCR-ABL positive cells. We show that K-562 cells and Gleevec can be used as a model system for the *in vitro* analysis of differentiation-inducible modification of blastic leukemia cells.

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Chapter 1

Review of the Literature

Introduction

Chronic myelogenous leukemia (CML) is a malignant clonal disease, arising from a single pluripotent hematopoietic stem cell. The annual incidence of this myeloproliferative disease is approximately 1 per 100,000 population. The disease is essentially one of adult life and is uncommon in children. This tri-phasic disease begins with a prolonged chronic phase, followed by an accelerated phase of variable length, and then develops into a blast phase. The chronic phase is characterized by the marked expansion of the granulocytic series, but with differentiation relatively unaffected. The transformation into accelerated and blast phases are marked by a breakdown in the differentiation process. Due to chromosomal changes, individuals in late stages of CML may have more than one leukemic cell clone. Accordingly, malignant cells may include stem cells as well as progenitors, precursors, and mature cells of the granulocytic, monocytic, erythrocytic, lymphocytic, and/or megakaryocytic series[1,2].

CML was the first neoplastic process to be linked to a consistent acquired genetic abnormality. The crucial genetic event in CML is the generation of a t(9;22)(q34;q11) reciprocal chromosomal translocation. This translocation creates a

fusion gene, BCR-ABL on the 22q-, the Philadelphia (Ph^1) chromosome, and the reciprocal fusion gene ABL-BCR on the derivative 9q+. While the latter gene does not appear to have any functional role in the disease, the BCR-ABL protein is leukemogenic because its ABL-derived tyrosine kinase is constitutively active. Since the BCR-ABL protein is a novel intracellular protein with increased tyrosine kinase activity, an inhibitor of the BCR-ABL protein tyrosine kinase has been sought as a potentially useful agent to block its activity. Compounds which inhibit phosphotyrosine kinase activity include quercetin,[3] and the isoflavone genestein,[4] and other naturally occurring compounds such as erbstatin[5] and hebmycin A.[6] While these compounds displayed some ability to revert cells transformed by tyrosine kinase oncogenes, they demonstrated little specificity among tyrosine kinases. To improve target specificity, synthetic compounds (tryphostins) were modeled after the naturally occurring kinase inhibitors.[7] AG1112, one of the early tryphostins that showed specificity and was capable of inhibiting the ABL protein tyrosine kinase, was reported to induce the differentiation and death of K-562 cells.[8,9] As the crystalline structures of various protein kinases were determined, it was then possible to rationally design compounds based on the structure of the ATP binding site or the active site of the enzyme. One tyrosine kinase inhibitor that has shown increased potency and selectivity for the ABL protein is Gleevec (CGP57148B, STI571, Glivec), a 2-phenylaminopyrimidine derivative. [10,11]

This thesis provides evidence that Gleevec has profound effects on the K-562 cell line, including altering the expression of adhesion markers, mediating homotypic aggregation and inducing differentiation towards a "dendritic" pathway. Further

evidence shows that K-562 sublines can be useful models for understanding the mechanisms of resistance to Gleevec. Additional results demonstrate that cell surface antigen expression of K-562 cells is influenced by treatment with Gleevec. Also shown are the effects of Gleevec on cell proliferation, karyotype, BCR-ABL mRNA, and the BCR-ABL tyrosine kinase protein. A discussion follows regarding the observations of induced cellular adhesion and homotypic aggregation by Gleevec and explores the implication of morphological change with respect to cellular differentiation. Subsequent conclusions illustrate that the K-562 cell line can be a model system for understanding the basis for Gleevec resistance.

K-562 Cells

Established in 1970 from the pleural effusion of a patient with CML in blastic crisis, the K-562 cell line provides a useful pluripotential hematopoietic leukemia cell line for the study of leukemia cell pathology and differentiation.[12-15] Significantly, the K-562 cell line was the first successful long-term culture of malignant cells from a patient with CML that retained the Philadelphia chromosome (Ph^1). K-562 cells grow in suspension as nonaggregated, highly undifferentiated cells. The nuclei are round or slightly indented, with each nucleolus containing several nucleoli with irregularly granular chromatin.[16] Nowell and Hungerford first identified the association between a truncated chromosome 22 and CML, in Philadelphia, 1960.[17] The Philadelphia chromosome (Ph^1), as noted by Rowley, indicates a reciprocal translocation between the long arms of chromosome 9 and 22 [t(9:22)(q34:q11)] **(Figure i)**.[18] The Ph^1

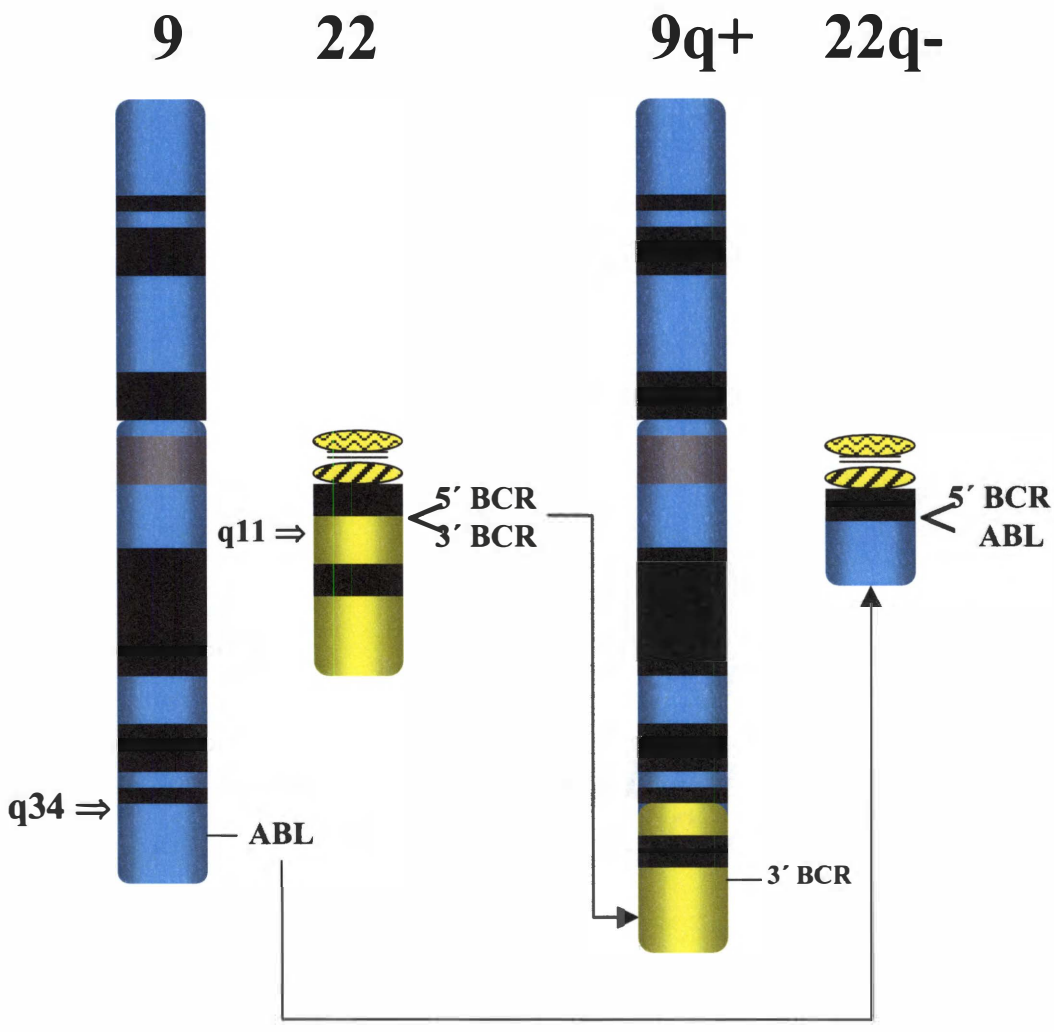


Figure i. CML chromosomal rearrangements. Translocation of ABL and BCR genes. Note that the 5' portion of the BCR remains on the 22q- whereas the 3' portion of BCR moves to the 9q+. Figure adapted from Hughes, T. and Goldman, J. *Hematology:Basic Principles and Practice*, 2nd Edition, 1995.

chromosome can be detected in >90% of CML patients[19] and is variably present in all K-562 cell clones.[13]. This recombination generates the biologically active BCR-ABL fusion gene, where the 3′ segment of the c-ABL gene from chromosome 9q34 is translocated to the 5′ part of the BCR gene located on chromosome 22q11.[13-16,20] The breakpoints within the ABL gene at 9q34 can occur at various points over a large area at its 5′ end. These breakpoints occur either upstream of the first alternative exon Ib, downstream of the second alternative exon Ia, or between the two.[21] Regardless of the exact location, the BCR sequence fuses to the ABL exon a2 **(Figure ii)**. In contrast to ABL, the breakpoints within BCR are specific to one of three locations, referred to as breakpoint cluster regions. In most CML patients, the break occurs within a 5.8 kb region spanning BCR exons 12-16 (originally referred to as exons b1-b5), defined as the major breakpoint region (M-BCR). The break at this point creates fusion transcripts with either b2a2 or b3b2 junctions, due to alternative splicing, which is translated into a 210 kd chimeric protein ($P210^{BCR-ABL}$). Breakpoints further upstream result in the e1a2 fusion. The e1a2 mRNA is translated into a190 kd protein ($P190^{BCR-ABL}$). A third breakpoint was identified downstream of exon 19, giving rise to a 230 kd protein ($P230^{BCR-ABL}$), associated with Ph-positive chronic neutrophilic leukemia.

The transforming capability of the hybrid BCR-ABL gene is profound. It is widely accepted that the acquisition of the Ph^1 translocation and expression of the BCR-ABL tyrosine kinase is the initiating and rate-limiting event in CML.[22] In transgenic mice, it was found that transfection with either $P210^{BCR-ABL}$ [23] or $P190^{BCR-ABL}$ [24] can induce CML. Laboratory studies demonstrate that the BCR-ABL protein can induce the survival of hematopoietic progenitors in the absence of cytokines and growth factors, [25]

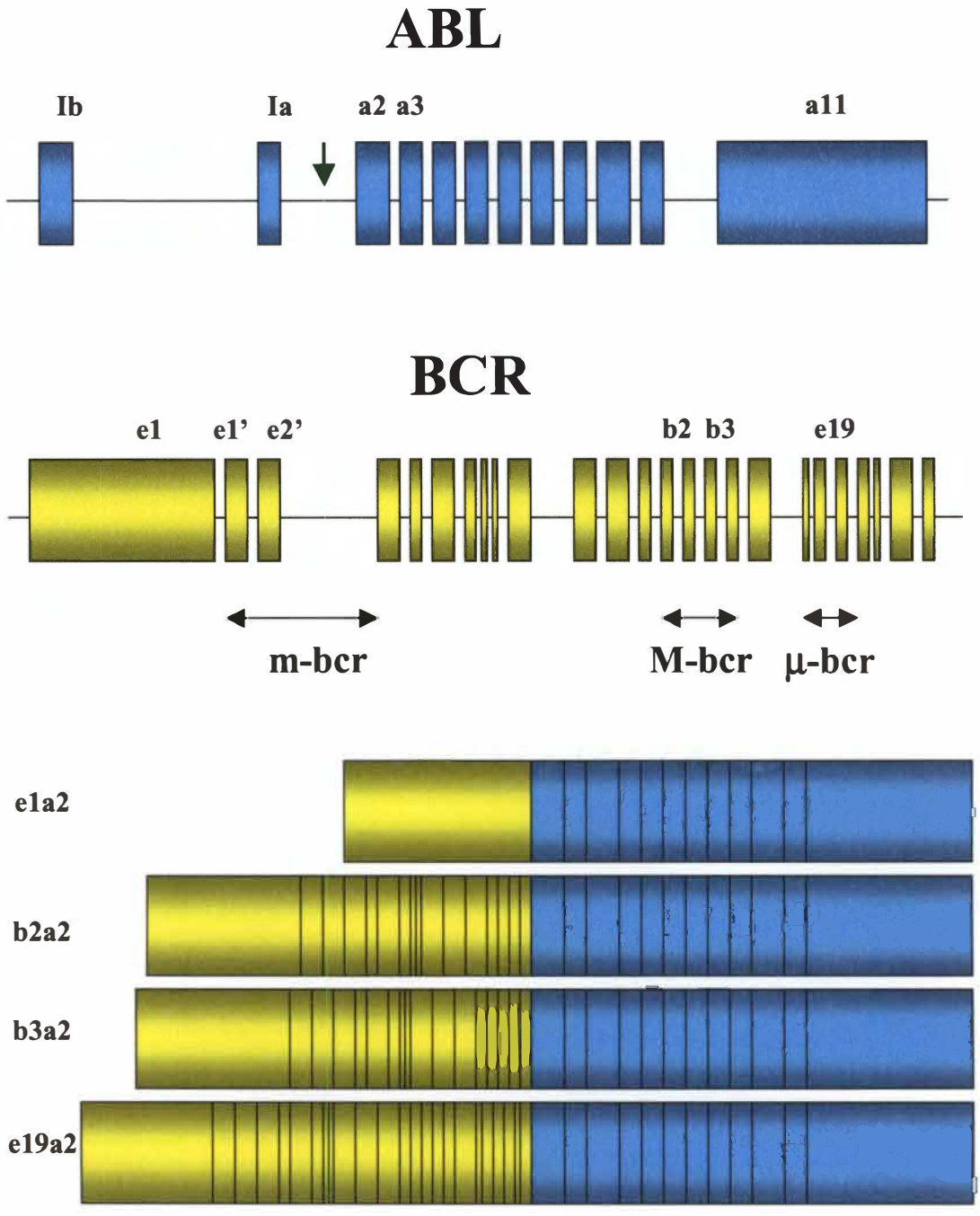


Figure ii. Breakpoint regions in ABL and BCR genes. The ABL, BCR minor (m-bcr), BCR major (M-bcr), and μ-bcr breakpoint regions are shown, as well as the structure of the chimeric mRNAs derived from the various breakpoints. Figure adapted from Deininger (2000).

possibly by providing protection from apoptotic pathways.[26] Based on the McGahon *et al.* observation that the BCR portion of the chimeric protein has some variability while the ABL part is almost always constant, it can be deduced that ABL is likely to be the transforming principle; whereas the BCR sequence may dictate the phenotype of the disease.[27]

While K-562 cells remain highly undifferentiated, they do express phenotypes typically associated with cells of several hematopoietic lineages. K-562 cells are capable of spontaneous differentiation into recognizable progenitors of the erythrocytic, [28] monocytic[13], and granulocytic series.[13] K-562 cells also can be further manipulated to display the characteristics of erythrocytic, monocytic, granulocytic, and megakaryocytic differentiation.[29,30] Depending on the treatment involved, K-562 cells express antigens associated with these multiple lineages. Among prominent inducible markers enhanced by differentiation is the expression of IL-6[31,32], IL-1β and CD41,[33] CD61,[34] and MHC class I molecules.[35] For this reason, K-562 cells serve as an important tool for studying hematopoiesis.

Gleevec

The biological translation of the many protein interactions confers the altered phenotype of CML cells. CML is marked by a constitutively active BCR-ABL tyrosine kinase. The development of Gleevec represents a novel approach for molecular targeting for therapy of CML. Gleevec was developed by scientists at Novartis as a specific platelet-derived growth factor receptor α and β (PDGFR) inhibitor, but using

structure activity relations and high throughput screenings, a series of compounds were synthesized and optimized against a variety of targets **(Figure iii)**.[11,36] Its specificity is remarkable. It inhibits all ABL kinases while numerous other tyrosine and serine/threonine protein kinases, except PDGFR and the c-Kit tyrosine kinases, remain unaffected.[10,37] Its specificity is attributed Gleevec's mechanism of action. Gleevec blocks the binding of ATP to the BCR-ABL tyrosine kinase, thus inhibiting the activity of the kinase **(Figure iv)**.[11,38] Schindler *et al.* reported that ABL, like many other kinases, has an "activation loop" that has to have a phosphate group added before the enzyme can add phoshate groups to other proteins.[39] This alters the shape of the enzyme so that it adopts an "open" conformation enabling the kinase to bind ATP and its target protein.[39] They further suggested that Gleevec binds to the inactive conformation of ABL, presumably preventing it from acquiring the activating phosphate group.[39]

Prior to the development of Gleevec, patients in blast phase were often refractory to treatment while those in chronic phase did show better prognosis. Non-transplant treatment options for CML included interferon-alpha, a glycoprotein that has been shown to prolong survival, achieving major cytogenetic responses, particularly for patients in chronic phase.[40-42] In an effort to improve the treatment results, trials have focussed on combining IFN-α with cytotoxic drugs such as cytarabine. Other non-transplant treatment options include chemotherapy with hydroxyurea, busulphan with all-trans retinoic acid, and homoharring-tonine. Allogenic stem cell translation using blood- or marrow-derived stem cells from an HLA-identical sibling donor performed during chronic phase has proven successful.[43]

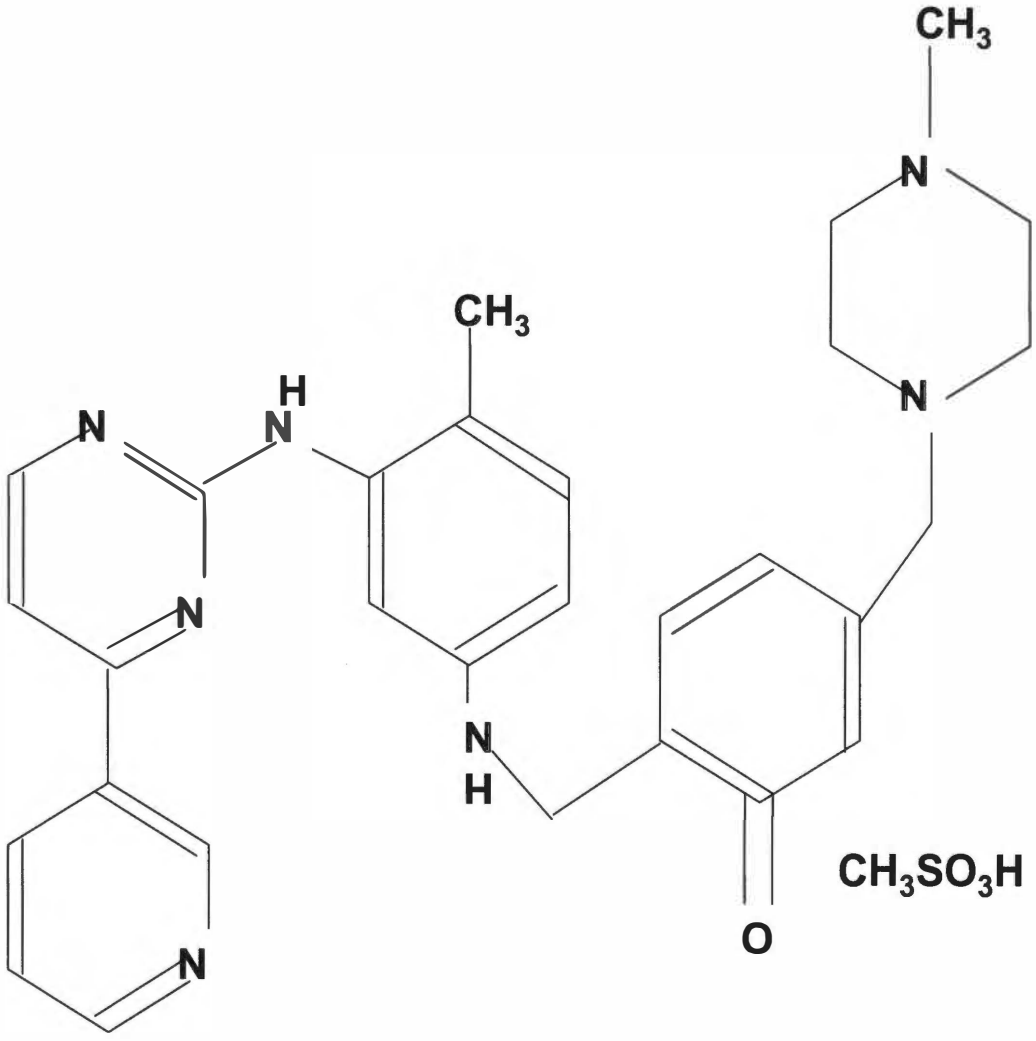


Figure iii. Gleevec structural formula. Gleevec is imatinib mesylate, chemical formula 4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate. Its molecular formula is $C_{29}H_{31}N_7O \cdot CH_4SO_3$ and its relative molecular mass is 589.7. Figure from Novartis.

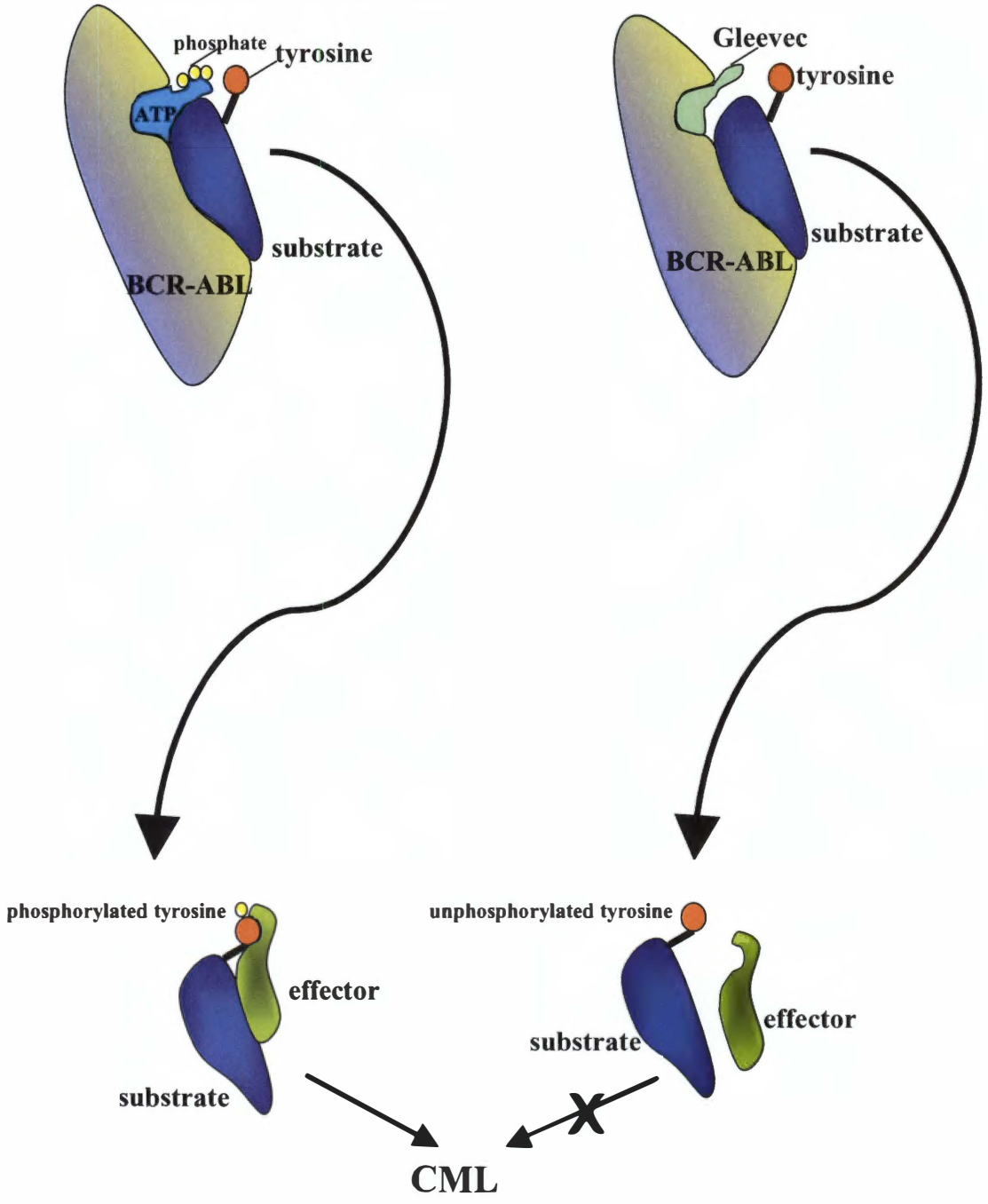


Figure iv. Mechanism of action of Gleevec. Gleevec binds to the ATP binding site of the BCR-ABL tyrosine kinase thus blocking its ability to bind ATP. By preventing the protein from binding ATP, it is unable to transfer a phosphate to tyrosine residues on various substrates. Thus the kinase activity is thwarted. Figure adapted from Mauro Curr Opin Oncol. 2001;13:3-7.

Pre-clinical experiments with Gleevec showed that the proliferation of cells expressing BCR-ABL is suppressed in vitro and in vivo, whereas cell lines that do not express BCR-ABL are not sensitive.[10] In 1997, Deininger *et al.* observed that a dose of 1.0 μM of Gleevec was sufficient to inhibit colony formation and induce apoptosis in cell lines derived from patients in chronic phase.[44] Gleevec has been shown to have selective effects on CML progenitors, compared with normal progenitors, in long-term marrow culture studies.[45] In 1999, le Coutre *et al.* determined that with dosing providing continuos exposure, Gleevec was capable of eradicating BCR-ABL-positive tumors in nude mice.[46] This new drug has also been shown to down-regulate Bcl-x_L, XIAP, and cIAP1 levels as well as inhibiting Akt kinase and $NF_\kappa B$ activities.[47]

As previously stated, blast crisis is highly refractory to treatment. The rate of response to conventional induction chemotherapy in patients with myeloid blast crisis is approximately 20 percent, and the rate of total remission is less than 10 percent.[48] After allogenic stem-cell transplantation during blast crisis, the five year survival rate is only 6 percent.[49,50] Gleevec, however, has shown considerable efficacy. The overall response rate in myeloid blast crisis of CML was 55 percent, and the rate of remission was 11 percent.[48] In contrast, more than 98 percent of CML patients in chronic phase, refractory to IFN-α-treatment, obtained complete hematological responses during treatment with Gleevec; about 54 percent achieved cytogenetic responses.[51]

Homotypic Aggregation

Intercellular adhesive events are involved in a wide range of biological processes, including pattern formation and morphogenesis during development, immune responses, leukocyte recirculation, wound repair, tumor growth and metastasis.[52,53] It has been suggested that BCR-ABL affects cytoskeletal function. Previous observations have shown that BCR-ABL localizes in the cytoskeleton by binding to actin through a COOH terminus actin-binding domain.[54,55] Several prominent tyrosine kinase substrates for BCR-ABL are cytoskeletal proteins, including Paxillin, Vinculin, Tensin, focal adhesion kinase (p125 FAK), p120 CBL, cCRK and CRK-L.[56-60] Since BCR-ABL mutants that fail to localize BCR-ABL in the cytoplasm have reduced transforming potential, it is thought that this particular location is significant.[61] CML cells have been shown to have defective integrin-dependant adhesion and signaling function.[62-64] Further observations have shown that while adherence to stromal layers[65] and fibronectin is diminished, adhesion to laminin and collagen type IV is increased in CML cells.[66] These adherence complexities may help to explain the early release of myeloid cells from the bone marrow and accumulation of myeloid cells at all stages of differentiation.[56,60,66]

Homotypic aggregation is an adhesive phenomenon and is related to intercellular activation. Several adhesion molecules have been characterized and shown to play critical roles in the maintenance of tissue architecture and cell migration through specific recognition of extracellular matrix components. Axelsson *et al.* observed that K-562 cells were strongly stained by CD43 and showed evidence for aggregation. CD43 triggered cell adhesion by a pathway which was inhibited by antibodies to CD18

(β2 Integrin), but not CD11a.[67] A 20-kDa protein, known as JKT.M1, has been shown to induce adhesion and appears to be independent of CD43, CD44, and VLA4 (CD29/CD49d).[68] Additionally, phorbol esters, known activators of protein kinase C, have been shown to increase cell-cell adherance.[69]

Many studies have transfected K-562 cells with genes for various adhesion molecules in order to further elucidate their role in cell-cell interactions. Hickstein *et al.* showed that K-562 cells expressing CD11b/CD18 complex stimulated with the phorbol ester PMA for 24 to 48 hours homotypically aggregated.[70] LFA-1 (CD11a/CD18)-mediated cell adhesion to ICAM-1 is predominantly regulated by receptor clustering, although the actual affinity for ICAM-1 remains unchanged.[71] Functional analysis of K-562 transfectants has demonstrated that the β1 integrins CD49b/CD29, CD49d/CD29, and CD49e/CD29 are constitutively expressed and that their adhesive functions can be induced by treatment with cellular antagonists or activating monoclonal antibodies.[72-74] K-562 cells can produce and release a soluble factor that inhibits neutrophil adhesive function, including the neutrophil binding of iC3b-coated particles,[75] which is a CD11b/CD18-dependant process.[76] This suggests a specific effect of the K-562-derived soluble factor on the activity of the β2 integrins.[77] Cho *et al.* observed that CD98 is a central component within a multimolecular complex, containing β1 integrins, CD147 and their associated transporter molecules, that can regulate adhesion, differentiation and antigen presentation.[78]

Differentiation

Hematopoiesis is a continuous progression that generally maintains a steady state in which the production of mature blood cells equals their loss. As with other leukemias, CML is characterized by persistent proliferation of hematopoietic progenitor cells, together with maturation arrest at various stages of development. The finding that some cell lines can be induced by various compounds to terminally differentiate has suggested an alternative approach to the therapy of certain leukemias. Thus, discernment of the pathways of differentiation from pluripotent stem cell to mature cells is crucial to the understanding of blast phase CML. K-562 cells treated with inducers of differentiation can develop properties associated with early erythroid, megakaryocytic, monocytic lineages, and further differentiated myelocytic subsets.[13-16]

The ability of the cells to produce embryonic and fetal hemoglobin shows the aptitude to differentiate along the erythroid lineage.[28,79,80] Further studies showed that K-562 had the ability to produce fetal[80,81] and embryonic[79,80] hemoglobin upon stimulation with sodium butyrate and hemin, respectively.

Vainchenker *et al.* first reported the presence of K-562 megakaryoblastoid properties.[29] They observed the presence of promegakaryoblastic peroxidase activity in 1-2% of uninduced cells, and in 20% of cells induced to differentiate with hemin. Subsequently, it was found that phorbol esters induce the upregulation of glycoprotein IIIa (GPIIIa), part of the megakaryoblastic marker complex IIb/IIIa (β_3 integrin, CD61),[34,82] and DNA ploidy from 4-16N.[83] Nanomolar concentrations of phorbol esters

have also been found to upregulate the expression of the thromboxane A_2 receptors and the megakaryocytic-specific antibody FMC-27.[84]

Original indications that K-562 cells can differentiate into the monocyte series came from studies by Lozzio *et al.* demonstrating that 80-90% of cultured K-562 cells react with α-naphthyl acetate esterase, which is typical of monocytic cells.[13] However, the absence of myeloperoxidase in some K-562 cell lines calls into question their role as precursors of the granulocytic-monocytic series.[85] K-562 cells do react with antibodies directed towards monocyte-granulocytic precursors, with dependent differences correlating with induction by phorbol esters, sodium butyrate, or hemin.[85] O'Keefe and Ashman showed that 100% of K-562 cells have receptors for peanut agglutinin, a monocytic-specific receptor.[86] This observation is supported by studies showing that phorbol ester can cause K-562 cells to partially differentiate into myelomonocytic cells,[87] but is refuted by other findings that failed to observe phorbol ester-induced monocytic differentiation.[1,2,88,89] However, there is evidence that the expression of IL-1α and IL-1β[90]and IL-6,[31] both associated with monocytes, are induced by PMA and may be involved in the ability of PMA-treated cells to replace activated monocytes in stimulating resting T cells.[91]

Transfection of K-562 cells with the genomic c-*fes* sequence induces functional maturation of these cells to the granulocytic type,[92,93] suggesting that $p93^{c\text{-}fes}$ may serve as an important effector for initiating myelopoiesis.[94] These transfected cells have been shown to exhibit superoxide formation (NBT reduction), phagocytosis, and increased expression of the myeloid surface antigens CD13 and CD33.[95] Additionally, Fang *et al.* showed that there was a reduction of $p210^{bcr\text{-}abl}$ upon myeloid differentiation, yet the

level of other phosphotyrosine-containing proteins remained unchanged in p93$^{c\text{-}fes}$ transfected K-562 cells.

Differentiation can often be studied by observation of parameters, such as changes in morphological and adherence properties. Inducers, such as phorbol esters (phorbol 12myristate 13-acetate [PMA]), cause some of the cells to adhere to the flask with cytoplasmic extrusions, phagocytotic activity and pseudopodial whip-like extensions.[96] BCR-ABL tyrosine kinase protein localizes in the cytoskeleton by binding to actin through a COOH terminus actin-binding domain and many of the prominent tyrosine kinase substrates for BCR-ABL are cytoskeletal proteins.[55] It is thought that the cytoplasmic location for BCR-ABL is important for transformation, since mutants that fail to localize BCR-ABL have reduced transforming activity.[61] Further studies suggest that BCR-ABL affects normal function of the cytoskeleton. Gordon *et al.* have shown that CML cells have a diminished capacity to adhere to stromal layers[65].

Chapter 2

Methods/Materials

K-562 Cell Line Culture

The K-562 cell line was derived from the pleural effusion of a CML patient in blast crisis.[16] Cells from sublines F(1), F-1-1, and C-1-1[97,98] were passaged weekly in Eagle's minimal essential medium supplemented with 15% fetal bovine serum (FBS) (GIBCO BRL, Grand Island, NY), plus nonessential amino acids with an inoculum of 1.5×10^4 cells/ml.[16] The cultures were incubated at 37°C in 5% CO_2. Cells from 10 ml cultures were pelleted, the supernatants were removed, and the pellets were suspended in RPMI 1640 medium, 10 ml, with glutamine (GIBCO BRL), 10% FBS, and 10 µl/ml Gentamicin (Sigma, St. Louis, MO) at 37°C in 5% CO_2.

Proliferation

Proliferation, as determined by DNA synthesis, was assayed by the incorporation of tritiated thymidine ([^{3}H]-thymidine) (ICN, Newport Beach, CA), 0.25 µCi in 50 µl RPMI media. K-562 cells, in 100 µl aliquots, from sublines F(1), F-1-1 and C-1-1 were co-cultured with Gleevec and 100 µl media, in triplicate at concentrations ranging from 0.1 µM to 10 µM for 4 hours. The cultures were

harvested with a cell harvester (Skatron Instruments, Inc., Sterling, VA) and radioactivity was expressed as counts per minute (cpm) in a Packard liquid scintillation analyzer (Downer's Grove, IL).

Visual counts, using a hemocytometer, were determined after 48 hours with or without treatment with Gleevec. The cell count was expressed as the number of cells/ml.

Microscopy

The non-adhering and adhering cells were examined by inverted light microscopy and/or Nomarski/phase contrast microscopy with or without laser confocal microscopy (Leica, Germany). Cells were grown in flasks at an initial concentration of 1×10^6 cells/ml. Non-adherent cells were removed and the flasks were washed three times with 3.0 ml media. The cells were cytocentrifuged and stained with Wright-Giemsa. Adherent cells were fixed with Carson-Millonig-buffered formalin (Biochemical Sciences, Swedesboro, NJ) and stained with Wright-Giemsa.

Both non-adherent and adherent cells were stained for f-actin with Alexa Fluor 488 phalloidin (Molecular Probes, Eugene OR) (1:40 dilution in phosphate buffered saline, PBS) to determine cytoskeletal involvement in morphological and adhesive changes. Propidium iodide (Molecular Probes, Eugene OR) (1:3000 dilution in PBS for adherent cells; 1:500 dilution for non-adherent cells) was used as a nuclear stain. Cells were fixed with either Carson-Millonig-buffered formalin (Biochemical Sciences) or Cytofix/Cytoperm (Pharmingen, San Diego, CA).

Gleevec

Gleevec, imatinib mesylate, or 4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl]benzamide methanesulfonate was a generous gift from Novartis (Basel, Switzerland). Its molecular formula is $C_{29}H_{31}N_7O \cdot CH_4SO_3$ and its relative molecular mass is 589.7.

Cells, 1 x 10^5/ml, were co-cultured with Gleevec at concentrations ranging from 0.1 μM to 10 μM. For each subline, cell lines resistant to Gleevec were developed by initially culturing them in Gleevec, 0.1 μM. Resistance was determined when the proliferation of the treated cell line was equivalent to control cells. These proliferative adaptations coincided with resistance to morphologic change following subsequent treatment with Gleevec, 0.1 μM. The C-1-1 subline resistant to Gleevec, 0.1 μM, was subsequently subjected to increased concentrations in a step-wise manner. Sublines resistant to Gleevec, 1.0 μM, and 3.0 μM were similarly developed.

PMA

Phorbol 12-myristate 13-acetate (PMA) is a tumor promoter that activates isoforms of the phospholipid /calcium-dependant serine-threonine kinase, protein kinase C (PKC). The downstream effects of PKC activation included events in the MEK/MAPK pathway,[99,100] providing necessary and sufficient signaling for

megakaryocytic differentiation.[101] It was determined that PMA, 0.2 nM, (Sigma, St. Louis, MO) was optimal for cell killing and morphologic change.[102]

Reverse Transcript-Polymerase Chain Reaction (RT-PCR)

Cells, 1-8 x 10^6, co-cultured with Gleevec, 0.1 μM, were harvested after 4, 24, and 48 hours of incubation and total RNA was extracted with TRIzol reagent (GIBCO BRL) according to the manufacturer's methods. cDNA was synthesized from 1 μg mRNA, using reagents from a GeneAmp RNA PCR kit with MuLV reverse transcriptase (Perkin Elmer, Foster City, CA) according to the manufacturer's procedures. PCR was then utilized to amplify the desired β-actin control target. The effects of Gleevec on BCR-ABL mRNA expression were determined by RT-PCR Amplification reagents, including RedTaq DNA polymerase (Sigma, St. Louis, MO). Primers were subjected to 28 cycles at 94°C for 45 sec, 55°C for 45 sec, and 72°C for 60 sec. β-actin primers included the 5' primer [5'-TCATGCCATCCTGCGTCT-3'] and 3' primer [5'-CCGGACTCATCGTACTCC-3']. The β-actin product was 514 bp. BCR-ABL primers included the 5' primer [5'-TTC AGA AGC TTC TCC CTG-3'], which recognized an exon sequence in the Bcr-locus, while the 3' primer [5'-CTC CAC TGG CCA CAA AAT-3'] recognized an exon sequence of the Abl-locus yielding a PCR product of 327 bp.[103] The PCR products were electrophoresed in an agarose gel containing ethidium-bromide for the visualization of DNA bands under UV light.

Further experiments that assessed changes in BCR-ABL mRNA included cells co-cultured in Gleevec concentrations ranging from 0.1 μM to 10.0 μM, and Gleevec-resistant cells including sublines resistant to 0.1 μM, 0.3 μM and 3.0μM.

Flow Cytometry

Control K-562 cells or those treated with Gleevec, 0.1 μM, were immunophenotyped with a panel of the following conjugated monoclonal antibodies: FITC-conjugated anti-CD45/PE-conjugated anti-14, Simultest Control γ_1/ γ_{2a}, and PE-conjugated anti-CD 11b (Becton Dickinson, Mountain View, CA); FITC-conjugated anti-HLA-DR, DP, DQ, FITC-conjugated anti-CD61 and PE-conjugated anti-CD33 (Pharmingen, San Diego, CA); FITC-conjugated anti-HLA-COM (Chemicom, Temecula, CA), and PE-conjugated anti-HLA-ABC (Biodesign, Kennebuck, ME). Cells, 0.1ml, were added to the antibody preparations, incubated for 15 minutes, washed with PBS, fixed with 1.0% formalin, and analyzed by flow cytometry, using a FACScan (Becton Dickinson) with CellQuest v1.2 software, at the Flow Cytometry Laboratory, Dr. J. Fuhr, Director. Statistical analyses were performed using SigmaStat software v2.03.

Western Blot

The level of BCR-ABL protein was demonstrated by Western blot. The K-562 cells were treated with Gleevec, 0.1 μM, for 2, 4, 6, 24 and 48 hours. Protein was extracted using NE-PER Cell Lysing Protein Extraction reagents (Pierce Chemical, Rockford, IL). The lysates, 30 μg protein, were electrophoresed on a Tris-Glycine polyacrylamide gel with a 4% to 20% gradient (Invitrogen, Carlsbad, CA). The gel was then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and blocked with 5% milk in Tris-buffered saline with $Tween_{20}$ ($TBST_{20}$) (Ameresco, Solo, OH). The membrane was washed three times with $TBST_{20}$ following incubation with the mouse monoclonal antibody directed against c-ABL (ab-3) (Calbiochem, SanDiego, CA) (1:100 dilution in BLOTTO). The membrane was washed three times with $TBST_{20}$ and then incubated with goat anti-mouse horse radish peroxidase (HRP)-conjugated antibody (Santa Cruz, Carlsbad, CA) (1:10,000 dilution in BLOTTO). Binding of anti-c-ABL (ab-3) was detected by the SuperSignal West Pico Chemiluminescent substrate for HRP (Pierce Chemical, Rockford, IL), according to the manufacturer's procedure and developed on Hyperfilm ECL (Amersham Pharmacia Biotech, UK).

Karyotype and FISH Analysis

Control K-562 cells, K-562 cells, sublines F-1-1 and C-1-1, resistant to Gleevec, 0.1 μM, and K-562 cells, subline C-1-1, resistant to Gleevec, 3.0 μM, were prepared with Colcemid Solution (GIBCO BRL). Light microscopy was used in order to view

metaphase spreads and develop a karyotype. Fluorescence *in situ* hybridization (FISH) was utilized using loci-specific probes (Vysis, Downers Grove, IL), and analyzed with Cytovision v4.1 software (Applied Imaging, Santa Clara, CA) for the same cell lines. The hybridized metaphases and interphases were captured under the optimal excitation frequency, revealing the BCR-ABL fusion. The ABL probe and the BCR probe are approximately 650 kb and 300 kb, respectively. In a cell possessing the typical CML translocation, t(9;22), the probe produces one large red signal (native ABL) and one green signal (native BCR) and one fused yellow signal (5′ BCR/5′ ABL).

Chapter 3

Results

Proliferation

The proliferative [^{3}H]-thymidine uptake assay was performed after 1, 2, 3 and 4 days of incubation over a range of Gleevec concentrations from 0.1 μM to 10.0 μM. The proliferation of the cells co-cultured with Gleevec, 0.1 μM, was significantly decreased compared to the control cells **(Figure 1)**. Cells co-cultured with Gleevec, 1.0 μM and 10 μM, did not proliferate. These results coincided with concurrent visual counts for all three sublines **(Table 1, Figure 2)**. Hence, K-562 cells were co-cultured with Gleevec, 0.1 μM, for the majority of the studies. The most effective concentration (0.1 μM) was then studied to determine the effective duration of Gleevec exposure. The maximal effect was determined to be at 2 days for all three sublines **(Figure 3)**. [^{3}H]-thymidine uptake assays and visual count analyses corroborated this finding.

For all three sublines, cell lines resistant to Gleevec were developed by initially exposing them to 0.1 μM. Resistance was determined when the proliferation profile of the treated cell line met that of the profile of the control cell line. Proliferation results showed that the F(1) subline reached resistance after 19 weeks, F-1-1 after 16 weeks and the C-1-1 after 15 weeks, coinciding with resistance to morphological changes.

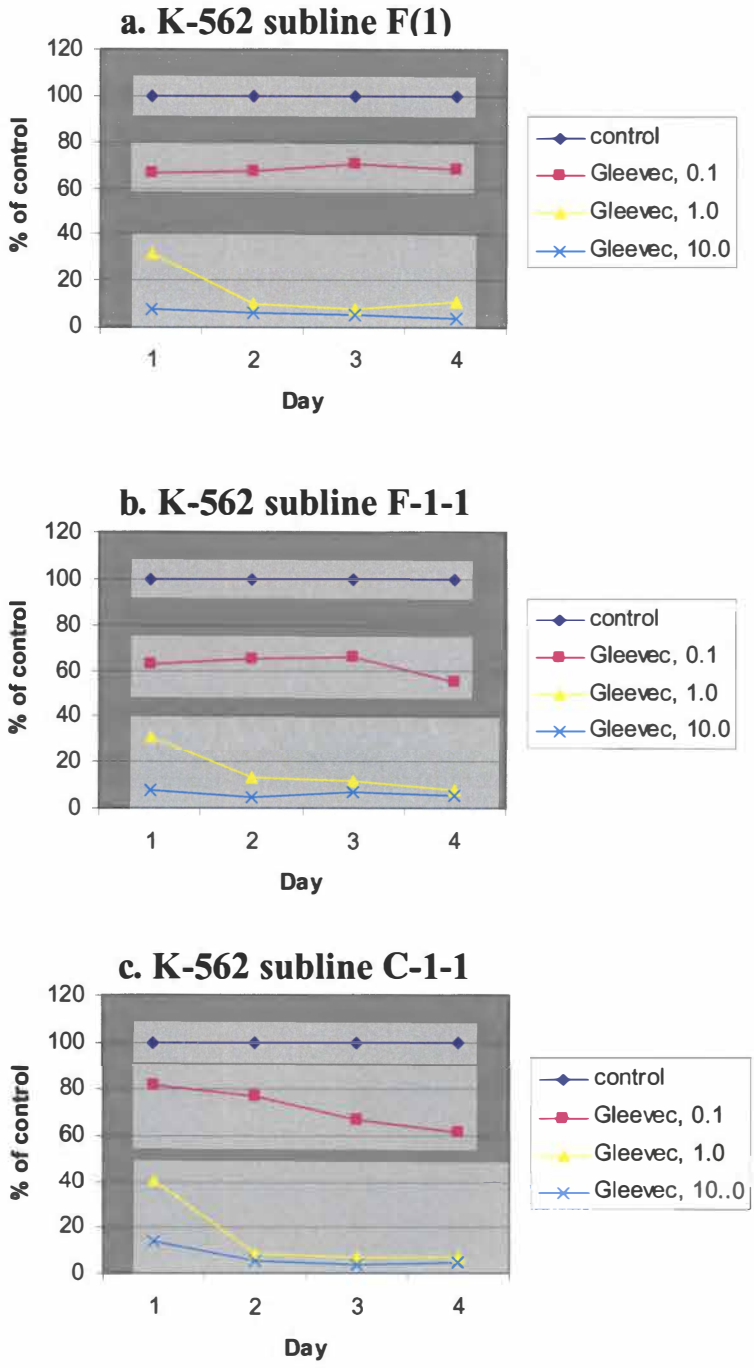


Figure 1. Effect of Gleevec on K-562 proliferation. K-562 cells, $1x10^4$, from sublines **a)** F(1), **b)** F-1-1 and **c)** C-1-1were cocultured with Gleevec in triplicate at concentrations of 0.1 µM, 1.0 µM and 10 µM. The proliferation of the cells co-cultured with 0.1 µM Gleevec were significantly decreased compared to the control cells. Cells co-cultured with Gleevec, 1.0 µM and 10 µM, did not proliferate.The proliferative [^{3}H]-thymidine uptake assay was performed after 1, 2, 3 and 4 days of incubation.

Table 1. Cell counts/ml (10^{-4})

Gleevec	control	0.1 μM	0.3 μM	0.5 μM	1.0 μM	3.0 μM	10.0 μM
F(1)	19.2	9.2	2.5	2.1	n.d.	0.3	n.d.
F1-1	28.5	15.1	9.3	3.7	2.5	0.6	n.d.
C1-1	27.6	17.3	6.2	2.5	0.6	1.2	n.d.

K-562 cells, $1x10^5$/ml, were incubated in triplicate in the presence of various concentrations of Gleevec ranging from 0.1 μM to 10 μM. Counts, using a hemocytometer were taken after 48 hours. *n.d.(non-detectable)

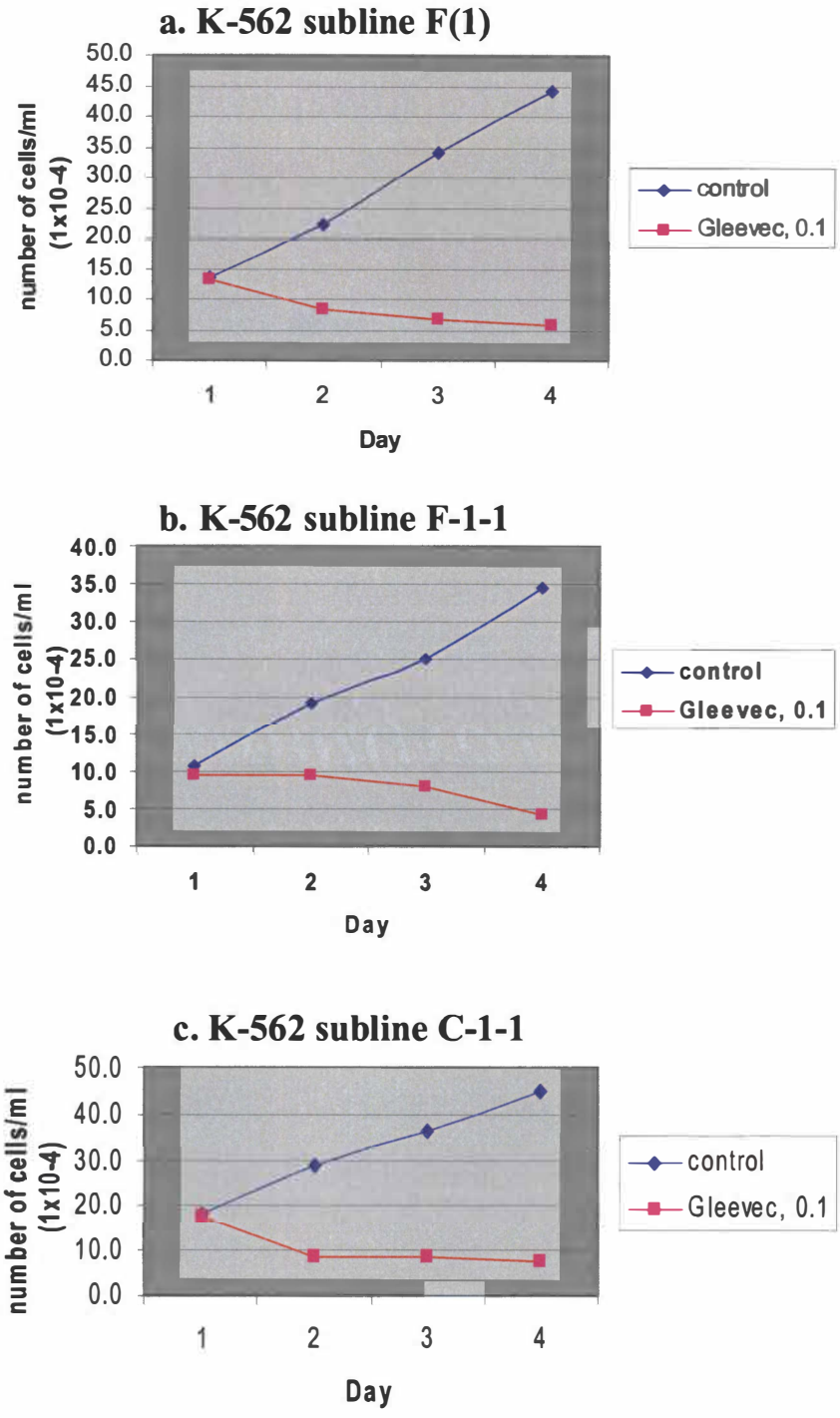


Figure 2. Cell counts as determined by visual analysis. K-562 cells, $1x10^5$ml, were incubated in triplicate in the presence of various concentrations of Gleevec ranging from 0.1 μM to 10μM. Visual counts using a hemocytometer were performed after 24, 48, 72 and 96 hours for **a)** subline F(1) **b)** subline F-1-1 and **c)** subline C-1-1.

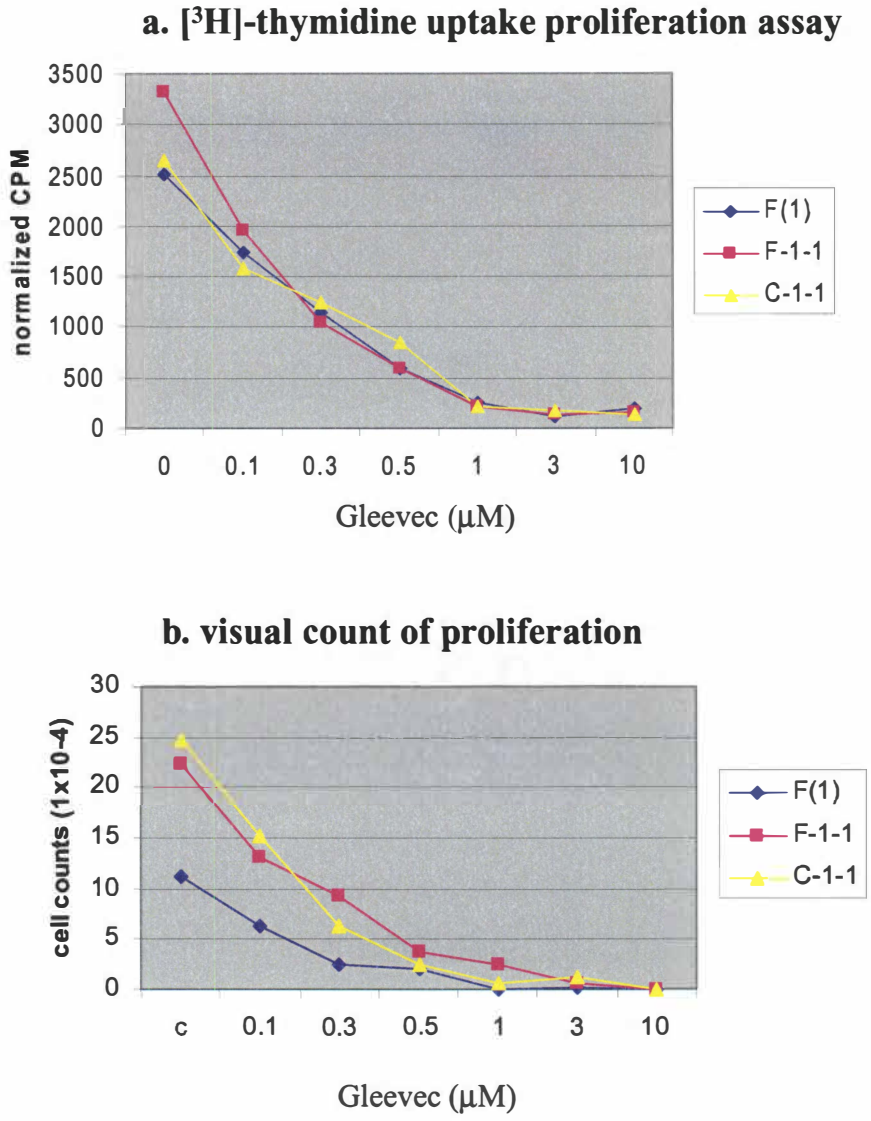
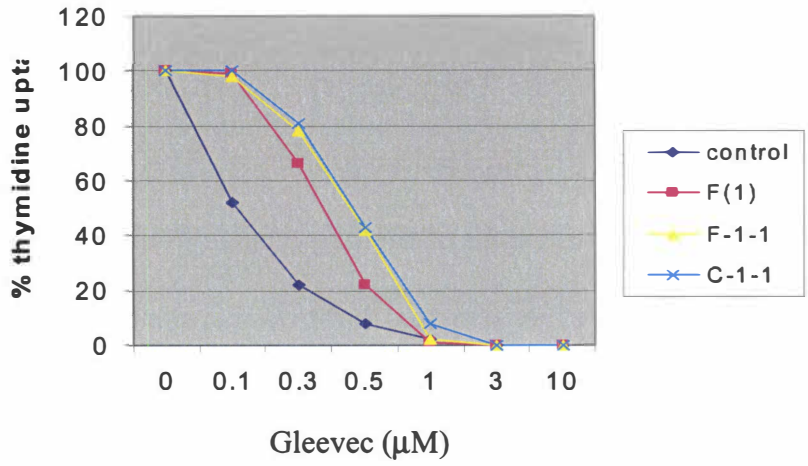


Figure 3. Proliferative activity at 48 hours. K-562 cells were cultured in the presence of various concentrations of Gleevec ranging from 0.1 μM to 10 μM. **a)** Cells, $1x10^4$, were incubated in triplicate and analyzed by [^{3}H]-thymidine uptake assay. **b)** Cells, $1x10^5$/ml were co-cultured with Gleevec and analyzed by visual analysis. Counts, using a hemocytometer, were determined after 48 hours.

The C-1-1 subline resistant to 0.1 μM was subsequently subjected to increased concentrations of Gleevec in a step-wise manner. By this method, a subline resistant to 0.3 μM was developed within 4 weeks. Sublines resistant to Gleevec, 1.0 μM and 3.0 μM, were developed similarly. The proliferative activity of the 0.1 μM-resistant cell lines was determined by [^{3}H]-thymidine uptake assay. This was done in triplicate in the presence of various concentrations of Gleevec, ranging from 0.1 μM to 10 μM. The proliferation of all sublines resistant to 0.1 μM was not significantly decreased when co-cultured with Gleevec, 0.1 μM, as compared to the control cells. All three sublines show little to no decrease in proliferation when cocultured in 0.1 μM **(Figure 4a)**. Proliferation was not as diminished by Gleevec in comparison to control cells through co-cultures treated with Gleevec, 0.5 μM. As the concentration reached 1.0 μM, the 0.1 μM-resistant cells were no longer proliferating. Visual cell counts showed similar results **(Figure 4b)**.

The proliferative activity of progressively increased cell lines resistant to Gleevec concentrations above 0.1μM was determined **(Figure 5)**. K-562 cells were initially cultured in the presence of Gleevec, 0.1 μM, then cultured in higher concentrations. The concentration was subsequently increased as the cells adapted and proliferated similarly to controls. When cells adapted to certain concentrations they proliferated, but proliferation decreased when the concentration of Gleevec reached higher levels. The proliferation of the C-1-1 subline resistant to 0.3 μM was not significantly decreased when co-cultured with Gleevec, 0.3 μM, as compared to the control cells. Apparent proliferation diminution by increased Gleevec concentrations

a.



b.

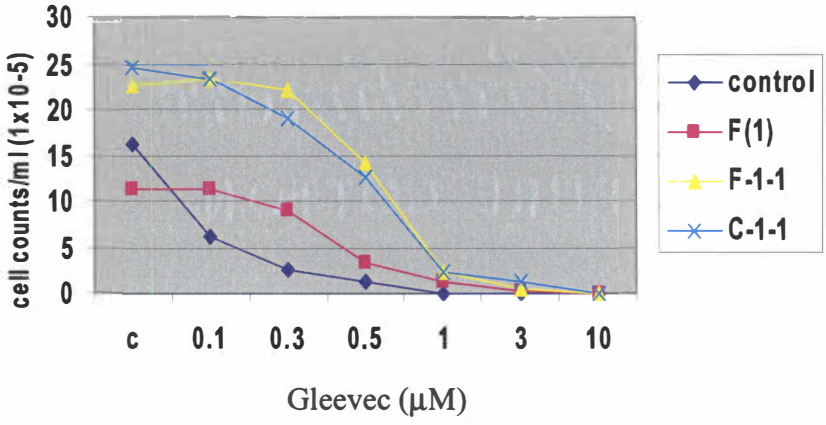


Figure 4. Proliferative activity of resistant cell lines at 48 hours. K-562 cells resistant to Gleevec, 0.1 μM, were further passaged 8 times. Cells were then co-cultured in the presence of various concentrations of Gleevec ranging from 0.1 μM to 10 μM. **a)** Cells, 10^4, from sublines F(1), F-1-1, and C-1-1 resistant to Gleevec, 0.1 μM, were incubated in triplicate. The proliferation of all sublines resistant to 0.1 μM were not decreased when co-cultured with Gleevec, 0.1 μM, as compared to the control cells. Proliferation was not as diminished by Gleevec in comparison to control cells through co-cultures treated with Gleevec, 0.5 μM. The proliferative [^{3}H]-thymidine uptake assay was performed after 48 hours of incubation with Gleevec. **b)** K-562 cells, 1x10^5/ml, were co-cultured with Gleevec and analyzed by visual analysis. Counts, using a hemocytometer were determined after 48 hours.

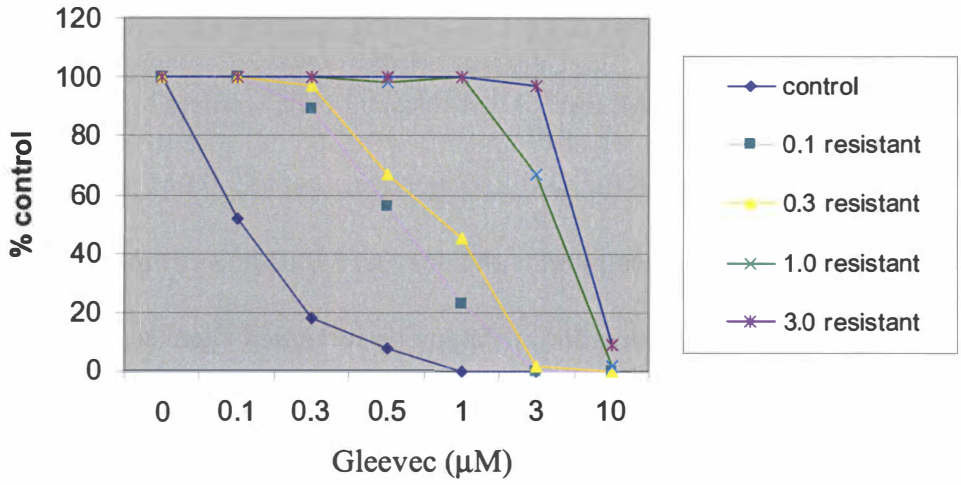


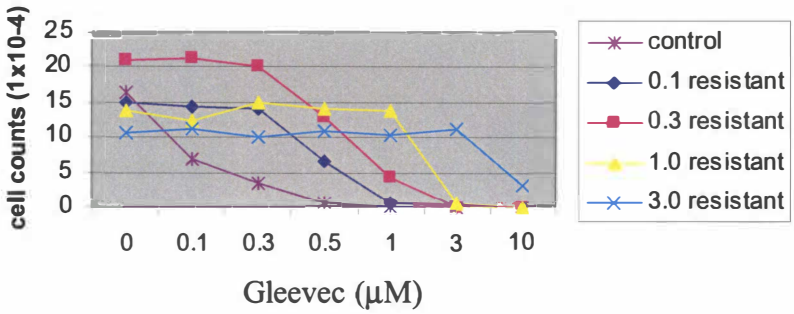
Figure 5. Proliferative activity of progressively increased resistance cell lines at 48 hours. K-562 cells were initially cultured in the presence of Gleevec, 0.1 μM. The concentration was subsequently increased as the cells adapted and proliferated similarly to controls. Cells, 10^4, from four C-1-1 sublines resistant to 0.1 μM, 0.3 μM, 1.0 μM, and 3.0 μM were incubated in triplicate in the presence of various concentrations of Gleevec ranging from 0.1 μM to 10 μM. The proliferation of the subline resistant to 0.1 μM was not decreased when co-cultured with Gleevec, 0.1 μM, as compared to the control cells. The proliferation of the subline resistant to 0.3 μM was not decreased when co-cultured with Gleevec, 0.3 μM, as compared to the control cells. The proliferation of the subline resistant to 1.0 μM was not decreased when co-cultured with Gleevec, 1.0 μM, as compared to the control cells. The proliferation of the subline resistant to 3.0 μM was not decreased when co-cultured with Gleevec, 3.0 μM, as compared to the control cells. The proliferative [^{3}H]-thymidine uptake assay was performed after 48 hours of incubation.

did not occur until reaching a level of 1.0 μM. As the concentration reached 3.0 μM, the 0.3 μM-resistant cells were no longer proliferating. The proliferation of the C-1-1 subline resistant to 1.0 μM was not significantly decreased when co-cultured with Gleevec, 1.0 μM, as compared to the control cells. Apparent proliferation diminution by increased Gleevec concentrations did not occur until reaching a level of 3.0 μM. As the concentration reached 10.0 μM, the 1.0 μM-resistant cells were no longer proliferating. The proliferation of the C-1-1 subline resistant to 3.0 μM was not significantly decreased when co-cultured with Gleevec, 3.0 μM, as compared to the control cells. Apparent proliferation diminishment by increased Gleevec concentration did not occur until reaching a level of >3.0 μM. As the concentration reached 10.0 μM, the 3.0 μM-resistant cells were no longer proliferating. Visual cell counts showed similar results **(Figure 6)**.

Morphologic Change

The induction of morphologic changes recurrent with additional cellular modifications was assessed by daily temporal observations of treated and untreated cells. Cells, 10ml, initially cultured at $1x10^5$/ml, were grown in $25cm^2$ flasks. Untreated cells were analyzed by wet-mount or in culture by inverted microscopy **(Figure 7)**. The cells remained spherical and blastic. The cells adhering to the flask were grouped as they were observed each day as follows: blastic, apoptotic-like, <10 dendritic hairs, >10 dendritic hairs, single pseudopodia, single/multiple filopodia, conical with or without a tail, and elongated and whip-like **(Figure 8, 9, 10)**. Pseudopodia were characterized as

a.



b.

Gleevec	control	0.1 μM	0.3 μM	0.5 μM	1.0 μM	3.0 μM	10.0 μM
0.1 resistant	15.00	14.38	14.06	7.19	3.75	0.31	0.00
0.3 resisant	20.94	21.25	20.00	12.81	4.38	0.00	0.00
1.0 resistant	13.75	12.50	15.00	14.06	13.75	0.63	0.00
3.0 resistant	10.63	11.25	10.00	10.94	10.31	11.25	3.13

Figure 6. Proliferative activity of progressively increased resistance cell lines as determined by visual counts. K-562 cells, $1x10^5$/ml, from four C-1-1 sublines resistant to 0.1 μM, 0.3 μM, 1.0 μM, and 3.0 μM were incubated in triplicate in the presence of various concentrations of Gleevec ranging from 0.1 μM to 10 μM. **a)**The proliferation of the subline resistant to 0.1 μM was not decreased when cocultured with Gleevec, 0.1 μM, compared to the control cells The proliferation of the subline resistant to 0.3 μM was not decreased when co-cultured with Gleevec, 0.3 μM, as compared to the control cells. The proliferation of the subline resistant to 1.0 μM was not decreased when co-cultured with Gleevec, 1.0 μM, as compared to the control cells. The proliferation of the subline resistant to 3.0 μM was not decreased when co-cultured with Gleevec, 3.0 μM, as compared to the control cells. Visual counts using a hemocytometer were performed after after 48 hours of incubation. **b)** Table of cell counts.

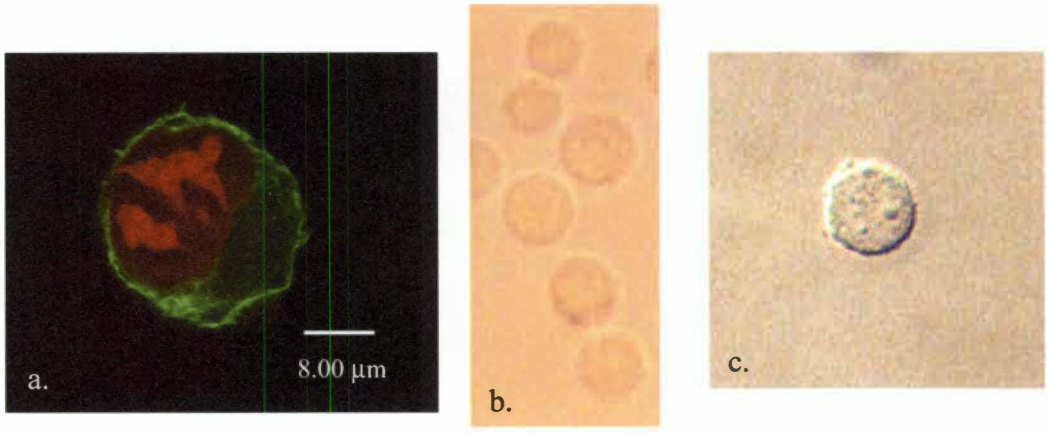


Figure 7. Untreated non-adherent K-562 cells from the designated subline C-1-1. a) Non-adherent untreated control cell stained for f-actin and for nuclear staining, propidium iodide. Cells were examined by inverted fluorescent microscopy. **b)** Non-adherent untreated control cells examined by inverted microscopy (200x). **c)** Non-adherent untreated control cell examined by phase-contrast microscopy (200x).

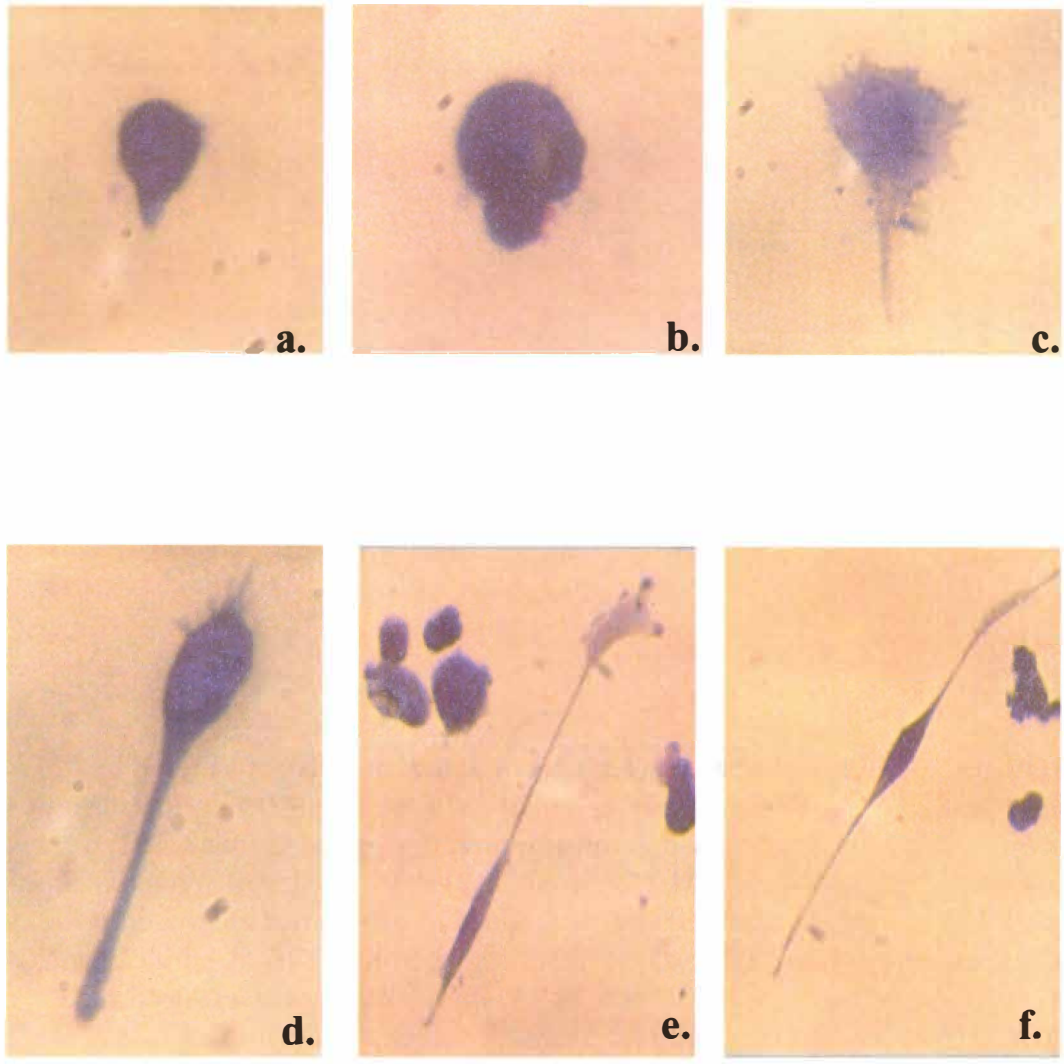


Figure 8. Morphological characteristics of adherent cells determined by temporal analysis. A proposed progression of cell maturation (Filipodia "Elongated" Pathway) by sequential observations. K-562 cells from the designated subline C-1-1, were co-cultured with PMA, 0.2 nM . After removing the non-adherent cells, the attached cells were fixed and then stained with Wright-Giemsa stain. The stained adherent cells were examined by inverted microscopy (400x) and grouped according to morphologic characteristics. The cells were grouped as follows: blastic, apoptotic-like, single cytoplasmic protrusion, multiple protrusions, conical with or without a tail, elongated, or whip-like when observed daily for 5 days. **a)** pseudopodia formation (cytoplasmic extrusion) **b)** broad-based protrusion **c)** conical shape with tail (evidence for filipodia), cell maintained nucleus **d)** single filipodia, nucleus separation **e)** evidence for secondary filipodia, separate nuclear regions **f)** two filopodia.

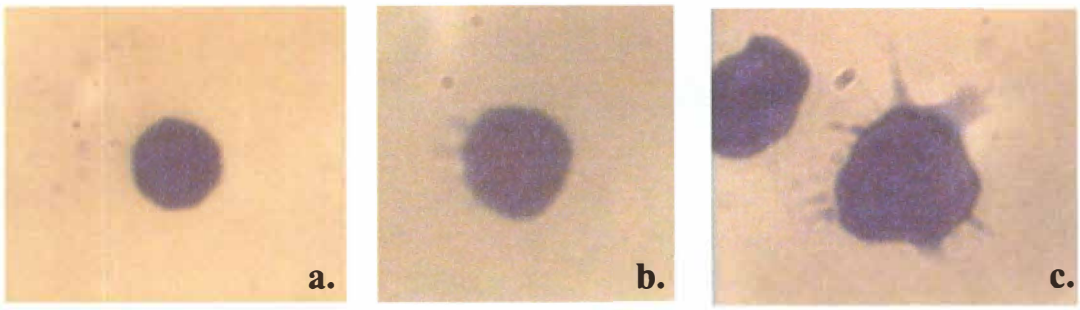


Figure 9. Morphological characteristics of adherent cells determined by temporal analysis. Proposed progression of cell maturation (Dendritic Pathway) by sequential observations. K-562 cells from the designated subline C-1-1, were co-cultured with PMA, 0.2 nM. Cells were fixed and stained with Wright-Giemsa stain. The stained adherent cells were examined by an inverted microscope (400x). The cells were grouped as follows: blastic, apoptotic-like, <10 "dendritic" hairs, >10 "dendritic" hairs. **a)** blastic cell **b)** "dendritic" hairs (<10) **c)** "dendritic" hairs (>10) when observed daily for 5 days. K-562 cells from the designated subline C-1-1, were co-cultured with PMA, 0.2 nM.

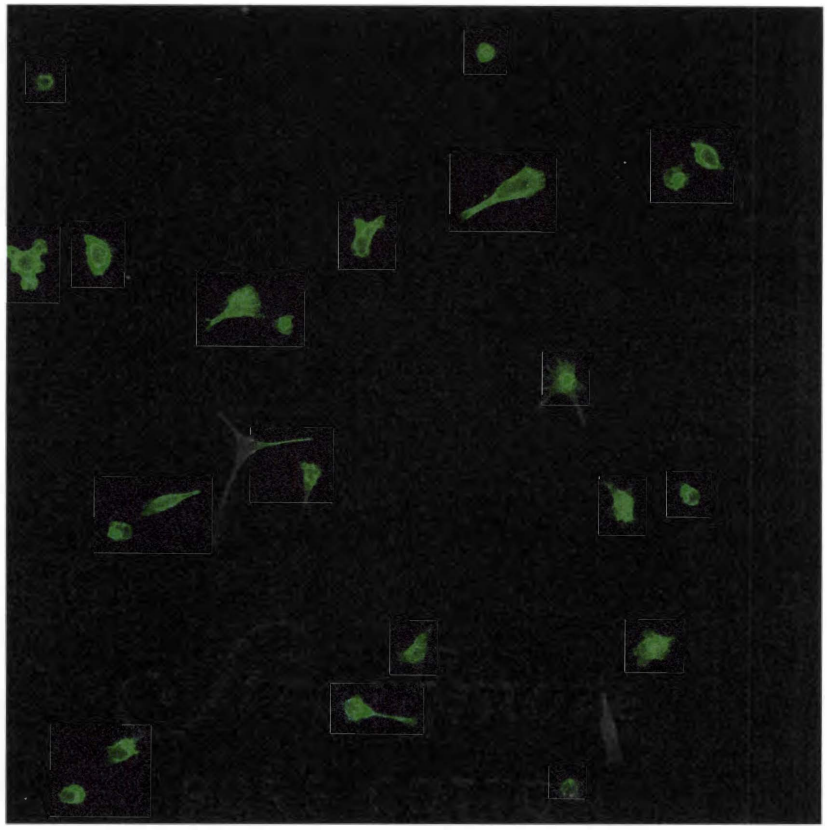


Figure 10. Filamentous actin staining of cells exhibiting various morphologies. K-562 cells from the designated subline C-1-1, were co-cultured with PMA, 0.2 nM. After removing the non-adherent cells, the attached cells were fixed and stained for f-actin. The stained adherent cells were examined by inverted fluorescence microscopy (400x). f-actin is involved in the numerous morphological shapes alterations.

having a rounded cytoplasmic protrusion. Filopodia were characterized as cytoplasmic extensions with an unambiguous point. Dendritic hairs were defined as slender projections from the edge of the cell membrane. Observations were made daily for five days.

Studies showed K-562 cells co-cultured with PMA, 0.2 nM, demonstrated dendritic, pseudopodic, and filopodic characteristics. While the non-adherent cells showed some of these changes, the population of adherent cells with induced morphologic change was more significant. PMA studies were performed since several previous studies indicated that PMA causes cell adherence and morphologic changes.

It was observed that cells co-cultured with Gleevec were predominantly dendritic in appearance with many having >10 hairs **(Figure 11f)**. Observations were made for five days. The dendritic pathway showed characteristics of developing dendritic cells **(Figure 11)**. As the duration of incubation with Gleevec lengthened, an increasing number of dendritic hairs were observed. By day 2, the predominant adherent cell type were those with dendritic appearance with <10 ten hairs. The sub-population of cells with >10 hairs were not observed until day 3, on which they constituted roughly the same proportion of adherent cells as those with <10 hairs. On day 4 more than half of the adherent cells had >10 hairs. No further changes in the proportion of cell types were evident beyond day 4.

On occasion elongated cells were observed **(Figure 12)**; however, the majority of morphologies observed were of the dendritic pathway. The elongation pathway was characterized by an initial single cytoplasmic extrusion while the single nucleus remained intact. As the extrusion became more pronounced and showed evidence for

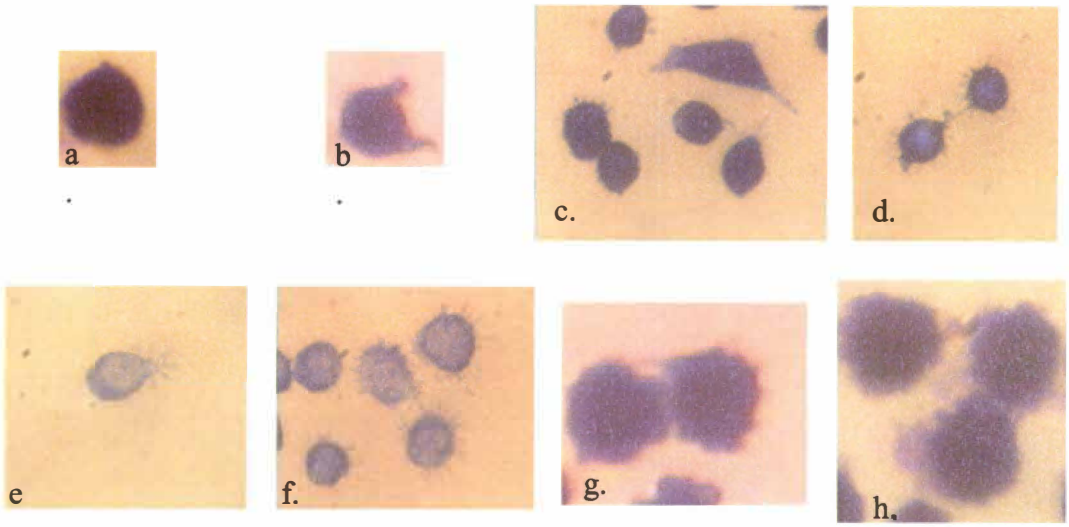


Figure 11. Morphological characteristics of the adherent cells determined by temporal analysis. Spectrum of morphological changes in adherent K-562 cells co-cultured with Gleevec, 0.1μM, were assessed by sequential observation for 5 days. After removing the non-adherent cells, the attached cells were fixed and stained with Wright-Giemsa. Cells were examined by inverted microscopy (400x). Cells with "dendritic" hairs and/or short filopodia were the predominant cell type of those that remained adherent after wash treatment. **a)** Adhering cell with no apparent morphological changes (enlarged). **b)** Cell with two pseudopodia (cytoplasmic extrusion). **c)** Larger cell with two filopodia and at least three cells exhibiting "dendritic" hairs. **d, e, f)** Cells with multiple "dendritic" hairs. **g, h)** Cells with multiple pseudopodia (cytoplasmic extrusions).

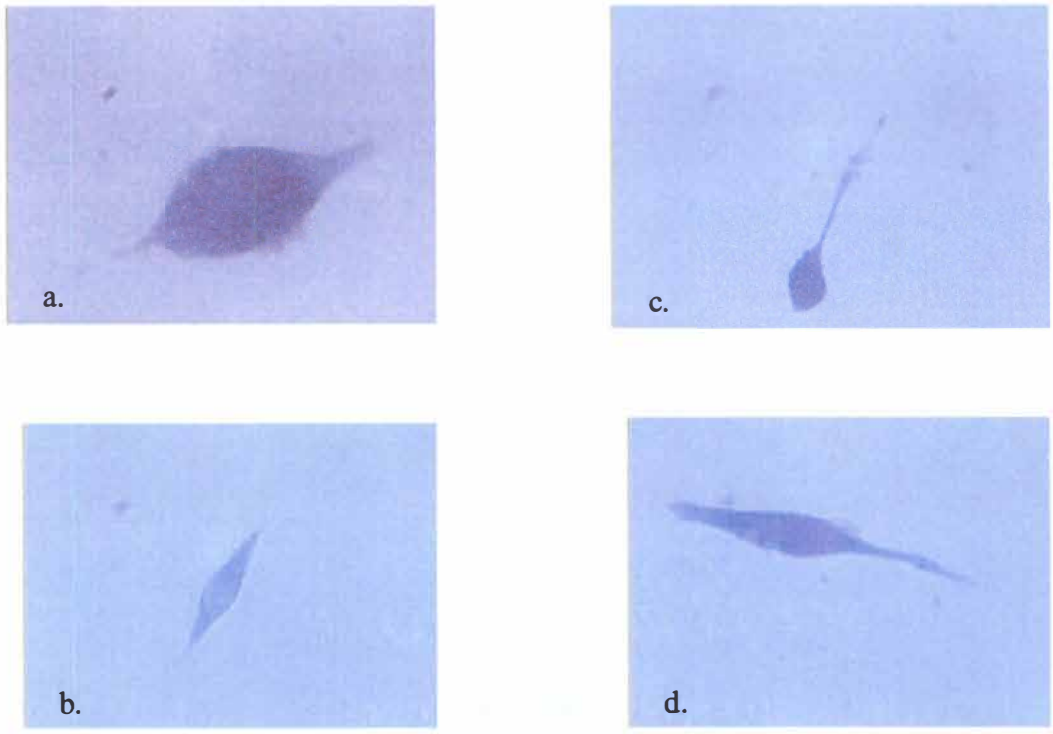


Figure 12. Morphological characteristics of the adherent cells determined by temporal analysis. Adherent K-562 cells co-cultured with Gleevec, 0.1μM, for 48h. Adherent Gleevec-treated cells (0.1μM) from the designated subline C-1-1, with cytoplasmic projections/extrusions. Cells were fixed and stained with Wright-Giemsa stain. These cells represented a minority subset. Cells examined by inverted microscopy (400x). **a)** Cell with apparent filopodia, one more splayed than the other (image enlarged). **b)** More apparent filopodia **c)** double filopodia **d)** Apparent double filopodia

filopodia, it was observed that additional nuclear regions developed. It appeared that, in the later stages of this proposed pathway, secondary filopodia were formed producing a cell with a length many times that of the diameter of an original cell.

While the non-adherent cells showed some of these changes **(Figure 13)**, most of the cells observed with induced morphologic change were adherent cells. Studies with Gleevec, 0.1 μM, showed similar morphological changes in adherent cells as compared with PMA. It was also observed that the Gleevec-induced adherence to plastic was not as apparent as adherence induced by PMA treatment.

Cells that were co-cultured for multiple weeks in the presence of Gleevec, 0.1 μM, initially showed substantial morphologic change. For several weeks the non-adhering cells continued to show considerable morphologic change following passaging of the cells. These changes were usually observed after 48 hours of culture. Following weeks of exposure to Gleevec, 0.1 μM, the induction of morphologic change was decreased. Resistance was determined when no further morphological changes were induced by treatment with Gleevec. The F(1) subline reached resistance after 19 weeks, F-1-1 after 16 weeks, and the C-1-1 after 15 weeks.

Additional studies with Gleevec-resistant sublines showed similar morphologic changes when co-cultured with concentrations of Gleevec above which they had become resistant. However, as with the control sublines, these resistant sublines also became resistant to increasing concentrations of Gleevec.

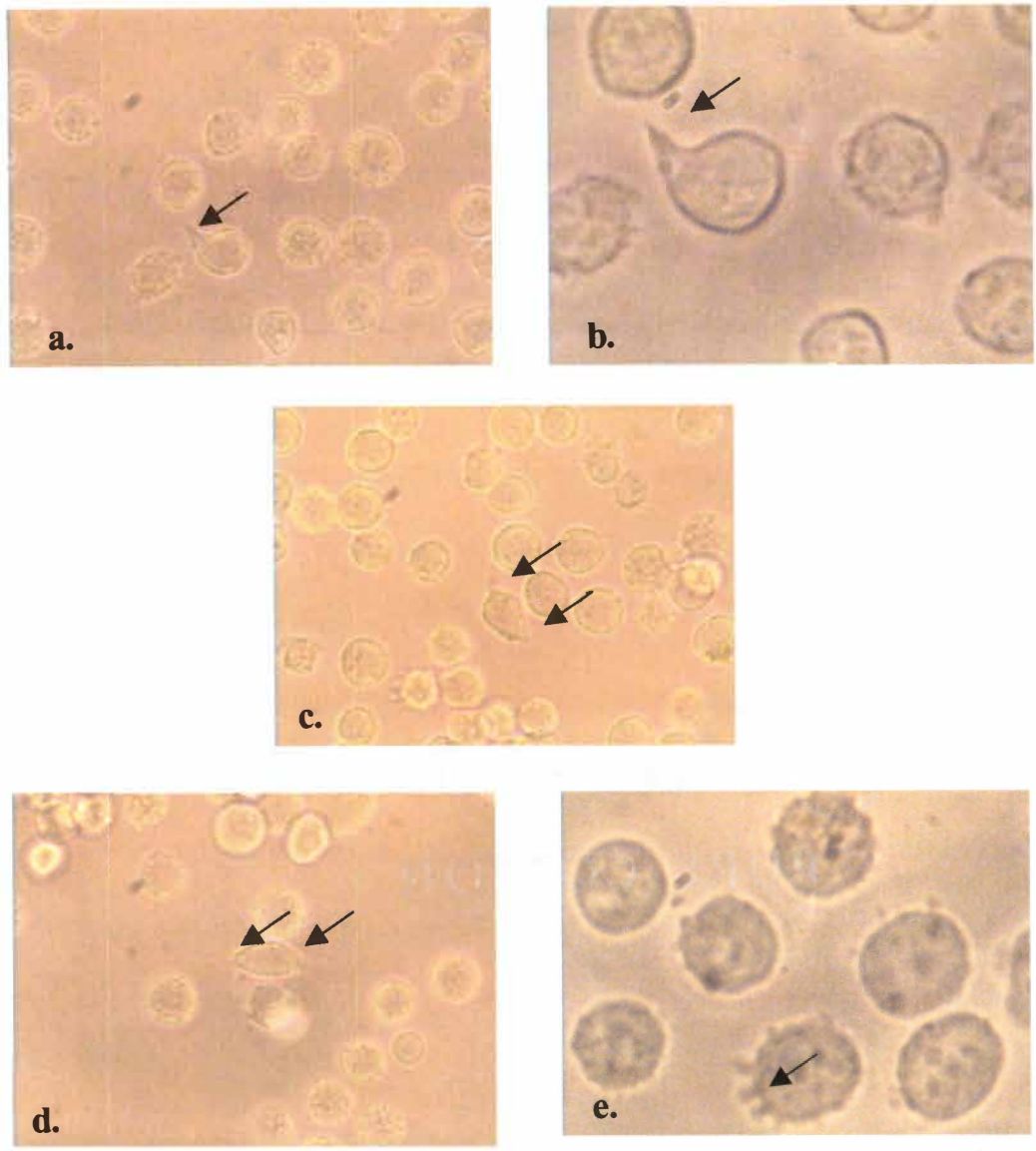


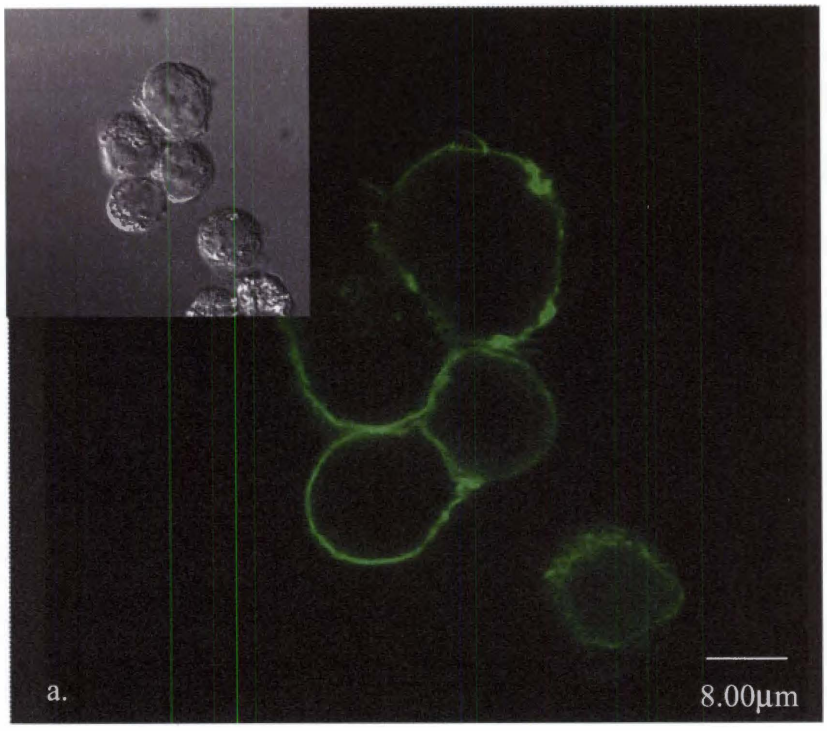
Figure 13. Morphological characteristics of the non-adherent cells determined by temporal analysis. K-562 cells co-cultured with Gleevec, 0.1μM, were assessed by sequential observation for 5 days. **a)** Gleevec-treated cells (0.1μM), in suspension from the designated subline C-1-1, with single cytoplasmic projection. Cells examined by inverted microscopy (200x). **b)** Enlargement of figure 13 a. (400x). **c, d)** Gleevec-treated cells (0.1μM) in suspension from the designated subline F-1-1 with double cytoplasmic projections, examined by inverted microscopy (200x). **e)** Gleevec-treated cells (0.1μM) in suspension from the designated subline C-1-1 with multiple cytoplasmic, "dendritic" hairs, examined by inverted microscopy (400x).

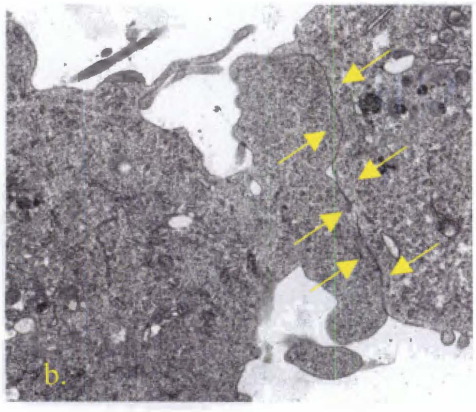
Homotypic Aggregation

While the observations were made of the changes in adherence properties to plastic following treatment with Gleevec, 0.1 μM, it was also observed that cell-cell adherence was also augmented. Homotypic aggregation was observed within 24 hours after treatment with Gleevec, 0.1 μM **(Figure 14, 15)**. After this initial change in cell-cell adherence, the number and size of aggregated cells remained constant through 4 days. After an extended exposure to Gleevec, 0.1 μM, (F(1) 19 weeks, F-1-1 16 weeks and C-1-1 15 weeks), homotypic aggregation was not induced upon weekly re-culturing. Similar observations were seen when the Gleevec-resistant sublines were co-cultured with higher doses of the drug.

Cells induced to homotypically aggregate were then stained for f-actin and with the nuclear stain propidium iodide. Visual observations showed involvement of f-actin in cell-cell contact, which was supported by the increased staining intensity at points of contact **(Figure 16)**.

Upon observation of cells adherent to plastic following treatment with Gleevec, 0.1 μM, a clustering effect was evident. The clustering was frequently observed concurrent with the morphologic changes caused by weekly Gleevec re-exposure **(Figure 17)**.





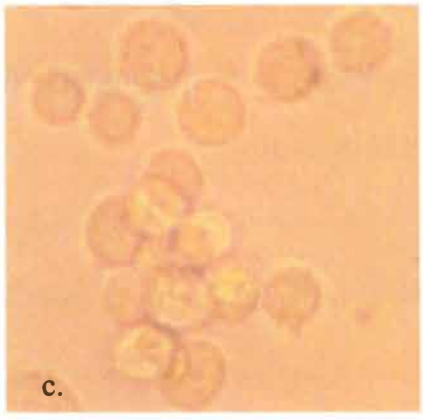


Figure 14. Cell-cell interactions and homotypic aggregation. K-562 cells from the designated subline C-1-1, were co-cultured with Gleevec or T cell conditioned media (TCCM). **a)** Cells were fixed and stained for f-actin. The stained cells were examined by laser confocal microscopy (1000x). The inset is an Nomarski image of the same cells. **b)** K-562 cells were co-cultured in TCCM for 48 h. The cells were then examined by electron microscopy (6000x). The yellow arrows emphasize the cell-cell contact. **c)** K-562 cells resistant to Gleevec, 0.1 µM, then subsequently treated with 1.0 µM Gleevec for 48 h. The stained cells were examined by inverted microscopy (200x).

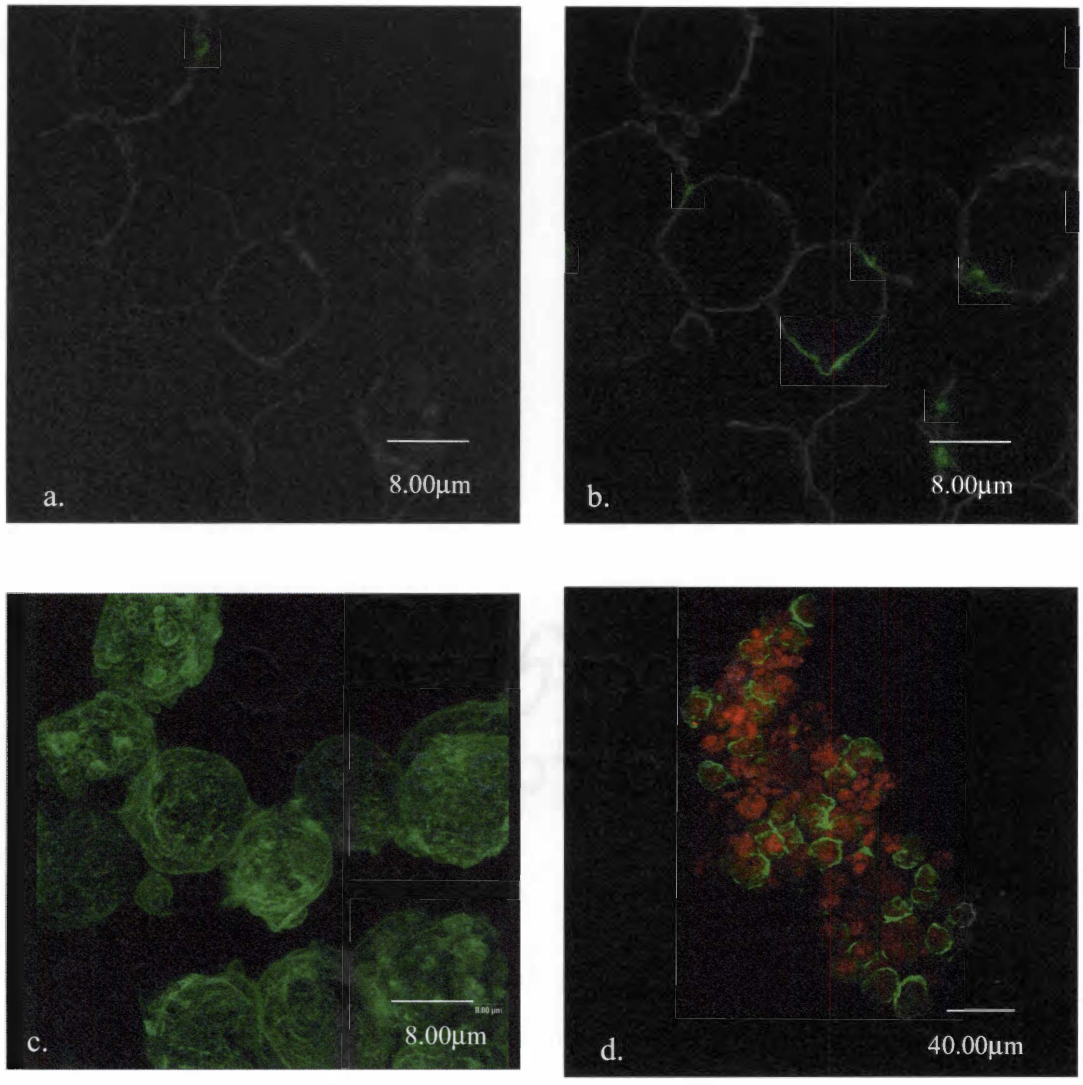


Figure 15. Cell-cell interactions and homotypic aggregation. K-562 cells, subline C-1-1, resistant to Gleevec, 0.1 µM, treated with 1.0 µM Gleevec for 48 h. Cells were fixed and stained for f-actin. **a, b)** Cells induced to homotypically aggregate. The stained cells were examined in serial selections by laser confocal microscopy. **c)** A composite picture of all serial selections. **d)** Cells induced to homotypically aggregate then stained for f-actin and the nuclear stain, propidium iodide.

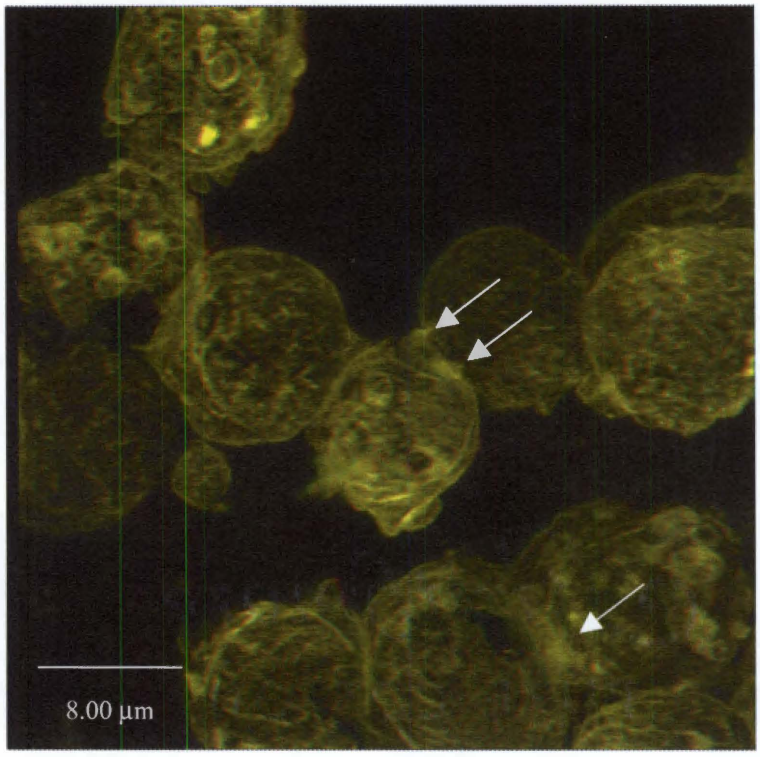


Figure 16. Cell-cell interactions and homotypic aggregation. K-562 cells, subline C-1-1, resistant to Gleevec, 0.1 µM, then treated with 1.0 µM Gleevec for 48 h. Cells were fixed and stained for f-actin. Cells induced to homotypically aggregate were examined in serial selections by inverted fluorescent microscopy. The image was then converted into three-dimensional image. Arrows point to apparent focal adhesion points.

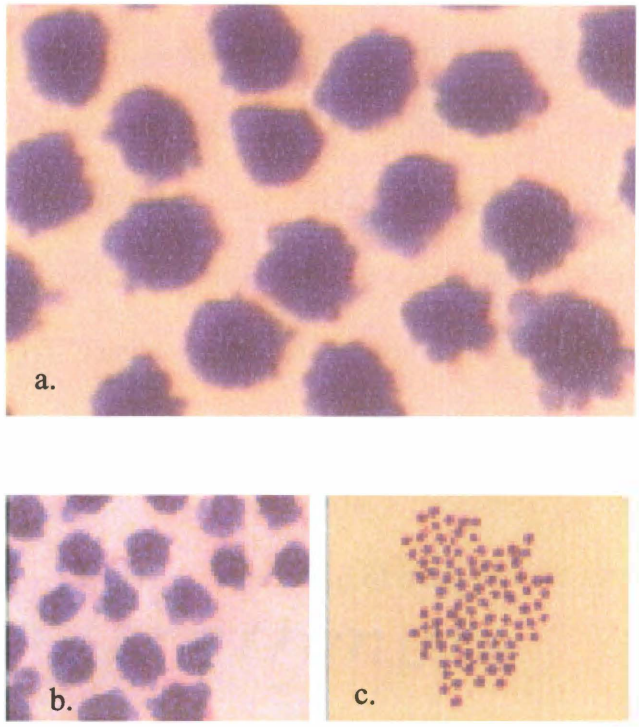


Figure 17. Clustering adherent Gleevec-treated cells (0.1μM) from the designated subline C-1-1. After removing the non-adherent cells, the attached cells were fixed and stained with Wright-Giemsa. Cells were examined by an inverted microscope **a)** 400x. **b)** 200x. **c)** 100x. The clustering was frequently observed concurrent with the morphologic changes caused by Gleevec exposure.

Karyotype and FISH analysis

Karyotyping for each subline showed consistent archetypal chromosomal characteristics. Differences in chromosomal markers were present **(Figure 18 a, c, g)**. Multiple BCR-ABL fusions and chromosomal rearrangements were seen in K-562 cells in contrast to the single BCR-ABL fusion typically found in CML patients. The karyotype of control K-562 cells, subline C-1-1, was indistinguishable from the karyotype of K-562 cells resistant to 0.1 μM. FISH analysis shows no visual differences between the metaphase spreads of control K-562 cells, subline C-1-1 and K-562 cells resistant to Gleevec 1.0μM. Thus, the high degree of BCR-ABL gene amplification within the untreated K-562 cells made it difficult to detect changes in signal strength compared to the resistant lines **(Figure 18 b, d, f)**.

Flow Cytometry

The results of immunophenotyping K-562 cells cultured in the absence or presence of Gleevec for days 1 through 4 showed similar results for all three sublines. Gleevec induced the apparent expression of HLA-common antigen and CD11b, the differentiation antigen associated with immature dendritic cells in the F-1-1 **(Figure 19)**, C-1-1 **(Figure 20)**, and F(1) (results not shown**)**. Although the induction of CD11b is discernible in the F-1-1 subline results, it is not statistically significant.

In the Gleevec 0.1 μM-resistant cell lines, similarities and differences existed between sublines (data not shown). The HLA-common antigen expression remained up-reglated in the F-1-1 and C-1-1 Gleevec-resistant sublines in comparison to control

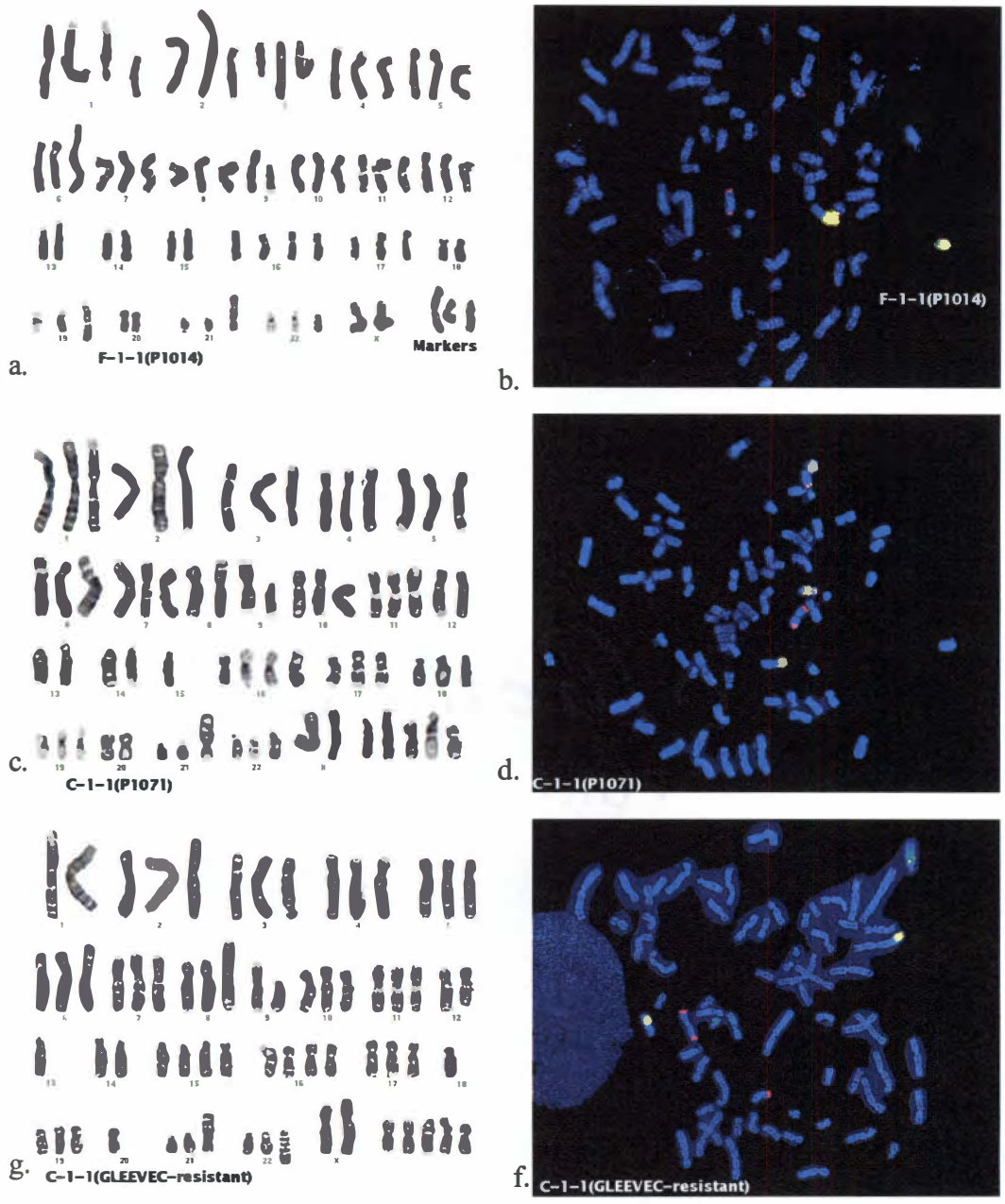


Figure 18. Karyotype and FISH analysis of control and Gleevec-resistant K-562 cell lines. FISH chromosomal analyses detected the BCR gene (green), ABL gene (red), and the fusion gene (yellow) from cells in metaphase. **a)** karyotype of control K-562 cells (subline F-1-1) **b)** metaphase spread of control K-562 cells (subline F-1-1) **c)** karyotype of control K-562 cells (subline C-1-1) **d)** metaphase spread of control K-562 cells (subline C-1-1) **e)** karyotype of K-562 cells (subline C-1-1) resistant to Gleevec, 0.1μM **f)** metaphase spread of K-562 cells (subline C-1-1) resistant to Gleevec, 0.1μM, shows similar signal compatible with amplified element.

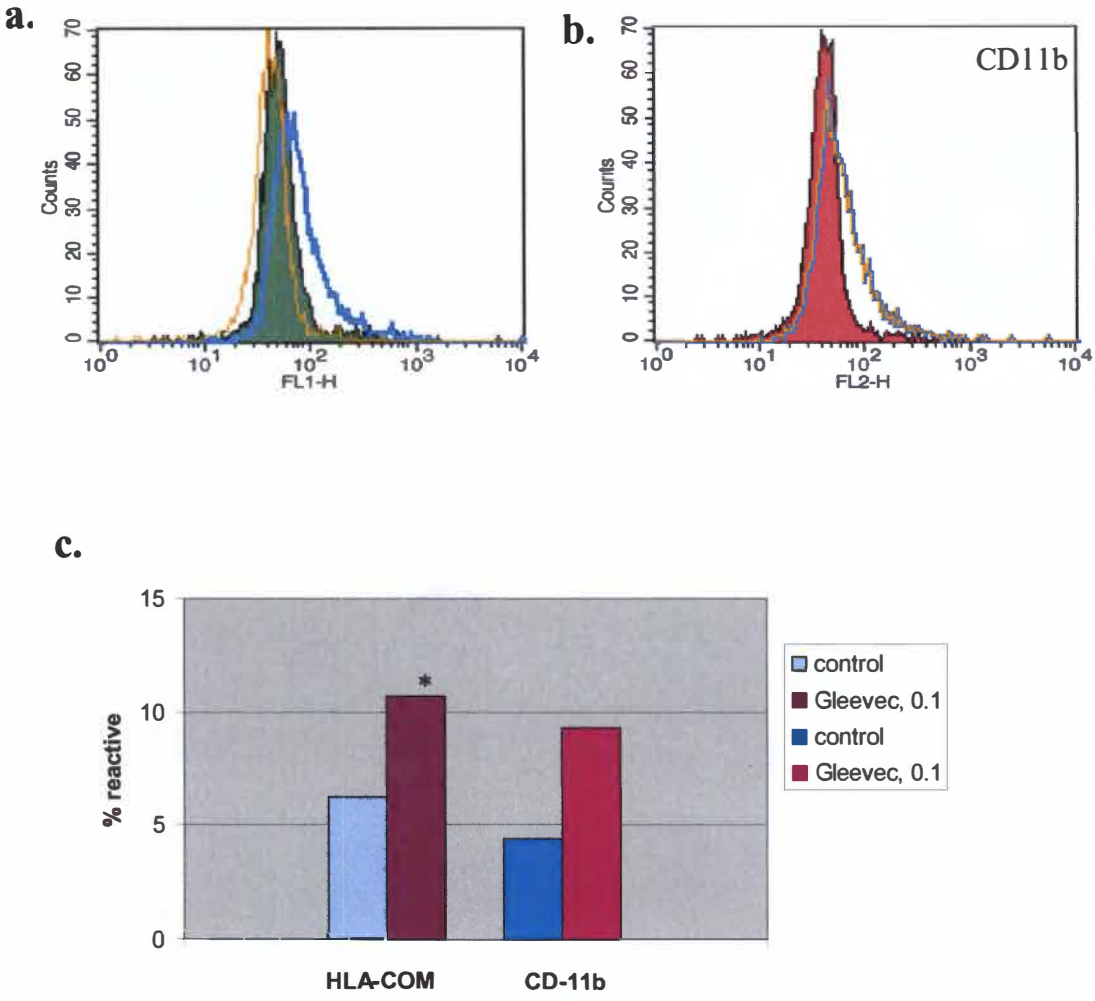


Figure 19. Antibody reactivity of K-562 cells after 48hr incubation cocultured with Gleevec, 0.1 μM, subline F-1-1. Gleevec induced the expression of HLA-common antigen as determined by flow cytometry. Solid histograms represent cells without Gleevec treatment, open orange histograms represent isotype controls and the open blue histograms represent Gleevec-treated cells after 48 h incubation **a)** HLA-common **b)** CD11b **c)** Graph of percent change in reactivity of surface expression. *statistically significant $p<0.05$ compared to controls.

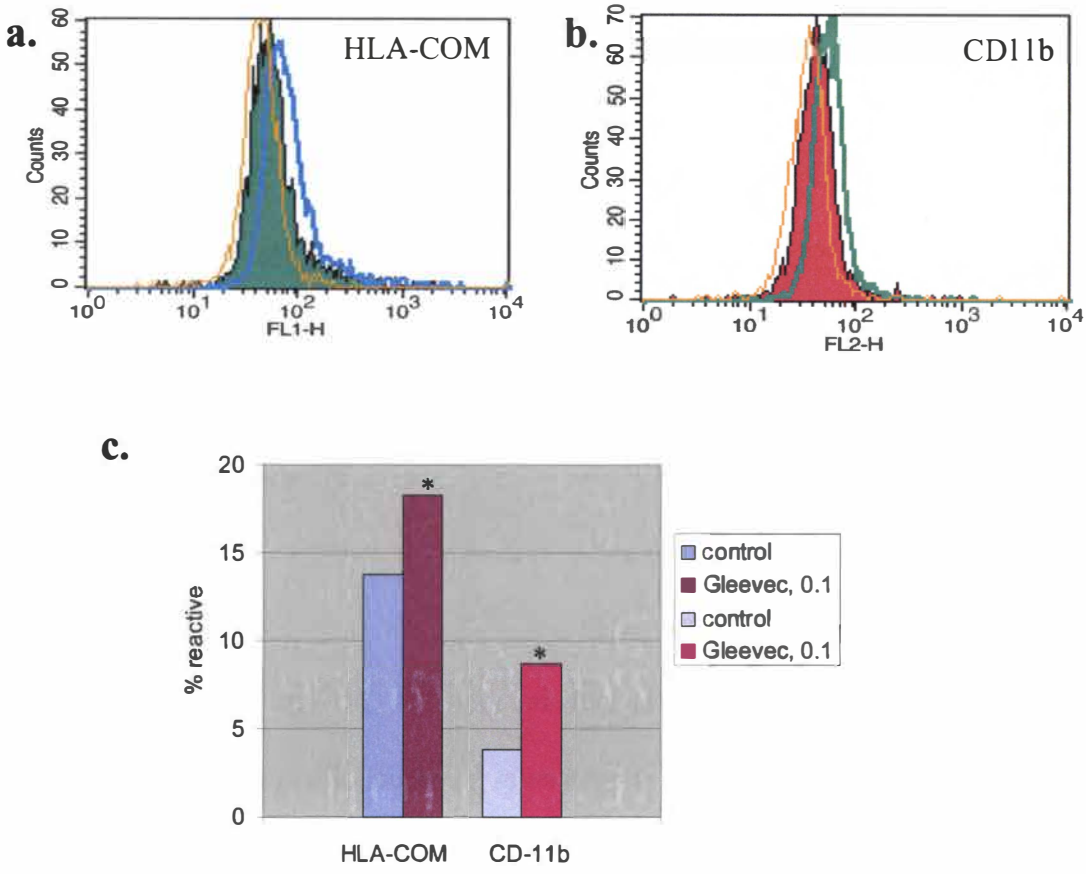


Figure 20. Antibody reactivity of K-562 cells after 48hr incubation co-cultured with Gleevec 0.1μM, subline C-1-1. Gleevec induced the expression of HLA-common antigen and the de novo expression of CD11b, the differentiation antigen associated with immature dendritic cells as detected by flow cytometry. Solid histograms represent cells without Gleevec treatment and the open orange histograms represent isotype controls. The open blue and open green histograms represent Gleevec-treated cells after 48 h incubation **a)** HLA-common (open blue) **b)** CD11b (open green) **c)** Graph of percent change in reactivity of surface expression. *statistically significant $p<0.05$ as compared to control cells.

cells. CD11b, which consistently showed increased expression in both the F-1-1 and C-1-1 sublines after initial treatment with Gleevec, 0.1 μM, continued to show increased expression in the F-1-1 Gleevec 0.1 μM-resistant subline, yet expression diminished in the resistant C-1-1 subline. The expression of HLA-ABC initially showed decreased expression in both the F-1-1 and C-1-1 Gleevec-treated sublines. While the F-1-1 showed increased expression once this subline had become resistant to Gleevec, 0.1 μM, the C-1-1 Gleevec-resistant subline showed null expression. The expression of CD33, which was abrogated after initial treatment with Gleevec, 0.1 μM, in both F-1-1 and C-1-1 sublines showed null expression in both Gleevec-resistant sublines.

RT-PCR

The BCR-ABL bands from the cells incubated in Gleevec, 0.1 μM and 1.0 μM, were less intense than the untreated control cells, by visual observation **(Figure 21)**. The BCR-ABL band from the cells incubated in Gleevec, 10.0 μM, was undetectable. These results were consistent with 6 other experiments. The BCR-ABL band from the cells incubated for 24 hr with Gleevec, 0.1 μM, was less intense than cells incubated for 4 hours or the untreated control cells and was undetectable in the cells co-cultured for 48 hr **(Figure 22)**. This suggested that Gleevec had transcriptional effects within 24 hours. These results were consistent in 5 experiments.

Resistant sublines showed similar mRNA expression with comparison to the controls by visual observation. The BCR-ABL bands from all three sublines resistant to Gleevec, 0.1 μM, were of equal intensity, compared to the control cells **(Figure 23)**.

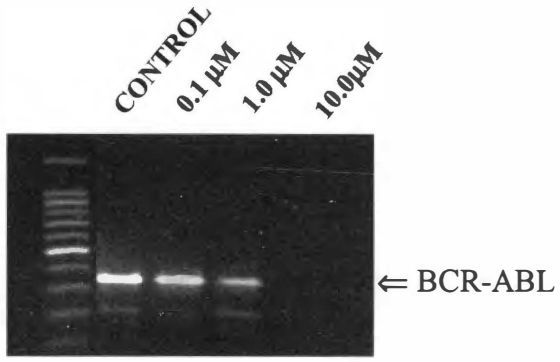


Figure 21. The effects of Gleevec on BCR-ABL mRNA expression as determined by RT-PCR. K-562 cells, subline C-1-1, co-cultured with Gleevec, 0.1 μM, 1.0 μM and 10.0 μM, were harvested after an incubation of 48 hr and the total RNA was extracted. Synthesized cDNA, 1 μg, was amplified. The 5' primer recognized an exon sequence in the BCR-locus, while the 3' primer recognized an exon sequence of the ABL-locus yielding a PCR product of 327 bp. By visual observation, the BCR-ABL band from the cells incubated in Gleevec, 0.1 μM and 1.0 μM were less intense than untreated control cells. The BCR-ABL band from the cells incubated in 10.0 μM Gleevec was undetectable. These results were consistent in 6 experiments.

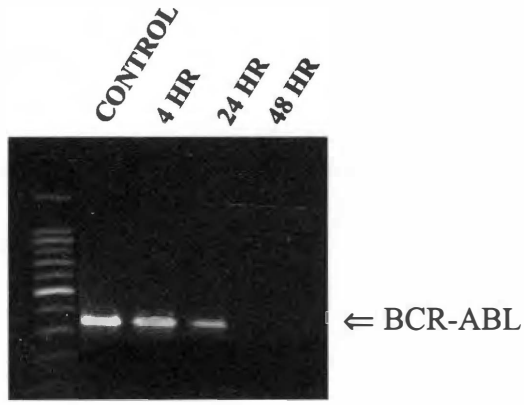


Figure 22. The effects of Gleevec on BCR-ABL mRNA expression as determined by RT-PCR. K-562 cells, subline C-1-1, co-cultured with Gleevec, 0.1 μM, were harvested after incubation for 4 hr and 24 hr and the total RNA was extracted. Synthesized cDNA, 1 μg, was amplified. The 5' primer recognized an exon sequence in the BCR-locus, while the 3' primer recognized an exon sequence of the ABL-locus yielding a PCR product of 327 bp. By visual observation, the BCR-ABL band from the cells incubated for 24 hr with Gleevec was less intense than cells incubated for 4 hours and less intense than the control cells. The BCR-ABL band from the cells incubated for 48 hr was undetectable. These results were consistent in 5 experiments.

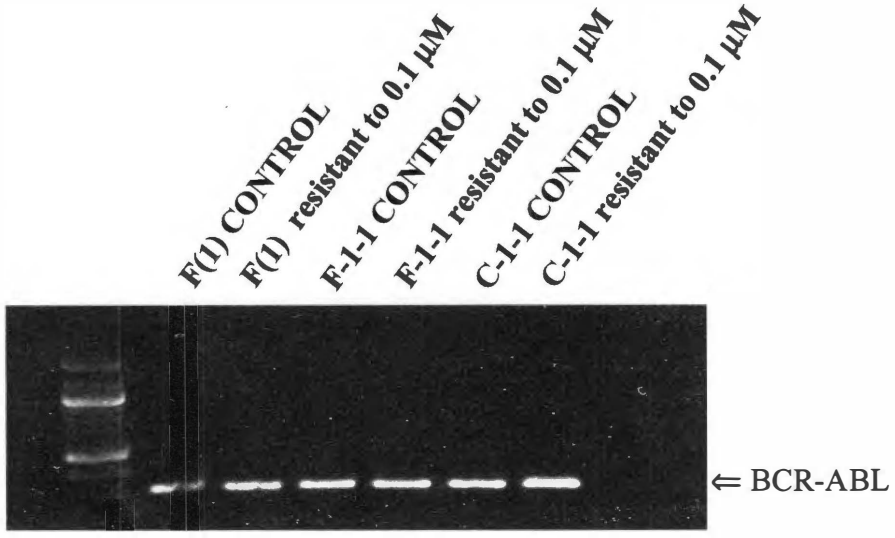


Figure 23. The effects of Gleevec on BCR-ABL mRNA expression of resistant cell lines determined by RT-PCR. K-562 cell sublines resistant to Gleevec, 0.1 μM, were harvested and the total RNA was extracted. Synthesized cDNA, 1 μg, was amplified. The 5' primer recognized an exon sequence in the BCR-locus, while the 3' primer recognized an exon sequence of the ABL-locus yielding a PCR product of 327 bp. The BCR-ABL band from the sublines resistant to Gleevec, 0.1 μM, appeared to be of equal intensity as compared to the control cells. These results were consistent in 3 experiments.

These results were consistent with 3 other experiments. The BCR-ABL band from the sublines resistant to 0.1 μM and 0.3 μM were of equal intensity, compared to the control cells **(Figure 24)**. The BCR-ABL band from the sublines resistant to 3.0 μM was of greater intensity, compared to the control cells **(Figure 25)**. These results were consistent in 3 experiments.

Western Blot

Results suggested the loss of BCR-ABL protein expression after 48 hours of incubation **(Figure 26)**. These results were consistent with 3 other experiments. These results suggested that Gleevec inhibited the expression of the BCR-ABL gene product. The amount of protein detected in the cells resistant to Gleevec, 3.0 μM, suggested an increase in BCR-ABL protein as compared to control cells **(Figure 27)**. The amount of protein between cells resistant to 0.1 μM and control cells was undetectable. These results were consistent with 6 other experiments.

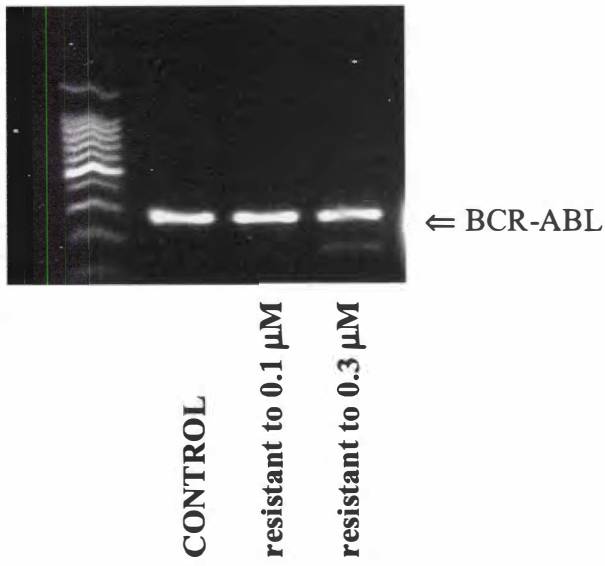


Figure 24. The effects of Gleevec on BCR-ABL mRNA expression of resistant cell lines determined by RT-PCR. K-562 cell sublines resistant to Gleevec, 0.1 μM and 0.3 μM, were harvested and the total RNA was extracted. Synthesized cDNA, 1 μg, was amplified. The 5' primer recognized an exon sequence in the BCR-locus, while the 3' primer recognized an exon sequence of the ABL-locus yielding a PCR product of 327 bp. The BCR-ABL band from the sublines resistant to 0.1 μM and 0.3 μM Gleevec were of similar intensity as compared to the control cells. These results were consistent in 3 experiments.

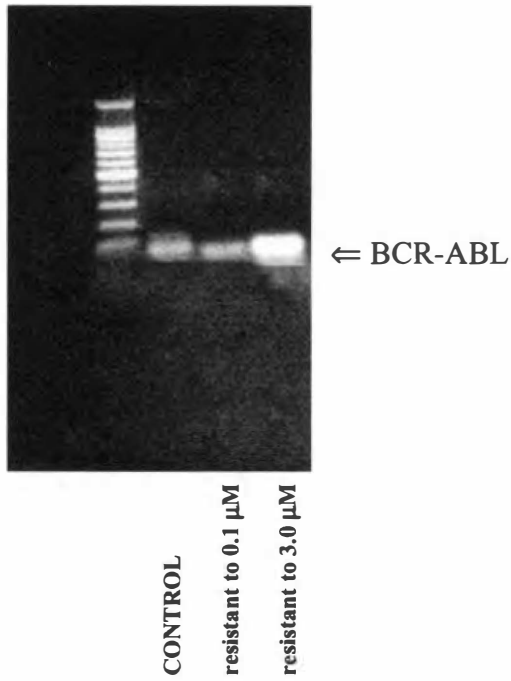


Figure 25. The effects of Gleevec on BCR-ABL mRNA expression of resistant cell lines determined by RT-PCR. K-562 cell sublines resistant to Gleevec, 0.1 μM and 3.0 μM, were harvested and the total RNA was extracted. Synthesized cDNA, 1 μg, was amplified. The 5' primer recognized an exon sequence in the BCR-locus, while the 3' primer recognized an exon sequence of the ABL-locus yielding a PCR product of 327 bp. The BCR-ABL band from the sublines resistant to Gleevec, 0.1 μM, was of similar intensity as compared to the control cells while the BCR-ABL band from the subline resistant to Gleevec, 3.0 μM, was of greater intensity. These results were consistent in 3 experiments.

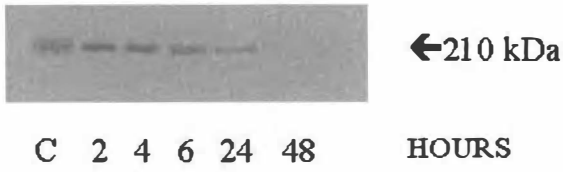


Figure 26. The level of BCR-ABL protein demonstrated by Western blot. Samples containing 30 μg of total RNA per lane were extracted from K-562 cells treated with Gleevec, 0.1 μM, for 2, 4, 6, 24 and 48 hr. The results suggested the loss of BCR-ABL protein expression after 48 hours of incubation, confirming that Gleevec inhibited the expression of the BCR-ABL gene product. These results were consistent in 3 experiments.

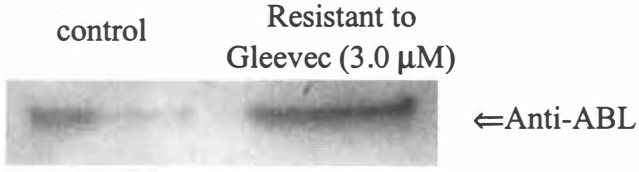


Figure 27. The level of BCR-ABL protein in resistant sublines demonstrated by Western blot. Samples containing 30 μg of total RNA per lane, extracted from K-562 cells resistant to Gleevec, 3.0 μM. The results suggested an increase in BCR-ABL protein expression after the cells have become resistant to Gleevec. These results were consistent in 3 experiments.

Chapter 4

Discussion

Gleevec represents a major therapeutic advance in the management of CML and gastrointestinal stromal tumors.[104,105] It is a small molecule that inhibits BCR-ABL tyrosine kinase activity and selectively kills BCR-ABL-expressing cells both *in vitro* and *in vivo*.[10,11] The response to Gleevec by CML patients in chronic phase, who have failed previous treatment with interferon alfa, has been long-lasting; complete hematological responses were observed in 98% of patients occurring within 4 weeks.[51] Complete cytogenetic responses were found in some individuals, while 54% of patients showed marked cytogenetic responses.[51] On the other hand, remissions observed in blast crisis patients have usually lasted only 2 to 6 months.[48]

Since K-562 cells were derived from a CML patient in blast crisis, they were used as a cell model to probe occurrences within the cells in the process of becoming resistant to Gleevec. We showed the initial effects of Gleevec upon cell-cell contact, morphologic change, and adherence to plastic. We then followed the properties of cell resistance throughout their progression towards resistance to Gleevec by establishing sublines that proliferate in concentrations of Gleevec that were cytotoxic to non-resistant cells.

Gleevec induced erythroid differentiation in K-562 cells, as detected by benzidine-positive cells.[10] Based on the sequential daily observations of the morphology of adherent Gleevec-treated cells, it was assessed that cells progress

towards a hairy cell appearance. For up to 24 hours of treatment with Gleevec, 0.1 μM, the adherent cells had less than 10 hairy appendages and after 24 hours the predominant cells had more than 10 hairs. Hence, we concluded that Gleevec appeared to have induced cells into the morphology of dendritic cells. Though cells with cytoplasmic protrusions, the appearance of a point, the extension of a tail, cell elongation, and/or the appearance of a whip-like extension were sometimes observed when treated with Gleevec, this was very infrequent. These cells had the appearance of the adherent PMA-treated cells, which included both the dendritic and elongated cell lineages. At this time we do not have an explanation for the adherence properties induced by Gleevec.

To further detect the presence of dendritic cells, monoclonal antibodies directed against cell surface markers of differentiation were used to immunophenotype Gleevec-treated cells. Previous reports have shown that Gleevec, 0.25μM, has the ability to augment expression in K-562 cells of CD11b, a marker for the expression of the differentiation antigen associated with immature dendritic cells.[47] They showed that cells co-cultured in Gleevec, 0.25μM, for 7 days increased the percentage of cells expressing CD11b-positive cells from 1.0% in the control cells to 37.1% in the treated cells. We determined that lower doses of Gleevec, 0.1μM, co-cultured for a shorter duration, may or may not have this same applicability as an alternative inducer of expression. In this study, CD11b results showed that two of the three sublines had only moderate increases in expression; however, the flow cytometry results analyzed were only the non-adherent cells. It could be possible that the adherent cells, those observed

with the most morphologic change, may have shown significant CD11b expression while the non-adherent cells would require higher concentrations of Gleevec to induce significant expression.

BCR-ABL has the ability to diminish cellular adhesive properties, including disruption of the integrin/cytoskeleton interactions which modulate receptor avidity within the bone marrow microenvironment.[56,60,66] Our results suggest that Gleevec resulted in *de novo* adhesive responses, since K-562 cells are not adherent under normal growth conditions. Homotypic aggregation has been reported previously by the addition of monoclonal antibodies,[67,68,106] by the transfection of cell adhesion molecules[70,71,77,78,107,108] and with PMA treatment.[69] Results presented herein showed that Gleevec induced similar cell-cell adhesive events. Homotypic aggregation was typically observed within 24 hours following treatment. Cells that had become resistant to Gleevec did not demonstrate homotypic aggregation upon passaging with Gleevec. K-562 cells grown in the growth-conditioned medium of peripheral blood mononuclear cells stimulated with phytohemagglutinin resulted in homotypic aggregation.[109] Homotypic aggregation occurred after increasing the concentration of Gleevec above that to which they had become resistant. These results were interpreted to indicate that Gleevec has direct influence upon the BCR-ABL adherence effects. We also noted the formation of adherent clusters of cells to plastic surfaces. This clustering may mirror differentiation-associated changes due to Gleevec.

Increased staining for filamentous actin (f-actin) and an enhanced rate of formation and retraction of actin-containing protrusions have been previously reported in cells transfected with BCR-ABL.[60] In this study, f-actin involvement within the

previously described morphological changes and cell-cell contact were examined. Induced morphological alterations, including dendritic hairs, pseudopodia and filopodia, were all directly associated with f-actin staining. Additionally, brighter fluorescence at points of cell-cell contact suggests that f-actin was involved at these adhesive junctions.

Adhesive events that we assessed included the increased affinity towards adherence to plastic and increased affinity for cell-cell contact. It was observed that adherent cells could not be removed from the plastic surface following rigorous washing; however, the homotypically aggregated cells could be disaggregated with gentle pipetting. This observation was interpreted to indicate that while both adhesive events were augmented, the binding strength of the cells that adhered to plastic surfaces was much greater than the cell-cell association. This observation indicated that the tenuous cell-cell adhesion involved in homotypic aggregation may precede the stronger adhesion observed in the attachment to plastic surfaces. As observed, most of the morphological changes were observed in the adherent cells. This observation indicated adherent cells were able to undergo morphologic change. Therefore, it appeared that Gleevec initially stimulated cell-cell contact, which promulgated homotypic aggregation. Based upon daily analysis, homotypic aggregation was observed prior to cell adherence. It is proposed that cells with susceptibility to homotypic aggregation may then be capable of additional adherence properties, which could include the attachment to plastic surfaces. These attached cells may then undergo morphological changes. Previous studies at this institution showed that metastatic dissemination of K-562 cells in nude mice in pulmonary capillaries was shown to involve interdigitating cytoplasmic processes, which possibly entails psuedopodia formation. Additional

evidence has shown that the intravascular arrest and extravasation of K-562 cells may have required cell-cell adhesion, formation of a nodule of cells, disruption of the endothelium and alteration of the basement membrane.[110] The characteristics of K-562 cells in culture would suggest that this microenvironment does not provide the necessary milieu for the arrest and extravasation of neoplastic cells. Gleevec may be a drug to further study the adhesive changes involved in the K-562 cell line including the intravascular extravasation of metastatic cells.

Our study examined proliferation, BCR-ABL mRNA and protein expression, and gene amplification in K-562 cells resistant to Gleevec. The proliferative results show that K-562 cell lines can become resistant to increasing concentrations of Gleevec up to 3.0 μM. However, when resistant sublines are exposed to concentrations that are higher than the concentration to which they have become resistant, they are susceptible to cell death. Previous studies indicate that BCR-ABL acts as an anti-apoptosis gene in K-562 cells and suggest that the effect is dependent on the kinase activity protein.[26] As previously stated, Gleevec specifically inhibits or kills BCR-ABL-expressing cells. Inhibition of BCR-ABL with Gleevec could render CML cells susceptible to apoptosis providing an effective strategy for the elimination of these cells. Studies have shown that high concentrations of Gleevec were able to induce apoptosis in K-562 cells as determined by annexin-V-positive cells[47,111] and in BCR-ABL-positive MO7/p^{210} as determined by caspase-3 activation and the regulation of the BCL-X protein.[112] While *in vitro* analysis shows that apoptosis may be an early event following treatment,[111] during clinical treatment with Gleevec, blood counts gradually returned to normal during the first month, suggesting that the drug does not rapidly induce apoptosis.[51]

Analogous with previous reports, our data indicates increased BCR-ABL mRNA when the cells become resistant. Additionally, K-562 sublines resistant to high concentrations of Gleevec (3.0 μM) contain more BCR-ABL tyrosine kinase protein compared to cells resistant to 0.1 μM or control cells. This suggests that increases in BCR-ABL tyrosine kinase may only be detectable in cell lines resistant to high concentrations of Gleevec. K-562 cells resistant to Gleevec 0.5μM, have been shown to express increased BCR-ABL protein without oncogene amplification,[113] although resistance has been shown to be mediated by BCR-ABL gene amplification, both in a CML blast crisis-derived cell line LAMA84,[114] and in BCR-ABL transformed Ba/F3 hemopoietic cells.[113] Results indicate that gene amplification was undetectable in cells resistant to low levels of Gleevec, 0.1 μM, nor was it detectable in cells resistant to higher concentrations of Gleevec, 3.0 μM. We determined that the K-562 cell line is not an optimal cell line in order to measure gene amplification due to the high degree of BCR-ABL gene amplification within the untreated K-562 cells which makes it difficult to detect quantitative signal strength in comparison to the resistant lines.

The development of Gleevec resistance presents new therapeutic challenges for the treatment of CML. This study characterizes some of the features of initial treatment with Gleevec and the progression towards resistance. We assert previously unreported homotypic aggregation and morphological occurrences induced by Gleevec. It is our perspective that individuals in blast crisis could harbor a sub-population of cells that are not sensitive to Gleevec. Mechanisms for resistance may include gene mutation and/or amplification.[113,115] This *in vitro* study may also provide an excellent model for *in vivo*

experiments. Further studies are needed to compare adhesive modifications within the marrow microenvironment and areas of neoplastic growth. Additional considerations include using multiple cell lines, including BCR-ABL transfected cells and fresh cells from a patient with CML. The development of an improved system for the analysis of attached cells would also facilitate future investigations.

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