CHARACTERIZATION OF THE ANTI-LEUKEMIA STEM CELL ACTIVITY OF CHAETOCIN

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By

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ABSTRACT

Chronic myelogenous leukemia is a myeloproliferative hematopoietic stem cell disease resulting from a reciprocal translocation that gives rise to BCR-ABL, a constitutively active tyrosine kinase. Imatinib and other tyrosine kinase inhibitors are currently standard therapy; however, point mutations often lead to drug resistance and disease relapse often occurs due to the persistence of quiescent leukemia stem cells that are shielded by stromal factors within the bone marrow microenvironment. In an effort to develop new therapies capable of eradicating these elusive cells, a novel approach has been proposed in which the biochemical properties of cancer cells are targeted. It has been established that one such property is oxidative stress due to the increased production of reactive oxygen species, which makes cancer cells especially dependent on their antioxidant systems to maintain redox homeostasis. Recent studies demonstrate that chaetocin, a mycotoxin produced by *Chaetomium* species fungi, possesses potent and specific antimyeloma activity due in part to its ability to inhibit thioredoxin reductase-1, a central oxidative stress remediation enzyme. In this study, the effectiveness of chaetocin against leukemia stem cells has been investigated using *in vitro* and *in vivo* murine chronic myelogenous leukemia models. Our results indicate that: chaetocin and imatinib function synergistically in decreasing cell viability, inducing apoptosis, and inhibiting the colony formation of chronic myelogenous leukemia cells *in vitro*; that chaetocin in combination with imatinib reduces leukemia stem cell frequency *in vivo*; that chaetocin increases intracellular reactive oxygen species levels; and that chaetocin does not disrupt the proliferation and differentiation of normal murine hematopoietic stem cells. Surprisingly, our results also show that while bone marrow stromal factors inhibit the activity of imatinib, they potentiate the activity of chaetocin, indicating that chaetocin could potentially be used to target leukemia stem cells within the bone marrow niche.

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for Will

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LIST OF ABBREVIATIONS

ABL	Abelson
BCR	Breakpoint Cluster Region
BFU-E	Burst-Forming Unit-Erythroid
BMSF	Bone Marrow Stromal Factor
CFC	Colony Forming Cell
CFU	Colony-Forming Unit
CFU-E	CFU-Erythroid
CFU-G	CFU-Granulocyte
CFU-GEMM	CFU-Granulocyte, Erthyroid, Macrophage, Megakaryocyte
CFU-GM	CFU-Granulocyte, Macrophage
CFU-M	CFU-Macrophage
CI	Combination Index
CJ	Craig Jordan
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
c-Kit	Mast/Stem Cell Growth Factor Receptor
CML	Chronic Myelogenous Leukemia
DMSO	Dimethyl Sulfoxide
dR6G	Dihydrorhodamine 6G
ED	Effective Dose
FBS	Fetal Bovine Serum
FITC	Fluorescein Isothiocyanate
Flt3	FMS-like Tyrosine Kinase 3 Ligand
GFP	Green Fluorescent Protein
GMP	Granulocyte-Macrophage Progenitor
H3K9	Histone 3 Lysine 9
HIF-1α	Hypoxia-Inducible Factor 1α
HOXA9	Homeobox Protein A9

HSC	Hematopoietic Stem Cell
IC_{50}	Half-Maximal Inhibitory Concentration
IL-3	Interleukin-3
IL-6	Interleukin-6
IMDM	Iscove's Modified Dulbecco's Medium
LDA	Limiting Dilution Analysis
Lin	Lineage
LSC	Leukemia Stem Cell
MEP	Megakaryocyte-Erythrocyte Progenitor
Nup98	Nuclear Pore Complex Protein 98
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PE	Phycoerythrin
Ph	Philadelphia Chromosome
PS	Phosphatidylserine
Rad	Radiation Absorbed Dose
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute Medium
Sca-1	Stem Cell Antigen 1
SCF	Stem Cell Factor
SD	Standard Deviation
SUV39H1	Suppressor of Variegation 3-9 Homolog 1
T315I	Threonine Substituted by Isoleucine at Position 315
TKI	Tyrosine Kinase Inhibitor
TrxR1	Thioredoxin Reductase-1
YFP	Yellow Fluorescent Protein

1. REVIEW OF THE LITERATURE

1.1 Introduction

Myeloid leukemias are heterogeneous life-threatening bone marrow neoplasms believed to derive from transformed normal hematopoietic cells (Bonnet and Dick, 1997; Blair *et al*., 1998; Holyoake *et al*., 2001). Chronic myelogenous leukemia (CML) in particular is known to arise after a reciprocal translocation between chromosomes 9 and 22, which results in formation of the BCR-ABL oncogene. In Canada, there are approximately 460 new cases of CML diagnosed each year and it is estimated that roughly 3,000 Canadians are currently living with this form of leukemia (http://www.cmlsociety.org). Although CML is a rare form of leukemia, accounting for only 20% of all adult and 2.6% of childhood leukemias (http://seer.cancer.gov/statfacts/html/cmyl.html) it has become a paradigm of successful cancer therapy based on a rational treatment approach. With the advent of imatinib and the second generation tyrosine kinase inhibitors, dasatinib and nilotinib, small molecule drugs have become mainstay for first-line CML management (Deininger *et al*., 2009; Kantarjian *et al*., 2010; Saglio *et al*., 2010; Nicolini *et al*., 2011). Although these therapies are highly effective, with overall survival of CML now at greater than 90% (Kantarjian *et al*., 2011; Signorovitch *et al*., 2011; Tauchi *et al*., 2011), they do not represent a cure since they are unable to eradicate the leukemia stem cells that are responsible for disease relapse upon drug discontinuation, meaning that CML patients are forced to continue this expensive drug therapy for the rest of their lives. This, among other factors, is believed to be responsible for the growing noncompliance seen among CML patients (Crews and Jamieson, 2012).

It is clear that in order to cure CML, the quiescent leukemia stem cells that reside primarily in the protective bone marrow niche must be eliminated. Since these cells are supported by bone marrow stromal factors and are therefore not reliant on BCR-ABL for survival, tyrosine kinase inhibitors are ineffective against them (Hu *et al*., 2006). One method of targeting these cells is to exploit their biochemical properties. It has been established that cancer cells are under oxidative stress due to increased metabolism and the production of reactive oxygen species (ROS) (Szatrovski *et al.,* 1991), which aids their growth and proliferation (Hu *et al*., 2005) but also makes them highly dependent on their antioxidant

defense systems to maintain redox homeostasis. This dependence could therefore be exploited by inhibiting one or more of these antioxidant systems, resulting in the accumulation of cytotoxic levels of ROS.

Recent reports have shown than chaetocin, a mycotoxin produced by *Chaetomium* species fungi has potent and specific *in vitro* and *in vivo* antimyeloma activity (Isham *et al*., 2007) due, at least in part, to its ability to inhibit thioredoxin reductase-1, a central ROS remediation enzyme (Tibodeau *et al*., 2009). The general aim of this study was to characterize the effectiveness of chaetocin against chronic myelogenous leukemia stem cells both *in vitro* and *in vivo* by investigating the ability of chaetocin to increase intracellular ROS levels, induce apoptosis, and prevent colony formation, as well as its ability to function synergistically with imatinib.

1.2 Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a myeloproliferative stem cell disease resulting from a reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11)] in a hematopoietic stem cell (HSC). This translocation fuses the breakpoint cluster region (BCR) gene on chromosome 22 with the Abelson oncogene (ABL) on chromosome 9, resulting in formation of the Philadelphia (Ph) chromosome which encodes the constitutively active tyrosine kinase, BCR-ABL (Woessner *et al*., 2011), as shown in Figure 1.1. Unlike the primarily nuclear c-ABL, the Bcr-Abl oncoprotein is distributed throughout the cytoplasm and interacts with various proteins involved in signal transduction pathways leading to deregulated proliferation, differentiation, and survival (Sattler and Griffin, 2003). The Ph chromosome occurs in over 90% of CML cases and its presence, along with other symptoms, is diagnostic of CML (Chen *et al*., 2010).

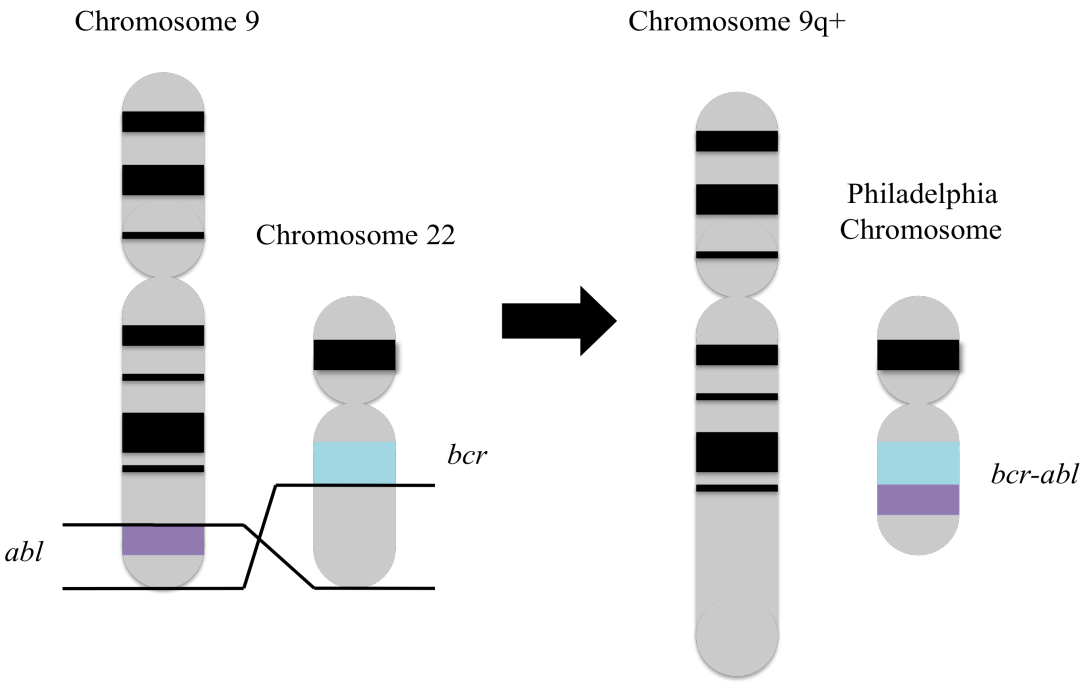

Figure 1.1 Chromosomal translocation resulting in formation of the Philadelphia chromosome. Reprinted with permission from Elsevier (Enright and Bond, 2008).

The disease course of CML is triphasic, starting with a chronic phase, progressing to an accelerated phase, and ultimately ending in a terminal phase known as blast crisis (Chen *et al*., 2010). Patients are typically diagnosed in the chronic phase and often present with splenomegaly and left-shifted neutrophilic leukocytosis (Woessner *et al*., 2011). In the absence of treatment, the patient inexorably progresses through to blast crisis, characterized by gradual or sudden loss of differentiation capacity, poor response to treatment, and short survival (Vardiman *et al*., 2002).

In the mid-1970s, allogeneic hematopoietic stem cell transplant was pioneered and was the first CML therapy shown to induce a state of Ph-negativity. Despite advancements that have been made since then, this is the only treatment considered to have the potential to cure CML but, due to the limited number of donors available, is reserved for patients which have progressed to blast crisis (Breccia *et al*., 2010; Venepalli *et al*., 2010; Oyekunle *et al*., 2011).

CML was the first cancer to be associated with a pathognomonic chromosomal translocation and is the most extensively studied cancer from a molecular standpoint (Daley *et al*., 1991; Pear *et al*., 1998; Deininger *et al*., 2000). Since the BCR-ABL oncoprotein has been

well defined and is not only required but also sufficient for transformation, CML has become a model disease in cancer biology (Rodrigues *et al*., 2008). This intensive study led to the development of imatinib, a BCR-ABL tyrosine kinase inhibitor (TKI) that is currently standard therapy for CML, yielding survival rates over 90%. However, imatinib-based therapy faces three major challenges. First, drug resistance occurs in approximately 40% of patients due to point mutations in the BCR-ABL kinase domain (Branford *et al*., 2003; Nardi *et al*., 2004). The development of more potent, second generation TKIs (e.g., nilotinib and dasatinib) allows for treatment of imatinib-resistant mutations but further mutations often develop later (Shah *et al*., 2004; Weisberg *et al*., 2005). Importantly, the T315I mutation has been found to be resistant to all currently approved TKIs and poses a significant threat since it is reported to represent 15-20% of all BCR-ABL mutations (Burke *et al*., 2011). Second is the limited response in patients who have progressed to blast crisis (Sawyers *et al*., 2002). Additional chromosomal and molecular changes likely free these cells from their dependence on BCR-ABL for survival and thus they do not respond to TKI therapy (Calabretta and Perrotti, 2004). Third, CML stem cells are insensitive to all TKIs (Grahm *et al*., 2002; Copland *et al*., 2006). Although CML patients may be in complete cytogenetic remission due to TKI therapy, they still contain malignant hematopoietic progenitor cells (Bhatia *et al*., 2003) and drug discontinuation usually results in disease recurrence. Thus, CML patients must continue drug treatment indefinitely (Woessner *et al*., 2011). However, due in part to the spiraling costs of full dose TKI therapy, a growing proportion of CML patients are non-compliant and thus progress to advanced phase disease with a five year survival rate of less than 30% (Crews and Jamieson, 2012).

1.3 Cancer Stem Cells

Recurrence of cancer after chemotherapy or radiotherapy is initiated by a subpopulation of residual malignant cells that are highly resistant to treatment and which are believed to be cancer-initiating cells or cancer stem cells (Eyler and Rich, 2008; Visvader and Lindeman, 2008). These cells share essential functional properties such as the capacity to become quiescent, acquire multi-lineage differentiation potential, survive, and self-renew (Jamieson *et al*., 2008).

Leukemia stem cells (LSCs) reside at the apex of a hierarchy of malignant cells that is analogous to the hierarchy found in normal hematopoiesis (Fig. 1.2 and 1.3) and are stringently defined by functional attributes such as the ability to instigate, maintain, and serially propagate leukemia *in vivo* while retaining the capacity to differentiate into committed progeny that lack these properties (Bonnet and Dick, 1997; Jordan *et al*., 2000; Huntly *et al*., 2004). More specifically, it has been determined that BCR-ABL-expressing Lin^- c-Kit^+ Sca-1^+ cells function as LSCs in chronic phase CML (Hu *et al*., 2006) whereas granulocyte macrophage progenitors function as LSCs in blast crisis CML (Wang and Dick, 2005), the different properties of which may account for the difference between these two phases.

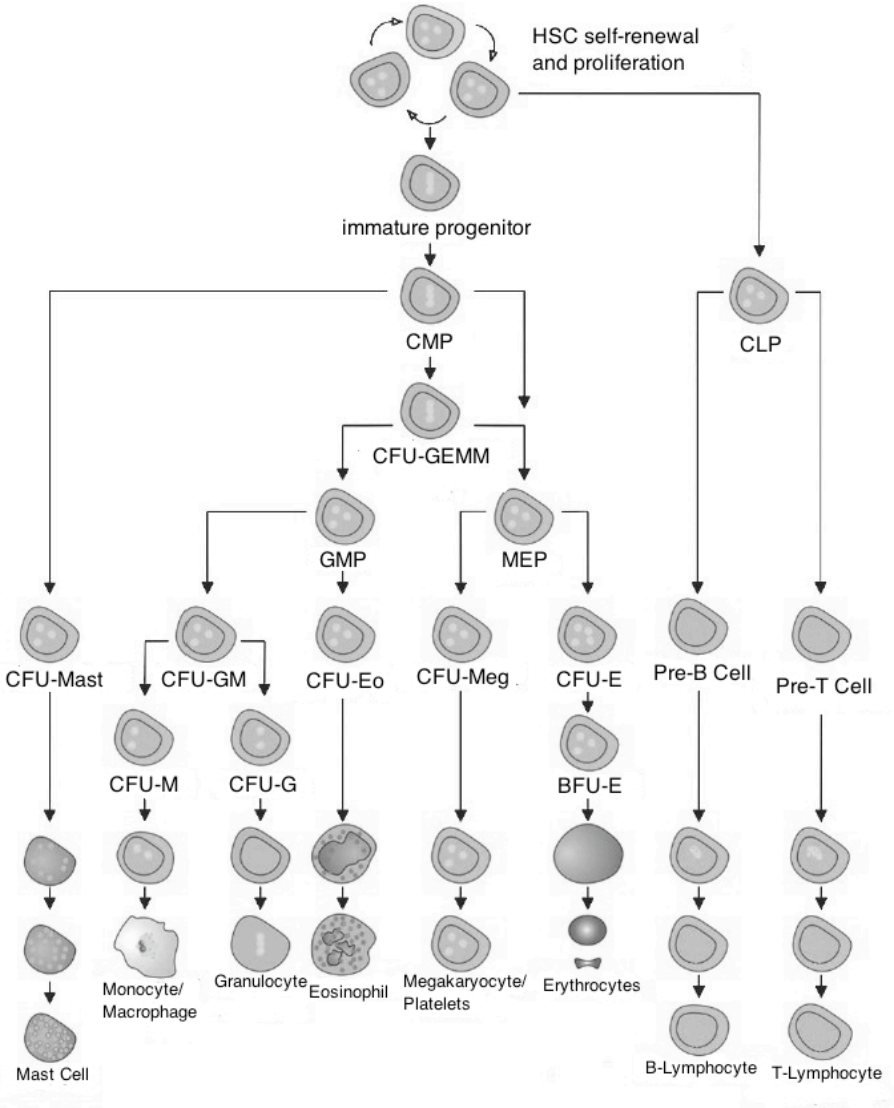

Figure 1.2 Hierarchy of normal hematopoiesis in humans. Self-renewing HSCs give rise to several multipotent progenitors, including common myeloid progenitors (CMP) and common lymphoid progenitors (CLP), which produce oligopotent progenitors, unipotent progenitors, and finally, fully differentiated cells. The CMP is able to produce granulocyte-macrophage progenitors (GMP) and megakaryocyte/erthrocyte progenitors (MEP) giving rise to monocytes/macrophages/granulocytes and megakaryocytes/platelets/erthryocytes, respectively. Pro-erythroblast colony forming unit-erythroid (CFU-E) gives rise to erythrocytes and the CLP gives rise to pre-B and pre-T cells, which continue to mature B and T lymphocytes. Reprinted with permission from Pulmonary Circulation (Firth and Yuan, 2012).

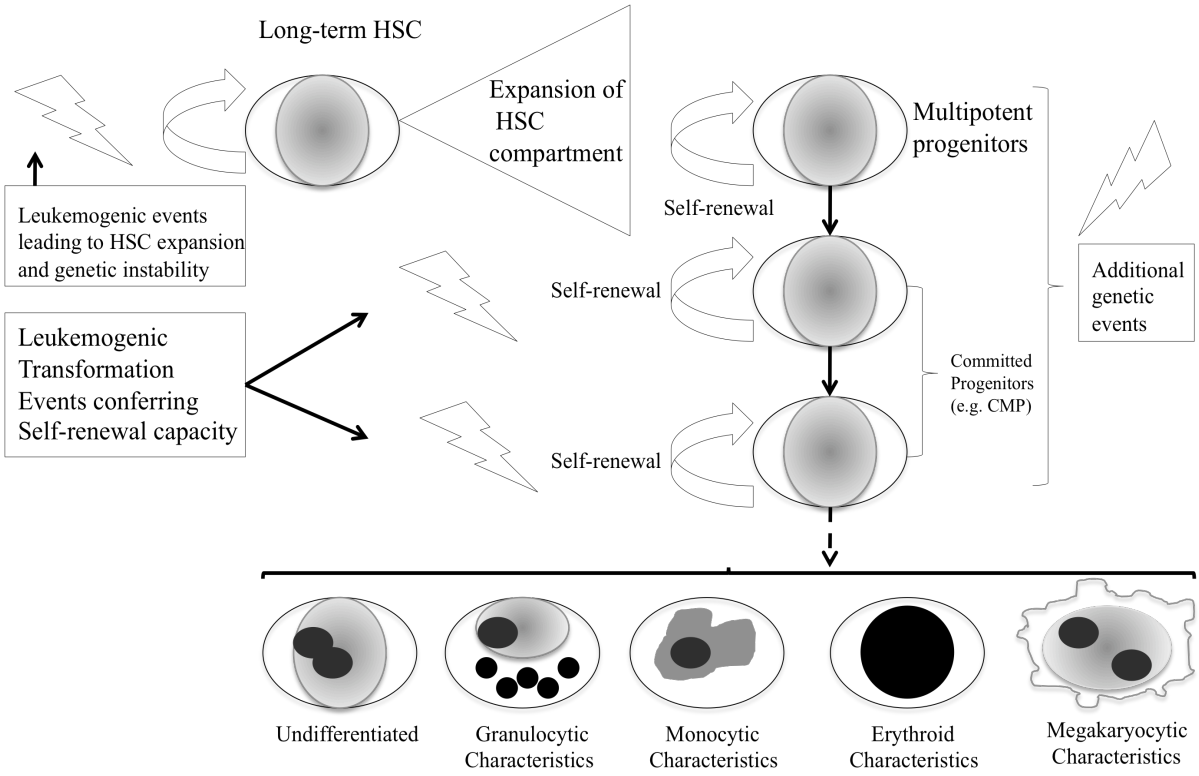

Figure 1.3 Hierarchy of leukemia stem cells (LSCs). Oncogenic mutations may occur within long-term hematopoietic stem cells (HSCs) or in committed downstream progenitors. Mutations within long-term HSCs may give rise to a pre-leukemia state with expanded HSC numbers and genetic instability, leading to additional genetic events. Conversely, leukemogenic events that confer limitless self-renewal can transform HSCs or committed progenitors. Reprinted with permission from Elsevier (Lane and Gilliland, 2010).

LSCs, like their normal HSC counterparts, possess a range of characteristics that enable their long-term survival and facilitate their escape from the cytotoxic effects of chemotherapy (Mahadevan and List, 2004). By reducing this cytotoxic stress, LSCs become a reservoir for the selection of mutants that are resistant to targeted or conventional therapy (Heidel *et al*., 2006). For this reason, a significant percentage of CML patients are expected to develop TKI resistance driven by quiescent LSCs (Crews and Jamieson, 2012).

1.3.1 Bone Marrow Stromal Factors

Substantial emerging evidence indicates that LSCs do not exist primarily in blood circulation but instead reside within and utilize the bone marrow microenvironment, taking refuge in this niche during chemotherapy and later re-emerging to initiate disease relapse. LSCs home to and

engraft the bone marrow niche where they are somewhat protected from chemotherapy-induced apoptosis, potentially through niche-induced LSC quiescence (Ishikawa *et al*., 2007).

The bone marrow is a hematopoietic organ that resides within the protective confines of bones and is the major site of hematopoiesis. The primary function of bone marrow is to maintain the numbers of differentiated hematopoietic cells in the peripheral blood at a constant level (Fliedner *et al*., 2002). Under physiological conditions, normal HSCs residing in the bone marrow niche receive cell-extrinsic support from the heterogeneous cell populations that comprise the surrounding hematopoietic microenvironment, including primary cells of mesenchymal lineages, endothelial and perivascular cells, and adipocytes (Lane *et al*., 2009). Interactions between HSCs and the bone marrow niche can occur as a result of direct intracellular contact, secreted factors such as cytokines and chemokines, or through microenvironmental matrix proteins that regulate cellular adhesion and migration, as well as growth factor receptor and integrin signals, among others (Chiodoni *et al*., 2010) as illustrated in Figure 1.4.

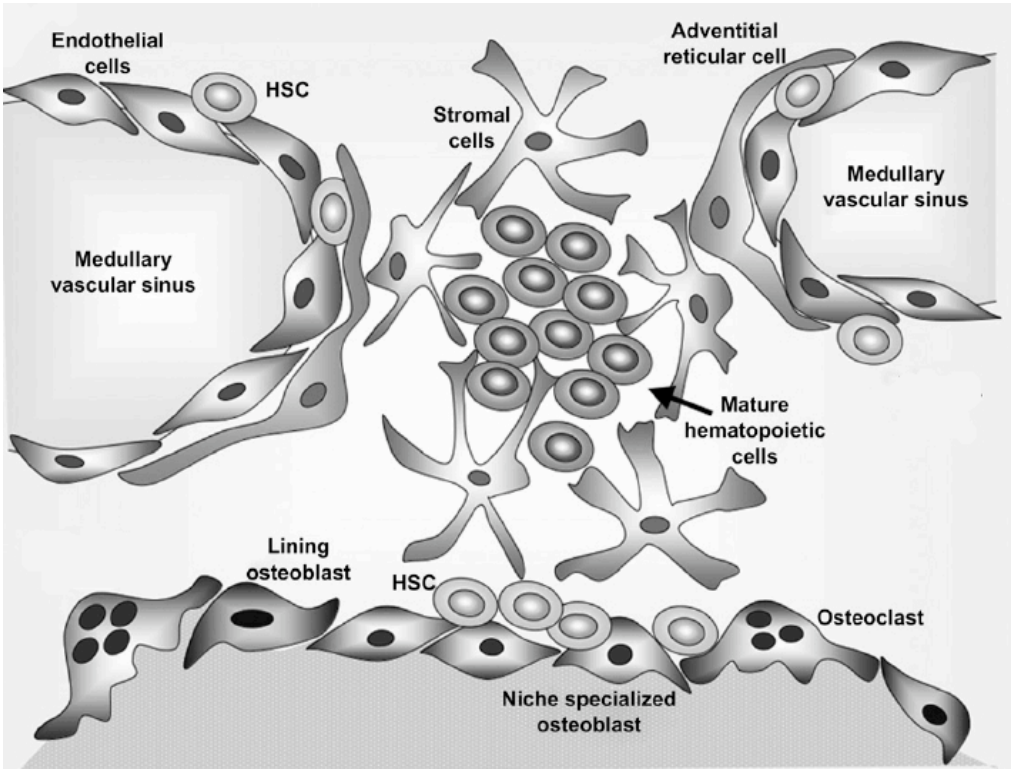

Figure 1.4 The bone marrow microenvironment. Hematopoietic stem cells receive support from stromal factors within the bone marrow microenvironment. Reprinted with permission from Elsevier (Di Maggio *et al*., 2011).

LSCs also receive vital cues from the bone marrow microenvironment that dictate their behavior and eventual disease phenotype. Evidence suggests that LSCs in turn have an effect on the niche and may even circumvent normal constraints to create their own distinct pathological niche at the expense of normal HSCs, leading to disproportionate impairment of HSC engraftment and hematopoietic function (Colmone *et al*., 2008). In addition, evidence suggests that normal HSCs can be altered by signals within a pathological niche, leading to hematopoietic dyscrasias (Walkley *et al*., 2007a, 2007b).

It has been determined that while TKIs are capable of inducing apoptosis in CML cells through the inhibition of BCR-ABL, LSCs are not dependent on BCR-ABL for survival and thus are not eliminated by TKIs. This indicates that LSCs rely on survival signals other than BCR-ABL and it is likely that these signals are provided by the bone marrow niche (Corbin *et al*., 2011). In support of this theory, it has been demonstrated that soluble factors secreted by HS-5 stromal cells are sufficient to cause resistance to TKIs in CML cells (Bewry *et al*., 2008). Furthermore, it has recently been shown that TKI therapy promotes the migration of LSCs to the bone marrow via activation of inflammatory signaling receptors, fostering the survival of quiescent LSCs in the bone marrow niche (Jin *et al*., 2008).

The development of therapeutic strategies capable of eradicating LSCs in supportive niches while sparing normal HSC function would represent an important complement to current TKI treatment but such therapies remain elusive to date (Essers and Trumpp, 2010).

1.4 Reactive Oxygen Species in Cancer

Reactive oxygen species (ROS) are broadly defined as oxygen-containing, reactive chemical species. Two types of ROS are present in biological systems: free radicals, which contain one or more unpaired electrons in their outer molecular orbits, and non-radical ROS, which do not contain unpaired electrons but are chemically reactive and can be converted to radical ROS (Trachootham *et al*., 2009).

ROS play essential roles in living organisms in that they regulate many signal transduction pathways by directly reacting with and modifying the structure of proteins and genes in order to modulate their functions. More specifically, ROS are involved in signaling cell growth and differentiation, regulating the activity of enzymes, mediating inflammation by

stimulating cytokine production, and eliminating pathogens and foreign particles. However, ROS can also have harmful effects within the cell since oxygen-containing free radicals are highly reactive with biological molecules; a mild increase in ROS may promote cell proliferation and differentiation (Schafer and Buettner, 2001; Boonstra and Post, 2004), whereas a severe increase in ROS can cause oxidative damage to lipids, proteins, and DNA (Perry *et al*., 2000), which may ultimately result in cell death (Trachootham *et al*., 2009). Maintenance of ROS homeostasis is therefore essential for normal cell growth and survival. Cellular ROS levels are controlled by ROS-scavenging systems such as superoxide dismutases, glutathione peroxidase, peroxiredoxins, glutaredoxin, thioredoxin, and catalase (Trachootham *et al*., 2009).

It has been established that cancer cells are under oxidative stress due to increased metabolic activity and the production of ROS (Szatrovski *et al*., 1991). Although the precise pathways involved remain unclear, it is known that the activation of oncogenes, aberrant metabolism, mitochondrial dysfunction, and loss of functional p53 are intrinsic factors responsible for increased ROS production in cancer cells (Irani *et al*., 1997; Brandon *et al*., 2006; Horn and Vousden, 2007; Rodrigues *et al*., 2008). For example, transformation of hematopoietic cells by the oncogenic tyrosine kinase, BCR-ABL is associated with a chronic increase in intracellular ROS (Sattler *et al*., 2000). This increased level of ROS is believed to play an important role in maintaining cancer phenotype through its stimulating effects on cell growth and proliferation (Hu *et al*., 2005), genetic instability (Radisky *et al*., 2005), and senescence evasion (Chen *et al*., 2005). This intrinsic oxidative stress associated with oncogenic transformation may render cancer cells highly dependent on their antioxidant systems in order to maintain redox balance (Trachootham *et al*., 2006). Supporting this theory, aberrant regulation of redox homeostasis and stress adaptation have been shown to occur in cancer cells, demonstrated by the significantly altered levels of ROS-scavenging enzymes seen in malignant cells (Oberley and Oberley, 1997) and primary cancer tissues (Saydam *et al*., 1997; Hu *et al*., 2005; Murawaki *et al*., 2008).

One ROS mitigating enzyme known to be upregulated in a variety of human cancers is thioredoxin reductase-1 (TrxR1) (Rundlöf and Arnér, 2004; Biaglow and Miller, 2005), a glutathione reductase-like flavoenzyme that participates in diverse metabolic reactions involving oxidation-reduction cycles (Fig. 1.5) and is widely believed to be central to

intracellular ROS mitigation (Arnér and Holmgren, 2000; Becker *et al*., 2000; Williams *et al*., 2000). Overexpression of TrxR1 has been linked to aggressive tumour growth and poorer prognosis (Kakolyris *et al*., 2001; Raffel *et al*., 2003), enhanced tumour proliferation via regulatory effects on the G_1 checkpoint during cell cycle progression (Smart *et al*., 2004), invocation of a pro-survival signaling cascade (Wei *et al*., 2000; Smart *et al*., 2004), and greater resistance against some anticancer agents (Kirkpatrick *et al*., 1998). This evidence suggests that upregulated TrxR1 activity may, at least in part, account for how cancer cells have adapted to their generally higher basal levels of cellular oxidative stress (Pelicano *et al*., 2004) and is therefore a potentially useful antineoplastic molecular target.

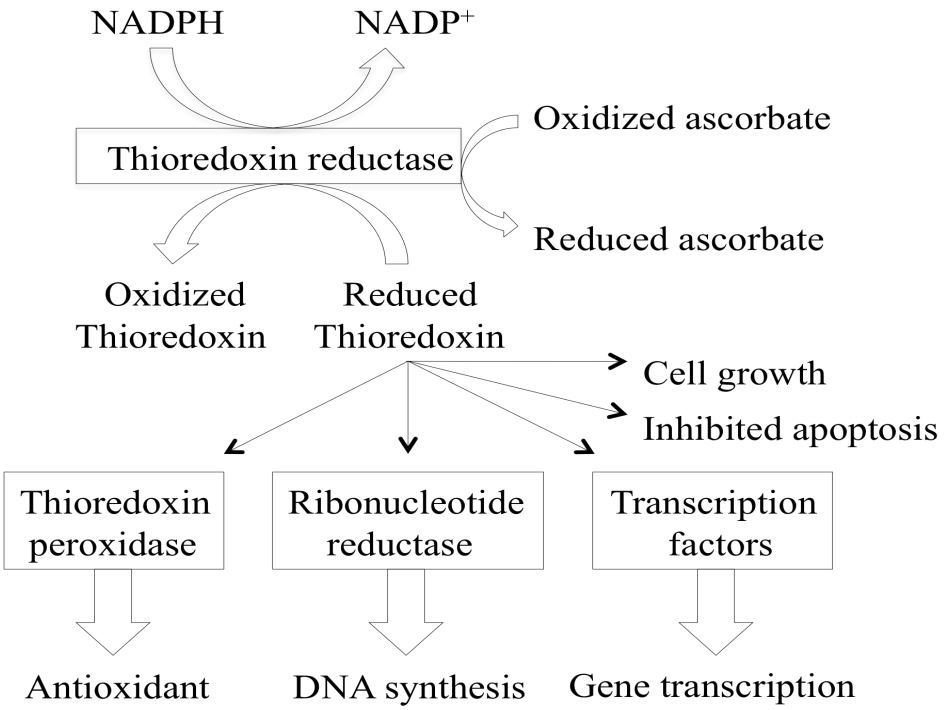

Figure 1.5 Reactions and functions of thioredoxin reductase in the cell. Reprinted with permission from the Biochemical Society (Mustacich and Powis, 2000).

1.5 Chaetocin

Chaetocin is a mycotoxin produced by *Chaetomium* species fungi and is a representative thiodioxopiperazine, a class of fungal secondary metabolites with structural similarities. The chemical structure of chaetocin was elucidated in 1970 and found to contain an unusual bridged

disulfide diketopiperazine core (Hauser *et al*., 1970), shown in Figure 1.6. Other dioxopiperazines have been reported to have antimicrobial (Katagiri *et al*., 1970), antiparasitic (Dong *et al*., 2005), antiviral (Neuss *et al*., 1968), immunosuppressive (Yamada *et al*., 2000), and/or anti-inflammatory effects (Kawahara *et al*., 1990) while the biological effects of chaetocin have remained largely unexplored (Isham *et al*., 2007).

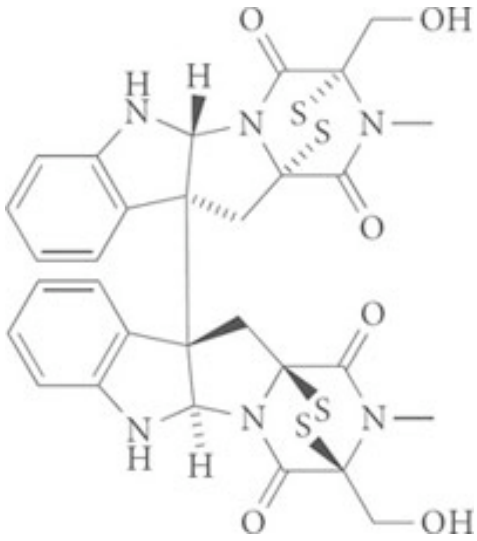

Figure 1.6 Chemical structure of chaetocin.

The recent discovery of the potent and selective *in vitro, in vivo*, and *ex vivo* anticancer activity of chaetocin (Isham *et al*., 2007) sparked great interest in the cancer research field. Research has shown that chaetocin dramatically accumulates in cancer cells via a process inhibited by glutathione and requiring unreduced disulfides for uptake. Once inside the cell, the anticancer activity of chaetocin appears to be mediated primarily through the imposition of oxidative stress and consequent induction of apoptosis. As discussed above, cancer cells have a heightened sensitivity to the cytotoxic effects of imposed oxidative stress, which likely contributes to the selective anticancer effects of chaetocin (Isham *et al*., 2007).

The ability of chaetocin to inflict oxidative stress has been specifically linked to its action as a competitive substrate for, and inhibitor of, the central oxidative stress remediation enzyme, thioredoxin reductase-1 (TrxR1), as illustrated in Figure 1.7. By competing with thioredoxin for reduction by TrxR1, chaetocin depletes levels of reduced cellular Thx, a

survival-critical ROS remediation substrate and downstream effector of TrxR1. The importance of ROS imposition to chaetocin-induced cytotoxicity has been further demonstrated by its abrogation when cells are co-treated with *N*-acetyl cysteine, a cell permeable reduced glutathione precursor (Isham *et al*., 2007).

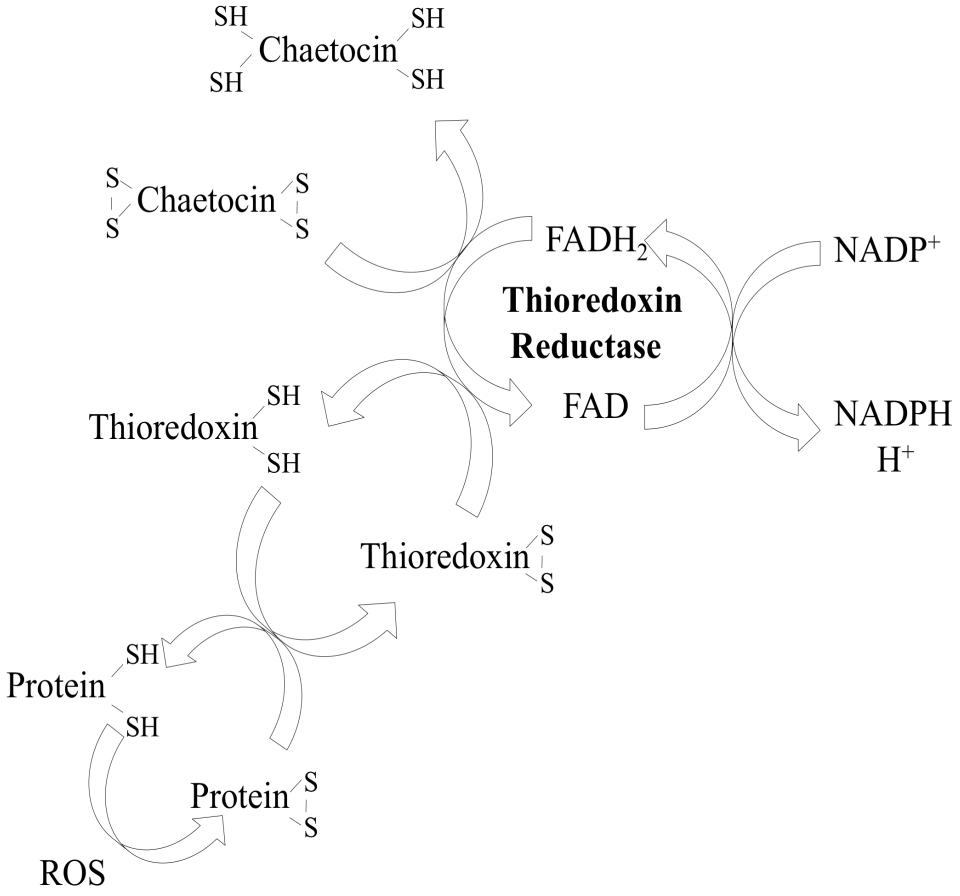

Figure 1.7 Model of the observed effects of chaetocin on the Trx/TrxR1 pathway. Chaetocin competes with thioredoxin as a substrate for thioredoxin reductase, thereby serving to attenuate reduction of the thioredoxin reductase downstream substrate and effector, thioredoxin. Reprinted with permission from Antioxidants and Redox Signaling (Tibodeau *et al*., 2009).

In addition to its ability to impose cytotoxic oxidative stress, chaetocin has been found to potently and specifically inhibit the suppressor of variegation 3-9 homolog 1 (SUV39H1) enzyme in an ROS-dependent manner (Greiner *et al*., 2005). SUV39H1 is the main histone methyltransferase responsible for the accumulation of histone H3 containing a tri-methyl group at its lysine 9 position (H3K9me3) in heterochromatin (Goyama *et al*., 2010) and its depletion

directly induces death receptor-mediated apoptosis (Chaib *et al*., 2012).

Other effects of chaetocin on cancer cells that have recently been reported include mitochondrial membrane depolarization, morphological changes including vacuole and condensed chromatin cluster formation (Illner *et al*., 2010), HIF-1α down-regulation caused by its deregulation of HIF-1α pre-messenger RNA splicing (Kung *et al*., 2004; Lee *et al*., 2011), and attenuation of endothelial cell proliferation and tumour-associated vascularity *in vivo*, which demonstrates the potential of chaetocin to act directly in the tumour microenvironment. Importantly, it has also recently been uncovered that chaetocin is capable of inducing necrotic cell death, largely independent of reliance on intact programmed cell death (PCD) pathways. This discovery indicates that chaetocin has the potential to be effective in killing cancer cells harbouring defective PCD pathways that might otherwise be resistant to other therapeutics. It has been proposed that the imposition of oxidative stress by chaetocin may serve as a catastrophic cellular insult capable of killing cells by necrosis even if apoptotic and autophagic pathways are disregulated (Isham *et al*., 2012).

2. HYPOTHESIS AND SPECIFIC AIMS

We hypothesized that chaetocin induces apoptosis through the imposition of cytotoxic levels of oxidative stress in chronic myelogenous leukemia stem cells both *in vitro* and *in vivo* while sparing normal hematopoietic stem cells and that chaetocin functions synergistically with imatinib. We developed the following aims to test our hypothesis.

2.1 Assess the Activity of Chaetocin in the Murine CML Model Cell Line TonB210

TonB210 is a murine hematopoietic cell line with doxycycline-inducible BCR-ABL expression (Klucher *et al*., 1998). This cell line shows an absolute dependence on doxycycline for cell survival and proliferation *in vitro* in the absence of IL-3, correlating with BCR-ABL expression. This cell line was used to compare the activity of chaetocin in the presence and absence of BCR-ABL expression by measuring cell viability, apoptosis, and colony formation. In addition, the activity of chaetocin was evaluated in the presence of bone marrow stromal factors (BMSFs).

2.2 Assess the Activity of Chaetocin in the Murine CML Model CJ Cells

Neering *et al.* have developed a CML model using retroviral vectors to co-express the BCR/ABL and Nup98/HOXA9 translocation products, both of which have been previously documented in leukemia patients. The Nup98/HOXA9 translocation is a transcription factor-based anomaly that inhibits cellular differentiation. This CML model, which we refer to as Craig Jordan (CJ) cells, provides a platform from which the properties of normal versus malignant stem cells can be examined and the relative effects of therapeutic regimens evaluated (Neering *et al.*, 2007). As with the cell line TonB210, CJ cells were used to assess the effects of chaetocin, both alone and in combination with imatinib, on cell viability, apoptosis, and colony formation, both in the absence and presence of BMSFs.

2.3 Assess the Activity of Chaetocin in Normal Murine Hematopoietic Stem Cells

Others have speculated that since cancer cells have a higher basal level of oxidative stress compared to normal cells, chaetocin will induce cytotoxic levels of oxidative stress in cancer cells, while normal cells will be able to withstand this assault (Isham *et al*., 2007). In order to test this theory, we isolated normal murine hematopoietic stem cells and treated them with chaetocin, both alone and in combination with imatinib, and evaluated their effects on cell viability and colony formation.

2.4 Determine the Effect of Chaetocin on Chronic Myelogenous Leukemia Disease Latency and Stem Cell Frequency *In Vivo*

It has been shown that CJ cells are capable of establishing a CML phenotype when injected intravenously into recipient mice (Neering *et al.*, 2007). In order to test the ability of chaetocin to interfere with this process, CJ cells were treated with chaetocin, both alone and in combination with imatinib, before injection. Since mice afflicted with CML display a specific set of symptoms, it was possible to determine the differences in time required for the establishment of CML among each treatment group. Mice were euthanized once symptoms of CML became apparent.

In order to determine the effect of chaetocin on stem cell frequency, a limiting dilution assay was performed in which CJ cells were treated with chaetocin both alone and in combination with imatinib before being injected into mice at a range of different cell concentrations. As before, the mice were euthanized once symptoms of illness became apparent. The results were then analyzed using L-Calc software (StemCell Technologies) in order to determine the stem cell frequency in each treatment group.

2.5 Assess the Effect of Chaetocin on Intracellular Reactive Oxygen Species

In order to determine the effects of chaetocin on intracellular ROS levels, cells were treated with chaetocin or imatinib and the levels of ROS were measured using an ROS-sensitive dye and flow cytometry.

2.6 Investigate the Synergy/Antagonism of Chaetocin and Imatinib

In order to determine whether chaetocin and imatinib function synergistically or antagonistically, cells were treated with chaetocin and imatinib, both alone and in combination, at a range of different concentrations. CalcuSyn software (Biosoft) was then used to analyze the results.

3. MATERIALS AND METHODS

3.1 Reagents and Suppliers

The reagents used for experiments in this thesis were all molecular biology or reagent grade and are listed in Table 3.1. Several of the procedures used in this study were performed using commercially available kits, which are listed in Table 3.2. Table 3.3 lists the companies from which all reagents and kits were obtained.

Table 3.1 List of Reagents and Suppliers

Reagent	Supplier
Chaetocin	Sigma-Aldrich
DMSO	Sigma-Aldrich
Doxycycline Hyclate	Sigma-Aldrich
dR6G	Invitrogen Life Technologies
FBS	Invitrogen Life Technologies
Flt3	R&D Systems
IL-3	R&D Systems
IL-6	R&D Systems
Imatinib Mesylate	LC Labs
IMDM	Invitrogen Life Technologies
Penicillin/Streptomycin, 100X Mix	Invitrogen Life Technologies
RPMI	Invitrogen Life Technologies
SCF	R&D Systems
Trypan Blue, 0.4%	Invitrogen Life Technologies
Trypsin	Invitrogen Life Technologies
MethoCult® GF M3434	StemCell Technologies

Table 3.2 List of Commercially Available Kits

Commercially Available Kit	Company
Annexin V : FITC Apoptosis Detection Kit I	BD Pharmingen
Annexin V : PE Apoptosis Detection Kit I	BD Pharmingen
Lineage Cell Depletion Kit	Miltenyi Biotec

Table 3.3 Names and Addresses of Suppliers

Supplier	Address
BD Pharmingen	Mississauga, Ontario, Canada
Biosoft	Cambridge, United Kingdom
Invitrogen Life Technologies	Burlington, Ontario, Canada
LC Labs	Woburn, Massachusetts, USA
Miltenyi Biotec	Auburn, California, USA
R&D Systems	Minneapolis, Minnesota, USA
Sigma-Aldrich	Oakville, Ontario, Canada
StemCell Technologies	Vancouver, British Columbia, Canada

3.2 Cell Lines and Tissue Culture

All cultures contained 1% (v/v) penicillin/streptomycin solution (Gibco) and were maintained at 37°C with 5% CO_2. All cell culture media were supplied by Invitrogen. CJ cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 20% (v/v) fetal bovine serum (FBS, Invitrogen). TonB210 cells were cultured in Roswell Park Memorial Institute Medium (RPMI) with 10% (v/v) FBS supplemented with either 10 ng/mL IL-3 or 2 μg/mL doxycycline. M2-10B4 cells were cultured in RPMI with 10% (v/v) FBS. Lineage depleted murine HSCs were cultured in IMDM containing 20% (v/v) FBS and cytokine cocktail (25 ng/mL SCF, 25 ng/mL Flt3, 10 ng/mL IL-6, and 10 ng/mL IL-3).

3.2.1 Bone Marrow Stromal Factor Pretreatment

M2-10B4 cells were cultured as described above for several days before collecting the culture medium, known to contain secreted BMSFs (Sutherland *et al*., 1991), for storage at -80°C. Cells were pretreated with this BMSF-containing medium diluted 1:1 with fresh culture medium for four hours at 37°C with 5% CO_2 before the addition of drug(s).

3.3 Trypan Blue Exclusion Assay

Cell viability was determined by Trypan Blue exclusion assay. Briefly, cells were cultured as above and treated with chaetocin and/or imatinib. A culture sample of 10 μLs was then diluted 2X in 0.4% Trypan Blue staining reagent (Invitrogen) and the percentage of viable cells

determined using a hemocytometer (Spencer). Trypan Blue only traverses the membranes of dead cells, staining them blue while viable cells remain clear; therefore, the percentage of viable cells remaining after drug treatment may be calculated.

3.4 Annexin-V Assay

Apoptosis was detected in CJ cells following chaetocin and/or imatinib treatment using the Annexin-V PE Apoptosis Detection Kit I (BD Pharmingen) and flow cytometry as per manufacturer's instructions.

Apoptosis was detected in TonB210 cells following chaetocin and/or imatinib treatment using the Annexin-V FITC Apoptosis Detection Kit I (BD Pharmingen) and flow cytometry as per manufacturer's instructions.

3.5 Methylcellulose Assay

Colony formation was quantitated using MethoCult® GF M3434 methylcellulose (StemCell Technologies) as per manufacturer's instructions. Cells were plated in triplicate at a density of 250-1000 cells per 150 μL methylcellulose and incubated at 37°C with 5% CO_2 for 6-10 days before quantifying the resulting colonies *in situ* by light microscopy.

3.6 Reactive Oxygen Species Assay

ROS were detected in CJ cells using dihydrorhodamine 6G (dR6G, Invitrogen Life Technologies) and flow cytometry. Briefly, cells were treated with chaetocin or imatinib for 24 hours, an aliquot of cells washed with PBS and resuspended in 100 μM dR6G and incubated at room temperature (RT) for 60 min before being washed, resuspended in PBS, incubated at 37°C with 5% CO_2 for 60 min, and analyzed by flow cytometry.

3.7 CalcuSyn

Synergy calculations were performed via CalcuSyn software (Biosoft). Briefly, cells were

treated with chaetocin and/or imatinib for 24 hours, the percent viability determined by Trypan Blue exclusion assay, and results analyzed using CalcuSyn.

3.8 L-Calc

Stem cell frequencies were calculated using L-Calc software (StemCell Technologies). Briefly, CJ cells were treated with 100 nM chaetocin for 24 hours and/or 1 μM imatinib for 48 hours before being injected intravenously into sublethally irradiated recipient mice at a range of different cell numbers per mouse (7,500, 15,000, 30,000, 60,000, 120,000, or 240,000) with five mice per group. Once leukemic symptoms became apparent (e.g., ruffled fur, lethargy, splenomegaly), the mice were euthanized using CO_2 and their spleens removed to confirm disease by histological analysis and detection of BCR-ABL-GFP/Nup98-HOXA9-YFP expression by flow cytometry. Values corresponding to dose (i.e., the number of cells injected), total number of positive results obtained per dose (development of CML), and total number of replicates tested (5) were then entered into the program to determine the LSC frequency of each treatment group.

3.9 Murine Chronic Myelogenous Leukemia Models

All animal experiments were performed in accordance with the Canadian Council on Animal Care (CCAC) guidelines using C57BL/6N mice (Charles River Laboratories).

Mice were sublethally irradiated by exposure to 600 rad 24 hours or less before intravenous injection of untreated or chaetocin and/or imatinib-treated CJ cells and were euthanized using CO_2 upon presentation of leukemic symptoms (e.g., ruffled fur, lethargy, splenomegaly).

3.10 Lineage Depletion of Normal Murine Hematopoietic Stem Cells

Bone marrow was obtained from untreated mice by flushing the femora and tibiae with IMDM using a syringe and 26G needle. Cells were lineage depleted using the Lineage Cell Depletion Kit (Miltenyi Biotec) as per manufacturer's instructions.

3.11 Statistical Analysis

P-values for viability, apoptosis, and colony formation assay data were determined by performing an unpaired *t* test using the Graphpad online calculator. P-values for disease latency data were determined by performing a logrank test using Prism software. P-values for LSC frequency data were determined based on the results of a Pearson Chi-square test using L-Calc software. Drug interaction data were analyzed by performing combination index and isobologram analysis using CalcuSyn software.

4. RESULTS

4.1 Effects of Chaetocin and Imatinib on TonB210 Cells

TonB210 is a murine hematopoietic cell line with doxycycline-inducible BCR-ABL expression (Klucher *et al*., 1998). This cell line shows an absolute dependence on doxycycline for cell survival and proliferation *in vitro* in the absence of IL-3, correlating with BCR-ABL expression. Therefore, these cells allow investigation of drug effects both in the absence and presence of BCR-ABL.

4.1.1 Determination of the IC_{50} of Chaetocin and Imatinib on TonB210 Cell Viability

In order to determine the half-maximal inhibitory concentrations (IC_{50}) of chaetocin and imatinib for TonB210 cell viability, both with and without doxycycline-induced BCR-ABL expression, cells were treated with a range of drug concentrations and Trypan Blue exclusion assays were performed after 48 hours.

Trypan Blue is a diazo dye that only traverses membranes of dead cells, staining them blue while viable cells remain clear. Thus, Trypan Blue allows for calculation of the percentage of viable cells remaining after drug treatment.

Imatinib is a BCR-ABL inhibitor and thus had no effect on the viability of IL-3 dependent TonB210 cells where BCR-ABL was not induced (Fig. 4.1). Chaetocin decreased the viability of IL-3 dependent TonB210 cells with an IC_{50} of 175 nM (Fig. 4.2). Imatinib inhibited the viability of doxycycline treated TonB210 cells, which express BCR-ABL, with an IC_{50} of 1.5 μM (Fig. 4.3). Chaetocin inhibited the viability of doxycycline treated TonB210 cells with an IC_{50} of 175 nM (Fig. 4.4).

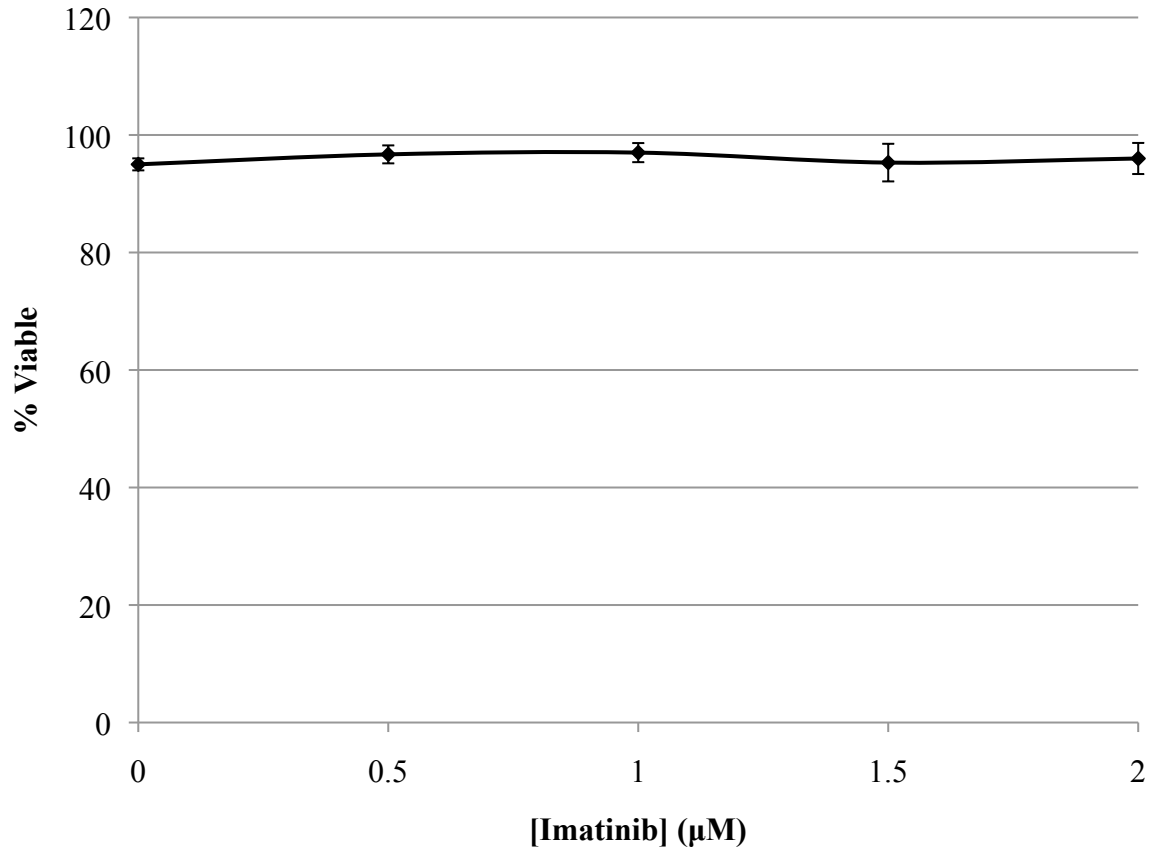

Figure 4.1: Effect of imatinib on the viability of IL-3 treated TonB210 cells, which do not express BCR-ABL. Cell viability was determined using Trypan Blue exclusion assays after 48 hr treatment with indicated concentrations of imatinib. Error bars represent standard deviation from three independent measurements.

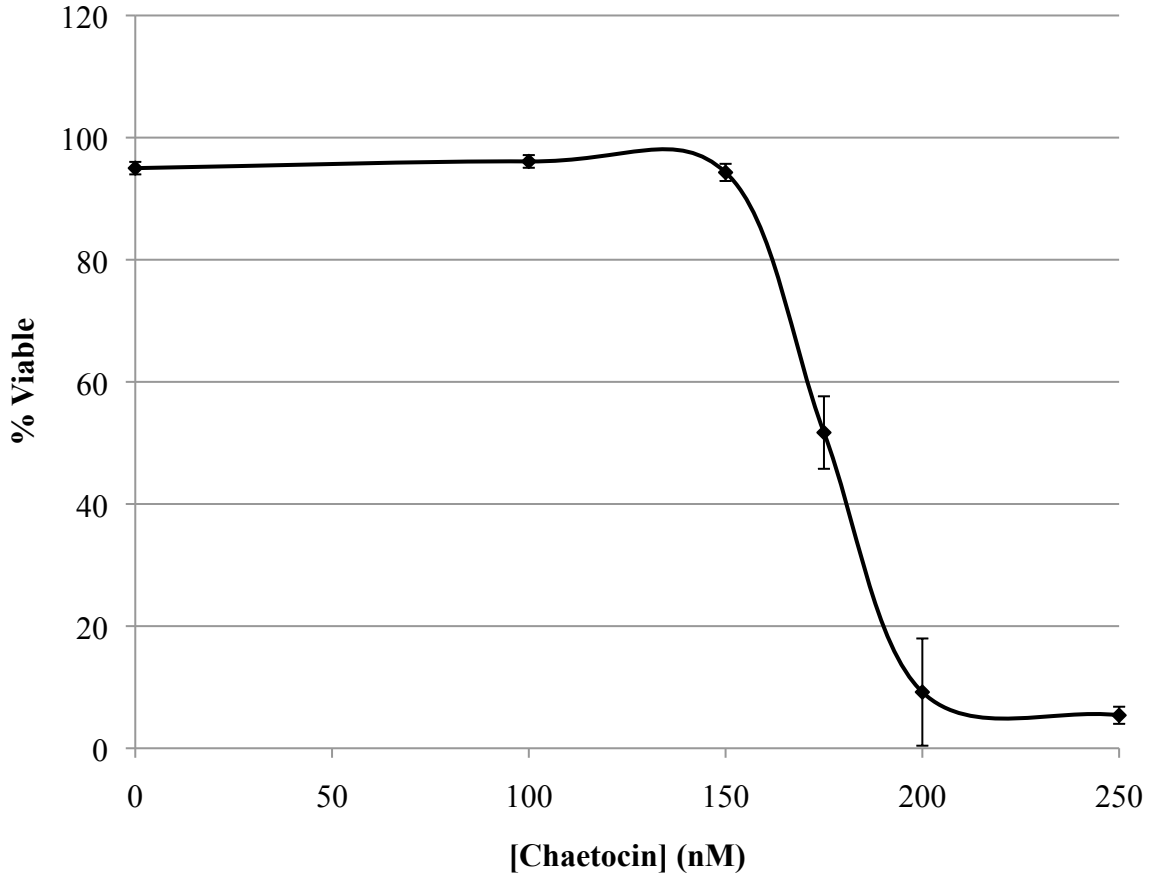

Figure 4.2: Effect of chaetocin on the viability of IL-3 treated TonB210 cells, which do not express BCR-ABL. Cell viability was determined using Trypan Blue exclusion assays after 48 hr treatment with indicated concentrations of chaetocin. Error bars represent standard deviation from three independent measurements.

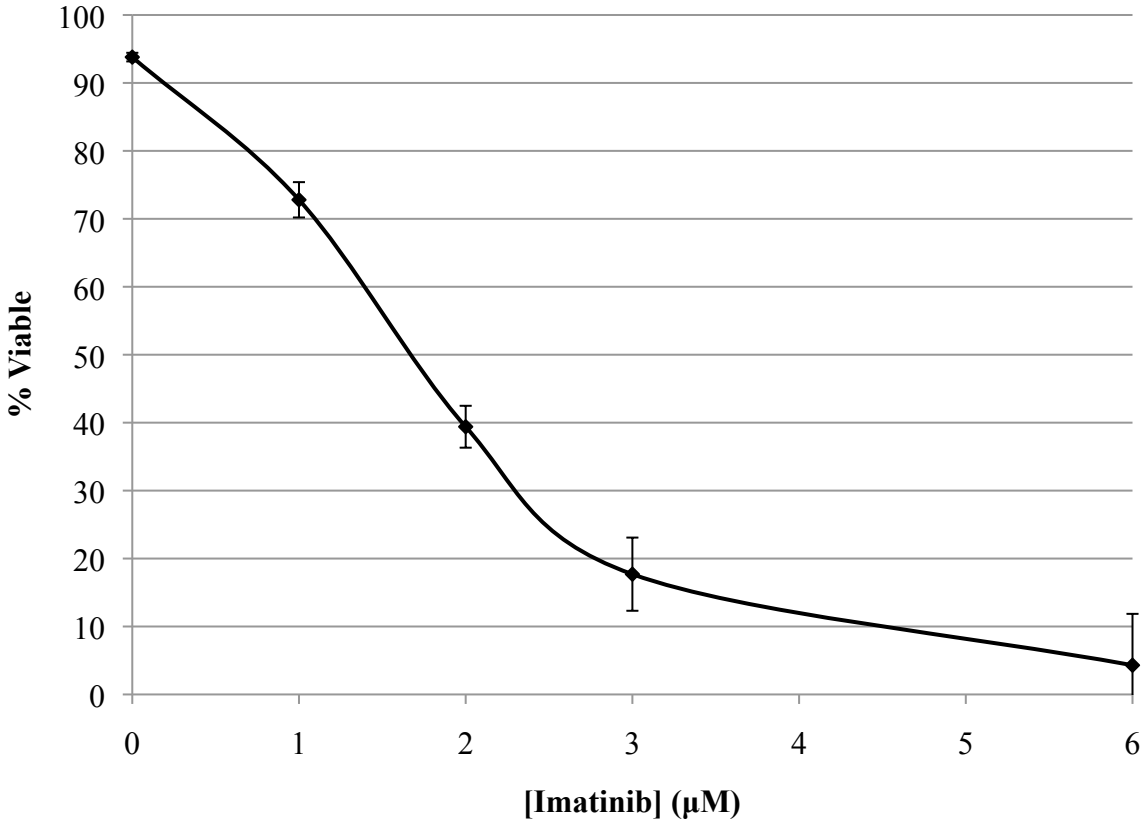

Figure 4.3: Effect of imatinib on the viability of doxycycline treated TonB210 cells, which express BCR-ABL. Cell viability was determined using Trypan Blue exclusion assays after 48 hr treatment with indicated concentrations of imatinib. Error bars represent standard deviation from three independent measurements.

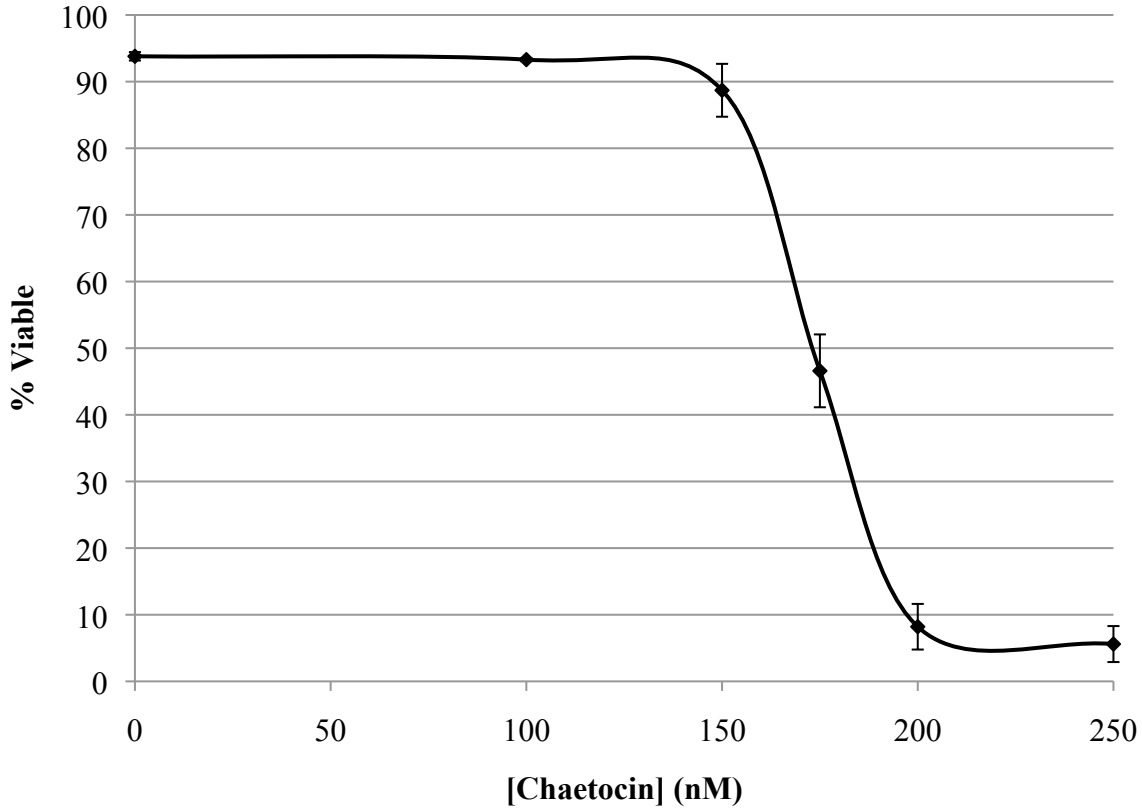

Figure 4.4: Effect of chaetocin on the viability of doxycycline treated TonB210 cells, which express BCR-ABL. Cell viability was determined using Trypan Blue exclusion assays after 48 hr treatment with indicated concentrations of chaetocin. Error bars represent standard deviation from three independent measurements.

4.1.2 Effects of Chaetocin and Imatinib on TonB210 Cell Viability

In order to determine the effect of chaetocin and imatinib on the viability of TonB210 cells, both in the presence and absence of BCR-ABL expression, cells were treated with IC_{50} and IC_{90} concentrations of each drug, both alone and in combination, and the resulting viabilities were determined by Trypan Blue exclusion assay. Imatinib had no effect on the viability of TonB210 cells not expressing BCR-ABL and had no effect on the activity of chaetocin (Fig. 4.5). Meanwhile, TonB210 cells expressing BCR-ABL responded to both chaetocin and imatinib and the combination of these drugs resulted in a dramatic decrease in cell viability (Fig. 4.6).

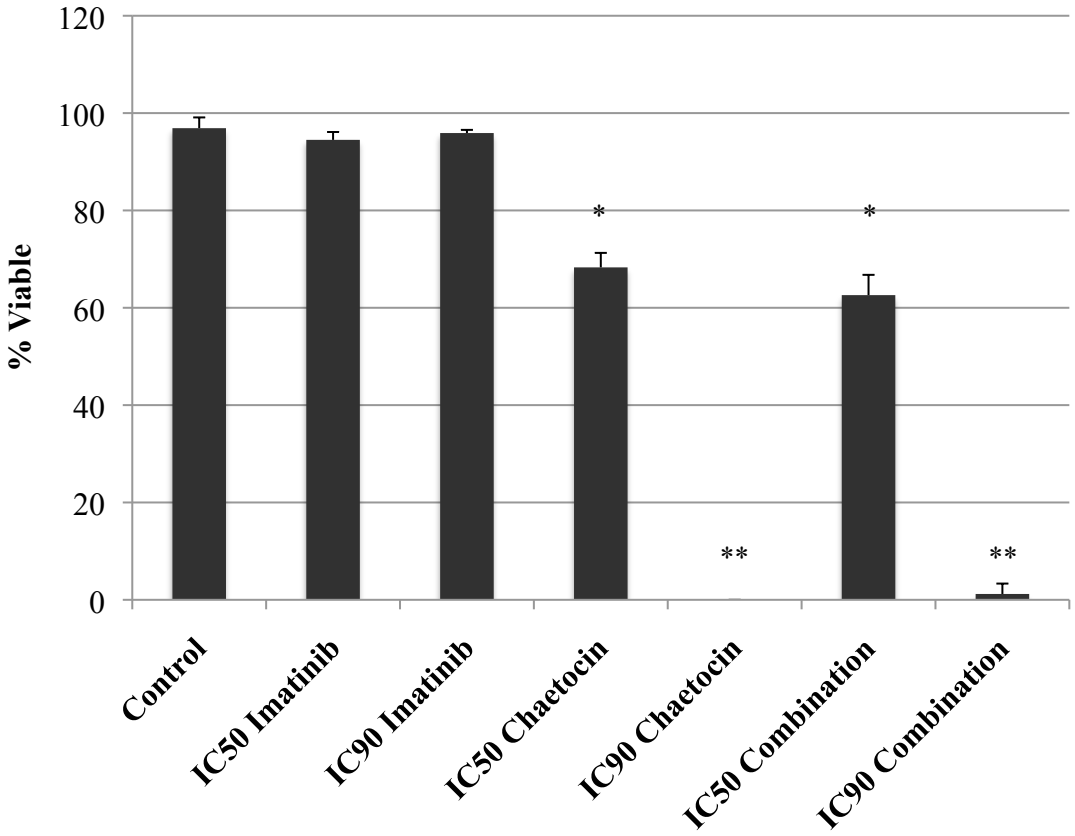

Figure 4.5: Effects of chaetocin and imatinib on the viability of IL-3 treated TonB210 cells, which do not express BCR-ABL. Cells were treated with chaetocin and/or imatinib at both IC_{50} and IC_{90} concentrations for 48 hr and viability was determined using Trypan Blue exclusion assays. Error bars represent standard deviation from three independent measurements. *p-value <0.0005 (IC_{50} chaetocin and IC_{50} combination vs. control) **p-value <0.0001 (IC_{90} chaetocin and IC_{90} combination vs. control)

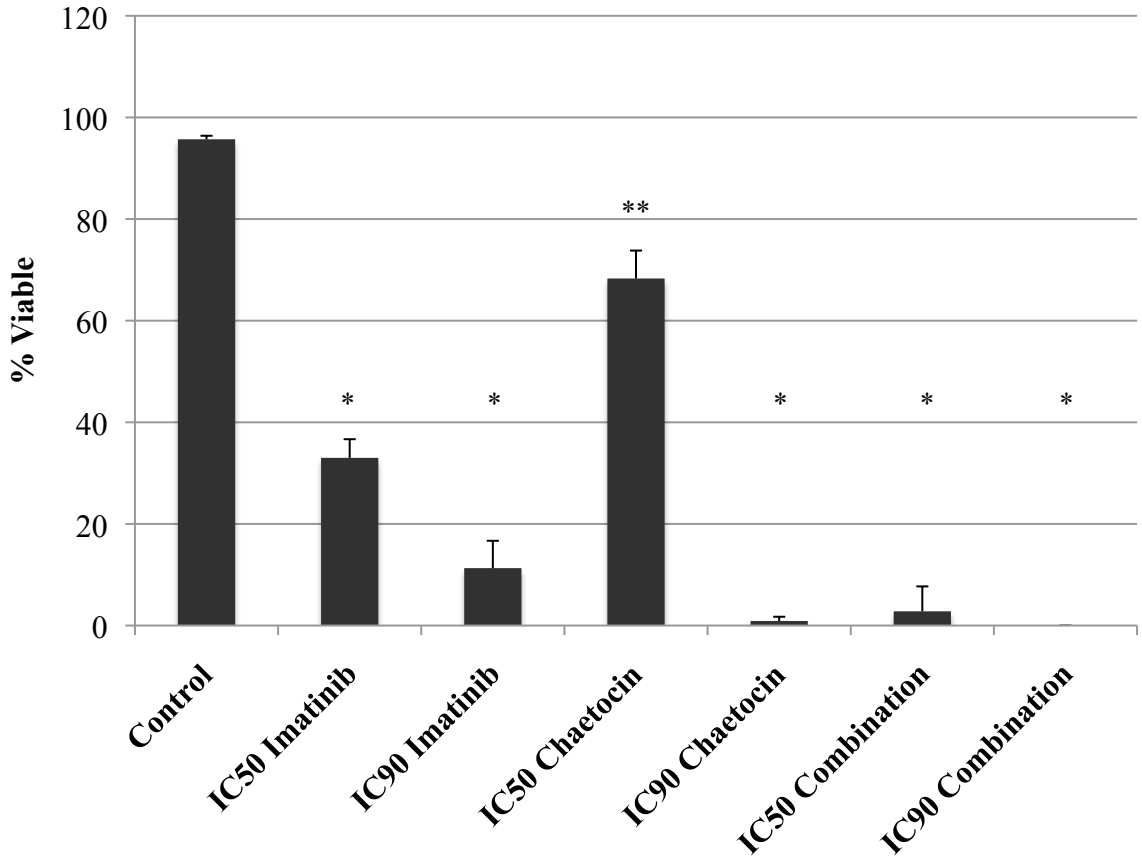

Figure 4.6: Effects of chaetocin and imatinib on the viability of doxycycline treated TonB210 cells, which express BCR-ABL. Cells were treated with chaetocin and/or imatinib at both IC_{50} and IC_{90} concentrations for 48 hr and viability was determined using Trypan Blue exclusion assays. Error bars represent standard deviation from three independent measurements.
*p-value <0.0001 (IC_{50} and IC_{90} imatinib, IC_{90} chaetocin, and IC_{50} and IC_{90} combination vs. control) **p-value <0.005 (IC_{50} chaetocin vs. control)

4.1.3 Effects of Chaetocin and Imatinib on the Induction of Apoptosis in TonB210 Cells

In order to determine if chaetocin and imatinib reduced the viability of TonB210 cells through the induction of apoptosis, cells were treated with IC_{50} and IC_{90} concentrations of each drug, both alone and in combination, and the percentage of cells undergoing apoptosis was determined using the Annexin-V assay.

Annexin-V is a phospholipid-binding protein with a high affinity for phosphatidylserine (PS) in the presence of physiological concentrations of Ca^{2+} (Moss *et al*., 1991). The negatively charged phospholipid PS is located in the cytosolic leaflet of the plasma membrane lipid bilayer of most normal, viable eukaryotic cells and its redistribution from the inner to the outer leaflet is an early, widespread event during apoptosis (Koopman *et al*., 1994; Martin *et al*., 1995). Thus, apoptotic cells can be directly detected through their staining with fluorochrome-conjugated Annexin-V.

Chaetocin induced apoptosis in TonB210 cells not expressing BCR-ABL while imatinib had no effect (Fig. 4.7). This also showed that imatinib had no effect on the ability of chaetocin to induce apoptosis in cells not expressing BCR-ABL. Both chaetocin and imatinib induced apoptosis in TonB210 cells expressing BCR-ABL and a greater percentage of cells are affected when these drugs are combined (Fig. 4.8).

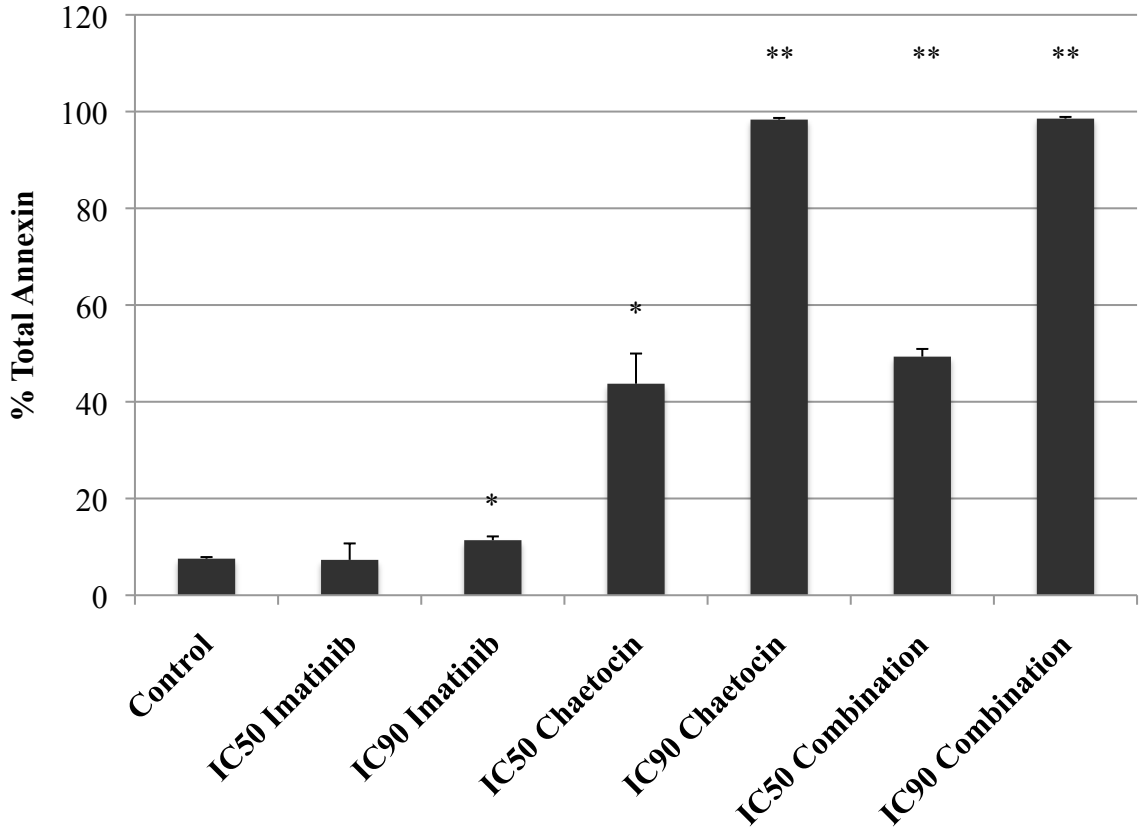

Figure 4.7: Effects of chaetocin and imatinib on the induction of apoptosis in IL-3 treated TonB210 cells, which do not express BCR-ABL. Cells were treated with chaetocin and/or imatinib at both IC_{50} and IC_{90} concentrations for 48 hr and the percentage of cells undergoing apoptosis was determined using Annexin-V assays. Error bars represent standard deviation from three independent measurements. *p-value <0.005 (IC_{90} imatinib and IC_{50} chaetocin vs. control) **p-value <0.0001 (IC_{90} chaetocin and IC_{50} and IC_{90} combination vs. control)

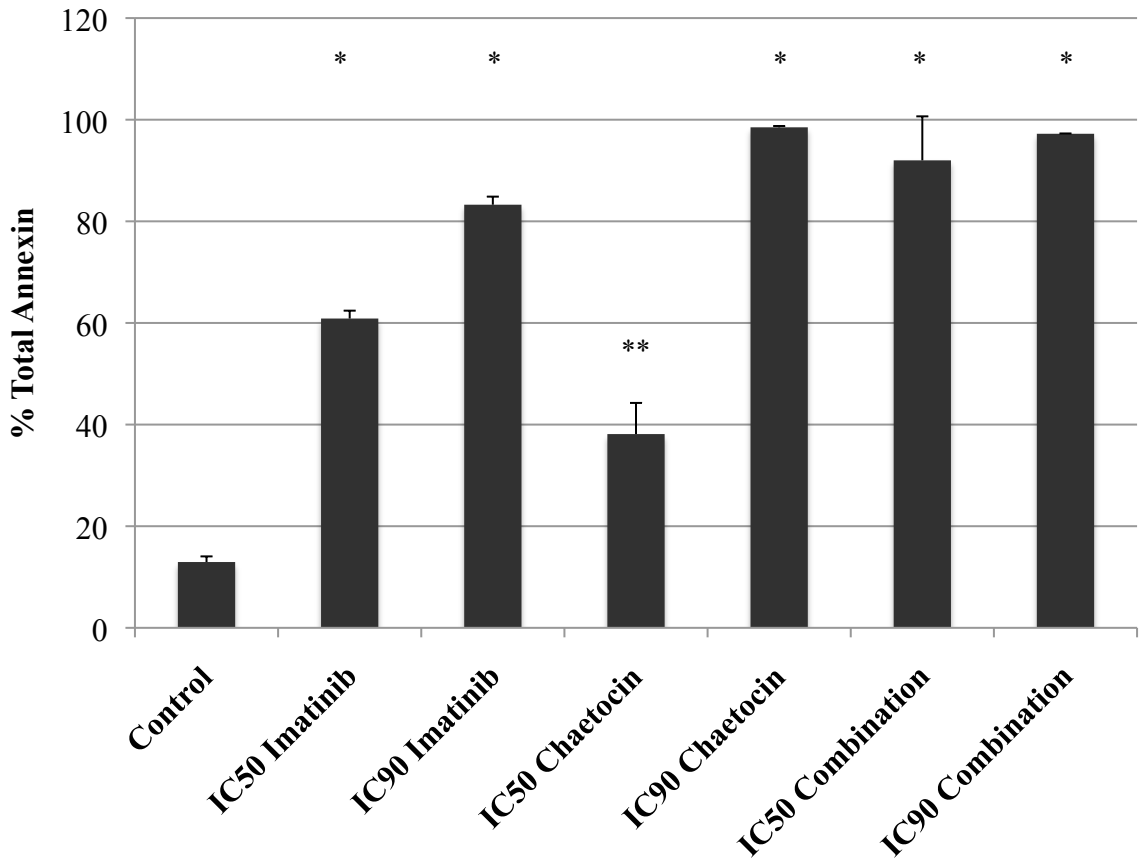

Figure 4.8: Effects of chaetocin and imatinib on the induction of apoptosis in doxycycline treated TonB210 cells, which express BCR-ABL. Cells were treated with chaetocin and/or imatinib at both IC_{50} and IC_{90} concentrations for 48 hr and the percentage of cells undergoing apoptosis was determined using Annexin-V assays. Error bars represent standard deviation from three independent measurements. *p-value <0.0001 (IC_{50} and IC_{90} imatinib, IC_{90} chaetocin, and IC_{50} and IC_{90} combination vs. control) **p-value <0.005 (IC_{50} chaetocin vs. control)

4.1.4 Effects of Chaetocin and Imatinib on TonB210 Colony Formation

In the bone marrow, a small number of hematopoietic stem cells produce heterogeneous populations of actively dividing hematopoietic progenitors whose proliferation and differentiation results in the generation of millions of mature blood cells daily. When cultured in a suitable semi-solid matrix such as methylcellulose, a relatively inert polymer that forms a stable gel with good optical clarity, individual progenitors called colony-forming cells (CFCs) proliferate to form discrete cell clusters or colonies. CFCs can then be classified and

enumerated *in situ* by light microscopy based on the morphological recognition of one or more types of hematopoietic lineage cells within the colony.

Effects of chaetocin and imatinib on the colony forming ability of IL-3 or doxycycline treated TonB210 cells were investigated by treating these cells with IC_{50} and IC_{90} concentrations of each drug for 48 hours before plating cells in methylcellulose at a density of 250 cells per 150 μL. The numbers of colonies formed after 7 days of incubation were counted *in situ* using light microscopy.

Chaetocin dramatically reduced the ability of IL-3 treated TonB210 cells to form colonies while imatinib had no effect. In addition, imatinib had only a modest effect on the activity of chaetocin in these cells (Fig. 4.9). In doxycycline treated TonB210 cells, both chaetocin and imatinib severely limited colony formation and when these drugs were combined, colony formation was obliterated (Fig. 4.10).

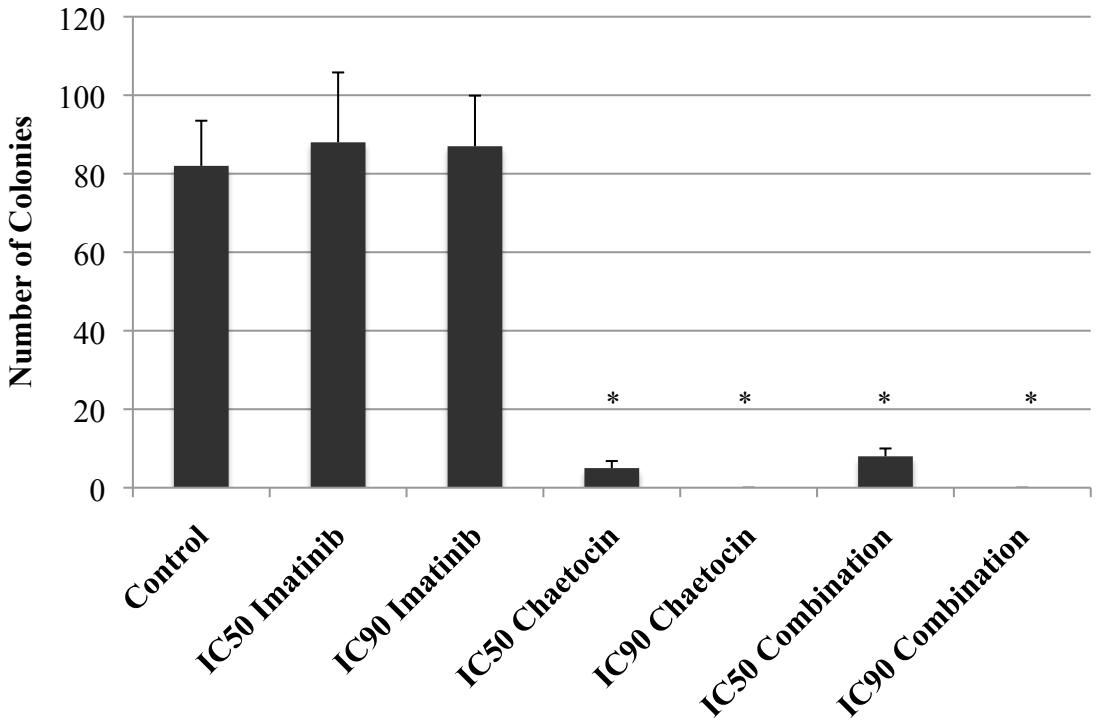

Figure 4.9: Effects of chaetocin and imatinib on the colony formation of IL-3 treated TonB210 cells, which do not express BCR-ABL. Cells were treated with chaetocin and/or imatinib at both IC_{50} and IC_{90} concentrations for 48 hr before plating in methylcellulose. Resulting colonies were counted after 7 days incubation using light microscopy. Error bars represent standard deviation from three independent measurements. *p-value <0.0001 (IC_{50} and IC_{90} chaetocin and IC_{50} and IC_{90} combination vs. control)

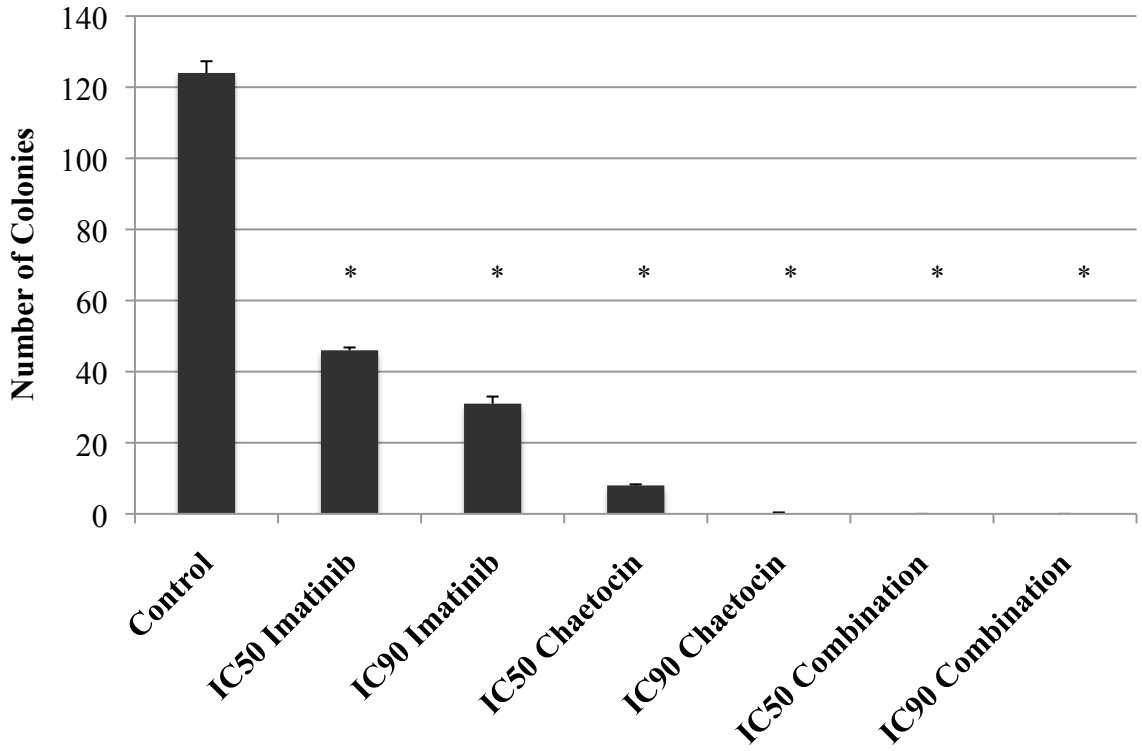

Figure 4.10: Effects of chaetocin and imatinib on the colony formation of doxycycline treated TonB210 cells, which express BCR-ABL. Cells were treated with chaetocin and/or imatinib at both IC_{50} and IC_{90} concentrations for 48 hr before plating in methylcellulose. Resulting colonies were counted after 7 days incubation using light microscopy. Error bars represent standard deviation from three independent measurements. *p-value <0.0001 (IC_{50} and IC_{90} imatinib, IC_{50} and IC_{90} chaetocin, and IC_{50} and IC_{90} combination vs. control)

4.1.5 Effects of Bone Marrow Stromal Factors on the Activity of Chaetocin and Imatinib in TonB210 Cells

The heterogeneous cell populations that comprise the bone marrow niche support and interact with HSCs in part through the secretion of cytokines, chemokines, and growth factors (Chiodoni *et al*., 2010), collectively referred to as bone marrow stromal factors (BMSFs) in this study. Importantly, it has been shown that BMSFs enable the survival of LSCs despite the inhibition of BCR-ABL by TKIs such as imatinib (Corbin *et al*., 2011). Therefore, we thought it pertinent to investigate effects of BMSFs on the activity of chaetocin and imatinib in IL-3 and doxycycline treated TonB210 cells.

BMSFs were obtained by culturing M2-10B4 cells for several days before collecting the culture medium. M2-10B4 is a murine stromal cell line that has been shown to secrete BMSFs (Sutherland *et al*., 1991). TonB210 cells were then incubated with M2-10B4 culture medium for 4 hours before the addition of chaetocin and/or imatinib in order to test its effects on the ability of these drugs to decrease viability, induce apoptosis, and inhibit colony formation.

Imatinib had no effect on the viability of IL-3 treated TonB210 cells, regardless of the presence of BMSFs while the activity of chaetocin was potentiated by BMSFs, both with and without imatinib co-treatment (Fig. 4.11). The presence of BMSFs significantly inhibited the ability of imatinib to decrease the viability of doxycycline treated TonB210 cells expressing BCR-ABL while BMSFs increased the potency of chaetocin, causing a significantly larger decrease in viability. In addition, the combination of chaetocin and imatinib resulted in a dramatic decrease in cell viability, regardless of the presence of BMSFs (Fig. 4.12).

Imatinib did not induce apoptosis in IL-3 treated TonB210 cells, regardless of the presence of BMSFs while the induction of apoptosis in IL-3 treated TonB210 cells by chaetocin was dramatically potentiated by BMSFs, both with and without imatinib co-treatment (Fig. 4.13). BMSFs did not have a significant effect on the ability of imatinib to induce apoptosis in doxycycline treated TonB210 cells expressing BCR-ABL but the ability of chaetocin to induce apoptosis was found to be dramatically increased by the presence of BMSFs (Fig. 4.14).

Finally, imatinib had no effect on the colony forming ability of IL-3 treated TonB210 cells, regardless of the presence of BMSFs while chaetocin drastically inhibited the colony formation of IL-3 treated TonB210 cells, both with and without BMSFs, regardless of imatinib co-treatment (Fig. 4.15). BMSFs were found to interfere with the ability of imatinib to inhibit colony formation of doxycycline treated TonB210 cells while the activity of chaetocin was unaffected. In addition, the combination of chaetocin and imatinib was found to drastically reduce the colony forming ability of doxycycline treated TonB210 cells, regardless of the presence of BMSFs (Fig. 4.16).

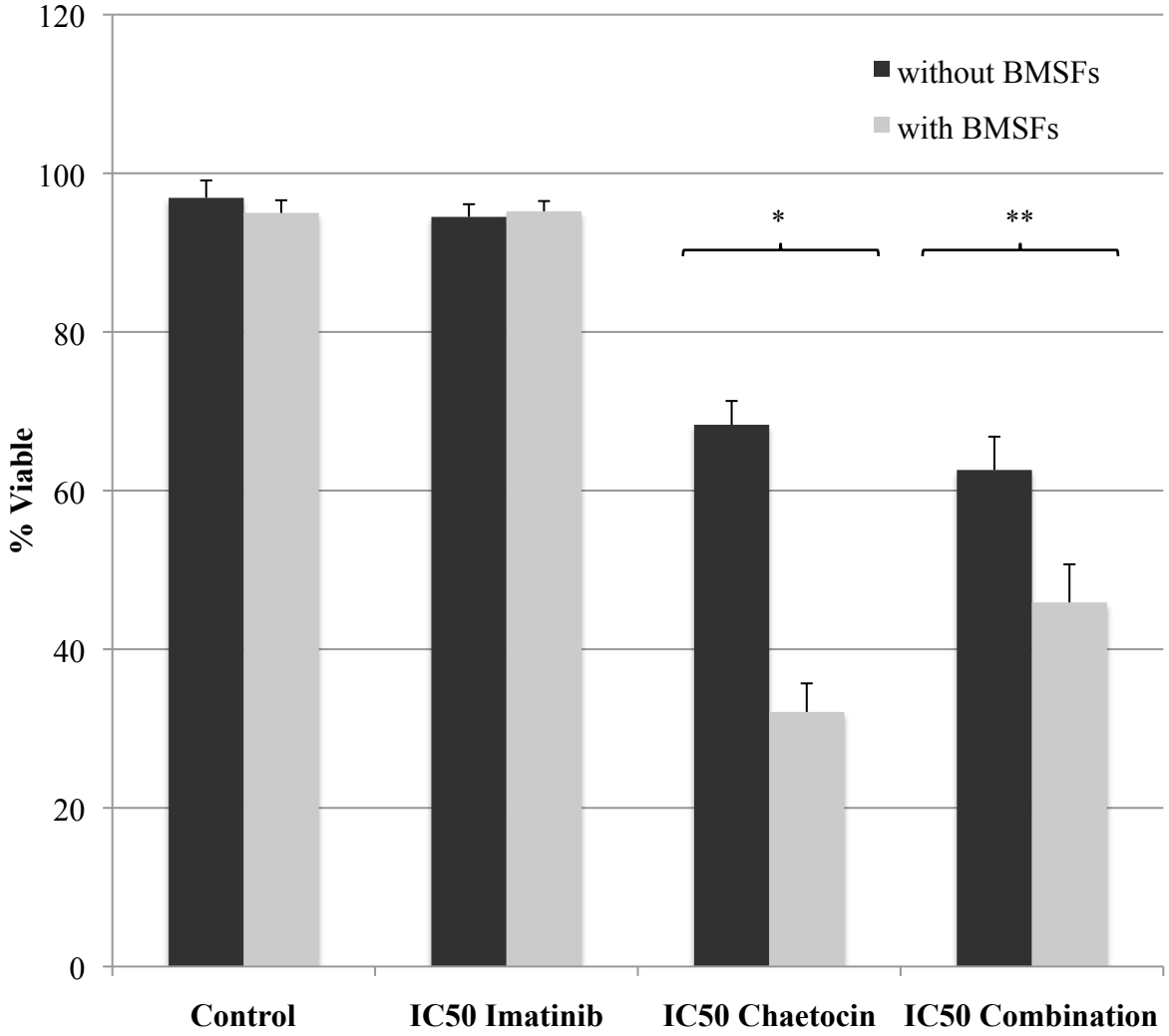

Figure 4.11: Effects of bone marrow stromal factors on the activity of chaetocin and imatinib in IL-3 treated TonB210 cells, which do not express BCR-ABL. Cells were incubated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with chaetocin and/or imatinib at IC_{50} concentrations for 48 hrs and viability was determined using Trypan Blue exclusion assays. Error bars represent standard deviation from three independent measurements. *p-value <0.0005 (IC_{50} chaetocin without BMSFs vs. IC_{50} chaetocin with BMSFs) **p-value <0.05 (IC_{50} combination without BMSFs vs. IC_{50} combination with BMSFs)

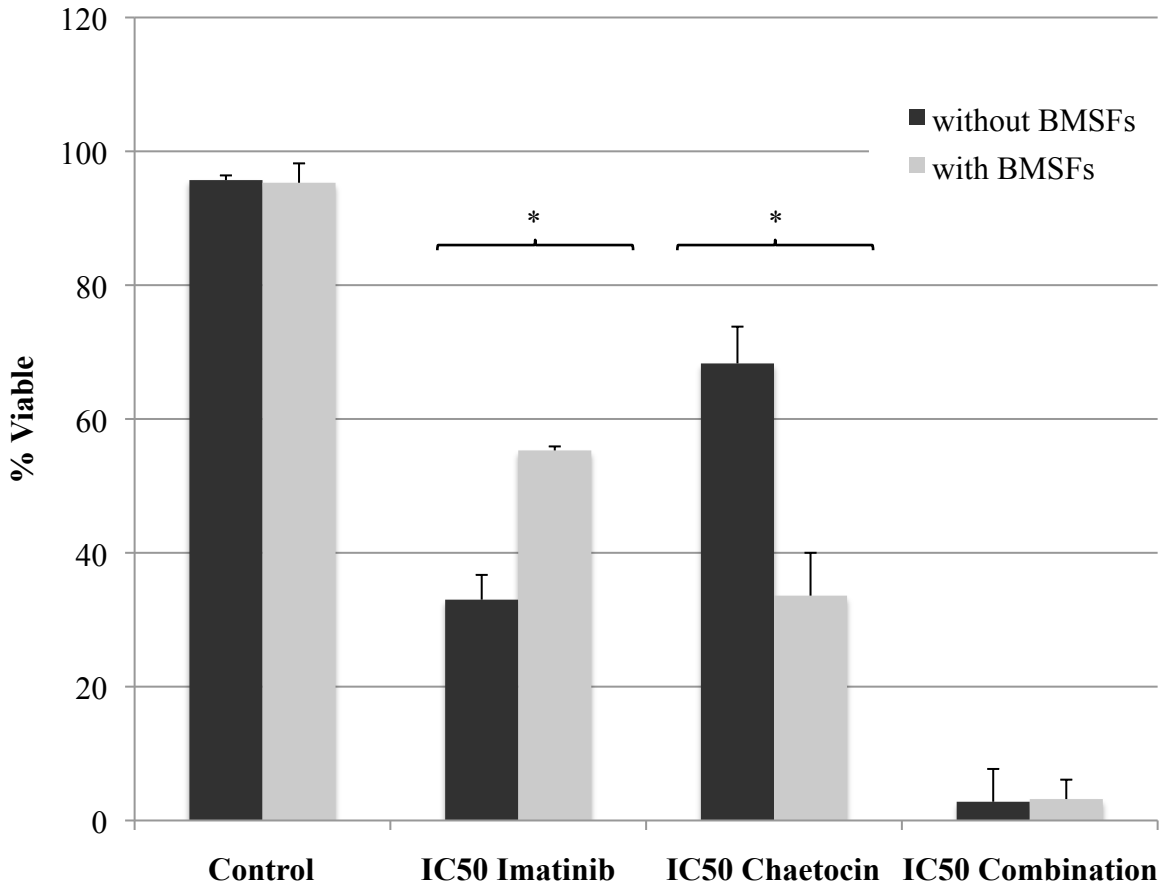

Figure 4.12: Effects of bone marrow stromal factors on the activity of chaetocin and imatinib in doxycycline treated TonB210 cells, which express BCR-ABL. Cells were incubated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with chaetocin and/or imatinib at IC_{50} concentrations for 48 hours and viability was determined using Trypan Blue exclusion assays. Error bars represent standard deviation from three independent measurements. *p-value <0.005 (IC_{50} imatinib without BMSFs vs. IC_{50} imatinib with BMSFs and IC_{50} chaetocin without BMSFs vs. IC_{50} chaetocin with BMSFs)

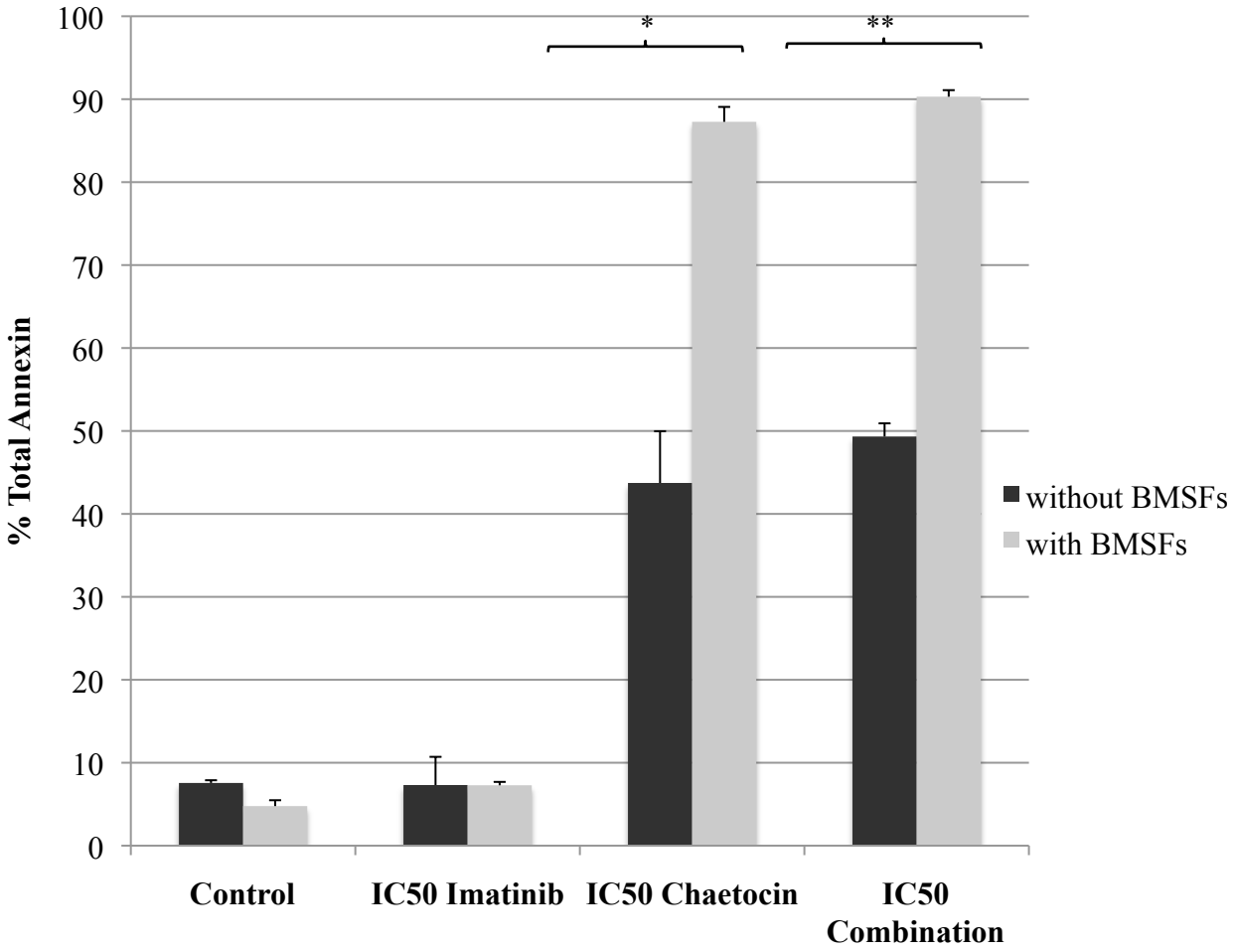

Figure 4.13: Effects of bone marrow stromal factors on the induction of apoptosis by chaetocin and imatinib in IL-3 treated TonB210 cells, which do not express BCR-ABL. Cells were incubated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with chaetocin and/or imatinib at IC_{50} concentrations for 48 hrs and apoptosis was detected using Annexin-V assays. Error bars represent standard deviation from three independent measurements. *p-value <0.0005 (IC_{50} chaetocin without BMSFs vs. IC_{50} chaetocin with BMSFs) **p-value <0.0001 (IC_{50} combination without BMSFs vs. IC_{50} combination with BMSFs)

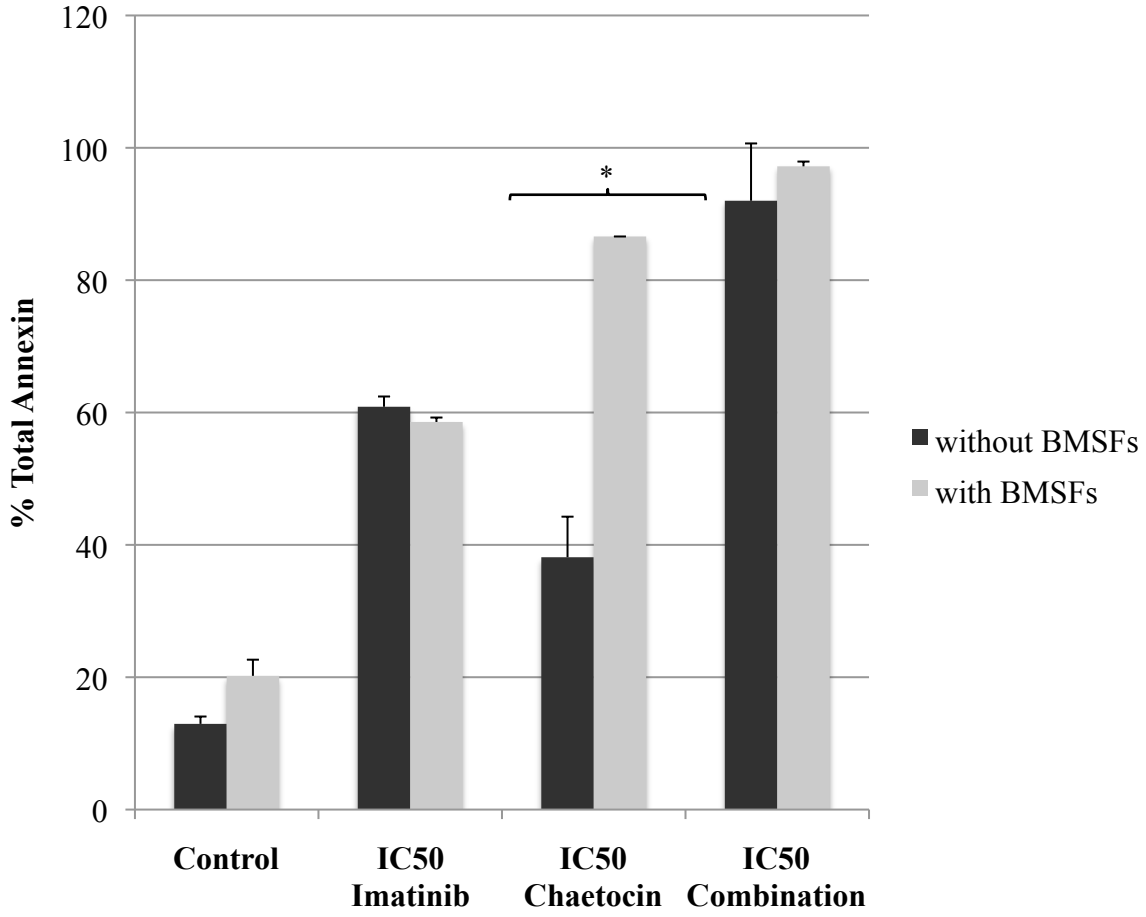

Figure 4.14: Effects of bone marrow stromal factors on the induction of apoptosis by chaetocin and imatinib in doxycycline treated TonB210 cells, which express BCR-ABL. Cells were incubated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with chaetocin and/or imatinib at IC_{50} concentrations for 48 hrs and apoptosis was detected using Annexin-V assays. Error bars represent standard deviation from three independent measurements. *p-value <0.0005 (IC_{50} chaetocin without BMSFs vs. IC_{50} chaetocin with BMSFs)

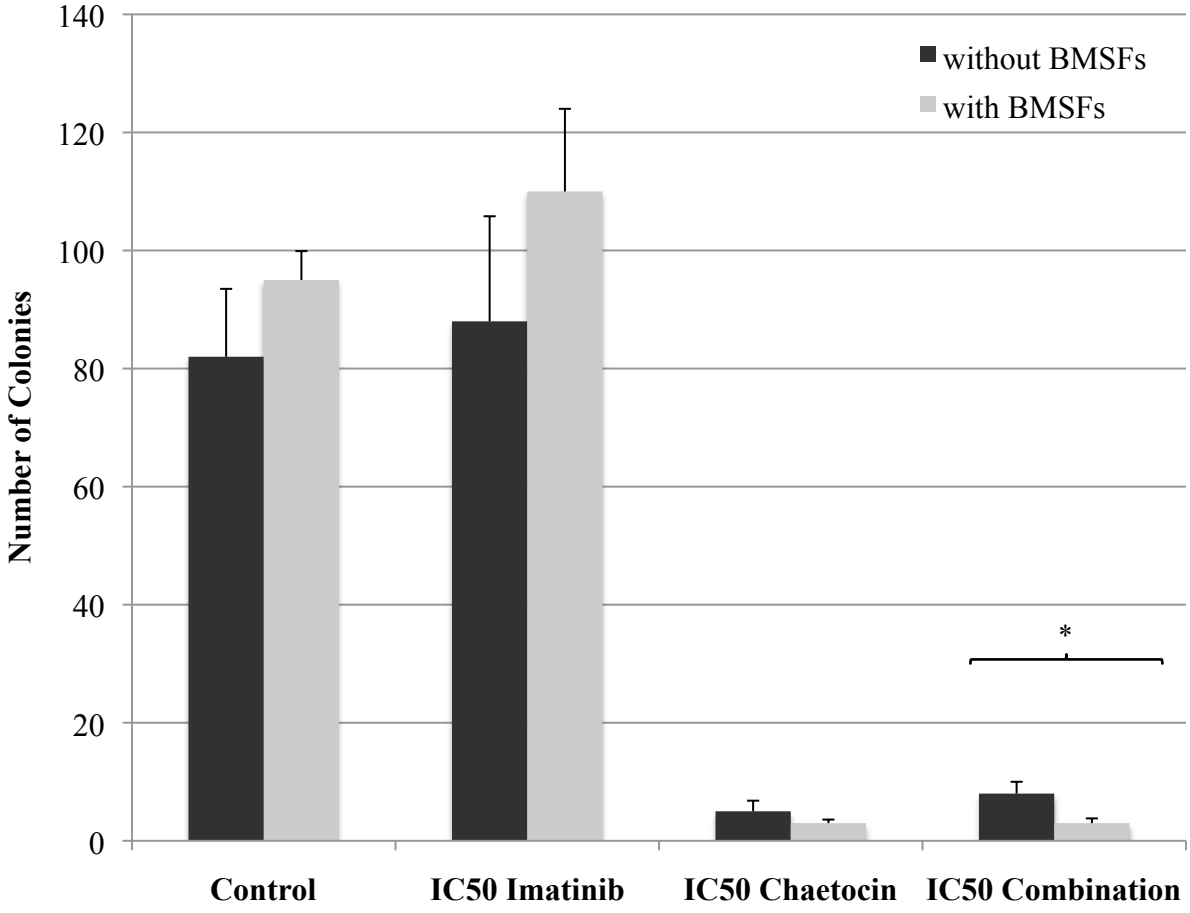

Figure 4.15: Effects of bone marrow stromal factors on the inhibition of colony formation by chaetocin and imatinib in IL-3 treated TonB210 cells, which do not express BCR-ABL. Cells were incubated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with chaetocin and/or imatinib at IC_{50} concentrations for 48 hrs before plating in methylcellulose. Colonies were counted after 7 days incubation using light microscopy. Error bars represent standard deviation from three independent measurements. *p-value <0.05 (IC_{50} combination without BMSFs vs. IC_{50} combination with BMSFs)

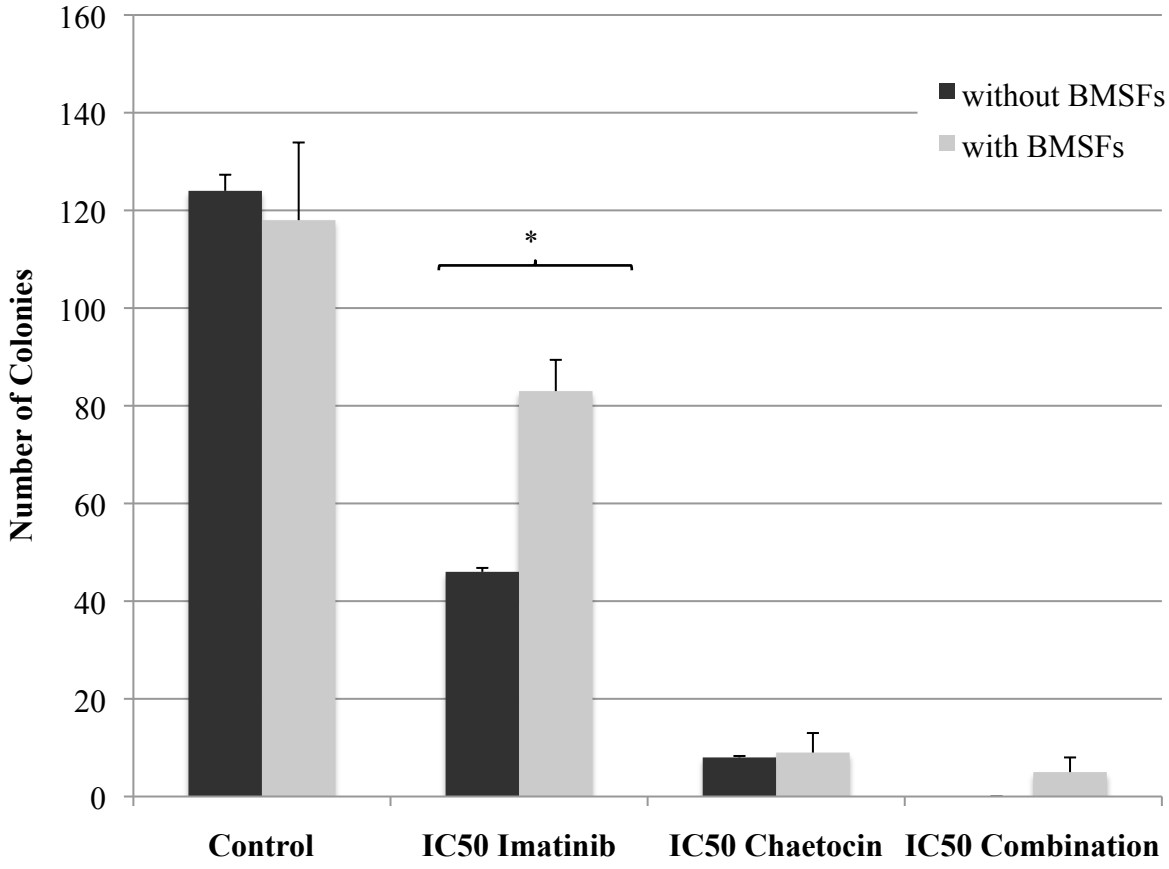

Figure 4.16: Effects of bone marrow stromal factors on the inhibition of colony formation by chaetocin and imatinib in doxycycline treated TonB210 cells, which express BCR-ABL. Cells were incubated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with chaetocin and/or imatinib at IC_{50} concentrations for 48 hrs before plating in methylcellulose. Colonies were counted after 7 days incubation by light microscopy. Error bars represent standard deviation from three independent measurements. *p-value <0.0001 (IC_{50} imatinib without BMSFs vs. IC_{50} imatinib with BMSFs)

4.2 Effects of Chaetocin and Imatinib on CJ Cells

Neering *et al*. have developed a murine model of blast crisis CML that closely mimics the biological features of human LSCs. Briefly, this model was created by modifying primitive normal hematopoietic cells such that they express both BCR/ABL and Nup98/HOXA9 translocation products and identifying a distinct LSC population with the aberrant immunophenotype of lin^-, $Kit^{+/-}$, $Flt3^+$, Sca^+, $CD34^+$, and $CD150^-$ (2007).

This system provides a powerful means by which the *in vivo* behavior of LSCs versus

HSCs can be characterized and candidate treatment regimens optimized for maximal specificity toward primitive leukemia cells (Neering *et al*., 2007). In this study, this murine CML model, referred to as "CJ cells," has been used to characterize the activity of chaetocin, both alone and in combination with imatinib, in regards to viability, apoptosis, and colony formation.

4.2.1 Determination of the IC_{50} of Chaetocin and Imatinib on CJ Cell Viability

In order to determine IC_{50} concentrations of chaetocin and imatinib for inhibiting the viability of CJ cells, these cells were treated with a range of drug concentrations and the resulting viabilities determined by Trypan Blue exclusion assay after 48 hours. The IC_{50} of chaetocin was determined to be 350 nM (Fig. 4.17) and the IC_{50} of imatinib was determined to be 2 μM (Fig. 4.18).

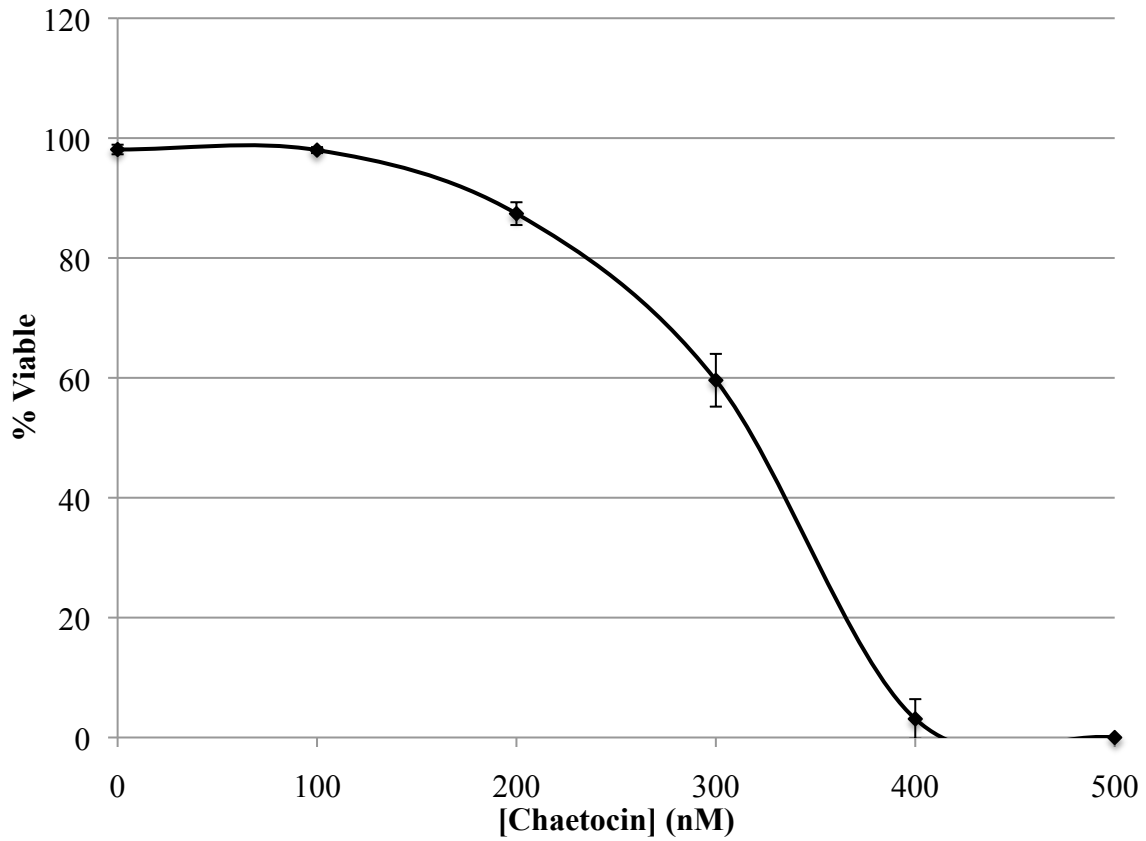

Figure 4.17: Effect of chaetocin on the viability of CJ cells. Cell viability was determined using Trypan Blue exclusion assays after 48 hr treatment with indicated concentrations of chaetocin. Error bars represent standard deviation from three independent measurements.

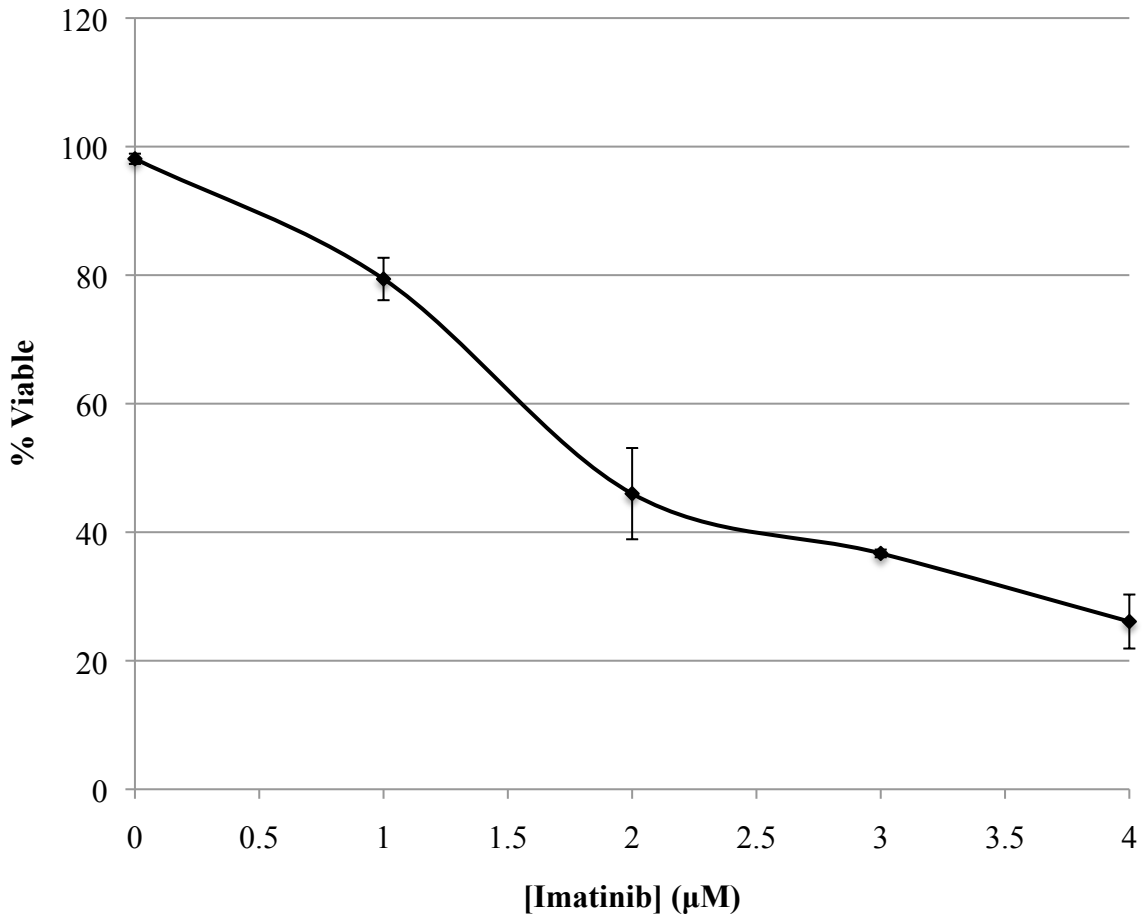

Figure 4.18: Effect of imatinib on the viability of CJ cells. Cell viability was determined using Trypan Blue exclusion assays after 48 hr treatment with indicated concentrations of imatinib. Error bars represent standard deviation from three independent measurements.

4.2.2 Effects of Chaetocin and Imatinib on CJ Cell Viability

In order to determine the effect of chaetocin and imatinib on the viability of CJ cells, cells were treated with IC_{50} and IC_{90} concentrations of each drug, both alone and in combination, and the resulting viabilities were determined by Trypan Blue exclusion assay. The combination of chaetocin and imatinib resulted in a greater decrease in viability than either drug individually, indicating that chaetocin and imatinib function synergistically in CJ cells (Fig. 4.19).

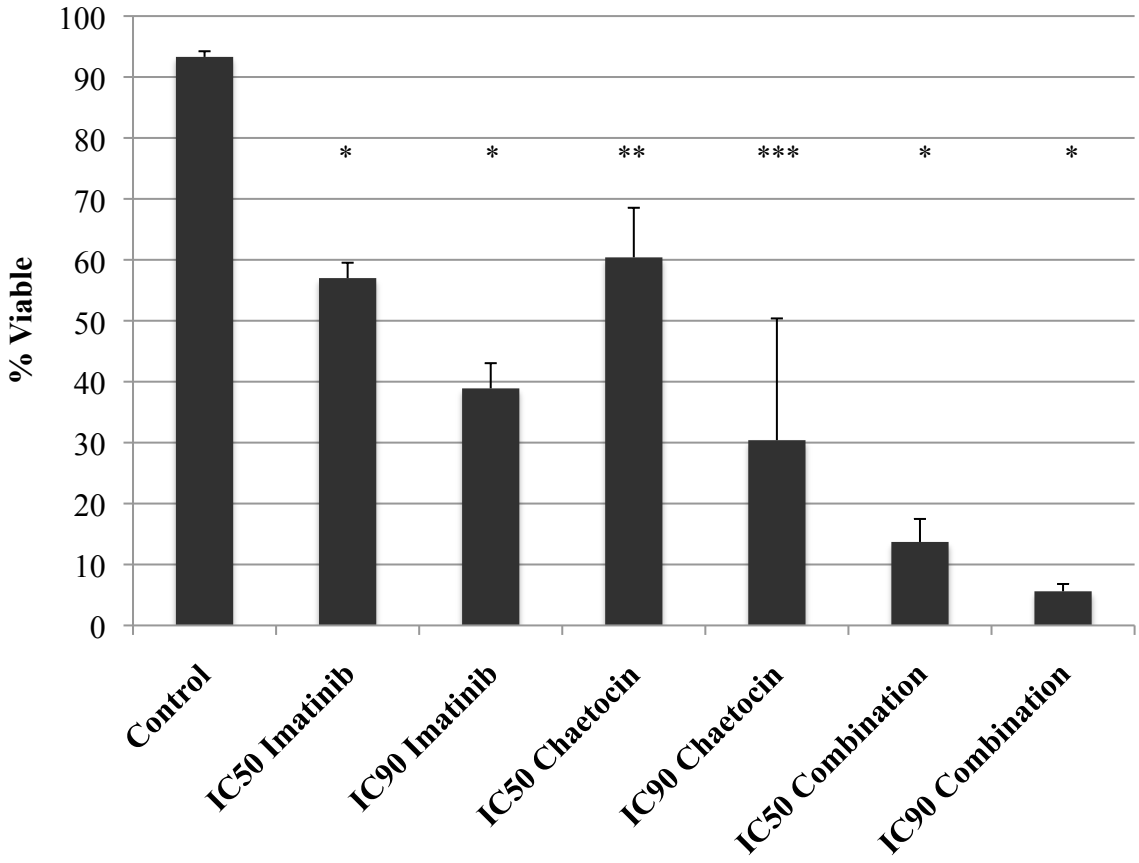

Figure 4.19: Effects of chaetocin and imatinib on the viability of CJ cells. Cells were treated with IC_{50} and IC_{90} concentrations of chaetocin and/or imatinib and viability was determined using Trypan Blue exclusion assays after 48 hrs. Error bars represent standard deviation from three independent measurements. *p-value <0.0001 (IC_{50} and IC_{90} imatinib and IC_{50} and IC_{90} combination vs. control) **p-value <0.005 (IC_{50} chaetocin vs. control) ***p-value <0.01 (IC_{90} chaetocin vs. control)

4.2.3 Effects of Chaetocin and Imatinib on the Induction of Apoptosis in CJ Cells

In order to determine if chaetocin and imatinib reduced the viability of CJ cells through the induction of apoptosis, cells were treated with IC_{50} and IC_{90} concentrations of each drug, both alone and in combination, and the percentage of cells undergoing apoptosis was determined using the Annexin-V assay. Both chaetocin and imatinib were found to induce apoptosis in CJ cells and a higher percentage of cells were found to be apoptotic when these drugs were used in combination (Fig. 4.20).

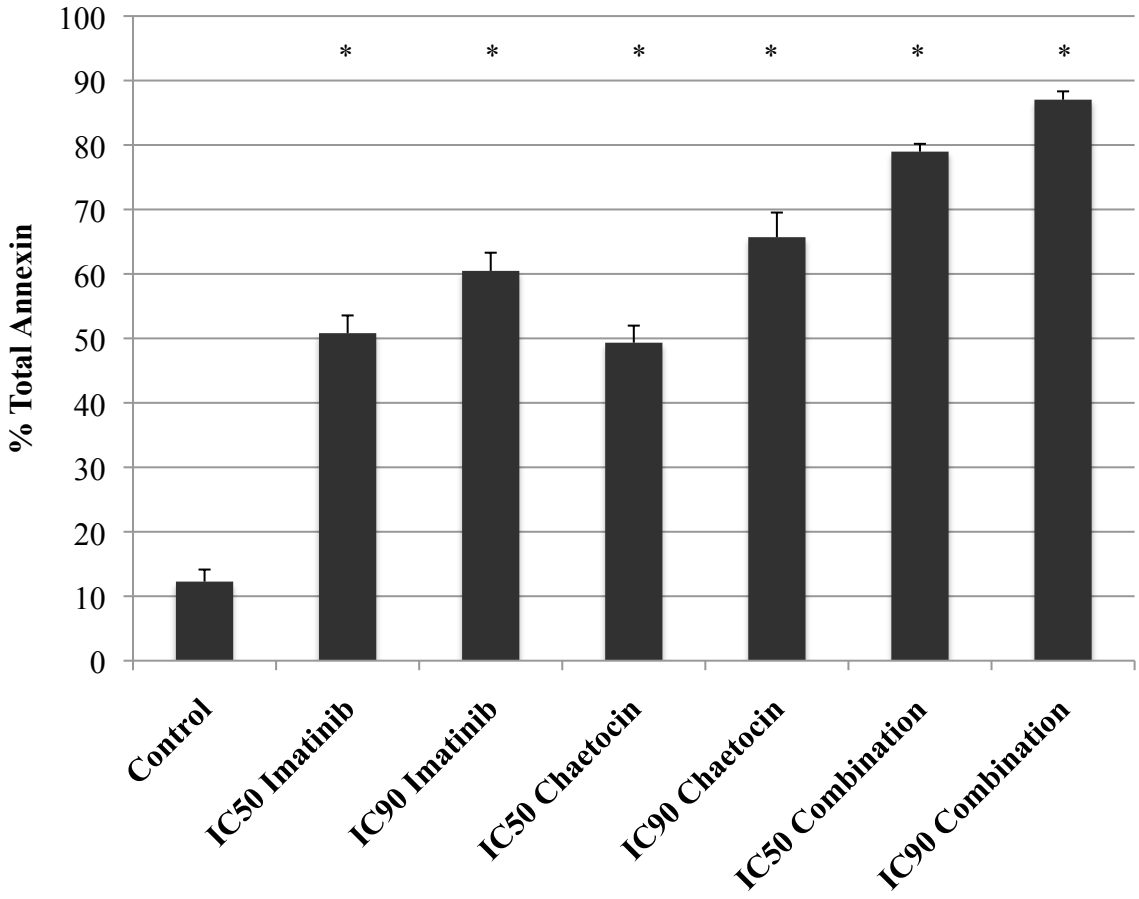

Figure 4.20: Effects of chaetocin and imatinib on the induction of apoptosis in CJ cells. Cells were treated with IC_{50} and IC_{90} concentrations of chaetocin and/or imatinib and the induction of apoptosis was detected using Annexin-V assays after 48 hrs. Error bars represent standard deviation from three independent measurements. *p-value <0.0001 (IC_{50} and IC_{90} imatinib, IC_{50} and IC_{90} chaetocin, and IC_{50} and IC_{90} combination vs. control)

4.2.4 Effects of Chaetocin and Imatinib on CJ Cell Colony Formation

Effects of chaetocin and imatinib on the colony forming ability of CJ cells were investigated by treating these cells with IC_{50} and IC_{90} concentrations of each drug, both alone and in combination, for 48 hours before plating cells in methylcellulose at a density of 1000 cells per 150 μL. The numbers of colonies formed after 6 days of incubation were counted *in situ* using light microscopy.

Both chaetocin and imatinib caused a dramatic decrease in the number of colonies formed and when these drugs were used in combination, the colony forming ability of these

cells was nearly obliterated (Fig. 4.21).

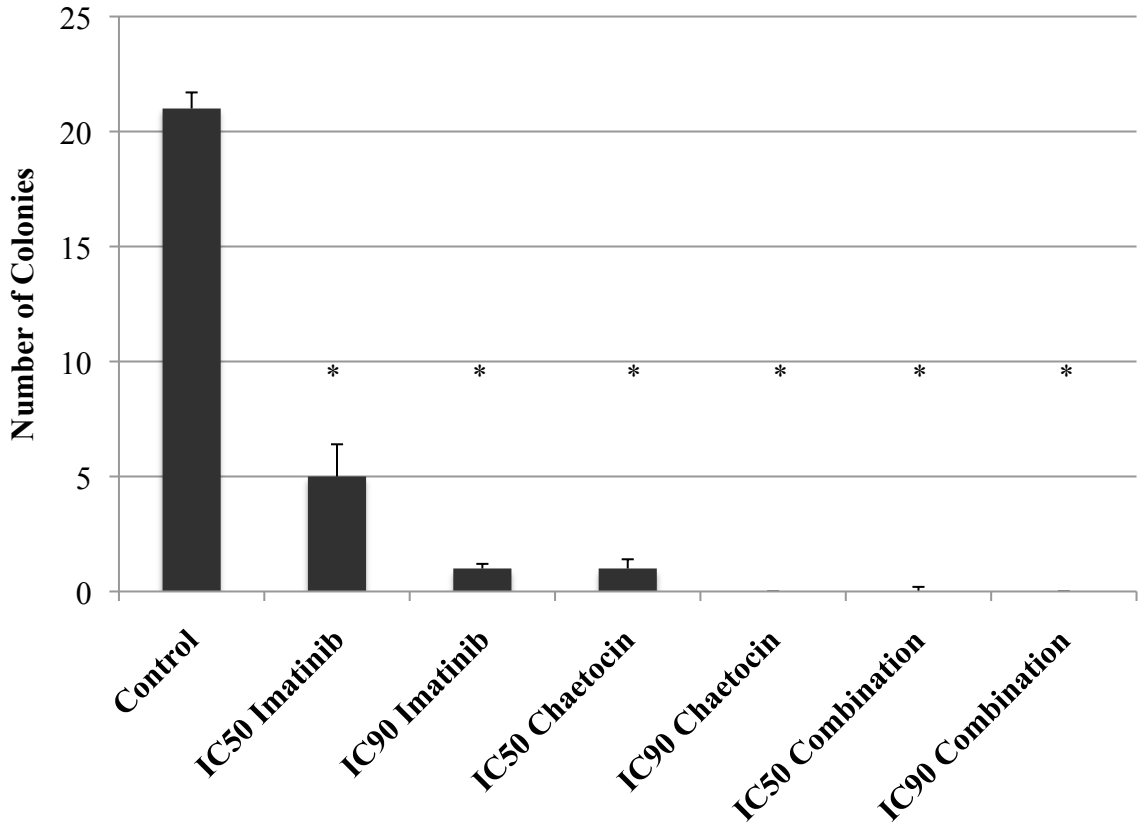

Figure 4.21: Effects of chaetocin and imatinib on the colony formation of CJ cells. Cells were treated with IC_{50} and IC_{90} concentrations of chaetocin and/or imatinib for 48 hrs before plating in methylcellulose. Resulting colonies were counted after 6 days incubation using light microscopy. Error bars represent standard deviation from three independent measurements. *p-value <0.0001 (IC_{50} and IC_{90} imatinib, IC_{50} and IC_{90} chaetocin, and IC_{50} and IC_{90} combination vs. control)

4.2.5 Effects of Bone Marrow Stromal Factors on the Activity of Chaetocin and Imatinib in CJ Cells

CJ cells were incubated with M2-10B4 culture medium for 4 hours before the addition of chaetocin and/or imatinib in order to test effects of BMSFs on the ability of these drugs to decrease viability, induce apoptosis, and inhibit colony formation.

BMSFs were found to inhibit the ability of imatinib to decrease CJ cell viability but potentiated the activity of chaetocin, resulting in a significantly greater decrease in cell viability. In addition, the combination of chaetocin and imatinib resulted in a greater decrease of cell viability compared to either drug separately, regardless of the presence of BMSFs (Fig. 4.22).

In agreement with these findings, BMSFs inhibited the ability of imatinib to induce apoptosis while potentiating the induction of apoptosis by chaetocin. Once again, the presence of BMSFs did not have a significant effect on the activity of chaetocin and imatinib when these drugs were used in combination (Fig. 4.23).

Finally, BMSFs significantly reduced the ability of imatinib to inhibit colony formation of CJ cells but did not reduce the ability of chaetocin to inhibit colony formation. In addition, the combination of chaetocin and imatinib nearly obliterated the colony forming ability of CJ cells, regardless of the presence of BMSFs (Fig. 4.24).

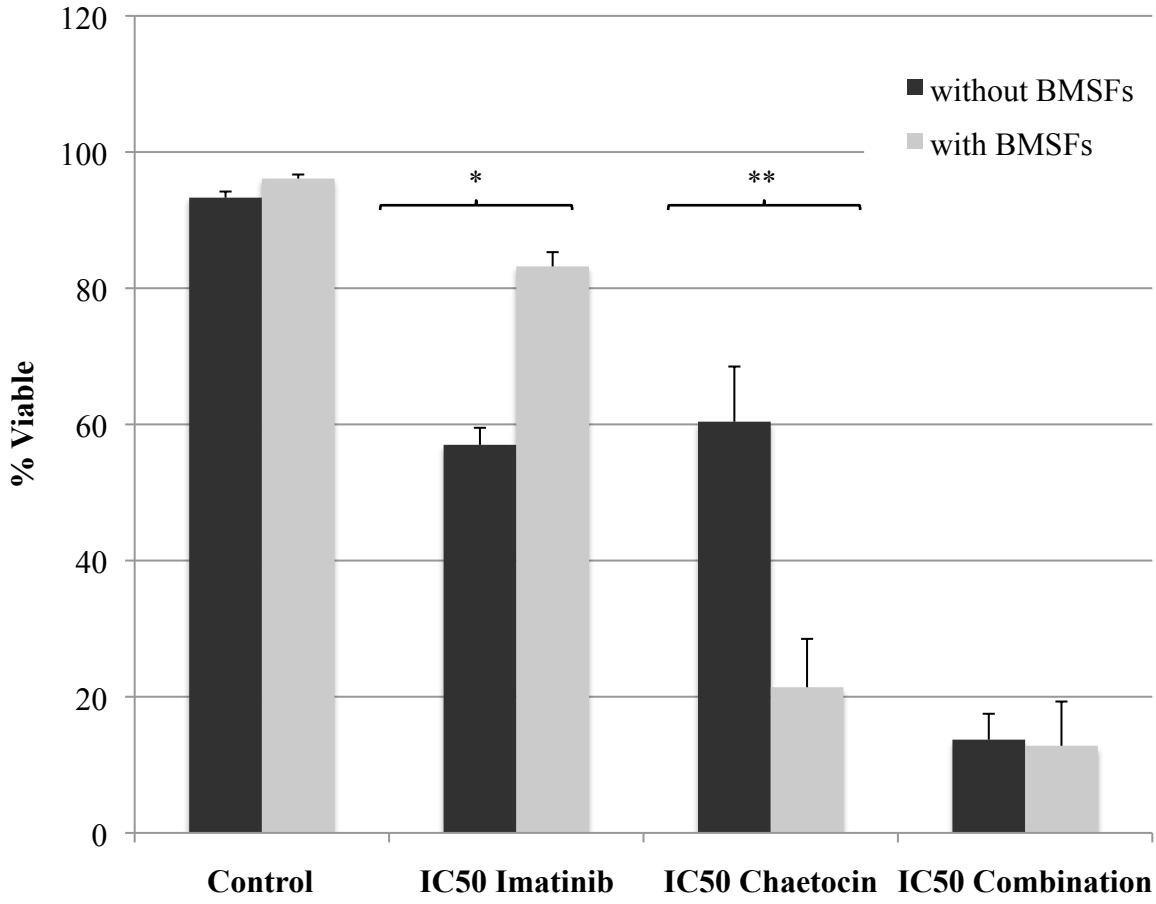

Figure 4.22: Effects of bone marrow stromal factors on the activity of chaetocin and imatinib in CJ cells. Cells were pretreated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with IC_{50} concentrations of chaetocin and/or imatinib and viability was determined using Trypan Blue exclusion assays after 48 hrs. Error bars represent standard deviation from three separate measurements. *p-value <0.0005 (IC_{50} imatinib without BMSFs vs. IC_{50} imatinib with BMSFs) **p-value <0.005 (IC_{50} chaetocin without BMSFs vs. IC_{50} chaetocin with BMSFs)

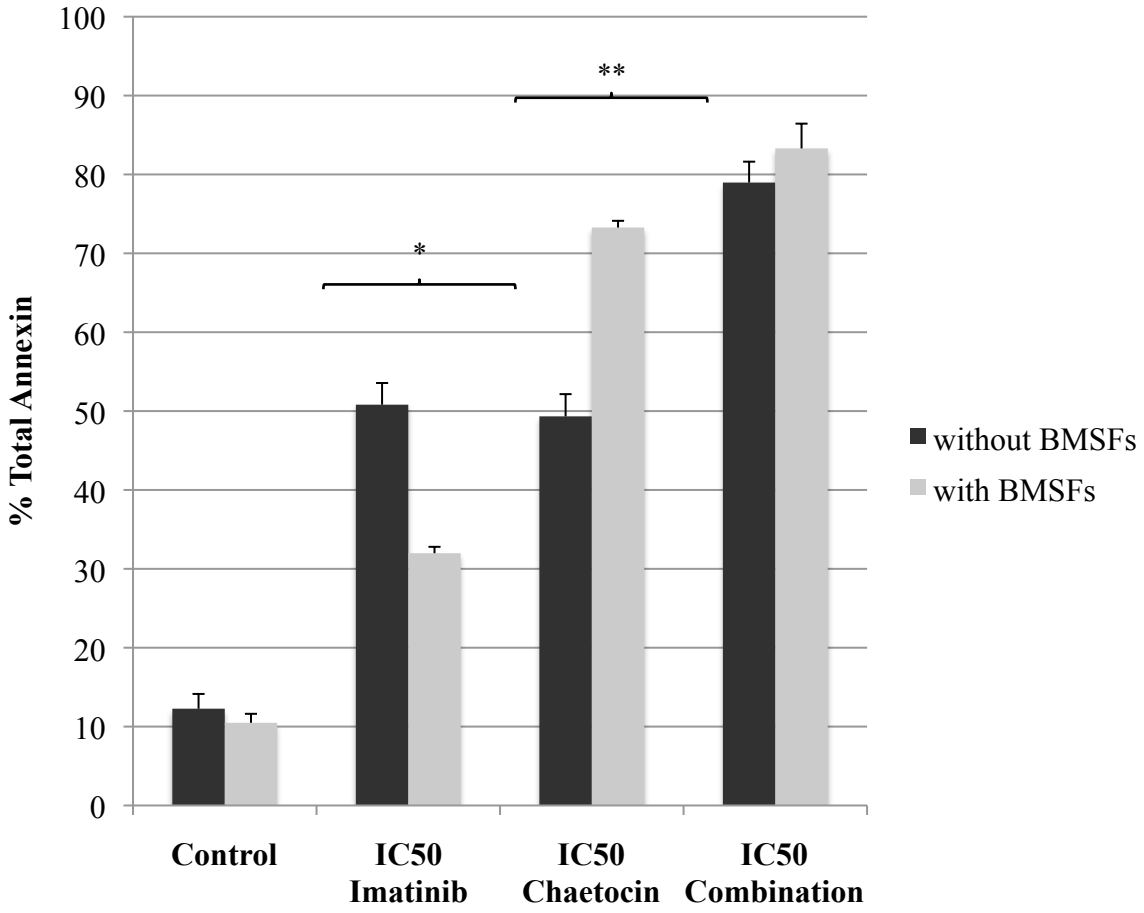

Figure 4.23: Effects of bone marrow stromal factors on the induction of apoptosis by chaetocin and imatinib in CJ cells. Cells were pretreated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with IC_{50} concentrations of chaetocin and/or imatinib and apoptosis was detected using Annexin-V assays after 48 hrs. Error bars represent standard deviation from three separate measurements. *p-value <0.0005 (IC_{50} imatinib without BMSFs vs. IC_{50} imatinib with BMSFs) **p-value = 0.0005 (IC_{50} chaetocin without BMSFs vs. IC_{50} chaetocin with BMSFs)

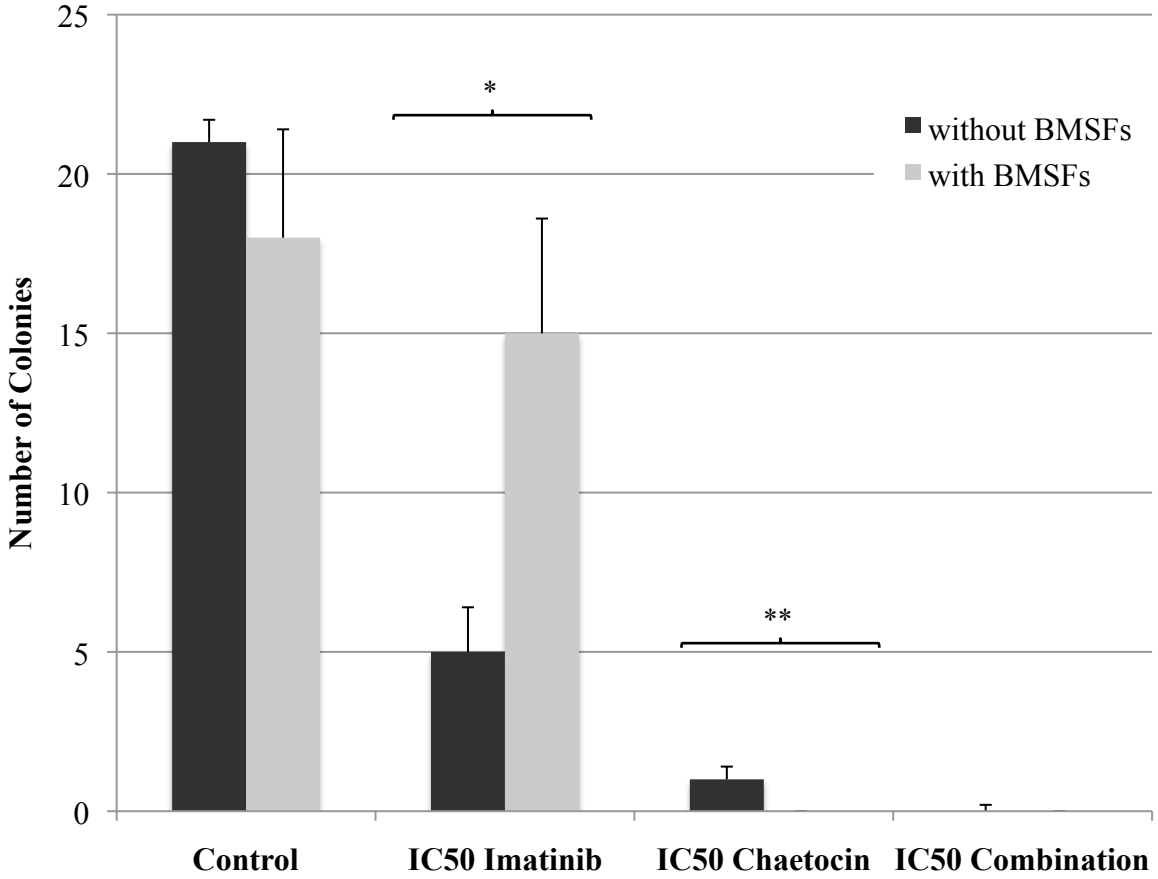

Figure 4.24: Effects of bone marrow stromal factors on the inhibition of colony formation by chaetocin and imatinib in CJ cells. Cells were pretreated with BMSFs for 4 hrs before the addition of drug(s). Cells were treated with IC_{50} concentrations of chaetocin and/or imatinib for 48 hrs before plating in methylcellulose. Colonies were counted after 6 days incubation using light microscopy. Error bars represent standard deviation from three independent measurements. *p-value <0.0001 (IC_{50} imatinib without BMSFs vs. IC_{50} imatinib with BMSFs) **p-value <0.05 (IC_{50} chaetocin without BMSFs vs. IC_{50} chaetocin with BMSFs)

4.3 Effects of Chaetocin and Imatinib on Normal Murine Hematopoietic Stem Cells

While the data above shows effects of chaetocin and imatinib on leukemic cells, it is equally important to investigate effects of these drugs on normal hematopoietic cells to ensure that normal hematopoiesis will not be affected during *in vivo* treatment. To do this, normal HSCs were obtained from the bone marrow of untreated mice and the immature HSCs isolated and treated with 100 and 180 nM chaetocin and/or 0.5 and 0.9 μM imatinib before assessing the

effects of these drugs on cell viability, induction of apoptosis, and colony formation after 48 hours.

4.3.1 Effects of Chaetocin and Imatinib on Normal Murine Hematopoietic Stem Cell Viability and Apoptosis

After 48 hours, viabilities of the treated HSCs were determined using Trypan Blue exclusion assays. Both chaetocin and imatinib had only a small effect on normal HSC viability (Fig. 4.25). Also, neither chaetocin nor imatinib caused significant induction of apoptosis in these cells, as determined using Annexin-V assays (Fig. 4.26).

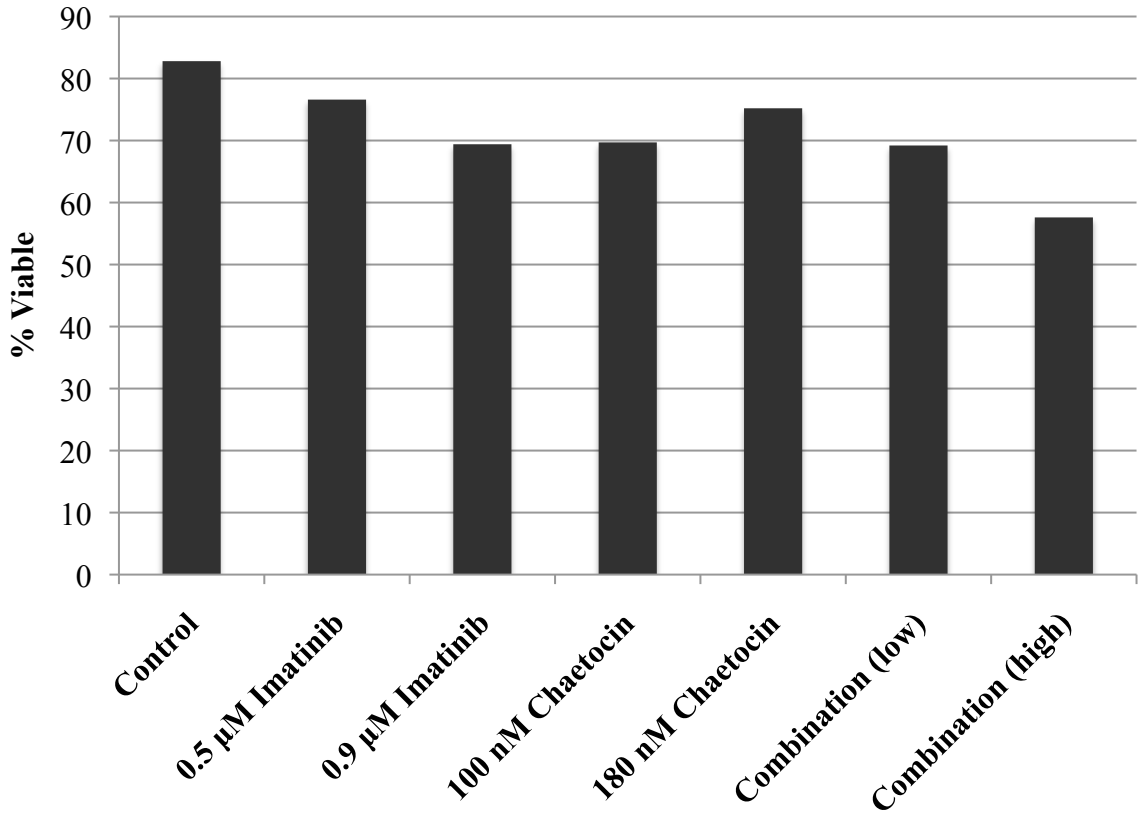

Figure 4.25: Effects of chaetocin and imatinib on the viability of normal murine hematopoietic stem cells. Cells were treated with 0.5 μM or 0.9 μM imatinib and/or 100 nM or 180 nM chaetocin for 48 hrs and viability was determined using Trypan Blue exclusion assays.

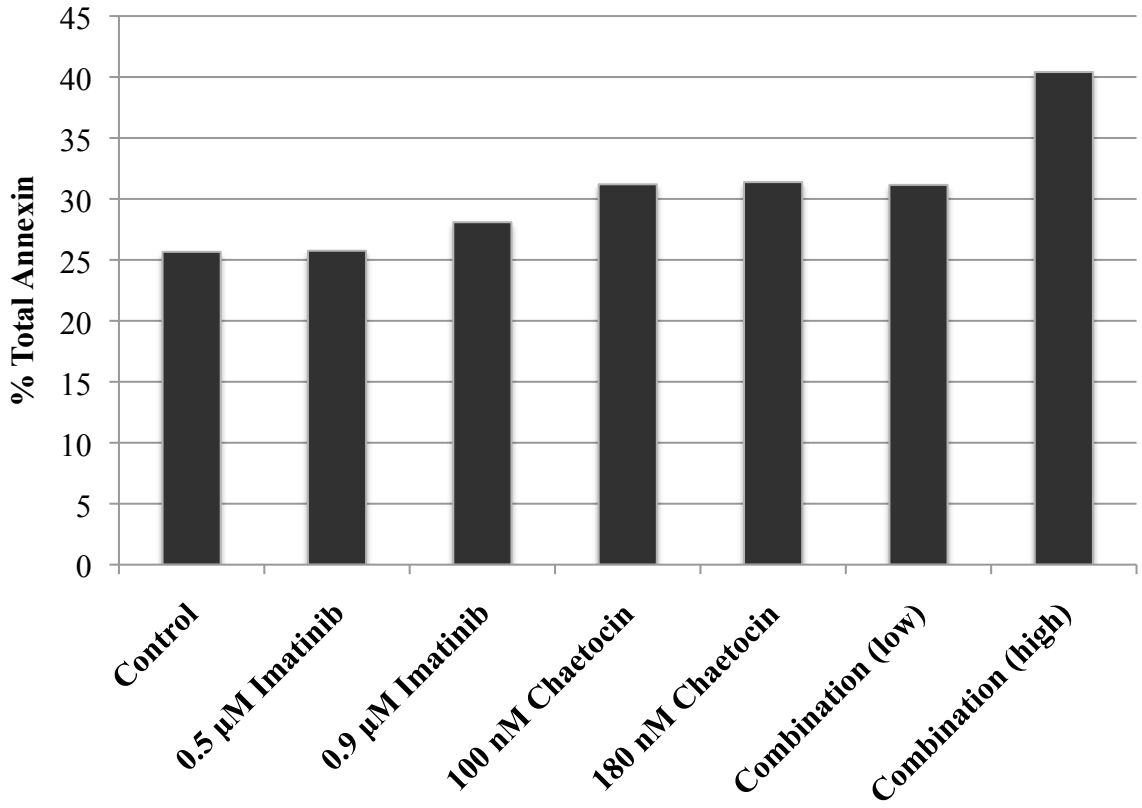

Figure 4.26: Effects of chaetocin and imatinib on the induction of apoptosis in normal murine hematopoietic stem cells. Cells were treated with 0.5 μM or 0.9 μM imatinib and/or 100 nM or 180 nM chaetocin for 48 hrs and apoptosis detected using Annexin-V assays.

4.3.2 Effects of Chaetocin and Imatinib on the Colony Formation of Normal Murine Hematopoietic Stem Cells

Effects of chaetocin and imatinib on the colony forming ability of normal lineage-depleted murine HSCs was determined by treating these cells with 100 and 180 nM chaetocin and/or 0.5 and 0.9 μM imatinib, plating 1000 cells per 150 μL methylcellulose after 48 hours treatment, incubating for 10 days, and manually counting the resulting colonies *in situ* using light microscopy.

Since methylcellulose assay conditions allow the proliferation and differentiation of progenitor cells into mature cell types, resulting colonies can be differentiated by their morphology. Neither chaetocin nor imatinib had a significant effect on the colony forming

ability of normal lineage-depleted murine HSCs. In addition, neither chaetocin nor imatinib had a significant effect on the types of colonies formed (Fig. 4.27).

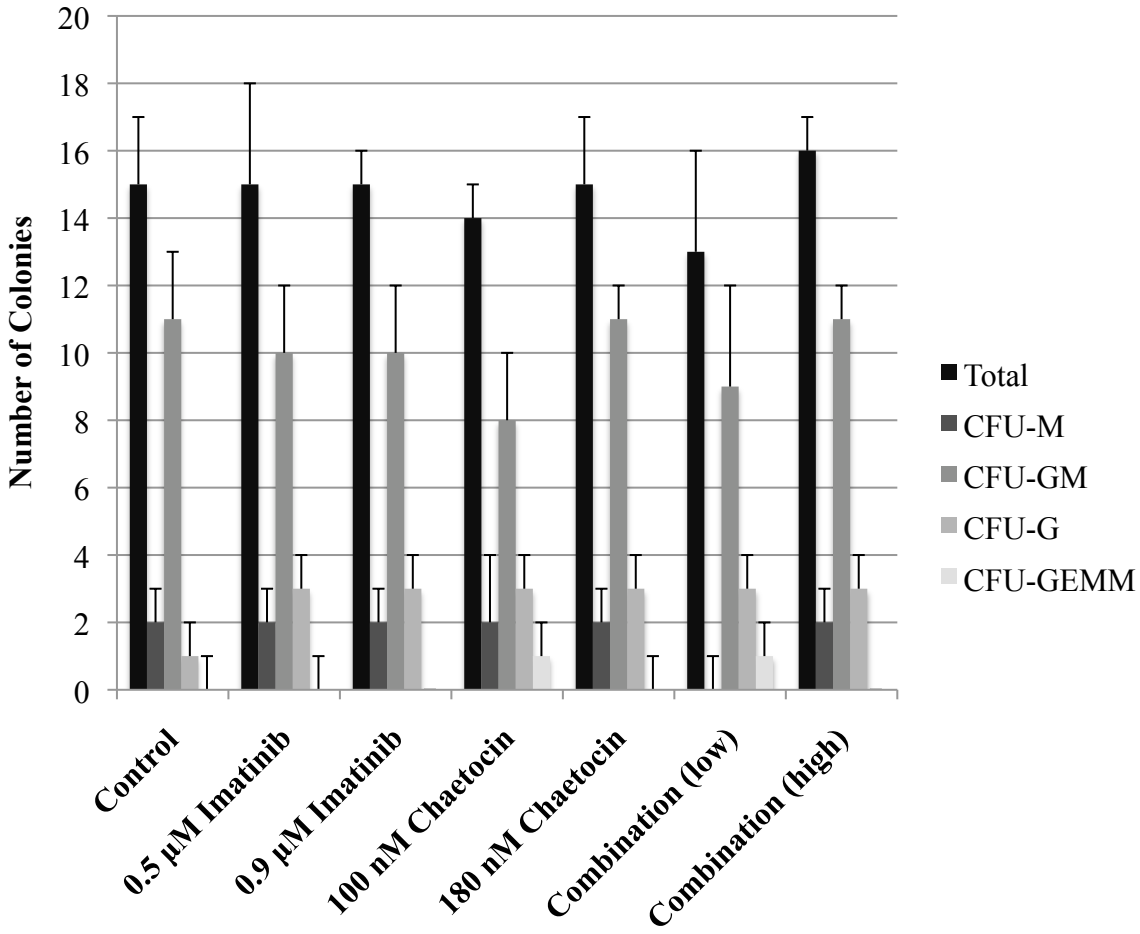

Figure 4.27: Effects of chaetocin and imatinib on the colony formation of normal murine hematopoietic stem cells. Cells were treated with 100 nM or 180 nM chaetocin and/or 0.5 μM or 0.9 μM imatinib for 48 hrs before being plated in methylcellulose and incubated for 10 days. Colonies were counted *in situ* using light microscopy and colony types differentiated by morphology, specifically CFU-M (Colony Forming Unit-Macrophage), CFU-GM (CFU-Granulocyte, Macrophage), CFU-G (CFU-Granulocyte), and CFU-GEMM (CFU-Granulocyte, Erythroid, Macrophage, Megakaryocyte). Error bars represent standard deviation from three independent measurements. All p-values >0.07 relative to untreated control.

4.4 Effects of Chaetocin and Imatinib on Leukemia Stem Cells *In Vivo*

A genetically and biologically accurate model of *in vivo* leukemogenesis has been created by Neering *et al*. (2007) through the modification of primitive normal hematopoietic cells to express the translocation products of the BCR/ABL and Nup98/HOXA9 translocations, two genetic abnormalities that have previously been documented in leukemia patients (Yamamoto *et al*., 2000; Ahuja *et al*., 2001). It has been demonstrated that these modified cells, referred to in this study as "CJ cells," are capable of initiating leukemogenesis, resulting in blast crisis CML in recipient mice when injected intravenously. This *in vivo* CML model was utilized in this study to investigate the effects of chaetocin, both alone and in combination with imatinib, on both disease latency and leukemia stem cell frequency.

4.4.1 Effects of Chaetocin and Imatinib on CML Disease Latency

In order to determine the effect of imatinib and chaetocin on CML disease latency *in vivo*, CJ cells were treated with 100 nM chaetocin for 24 hours and/or 1 μM imatinib for 48 hours and intravenously injected into sublethally irradiated mice. At the time of injection, cells treated with chaetocin were 83.8% viable, cells treated with imatinib were 46.1% viable, and cells treated with chaetocin and imatinib were 46.9% viable as determined using Trypan Blue exclusion assays. The mice were then monitored for signs of illness (e.g., ruffled fur, lethargy, splenomegaly) and euthanized with CO_2 once illness became apparent. Spleens were removed after euthanization in order to confirm the development of CML by histological analysis as well as by detection of BCR-ABL-GFP/Nup98-HOXA9-YFP expression using flow cytometry.

Mice injected with CJ cells treated with either chaetocin or imatinib survived significantly longer than the untreated control (p-value = 0.0027). In addition, mice injected with CJ cells treated with both chaetocin and imatinib survived significantly longer than the mice injected with chaetocin alone (p-value = 0.0027). However, mice injected with CJ cells treated with both chaetocin and imatinib did not survive significantly longer than the mice injected with CJ cells treated with imatinib alone (p-value = 0.1231) (Fig. 4.28).

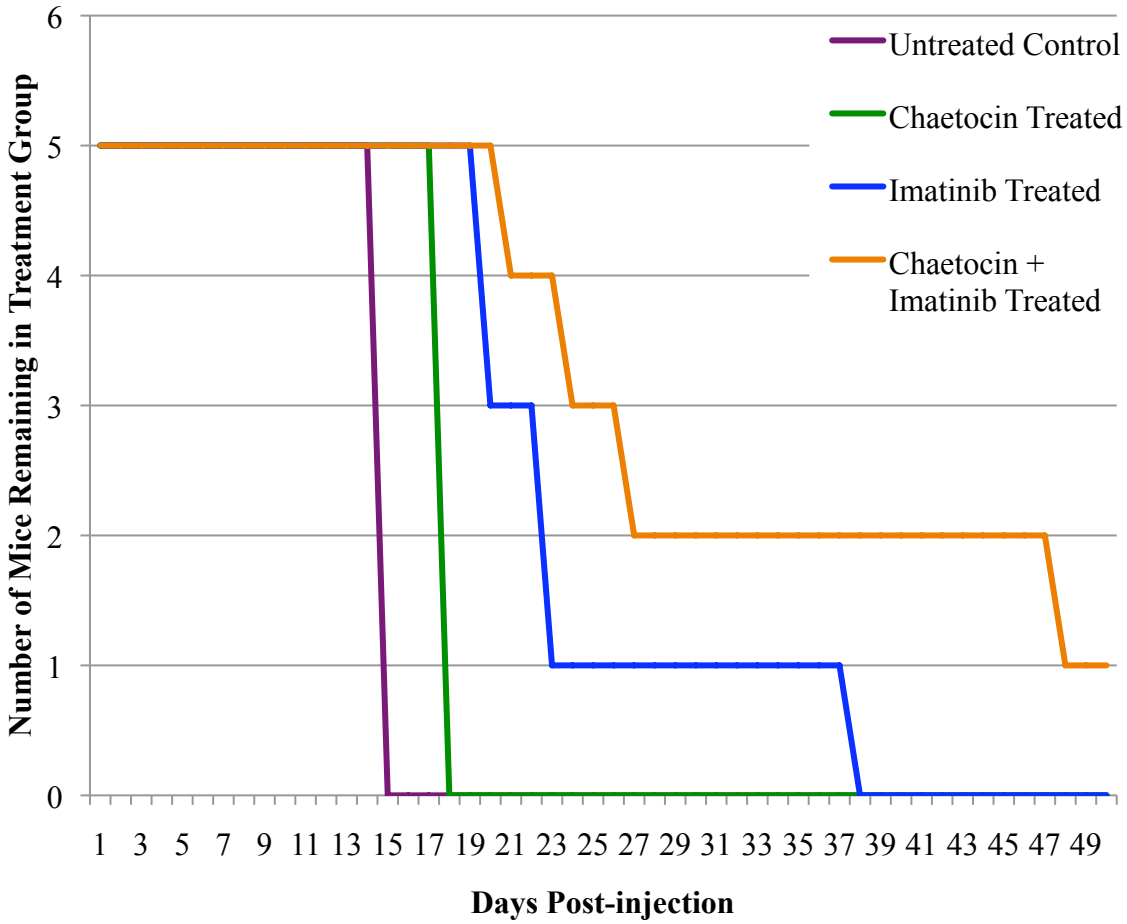

Figure 4.28: Effects of chaetocin and imatinib on chronic myelogenous leukemia disease latency. CJ cells (120,000) were treated with 100 nM chaetocin for 24 hrs and/or 1 μM imatinib for 48 hours before being injected intravenously into sublethally irradiated mice. Mice were euthanized once symptoms of leukemia became apparent. All p-values <0.003 relative to untreated control.

4.4.2 Effects of Chaetocin and Imatinib on Leukemia Stem Cell Frequency

Limiting dilution analysis (LDA) is applied in many areas of research to estimate the frequency of a specific cell type within a population capable of a defined response. In hematology, the LDA has been applied to the *in vitro* quantification of HSCs and has also been adapted to allow the quantification of murine HSCs with the capacity to produce mature cells of all hematopoietic lineages *in vivo* using software such as L-Calc (StemCell Technologies).

In this study, LDA was performed in order to determine the frequency of LSCs *in vivo* using CJ cells treated with chaetocin and/or imatinib. CJ cells were treated *in vitro* with 100

nM chaetocin for 24 hours and/or 1 μM imatinib for 48 hours before being injected intravenously into sublethally irradiated mice at a range of different cell numbers. Once CML symptoms became apparent, the mice were euthanized using CO_2 and the spleens removed for histological analysis and confirmation of BCR-ABL-GFP/Nup98-HOXA9-YFP expression by flow cytometry. The amount of time required for the development of CML symptoms was then analyzed using L-Calc software to determine the LSC frequency of each treatment group.

Both chaetocin and imatinib treatment alone was found to decrease LSC frequency (1 in 10,143 and 1 in 95,782, respectively) but the combination of these drugs caused a much more dramatic decrease in LSC frequency (1 in 293,628) (Table 4.1). In addition, the combination of chaetocin and imatinib resulted in a significantly greater decrease in LSC frequency relative to imatinib treatment alone (p-value = 0.0034).

Table 4.1: Effects of chaetocin and imatinib on leukemia stem cell frequency.

Treatment	LSC Frequency	P-value
Untreated Control	1 in 4,036	
Imatinib	1 in 95,782	0.0001
Chaetocin	1 in 10,143	0.0998
Chaetocin + Imatinib	1 in 293,628	0.0001

4.5 Effects of Chaetocin and Imatinib on Intracellular Reactive Oxygen Species

Isham *et al*. have determined that chaetocin is capable of increasing intracellular levels of ROS to cytotoxic levels by inhibiting the central ROS mitigating enzyme, TrxR1 (2007). It has also been established that expression of the constitutively active tyrosine kinase, BCR-ABL results in increased levels of intracellular ROS (Sattler *et al*., 2000) and that inhibition of c-Abl signaling by imatinib attenuates mitochondrial dysfunction and cell death in the cellular response to oxidative stress (Kumar *et al*., 2003). In this study, effects of chaetocin and imatinib on intracellular ROS as well as the ability of imatinib to affect chaetocin-induced oxidative stress were investigated using an ROS-sensitive fluorescent dye.

To determine effects of chaetocin and imatinib on intracellular ROS levels, CJ cells were treated with either 1 μM imatinib or 200 nM chaetocin for 24 hours and intracellular ROS were detected using dihydrorhodamine 6G (dR6G) and flow cytometry.

dR6G is an uncharged, non-fluorescent ROS indicator that passively diffuses across cellular membranes where it is oxidized to cationic rhodamine 6G, which localizes to the mitochondria and exhibits orange fluorescence.

While imatinib had no apparent effect on intracellular ROS, chaetocin increased the levels of intracellular ROS (Fig. 4.29).

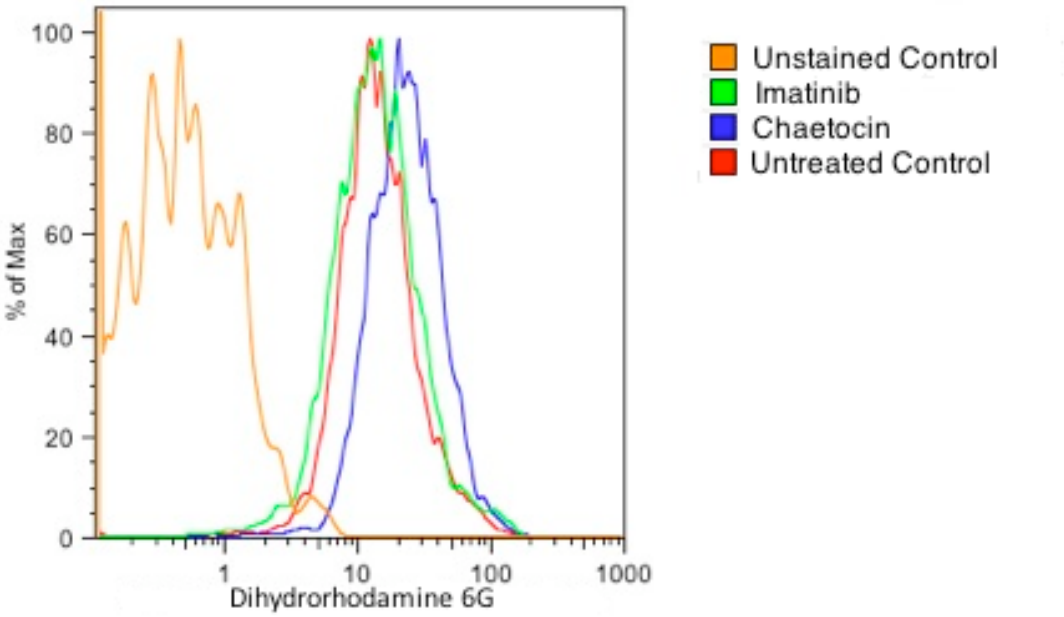

Figure 4.29: Effects of chaetocin and imatinib on intracellular reactive oxygen species in CJ cells. Cells were treated with chaetocin or imatinib for 24 hrs and intracellular ROS levels were determined using dihydrorhodamine 6G and flow cytometry. Results shown are representative of three independent measurements.

4.6 The Synergy/Antagonism of Chaetocin and Imatinib

The results presented in this thesis indicate that chaetocin and imatinib may function synergistically. To investigate this possible synergism further and determine whether chaetocin and imatinib would function more effectively if added non-simultaneously, multiple drug dose-effect experiments were performed using CJ cells and the results analyzed using CalcuSyn software.

CalcuSyn is described as the definitive analyzer of combined drug effects due to its ability to automatically quantify phenomena such as synergism and antagonism by performing multiple drug dose-effect calculations using the Median Effect methods described by Chou and Talalay (1983). More specifically, this software performs combination index (CI) analysis, which provides qualitative information on the nature of the drug interaction in the form of a numerical value. CI values are calculated as described in the equation below (4.1), in which $C_{A,x}$ and $C_{B,x}$ are the concentrations of drug A and B used in combination to achieve x% drug effect, while $IC_{x,A}$ and $IC_{x,B}$ are the concentrations for single agents that achieve the same effect (Zhao *et al*., 2004).

$$CI = \frac{C_{A,x}}{IC_{x,A}} + \frac{C_{B,x}}{IC_{x,B}} \tag{4.1}$$

Each range of CI values has been designated a symbol that describes the nature of the observed drug interaction (Table 4.2). The interaction between two drugs can be characterized as synergistic, additive, or antagonistic. "Synergy" denotes a combined effect that is above what would be expected from the individual potencies and efficacies of each drug, whereas "additive" is used to describe a combination effect that is consistent with the individual drug potencies, and finally, "antagonistic" describes a combined effect that is less than the effects produced by each drug separately. In brief, a CI of less than, equal to, or more than 1 indicates synergy, additivity, or antagonism, respectively (Zhao *et al*., 2004; Tallarida, 2011).

Table 4.2: Combination Index (CI) Symbols.

Range of CI	Symbol	Description
<0.1	+++++	very strong synergism
0.1-0.3	++++	strong synergism
0.3-0.7	+++	synergism
0.7-0.85	++	moderate synergism
0.85-0.90	+	slight synergism
0.90-1.10	+/-	nearly additive
1.10-1.20	-	slight antagonism
1.20-1.45	--	moderate antagonism
1.45-3.3	---	antagonism
3.3-10	----	strong antagonism
>10	-----	very strong antagonism

CJ cells were treated with a range of chaetocin and imatinib concentrations, with two concentrations above and two below the IC_{50} values of each, both alone and in combination. In addition, this experiment was also performed using the same drug concentrations with the addition of chaetocin and imatinib staggered by 24 hours. The percentage of cell death was determined after 48 hours using Trypan Blue exclusion assays and the results analyzed using CalcuSyn software. The results of this experiment indicate that chaetocin and imatinib function in an additive manner when administered simultaneously. Conversely, chaetocin and imatinib were found to function antagonistically when chaetocin was administered before imatinib and vice versa. In addition, chaetocin and imatinib were found to function more antagonistically when imatinib was administered before chaetocin (Table 4.3).

Table 4.3: Drug combination analysis of chaetocin and imatinib in CJ cells.

	CI values ± S.D.		
	ED_{50}	ED_{75}	ED_{90}
Simultaneous Addition	0.968 ± 0.42	0.869 ± 0.34	0.816 ± 0.30
Chaetocin before Imatinib	2.195 ± 0.62	1.841 ± 0.50	1.615 ± 0.45
Imatinib before Chaetocin	2.951 ± 0.61	2.869 ± 0.62	2.917 ± 0.71

Another method used to evaluate the nature of interaction between two drugs is isobologram analysis in which the concentrations of each drug required to produce a defined single agent effect (e.g., IC_{50}) when used as single agents are placed on the *x* and *y* axes of a two-coordinate plot and a line is drawn between these two points; this line is known as the line of additivity. A coordinate representing the concentrations of the two drugs used in

combination required to obtain the same effect are then placed on the plot and the location of this point relative to the line of additivity indicates the nature of the interaction between these drugs with synergy, additivity, or antagonism being indicated by a point located below, on, or above the line of additivity, respectively (Zhao *et al*., 2004).

CalcuSyn software was used to generate isobolograms describing the nature of the interaction between chaetocin and imatinib when these drugs were administered simultaneously, when chaetocin was administered 24 hours before imatinib, and when imatinib was administered 24 hours after chaetocin. The results of these analyses show that chaetocin and imatinib function synergistically when administered simultaneously and that these drugs function antagonistically when chaetocin is administered before imatinib and vice versa. In addition, this data shows that chaetocin and imatinib function more antagonistically when imatinib is administered before chaetocin (Fig. 4.30).

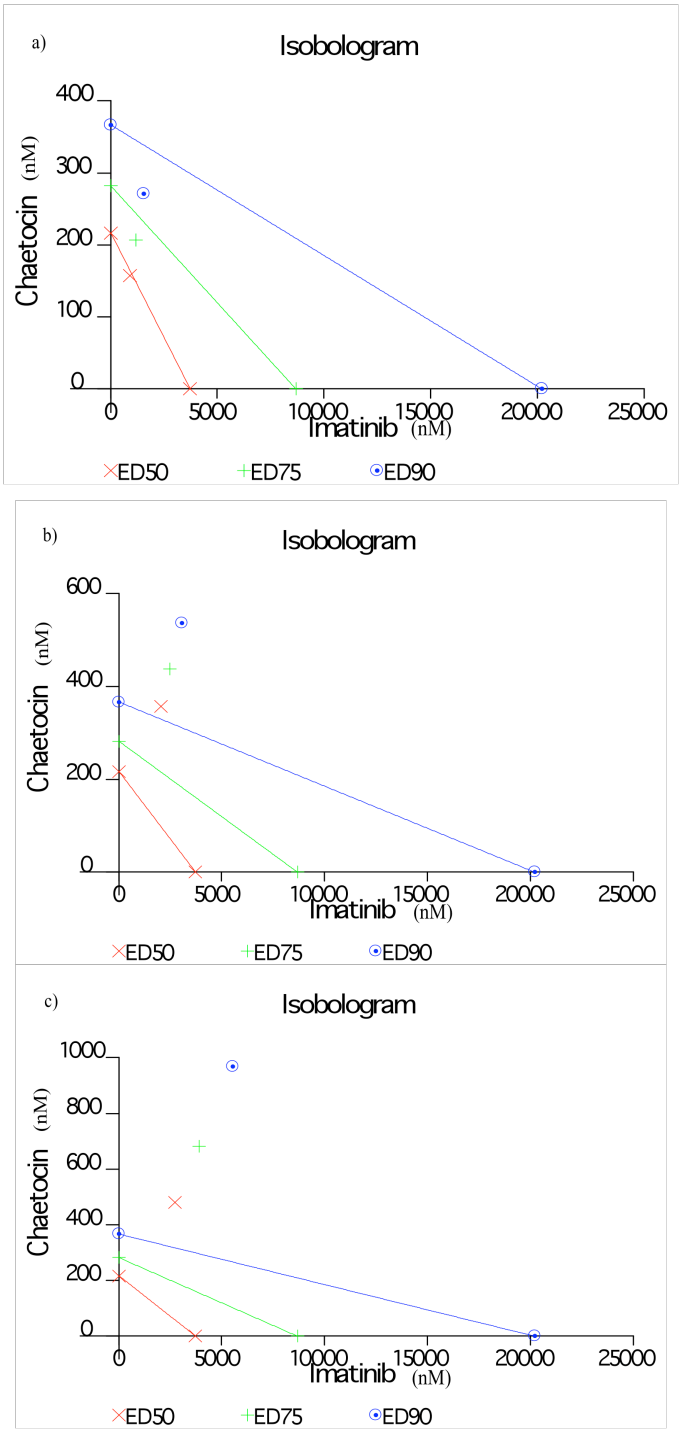

Figure 4.30: Isobologram analysis of the interaction between chaetocin and imatinib in CJ cells. Cells were treated with chaetocin and imatinib for 48 hrs either (a) simultaneously, (b) with chaetocin 24 hrs before the addition of imatinib, or (c) with imatinib 24 hrs before the addition of chaetocin. Cell viabilities were determined using Trypan Blue exclusion assays and the results analyzed using CalcuSyn software. Each diagonal line represents the line of additivity at the indicated dose and the data points indicate synergy, additivity, or antagonism by their location below, on, or above the line, respectively.

5. DISCUSSION

In this study, the effectiveness of chaetocin against leukemia stem cells (LSCs) was investigated using *in vitro* and *in vivo* murine chronic myelogenous leukemia (CML) models. The results of this study indicate that chaetocin and imatinib function synergistically in decreasing cell viability, inducing apoptosis, and inhibiting the colony formation of CML cells *in vitro*, that chaetocin in combination with imatinib reduces LSC frequency *in vivo*, that chaetocin increases intracellular reactive oxygen species levels, and that chaetocin does not disrupt the proliferation and differentiation of normal murine hematopoietic stem cells (HSCs). Surprisingly, BMSFs enhanced the activity of chaetocin while they reversed the activity of imatinib. These findings strongly suggest that chaetocin could potentially be used as a complement to standard tyrosine kinase inhibitor (TKI) therapy for the treatment of CML and may be especially effective against LSCs within the bone marrow niche.

5.1 Chaetocin and Imatinib Decrease Cell Viability, Induce Apoptosis, and Inhibit the Colony Formation of CML Cells *In Vitro*

The first *in vitro* CML model used in this study to characterize the activity of chaetocin was TonB210, a murine hematopoietic cell line with doxycycline-inducible BCR-ABL expression. In the absence of IL-3, this cell line shows an absolute dependence on doxycycline induced BCR-ABL expression for cell survival and proliferation (Klucher *et al*., 1998).

In TonB210 cells, imatinib only inhibited viability when BCR-ABL was expressed (Fig. 4.1 and 4.3), whereas chaetocin inhibited cell viability in the presence or absence of BCR-ABL expression (Fig. 4.2 and 4.4). When TonB210 cells were treated with both chaetocin and imatinib, the drugs' effects were additive in decreasing viability at the IC_{50} concentrations. At concentrations above IC_{50} values, the doses were too toxic to determine whether the drugs were acting in a synergistic manner (Fig. 4.6).

Similar results were also observed for effects of chaetocin and imatinib on the induction of apoptosis in TonB210 cells. Chaetocin induced apoptosis in TonB210 cells, both with and without BCR-ABL expression, while imatinib initiated apoptosis only in the presence of BCR-ABL. Co-treatment of TonB210 cells with chaetocin and imatinib resulted in a greater percentage of cells undergoing apoptosis when BCR-ABL was expressed and imatinib had no

effect on the ability of chaetocin to induce apoptosis in cells not expressing BCR-ABL (Fig. 4.7 and 4.8).

Imatinib and chaetocin had a dramatic effect on the number of colonies formed by TonB210 cells expressing BCR-ABL and, when these drugs were combined, almost no colonies formed. In addition, imatinib had no effect on the proliferation of TonB210 cells not expressing BCR-ABL and imatinib did not affect the ability of chaetocin to inhibit the proliferation of these cells (Fig. 4.9 and 4.10). Since colony formation is a measure of stem cell proliferation and differentiation, these results indicate that chaetocin may have the ability to target the CML stem cell population.

The second *in vitro* CML model used in this study to characterize the effects of chaetocin was CJ cells, a murine model of blast crisis CML created through the modification of primitive normal hematopoietic cells such that they express both the BCR/ABL and Nup98/HOXA9 translocation products (Neering *et al*., 2007). As with TonB210 cells, chaetocin and imatinib decreased cell viability, induced apoptosis, and inhibited colony formation.

In CJ cells treated with either chaetocin or imatinib alone, viability was inhibited and when these cells were treated with chaetocin and imatinib simultaneously, the effects of these drugs were found to function in an additive manner at both the IC_{50} and IC_{90} concentrations (Fig. 4.19).

In addition, we sought to determine whether the cell death observed after drug treatment was due to the induction of apoptosis. Our results show that both chaetocin and imatinib initiate apoptosis in CJ cells and that a greater percentage of cells are found to be apoptotic when these drugs are used in combination (Fig. 4.20).

Finally, the effects of chaetocin and imatinib on the proliferation and differentiation of CJ cells were investigated. Both chaetocin and imatinib severely inhibited the colony forming ability of these cells and when these drugs were used in combination, almost no proliferation occurred (Fig. 4.21). This finding further supports our hypothesis that chaetocin is capable of targeting CML stem cells in combination with imatinib.

To determine if chaetocin and imatinib function synergistically and to investigate if their synergy could be enhanced by adding each drug non-simultaneously, multiple drug dose-effect experiments were performed using CJ cells. The results of these experiments showed

that chaetocin and imatinib function synergistically when CJ cells are treated with these drugs simultaneously. Conversely, chaetocin and imatinib were found to function antagonistically when chaetocin was added 24 hours after the addition of imatinib and vice versa (Table 4.3 and Fig. 4.30).

It has been established that cancer cells are under oxidative stress due to an increased production of ROS (Szatrovski *et al*., 1991). More specifically, transformation of hematopoietic cells by the oncogenic tyrosine kinase, BCR-ABL has been associated with a chronic increase in intracellular ROS (Sattler *et al*., 2000). Since it has been established that the antimyeloma activity of chaetocin strongly relies on its ability to impose cytotoxic levels of oxidative stress (Isham *et al*., 2007), one explanation for why these drugs function antagonistically when cells are treated with imatinib before chaetocin could be that since imatinib treatment reduces the levels of intracellular ROS through the inhibition of BCR-ABL (Landry *et al*., 2013), imatinib likely counteracts the cytotoxic effects of chaetocin.

These results indicate that if chaetocin is to be used as a complement to TKI treatment, it is important that chaetocin be administered at the same time as the TKI in order to maximize the effectiveness of chaetocin against LSCs.

To investigate the effects of chaetocin and imatinib on intracellular ROS levels, CJ cells were treated with chaetocin or imatinib and ROS were detected using a fluorescent ROS indicator and flow cytometry. The results of this experiment showed that imatinib had no effect on the intracellular levels of ROS in these cells while chaetocin caused a slight increase (Fig. 4.29).

This finding supports the theory proposed by Isham *et al*. which states that chaetocin increases intracellular ROS levels by acting as a competitive inhibitor of thioredoxin reductase-1 (TrxR1), a central ROS-mitigating enzyme (2007). However, these results do not support the finding by others that inhibition of BCR-ABL by imatinib decreases intracellular ROS levels (Landry *et al*., 2013).

In summary, these results show that chaetocin is capable of inducing apoptosis and severely limiting the proliferation and differentiation of two different *in vitro* CML models, that this activity is enhanced by co-treatment with imatinib, and that chaetocin and imatinib function synergistically when cells are treated with these drugs simultaneously, while these drugs act antagonistically when cells are treated with imatinib before chaetocin and vice versa.

5.2 Bone Marrow Stromal Factors Potentiate the Anti-Leukemia Stem Cell Activity of Chaetocin

To investigate the effects of BMSFs on the activity of chaetocin and imatinib *in vitro*, TonB210 and CJ cells were cultured in the medium of M2-10B4 cells for several hours before the addition of these drugs. Since M2-10B4 cells are known to secrete BMSFs, their culture medium can be used to simulate the environment of the bone marrow niche (Sutherland *et al*., 1991). As in the previous section, the effects of chaetocin and imatinib on cell viability, induction of apoptosis, and inhibition of colony formation were then examined.

BMSFs were found to effectively inhibit the ability of imatinib to reduce cell viability but potentiated the activity of chaetocin to reduce cell viability in both TonB210 and CJ cells. In addition, chaetocin and imatinib were found to produce a greater amount of cell death in combination, regardless of the presence of BMSFs (Fig. 4.11, 4.12, and 4.22).

While BMSFs did not have a significant effect on the ability of imatinib to induce apoptosis in TonB210 cells, they did significantly inhibit the induction of apoptosis by imatinib in CJ cells and significantly potentiated the ability of chaetocin to induce apoptosis in both TonB210 and CJ cells. In addition, chaetocin and imatinib were found to produce a greater percentage of apoptotic cells in combination in both TonB210 and CJ cells, regardless of the presence of BMSFs (Fig. 4.13, 4.14, and 4.23).

Finally, while BMSFs did not significantly potentiate the ability of chaetocin to inhibit the colony formation of TonB210 cells, they did significantly potentiate the ability of chaetocin to inhibit the colony formation of CJ cells and significantly reduced the ability of imatinib to inhibit the colony formation of both TonB210 and CJ cells. In addition, chaetocin and imatinib in combination were found to dramatically inhibit the proliferation and differentiation of both TonB210 and CJ cells, regardless of the presence of BMSFs (Fig. 4.15, 4.16, and 4.24).

In summary, BMSFs were found to effectively inhibit the ability of imatinib to induce apoptosis and inhibit colony formation in TonB210 and CJ cells, while BMSFs were found to potentiate the activity of chaetocin. In addition, the potency of chaetocin was found to be increased by imatinib, regardless of BMSF pretreatment. This suggests that these drugs function synergistically and that the presence of chaetocin overcomes the protective effects of BMSFs, allowing imatinib to function more effectively.

These findings support the theory that LSCs are not reliant on BCR-ABL for survival in the presence of BMSFs (Corbin *et al*., 2011) and that BMSFs effectively lead to imatinib resistance (Bewry *et al*., 2008). Importantly, these findings also show that BMSFs do not protect LSCs from the toxic effects of chaetocin and appear to potentiate its cytotoxic effects, suggesting that chaetocin is capable of targeting LSCs within the protective confines of the bone marrow niche.

5.3 Chaetocin does not disrupt the Proliferation and Differentiation of Normal Murine Hematopoietic Stem Cells *In Vitro*

In order to ensure that normal hematopoiesis will not be affected by chaetocin treatment, normal hematopoietic stem cells (HSCs) were isolated from the bone marrow of healthy mice by lineage depletion and treated with chaetocin and/or imatinib *in vitro* before evaluating the effects of chaetocin on cell viability, induction of apoptosis, and colony formation.

The results presented in this study show that chaetocin has little to no effect on the viability of normal murine HSCs (Fig. 4.25). Our finding that only a small percentage of cells were apoptotic after treatment with chaetocin and imatinib (Fig. 4.26) further supports this result. Importantly, our results showed that chaetocin, both alone and in combination with imatinib, had no significant effect on the proliferation and differentiation of normal HSCs (Fig. 4.27). These findings suggest that chaetocin will not affect normal hematopoiesis *in vivo* and could therefore potentially serve as a safe and effective treatment for CML in combination with imatinib.

5.4 Chaetocin in Combination with Imatinib Reduces Leukemia Stem Cell Frequency *In Vivo*

The effects of chaetocin, both alone and in combination with imatinib, on CML disease latency were investigated by injecting chaetocin and/or imatinib treated CJ cells intravenously into sublethally irradiated mice, monitoring for symptoms of illness, and euthanizing the mice once symptoms of CML became apparent. The development of CML was confirmed by histological analysis of spleen tissue and detection of BCR-ABL-GFP/Nup98-HOXA9-YFP expression.

The results presented in this study show that mice injected with CJ cells treated with

chaetocin and/or imatinib *in vitro* survived significantly longer than mice injected with untreated CJ cells (Fig. 4.28). This finding indicates that chaetocin effectively delays the onset of CML, especially in combination with imatinib.

In addition, limiting dilution analysis (LDA) was performed to determine the effect of chaetocin on LSC frequency *in vivo*, both alone and in combination with imatinib. The results of this experiment showed that chaetocin in combination with imatinib caused a significant and dramatic decrease in LSC frequency, compared to both untreated cells and cells treated with imatinib alone (Table 4.1). This finding indicates that chaetocin and imatinib function synergistically in effectively targeting LSCs and further supports our claim that chaetocin could serve as a valuable complement to standard TKI therapy.

5.5 Significance and Future Directions

The results of this study indicate that chaetocin and imatinib function synergistically when administered simultaneously to decrease cell viability, induce apoptosis, and inhibit the proliferation and differentiation of two different murine CML models *in vitro*, that chaetocin in combination with imatinib reduces LSC frequency *in vivo*, that chaetocin increases intracellular ROS levels in CML cells, and that chaetocin does not disrupt the proliferation and differentiation of normal HSCs, indicating that normal hematopoiesis will be unaffected by chaetocin *in vivo*. The most intriguing finding in this study was that while BMSFs diminish the effectiveness of imatinib against CML cells, they appear to potentiate the cytotoxic activity of chaetocin, indicating that chaetocin may be capable of targeting LSCs within the bone marrow niche, which are thought to be responsible for CML disease relapse.

Despite the insights that the results of this study have provided, many questions remain regarding the cytotoxic activity of chaetocin. For example, it has been established that chaetocin inhibits TrxR1 but other molecular targets of chaetocin remain unclear and it is possible that chaetocin inhibits other cellular reductases. To address this question, a comprehensive study of the effects of chaetocin across a wider spectrum of cellular reductases should be performed to determine if chaetocin affects other antioxidant systems such as glutathione, catalase, and superoxide dismutases.

Another interesting study that should be performed is the investigation of the synergy or

antagonism between chaetocin and other TKIs, such as dasatinib and nilotinib. As was performed in this study, *in vitro* CML models can be used to test these different drug combinations. Since these TKIs share a molecular target with imatinib, it is likely that they will also function synergistically with chaetocin.

Furthermore, the potentiation of the activity of chaetocin by BMSFs should be investigated. It is possible that BMSFs are enhancing the ability of chaetocin to increase ROS to cytotoxic levels and the pathways through which this may be occurring should be examined. One approach to this question would be to first invistigate the effects of individual cytokines, such as IL-6 and SCF, on the activity of chaetocin using *in vitro* CML models and monitoring their effects on the ability of chaetocin to decrease cell viability, induce apoptosis, and inhibit the proliferation and differentiation of CML cells.

Finally, further *in vivo* chaetocin experiments should be performed using murine CML models. For example, the oral or intraperitoneal administration of chaetocin could be tested to further investigate the *in vivo* efficacy of chaetocin against LSCs, both alone and in combination with TKIs.

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