Novel Drug Combinations for the Eradication of $Ph^+/BCR\text{-}ABL^+$ Haemopoietic Stem Cells in Chronic Myeloid Leukaemia

by

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Abstract

INTRODUCTION: Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of the haemopoietic stem cell (HSC). It results from acquisition of the Philadelphia (Ph) chromosome and expression of the oncogenic fusion protein BCR-ABL. Imatinib mesylate (IM) is a tyrosine kinase inhibitor (TKI) which competitively inhibits ATP binding to BCR-ABL, resulting in inhibition of downstream signal transduction pathways. Despite inducing a complete cytogenetic response in the majority of CML patients in chronic phase (CP), nearly all patients treated with IM have detectable disease at the molecular level by quantitative RT-PCR and, therefore, are unlikely to be cured. It has been demonstrated that this molecular persistence results from a population of quiescent CML stem cells which are not effectively targeted by IM. In addition, a minority of CML patients harbour BCR-ABL kinase domain mutations, rendering them IM-resistant. We set out to investigate different therapeutic strategies for targeting the quiescent CML stem cell population *in vitro*. These strategies were treating with continuous or interrupted IM in combination with recombinant human granulocyte-colony stimulating factor (rHu-G-CSF), comparing the efficacy of IM with the novel, dual SRC/BCR-ABL TKI, dasatinib, and combining either IM or dasatinib with the cytotoxic farnesyltransferase inhibitor (FTI), BMS-214662.

METHODS: $CD34^+$ CML cells isolated from newly diagnosed CP CML patients, normal $CD34^+$ cells, total mononuclear cells (MNC) from blast crisis (BC) CML patients and acute myeloid leukaemia (AML) patients, and wild-type and mutant BCR-ABL-expressing Ba/F3 cells were cultured in the described conditions. To assess efficacy against the primitive progenitor population, carboxy-fluorescein diacetate succinimidyl diester (CFSE) was used to track cell division and caspase-3 activity to assess apoptosis in primary cell samples. To determine the inhibitory

effect of different agents on BCR-ABL kinase activity a flow-cytometry based CrKL phosphorylation assay was developed and used. The relevance of SRC kinases in the different phases of CML was assessed by measuring expression of phosphorylated SRC (p-SRC) and BCL-2 at baseline and again following treatment with different TKIs (IM, dasatinib and PP2). Previous studies have indicated that alterations in BCL-2 expression may be linked to SRC family kinase activity in CML cells *in vitro*. Long-term culture-initiating cell (LTC-IC) assays were used to determine the efficacy of BMS-214662 against CML and normal HSC. Tritiated thymidine proliferation assays were employed to assess concentration-response curves and determine the efficacy of BMS-214662 in Ba/F3 cell lines with BCR-ABL kinase mutations. Fluorescence in-situ hybridisation (FISH) confirmed the proportion of BCR-ABL$^+$ CD34$^+$ cells at baseline and again after culture in the different treatment conditions.

RESULTS: Interrupted IM therapy did not enhance the eradication of total viable cells or non-proliferating CFSE^{max} CD34$^+$ CML cells compared to continuous IM. The combination of continuous IM with intermittent rHu-G-CSF more effectively reduced total viable cells than other IM/rHu-G-CSF combinations. Imatinib mesylate had no significant effect on non-proliferating CFSE^{max} CD34$^+$ CML cells. Intermittent rHu-G-CSF significantly reduced non-proliferating CFSE^{max} CD34$^+$ CML cells compared to either no rHu-G-CSF or continuous rHu-G-CSF by increasing the number of cells which left the quiescent fraction by cell division. The addition of IM did not enhance this effect.

At therapeutically achievable concentrations (5μM and 150nM, respectively) IM and dasatinib had equivalent cytotoxicity on primary CD34$^+$ CML cells. Compared to no drug control, neither IM nor dasatinib reduced the non-proliferating CFSE^{max} CD34$^+$ CML cell population. Caspase-3 activity was not significantly increased in

the treatment arms, indicating that the cells exiting the undivided gate did so by cell division and not apoptosis. Using the CrKL phosphorylation assay, dasatinib was shown to be a more effective BCR-ABL kinase inhibitor than IM. Activated SRC expression, measured by p-SRC and BCL-2 levels, was significantly increased in $CD34^+$ CML cells compared to normal $CD34^+$ cells. There was a trend for higher p-SRC, but not BCL-2 expression, in BC compared to CP CML. Further results suggested that p-SRC expression was mediated by BCR-ABL, as IM or dasatinib, but not the SRC-specific kinase inhibitor PP2, reduced p-SRC expression; albeit only temporarily with IM.

BMS-214662 was very potent against $CD34^+$ CML cells at clinically achievable concentrations (250nM). When used in combination with IM or dasatinib, BMS-214662 significantly enhanced overall cytotoxicity compared to either IM or dasatinib alone. BMS-214662 targeted non-proliferating $CFSE^{max}$ $CD34^+$ cells in short-term culture experiments, and had minimal anti-proliferative effect. Apoptosis studies demonstrated that BMS-214662 increased caspase-3 activity in proliferating and non-proliferating $CFSE^{max}$ $CD34^+$ CML cells. BMS-214662 overcame the anti-proliferative effects of IM or dasatinib to significantly reduce non-proliferating $CFSE^{max}$ $CD34^+$ cells present after treatment. The LTC-IC assays confirmed that BMS-214662 was targeting primitive CML stem cells and provided evidence of selectivity for CML versus normal HSCs. In 2 of 3 patients, FISH confirmed that more than 90% of the cells surviving LTC-IC were Ph^-; further evidence for the selectivity of BMS-214662 for Ph^+ cells. Preliminary experiments presented here indicate that BMS-214662 is likely to be effective in BC CML, patients with BCR-ABL kinase mutations resistant to TKIs and also AML.

CONCLUSIONS: Intermittent exposure to rHu-G-CSF can enhance the effect of IM on CML cells by specifically targeting the non-proliferating $CD34^+$ sub-

population. A pilot study to treat CP CML patients with IM and intermittent rHu-G-CSF is currently underway to determine if this strategy will reduce molecular persistence *in vivo*. Dasatinib was more effective than IM within the CML stem cell compartment, however the most primitive quiescent CML cells appear to be inherently resistant to both drugs, indicating that BCR-ABL and SRC kinases may not be relevant targets in the most primitive quiescent CML stem cell population. BMS-214662 is the first agent assessed using the CFSE method to show a significant reduction in non-proliferating $CFSE^{max}$ $CD34^{+}$ cells compared to the no drug control in CML. LTC-IC confirmed that BMS-214662 was targeting CML stem cells and highlighted specificity for Ph^{+} versus Ph^{-} cells. Further studies are ongoing to elucidate the mode of action of BMS-214662 which may have utility not only in CML but also other malignancies in which cancer stem cells have been identified.

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Author's Declaration

Unless otherwise stated, I declare that all the work presented in this thesis is my own.

Definitions and Abbreviations

^{3}H	Tritiated
7-AAD	7-Aminoactinomycin D
17AAG	17-(Allylamino)-17-demethoxygeldanamycin
ABCG2	ATP binding cassette subfamily member 2
ABL	Abelson proto-oncogene
ALL	Acute lymphoblastic leukaemia
alloSCT	Allogeneic stem cell transplant
AML	Acute myeloid leukaemia
AP	Accelerated phase
APC	Allophycocyanin
Ara-C	Cytosine arabinoside
ATP	Adenosine triphosphate
BC	Blast crisis
BCA	Bicinchoninic acid
BCR	Breakpoint cluster region gene
BCR-ABL	BCR-ABL fusion gene
BCR-ABL	BCR-ABL protein tyrosine kinase
BIT	Bovine serum albumin/insulin/transferrin serum substitute
BSA	Bovine serum albumin
CCR	Complete cytogenetic response
CD	Cluster of differentiation; cell surface molecules recognised by specific sets of antibodies
CENP	Centromere protein
CFC	Colony-forming cell assay
CFSE	Carboxy-fluorescein diacetate succinimidyl diester
CFU-GM	Granulocyte macrophage-colony forming unit
CHR	Complete haematologic response
CIS	Cytokine-induced SH2-containing family of proteins
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CMolR	Complete molecular response
CP	Chronic phase
CrKL	v-Crk avian sarcoma virus CT10 oncogene homolog-like
CXCR-4	Chemokine (C-X-C motif) receptor 4
DLI	Donor lymphocyte infusion
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FGF-R	Fibroblast growth factor receptor
FISH	Fluorescence in-situ hybridisation
FITC	Fluorescein isothiocyanate
FL	Fluorescence channel

FSC	Forward scatter
FT	Farnesyltransferase
FTI	Farnesyltransferase inhibitor
G_0	Gap phase 0 (of cell cycle; quiescent)
G_1	Gap phase 1 (of cell cycle)
G_2	Gap phase 2 (of cell cycle)
G-CSF	Granulocyte-colony stimulating factor
G-CSF-R	Granulocyte-colony stimulating factor receptor gene
G-CSF-R	Granulocyte-colony stimulating factor receptor protein
G-proteins	Guanine nucleotide binding proteins
GIMI	G-CSF with Imatinib Mesylate Intermittently
GM-CSF	Granulocyte macrophage-colony stimulating factor
GGT	Geranylgeranyltransferase
GvHD	Graft versus host disease
GvL	Graft versus leukaemia
GSK-3β	Glycogen synthase kinase-3β
HBSS-CMF	Hank's buffered salt solution – calcium and magnesium free
HLA	Human leukocyte antigen
HSC	Haemopoietic stem cell
IAP	Inhibitor of apoptosis protein
IC_{50}	Inhibitory concentration$_{50}$
IFN-α	Interferon-α
Ig	Immunoglobulin
IκK	IκB kinase
IL	Interleukin
IM	Imatinib mesylate
IMDM	Iscove's Modified Dulbecco's Medium
iNOS	Inducible nitric oxide synthase
IRIS	International study of Interferon versus STI571
JAK	Janus kinase
K562-R	IM-resistant K562 cell line
kbp	Kilobase pairs
kD	KiloDalton
LAMA-R	IM-resistant LAMA cell line
LDAC	Low dose cytosine arabinoside
LSC	Leukaemic stem cell
LTBMC	Long term bone marrow culture
LTC-IC	Long term culture-intitiating cell assay
M	Mitosis phase (of cell cycle)
MAPK	Mitogen-activated protein kinase
MCR	Major cytogenetic response
mCR	Minor cytogenetic response
MDR	Multidrug resistance protein
MFI	Mean fluorescence intensity
mg	Milligrams
mL	Millilitres
MMolR	Major molecular response
MNC	Mononuclear cell
MRC	Medical Research Council
MRD	Minimal residual disease
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MW	Molecular weight
µg	Micrograms

µM	Micromolar
NA	Not applicable/not available
NCI	National Cancer Institute
NCRN	National Cancer Research Network
ND	Not determined
NF-κB	Nuclear factor- κB
ng	Nanograms
nM	Nanomolar
NK cells	Natural killer cells
NO	Nitric oxide
NOD	Non-obese diabetic
p	Phosphorylated
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Partial cytogentic response
PDGF-R	Platelet-derived growth factor receptor
PE	Phycoerythrin
PFS	Progression free survival
Ph	Philadelphia chromosome
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PMSF	Phenoxymethylsulphonylfluoride
PTEN	Phosphatase and tensin homolog
PTPases	Protein tyrosine phosphatases
qRT-PCR	Quantitative real-time RT-PCR
Ras-GAP	Ras-GTPase activating protein
Ras-GDP	Ras-guanidine diphosphate
Ras-GTP	Ras-guanidine triphosphate
rHu-G-CSF	Recombinant human G-CSF
RISCT	Reduced intensity stem cell transplant
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Synthesis phase (DNA; of cell cycle)
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SCT	Stem cell transplant
SDF-1	Stromal-derived factor-1
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFM	Serum free medium
SFM + 5GF	Serum free medium supplemented with a 5 growth factor cocktail (IL-3, IL-6, Flt-3 ligand, G-CSF, SCF)
SH	SRC homology domain
siRNA	Short interfering RNA
SKI	SRC kinase inhibitor
SOCS	Suppressors of cytokine signalling family of proteins
SOS	Son of sevenless
SSC	Side scatter
STAT	Signal transducers and activators of transcription
STI	Signal transduction inhibitor 571
t	Translocation
TDZD-8	4-benzyl, 2-methyl, 1,2,4-thiadiazolidine 3,5-dione
TG buffer	Tris/glycine buffer

TGS buffer	Tris/glycine/SDS buffer
TKI	Tyrosine kinase inhibitor
VEGF-R	Vascular endothelial growth factor receptor
WCC	White cell count
WHO	World Health Organisation
WT	Wild type
Y	Tyrosine

1 Introduction

1.1 Haemopoietic stem cells and normal haemopoiesis

Stem cells are defined as cells that can differentiate into multiple different cell types and have the ability to self-renew. There are two broad categories of stem cells: (1) the pluripotent stem cell which can differentiate into endoderm, mesoderm and ectoderm, e.g. embryonic stem cells; and (2) multipotent stem cells which are lineage-specific and include haemopoietic stem cells (HSC). The HSC is a relatively rare cell within the bone marrow, and it is estimated that there are between $3x10^5$ and $4x10^6$ HSC in the human, based on studies using limiting dilution analysis in NOD-SCID mice (Wang et al., 1997) and long-term culture-initiating cell (LTC-IC) assays (Pettengell et al., 1994). Further studies indicate that each HSC divides approximately 70 times during its lifetime (Vickers et al., 2000) so its self-renewal capacity is finite. By this process of cellular amplification, it is estimated that if one stem cell divides 20 times then $1x10^6$ mature cells are produced.

Haemopoiesis is the process of blood cell production. As the majority of mature blood cells only live for a short time in the circulation (a few hours in the case of granulocytes) before destruction by the spleen, it is necessary for the bone marrow to produce up to 10^{13} cells per day to maintain the haemopoietic system. The process of haemopoiesis begins with the multipotent HSC which has self-renewal capacity and the ability to differentiate into all types of mature blood cell (myeloid, erythroid, lymphoid etc) through a range of lineage-committed progenitor cells (**Figure 1-1**).

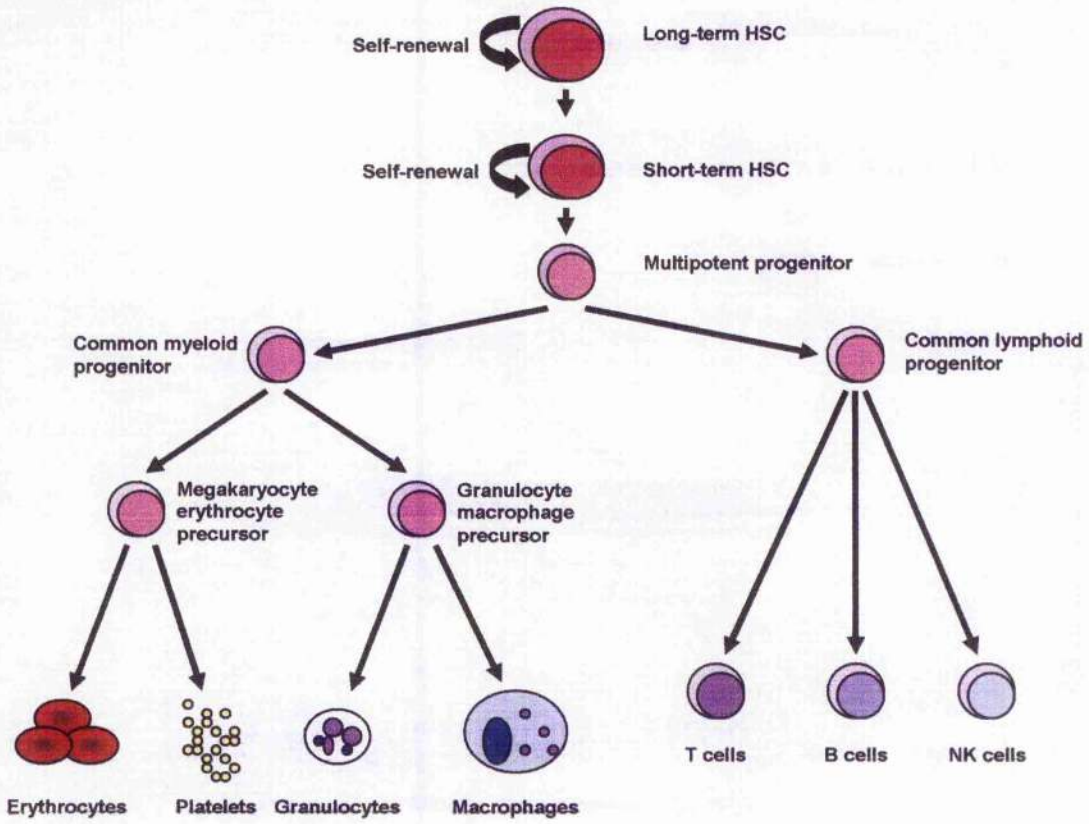


Figure 1-1. Diagrammatic representation of the HSC and the cell lines produced from it. Only long-term and short-term stem cells undergo self-renewal. The multipotent progenitors do not undergo self-renewal, but give rise to common myeloid and common lymphoid progenitors, which through several proliferation and differentiation steps, give rise to the mature cells of the haemopoietic system.

The ability of HSCs to self-renew is heterogeneous and studies on mouse bone marrow cells indicate that 0.05% of bone marrow cells are multipotent progenitors. This HSC population can be divided into three distinct maturational sub-populations: long-term self-renewing HSCs that produce mature haemopoietic cells for the lifetime of the mouse; short-term self-renewing HSCs; and multipotent progenitors which have lost the ability to self-renew, reconstitute lethally irradiated mice for less than eight weeks and have increased mitotic activity (Morrison et al., 1997).

It is believed that, in steady state, only a minority of HSCs reconstitute the haemopoietic system, with the vast majority of HSCs existing in a quiescent state (i.e.in G_0; out of the cell cycle). This extended period in G_0 allows the resting HSCs time to repair any DNA damage and maintain their genetic integrity (Lajtha, 1979). Evidence for the existence of HSCs in a quiescent state came from culture studies in which primitive human progenitor cells remained as single cells for as long as two weeks and only began proliferation after stimulation with a cytokine cocktail (Leary et al., 1989; Leary et al., 1992).

Potential human HSCs are characterised as $CD34^{+}lin^{-}CD38^{-}$ (Miller et al., 1999) and these cells have the ability to repopulate SCID mice. However, for clinical and the majority of research purposes, HSCs tend to be isolated on the basis of CD34 expression only, resulting in a heterogeneous population of which only a small proportion are multipotent HSCs. The CD34 antigen is a transmembrane glycoprotein and member of the sialomucin family (Simmons et al., 2001). Although its precise function is unknown, CD34 is believed to be involved in cell adhesion.

The *in vitro* study of HSCs is difficult for a number of reasons. Firstly, these are rare cells with only relatively small numbers present in any individual. Secondly, *in vitro* culture results in varying degrees of expansion and differentiation of these cells depending on the culture conditions. This results in the loss of stem cell phenotype as the cells mature and acquire lineage-specific markers, and the stem cells become diluted by their more mature progeny. Thirdly, despite extensive studies into the functional and phenotypic properties of HSCs (Weissman, 2000), the mechanisms which control self-renewal versus proliferation and differentiation remain unexplained. The HSC achieves both these functions via asymmetric cell division in which one new HSC is produced along with one daughter which then

undergoes symmetrical division and differentiates. However, HSCs can also undergo symmetrical cell division to produce two daughter cells. It is believed that stem cell fate is decided by, as yet unidentified, factors in the stem cell niche.

Following the identification and characterisation of the HSC, comparisons were drawn with the behaviour of cancer cells, in particular leukaemias, with the first evidence for the 'cancer stem cell' being described in acute myeloid leukaemia (AML), (Bonnet and Dick, 1997). The leukaemic stem cell (LSC) shares a number of properties with normal HSC including self-renewal capacity, similar phenotype (i.e. $CD34^{+}38^{-}$) and the ability to reconstitute NOD-SCID mice, with the recipient mice developing leukaemias of the same phenotype as the transplanted cells. It is now believed that the LSC is derived from an HSC following one or more leukaemogenic events (**Figure 1-2**). Chronic myeloid leukaemia (CML) and acute lymphoblastic leukaemia (ALL) have also been described as HSC disorders (Eaves et al., 1998; Cobaleda et al., 2000).

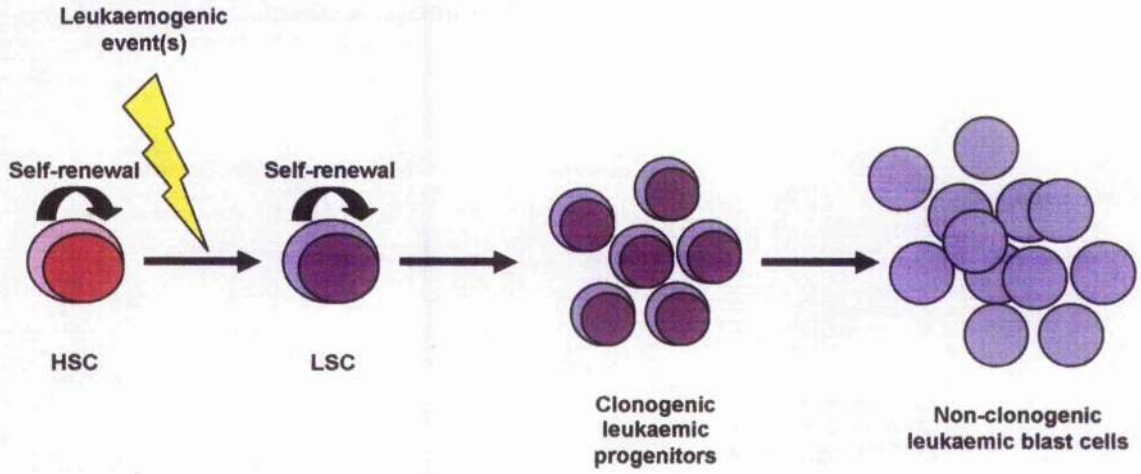


Figure 1-2. Schematic diagram of the transformation of an HSC to a LSC which also retains the capacity to self-renew. Following a number of intermediate progenitor stages, the leukaemic blast cells are produced and these blast cells form the vast majority of leukaemic cells present.

1.2 Chronic myeloid leukaemia (CML)

Chronic myeloid leukaemia develops when a single, multipotent HSC acquires the Philadelphia (Ph) chromosome (**Figure 1-3**) which is an abnormal, shortened chromosome 22 that results from a reciprocal translocation between the long arms of chromosomes 9 and 22 and is designated t(9;22)(q34;q11) (Rowley, 1973). In the 1980s, it was shown that this translocation resulted in the *ABL* proto-oncogene, normally on chromosome 9, becoming juxtaposed with the *breakpoint cluster region* (*BCR*) on chromosome 22 (Bartram et al., 1983; Groffen et al., 1984), resulting in production of the unique fusion gene product BCR-ABL, a 210kD oncoprotein, often referred to as $p210^{BCR-ABL}$, which is a constitutively active tyrosine kinase (Lugo et al., 1990). The Ph chromosome appears in the myeloid, erythroid, megakaryocytic and lymphoid cells of CML patients.

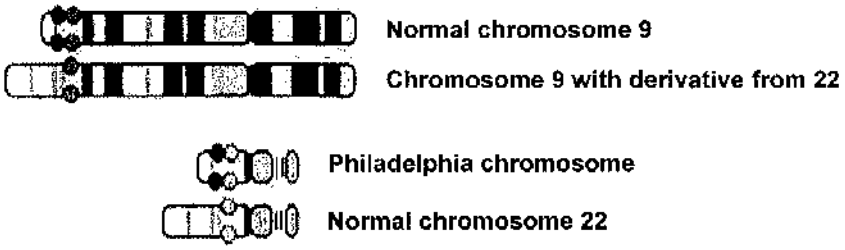


Figure 1-3. The Philadelphia (Ph) chromosome results from a reciprocal translocation between the long arms of chromosome 9 and 22, t(9;22)(q34;q11). The Ph chromosome is the shortened chromosome 22 and results in production of the fusion gene product BCR-ABL which has unregulated tyrosine kinase activity. A reciprocal fusion, *ABL-BCR*, is formed on the long arm of chromosome 9; however, this is not thought to play a role in the pathogenesis of CML. In addition to the Ph chromosome, a minority of patients (~15%) have deletions on the long arm of chromosome 9 which occur at the time of the reciprocal translocation, involve the derivative chromosome 22 and are associated with a poor prognosis (Huntly et al., 2001).

Chronic myeloid leukaemia is a myeloproliferative disorder and accounts for 15-20% of all leukaemias in adults (Faderl et al., 1999). The incidence of CML is constant worldwide at 1.0 to 1.5 per 100,000 of the population. The median age of onset is 40-60 years; however, it may occur in children and the very old. There is a slight male predominance (1.4:1). The risk of CML is slightly increased by exposure to high doses of irradiation as seen in atomic bomb survivors and patients irradiated for ankylosing spondylitis. Most cases of CML are sporadic and no predisposing factors or familial predispositions have been identified.

Chronic myeloid leukaemia has three stages: chronic phase (CP); accelerated phase (AP); and blast crisis (BC). It is usually diagnosed in CP, which is characterised by a leucocytosis due to increased granulopoiesis with hepatosplenomegaly as a result of leukaemic infiltration and can last from several months to several years. In 50% of patients, the disease evolves gradually, through an intermediate stage, AP, which may last for months or occasionally years before frank BC. In the remaining 50%, the CP transforms unpredictably and abruptly to BC. In AP there is an increase in the number of immature cells in the bone marrow or peripheral blood and it may be associated with additional cytogenetic abnormalities (i.e. clonal evolution). Blast crisis may be of myeloid or lymphoid lineage and behaves like an acute leukaemia; it carries a very poor prognosis. The WHO criteria for the diagnosis of AP and BC CML are shown in **Table 1-1** (Vardiman et al., 2002).

AP CML Diagnosis if one or more of the following present: Blasts 10-19% of peripheral white cell count (WCC) or bone marrow cells; Peripheral blood basophils ≥ 20%; Persistent thrombocytopenia (< $100x10^9$/L) unrelated to therapy, or persistent thrombocytosis > $1000x10^9$/L unresponsive to therapy; Increasing spleen size or WCC unresponsive to therapy; Cytogenetic evidence of clonal evolution.
BC CML Diagnosis if one or more of the following present: Blasts ≥ 20% of peripheral WCC or bone marrow cells; Extramedullary blast proliferation; Large clusters or foci of blasts in bone marrow trephine.

Table 1-1. Criteria for the diagnosis of AP and BC CML.

The peripheral blood and bone marrow in CP CML usually have a distinct morphological appearance. There is a leucocytosis, usually > $50x10^9$/L, but occasionally > $500x10^9$/L with a complete spectrum of myeloid cells present in the peripheral blood. The white cell differential count is characteristic with myelocytes and mature neutrophils predominating. Blast cells and promyelocytes are increased to a lesser extent. Nearly all patients have a basophilia and the majority also have an eosinophilia. There is often an associated normochromic normocytic anaemia. The platelet count may be normal, increased or decreased. The bone marrow is usually very hypercellular with marked granulocytic hyperplasia and the myeloid:erythoid ratio often exceeds 10:1. Occasionally there may be associated megakaryocyte hyperplasia or marrow fibrosis at presentation.

1.2.1 BCR-ABL and its mechanisms of action

The fusion gene product, BCR-ABL, has a central role in the pathogenesis of CML and this makes it an attractive target for drug therapy. A number of features of BCR-ABL are essential for cellular transformation. In BCR these include the

coiled-coil motif present in amino acids 1-63, the tyrosine at position 177 (Y177), and phosphoserine-threonine-rich sequences between amino acids 192-242 and 298-413; and in ABL, the SH1, SH2, and actin-binding domains (Pendergast et al., 1991; McWhirter et al., 1993; Pendergast et al., 1993). The most important functional domains of BCR and ABL proteins are shown in **Figure 1-4**. Autophosphorylated BCR-ABL tyrosine kinase phosphorylates a range of substrates and activates a range of intracellular signalling pathways which alter the behaviour of CML stem cells in a number of ways. Substrates of BCR-ABL can be grouped into three broad categories (1) adaptor molecules such as CrKL and $p62^{DOC}$; (2) proteins associated with the cell membrane and cytoskeleton such as talin and paxillin and (3) proteins with catalytic function such as Ras-GAP and phospholipase Cγ (Deininger et al., 2000). **Figure 1-5** shows the main pathways activated by BCR-ABL in CML cells.

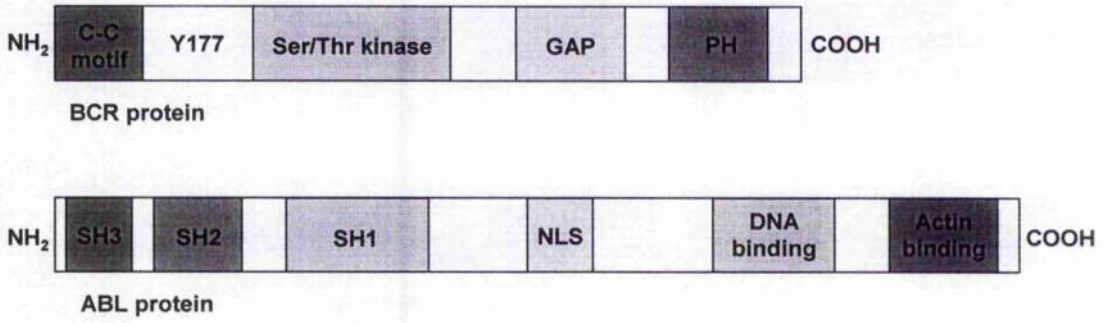


Figure 1-4. The important functional domains of BCR and ABL proteins. In BCR, the coiled-coil (C-C) motif is at the amino (NH_2) terminus. This is followed by the tyrosine at position 177, and phosphoserine-threonine-rich sequences between amino acids 192-242 and 298-413. The serine-threonine rich kinase domain of BCR contains an SH2 binding domain and can bind the SH2 domain of ABL in a non-phosphotyrosine-dependent manner. BCR also has a GTPase-activating protein (GAP)-homology domain and a pleckstrin (PH) domain at the carboxy (COOH) terminus. In ABL, there are SH3, SH2 and SH1 domains towards the amino terminus followed by a nuclear localisation site (NLS). At the carboxy terminus there are DNA and actin binding domains. C-C, coiled coil; Y177, tyrosine 177; Ser/Thr kinase, serine-threonine rich kinase domain; GAP, GTPase-activating protein; PH, pleckstrin

homology; SH, Src homology domain; NLS, nuclear localisation site. Adapted from Holyoake (Holyoake, 2001).

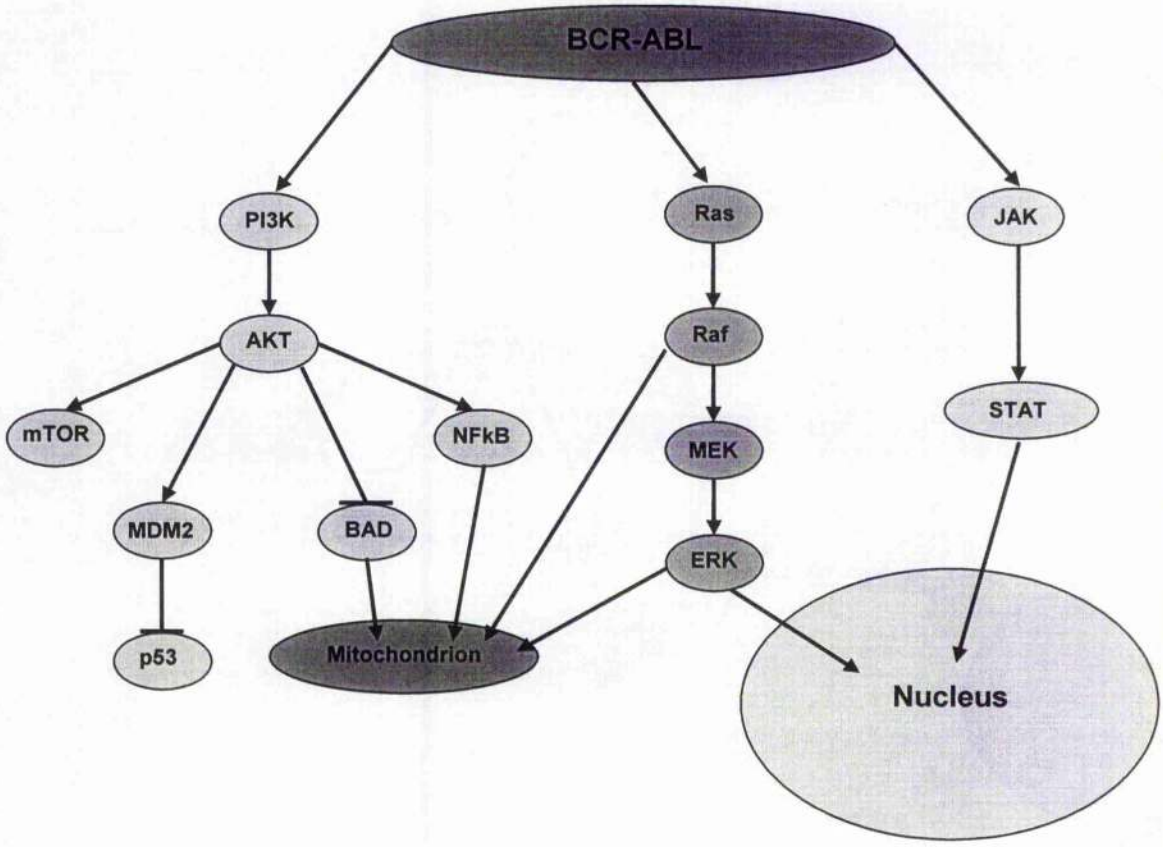


Figure 1-5. Schematic diagram of the three main signal transduction pathways (PI3K-AKT, Ras-Raf-MEK-ERK and JAK-STAT) activated by BCR-ABL in CML cells. In normal HSC, these pathways are activated by the cytokine interleukin-3 (IL-3) and promote survival, growth and differentiation. However, BCR-ABL activates these pathways in the absence of IL-3, resulting in increased proliferation and prolonged survival by inhibiting apoptosis (Steelman et al., 2004).

BCR-ABL has transforming activity both *in vitro* and *in vivo* which was originally demonstrated in a mouse model (Daley et al., 1990). Briefly, in these studies, murine bone marrow was infected with a retrovirus encoding $p210^{BCR-ABL}$ and transplanted into irradiated syngeneic recipients. The transplant recipients developed haematological malignancies, including a CML-like myeloproliferative disorder in the majority.

In vitro cell culture studies (Eaves et al., 1986; Eaves et al., 1987) have shown that BCR-ABL results in marked clonal expansion (i.e. proliferation) in association with increasing differentiation. However, this is heterogeneous, with proliferation rates varying between patients. In addition, in CML, the mechanisms which normally maintain the majority of HSCs in a quiescent state are deregulated, contributing to the increased proliferation, although a deeply quiescent population of CML stem cells has been identified (Holyoake et al., 1999). Further evidence for an increased proliferation rate in CML versus normal progenitor cells comes from studies of telomere dynamics which showed that there was accelerated replication-dependent telomere shortening in Ph^+ versus Ph^- leucocytes (Brummendorf et al., 2000). In addition, very short telomeres predicted early progression to BC, suggesting a link between the CML stem cell, genetic instability and disease progression. An alternative hypothesis for the cell expansion seen in CML called the 'discordant maturation hypothesis' has also been proposed in which the most mature proliferating cells in CP CML are responsible for expansion of the Ph^+ population (Strife et al., 1988). However, further studies have demonstrated that the myeloid expansion observed in CML is far more likely to be a result of increased numbers of primitive CML progenitor cells (Marley et al., 1996).

The increased proliferation and abnormal circulation of primitive CML progenitors can, in part, be explained by abnormal adhesive interactions between the bone marrow microenvironment and the CML cells. Studies have shown that CML progenitors fail to adhere to normal stromal layers (Gordon et al., 1987; Verfaillie et al., 1992) and that this may be related to abnormal function of adhesion molecules. Further studies using LTC-IC and colony forming cell (CFC) assays have demonstrated that treatment with interferon-α (IFN-α) restores normal adhesive interactions between CML cells and the bone marrow stroma in a concentration-dependent manner (Dowding et al., 1991; Bhatia et al., 1994). In

addition, in CML cells, restoration of normal adhesive properties in the presence of IFN-α was inhibited by antibodies to α_4, α_5 and β_1 integrins. These integrins are present at normal levels on CML progenitors and the levels are unchanged by IFN-α treatment (Bhatia et al., 1994). This indicates that abnormal adhesion between CML progenitors and bone marrow stroma may result from defective $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin receptor function which results in a failure in the transmission of signals that inhibit proliferation from the bone marrow microenvironment to the primitive CML progenitors. Normalisation of progenitor-stromal interactions by IFN-α in CML may result in normal progenitor proliferation and partly explain the therapeutic efficacy of IFN-α in CML.

In addition to the deregulation of cell proliferation in CML, there is an imbalance between the rate of cell production and cell death. As shown in **Figure 1-5**, BCR-ABL triggers multiple signal transduction pathways including the PI3K-AKT, Ras-Raf-MEK-ERK and JAK-STAT pathways (Steelman et al., 2004) which inhibit apoptosis and prolong growth factor-independent survival of CML cells (Bedi et al., 1994). Inhibition of BCR-ABL by antisense oligonucleotides reversed this anti-apoptotic effect resulting in increased cell death in response to serum deprivation or treatment with cytotoxic agents (Bedi et al., 1994; McGahon et al., 1994). Further studies have shown that the anti-apoptotic effect of BCR-ABL is associated with prolongation of cell cycle arrest at the G_2M checkpoint (Bedi et al., 1995). In this study, BCR-ABL$^+$ Ba/F3 cells were exposed to ionising radiation and cell cycle distribution was assessed. The irradiated Ba/F3 cells remained viable and had a prolonged G_2 arrest compared to non-irradiated cells. It was hypothesised that this transient delay at the G_2 checkpoint promoted cell survival by permitting repair of otherwise lethal DNA damage caused by cytotoxic agents, thus allowing successful completion of DNA replication and explaining, at least in

part, the resistance to chemotherapeutic agents seen in CML. These repaired CML cells were then capable of proliferating through subsequent cell divisions.

1.2.1.1 Phosphatidylinositol-3 kinase-AKT pathway

Phosphatidylinositol-3 kinase (PI3K) activity is associated with cell division, glucose transport/metabolism, cytoskeletal organisation and inhibition of apoptosis. In CML, BCR-ABL forms a complex with PI3K, Cbl and the adaptor molecules Crk and CrKL via which PI3K is activated (Sattler and Salgia, 1998). AKT exerts it anti-apoptotic effects through phosphorylation of downstream target molecules. The first downstream target of AKT to be identified was the BCL-2 family member protein BAD (Datta et al., 1997; Andreeff et al., 1999). The phosphorylation of BAD by AKT resulted in the association of BAD with 14-3-3 proteins, which enhanced cell survival by inhibiting the binding of BAD to BCL-2 and BCL_{XL}, thus allowing BCL_{XL} to bind pro-apoptotic BAX molecules and preventing the formation of pro-apoptotic BAX homodimers. Studies have shown that BAD-dependent and independent anti-apoptotic pathways are active in CML (Neshat et al., 2000).

AKT also phosphorylates IκB kinase (IκK), which induces degradation of the nuclear factor-κB (NF-κB) inhibitor IκB. NF-kB is a transcription factor that inhibits apoptosis and may also regulate cellular transformation (Romashkova and Makarov, 1999). Expression of BCR-ABL, via up-regulation of AKT, leads to activation of NF-κB-dependent transcription by causing nuclear translocation of NF-κB and increasing the trans-activation function of the RelA/p65 subunit of NF-κB (**Figure 1-6**). This activation is dependent on the tyrosine kinase activity of BCR-ABL and partially requires Ras (Reuther et al., 1998). The mechanism by which Ras activates NF-κB is not known, but may be through AKT which is

downstream of Ras as well as BCR-ABL. However, inhibition of Ras blocks NF-κB activation.

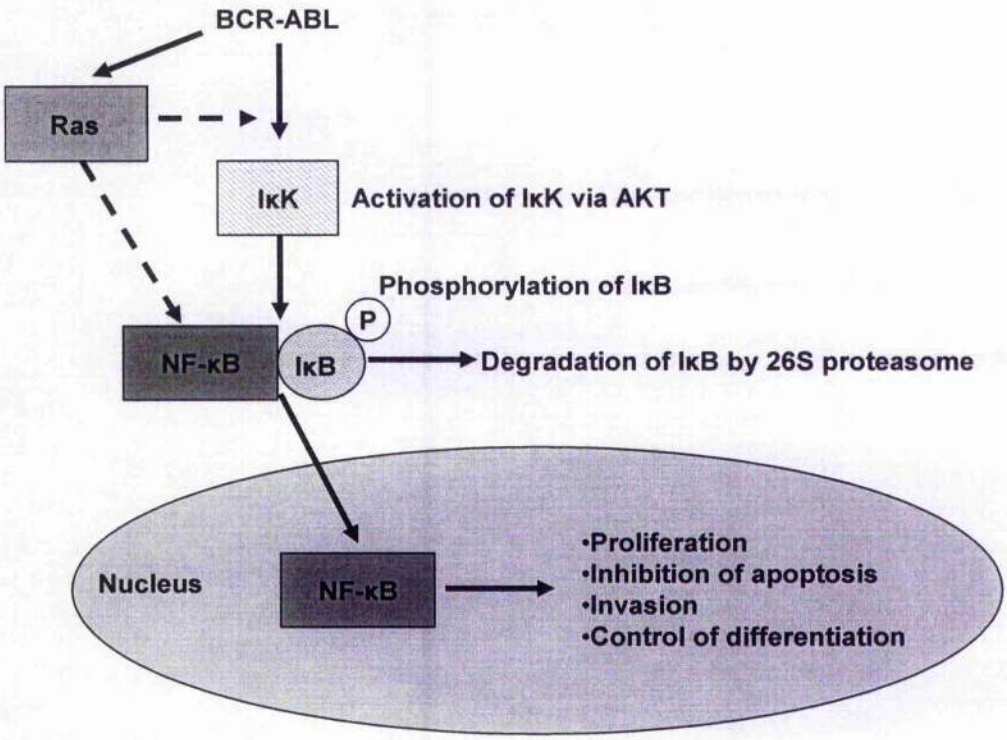


Figure 1-6. Schematic diagram of the upregulation of NF-κB by BCR-ABL. AKT phosphorylates IκK, resulting in the subsequent phosphorylation and degradation of IκB. Without the inhibitory effect of IκB, NF-κB is activated and translocated to the nucleus where it exerts effects on proliferation, inhibition of apoptosis, tumour invasion and control of differentiation. The mechanism of activation of NF-κB by Ras is unknown.

Further downstream targets of AKT include MDM2 and mammalian target of rapamycin (mTOR). MDM2 is a negative regulator of the function of the tumour suppressor gene p53 that targets p53 for degradation by the proteasome (Mayo and Donner, 2001). Studies have shown that BCR-ABL activates MDM2 resulting in increased MDM2 expression and enhanced resistance to apoptosis (Trotta et al., 2003). The serine threonine kinase mTOR is a central regulator of cell growth and recent studies have shown that BCR-ABL induces expression of vascular endothelial growth factor via the PI3K pathway and mTOR, contributing to the

increased angiogenesis seen in CML (Mayerhofer et al., 2002; Mayerhofer et al., 2005).

1.2.1.2 Ras-Raf-MEK-ERK pathway

The Ras proteins are a group of guanine nucleotide binding proteins (G-proteins) that, following isoprenylation in the cytosol, associate with the inner plasma membrane of the cell and transduce external signals to the interior of the cell. They belong to the protein group termed the 'Ras superfamily of monomeric GTPases' which also contains the Rho and Rab protein families which are involved in the signal transduction between cell-surface receptors and the actin cytoskeleton and regulation of intracellular transport vesicles, respectively. The Ras protein superfamily are substrates for post-translational modification and undergo prenylation via farnesyltransferase (FT) and geranylgeranyltransferase (GGT). Ras proteins exist in specific isoforms (H-Ras, K-Ras and N-ras) that differ in their affinity for specific isoprenyl groups and are either in an active form (Ras-GTP) or inactive form (Ras-GDP) which is tightly regulated (**Figure 1-7**). The Ras-Raf-MEK-ERK pathway, which is often referred to as the mitogen-activated protein kinase (MAPK) pathway, transmits signals from cell surface receptors to transcription factors in the nucleus and is able to interact with other signal transduction pathways including PI3K-AKT and JAK-STAT. Activation of Ras by cytokines such as IL-3 leads to recruitment of the serine-threonine kinase Raf to the cell membrane which results in a signalling cascade through MEK1/2 and ERK culminating in the activation of gene transcription (Marais et al., 1995; Cahill et al., 1996). It is believed that when mutated, Ras is no longer under the control of its regulators such as the positive regulator Son of sevenless (SOS; a guanine nucleotide exchange factor) (**Figure 1-7**), resulting in constitutive activation of Ras-GTP (Lowy and Willumsen, 1993).

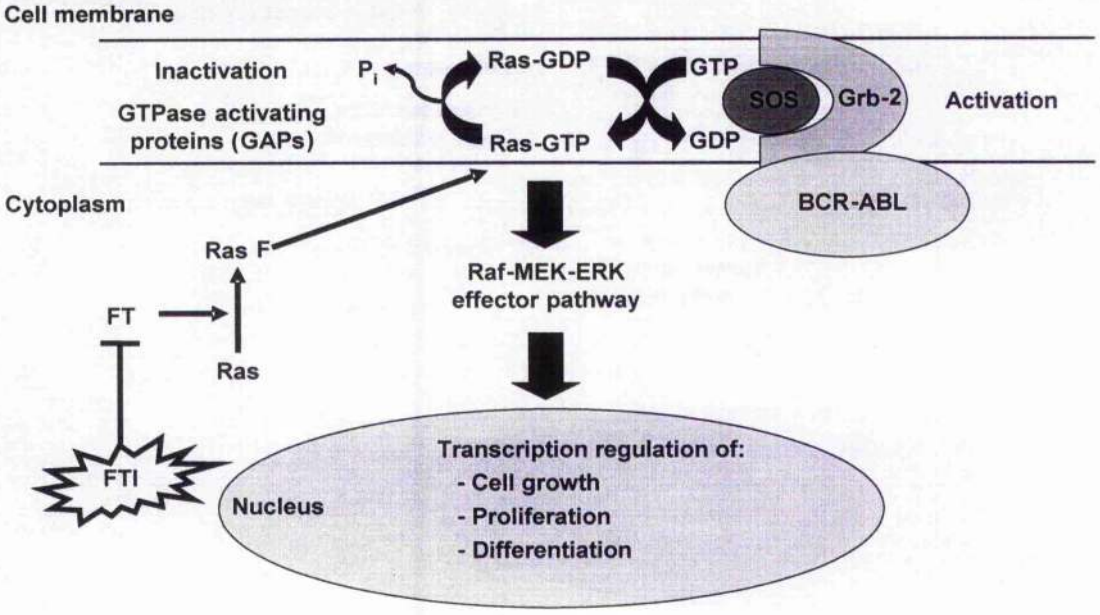


Figure 1-7. Schematic diagram of Ras-mediated signalling. Ras is farnesylated in the cytoplasm by farnesyltransferase (FT) and this process can be inhibted by farnesyltransferase inhibitors (FTIs). Farnesylated Ras (Ras-F) then translocates to the cell membrane where it interacts with the regulatory proteins GAP and SOS. Farnesylated Ras cycles between active GTP-bound and inactive GDP-bound forms. Activated farnesylated Ras-GTP stimulates the Raf-MEK-ERK effector pathway resulting in alterations in the transcriptional control of cell growth, proliferation and differentiation. The association of BCR-ABL with the Grb-2/SOS complex stabilises Ras in its activated GTP-bound form resulting in increased cell growth and proliferation.

A number of associations between BCR-ABL and Ras have been identified. Autophosphorylation of BCR-ABL on Y177 allows direct interaction with the adaptor molecule Grb-2 (Pendergast et al., 1993). This enables Grb-2 to bind SOS and stabilise Ras in its activated GTP-bound form. The adaptor molecules Shc and CrKL can also activate Ras (Oda et al., 1994; Pelicci et al., 1995; Senechal et al., 1996). In CML, BCR-ABL activates Ras, and inhibition of Ras results in arrest of cell growth. This was demonstrated in cell line models in which the introduction of a dominant-negative Ras into BCR-ABL-transfected cells inhibited malignant transformation (Sawyers et al., 1995). Further studies have shown that disrupting the Grb-2/SOS complex inhibits Ras-mediated cell proliferation in BCR-ABL

expressing cell lines and primary CML cells (Kardinal et al., 2001). Therefore, the Ras-Raf-MEK-ERK axis appears to be important in the pathogenesis of BCR-ABL$^+$ leukaemias and inhibition of this pathway may represent a valuable treatment option for patients with CML (**Section 1.3.3.1**).

1.2.1.3 JAK-STAT pathway

The JAK-STAT pathway is activated by cytokines such as IL-3, IFN-α and granulocyte macrophage-colony stimulating factor (GM-CSF). The Janus kinases (JAKs) are a class of tyrosine kinases which phosphorylate and activate a group of transcription factors called the signal transducers and activators of transcription (STATs). The STATs are then translocated to the nucleus where they upregulate the transcription of specific genes which are predominantly associated with the regulation of cell survival, proliferation and differentiation. The JAK-STAT pathway is regulated by the suppressors of cytokine signalling (SOCS) and cytokine-induced SH2-containing (CIS) family of proteins. To date, four different JAK (JAK 1-3 and TYK1), seven different STAT (STAT 1, 2, 3, 4, 5a, 5b, and 6) and six different SOCS/CIS (SOCS 1-5 and CIS1) molecules have been described (Kisseleva et al., 2002). The STATs are constitutively phosphorylated in CML cells and appear to contribute to the malignant transformation seen in CML (Chai et al., 1997; de Groot et al., 1999). The constitutive activation of STAT5 is not always associated with the activation of JAK, indicating that BCR-ABL may be able to directly activate STAT5 (Chai et al., 1997). The effect of STAT activation on BCR-ABL-transformed cells is predominantly anti-apoptotic and involves the transcriptional activation of BCL_{XL} (Horita et al., 2000; Sillaber et al., 2000).

1.2.2 Growth factor independence in CML

1.2.2.1 Interleukin-3 (IL-3)

Interleukin-3 is a highly glycosylated 26kD cytokine. It binds to the IL-3 receptor which is a member of the cytokine receptor gene family. Activation of the IL-3 receptor induces the activity of the JAK-STAT, PI3K-AKT and Ras signalling pathways promoting survival, growth and differentiation of HSCs (Steelman et al., 2004).

1.2.2.2 Granulocyte-colony stimulating factor (G-CSF)

Like IL-3, granulocyte-colony stimulating factor (G-CSF) is a 25kD glycoprotein that acts as a cytokine. It binds to the G-CSF receptor (G-CSF-R), another member of the cytokine receptor gene family, resulting in ligation of the extracellular domain of the G-CSF-R. The G-CSF-R is expressed by all cells in the granulocyte series, including HSCs (McKinstry et al., 1997). In addition, the G-CSF-R is also expressed on endothelial cells, placental cells, activated T lymphocytes and many non-haemopoietic tumour cell lines (Roberts, 2005). Granulocyte-colony stimulating factor affects survival, proliferation and differentiation of all cells of myeloid lineage, from HSCs to mature neutrophils through activation of downstream signal transduction pathways including JAK-STAT, PI3K-AKT and Ras as well as the SRC family of proteins. Stimulation with G-CSF alone is sufficient for committed myeloid progenitors to proliferate and differentiate. However, for normal HSC proliferation, additional stimulation with other cytokines is also required. Further functional roles for G-CSF include speeding up the maturation of metamyelocytes, enhancing the function of mature neutrophils by increasing superoxide production, bacteriocidal killing and phagocytosis, mobilisation of HSCs from the bone marrow into the peripheral

blood and, possibly regulation of immune responses (Roberts, 2005). All tissues in the body are capable of producing G-CSF after stimulation, in particular, macrophages, endothelial cells, fibroblasts, other mesenchymal cells and bone marrow stroma.

In humans, the serum concentration of G-CSF is very low (1.4±0.7 pg/mL) in healthy subjects (Jorgensen et al., 2003). However, levels significantly increase in response to infection or inflammation and fall again with recovery (Kawakami et al., 1990; Jorgensen et al., 2003). Inflammatory stimuli such as IL-1, lipopolysaccharide and tumour necrosis factor-α increase the production of G-CSF by macrophages, fibroblasts and endothelial cells (Roberts, 2005). Interleukin-17 is thought to be a major upstream regulator of G-CSF production, particularly by bone marrow cells and, *in vitro*, results in a concentration-dependent increase in G-CSF expression (Fossiez et al., 1996). *In vivo*, in a murine model deficient in leucocyte adhesion molecules which resulted in abnormal haemopoiesis and neutrophilia, inhibition of IL-17 reduced serum G-CSF concentrations and neutrophil count, thereby providing evidence for a feedback loop between IL-17 and G-CSF (Forlow et al., 2001).

Recently, SOCS-3 was shown to be rapidly up-regulated in response to G-CSF in mature neutrophils and the myeloid cell line U937 (Hortner et al., 2002). SOCS-3 negatively regulates STAT activation. In addition, this study showed that recruitment of SOCS-3 to the G-CSF-R was phosphorylation dependent and pY729 was the major recruitment site on the G-CSF-R for SOCS-3. An *in vivo* mouse model in which the haemopoietic cells had a SOCS-3 deletion confirmed that SOCS-3 was an important negative regulator of G-CSF signalling in myeloid cells (Croker et al., 2004). When given exogenous G-CSF, these mice developed neutrophilia, splenomegaly and inflammatory disorders.

1.2.2.3 The roles of autocrine IL-3 and G-CSF production in CML

Research in the late 1980s first demonstrated that the production of autocrine growth factors in myeloid malignancies resulted in autonomous growth (Young et al., 1987; Oster et al., 1989). Studies in murine bone marrow transplantation models showed that in mice, in whom the bone marrow cells had either been retrovirally transduced or transgenically engineered to produce IL-3, there was development of a myeloproliferative disorder with leukaemic transformation of primitive progenitors (Chang et al., 1989; Wong et al., 1989; Just et al., 1993). Further *in vitro* studies have demonstrated that continuous expression of BCR-ABL in cytokine-dependent cell lines resulted in growth factor-independent proliferation in association with autocrine production of IL-3 and GM-CSF (Li et al., 1999; Li et al., 2001; Peters et al., 2001b). In addition, in one of these studies, transplant of these cells into a mouse model induced a fatal leukaemia (Peters et al., 2001b). However, the other study showed that IL-3 and GM-CSF were not required for induction of a CML-like myeloproliferative disorder in mice by BCR-ABL (Li et al., 2001), but may be required for disease maintenance. Research has also shown that primitive $CD34^+$ CML stem cells produce IL-3 and G-CSF via an autocrine mechanism which results in increased STAT5 phosphorylation that is highly dependent on IL-3 but not G-CSF (Jiang et al., 1999; Jiang et al., 2000b). It is believed that this contributes to their resistance to apoptosis by prolonging the growth factor independent survival of CML progenitors. This hypothesis was further corroborated by *in vitro* studies which showed that, after prolonged culture, $IL\text{-}3^{null}$ mouse bone marrow cells transduced with BCR-ABL required rescue with an IL-3 transgene to maintain growth factor-independence and STAT5 activation. In addition, these BCR-ABL-transduced cells lacked *in vivo* leukaemogenic activity (Jiang et al., 2002). These data are also supported by results from an *in vivo* mouse model which used a murine retroviral stem cell vector to transduce the

BCR-ABL oncogene into mouse bone marrow cells (Zhang and Ren, 1998). A myeloproliferative disorder resembling human CP CML was induced in 100% of transplanted mice in association with excess production of IL-3. Overall, these studies suggest that the combined expression of BCR-ABL and IL-3 may be vital to leukaemia stem cell survival in CML. However, the role of G-CSF is less clear.

It was first demonstrated in 1990 that G-CSF transcripts were increased in CML (Klein et al., 1990). This study also showed that the G-CSF produced from these cells was functional, as supernatants taken from cultures of these cells were capable of stimulating growth of granulocyte-forming colonies from normal bone marrow cells. Additionally, the growth of colonies obtained from these CML cells could be inhibited by neutralising G-CSF antibodies. Therefore, this study concluded that CML-derived progenitors secrete autocrine G-CSF which drives terminal cell divisions. Further studies have confirmed that purified populations of $CD34^+$ CML cells can survive and proliferate *in vitro* in the absence of exogenous growth factors, however, normal $CD34^+$ cells rapidly die under the same culture conditions (Bedi et al., 1994; Maguer-Satta et al., 1998). Jiang *et al* have also shown that when $CD34^+$ cells differentiate and become $CD34^-$, IL-3 and G-CSF production decrease, and the cells lose their growth factor independence *in vitro*, despite the continued expression of BCR-ABL (Jiang et al., 1999). In addition, these autonomously proliferating $CD34^+$ CML cells are capable of producing clonogenic progenitors *in vitro* for all the lineages (erythroid, granulocyte-macrophage and megakaryocyte) that normal $CD34^+$ cells generate *in vivo* in the presence of haemopoietic growth factors (Jiang et al., 2000b). These findings highlight the role of autocrine growth factor production in the multi-lineage expansion of $CD34^+$ CML cells that occurs *in vivo*.

1.2.3 Quiescent LSCs in CML

Cells with a mechanism of autocrine IL-3 production would be expected to have increased cell cycle activity which would make them susceptible to eradication by conventional chemotherapy agents. However, CML is incurable using repeated AML-type chemotherapy (Kantarjian et al., 1991) suggesting that there is a pool of quiescent CML stem cells which are resistant to treatment. Studies have now confirmed the existence of a highly quiescent population of LSC in CML (Holyoake et al., 1999). Viable BCR-ABL$^+$ G_0 cells were isolated using Hoescht 33342 and Pyronin Y staining from total CD34$^+$ CML cells by fluorescence activated cell sorting (FACS) and were demonstrated to have *in vitro* progenitor activity by LTC-IC assay and the capability of engrafting immunodeficient mice. The ability of these quiescent CML stem cells to produce leukaemic progeny also illustrates the reversibility of this quiescent state. Further research showed that the entry of BCR-ABL$^+$ progenitors into a quiescent state *in vivo* was greatest in the most primitive leukaemia cell populations. This was associated with down-regulation of IL-3 and G-CSF gene expression, and spontaneously reversed in association with up-regulation of IL-3 expression and entry of cells into a continuously cycling state (Holyoake et al., 2001). The phenomenon of quiescence in CML is important because it would be predicted that the growth factor-independent quiescent CML stem cells would have a proliferative advantage over normal quiescent HSCs when the concentration of cytokines is low.

1.2.4 The historical treatment of CML

There have been great advances in the management of CML over the past half century. Prior to this, a range of therapies including arsenic, benzene, radiotherapy and splenectomy were used as palliative treatment in small numbers of patients with limited success (Geary, 2000). From the 1950s to the 1980s, patients with CP

CML were treated with the oral alkylating agent busulphan as this was relatively specific for haemopoietic tissue (Galton, 1953). However, this frequently resulted in permanent sterility and bone marrow failure, and was later reserved for progressive disease.

In the 1980s, IFN-α, and the ribonucleotide reductase inhibitor hydroxycarbamide (formerly hydroxyurea), were introduced for the management of CML. Interferon-α is given by subcutaneous injection and is effective at reducing the leukocyte count and reversing the clinical and laboratory features of CML. In addition, it has been observed that 5-15% of patients receiving IFN-α sustain a very significant reduction in Ph^+ marrow metaphases (cytogenetic response) with restoration of Ph^- 'normal' haemopoiesis (Talpaz et al., 1986). However, IFN-α is not without side effects; almost all patients experience rigors, fevers, muscle aches and general 'flu-like' symptoms on commencing therapy. These symptoms usually last 1–2 weeks and can be relieved by paracetamol, but they recur whenever the dose is increased. A significant proportion of patients are unable to tolerate IFN-α as a result of lethargy, malaise, weight loss, depression and other psychological disorders.

Hydroxycarbamide targets relatively mature myeloid progenitors. It is given orally and the leukocyte count begins to fall within a few days of commencing therapy. Hydroxycarbamide therapy can be continued indefinitely, but does not induce a cytogenetic response and has no effect on the natural history of the disease, with patients eventually progressing to more advanced phases of CML (Kantarjian et al., 1993). Hydroxycarbamide has relatively few side effects.

The 1980s also saw the introduction of allogeneic stem cell transplantation (alloSCT) which, for the first time, offered the possibility of cure for younger CML

patients (<50-55 years of age) with HLA-matched donors (Goldman et al., 1986). This accounts for approximately 20% of CML patients'; however, the procedure is associated with significant morbidity and a mortality of between 20 and 40%, most commonly resulting from opportunistic infections and graft-versus-host disease (GvHD) (Sawyers, 1999; Silver et al., 1999). For those patients' who receive an alloSCT, a leukaemia-free survival rate of up to 70% is achieved (Clift and Anasetti, 1997). The success of alloSCT in CML is related to the power of the "graft-versus-leukaemia" (GvL) effect in addition to myeloablation. The GvL effect is greatest in those patients who have post-transplant GvHD, with GvL and GvHD being driven by major and minor HLA mismatches between donor and recipient (Spierings et al., 2004), although donor T lymphocytes may also recognise and destroy as yet unidentified leukaemia-specific antigens (Barrett and van Rhee, 1997). Donor T cell responses to minor histocompatibility antigen differences may contribute up to 35% of the total GvL response in leukaemic patients (Kloosterboer et al., 2004).

For patients that relapse after alloSCT, donor lymphocyte infusions (DLI) are given in incremental doses with the aim of harnessing the GvL effect. A response to DLI can be expected between 3 and 12 months post-infusion. The success rate of DLI for relapse of CML post-transplant is at least 70% for patients with haematologic relapse and as high as 90% in patients with molecular relapse only (Kolb et al., 1995).

With the success of alloSCT/DLI in curing CML by exploiting the GvL effect, interest in the late 1990s focused on reduced intensity "mini" stem cell transplant regimens (RISCT). These less intensive transplant conditioning regimens allow transplantation of older patients (up to age 70) and reduce regimen-related toxicities compared to standard alloSCT (Slavin et al., 1998; Champlin et al.,

2000). A recent study (Or et al., 2003) showed that of 24 patients with CML who received a RISCT in first CP, 21 remained alive and disease free after a median of 42 months follow up. The GvL effects of donor immunocompetent lymphocytes eradicated all host haemopoietic cells, as evidenced by molecular testing.

Because only a minority of patients are suitable for alloSCT, interest in the 1990s focused on autologous SCT. It was hoped that autografting would result in durable Ph^- haemopoiesis and, in 2000, the Medical Research Council (MRC) in the UK initiated one study comparing autografting with IFN-α therapy (The MRC CML 2000 trial), and another trial (the CML IV trial) to define the relative roles of IFN-α, autologous and alloSCT in the younger patient. However, with the introduction of the BCR-ABL tyrosine kinase inhibitor (TKI) imatinib mesylate (IM; Glivec™; Gleevec™, formerly STI571, Novartis Pharmaceuticals, Basel, Switzerland), these trials were abandoned as patients opted to commence IM treatment rather than start or continue on IFN-α therapy or undergo autologous SCT. Even the role of a potentially curative alloSCT has been called into question in the IM era.

1.2.5 The development of IM

1.2.5.1 Early studies of protein TKIs

The first step towards development of a useful TKI was synthesis of compounds called tyrphostins which were low molecular weight TKIs that blocked epidermal growth factor (EGF)-dependent cell proliferation (Yaish et al., 1988). It was later reported that two tyrphostins, AG568 and AG1112, which inhibited the ABL protein tyrosine kinase, inhibited $p210^{BCR\text{-}ABL}$ tyrosine kinase activity in the BC-derived CML cell line, K562, in association with erythroid differentiation (Anafi et al., 1993). Following this, it was suggested that these compounds would be useful for purging Ph^+ cells in preparation for autologous SCT. Another tyrphostin, AG957, was

reported to restore β_1 integrin-mediated adhesion and inhibitory signalling in primary CML ($CD34^+$) stem cell cultures (Bhatia et al., 1998), suppress Ph^+ colony growth in a concentration-dependent manner and induce apoptosis in combination with an anti-Fas receptor antibody (Carlo-Stella et al., 1999).

Parallel studies showed that the benzoquinonoid ansamycin antibiotic herbimycin A also had inhibitory activity against v-ABL and the SRC family of protein tyrosine kinases and reversed the transformation of susceptible cell lines with a reduction in phosphotyrosine content (Uehara et al., 1988). Herbimycin A was also reported to inhibit the growth and tyrosine phosphorylation of K562 cells and induce erythroid differentiation (Honma et al., 1989). In addition, herbamycin A induced growth inhibition in a variety of $p210^{Bcr\text{-}Abl}$ transformed cell lines but did not inhibit the growth of a broad spectrum of Ph^- haemopoietic cell lines (Okabe et al., 1992) and prolonged survival in a SCID mouse model with a disease resembling human Ph^+ ALL (Honma et al., 1995). Taken together, these studies using tyrphostins and herbimycin A suggested that the development of an agent with the potential to specifically inhibit BCR-ABL would be of therapeutic benefit in Ph^+ leukaemias.

1.2.5.2 The pre-clinical development of IM

The studies leading to the development of IM began in the early 1990s. A large number of adenosine triphosphate (ATP)-competitive 2-phenylaminopyrimidine compounds were synthesized and screened for protein tyrosine kinase inhibitory activity. One of these compounds, CGP57148 (CG = Ciba-Geigy), was found to be a potent inhibitor of ABL, c-KIT and platelet-derived growth factor receptor (PDGF-R) acting via competitive inhibition of the protein kinases with respect to ATP (Druker et al., 1996). The structure of this molecule is shown in **Figure 1-8**. CGP57148 was evaluated for its effects on cells containing the BCR-ABL fusion

protein. Cellular proliferation and tumour formation by BCR-ABL-expressing cells were specifically inhibited by this compound. In the CFC assays of peripheral blood or bone marrow from patients with CML and non-CML controls, there was a 92–98% decrease in the number of BCR-ABL$^+$ colonies formed from CML cells, but only 15–20% inhibition of normal colony formation, and it was concluded that this compound might be useful in the treatment of BCR-ABL$^+$ leukaemias. In addition, a mouse model demonstrated a concentration-dependent inhibition of tumour growth in BCR-ABL-inoculated animals treated with CGP57148, with no effects on animals inoculated with a non-BCR-ABL-expressing cell line. CGP57148 later became known as STI571 (Signal Transduction Inhibitor 571) or imatinib mesylate and is now marketed as Glivec^{TM} in Europe and Gleevec^{TM} in the USA by Novartis Pharmaceuticals, a company formed in 1996 by the merger of the Swiss life sciences companies Ciba-Geigy and Sandoz.

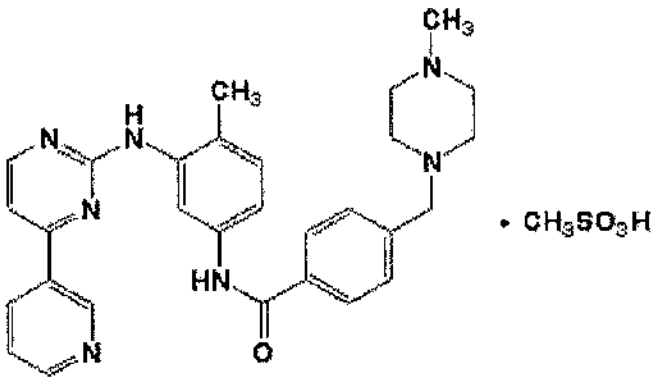


Figure 1-8. Structure of IM (Glivec^{TM}, Gleevec^{TM}; formerly CGP57148 and STI571).

The selective inhibition of BCR-ABL tyrosine kinase is mediated via interaction between IM and the amino acids that constitute the ATP binding cleft of BCR-ABL. IM is a competitive inhibitor of ATP at its specific binding site in the kinase domain of the BCR-ABL protein (Deininger et al., 2000). When ATP binds to its specific binding site in the kinase domain, tyrosine phosphorylation of downstream target proteins occurs. However, when the synthetic ATP mimic IM is bound, it blocks the

tyrosine phosphorylation of target proteins downstream of BCR-ABL, inhibiting effector pathways. IM has an inhibitory concentration$_{50}$ (IC_{50}) of 0.25 μM on BCR-ABL kinase activity (Druker et al., 1996). The IC_{50} can be measured as either inhibition of biochemical kinase activity or cellular proliferation. The IC_{50} is the concentration of drug which results in 50% inhibition of either kinase activity (biochemical IC_{50}) or cellular proliferation (cellular IC_{50}) compared to a no drug control. These initial results were swiftly confirmed in a number of other studies (Carroll et al., 1997; Deininger et al., 1997; le Coutre et al., 1999). Importantly, le Coutre *et al* compared dosing nude mice previously injected with BCR-ABL$^+$ human leukaemia cells once, twice and three times daily with IM over an 11-day period (le Coutre et al., 1999). In the mice dosed three times daily, there was an 87-100% cure rate; however, mice dosed once or twice daily were not cured of their Ph$^+$ leukaemia. This study supported the hypothesis that continuous therapy was likely to be necessary for IM to be effective in patients with Ph$^+$ leukaemias.

Preliminary toxicity testing of the oral IM compound revealed occasional renal calcification and mild bladder mucosal hyperplasia in rats at a dose of 6 mg/kg, and liver toxicity, vomiting, diarrhoea, anaemia and pancytopenia in dogs at the highest dose of 100 mg/kg (Druker and Lydon, 2000).

1.2.6 The introduction of IM into clinical trials

Phase 1 dose escalating trials of IM were undertaken in patients with CP CML who had failed IFN-α and patients with BC CML or Ph$^+$ ALL (Druker et al., 2001a; Druker et al., 2001b). Serious adverse events were minimal; the most common side effects seen were nausea, myalgia, oedema and diarrhoea. Doses of up to 1000mg/day were given; however, a maximum tolerated dose was not reached. In patients with CP CML who had failed IFN-α, complete haematologic responses (CHR) were seen in 53 of 54 patients treated with daily doses greater than 300mg

(Druker et al., 2001b). See **Table 1-2** for definitions of response. In addition, cytogenetic responses were seen in 29 of the 54 treated with more than 300mg per day and, of these, 7 patients achieved a complete cytogenetic response (CCR). In patients with myeloid BC CML responses were seen in 21 of 38 patients of which 4 had a CHR and, in patients with lymphoid BC or Ph^+ ALL, 14 of 20 patients responded , including a further **4** patients who had a CHR (Druker et al., 2001a). However, in more advanced disease, responses were not durable, with the majority of patients relapsing within 6 months of commencing therapy. These studies concluded that IM was well tolerated and had significant anti-leukaemic activity in all phases of CML. In addition, these studies demonstrated the essential role of BCR-ABL tyrosine kinase activity in CML and highlighted the potential for the development of anti-cancer drugs based on a specific molecular abnormality.

Response	**Definition**
Complete haematologic response (CHR)	Reduction of the WCC to less than 10 x 10^9/L and platelet count to less than 450 x 10^9/L, with no immature cells in the peripheral blood and disappearance of all symptoms and signs related to leukaemia including splenomegaly, maintained for at least 4 weeks
Complete cytogenetic response (CCR)	No Ph^+ cells detectable by cytogenetics in the bone marrow
Partial cytogenetic response (PCR)	1-34% of bone marrow cells have a detectable Ph chromosome
Major cytogenetic response (MCR)	Includes those that have achieved a CCR or PCR (i.e < 35% cells Ph^+
Minor cytogenetic response (mCR)	35-90% of bone marrow cells have a detectable Ph chromosome
Complete molecular response (CMolR)	Either BCR-ABL undetectable by qRT-PCR or ≥ 4.5 log reduction in BCR-ABL transcript levels compared to starting value
Major molecular response (MMolR)	Either ≥ 3 log reduction in BCR-ABL transcript levels or a BCR-ABL transcript level < 0.1% by qRT-PCR compared to starting value

Table 1-2. Definitions of response to IM therapy in CML

Imatinib mesylate progressed rapidly through Phase 2 clinical trials in which the efficacy and good toxicity profile of the drug were confirmed (Kantarjian et al., 2002; Ottmann et al., 2002; Sawyers et al., 2002; Talpaz et al., 2002). In these studies, the standard dose of IM was established as 400mg/daily for CP CML, but its favourable toxicity profile allowed IM to be used at higher doses (600-800mg daily) with the standard dose in AP and BC CML increasing to 600mg/daily and the possibility of combining IM with other chemotherapeutic interventions emerged. However, the appearance of the clinical phenomenon of IM resistance, particularly in lymphoid BC and Ph^+ ALL was concerning (Ottmann et al., 2002).

Following the success of the Phase 1 and 2 trials of IM in all phases of CML, a prospective, multicentre, open-label, Phase 3, randomised study was designed called the IRIS trial (International Randomised Study of Interferon versus STI571). This study set out to compare the efficacy of IM with that of IFN-α combined with low dose cytosine arabinoside (LDAC) in newly diagnosed CP CML patients and determine the haematologic and cytogenetic response rates, toxicity and rates of progression. Crossover between the two arms was allowed for lack of response, loss of response or intolerance to treatment. Interferon-α in combination with LDAC was chosen for the control arm because this combination was shown to result in superior rates of cytogenetic response (Guilhot et al., 1997; Baccarani et al., 2002) and possibly survival compared to IFN-α alone (Guilhot et al., 1997). However, the combination of IFN-α plus LDAC required daily subcutaneous injection of both drugs with frequent and troublesome side effects.

The rate of MCR at 18 months was 87.1% in the IM group compared with 34.7% in the IFN-α plus LDAC group ($P<0.001$) (O'Brien et al., 2003). The rates of CCR were 76.2% and 14.7% respectively ($P<0.001$). In addition, at 18 months, progression free survival (PFS) in the IM arm was 96.7% compared to 91.5% in

the control arm. IM was also better tolerated than IFN-α plus LDAC (Hahn et al., 2003). At the most recent update of this study, the CCR and MCR rates in the IM arm were 87% and 92%, respectively, with a PFS of 93% at 60 months (Druker et al., 2006). Only 3% of patients commenced on IFN-α plus LDAC continued on this arm compared to 69% of patients who initially commenced IM. Therefore, this trial demonstrated the superiority of IM to IFN-α plus LDAC in terms of disease response, PFS and tolerability. During the course of this study, molecular responses were assessed at regular intervals by qRT-PCR of peripheral blood leucocytes for BCR-ABL transcripts (Hughes et al., 2003). Patients responding to IM had a 3-4 log reduction in transcript levels compared to their starting value. Therefore, other than alloSCT, IM induced a much higher rate of CCR than any other licensed therapy and, indeed, has brought the role of alloSCT in CML into question. However, alloSCT remains the only proven curative option for CML as, after successful alloSCT, the majority of patients are BCR-ABL$^-$ by qRT-PCR. Conversely, even patients who have an optimal response to IM, i.e. achieve CCR, continue to have low levels of BCR-ABL transcripts detectable by qRT-PCR. In addition, there is increasing evidence for disease resistance at the stem cell level (Graham et al., 2002); a phenomenon termed molecular persistence, and the emergence of IM resistance, particularly in advanced disease.

1.2.7 Imatinib mesylate resistance

Although more common in AP and BC, resistance to IM may also be seen in CP CML. This phenomenon has resulted in IM being less successful in advanced disease (Druker et al., 2001a; Ottmann et al., 2002; Sawyers et al., 2002; Talpaz et al., 2002). Some patients in AP do respond well, and although many patients treated in BC achieve some haematological improvement, these benefits are not maintained, with the majority of patients in the advanced stages of CML

developing resistance to IM. Resistance can be primary, i.e. no response to IM after initial therapy, and is defined as failure to achieve CHR within 3 months and failure to achieve MCR by 6 months or CCR by 12 months, or acquired, i.e. loss of established haematological, cytogenetic or molecular response to IM or progression to AP or BC.

There are 2 broad categories of IM resistance: BCR-ABL-independent and BCR-ABL-dependent. In BCR-ABL-independent resistance, the leukaemia cells are no longer reliant on BCR-ABL to drive proliferation; their growth being dependent on additional oncogenic mutations (Donato et al., 2004) and this represents clonal evolution. Thus, in these cells, BCR-ABL is no longer a relevant target for IM and any specific BCR-ABL inhibitor would be ineffective in this situation. This may occur in primary or acquired resistance.

Acquired resistance in Ph^+ leukaemias is usually BCR-ABL-dependent and is the result of restoration of BCR-ABL kinase activity. This can occur via three different mechanisms. The first of these is *BCR-ABL* amplification which was first described in a landmark publication by Gorre *et al* in 2001 (Gorre et al., 2001). In this study, dual-colour fluorescence *in-situ* hybridisation (FISH) was performed to determine if *BCR-ABL* gene amplification could be implicated in IM resistance in primary CML samples. In a number of patients with advanced phase disease, multiple copies of the *BCR-ABL* gene were detected. It is presumed that, in the presence of BCR-ABL amplification, there is insufficient IM present in the leukaemic cell to inhibit the increased level of BCR-ABL protein.

In addition, this study by Gorre *et al* (Gorre et al., 2001) was one of the first to consider the possibility that mutations in BCR-ABL might result in IM resistance. Indeed, this was the first study to report the single nucleotide substitution (C to T)

at position 315 of *ABL*, resulting in replacement of a threonine residue with isoleucine (T315I). To date, more than 40 different *BCR-ABL* kinase domain mutations which result in intrinsic changes in the kinase that can affect drug binding or kinase activity have been reported (Branford et al., 2002; Shah et al., 2002; von Bubnoff et al., 2002; Branford et al., 2003). These mutations may involve the ATP-binding site (P-loop), the activation loop or the carboxy terminus of the BCR-ABL molecule. However, the T315I mutation remains the most clinically significant as it is resistant to high concentrations of IM and also the second generation inhibitors described later (**Section 1.3.2**).

Recent studies suggest that BCR-ABL protein conformation is absolutely critical for IM binding and function (Shah et al., 2002). Active BCR-ABL exists in an open (non-accessible) conformation and inactive BCR-ABL in a closed (accessible) conformation; thus sensitivity to IM in CML is presumed to result from a dynamic switch between open and closed conformations possibly linked to cell cycle progression. Indeed, IM has been shown to bind to the closed conformation of BCR-ABL and results in a number of structural changes to the protein after binding (Schindler et al., 2000). Therefore, in the presence of BCR-ABL kinase domain mutations, it is thought that IM is unable to bind due to either interruption of critical contact points between IM and BCR-ABL or induction of an inaccessible BCR-ABL conformation (Shah et al., 2002). The clinical significance of BCR-ABL kinase domain mutations is variable. Apart from the T315I mutation, those affecting the P-loop are associated with the poorest outcomes (Branford et al., 2003). In addition, studies have shown that some mutations confer only a moderate degree of IM resistance which can be overcome by increasing the dose of IM (Corbin et al., 2003).

The clinical significance of the T315I mutation is related to its position in the BCR-ABL molecule and it is described as a 'gatekeeper' of the ATP binding site. This is because it separates the ATP binding site from an internal cavity and the nature of the gatekeeper molecule is a vital determinant of inhibitor specificity (Liu et al., 1999; Schindler et al., 1999). Threonine^{315} forms critical hydrogen bonds with IM, and the absence of the oxygen molecule provided by the side chain of threonine^{315} prevents the formation of a vital hydrogen bond with IM (Gorre et al., 2001). In addition, compared to threonine, isoleucine contains an extra hydrocarbon group, resulting in a steric clash with IM and preventing IM binding to BCR-ABL.

The third proposed mechanism of BCR-ABL-dependent IM resistance is that intracellular levels of IM are reduced. This may occur via multidrug resistance proteins, e.g. MDR1 overexpression which increases drug efflux (Mahon et al., 2003; Thomas et al., 2004) and alpha-1-acid glycoprotein which reduces the availability of the drug *in vivo* (Gambacorti-Passerini et al., 2002). Further studies have shown that expression levels of the cellular transporter OCT-1 are low in some patients with CML on IM, resulting in low intracellular concentrations of IM (Thomas et al., 2004; White et al., 2005). The role of other transporter molecules is more contentious, in particular the role of ABCG2 with some groups showing IM to be a substrate of ABCG2 (Burger et al., 2004) and others an inhibitor (Houghton et al., 2004; Jordanides et al., 2006); although there is now increasing evidence that IM is an ABCG2 inhibitor.

In clinical practice, the most common mechanism of IM resistance is mutation of the BCR-ABL kinase domain that interferes with IM binding (P-loop mutations) and, in some patients, these mutations have been shown to be present prior to commencing treatment with IM (Roche-Lestienne et al., 2002; Kreuzer et al.,

2003). A major focus of current research interest is overcoming IM resistance and inducing CML stem cell apoptosis.

1.2.8 Molecular persistence

Despite an impressive rate of CCR in CP CML, the majority of patients have persistent detectable disease at the molecular level by qRT-PCR (Hughes et al., 2003; Branford et al., 2004). It is hypothesised that this minimal residual disease (MRD) is due to a dual population of primitive leukaemic cells. The first is a subset of CML cells which undergo cell cycle arrest and accumulate in G_0/G_1 *in vitro* in the presence of IM or other anti-proliferative agents, including conventional chemotherapy agents (Jorgensen et al., 2005a). Theoretically, these cells can be eradicated by giving pulsed sequential therapies to encourage the cells back into cycle. The second, and more significant population, postulated to contribute to MRD is the quiescent stem cell population (Holyoake et al., 1999) as, *in vitro*, quiescent CML stem cells were shown to be completely insensitive to IM at concentrations up to 10 times higher than those achievable *in vivo* (Graham et al., 2002), while proliferating cells remained exquisitely sensitive. In addition, an *in vivo* study to assess presence of BCR-ABL$^+$ cells by FISH and levels of BCR-ABL mRNA by qRT-PCR demonstrated that samples from different CML patients collected at different time points displayed persistence of BCR-ABL$^+$ progenitors despite continued IM therapy (Bhatia et al., 2003). This was further indication that IM did not eliminate malignant primitive progenitors in CML patients. Further clinical studies have shown that, over time, in patients who have achieved a CCR, BCR-ABL transcript levels slowly continue to decrease (Goldman et al., 2005). This finding supports the hypothesis that CML stem cells are less sensitive than more mature CML progenitors to IM *in vivo*. The quiescent CML stem cell population is gradually reduced as these cells enter the cell cycle and proliferate,

becoming IM-sensitive and undergoing apoptosis. As the quiescent CML stem cell population diminishes, the BCR-ABL transcript level falls. Therefore, despite its impressive clinical efficacy in CML, particularly CP, due to the quiescent CML stem cell population, IM does not cure CML.

One possible explanation for the relative insensitivity of quiescent CML stem cells to IM is the conformation of the BCR-ABL kinase domain in the quiescent versus proliferating stem cells (Shah et al., 2002). The switch between open (non-accessible) and closed (accessible) conformations may not be triggered in quiescent CML stem cells; hence IM may not be the optimal choice of agent to eradicate this population. Other proposed explanations for insensitivity of CML stem cells to IM include *BCR-ABL* amplification or increased *BCR-ABL* transcripts within the stem cell compartment and altered levels of drug influx and efflux pumps on the cell membrane of CML stem cells.

In some patients, no residual *BCR-ABL* transcripts were detected after prolonged treatment with IM (Kantarjian et al., 2004; Rosti et al., 2004; Cortes et al., 2005b). However, the reported frequency of this was very variable (4% to 34%), probably due to the different sensitivities of the test methods used and available technical skills (Baccarani et al., 2006). In a few patients who stopped IM after achieving a CMolR, disease rapidly recurred further highlighting the clinical importance of quiescent CML stem cells (Cortes et al., 2004b; Mauro et al., 2004). Despite this, IM has altered the natural history of the condition by increasing PFS in those patients who respond to it and therefore the overall median survival (Druker et al., 2006). However, the long term efficacy of IM remains unknown and the side effects of long term IM exposure remain to be assessed. A recent study brought attention to an increased risk of cardiac morbidity and mortality in patients treated with IM (Kerkela et al., 2006). It was reported that ten patients presented with

congestive cardiac failure while on IM therapy and, in addition, mice treated with IM developed left ventricular contractile dysfunction. This was found to be related to the endoplasmic reticulum stress response in cardiac myocytes which can be mediated by ABL. Therefore, cardiotoxicity may be an unexpected side effect of IM inhibition of ABL. However, this finding is controversial as there was no significant increase in cardiac morbidity or mortality reported in the IRIS trial (Druker et al., 2006).

Thus, in view of the potential for long term side effects and also the potential for molecular relapse, which may be due to the emergence of BCR-ABL kinase mutations, all CP CML patients, and particularly those with molecular persistence need to be clinically assessed regularly and monitored for signs of molecular relapse by qRT-PCR for *BCR-ABL*.

1.2.9 Assessment of BCR-ABL kinase activity

While measurement of *BCR-ABL* transcripts by qRT-PCR provides a valuable estimation of disease burden and how a patient is responding to treatment (Branford et al., 1999; Hughes et al., 2003; Hughes et al., 2006), it does not provide a direct assessment of the efficacy of a treatment against BCR-ABL kinase activity. In addition, there may be a discrepancy between BCR-ABL transcript and protein expression levels. Therefore it is important to be able to directly measure BCR-ABL kinase activity both *in vitro* and *in vivo*. The most direct measure of this would be determination of phosphorylated BCR-ABL protein expression which, although measurable in cell lines (Konopka et al., 1985; Clark et al., 1987), is difficult to measure in clinical specimens because BCR-ABL is subject to rapid degradation when conventional lysis buffer is used (Maxwell et al., 1987). This is because lysis of mature CML MNCs releases an enzyme, which is now thought to be an acid-dependent hydrolase (Patel et al., 2006), that

selectively destroys BCR-ABL and ABL, but not other proteins. Therefore, it is now accepted that measurement of phosphorylated CrKL, an adaptor molecule which is phosphorylated by BCR-ABL is an adequate surrogate (Nichols et al., 1994; Oda et al., 1994; ten Hoeve et al., 1994; Hochhaus et al., 2001). CrKL directly binds BCR-ABL and links BCR-ABL with downstream effector pathways (Senechal et al., 1996), playing a functional role in transformation. Phosphorylated CrKL can be measured reproducibly and quantitatively in clinical samples by Western blotting. In addition, it has been shown that CrKL phosphorylation is inhibited in a concentration-dependent fashion when CML cells were treated with IM, and this strongly correlated with inhibition of BCR-ABL phosphorylation (Gorre et al., 2001). Therefore assessment of CrKL phosphorylation status can be used to assess the degree and concentration-dependence of inhibition of BCR-ABL kinase activity by different TKIs in both cell lines and clinical samples.

1.3 Potential strategies to overcome IM resistance and molecular persistence

A number of potential strategies exist to overcome IM resistance and molecular persistence seen in CML both *in vitro* and *in vivo*. The three which I will concentrate on for the remainder of this thesis are (1) attempting to reverse the quiescent state and G_0/G_1 cell cycle arrest seen in primitive CML cells after treatment with IM; (2) the use of potent dual SRC/BCR-ABL TKIs; and (3) combining IM with other chemotherapeutic agents.

1.3.1 The rationale for reversing quiescence in CML stem cells

Studies have already shown that increasing the dose of IM does not eliminate CML cells *in vitro* (Graham et al., 2002) or *in vivo*. In fact, while IM effectively reduced dividing CML cells *in vitro*, it resulted in an increase in the number of non-

dividing (quiescent) CML cells due to a marked anti-proliferative effect (Graham et al., 2002) which was not anticipated and the mechanism behind it remains to be elucidated. In addition, many drug combinations do not eliminate quiescent CML cells *in vitro* (Holtz et al., 2005; Jorgensen et al., 2005a) resulting in the molecular persistence seen *in vivo*. Thus, reversal of the quiescent state may restore IM sensitivity in CML stem cells.

Cytokines, including G-CSF can trigger normal quiescent primitive progenitors to enter the cell cycle (Ogawa, 1993). In addition, exogenous G-CSF has been shown to promote rapid progression into S-phase of initially quiescent, primitive (c-kit$^+$, Sca-1$^+$, lin$^-$) progenitors in a murine model (van Pelt et al., 2003). Further studies have shown that G-CSF-R transcript levels are two-fold higher in cycling (G_1/S/G_2/M phases) normal human bone marrow cells in comparison to quiescent (G_0) cells (Jorgensen et al., 2006) . Of specific relevance, haemopoietic growth factors have been shown to stimulate AML cells *in vitro*, and the simultaneous administration of chemotherapy with growth factors resulted in increased cytotoxicity in AML, in particular with the cell cycle-specific agent cytosine arabinoside (Ara-C) (Cannistra et al., 1989; Bhalla et al., 1991; te Boekhorst et al., 1993). In addition, there are case reports of occasional patients with leukaemia achieving remission when treated with recombinant human G-CSF (rHu-G-CSF) alone (Nimubona et al., 2002).

A recent clinical trial examined the effect of priming with rHu-G-CSF in combination with conventional chemotherapy including Ara-C in the induction therapy phase on the outcome of AML (Lowenberg et al., 2003). The results demonstrated that although there was no significant difference in response rate after induction therapy, after a median follow-up of 55 months, the disease-free survival rate was higher in those patients receiving rHu-G-CSF with their

chemotherapy compared to those who did not receive rHu-G-CSF (42% versus 33%; P=0.02). In addition, chemotherapy including rHu-G-CSF showed a trend towards improved overall survival (P=0.016). However, in the subgroup of patients with poor risk AML, the administration of rHu-G-CSF did not improve outcome. Therefore, induction therapy including rHu-G-CSF with Ara-C improved overall and disease-free survival in standard risk AML (Lowenberg et al., 2003). Importantly, this study provided proof of the principle that combining haemopoietic growth factors to sensitise leukaemia cells with conventional chemotherapy was a valid potential therapeutic strategy for improving the response to therapy.

Recombinant human G-CSF has also been safely and successfully used for peripheral blood stem cell mobilisation in healthy donors (Goldman et al., 2006) and CML patients treated with IM with no significant rise in *BCR-ABL* transcript levels by qRT-PCR (Drummond et al., 2003; Hui et al., 2003). Furthermore, it is current practice to use rHu-G-CSF in combination with IM in CML patients with IM-induced neutropenia because myelosuppression during IM therapy is associated with a poorer cytogenetic response (Marin et al., 2003a; Sneed et al., 2004). It has been suggested that the improved cytogenetic responses observed in these patients result from an increased exposure to IM (Heim et al., 2003; Marin et al., 2003b; Quintas-Cardama et al., 2004). However, another possible action of G-CSF in this situation may be to promote re-entry of quiescent CML stem cells into the cell cycle and hence increase their sensitivity to IM (Jorgensen et al., 2005b).

Previous studies had shown that, *in vitro*, Ph^+ CML cells lost LTC-IC activity when cultured regardless of the presence of growth factors (Udomsakdi et al., 1992; Petzer et al., 1997). However, at the same time, CFC numbers were amplified and there was accumulation of increasingly differentiated cells of mixed lineages (Maguer-Satta et al., 1998; Jiang et al., 1999). These results indicate that the rapid

loss of LTC-IC activity seen in CML cells *in vitro* may be due to an enhanced propensity for primitive CML progenitors to differentiate into more mature cells which no longer have multipotent stem cell potential. Therefore, it may be possible to increase the differentiation of CML progenitor cells by the addition of rHu-G-CSF *in vitro* or *in vivo* to force quiescent cells to enter the cell cycle. In effect, is it possible to eradicate CML by forcing all the quiescent CML stem cells to proliferate, differentiate and lose their stem cell properties?

If this hypothesis is correct, it may be possible to induce cell cycle activation in quiescent CML stem cells with rHu-G-CSF, thereby escaping the IM-insensitive quiescent state. Additionally, continuous IM resulted in accumulation of IM-insensitive quiescent CML stem cells in G_0 (Graham et al., 2002), so it may be possible to bring the cells back into cycle by interrupting IM therapy, a so called 'IM holiday'. It is possible that combining these two approaches may be synergistic in eradicating quiescent CML stem cells. Thus, by inducing cell cycle activation with rHu-G-CSF and/or interruption of IM therapy, the aim is to reverse quiescence and sensitise cells to IM, thereby eliminating disease.

1.3.2 The rationale for using potent dual SRC/BCR-ABL TKIs

A significant minority of patients, particularly those with advanced disease become resistant to IM through the development of BCR-ABL kinase domain mutations which interfere with the binding of IM to the ATP binding pocket of BCR-ABL (Gorre et al., 2001; Branford et al., 2002; Shah et al., 2002; von Bubnoff et al., 2002; Branford et al., 2003). In addition, the mechanisms which contribute to the molecular persistence present in CML patients after treatment with IM are unclear (Hughes et al., 2003; Branford et al., 2004). Therefore, there is a requirement for the development of new agents which are more powerful inhibitors of BCR-ABL and bind to BCR-ABL regardless of conformational status, reducing the likelihood

of developing resistance. A new generation of combined SRC/BCR-ABL kinase inhibitors that do not appear to be conformation sensitive and are at least 10-20-fold more potent than IM are now available (Nagar et al., 2002). These novel agents are active against many of the frequently observed kinase domain mutations that cause resistance towards IM (von Bubnoff et al., 2003). Dasatinib (Sprycel™; formerly BMS-354825, manufactured by Bristol-Myers Squibb, Princeton, NJ, USA) is one such agent and was the first of these compounds to enter clinical trials in Ph^+ leukaemias. The structure of dasatinib is shown in **Figure 1-9.**

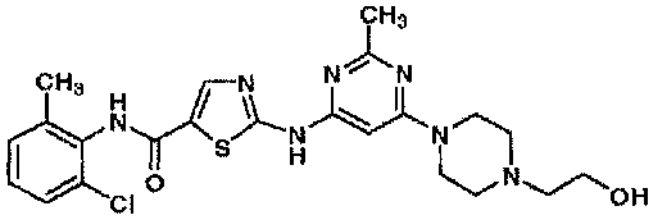


Figure 1-9. Structure of dasatinib (Sprycel™; formerly BMS-354825).

1.3.2.1 Dasatinib

Dasatinib was selected from a panel of substituted 2-(aminopyridyl) and 2-(aminopyrimidinyl) thiazole-5-carboxamides which were all shown to have substantial anti-proliferative activity against haematological and solid tumour cell lines, including the BC CML cell line K562 (Lombardo et al., 2004). Dasatinib was chosen on the basis of broad-spectrum anti-proliferative activity and suitable circulating plasma levels following oral dosing in a mouse screening assay with only moderate plasma protein interactions and maintained blood levels. Three-dimensional X-ray crystallography studies showed that ABL kinase complexed with dasatinib in an active conformation with the presence of three hydrogen bonds between dasatinib and ABL (Lombardo et al., 2004). These specific hydrogen bonds between dasatinib and ABL may account for the activity of

dasatinib against mutant BCR-ABL kinases (except T315I). A similar pattern of binding was observed between dasatinib and SRC kinases.

A kinase selectivity panel showed that dasatinib was a potent competitive inhibitor of ATP binding to SRC (IC_{50} ~0.5nM) and BCR-ABL (IC_{50} < 1.0nM) as well as PDGF-R-β (IC_{50} 28nM) and c-KIT (IC_{50} 5nM)(Lombardo et al., 2004). *In vivo* activity against wild-type BCR-ABL was evaluated using a K562 xenograft assay in nude mice (Lombardo et al., 2004). After the establishment of detectable tumour burden, daily treatment with dasatinib resulted in complete disappearance of tumour cells with no observed toxicity in the animals, showing, for the first time the potency of dasatinib *in vivo* and the favourable toxicity profile, at least in animal models of CML.

Further *in vitro* and *in vivo* experiments were undertaken to determine the efficacy of dasatinib in overcoming IM resistance caused by BCR-ABL kinase domain mutations (Shah et al., 2004). Firstly, using a cell-based assay, this study confirmed that dasatinib was more effective than IM at inhibiting wild-type BCR-ABL. Further cell-based assays using the murine pro-B cell line Ba/F3 which had been engineered to express different clinically significant mutant BCR-ABL kinases showed BCR-ABL inhibition and suppression of growth when dasatinib was used in the low nM range. Interestingly, even within this very low concentration range, there were differences in the sensitivities of selected BCR-ABL mutants (Shah et al., 2004; O'Hare et al., 2005). However, the T315I expressing Ba/F3 cell line was resistant to dasatinib, even in the μM range.

To assess therapeutic potential of dasatinib in IM-resistant Ph^+ leukaemias, SCID mice were inoculated with Ba/F3 cells expressing different BCR-ABL kinase mutations (Shah et al., 2004). Assessment of BCR-ABL activity by determination

of CrKL phosphorylation status on splenocyte lysates from the affected mice showed inhibition of CrKL phosphorylation for up to seven hours after administration of dasatinib. Another cohort of mice were treated with dasatinib for two weeks beginning three days after inoculation with Ba/F3 cells. The untreated control mice developed progressive disease as did those treated with dasatinib but inoculated with cells containing the T315I mutation. However, mice inoculated with cells expressing wild-type BCR-ABL or the IM-resistant mutant M351T remained healthy. Further *in vitro* studies using human bone marrow progenitor cells were undertaken to further assess the efficacy and safety of dasatinib. Dasatinib at a concentration of 5nM failed to inhibit the growth of CFCs from healthy donors. However, CFCs were inhibited by 60-80% in bone marrow progenitor cells taken from patients with either wild-type or mutant BCR-ABL (Shah et al., 2004). Based on the results of these studies, it was hypothesised that a significant number of patients with IM-resistant Ph^+ leukaemias would benefit from dasatinib if it could be given safely in the low nM range. Thereafter, dasatinib progressed rapidly to clinical trial.

Other dual SRC/BCR-ABL kinase inhibitors including PD173955, PD166326 and SKI-606 have been shown to be potent at picomolar concentrations in both cell line (Nagar et al., 2002; Golas et al., 2003; Huron et al., 2003; von Bubnoff et al., 2003; Konig et al., 2006) and animal models (Golas et al., 2003; Wolff et al., 2003). In addition, newer agents such as INNO-406, a BCR-ABL/LYN TKI are currently under evaluation (Kimura et al., 2005; Yokota et al., 2006) . However, as yet, only SKI-606 and INNO-406 have entered phase 1 clinical trials (Cortes et al., 2006).

The results of a Phase 1 dose-escalation study using dasatinib in patients with Ph^+ leukaemias who had either IM-resistance or IM-intolerance have recently been

published (Talpaz et al., 2006). Dasatinib was administered orally at doses of 15 to 240mg per day in monthly treatment cycles. Thirty seven of 40 patients (92.5%) with CP CML achieved a CHR, and a major haematologic response (< 5% blasts) was seen in 31 of 44 patients (70%) with advanced disease (AP, BC or Ph^+ ALL). The MCR rate was 45% in CP and 25% in advanced disease. These responses were sustained in 95% of CP patients and 82% of AP patients. However, nearly all patients with lymphoid BC or Ph^+ ALL relapsed within six months. The main side effects seen were reversible myelosuppression, pericardial effusion, and oedema. Dasatinib is currently completing Phase 2 clinical trials (Guilhot et al., 2005; Hochhaus et al., 2005; Ottmann et al., 2005; Talpaz et al., 2005) and has recently been licensed for use in the UK and the US.

Another agent also in clinical trials in IM-resistant Ph^+ leukaemias is nilotinib (Tasigna™; formerly AMN107, manufactured by Novartis). Nilotinib is a rationally designed selective inhibitor of BCR-ABL, which was based on the crystal structure of the ABL kinase domain in complex with IM (Weisberg et al., 2005). *In vitro*, nilotinib is approximately 20-fold more potent than IM against wild-type BCR-ABL and is also effective against the majority of BCR-ABL kinase domain mutations (except T315I) (O'Hare et al., 2005). In a phase 1 dose-escalation study, nilotinib was active in IM-resistant CML with a reasonable safety profile (Kantarjian et al., 2006). The commonest side effects were rash, gastrointestinal disturbances, hyperbilirubinaemia and reversible myelosuppression.

While dasatinib and nilotinib are effective against the majority of BCR-ABL kinase mutations, their major limitation is the inability to target the relatively common mutation T315I which is increasingly being seen (Shah et al., 2002), particularly in advanced disease. The small molecule aurora kinase inhibitor MK-0457 (VX-680; manufactured by Merck and Co., Inc, Blue Bell, PA, USA) has *in vitro* activity

against cells expressing wild type and mutant BCR-ABL including T315I (Giles et al., 2006) and has been shown to block cell cycle progression and inhibit apoptosis in different human tumours (Harrington et al., 2004). Aurora kinases are essential for the regulation of mitotic chromosome separation and cytokinesis. In addition, MK-0457 has been used in three patients with Ph^+ leukaemias associated with the T315I mutation (Giles et al., 2006). All three patients had clinical responses and no significant adverse events were reported, providing the first observation of response in patients with T315I mutations. Clinical trials with MK-0457 in patients with T315I mutations are currently underway.

1.3.2.2 The role of SRC kinases in normal haemopoiesis

The SRC kinases are a family of eight non-receptor protein tyrosine kinases (SRC, LYN, HCK, LCK, FYN, BLK, FGR and YES) which are expressed in haemopoietic and non-haemopoietic cells (Korade-Mirnics and Corey, 2000). SRC kinases are involved in a range of cellular processes including cell migration, adhesion, proliferation, survival, DNA synthesis, the cell cycle and phagocytosis. These responses are mediated via the Ras, PI3K and focal adhesion kinase (FAK) pathways. SRC kinase activity is modulated by post-translational changes in phosphorylation status and ubiquitination (Oda et al., 1999). The SH1 domain of SRC molecules forms the catalytic domain, consisting of ~250 amino acids at the carboxy terminus. Within the catalytic domain are a positive autophosphorylation site (Y416) which becomes phosphorylated during enzyme activation and a negative autophosphorylation site (Y527) which negatively regulates tyrosine kinase activity (Cooper and Howell, 1993). Dephosphorylation of Y527 results in a conformational change, increasing enzyme autophosphorylation and kinase activity.

LYN, HCK and FGR are the SRC kinases which have been shown to be expressed in myeloid cells (Korade-Mirnics and Corey, 2000). In myeloid cell development, SRC kinases promote growth factor-dependent cell cycle progression, and the presence of LYN has been described as being necessary for induction of DNA synthesis and hence proliferation by G-CSF in haemopoietic cells (Corey et al., 1998). In addition, SRC kinases may also contribute to the differentiation of myeloid cells, improve neutrophil adhesion and migration, increase phagocytosis and prolong survival by preventing apoptosis (Korade-Mirnics and Corey, 2000).

1.3.2.3 Evidence for abnormal function of SRC kinases in CML

SRC kinases play a major role in the development, growth, progression and metastasis of a range of human cancers (Frame, 2002) and increased SRC kinase activity and protein expression have been demonstrated in a variety of cancer types including colon, breast, pancreas, lung and brain. SRC kinases affect signal transduction through a number of oncogenic mechanisms including EGF-R, Her2/neu, PDGF-R, FGF-R and VEGF-R. Therefore it would be anticipated that inhibiting SRC kinase activity would be effective at blocking the abnormal pathways that stimulate oncogenic transformation of cells.

The first evidence for the activation for SRC kinases in leukaemia came from a study which showed that LYN was specifically activated in myeloid leukaemia cell lines in response to IL-3 (Torigoe et al., 1992), indicating that LYN may be important in IL-3 signalling cascades, at least, in some myeloid leukaemias. A later study demonstrated that proliferation of the myeloid leukaemia cell line M07e in response to GM-CSF was decreased by a LYN antisense oligonucleotide or culture in the presence of the TKI PD166285 (Roginskaya et al., 1999). More

pertinent was a study which showed that the SRC kinases LYN and HCK were activated in a number of cell lines expressing $p210^{BCR-ABL}$ (Danhauser-Riedl et al., 1996).

In normal haemopoiesis, CXCR-4, the G protein-coupled receptor of stromal-derived factor (SDF)-1, stimulates LYN and activates the PI3K pathway with effects on cell motility and migration. It has been demonstrated that binding of phosphorylated BCR-ABL to LYN results in constitutive activation of LYN and PI3K. In addition, these kinases become completely unresponsive to SDF-1 in the presence of phosphorylated BCR-ABL (Ptasznik et al., 2002). Moreover, inhibition of BCR-ABL with IM restored the regulation of LYN by SDF-1. This highlights a LYN-dependent mechanism via which BCR-ABL disrupts G protein-coupled receptor signalling and function and indicates that BCR-ABL disrupts normal LYN function, altering chemokine signalling and chemotaxis, resulting in altered cell motility and migration. Further studies have reported that *in vitro*, the IM-resistant cell line, K562-R, displays BCR-ABL independence in association with over-expression of LYN kinase (Donato et al., 2003). In addition, samples taken from patients with advanced phase CML who became IM-resistant showed similar levels of LYN kinase expression to the K562-R cell line (Donato et al., 2003). Up-regulation of BCL-2 by LYN has also been implicated as a potential mechanism of BCR-ABL-independent resistance in CML (Dai et al., 2004), although this study was conducted in cell lines only and may have been flawed as the phosphorylated LYN antibody used was targeted against the negative regulatory site (Y507) and not the activation site (Y411). These studies suggest that, in some cases, acquired IM-resistance may be associated with BCR-ABL independence and increased expression of additional tyrosine kinases, resulting in up-regulation of downstream signal transduction pathways.

HCK has also been implicated in the signalling pathways downstream of BCR-ABL. HCK is activated by BCR-ABL and studies have shown that the growth factor independence exhibited by BCR-ABL$^+$ cell lines is suppressed by a kinase-defective HCK (Lionberger et al., 2000). More recently, HCK has been implicated in a novel BCR-ABL-HCK-STAT5 signalling pathway, involved in transformation of myeloid cells by BCR-ABL (Klejman et al., 2002).

Taken together, these results indicate that SRC kinases are likely to be important in modulating signal transduction pathways downstream of BCR-ABL. In addition, there is evidence that activation of SRC kinases may result in BCR-ABL-independent IM-resistance in some patients. Therefore, dasatinib, which is 325 times more potent than IM at inhibiting BCR-ABL and also has the ability to inhibit SRC kinases may be useful for targeting quiescent CML stem cells.

1.3.3 The rationale for combining IM with other chemotherapeutic agents

To date, a wide range of conventional and novel agents (e.g. hydroxycarbamide, Ara-C, IFN-α, arsenic trioxide and FTIs) have been combined with IM *in vitro* to overcome IM resistance and eliminate CML cells (La Rosee et al., 2002; Topaly et al., 2002; Druker, 2003). The majority of *in vitro* data come from experiments with CML cell lines and not primary CML cells. Many of these studies showed significant *in vitro* responses in proliferating cells, however, they did not target the IM-insensitive quiescent stem cell population. Although many of these studies have been informative, none have addressed cytotoxicity at the stem cell level. A recent study (Jorgensen et al., 2005a) showed that many drug combinations inducing apoptosis in CML cell lines were ineffective in primary CML cells and, indeed, there was a trend towards further accumulation of quiescent stem cells in response to combination therapy. Indeed, in this study by Jorgenson *et* al

(Jorgensen et al., 2005a), the only combination to show superiority to IM alone on the quiescent stem cell population was the combination of IM with the FTI lonafarnib (formerly SCH66336; Schering-Plough, Kenilworth, NJ, USA), however, the results failed to reach statistical significance. Farnesyltransferase inhibitors have been used extensively in clinical trials of myeloid leukaemias including CML. Interestingly, a novel cytotoxic FTI, BMS-214662, has been described which has activity against non-proliferating cells *in vitro* (Lee et al., 2001).

1.3.3.1 The mode of action of FTIs

A critical pathway activated by BCR-ABL is the Ras pathway, and although not fully specific for Ras, a range of FTIs have been developed which inhibit Ras. The FTIs are also potent inhibitors of MDR1 (Wang et al., 2001). The signalling functions of both normal and oncogenic Ras are dependent on the membrane association of Ras which is achieved by post-translational processing of cytosolic Ras and involves three enzymatic steps. Firstly, there is farnesylation of a cysteine four residues from the C-terminus; secondly, hydrolysis of the C-terminal tripeptide; and, thirdly, methyl esterification of the new C-terminal farnesylcysteine (Hunt et al., 2000). The key step in this process is the farnesylation of Ras by the enzyme FT and, based on this finding, a number of FTIs have been developed for clinical use because, although Ras undergoes a number of post-translational modification steps, only farnesylation is necessary for membrane localisation and cell-transforming activity (Heimbrook and Oliff, 1998). In addition to Ras, a number of other intracellular proteins are substrates for prenylation by FT including RhoB, Rac and lamin (Rowinsky et al., 1999). In CML, the importance of Ras is as an obligatory signalling molecule downstream of BCR-ABL, and inhibitors of Ras may have potent anti-proliferative effects.

Ras proteins exist in the farnesylated form only in the absence of FTIs. The isoforms K-Ras and N-Ras, but not H-Ras, undergo geranylgeranylation when farnesylation is inhibited resulting in failure to completely inhibit Ras processing and it is hypothesised that the K-Ras resistance to FTIs explains the failure to inhibit growth or induce significant toxicity in normal cells as well as the relatively poor responses achieved in solid tumours (Reuter et al., 2000; Daley, 2003). Treatment of Ras transformed cells with FTIs selectively inhibits Ras-dependent signalling, including Ras processing resulting in the accumulation of unprenylated (inactive) Ras and inhibition of the Ras-Raf-MEK-ERK pathway.

However, the observed anti-tumour effects of FTIs are not solely due to Ras inhibition as these agents are also effective in Ras^{null} and Ras mutant cells (Nagasu et al., 1995; Sepp-Lorenzino et al., 1995; End et al., 2001; Rose et al., 2001); they may also act by inhibiting farnesylation of other proteins (Cox and Der, 1997; Lebowitz and Prendergast, 1998; Ashar et al., 2000; Prendergast and Rane, 2001); (**Discussion 7.3**). In addition, FTIs have also demonstrated preclinical activity in the treatment of eukaryotic pathogens such as plasmodium falciparum and trypanosomiasis brucei by inhibition of FT (Eastman et al., 2006). They may also have a role in the treatment of the Hutchinson-Gilford progeria syndrome which has recently been found to be associated with accumulation of an abnormal farnesylated form of prelamin A (Young et al., 2006). However, FTIs have yet to enter clinical trial for treatment of these conditions.

1.3.3.2 The use of FTIs in CML

Research has shown that lonafarnib inhibits the proliferation of IM-sensitive and resistant BCR-ABL$^+$ cell lines (Peters et al., 2001a; Hoover et al., 2002), is active against BCR-ABL-induced murine leukaemia (Peters et al., 2001a) and

suppresses haemopoietic colony formation from peripheral blood samples of CML patients resistant to IM (Hoover et al., 2002). In addition, lonafarnib enhances IM-induced apoptosis in IM-sensitive cells (Hoover et al., 2002; Nakajima et al., 2003) and, in patients with IM-resistance due to gene amplification cooperates with IM to induce apoptosis (Hoover et al., 2002). Tipifarnib (ZARNESTRA™; formerly R115777; Johnson and Johnson, Titusville, NJ, USA), another FTI in clinical use, has also been shown to act synergistically with IM to increase apoptosis and induce cell cycle arrest in BCR-ABL$^+$ cell lines (Miyoshi et al., 2005). However, in this study the problem of persistence of G_1 arrested leukaemia cells was highlighted as a potential barrier to the complete eradication of disease. In some cell lines (KCL22 and KCL22/SR) there was accumulation of cells in G_0/G_1; however, other cell lines (K562, K562SR, KU812 and KU812SR) showed a decrease in G_0/G_1 cells after treatment with IM plus tipifarnib, and it was hypothesised that apoptosis of these cells occurred before cell cycle arrest.

Phase 1 clinical trials of tipifarnib as a single agent have been conducted in CML (Cortes et al., 2003; Gotlib et al., 2003). In the first study of 22 patients with CML, 7 (6 CP and 1 AP) achieved a complete or partial haematologic response with 4 of these achieving a mCR (Cortes et al., 2003). However, the responses were transient with a median duration of 9 weeks. In the second study, of the 12 patients (11 AP and 1 BC) recruited, only 6 were evaluable at the time of interim analysis and of these, 3 had a haematological response (Gotlib et al., 2003). Therefore, tipifarnib was well tolerated and demonstrated moderate efficacy in IM-resistant patients. A pilot study has also demonstrated the safety and modest efficacy of lonafarnib as a single agent in IM-resistant CML (Cortes et al., 2002). More recently, studies combining both tipifarnib and lonafarnib with IM after failure of IM therapy demonstrated efficacy and a tolerable safety profile (Cortes et al., 2004a; Cortes et al., 2004c). However, the only data available on these studies

are interim analyses and final results remain unpublished. Nonetheless, taken as a group, these studies provide a rationale for examining other, more potent FTIs to treat IM-resistant CML and attempt to eradicate the quiescent CML stem cell population.

1.3.3.3 BMS-214662

BMS-214662 (manufactured by Bristol-Myers Squibb) is an atypical non-peptidomimetic FTI with a benzodiazepine core (Hunt et al., 2000) which inhibits H-Ras and K-Ras in the low nM range and is approximately 1000-fold selective for FT compared to GGT 1 (Rose et al., 2001). **Figure 1-10** details the structure of BMS-214662.

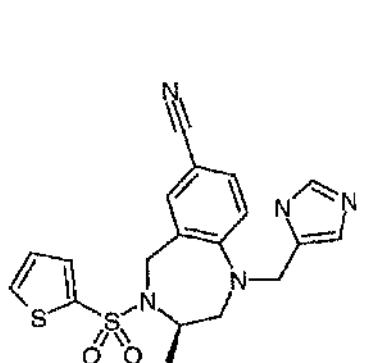


Figure 1-10. The structure of BMS-214662.

In preliminary experiments (Rose et al., 2001), BMS-214662 demonstrated efficacy against a range of tumour cell lines *in vitro* including the BC CML cell line K562 (IC_{50} 160nM using an *in vitro* cytotoxicity assay). *In vivo* studies using a nude mouse human tumour xenograft model demonstrated that BMS-214662 had curative potential in a range of human tumours including colon, pancreas, lung and bladder carcinoma (Hunt et al., 2000; Rose et al., 2001). More recently, BMS-214662 has also shown activity in the haemopoietic disorders myeloma, chronic lymphocytic leukaemia (CLL) and AML (Marzo et al., 2004; Cortes et al., 2005a;

Gomez-Benito et al., 2005). Very interestingly and pertinent to this project, BMS-214662 was also shown to preferentially target non-proliferating (quiescent) cells in solid tumour models *in vitro* (Lee et al., 2001). This study demonstrated that BMS-214662 was 20 times more potent in quiescent compared to proliferating cells from five different solid tumour cell lines. Based on these results, two potential therapeutic strategies incorporating BMS-214662 were investigated *in vivo* in animal models. In the first of these, BMS-214662 was combined with the chemotherapeutic agents, paclitaxel and epothilone, that preferentially kill proliferating cells and in the second, BMS-214662 was combined with manoeuvres to induce tumour cell quiescence such as tamoxifen in oestrogen-dependent breast cancer and surgical castration in androgen-dependent prostate cancer (Lee et al., 2001). Both strategies demonstrated synergy with BMS-214662 and significantly improved response and based on these findings, BMS-214662 was entered into clinical trials in solid tumours alone and in combination with other chemotherapeutic agents.

A number of Phase 1 dose-escalation clinical trials have been conducted in solid tumours with BMS-214662 given as a single agent for either 1 hour or 24 hours by intravenous infusion (Ryan et al., 2004; Papadimitrakopoulou et al., 2005; Tabernero et al., 2005; Eder et al., 2006). The commonest side effects reported in these studies were gastrointestinal disturbances, elevated liver transaminases, renal toxicity, fatigue, electrolyte imbalances and myelosuppression. However, disappointingly, despite reaching the maximum tolerated dose in all four studies, only one study demonstrated clinical benefit (Tabernero et al., 2005), and this was only in a minority of patients (5 of 31). However, the patients recruited to these studies had already failed standard treatments and in many cases had received multiple chemotherapy regimens.

Further Phase 1 studies were undertaken in which BMS-214662 was combined with either cisplatin or carboplatin and paclitaxel (Mackay et al., 2004; Dy et al., 2005). In combination with cisplatin, there were no objective responses, however disease stabilisation was observed in 15 of 29 patients over several cycles of treatment (Mackay et al., 2004). In combination with carboplatin and paclitaxel, 3 of 30 patients had a partial response and a further 8 had stable disease over several months therapy (Dy et al., 2005). These combination studies using BMS-214662 showed more promise than BMS-214662 as a single agent.

The only Phase 1 dose-escalation study undertaken in a bone marrow disorder used BMS-214662 as a single agent in patients with relapsed of refractory AML (n=19), ALL (n=3) or myelodysplastic syndrome (n=8)(Cortes et al., 2005a). Five of 30 patients had evidence of anti-leukaemic activity including 2 CHR (with incomplete platelet recovery) and, of these, one had a cytogenetic response. The remaining 3 patients that responded had an incomplete response with reduced bone marrow blast counts.

Therefore, BMS-214662 has demonstrated activity in HSC disorders (Cortes et al., 2005a) and appears to be more effective when used in combination with other therapies which target proliferating cells in solid tumours (Mackay et al., 2004; Dy et al., 2005). With *in vitro* evidence of activity against quiescent tumour cells (Lee et al., 2001), and the efficacy of other FTIs in combination with IM in CML (Cortes et al., 2004a; Cortes et al., 2004c; Jorgensen et al., 2005a), BMS-214662 appeared a logical choice to assess in combination with IM or dasatinib. It was hypothesised that IM or dasatinib would target the proliferating CML cells and BMS-214662 the deeply quiescent and G_0/G_1 arrested CML stem and progenitor cells.

1.4 Aims

Experiments were designed to address the following aims:

1. To determine if pulsed IM is more effective than continuous IM at eliminating $CD34^+$ CML stem cells *in vitro*;

2. To determine if the addition of rHu-G-CSF to either continuous or pulsed IM enhances the elimination of $CD34^+$ CML stem cells *in vitro*;

3. To assess the efficacy of dasatinib compared to IM against $CD34^+$ CML cells and determine if dasatinib would eradicate quiescent CML stem cells *in vitro*;

4. To determine the relevance of SRC kinases and BCL-2 in CP CML;

5. To determine the efficacy of BMS-214662 alone and in combination with either IM or dasatinib on the quiescent CML stem cell population *in vitro*;

6. To assess the efficacy of BMS-214662 in BC CML, cell lines expressing BCR-ABL kinase mutations and AML *in vitro*.

These questions are dealt with sequentially within the relevant Results Chapters. Each Results Chapter begins with a short introduction relevant to the proceeding data and finishes with a summary of the findings. The discussion of all the results and final conclusions is presented in a separate Discussion Chapter (**Chapter 7**).

2 Materials and Methods

2.1 Materials

2.1.1 *Novel small molecule inhibitors*

Imatinib mesylate (MW 590) was provided as a white powder under a Materials Transfer Agreement from Novartis Pharma (Basel, Switzerland). It was dissolved in sterile H_20 and stored as a 100mM stock solution at 4°C. Dasatinib (MW 489), BMS-214662 (MW 488) and BMS-225975 (MW 570) were provided as white powders under a Materials Transfer Agreement by Bristol-Myers Squibb (Princeton, NJ, USA). Each compound was dissolved in dimethyl sulfoxide (DMSO) to give a stock concentration of 10mg/mL and stored in multiple aliquots at -20°C prior to use. 'PP2 in solution™' (10mM in DMSO) was purchased from Calbiochem, Merck Biosciences Ltd, Nottingham, UK and stored at -20°C. All small molecule inhibitors were made up fresh and diluted to the appropriate concentration with PBS prior to use.

2.1.2 Tissue culture supplies (including CD34$^+$ selection)

Abbott Diagnostics Maidenhead, UK	LS1 BCR-ABL Dual Colour FISH probe
Amersham Pharmacia Biotech Ltd Buckinghamshire, UK	Tritiated (^{3}H) thymidine
Barloworld-Scientific Staffordshire, UK	Iwaki Type 1 Collagen coated 6-well plates
Baxter Healthcare Nottingham, UK	Sterile water
Becton Dickinson Plymouth, UK	Hypodermic needles Luer lock syringes
Bio-Rad Hercules, CA, USA	Triton-X-100
Chugai Pharma London, UK	Recombinant human G-CSF (rHu-G-CSF)
Fisher Scientific Loughborough, UK	Acetic acid Methanol

Greiner bio one Gloucestershire, UK	75cm^3 tissue culture flasks 96-well plates FACS tubes Pipette tips
Hendley Essex, UK	Multispot microscope slides
Invitrogen Paisley, UK	2-Mercaptoethanol Colcemid Foetal calf serum (FCS) L-glutamine 200mM (100X) PBS Penicillin-streptomycin solution 5000u/mL RPMI 1640 medium
Miltenyi Biotech Bisley, UK	CliniMACS CD34 reagent CliniMACS PBS/EDTA buffer CliniMACS tubing set
Nalge Nunc International Roskilde, Denmark	35mm non-adherent tissue culture dishes 75cm^3 non-adherent tissue culture flasks Cryotubes Cryofreezer container Vacubottles
Perkin Elmer Turku, Finland	Filter mats Wax scintillant
Sartorius Hannover, Germany	Minisart 0.2µM sterile filters
Scottish National Blood Transfusion Service Glasgow, UK	20% human albumin solution 4.5% human albumin solution (ALBA)
Sigma-Aldrich Dorset, UK	Ammonium chloride (NH_4Cl) Bovine serum albumin (BSA) Dimethyl sulphoxide (DMSO) Dulbecco's Modified Eagle Medium (DMEM) G418 disulphate salt solution Hank's buffered salt solution – calcium and magnesium free (HBSS-CMF) Hygromycin B Iscove's Modified Dulbecco's Medium (IMDM) Magnesium chloride ($MgCl_2$) Poly-L-lysine Potassium chloride (KCl) Sodium azide Trisodium citrate Trypan blue Trypsin-EDTA

Stem Cell Technologies British Columbia, Canada	Bovine pancreatic deoxyribonuclease (DNAse I) 1mg/mL Bovine serum albumin/insulin/transferrin (BIT) serum substitute Flt-3 ligand Hydrocortisone 21-hemisuccinate Interleukin-3 (IL-3) Interleukin-6 (IL-6) Methocult™ Myelocult™ Stem cell factor (SCF)
Sterilin Ltd Hounslow, UK	Pastettes 5mL, 10mL and 25mL disposable pipettes 15 and 50mL sterile plastic falcon tubes 90mm Petri dishes

2.1.3 Flow Cytometry reagents

Becton Dickinson Oxford, UK	7-aminoactinomycin D (7-AAD) FACS flow FACS clean Human anti-active caspase-3-PE Human anti-BCL-2-FITC kit Human anti-CD34-PE Human anti-CD34-APC Human anti-CD38-PE Human anti-Ki-67-FITC kit IgG FITC isotype control IgG PE isotype control IgG PE-Cy5 isotype control IgG APC isotype control
Caltag Laboratories Silverstone, UK	Fix and Perm® A and B
Molecular Probes Eugene, OR, USA	Carboxy-fluorescein diacetate succinimidyl diester (CFSE)
Sigma-Aldrich Dorset, UK	Anti-rabbit IgG-FITC conjugate Propidium iodide (PI) Formaldehyde
Upstate Dundee, UK	Human anti-p-Src family Alexa Fluor® 488 (Y416)

2.1.4 Molecular biology supplies

Amersham Pharmacia Biotech Ltd Buckinghamshire, UK	ECL™ Plus Western Blotting Detection Reagents Hyperfilm™ ECL™ (Xray film) Rainbow marker (RPN756)

Bio-Rad Hercules, CA, USA	4-15% Tris-HCl gradient gels Laemmli sample buffer Nitrocellulose membrane Tris/Glycine/SDS running buffer Tris/glycine transfer buffer Triton-X-100 Tween
Calbiochem, Merck Biosciences Ltd Nottingham, UK	Phenoxymethylsulphonylfluoride (PMSF) Sodium orthovanadate
Cell Signalling, New England Biolabs Hitchin, UK	Anti-rabbit IgG horseradish peroxidase-linked secondary antibody Human anti-p-CrKL antibody (rabbit) Human anti-pan-actin antibody (rabbit) Human anti-Mcl-1 antibody (rabbit)
Chemicon International Temecula, CA, USA	Re-Blot^{TM} Plus Strong Antibody Stripping Solution
Fisher Scientific Loughborough, UK	Methanol
Perbio Northumerland, UK	BCA kit
Sainsburys Supermarkets Ltd London, UK	Non-fat dry milk powder
Sigma-Aldrich Dorset, UK	0.1% Ponceau S solution Ethylenediaminetetraacetic acid (EDTA) NP-40 Protease inhibitor cocktail Sodium chloride Sodium deoxycholate Sodium fluoride Tris (hydroxymethyl)aminomethane hydrochloride (Tris-HCl)

2.2 Preparation of media and solutions

2.2.1 Tissue culture media

2.2.1.1 RPMI^{++}

RPMI 1640	500mL
FCS	50mL
L-glutamine	5mL
Penicillin/streptomycin solution	5mL

2.2.1.2 Serum free medium (SFM)

BIT	25mL
L-glutamine	1.25mL
Penicillin/streptomycin solution	1.25mL
2-Mercaptoethanol	250µL
IMDM	97.25mL
Make up in a Vacubottle and filter sterilise	

2.2.1.3 Serum free medium supplemented with growth factor cocktail (SFM + 5GF)

Serum free medium	50mL
IL-3 (50µg/mL)	20µL
IL-6 (50µg/mL)	20µL
G-CSF (50µg/mL)	20µL
Flt-3 ligand (50µg/mL)	100µL
SCF (50µg/mL)	100µL
Filter sterilise through 0.22µM filter	

2.2.1.4 RPMI for maintenance of stromal cell line M2-10B4 for LTC-IC

RPMI 1640	500mL
FCS	50mL
L-glutamine	10mL
Penicillin/streptomycin solution	10mL

2.2.1.5 DMEM for maintenance of genetically modified S1/S1 fibroblast cell line for LTC-IC

DMEM	500mL
FCS	75mL
L-glutamine	10mL
Penicillin/streptomycin solution	10mL

2.2.1.6 Myelocult

Myelocult™	100mL
Hydrocortisone hemisuccinate ($1x10^{-4}$M)	1mL

2.2.1.7 RPMI/WEHI medium for Ba/F3 cell lines

RPMI	500mL
WEHI-3B conditioned cell medium	50mL
FCS	10mL
L-glutamine	5mL
Penicillin/streptomycin solution	5mL

2.2.2 Tissue culture solutions

2.2.2.1 PBS/2% FCS

PBS	490mL
FCS	10mL

2.2.2.2 PBS/20% FCS

PBS	80mL
FCS	20mL

2.2.2.3 Ammonium chloride solution (10x)

Ammonium chloride (NH_4Cl)	8.33g
Sterile water (dH_2O)	100mL
Filter sterilise through 0.22µM filter	
Dilute stock solution 1:10 with dH_2O for lysis to give 1x strength	

2.2.2.4 'DAMP' solution for thawing cryopreserved $CD34^+$ or unmanipulated cell aliquots from liquid nitrogen

DNAse II solution (1mg/mL)	1 vial
Magnesium chloride (400X; 1M)	625µL
Trisodium citrate (0.155M)	26.5mL
Human serum albumin (20%)	12.5mL
PBS (magnesium/calcium free)	208mL

2.2.2.5 4.5% human albumin solution/20% DMSO

4.5% human albumin solution (ALBA)	80mL
DMSO	20mL

2.2.2.6 PBS/BSA/Azide

PBS	500mL
BSA	5g
Sodium azide	0.5g

2.2.2.7 IMDM/2% FCS

IMDM	98mL
FCS	2mL

2.2.3 *Flow cytometry solutions*

2.2.3.1 PBS/0.4% formaldehyde

PBS	48mL
10% formaldehyde solution	2mL

2.2.3.2 PBS/0.2% Triton-X-100

PBS	50mL
Triton-X-100	100µL

2.2.4 Molecular biology solutions

2.2.4.1 Lysis buffer for protein lysates

dH_2O	7.75mL
1.5M NaCl	1mL
1M Tris-HCl	0.5mL
150mM EDTA	333µL
NP-40	50µL
10%(w/v) Sodium deoxycholate	250µL
Immediately prior to use, protease and phosphatase inhibitors below added	
Protease inhibitor cocktail	100µL
PMSF	100µL
Sodium fluoride	20µL
Sodium orthovanadate	20µL

2.2.4.2 Running buffer

10X TGS buffer	100mL
PBS	900µL

2.2.4.3 Transfer buffer

10X TG buffer	80mL
PBS	720mL
Methanol	200mL

2.2.4.4 Wash buffer

PBS	1000mL
Tween	100µL

2.2.4.5 Blocking buffer

PBS	100mL
Non-fat dry milk	5g
Tween	100µL

2.2.4.6 Primary antibody buffer

PBS	100mL
BSA	5g
Tween	100µL

2.3 Methods

2.3.1 *Cell culture*

2.3.1.1 Collection of primary CML samples

All samples (peripheral blood and leucapheresis material) were collected using written informed patient consent with approval from the Local Research and Ethics Committee. See **Appendix 1** for the biological characteristics of the patients from whom the samples were collected.

2.3.1.2 Cell counting and assessment of cell viability

Cell counts and assessment of cell viability were performed using a haemocytometer with trypan blue exclusion. Trypan blue was diluted 1:10 with

PBS and either 40 or 90µL of trypan blue was added to 10µL of cell suspension to give a 1:5 or 1:10 dilution of cells. Eight microlitres of this mixture was transferred to a haemocytometer and a minimum of 100 viable cells were counted with dark blue-staining cells counted as non-viable. Further assessment of cell viability in $CD34^+$ CML cells after culture was performed using PI staining and FACS analysis (**Section 2.3.2**).

2.3.1.3 Red cell lysis

Peripheral blood samples or, in some instances, leucapheresis collections required lysis of red blood cells. Red cell lysis was performed using 0.083% NH_4Cl solution at a ratio of 5mL sample to 45mL NH_4Cl at 37°C for 10 minutes. This step was repeated to ensure complete red cell lysis. After completion of red cell lysis, leucocytes were recovered by centrifugation (1000RPM for 10 minutes) and resuspended in either PBS/2%FCS or 4.5% ALBA, for cryopreservation or $CD34^+$ selection.

2.3.1.4 Cryopreservation of cells

$CD34^+$ selected cells, total MNCs from BC CML and AML samples and leukaemia cell lines were cryopreserved in liquid nitrogen until use. Briefly, $4x10^6$ to 10^8 cells, as appropriate, were aliquoted into nunc cryotubes. To this cell suspension, an equal volume of 20% DMSO in 4.5% ALBA was added to give a final concentration of 10% DMSO. The cryotubes were transferred to a cryofreezer container and steadily cooled in isopropyl alcohol at -80°C before transfer to liquid nitrogen for long-term storage.

2.3.1.5 Recovery of frozen samples

Cell vials were removed from liquid nitrogen and thawed immediately at 37°C in a water bath until all ice crystals had disappeared. Using a pastette, the cells were then placed in a 15mL sterile tube (1 tube per vial of cells) and DAMP solution was added dropwise over 20 minutes. This step was performed at room temperature to enhance the activity of the DNAse II, with frequent agitation of the tubes to prevent clumping of cells. The cells were then centrifuged at 1000RPM for 10 mins, the supernatant discarded, and the pellet loosened by flicking the tube. The cells were then washed a further twice in DAMP to ensure that all DMSO and cell debris had been removed from the sample. Following this, the cells were resuspended in PBS/2% FCS, and the cell count and viability were assessed.

2.3.1.6 CLINIMACS $CD34^+$ cell selection

Samples were obtained from CML patients at diagnosis (n=22) who, as part of their initial clinical management, underwent leucapheresis to reduce the peripheral WCC and obtain $CD34^+$ cells for potential use for an autologous SCT at the time of disease progression. Further samples were obtained from patients with normal bone marrow (n=5) undergoing autologous stem cell collection for either non-Hodgkin's lymphoma or multiple myeloma who had been mobilised with rHu-G-CSF following chemotherapy and had excess $CD34^+$ cells remaining after those required for clinical use had been processed. All leucapheresis procedures were performed using the Cobe Spectra continuous blood flow separator (Cobe Laboratories, Quedgeley, UK). Samples were processed using the CliniMACS clinical system for $CD34^+$ cell selection within 24 hours of sample collection by either Dr Graham Templeton or Miss Ashley Hamilton according to the manufacturers' instructions. Briefly, total MNC were incubated with specific anti-

CD34 antibodies to which super-paramagnetic particles (~50nM in diameter) had been conjugated. This cell suspension was then exposed to a high-gradient magnet separation column and the $CD34^+$ cell-antibody-particle complex was captured and retained by the column. The $CD34^-$ cells which had not bound to the antibody-particle complex were not retained and were eluted with the waste. The retained $CD34^+$ cells were then eluted and collected by removing the magnetic field from around the column. The collected $CD34^+$ cells were then made up to a concentration of $1x10^7$/mL to $1x10^8$/mL with 4.5% ALBA and placed on ice prior to cryopreservation. After $CD34^+$ selection was completed an aliquot (~$5x10^4$ cells) was removed for flow cytometry assessment of CD34 purity and confirmed that, post-selection, all samples were >95% $CD34^+$. **Figure 2-1** shows an example of CD34 purity of a patient sample before and after CliniMACS $CD34^+$ cell selection. An additional sample of $1x10^6$ cells was removed for *BCR-ABL* assessment by FISH.

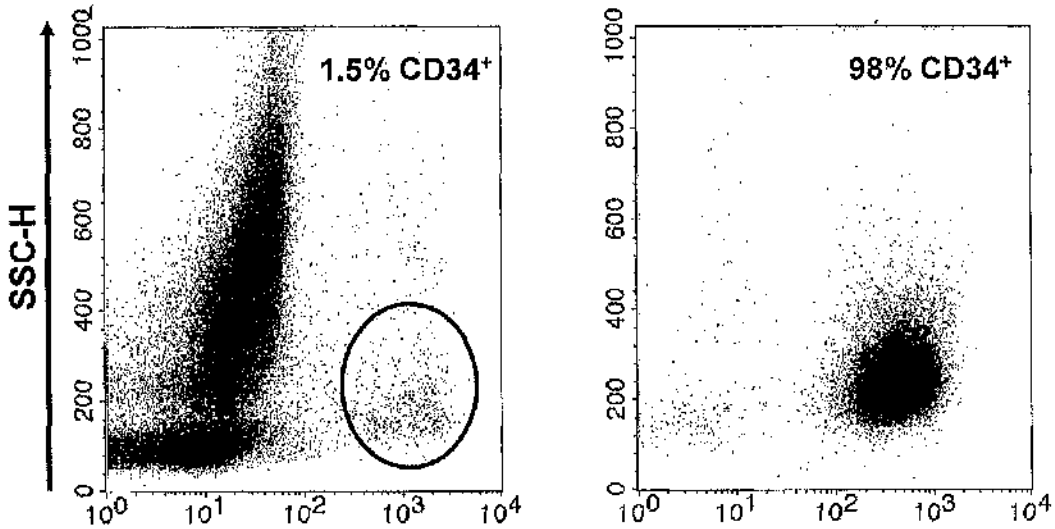


Figure 2-1. An example of flow cytometry dot plots of CD34 purity before (in the left panel, with $CD34^+$ population highlighted) and after (right panel) CliniMACS $CD34^+$ cell selection. SSC-H, side scatter.

2.3.1.7 CFSE staining

After recovery from liquid nitrogen and assessment of cell count and viability, $CD34^+$ CML cells were stained with 1μM CFSE according to previously described methods (Nordon et al., 1997; Holyoake et al., 1999; Graham et al., 2002). Before CFSE staining, an aliquot (~10%) of the $CD34^+$ cells was removed and set up in culture in SFM + 5GF as an unstained control. Carboxy-fluorescein diacetate succinimidyl diester is an intracellular stain fluorescent in FL1 by flow cytometry, the fluorescence intensity of which halves with each cell division. This allows high resolution tracking of cell division. Carboxy-fluorescein diacetate succinimidyl diester was dissolved in DMSO to a concentration of 5mM and stored in aliquots at -20°C. Prior to use, the CFSE was diluted to 500μM with PBS/2% FCS. Ten microlitres of this was then added to 5mL of cell solution to give a final concentration of 1μM. The cells were then incubated in a water bath at 37°C for 10 minutes exactly, after which the cells were removed and the CFSE was quenched by adding 10X volume of PBS/20% FCS. The cells were centrifuged at 1000RPM for 10 minutes and then washed once in PBS/2% FCS. The cells were then cultured overnight in 10mL SFM + 5GF in large non-adherent ($75cm^3$) tissue culture flasks (1-2x10^7 $CD34^+$ cells/flask) at 37°C, 5% CO_2. The following morning the CFSE-stained $CD34^+$ cells were removed from the tissue culture flask and placed into a sterile tube. The flask was then washed out with PBS/2% FCS and this was also added to the tube. The cells were then centrifuged at 1000RPM for 10 minutes and washed again in PBS/2% FCS. Following this, the CFSE-stained cells were resuspended in PBS/2% FCS and the cell count and viability were determined. An aliquot of cells (~2x10^4) was removed for flow cytometry to assess the position of the undivided ($CFSE^{max}$ $CD34^+$) cell population prior to culture in the different treatment conditions and an example of this is shown in **Figure 2-2.** For each experiment, a colcemid control was set up using CFSE-stained cells to

determine the position of the undivided $CFSE^{max}$ peak after culture. The colcemid stock (2mg/mL) was diluted 1:100 with PBS and then filter sterilised. Ten microlitres of this was added per mL of tissue culture medium in the colcemid control to give a final concentration of 200ng/mL.

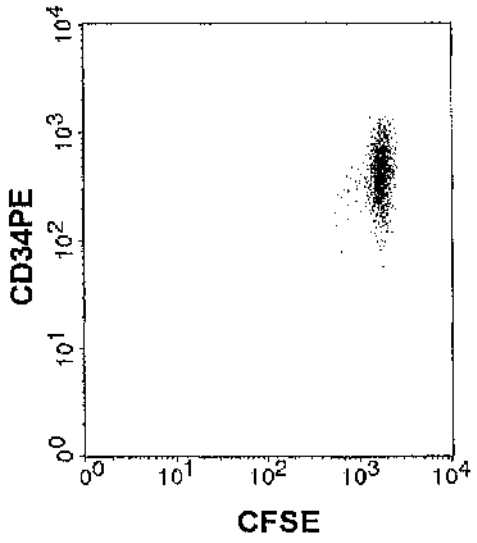


Figure 2-2. Representative FACS dot plot showing the position of the baseline undivided $CFSE^{max}$ $CD34^+$ cell population following overnight culture after staining with CFSE.

2.3.1.8 Culture of cell lines

The BC CML cell line K562 ($BCR\text{-}ABL^+$) and the AML cell line HL60 ($BCR\text{-}ABL^-$), which were both available 'in-house', were cultured in $RPMI^{1+}$ in tissue culture flasks. The cells were counted and medium changed every 48 to 72 hours to maintain the cell concentration between 0.4 and $1.2x10^5$ cells/mL. Ba/F3 cells containing wild-type or BCR-ABL with kinase domain mutations (T315I, M351T and H396P) were kindly provided under a Materials Transfer Agreement by Professor Brian Druker, Oregon Health Sciences University, Portland, Oregon, USA and Professor Junia Melo, Imperial College, London, UK. The cells were maintained in RPMI/WEHI medium which contained IL-3 from the WEHI-3B cells. The cells were counted and medium changed every 96 hours to maintain the cell concentration at ~$1x10^5$/mL.

2.3.1.9 Culture of $CD34^+$ cells

After either recovery from liquid nitrogen or CFSE staining, cells were cultured in SFM + 5GF in 35mm suspension dishes at an initial cell concentration of ~$5x10^5$/mL for 3 to 12 days at 37°C, 5% CO_2. The exception to this were the experiments using $CD34^+$ CML cells in Chapter 3, in which the cells were cultured in SFM only, to which an appropriate concentration of rHu-G-CSF was added. Drugs were added to each experiment as appropriate to the described conditions. The cells were harvested every 72 to 96 hours depending on the cycle length in each experiment, washed in PBS/2% FCS, and aliquots were removed for performing a cell count and FACS analysis. The remaining cells were re-set up in culture according to the conditions described in each experiment and drug was added as appropriate. At the end of the final cycle of each experiment, as well as performing a cell count and FACS analysis, an aliquot of cells was prepared for FISH to determine if the cells remaining after culture were BCR-ABL^+.

2.3.1.10 Long-term culture-initiating cell assay (LTC-iC)

Primitive haemopoietic cells with proliferative potential can be maintained in culture for extended periods of time, typically several months. These culture conditions have been called long-term bone marrow culture (LTBMC; (Coulombel et al., 1983a). Briefly, LTBMC requires the formation of a supportive stromal layer which supplies the necessary microenvironment to allow the primitive haemopoietic cells to proliferate over time. An application of LTBMC is an assay that measures the number of LTC-IC (Sutherland et al., 1991; Hogge et al., 1996). In this assay, the cells of interest are overlayed on pre-established, irradiated stromal layers. After 5 weeks culture the contents of each plate are set up in a committed progenitor assay for a further two weeks. At the end of this time, the

number of colonies formed is counted and this allows the frequency of LTC-IC to be determined. **Figure 2-3** shows a simplified schematic diagram for the method used in the LTC-IC assay.

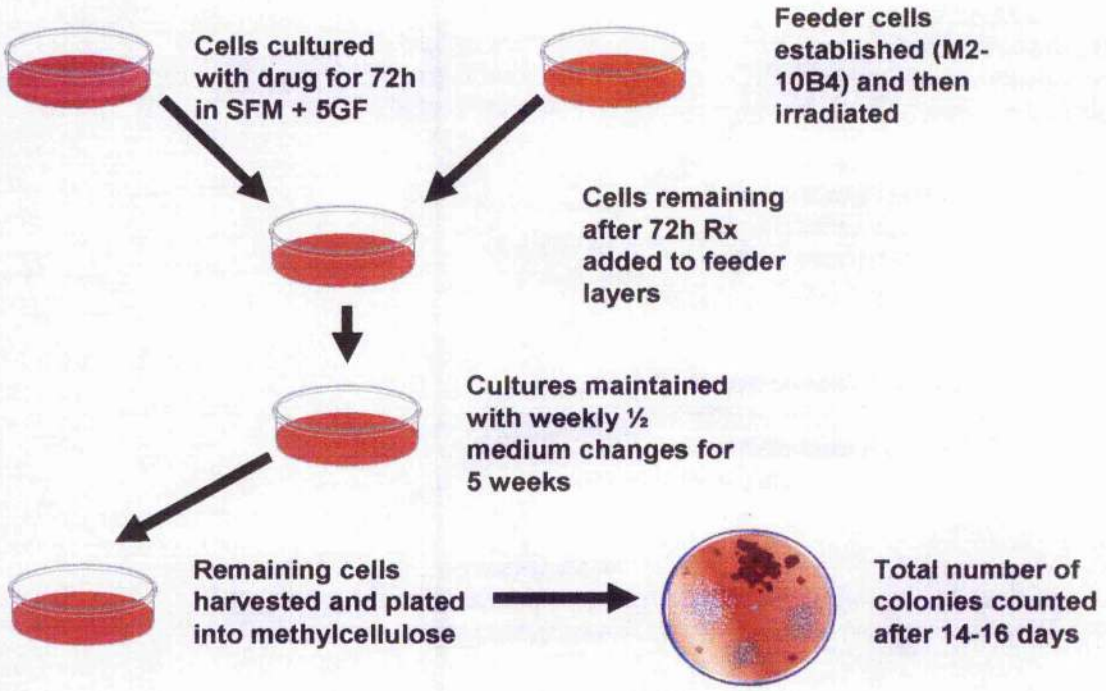


Figure 2-3. Simplified schematic diagram for the method used to assess LTC-IC after culture of $CD34^+$ cells in different conditions.

Two genetically-modified murine fibroblast cell lines, M2-10B4 and S1/S1 fibroblasts (both kindly provided by the Terry Fox Laboratories, Vancouver, BC, Canada) were used to provide the stromal support necessary for the LTC-IC. The M2-10B4 cells have been genetically modified to express G-CSF and IL-3, and S1/S1 fibroblasts SCF and IL-3. After thawing, these cell lines were maintained in culture at 37°C, 5% CO_2. The cells were trypsinised and passaged when the monolayer was semi-confluent to allow propagation of sufficient cells for LTC-IC. To minimise the proliferation of untransduced wild-type cells, the cultures were fed on alternate weeks with hygromycin B (final concentration 62.5µg/mL for M2-10B4

and 125µg/mL for S1/S1 fibroblasts) and G418 (final concentration 400µg/mL for M2-10B4 and 800µg/mL for S1/S1 fibroblasts).

Before the stromal layers were seeded with the test cells, it was necessary to irradiate the stromal cells to render then incapable of proliferation. After irradiation, equal numbers of the stromal cells (M2-10B4 and S1/S1 fibroblasts) were resuspended in Myelocult™ myeloid long-term culture medium and then inoculated onto Collagen Type 1 coated 6-well microtitre plates to facilitate stromal adherence. Over the next few days, these cells formed a monolayer onto which the test cells could be inoculated.

Prior to irradiation, the stromal layers were trypsinised and counted. A total of $1.5x10^5$ M2-10B4 and $1.5x10^5$ S1/S1 fibroblasts were required for each test well. The stromal cell layers were then irradiated at 80Gy. Following this, the M2-10B4 cells and S1/S1 fibroblasts were mixed and resuspended in Myelocult™ supplemented with hydrocortisone, to give a final cell concentration of $3x10^5$/mL. Two millilitres of this stromal cell suspension was then aliquoted into the wells of the Type 1 Collagen coated 6-well plate. The plates were then incubated at 37°C, 5% CO_2. After the stromal layers had been incubated for 24 to 48 hours, the test cells, which were $CD34^+$ CML cells treated under different drug conditions, were inoculated onto the stromal layers. Briefly, equal numbers of $CD34^+$ CML cells were treated under the treatment condition described in **Section 5.1.4**. After 72 hours culture, the remaining cells were washed in PBS/2% FCS and then resuspended in 310µL of PBS/2% FCS. Duplicate LTC-ICs were then set up with 5, 25 and 125µL of treated $CD34^+$ cell suspension. The cells were then incubated for 5 weeks at 37°C, 5% CO_2. Every week, 1mL of Myelocult™ medium was removed from each well and 1mL of fresh Myelocult™ medium was added and this constituted a half medium change.

At the end of 5 weeks culture, the LTC-IC were harvested and set up in committed progenitor assays. For each LTC-IC culture well, the culture supernatant (containing non-adherent cells) was pipetted into a sterile 15mL centrifuge tube (harvest tube). Two millilitres of HBSS-CMF was added to remove any serum-containing Myelocult™ medium and the plate swirled gently before the HBSS-CMF was transferred to the harvest tube. One millilitre of trypsin-EDTA was then added to each well and swirled gently at intervals until all the adherent cells had detached (up to 5 minutes). Detachment was facilitated by repeatedly pipetting the trypsin-EDTA over the surface of the well and this also helped generate a single cell suspension. The supernatant was transferred to the harvest tube. Immediately, 2mL of IMDM/2% FCS was added to the LTC-IC well and swirled gently and then transferred to the harvest tube. A further 2mL of HBSS-CML was added to the well and swirled gently before being transferred to the harvest tube. The harvest tubes were then centrifuged at 1000RPM for 10 minutes. Following this, the supernatant was gently poured off and the cells resuspended in the remaining supernatant. The volume of remaining cell suspension was recorded and a cell count performed.

Duplicate committed progenitor assays were set up for each cell volume of each treatment condition at two different cell concentrations ($1x10^4$ and $2.5x10^4$ cells/mL). The appropriate volume of cell suspension for duplicate wells was added to 2mL of Methocult™ medium for committed progenitor assays. The cell suspension and Methocult™ medium were thoroughly mixed and 1mL of this was aliquoted into a 35mm culture dish and then gently tapped so that the bottom of the dish was completely coated. The culture dishes were then incubated for 14 days at 37°C, 5% CO_2 and, at the end of this time, the number of viable colonies was counted in each dish and this allowed a comparison of the LTC-IC present in the different treatment conditions.

2.3.1.11 Tritiated (^{3}H) thymidine proliferation assays

Tritiated (^{3}H) thymidine proliferation assays were performed to determine the IC_{50} for the different small molecule inhibitors in cell lines and $CD34^+$ CML cells. These experiments were performed in 96 well plates and each well contained 90µL of cell suspension and 10µL of drug (final volume 100µL). There were five replicates of each drug concentration per experiment. Cell lines were seeded at a cell concentration of $2x10^5$ cells/mL in $RPMI^{++}$ for K562 and HL60 cells and RPMI/WEHI medium for the Ba/F3 cell lines. $CD34^+$ cells were seeded at a cell concentration of $5x10^5$ cells/mL in SFM only. Cell lines were cultured for 24 or 48 hours and $CD34^+$ cells for 72 hours prior to assessment of proliferation. Tritiated thymidine (37MBq per mL) was diluted 1:100 in medium and 20µL was added to each experimental condition 5 hours prior to reading the plate in the cell line experiments and 16 hours prior to reading the plate in the $CD34^+$ cell experiments. Beta counting was performed according to the manufacturers' instructions. Briefly, the cells were harvested using a FilterMate Harvester (Perkin Elmer, Turku, Finland) onto a filter mat, wax scintillant was melted onto the filter mat ready for beta counting using the Microbeta TriLux (Perkin Elmer).

2.3.1.12 Dual-colour fluorescence *in situ* hybridisation (FISH)

Chronic myeloid leukaemia cells, pre- and post-culture, and colonies from LTC-IC experiments were assessed for the presence of BCR-ABL by FISH. Aliquots of at least 5000 cells were required. The CML cells or pooled colonies were firstly put into a 5mL FACS tube and washed in PBS/2%FCS. The cells were then resuspended in 500µL of hypotonic solution (0.075M potassium chloride), transferred to a 1.5mL eppendorf and incubated at room temperature for 20 minutes. One hundred microlitres of freshly made fixative was added

(methanol:acetic acid [3:1]) and the cells incubated for a further 5 minutes at room temperature. The cells were then centrifuged at 5000RPM for 5 minutes, the supernatant removed and a further 500µL fixative added, the cells incubated for a further 5 minutes and then centrifuged for 5 minutes. This step was repeated a further twice. The cell pellet was finally resuspended in 50µL of fixative and then stored at -20°C prior to further preparation for FISH.

Aliquots of 20µL of cell solution were placed in wells of a poly-L-lysine coated multispot microscope slide and air-dried overnight. If the cells had been stained with CFSE, this was bleached under a bright light prior to performing FISH. The prepared slides were stored at -20°C wrapped in parafilm until FISH was performed according to the manufacturers' instructions using the LS1 BCR-ABL Dual Fusion probe. Interphase nuclei were evaluated using a Leica fluorescent microscope with a triple-band pass filter for DAPI, Spectrum Orange and Spectrum Green. All FISH slides were prepared and scored by Mrs Elaine Allan.

2.3.2 Flow cytometry

Flow cytometry also called FACS is used to sort cells according to their fluorescence. A flow cytometer consists of one or more lasers for supplying excitation energy and a series of filters and detectors for measuring the resultant fluorescent emissions. A flow cytometer can also measure the size of a cell using forward-angle light scatter (FSC) and the granularity of a cell using side-angle light scatter (SSC). Thus, flow cytometry allows the characterisation of individual cells using fluorochrome-labelled antibodies.

2.3.2.1 Surface antibody staining

All antibodies were titrated so that the minimum concentration which gave reliable staining and clear separation of different cell populations was used. This was

always less than the amount recommended by the manufacturer, thus reducing cost and facilitating compensation when using multiple channel flow cytometry in experiments. Prior to surface antibody staining, the cells were washed in PBS/2% FCS. The cells were then resuspended in 200µL PBS/2% FCS and the appropriate volume of antibody was used. The cells were then incubated in the dark for 15 minutes and washed twice in PBS/2% FCS (1200RPM for 5 minutes). If PI (1µg/mL) was being used to exclude dead cells, 1µL of this was added with the final wash. The cells were then analysed by FACS immediately. Appropriate isotype controls were included for all experiments. All FACS analysis was performed using the FACSCalibur flow cytometer (Becton Dickinson) with compensation set as required using log-linear scale.

2.3.2.2 Intracellular antibody staining

For cells requiring both surface and intracellular antibody staining, the surface staining was performed first. Propidium iodide could not be used in association with intracellular antibody staining. After surface staining, if appropriate, at least $1x10^5$ cells were resuspended in 100µL of Fix and Perm® Medium A (fixation medium) and incubated for 15 minutes (in the dark if also surface antibody stained). The cells were then washed with PBS, the supernatant completely removed using a graduated pipette and the cell pellet resuspended in 50µL Fix and Perm® Medium B (permeabilisation medium). To this the appropriate volume of intracellular antibody was added (Bcl-2-FITC, p-Src Alexa Fluor® 488 or anti-active-caspase-PE) and the cells incubated for 45 minutes at room temperature in the dark. Finally the cells were washed twice in PBS (1200RPM for 5 minutes) prior to FACS analysis.

Intracellular staining for CrKL phosphorylation was performed as above with the following modifications (Hamilton et al., 2006). All washes were performed using PBS/BSA/azide. After fixing with 100µL of Fix and Perm® Medium A, the cells were permeabilised with 25µL of Fix and Perm® Medium B, to which 2.5µL of p-CrKL antibody was added and the cell/antibody solution mixed by vortexing. The cells were then incubated at room temperature for 40 minutes. The cells were then washed twice and resuspended in 100µL PBS/BSA/azide to which 2µL of secondary antibody (anti-rabbit IgG-FITC conjugate) was added. The cells were then vortexed and incubated for a further 30 minutes at room temperature in the dark, washed twice and then analysed immediately by flow cytometry using the FACSCalibur.

The amount of p-CrKL in an untreated CML sample was assessed as the geometric mean fluorescence intensity (MFI) of the untreated CML sample minus the geometric MFI for P-CrKL of normal $CD34^+$ cells (negative control). The p-CrKL status of drug treated samples was expressed as a percentage of the untreated control (100%). To compare the amount of p-SRC or BCL-2 in a CML versus normal cells at baseline, the following calculation was used:

p-SRC or BCL-2 = ([CML MFI - isotype MFI] / [normal MFI – isotype MFI])*100%

As with p-CrKL, the amount of p-SRC or BCL-2 in drug treated samples was expressed as a percentage of the untreated control (100%).

2.3.2.3 High resolution cell cycle analysis

Different culture and treatment conditions may alter progression of cells through the cell cycle. Therefore, it was important to determine the effects of the different treatment conditions on progression of CML cells though the cell cycle. High

resolution cell cycle analysis was performed using the method described by Jordan *et al* (Jordan et al., 1996) which combines staining using the nuclear activation antigen Ki-67 (FITC) with 7-AAD staining of DNA. This method allows discrimination between G_0, G_1 and $S/G_2/M$.

Approximately $1x10^6$ cells were washed in PBS (1200RPM for 5 minutes), the supernatant discarded and then resuspended in 500µL of PBS/0.4% formaldehyde for fixing and incubated for 30 minutes on ice. Following this, 500µL of PBS/0.2% Triton-X-100 was added to permeabilise the cells and the cells were then incubated overnight at 4°C. The following morning, the cells were washed once in PBS and then resuspended in 1mL PBS. The cell suspension was then divided equally between two FACS tubes (500µL each) and either 20µL of Ki-67 FITC-labelled antibody or 20µL of FITC isotype control was added to the tubes. The cells were then vortexed and incubated for 40 minutes at room temperature in the dark. After one further wash, the cells were resuspended in 1mL of PBS and 5µL of 7-AAD (1µg/mL) was added and then incubated for at least 6 hours, but preferably overnight, at 4°C prior to FACS analysis. Before FACS analysis, the cells were washed once in PBS. Log-linear analysis was used in FL1 (Ki-67-FITC) and linear analysis on FL3 (7-AAD). To separate cells from debris, the cell population was gated on using FSC versus FL3. This gated cell population was then analysed in FL1 versus FL3 and this allowed calculation of the relative percentages of cells at each stage of the cell cycle. A minimum of 10,000 cell events were collected.

2.3.2.4 FACS for CFSE experiments

Carboxy-fluorescein diacetate succinimidyl diester stained cells were surface stained as described in **Section 2.3.2.1**. Flow cytometry analysis of CFSE-stained

cells was performed at baseline to determine the position of the undivided peak and confirm uniform staining of the cell population (which allowed resolution of the peaks at subsequent analyses), and again at the time points stated for each set of experiments. Isotype controls were used to correctly set the detectors so that the negative isotype population was placed in the first log decade for each flow cytometry channel. Propidium iodide staining in FL3 was used to identify the viable cell population. CFSE and CD34 positive controls were then run and the compensation adjusted. Because CFSE is a very bright fluorescent stain which has substantial spectral overlap into other FACS channels, considerable levels of compensation were required. To collect an adequate undivided $CFSE^{max}$ $CD34^{+}$ cell population in each experimental condition, a minimum of 50,000 (and preferably 100,000) cell events were collected.

Carboxy-fluorescein diacetate succinimidyl diester was also successfully combined with anti-active caspase-3-PE and CD34-APC to allow assessment of apoptosis in the undivided $CD34^{+}$ cell population. Surface and intracellular antibody staining were carried out as described (**Sections 2.3.2.1** and **2.3.2.2**, respectively). As the cells were fixed and permeabilised, PI could not be used, and the viable cell population was gated on FSC versus SSC to exclude debris and cell aggregates.

2.3.2.5 Calculation of the undivided ($CFSE^{max}$) cell population

To determine the anti-proliferative effect of different treatment conditions and assess the size of the non-proliferating primitive progenitor population, the percentage recovery of viable $CD34^{+}$ cells in the undivided population remaining after culture was assessed. At each analysis time point, the number of viable cells harvested from each culture condition was recorded, as was the percentage of

$CD34^+$ cells found in the undivided fraction ($CFSE^{max}$ $CD34^+$). Percentage recovery of input cells in the undivided peak could then be calculated by dividing the absolute number of $CFSE^{max}$ $CD34^+$ cells by the total number of input $CD34^+$ cells and multiplying by 100%. This allowed direct comparison of different treatment conditions on the non-proliferating primitive progenitor population.

2.3.3 Western blotting

This protein detection technique, which is also known as immunoblotting, is used to identify specific proteins. A specific protein can be identified after fractionation on either one or two-dimensional gels, by exposing all the proteins present to a specific antibody coupled to an easily detectable enzyme such as horseradish peroxidase, a radioactive isotope or a fluorescent dye. This is done after the proteins separated on the gel have been transferred or 'blotted' onto a sheet of nitrocellulose paper as this is more robust than the gel.

2.3.3.1 Preparation of protein lysate

The lysis buffer (**Section 2.2.4.1**) was prepared immediately prior to use. Equal cell numbers from different treatment condition were washed twice with ice cold PBS (1200RPM for 5 minutes). The cells were then transferred to a 1.5mL eppendorf and washed again in ice cold PBS (5000RPM for 5 minutes in a bench-top micro-centrifuge). The lysis buffer was added to the cells (1-2x10^5 cells/50µL lysis buffer), mixed by pipetting up and down and incubated for 15 minutes on ice. Following this, the cells were gently vortexed and then spun at 14000RPM for 10 minutes at 4°C to clarify the supernatant which was then saved as a detergent lysate and stored at -80°C until use.

2.3.3.2 Protein quantitation

Protein quantification was performed using the bicinchoninic acid (BCA) method for colorimetric detection and quantification of total protein according to the manufacturers' instructions. This method utilises the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium (the biuret reaction) with the colorimetric detection of the cuprous cation (Cu^{1+}) using a reagent containing BCA (Smith et al., 1985). The purple reaction product of this assay is formed by chelation of one cuprous ion with two molecules of BCA. The water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear over a broad range of protein concentrations (20-2,000µg/mL).

Firstly the BSA standards were prepared. BSA was dissolved in 0.9% saline to give a concentration of 2mg/mL. Serial dilutions of this stock were then made to produce a concentration gradient for the controls. Controls of the following concentrations were prepared: 1500; 1000; 750; 500; 250; 125; 50; 25; 5µg/mL; and a blank. These controls could be stored at -20°C for use on multiple occasions. To prepare the assay, 25µL of each control was pipetted into the well of a 96-well plate in duplicate. Protein lysate (2.5µL) was added to 22.5µL of PBS in duplicate test wells. BCA solutions A and B were combined (50A:1B) and 200µL of BCA solution A plus B was added to each well. The 96-well plate was then incubated at 37°C for 25 minutes and then the plate was read using an ELISA plate reader according to the manufacturers' instructions. This allowed accurate protein quantification so that equal amounts of protein could be used for Western blotting.

2.3.3.3 Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (SDS-PAGE)

This technique has revolutionised protein analysis. It uses a highly cross-linked gel as the inert matrix through which the proteins migrate. The proteins are in a solution which includes sodium dodecyl sulphate (SDS), a negatively charged detergent which binds to hydrophobic regions of the protein molecules, causing them to unfold into long polypeptide chains and become freely soluble in the solution. A reducing agent such as β-mercaptoethanol is also added to break any disulphide bonds present in the proteins, so that all the constituent polypeptides can be analysed separately. Each protein molecule binds many of the negatively charged detergent molecules. This masks the protein's inherent positive charge, resulting in migration of the protein towards the positive electrode when a voltage is applied. The speed of migration through the gel is dependent on the size of the polypeptide, with smaller polypeptides travelling more rapidly through the gel. This technique can be used to determine the approximate molecular weight of a polypeptide as well as the subunit composition of a protein.

To perform SDS-PAGE, equal volumes of protein lysate and Laemmli 2X sample buffer were mixed together in a 1.5mL eppendorf and then heated to 95°C for 5 minutes. The samples were then loaded onto a 4 to 15% gradient gel. Ten microlitres of rainbow ladder marker was loaded onto a lane of the gel to allow determination of protein size. The gel was electrophoresed in 1X TGS running buffer for 90 minutes at 130V using the Bio-rad Mini-PROTEAN^{TM} electrophoresis system.

2.3.3.4 Transfer to nitrocellulose membrane

The protein bands were then transferred from the gel to nitrocellulose membrane. Sponges, blotting paper and nitrocellulose membrane were soaked in transfer buffer. The gel was then removed from its holder, soaked in transfer buffer and the transfer sandwich was prepared as shown in **Figure 2-4** for wet transfer.

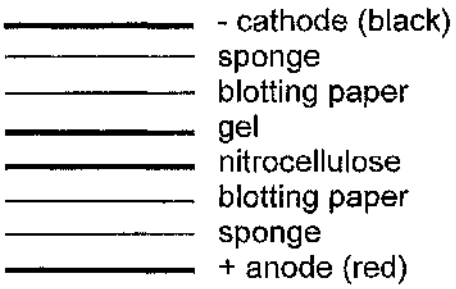


Figure 2-4. Preparation of sandwich for wet transfer of protein from gel to nitrocellulose membrane.

The sandwich then underwent electrophoresis for 1 hour at room temperature at 80V.

2.3.3.5 Immunolabelling

After transfer, the nitrocellulose membrane was stained with 0.1% Ponceau S for 30 to 60 seconds to assess protein transfer. The membrane was then de-stained with distilled water and washed in PBS-T for 5 minutes. Following this, the membrane was blocked in 5% non-fat dry milk in PBS-T at room temperature for 2 to 4 hours and then the blocking solution was discarded and the membrane incubated with the primary antibody solution (H-Ras, Mcl-1 or Actin in PBS-T/5% BSA) overnight at 4°C with gentle agitation. The H-Ras antibody was diluted 1:200, and the Mcl-1 and actin antibodies 1:1000 in PBS-T for immunolabelling. The following morning, the nitrocellulose membrane was washed 4 times (15 minutes per wash) in PBS-T and then incubated with horseradish peroxidase-

conjugated secondary antibody (anti-rabbit HRP 1:3000) in blocking solution for 1 hour at room temperature with gentle agitation. The membrane was again washed 4 times (15 minutes per wash) and then developed with the ECL plus™ kit according to the manufacturers' instructions. Briefly, a solution of ECL plus™ A and B (40A:1B) was prepared, and the membrane was soaked in this for 5 minutes. Excess ECL plus™ solution was then removed and the nitrocellulose membrane placed in an X-ray box for exposure onto Hyperfilm™ ECL™. Films were exposed for increasing lengths of time until satisfactory bands were seen. The films were developed using the Kodak X-omat™.

2.3.3.6 Stripping and re-blotting

The Re-Blot™ Plus Strong stripping solution was diluted 1:10 with distilled water. The nitrocellulose membrane was incubated in the stripping solution for 10 to 15 minutes and then washed twice in PBS-T for 10 minutes. The membrane was then re-blocked for 1 hour, prior to being incubated with a different primary antibody. This method allowed stripping and re-blotting of membranes 3 to 4 times.

2.3.4 Statistics

All statistical analyses were performed using the Student's T-test on the Minitab statistics software package. A level of $P \leq 0.05$ was taken to be statistically significant.

3 Results 1

3.1 Priming $CD34^+$ CML progenitor cells with rHu-G-CSF improves their eradication by IM *in vitro*

3.1.1 Introduction

In vitro studies have demonstrated that IM acts by inhibiting the growth of primitive CML progenitors through reversal of abnormally increased proliferation (Holtz et al., 2002). However, these growth arrested (quiescent) CML cells are not eradicated by IM *in vitro* (Graham et al., 2002; Bhatia et al., 2003) or *in vivo* as demonstrated by persistent detection of low level BCR-ABL transcripts by qRT-PCR (Hughes et al., 2003; Branford et al., 2004). In addition, recent case reports (Cortes et al., 2004b; Mauro et al., 2004) show that some patients who achieved a molecular remission on IM, relapsed rapidly after IM was stopped, but responded to re-institution of IM therapy. This disease behaviour is suggestive of an IM-insensitive LSC compartment.

In view of these observations, we decided to look for a treatment strategy which would promote re-entry of these quiescent CML progenitor cells back into the cell cycle, rendering them susceptible to eradication by IM. Two potentially successful approaches for eliminating the quiescent CML stem cell population could involve (1) interruption of IM therapy - an IM 'drug holiday'; or (2) priming with rHu-G-CSF. These therapeutic approaches could also be combined *in vitro* and *in vivo*. It is proposed that interruption of IM therapy would reverse the observed anti-proliferative effect of IM at the G_0/G_1 boundary, allowing these cells to re-enter the cell cycle and priming with rHu-G-CSF would promote re-entry of quiescent CML stem cells into the cell cycle, rendering them sensitive to IM.

Therefore, the aims of this part of the study were two fold. Firstly, to determine if interrupted (pulsed) IM therapy was more effective than continuous IM at eradicating CML stem cells *in vitro*; i.e. would an 'IM holiday' bring cells back into cycle? Secondly to assess if priming with rHu-G-CSF alone or in addition to interrupted or continuous IM therapy could improve eradication of CML stem cells *in vitro*; i.e. could exogenous G-CSF induce cell cycle activation?

3.1.2 Assessment of G-CSF-R protein expression in CD34$^+$ CML progenitor cells

Our collaborator, Dr Xiaoyan Jiang, had shown that *G-CSF-R* gene expression was up-regulated in the cycling (G_1/S/G_2/M) compared to the quiescent (G_0) fraction of both CML and normal CD34$^+$ progenitor cells by qRT-PCR. Furthermore, *G-CSF-R* gene expression was also significantly higher in both the quiescent (~2 fold) and cycling (~4 fold) fractions of CD34$^+$ CML progenitors compared to normal CD34$^+$ cells ($P<0.02$) (Jorgensen et al., 2006). Therefore, the aims of this experiment were to determine if G-CSF-R protein expression was increased in CML versus normal CD34$^+$ progenitor cells and if this expression was further enhanced by the addition of rHu-G-CSF.

Flow cytometry was used to assess G-CSF-R protein expression in CML (n=2) and normal (n=2) CD34$^+$ progenitor cells at 0, 24, 48, and 72 hours culture in SFM in the presence or absence of rHu-G-CSF at a concentration of 20 ng/mL (**Figure 3-1**). At baseline (0 hours), both CML and normal CD34$^+$ cells had low levels of G-CSF-R expression (~1% of cells G-CSF-R positive). After 24 hours culture, G-CSF-R protein expression had started to increase in the CD34$^+$ CML cells, reaching a peak (~12%) at 48 hours and still maintained at a high level at 72 hours culture (**Figure 3-2**). The addition of rHu-G-CSF did not affect G-CSF-R protein expression in these cells. In normal CD34$^+$ cells, G-CSF-R protein expression

remained at a low level (< 2%) at all time points in the presence or absence of rHu-G-CSF.

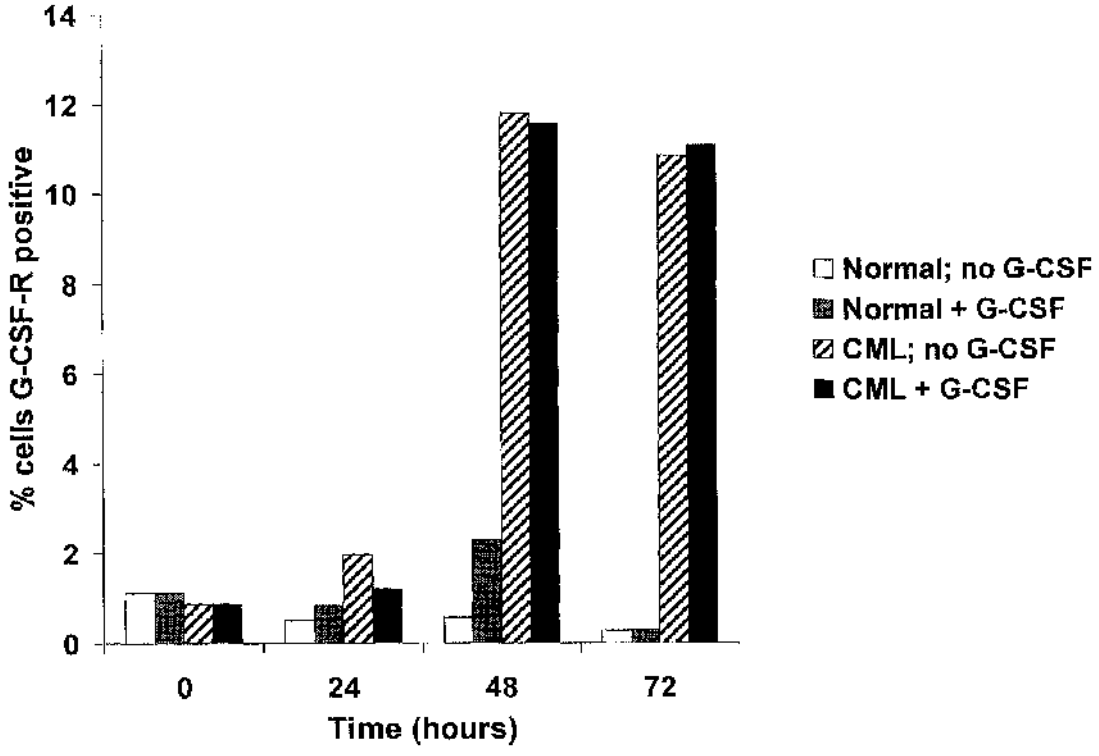


Figure 3-1. G-CSF-R protein expression (percentage of total cells G-CSF-R positive) in CML and normal CD34$^+$ cells after 0, 24, 48 and 72 hours culture in the presence or absence of rHu-G-CSF.

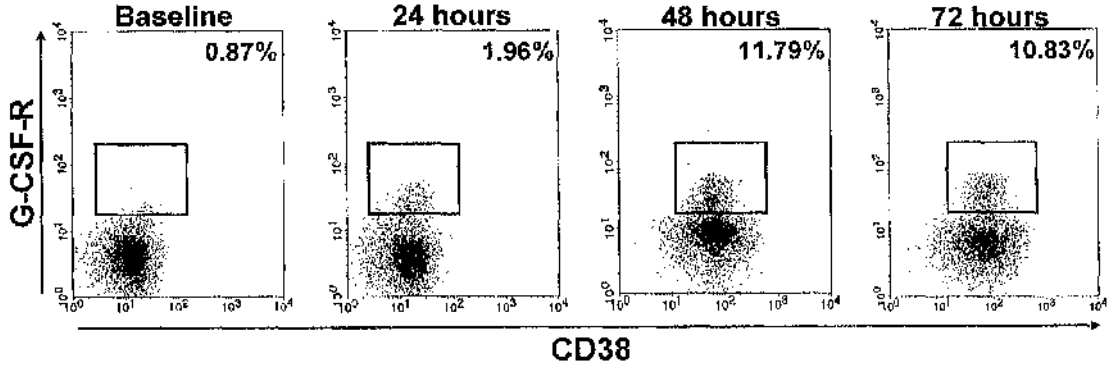


Figure 3-2. Representative FACS dot plots of G-CSF-R expression in CD34$^+$ CML cells at baseline (0 hours), 24, 48 and 72 hours culture in SFM only.

Therefore, G-CSF-R protein expression was low in both CML and normal $CD34^+$ cells immediately after thawing (time = 0 hours; baseline) and increased rapidly in the CML $CD34^+$ cells in culture. This indicates that the high *G-CSF-R* transcript levels seen in the cycling CML $CD34^+$ cells does translate into increased G-CSF-R protein expression. To confirm that the cell cycle profiles were similar in the CML and normal $CD34^+$ cells, Ki-67/7-AAD high resolution cell cycle analysis was performed on CML and normal $CD34^+$ cells after 72 hours culture in rHu-G-CSF (20 ng/mL). This verified that the ratio of cycling to quiescent cells was comparable in CML and normal $CD34^+$ cells (**Figure 3-3**). Thus, higher G-CSF-R expression in the CML cells was not due to a different cell cycle profile, with more cycling cells.

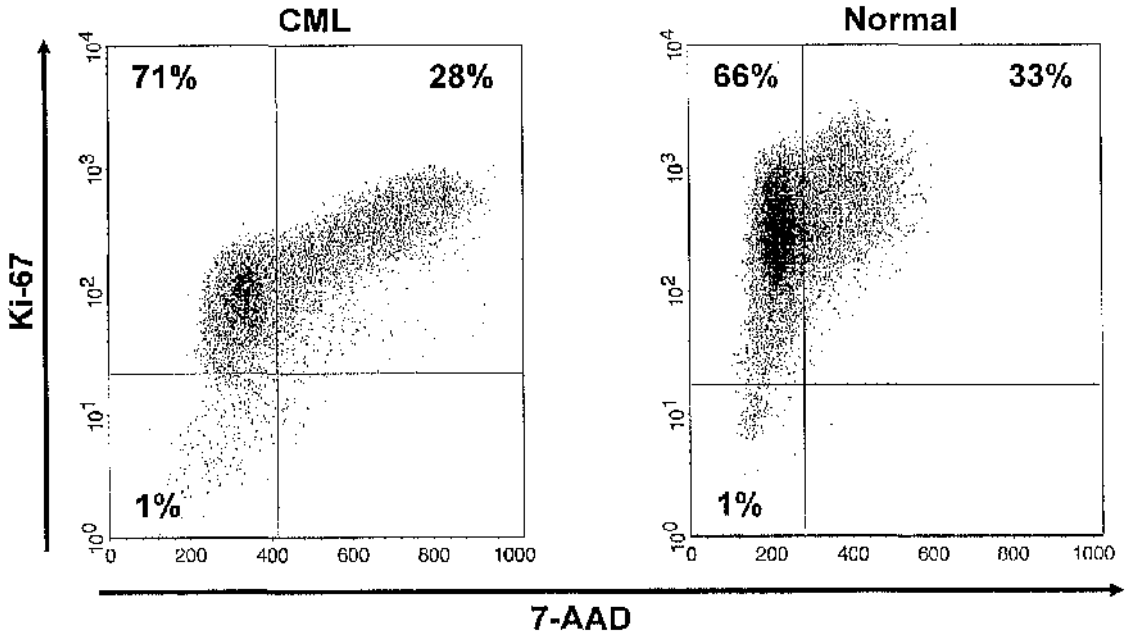


Figure 3-3. Representative FACS dot plots of Ki-67/7-AAD high resolution cell cycle analysis. These confirm that there was no difference in cell cycle status between CML and normal $CD34^+$ cells after 72 hours culture in the presence of rHu-G-CSF (20 ng/mL). The difference in appearance of the CML and normal FACS dot plots is for two reasons: firstly, the two samples were analysed on separate days and the FACSCalibur settings would have been different as the voltages and compensation were set with fresh control samples each time the FACSCalibur was used; and secondly, the CML sample was stained overnight with 7-AAD compared to the normal sample which was only stained for 6 hours.

Next, we needed to determine if the G-CSF-Rs present on the $CD34^+$ CML cells were functional. This was done by determining the concentration-dependent ability of rHu-G-CSF to enhance the growth of $CD34^+$ CML progenitor cells *in vitro*.

3.1.3 Assessment of the proliferative response of $CD34^+$ CML progenitor cells to rHu-G-CSF in vitro

This experiment set out to assess the proliferative response of $CD34^+$ CML cells to rHu-G-CSF and establish the optimum rHu-G-CSF concentration for further experiments. $CD34^+$ CML cells (n=2) were cultured for 6 days in medium containing a range of rHu-G-CSF concentrations (0-200ng/mL) or with 5GF (**Figure 3-4**). In the absence of any exogenous growth factors, the total number of cells increased in the first 2 days (~3-fold) and then remained constant for the next 4 days indicating a constant rate of cell production which balanced but did not exceed cell death, as expected from the known autocrine IL-3/G-CSF phenotype of these cells (Jiang et al., 1999; Jiang et al., 2000b). The addition of rHu-G-CSF did not significantly further enhance cell expansion during the first 2 days, although the addition of either rHu-G-CSF 200 ng/mL or 5GF showed a trend towards increased total viable cells on day 2. However, over the remaining 4 days, the addition of rHu-G-CSF stimulated an increase in total viable cells. At concentrations of 2 and 10 ng/mL, rHu-G-CSF produced a sub-optimal proliferative response. However, rHu-G-CSF concentrations of 20 to 200 ng/mL produced equivalent proliferative responses in $CD34^+$ CML cells, with no benefit gained from rHu-G-CSF concentrations above 20 ng/mL. A further increase in cell output could be obtained by using 5GF, a growth factor combination optimised to stimulate primitive normal haemopoietic cells (Petzer et al., 1996b; Nordon et al., 1997). Based on these results, a rHu-G-CSF concentration of 20 ng/mL was chosen for all subsequent experiments in this section.

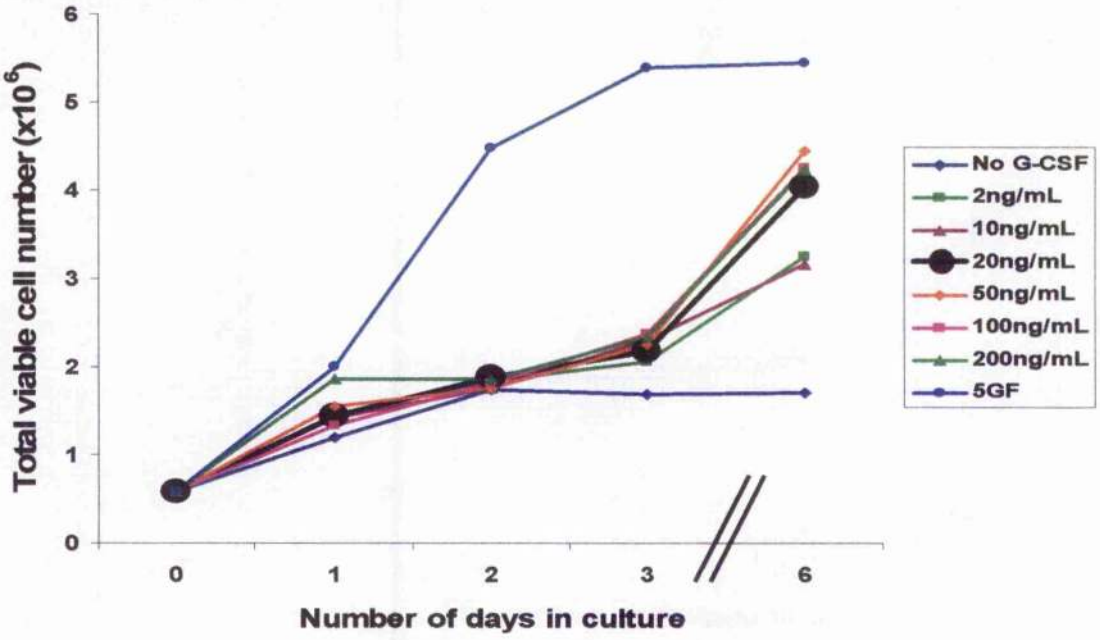


Figure 3-4. Proliferation of $CD34^+$ CML cells *in vitro* in response to different concentrations of rHu-G-CSF. Each well was seeded with 5 x 10^5 $CD34^+$ CML cells in SFM supplemented with 0, 2, 20 or 200 ng/mL rHu-G-CSF or 5GF. Total viable cell numbers were determined after 1, 2, 3 and 6 days. The rHu-G-CSF concentration of 20 ng/mL, chosen for subsequent experiments, has been highlighted (black line with circles).

3.1.4 Determination of total viable cell counts after rHu-G-CSF priming in combination with continuous or interrupted IM therapy

In this series of experiments (n=6), the effect of giving continuous, intermittent or no rHu-G-CSF (20 ng/mL) in combination with continuous, interrupted or no IM on total viable cell numbers was assessed in $CD34^+$ CML cells. The IM concentration used was 5 μM which is clinically achievable with standard doses of IM (Peng et al., 2004). Each experiment consisted of 3 cycles of 96 hours. Intermittent rHu-G-CSF was given for the first 24 hours of each 96 hour cycle and interrupted IM therapy was given for the last 72 hours of each 96 hour cycle. To account for all possible combinations of rHu-G-CSF with IM, there were 9 experimental arms (**Figure 3-5**): (1) no rHu-G-CSF, no IM; (2) no rHu-G-CSF, IM 72 hours; (3) no

rHu-G-CSF, IM 96 hours; (4) rHu-G-CSF 24 hours, no IM; (5) rHu-G-CSF 24 hours, IM 72 hours; (6) rHu-G-CSF 24 hours, IM 96 hours; (7) rHu-G-CSF 96 hours, no IM; (8) rHu-G-CSF 96 hours, IM 72 hours; and (9) rHu-G-CSF 96 hours, IM 96 hours. So that all conditions were treated equally, after the first 24 hours of each cycle, all experimental arms where washed three times in PBS and then re-set up ± IM and ± rHu-G-CSF for the remaining 72 hours.

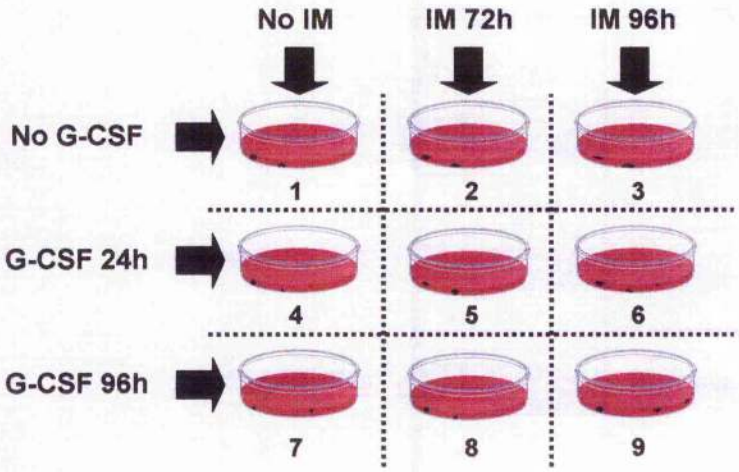


Figure 3-5. Schematic diagram of the nine possible combinations of rHu-G-CSF and IM used in the experimental protocol. G-CSF, rHu-G-CSF; h, hours.

Total viable cell counts were performed at the end of each 96 hour cycle (i.e. after 4, 8 and 12 days culture) and the results are shown in **Figure 3.6**. Taken as a group, the three experimental arms to which no IM was added showed the expected higher total viable cell counts relative to the IM containing arms ($P<0.001$ compared to both the IM 72 hours and IM 96 hours arms at the end of three treatment cycles). There were significantly fewer cells remaining after treatment with continuous IM (96 hours) compared to interrupted IM (72 hours; $P=0.038$) when no rHu-G-CSF was added. However, in the presence of either intermittent or continuous rHu-G-CSF, there was no significant difference between continuous or interrupted IM therapy.

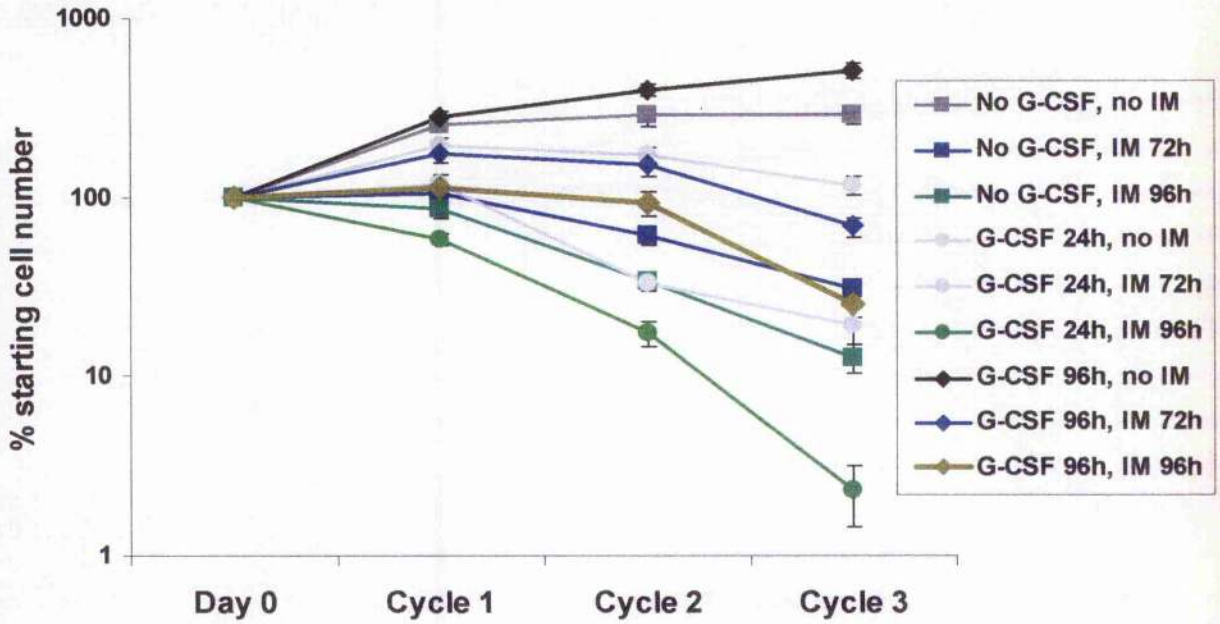
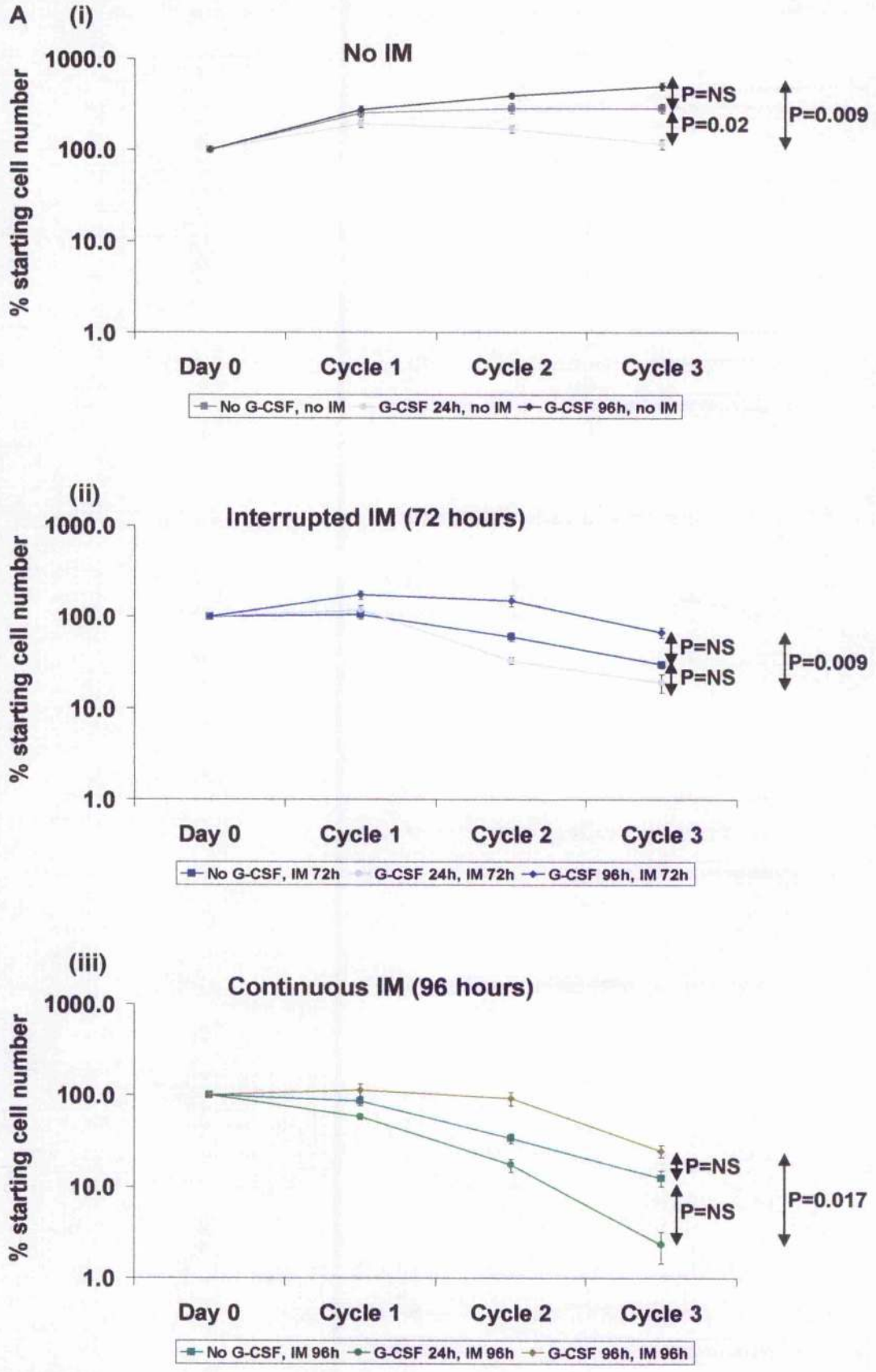


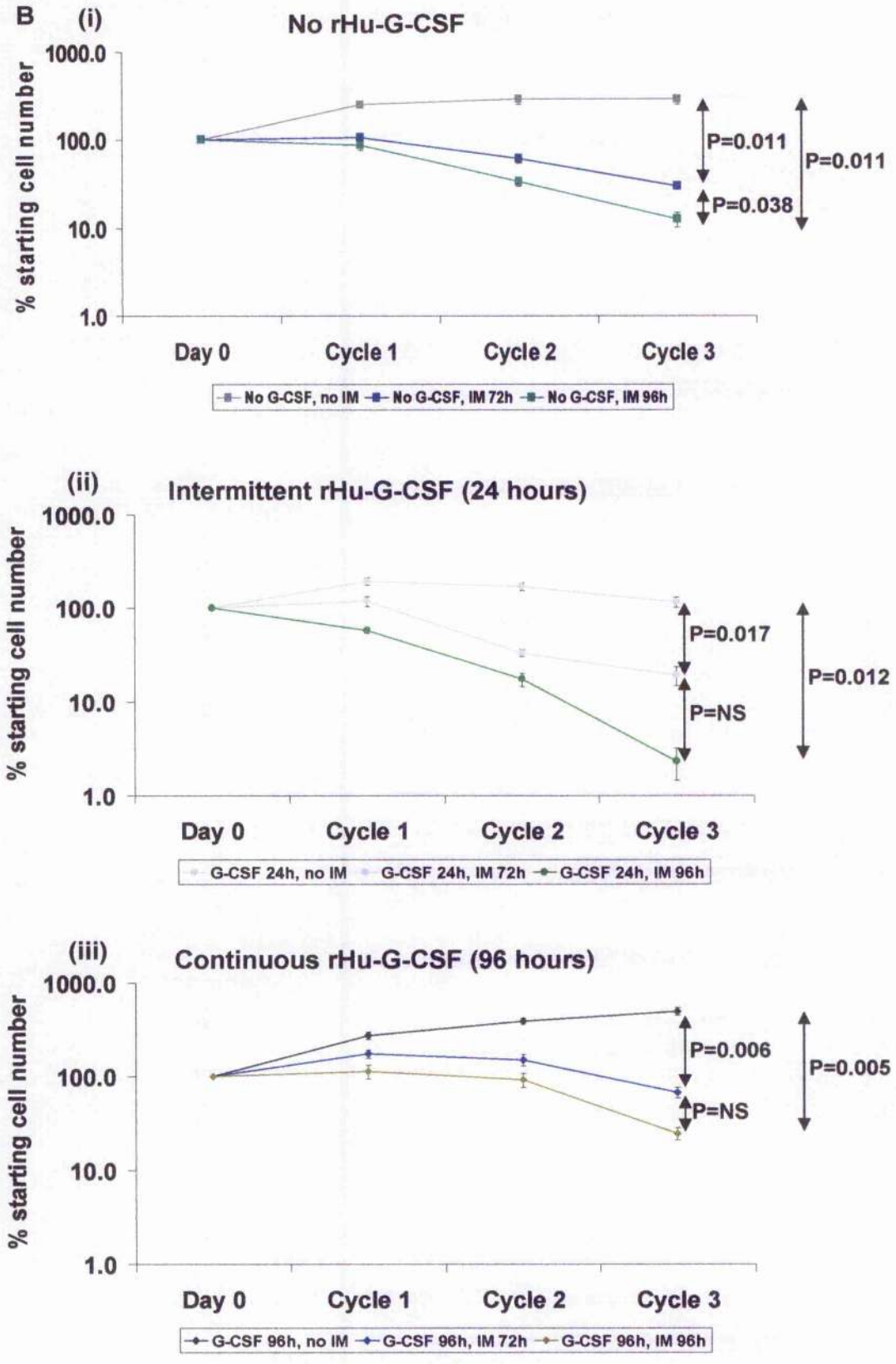
Figure 3-6. Total viable cell counts after each treatment cycle (4, 8 and 12 days) for all experimental conditions. Cells were cultured in SFM. Results are expressed as a percentage of the starting cell number (± SEM) which was the same for all experimental arms in an individual experiment. G-CSF, rHu-G-CSF.

As a group, the three arms to which continuous rHu-G-CSF (96 hours) was added had significantly more cells present at the end of three treatment cycles compared to the three arms containing either no rHu-G-CSF (P=0.036) or intermittent rHu-G-CSF (24 hours; P=0.007). Unexpectedly, the arms with no rHu-G-CSF had significantly more cells present than those to which intermittent rHu-G-CSF was added (P=0.019). The effect of IM on total cell numbers was consistently greater than that of rHu-G-CSF. This is because, even in the absence of rHu-G-CSF, $CD34^+$ CML cells produce G-CSF through an autocrine mechanism (Jiang et al., 1999), thus G-CSF is always present at a low level. Comparison of total viable cells in the individual arms is shown in **Figures 3-7A** and **3-7B** and demonstrates significant differences in total viable cells between the different treatment arms. Interestingly, the pattern of effect of IM (none, interrupted or continuous IM) on total cell numbers was similar regardless of the presence of rHu-G-CSF (none, intermittent or continuous) and vice versa. However, the effects were most marked

in the intermittent rHu-G-CSF arms with a reduction in total viable cells (relative to input) after 12 days (3 cycles) of ~5 fold and 50 fold in the interrupted and continuous IM arms, respectively.

Figure 3-7. Comparison of total viable cells (n=6) in the individual experimental arms together with P values. (A) Three plots comparing the three arms with (i) no IM, (ii) interrupted IM (iii) continuous IM. (B) Three plots comparing the three arms with (i) no rHu-G-CSF, (ii) intermittent rHu-G-CSF and (iii) continuous rHu-G-CSF. Results are expressed as a percentage of the starting cell number and represent the mean (± SEM). G-CSF, rHu-G-CSF; h, hours.


A (i)
No IM
% starting cell number
1000.0
100.0
10.0
1.0
P=NS
P=0.02
P=0.009
Day 0
Cycle 1
Cycle 2
Cycle 3
No G-CSF, no IM
G-CSF 24h, no IM
G-CSF 96h, no IM
(ii)
Interrupted IM (72 hours)
% starting cell number
1000.0
100.0
10.0
1.0
P=NS
P=NS
P=0.009
Day 0
Cycle 1
Cycle 2
Cycle 3
No G-CSF, IM 72h
G-CSF 24h, IM 72h
G-CSF 96h, IM 72h
(iii)
Continuous IM (96 hours)
% starting cell number
1000.0
100.0
10.0
1.0
P=NS
P=NS
P=0.017
Day 0
Cycle 1
Cycle 2
Cycle 3
No G-CSF, IM 96h
G-CSF 24h, IM 96h
G-CSF 96h, IM 96h


B
(i)
No rHu-G-CSF
% starting cell number
1000.0
100.0
10.0
1.0
P=0.011
P=0.038
P=0.011
Day 0
Cycle 1
Cycle 2
Cycle 3
No G-CSF, no IM
No G-CSF, IM 72h
No G-CSF, IM 96h
(ii)
Intermittent rHu-G-CSF (24 hours)
% starting cell number
1000.0
100.0
10.0
1.0
P=0.017
P=NS
P=0.012
Day 0
Cycle 1
Cycle 2
Cycle 3
G-CSF 24h, no IM
G-CSF 24h, IM 72h
G-CSF 24h, IM 96h
(iii)
Continuous rHu-G-CSF (96 hours)
% starting cell number
1000.0
100.0
10.0
1.0
P=0.006
P=NS
P=0.005
Day 0
Cycle 1
Cycle 2
Cycle 3
G-CSF 96h, no IM
G-CSF 96h, IM 72h
G-CSF 96h, IM 96h

3.1.5 Assessment of non-proliferating CD34$^+$ CML cells after priming with rHu-G-CSF in combination with continuous or interrupted IM therapy

After determination of total viable cell counts, we went on to assess the effects of continuous and intermittent rHu-G-CSF and continuous and interrupted IM therapy on the non-proliferating (quiescent) CD34$^+$ cell sub-population after 3 cycles (12 days) culture. These experiments were performed using CFSE to track cell division (**Figure 3-8**). A colcemid control was included to determine the location of the non-proliferating CFSE^{max} CD34$^+$ cells during FACS analysis. CFSE^{max} CD34$^+$ cells were assessed at the end of each 4-day cycle. The absolute number of these non-proliferating cells was calculated and then expressed as a percentage of non-proliferating cells present at the end of cycle 1 in the no rHu-G-CSF, no IM arm of the experiment. This calculation was performed for all three cycles and the results are shown in **Figure 3-9**. Representative FACS profiles for each of the experimental arms are shown in **Figure 3-10**.

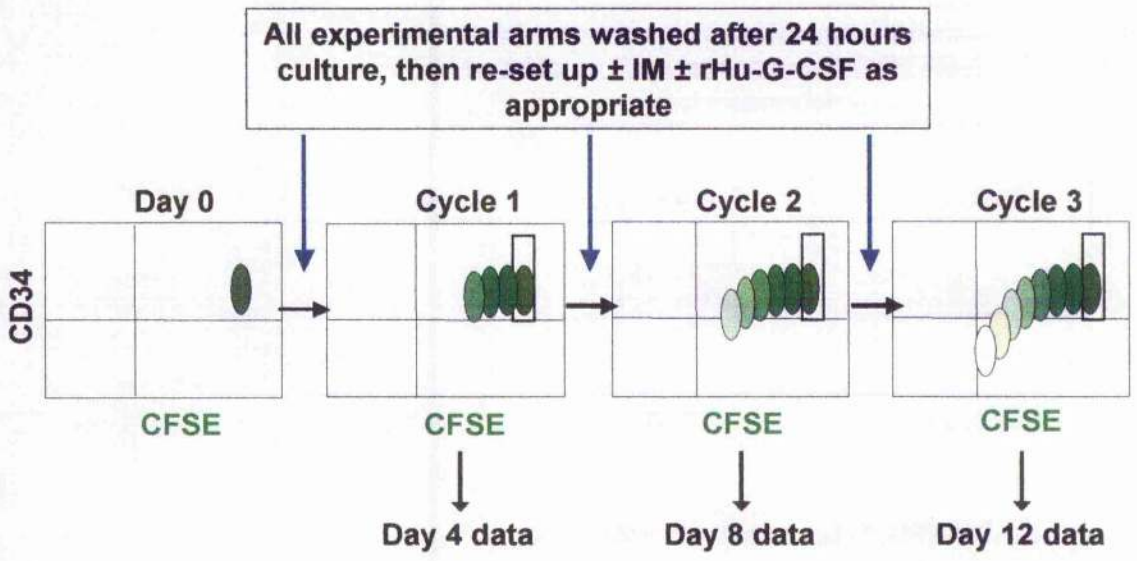


Figure 3-8. Schematic diagram of the protocol to detect the non-proliferating CFSE^{max} CD34$^+$ sub-population of CML cells (highlighted in rectangle) at the end of each treatment cycle ± rHu-G-CSF ± IM.

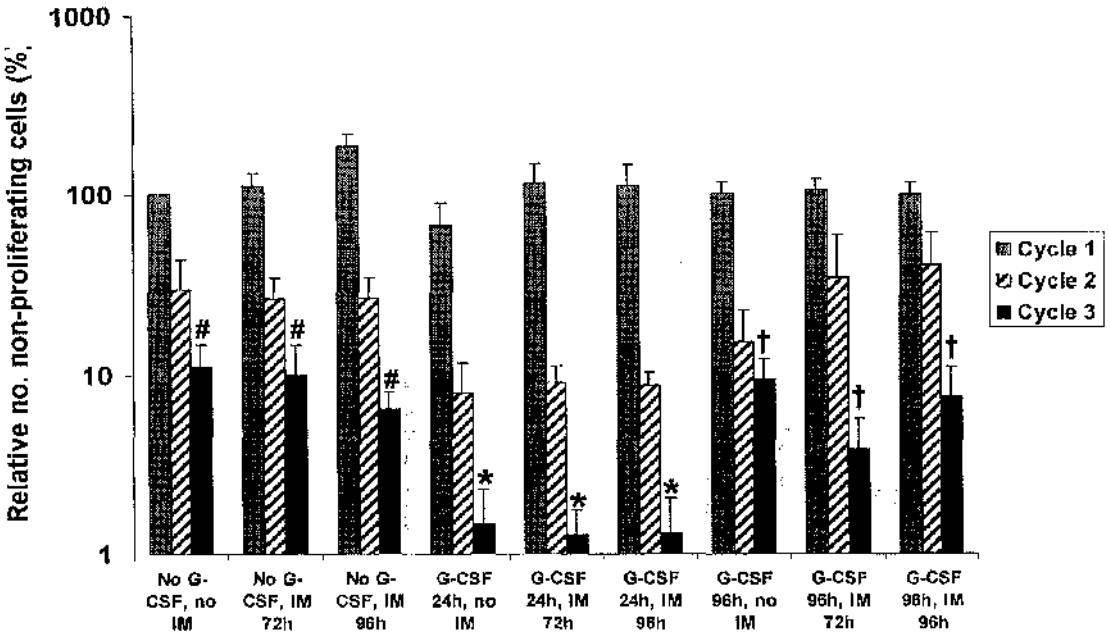


Figure 3-9. Relative size of non-proliferating CFSE^{max} CD34^{+} CML cell sub-population after cycles 1-3 (4, 8 and 12 days) in the described treatment conditions. Results are expressed as a percentage of the number of non-proliferating cells present at the end of cycle 1 in the no rHu-G-CSF, no IM arm (± SEM). Taken as a group the intermittent rHu-G-CSF arms (*) had significantly fewer undivided cells present at the end of 3 cycles compared to the no rHu-G-CSF (#; P=0.01) and continuous rHu-G-CSF (†; P=0.003) arms with no significant differences between the 3 individual intermittent rHu-G-CSF arms. IM alone had no significant effect on the non-proliferating population.

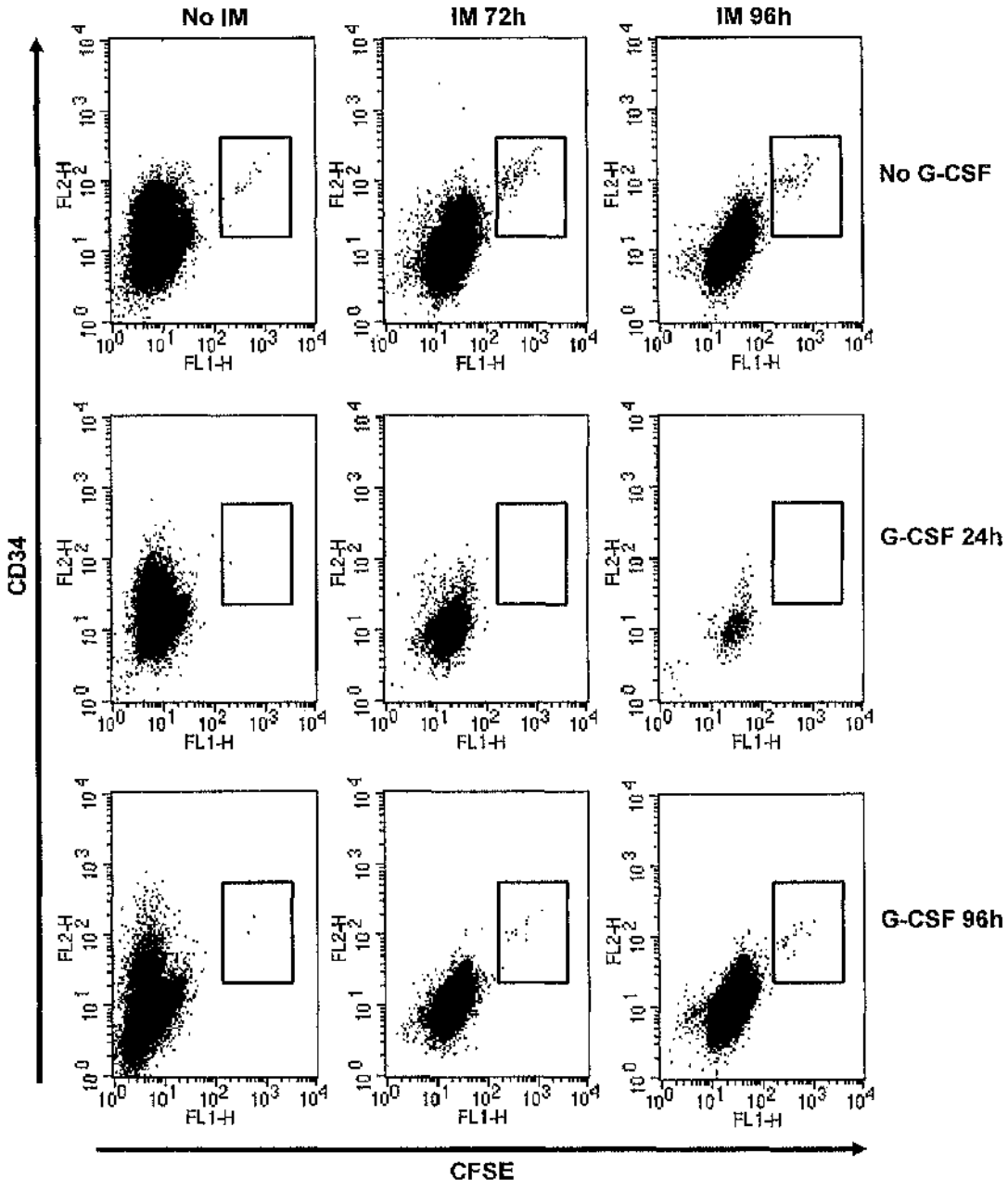


Figure 3-10. Representative FACS plots of CML cells cultured for 12 days under the different conditions of rHu-G-CSF and IM. Each panel shows a plot for the treatment arm indicated. The non-proliferating $CFSE^{max}$ $CD34^{+}$ CML cell population is highlighted in the box in each panel and the significant reduction in this population with intermittent rHu-G-CSF treatment is demonstrated. G-CSF, rHu-G-CSF; h, hours.

Taken as sets, there were no significant differences between the groups of three experimental arms in which either no IM, interrupted or continuous IM were present. These findings confirm those of previous experiments (Graham et al.,

2002) which showed that IM did not target the non-proliferating sub-population of $CD34^+$ CML cells. In addition, interrupted IM therapy did not improve eradication of the non-proliferating sub-population compared to continuous IM.

However, the results for rHu-G-CSF treatment were dramatically different. Intermittent rHu-G-CSF (24 hours) significantly reduced the non-proliferating sub-population of $CD34^+$ CML cells compared to the three arms containing no rHu-G-CSF ($P=0.01$) and, perhaps surprisingly, the three continuous rHu-G-CSF (96 hours) arms ($P=0.003$). Interestingly, there was no difference between the no rHu-G-CSF and continuous rHu-G-CSF arms. The addition of either interrupted or continuous IM to rHu-G-CSF did not significantly enhance eradication of the non-proliferating sub-population. This suggests that intermittent treatment with rHu-G-CSF may result in an enhanced initial mitogenic response in primitive $CD34^+$ CML cells which is not present in more mature myeloid cells.

In selected experiments, the cells remaining after 12 days culture were FACS sorted to isolate the non-proliferating sub-population of $CD34^+$ CML cells by Dr Martin Barow. The cells were sorted directly onto a microscope slide, the cells fixed, and FISH for the BCR-ABL fusion was kindly performed by Mrs Elaine Allan. This confirmed that the vast majority (>95%) of non-proliferating cells present were indeed $BCR\text{-}ABL^+$ after 12 days culture (**Figure 3-11**). Therefore, the insensitivity of the non-proliferating cells to rHu-G-CSF and IM was not due to enrichment of normal $CD34^+$ cells.

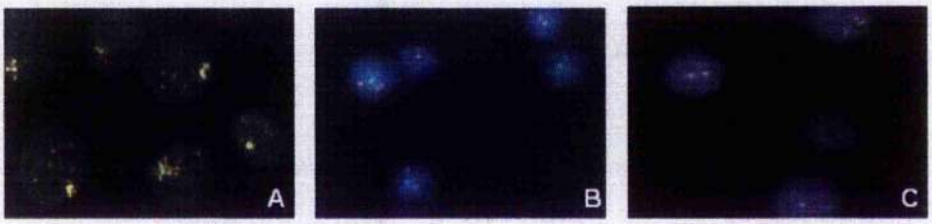


Figure 3-11. Representative FISH images of BCR-ABL$^+$ cells. As treatment with continuous rHu-G-CSF and IM was least effective in reducing non-proliferating CD34$^+$ cells relative to the no rHu-G-CSF no IM control, this treatment arm was selected for FACS sorting of the CFSE^{max} sub-population to provide sufficient cells for FISH analysis to determine Ph status of the non-proliferating cell population. (A) The K562 BC CML cell line which has multiple copies of BCR-ABL (positive control for FISH). (B) CML patient total CD34$^+$ cells prior to treatment. (C) CFSE^{max} Ph$^+$ interphase nuclei recovered after *in vitro* treatment with continuous rHu-G-CSF and IM. FACS sorting was performed by Dr Martin Barow and FISH by Mrs Elaine Allan.

To determine if cells leaving the non-proliferating CFSE^{max} CD34$^+$ sub-population did so by apoptosis or cell division, active caspase-3 was assessed in combination with CFSE and CD34 by flow cytometry in the different experimental arms at the end of cycle 1. The non-proliferating CFSE^{max} CD34$^+$ cells remaining at the end of cycle 1 were almost exclusively active caspase-3 negative, indicating that the cells leaving the non-proliferating CFSE^{max} CD34$^+$ sub-population had done so by cell division and not apoptosis (**Figure 3-12**). The addition of either rHu-G-CSF or IM to the cultures did not significantly increase apoptosis.

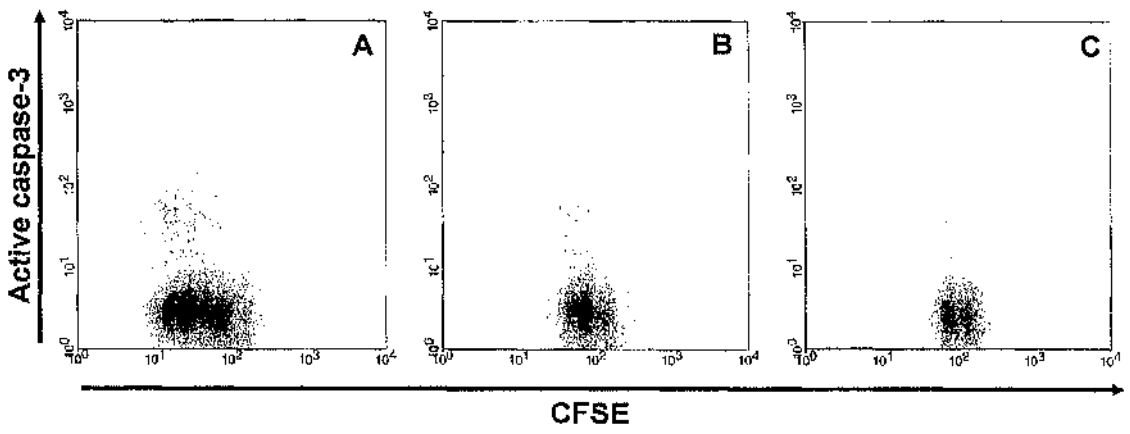


Figure 3-12. Representative FACS plots of active caspase-3 to measure apoptosis versus CFSE to track cell division in (A) rHu-G-CSF 24 hours , no IM; (B) rHu-G-CSF 24 hours, IM 72 hours; and (C) rHu-G-CSF 24 hours, IM 96 hours arms which demonstrate that non-proliferating $CFSE^{max}$ $CD34^{+}$ CML cells are not undergoing apoptosis. Active caspase-3 was measured after the first cycle (4 days). Apoptosis only occurs once the cells are dividing indicating that the cells leave the non-proliferating gate by cell division and neither rHu-G-CSF nor IM increases the rate of apoptosis.

3.1.6 Summary and conclusions

Continuous IM was more effective than interrupted IM in reducing total viable CML cells in the absence of rHu-G-CSF. In the presence of rHu-G-CSF, there was no significant difference between the effects of continuous and interrupted IM on total viable cells. IM most effectively reduced total viable cells when combined with intermittent rHu-G-CSF. As expected, IM had no significant effect on the non-proliferating sub-population of $CD34^{+}$ CML cells. However, intermittent rHu-G-CSF significantly reduced the non-proliferating sub-population compared to either no rHu-G-CSF or continuous rHu-G-CSF by increasing the number of cells which leave this quiescent pool by cell division. The addition of IM did not significantly enhance this effect.

These results suggest that the combination of intermittent rHu-G-CSF to stimulate cells to divide and reduce the non-proliferating pool, combined with continuous IM

to eradicate more mature myeloid cells may be particularly effective in treating CML and reducing the phenomenon of molecular persistence.

4 Results 2

4.1 A comparison of the efficacy of dasatinib and IM against quiescent primitive CML progenitor cells *in vitro*

4.1.1 Introduction

There is increasing evidence that, even after prolonged treatment, IM does not eradicate CML stem cells *in vivo*. Previous research has shown that patients in CCR after IM therapy continue to have detectable BCR-ABL$^+$ CD34$^+$ cells, CFCs and LTC-ICs (Bhatia et al., 2003). Therefore, it is unlikely that IM will cure CML and patients will require long-term monitoring of *BCR-ABL* levels by qRT-PCR. This is also supported by the observed rapid relapses in a number of patients who have stopped IM after a period of negative qRT-PCR results (Cortes et al., 2004b; Mauro et al., 2004) and the persistence of IM-insensitive, non-proliferating (quiescent) cells *in vitro* (Graham et al., 2002; Holtz et al., 2002). With continued IM therapy, there is also the risk of developing IM resistance through BCR-ABL kinase domain mutations (Shah et al., 2002), *BCR-ABL* amplification (Gorre et al., 2001), BCR-ABL independence with the acquisition of additional mutations (Donato et al., 2004) or reduced intracellular levels of IM (Mahon et al., 2003) as previously discussed (**Section 1.2.7**).

Recent work by our group has aimed to examine the mechanisms of IM resistance in non-proliferating (or quiescent) CD34$^+$ CML cells (Copland et al., 2006). These studies have shown that, before and after short term culture (72 hours) in the presence of IM, the majority of CD34$^+$ and the more primitive CD34$^+$38$^-$ cells are BCR-ABL$^+$ and have only a single copy of *BCR-ABL*; i.e. do not show gene

amplification. This research has also shown that $CD34^+$ CML cells have higher *BCR-ABL* transcript levels than the more mature total MNC population when measured by qRT-PCR. In addition, phosphorylated BCR-ABL, tyrosine and CrKL protein expression were highest in the most primitive $CD34^+38^-$ cell fraction, at an intermediate level in the $CD34^+$ population and expressed at a low level in total MNCs from CML patients. Furthermore, mutation analysis confirmed that kinase domain mutations were not detectable in the $CD34^+$ cells taken from early CP CML patients at diagnosis or in those cells surviving 5μM IM or 150nM dasatinib (Copland et al., 2006). Therefore, at present, the only confirmed explanation for the insensitivity of CML stem cells to IM is increased *BCR-ABL* gene and protein expression. Taken together, all these findings highlight the importance of finding a therapy which targets the primitive CML stem cell population.

Dasatinib is a novel multi-targeted kinase inhibitor of the SRC family and BCR-ABL kinases (Shah et al., 2004) which is currently in Phase 3 clinical trials in IM-resistant and IM-intolerant CML and Ph^+ ALL patients (Talpaz et al., 2006). *In vitro*, dasatinib is more potent (325 fold greater) than IM in cell lines and inhibits the majority of BCR-ABL kinase mutations that result in IM resistance (except T315I) (O'Hare et al., 2005). It is believed that the improved efficacy of dasatinib against CML cells is due to increased potency against BCR-ABL and not directly through the additional inhibition of SRC kinases (Donato et al., 2003; Donato et al., 2004; Hu et al., 2004). However, these studies have been conducted in cell lines and mouse models and the importance of SRC kinases in primary CML cells has not been directly assessed. In models of advanced phase CML, BCR-ABL has been shown to activate LYN and HCK, the SRC kinases most prevalent in myeloid cells (Corey and Anderson, 1999), and this contributes to the survival and proliferation of $BCR\text{-}ABL^+$ cells. Further *in vitro* studies have also shown that increased levels of activated LYN may up-regulate the anti-apoptotic protein BCL-

2 in IM-resistant BCR-ABL$^+$ cells, contributing to the IM resistance seen (Donato et al., 2003; Dai et al., 2004).

Therefore, the aims of this study were, firstly, to determine if dasatinib was more effective than IM in CD34$^+$ CML cells and if it would eradicate quiescent CML stem cells *in vitro*. Secondly, to determine the relevance of SRC kinases and BCL-2 in early CP CML.

4.1.2 Assessment of IC_{50} for dasatinib in the K562 cell line and primary CD34$^+$ CML cells

Concentration finding studies to determine the IC_{50} for dasatinib in K562 and primary CD34$^+$ CML cells were performed using ^3H thymidine cell proliferation assays and counting of total viable cells. K562 cells were assessed at 24 and 48 hours and compared to the BCR-ABL negative AML cell line HL60 to show the specificity of dasatinib for BCR-ABL. Primary CD34$^+$ CML cells were assessed after 72 hours culture in SFM only. Using proliferation assays (n=4), the IC_{50} for dasatinib in K562 cells was approximately 0.8nM at 48 hours culture (**Figure 4-1A**). These results were supported by total viable cell count results which showed a reduction in total viable cells to 80% of no drug control at 1nM and 35% at 5nM after 48 hours culture (**Figure 4-1B**). Dasatinib had no effect on proliferation or total viable cell counts in the BCR-ABL negative cell line HL60 at any of the concentrations assessed (**Figure 4-1A** and **B**). These results demonstrate that K562 cells are highly sensitive to dasatinib and that the drug selectively targets BCR-ABL expressing cells. Dasatinib had an IC_{50} of approximately 5nM as demonstrated by proliferation assay and confirmed by total viable cell count in primary CML cells (n=3; **Figure 4-2A** and **B**), indicating the potency of dasatinib in primary CML cells.

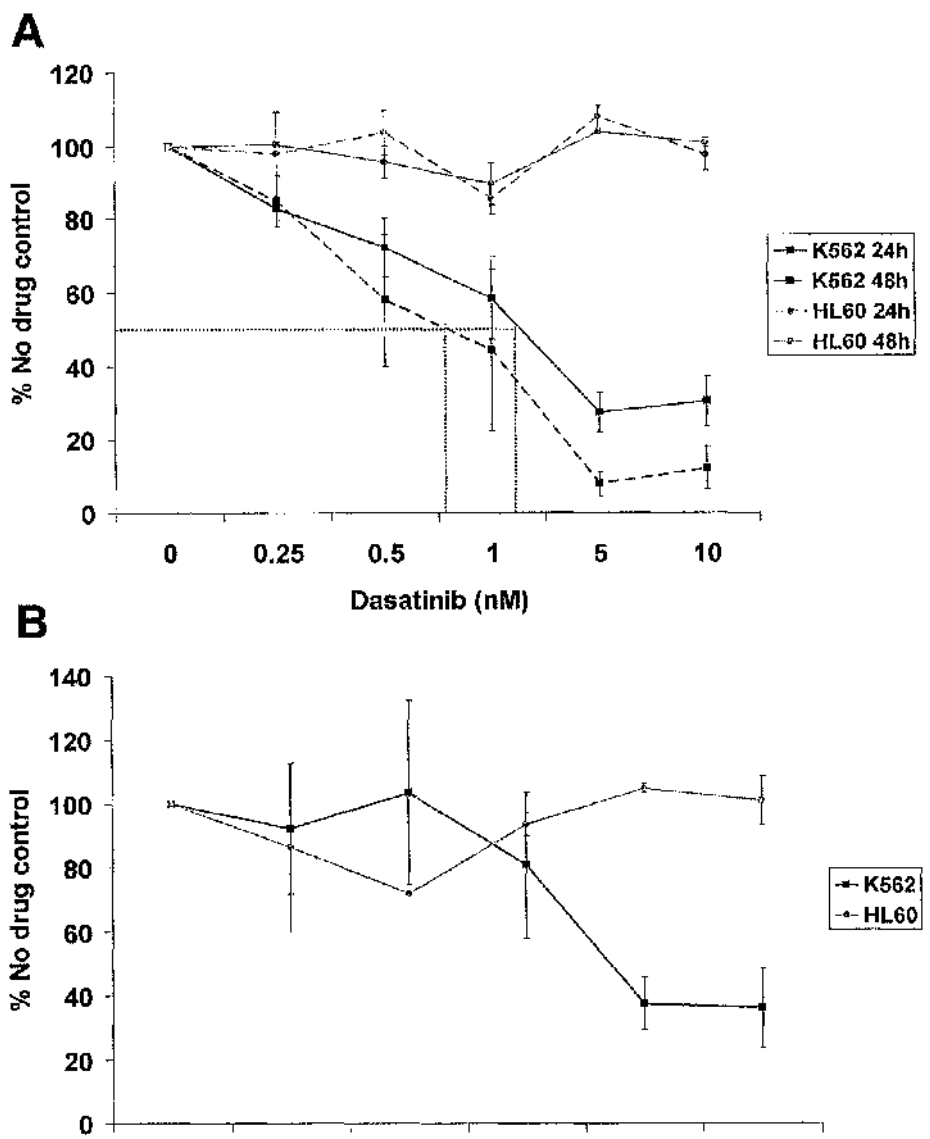


Figure 4-1. Determination of IC_{50} for dasatinib by ^3H thymidine proliferation assays and total viable cell counts in K562 cells (n=4 experiments) and HL60 cells (n=2 experiments). (A) ^3H thymidine proliferation assays after 24 and 48 hours in K562 and HL60 cell lines demonstrate that dasatinib has an IC_{50} of approximately 0.8nM at 48 hours in K562 cells and has no effect on HL60 cells. Five replicates of each condition were performed in each experiment. (B) Total viable cell counts in K562 and HL60 cells at 48 hours expressed as a percentage of the no drug control confirm the results of the proliferation assays. Results represent the mean ± SEM. h, hours.

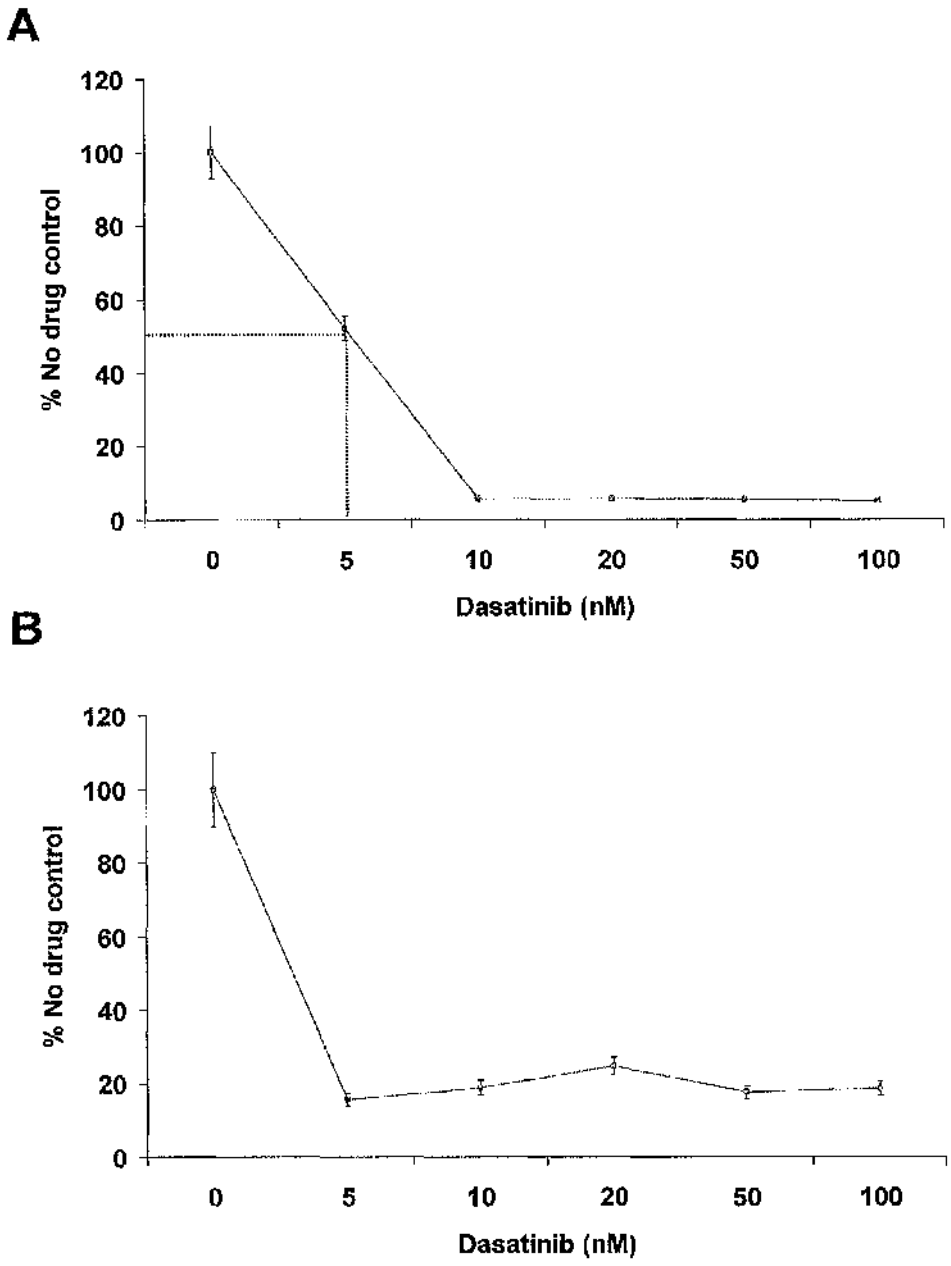


Figure 4-2. Determination of IC_{50} for dasatinib by ^3H thymidine proliferation assays and total viable cell counts in primary $CD34^+$ CML cells (n=3 experiments). (A) ^3H thymidine proliferation assays were performed after 72 hours and demonstrate that dasatinib has an IC_{50} of approximately 5nM in primary $CD34^+$ CML cells cultured in SFM only. Five replicates of each condition were performed in each experiment. (B) Total viable cell counts after 72 hours expressed as a percentage of the no drug control confirm the results of the proliferation assays. Results represent the mean ± SEM.

In primary $CD34^+$ CML cells, even at doses up to 100nM, there was no further reduction in proliferation or total viable cells compared to the 10nM dose. There are two possible explanations for this. Firstly, that there is enrichment of a normal

Ph^- cell population which is not affected by dasatinib. However, FISH on these and other patients samples after 6 days culture in the presence of dasatinib confirmed that >95% of cells remaining after culture were BCR-ABL^+, thus excluding this explanation. The second potential reason is that there is a population of cells present which is resistant to dasatinib and, as in the case of IM, this may be the primitive CML stem cell population.

These results for dasatinib are similar to those of other groups which have reported an IC_{50} of 0.6 to 0.8nM for cell lines expressing wild type BCR-ABL (Shah et al., 2004; O'Hare et al., 2005) and 60 to 80% inhibition of growth of bone marrow progenitors isolated from patients with IM-sensitive CML in CFU assays (Shah et al., 2004) . However, in Phase 1 clinical trials with CML patients (Talpaz et al., 2006), plasma concentrations of up to 180nM dasatinib have been achieved. Therefore, a concentration of 150nM dasatinib was chosen for further experiments as it more closely represented the clinical situation.

4.1.3 A comparison of IM and dasatinib in $CD34^+$ CML progenitor cells in short term culture

4.1.3.1 Assessment of total viable cell counts

In this series of experiments (n=8), the effects of IM or dasatinib, dosed either as single agents, sequentially or simultaneously on total viable cell counts was assessed in $CD34^+$ CML cells *in vitro.* As previously, an IM concentration of 5μM was chosen for these experiments, and dasatinib was used at 150nM as discussed. The cells were cultured in SFM + 5GF for a total of 6 days. After 72 hours, an aliquot of cells was removed for counting; the remaining cells in all treatment arms were washed and then re-set up in culture for a further 72 hours under the following conditions: (1) No drug control; (2) IM 5μM; (3) dasatinib

150nM; (4) IM for 72 hours then dasatinib for 72 hours; (5) dasatinib for 72 hours then IM for 72 hours; and (6) simultaneous IM and dasatinib. Therefore, at 72 hours, there were only 4 experimental arms (no drug control, IM 5μM, dasatinib 150nM and simultaneous IM and dasatinib). The total viable cell counts for 72 hours and 6 days are shown in **Figure 4-3 A** and **B**, respectively.

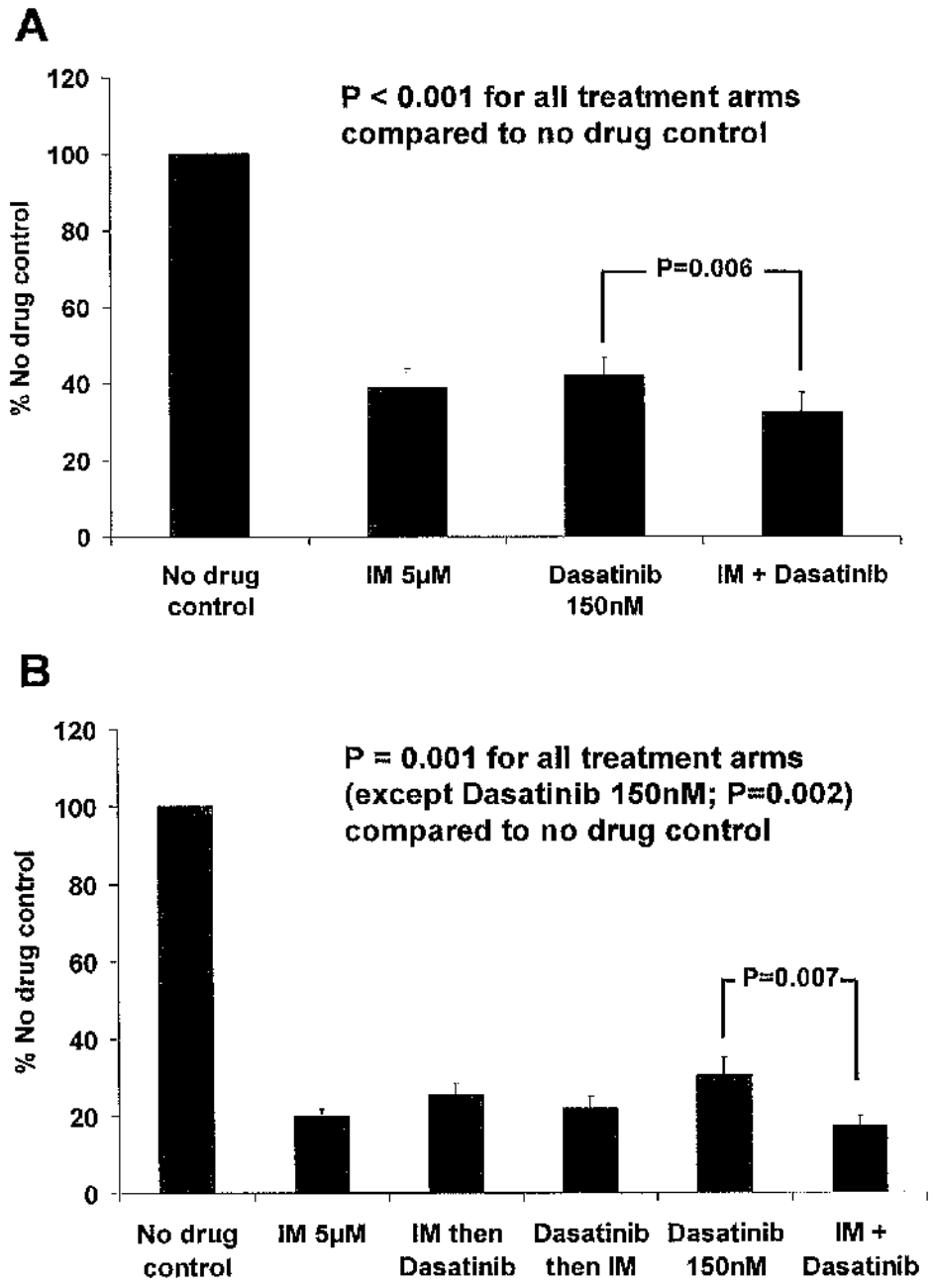


Figure 4-3. Total viable cell counts after 72 hours (A) and 6 days (B) for all experimental conditions. Cells were cultured in SFM + 5GF. Results are expressed as a percentage of the no drug control (± SEM).

At both time points, there was a significant reduction in total viable cells in all treatment arms compared to the no drug control (P<0.001 for all treatment arms at 72 hours, and P=0.001 for all treatment arms at 6 days except dasatinib 150nM which had a significance value of P=0.002). However, as the cells were cultured in the presence of 5GF, there was marked proliferation and expansion of total viable cells compared to the starting cell number in all treatment arms (**Figure 4-4**). The $CD34^+$ CML cells were cultured in 5GF for these experiments to aid identification of the quiescent progenitor population which has the greatest resistance to activation in the CFSE experiments described next (**4.1.3.2**).

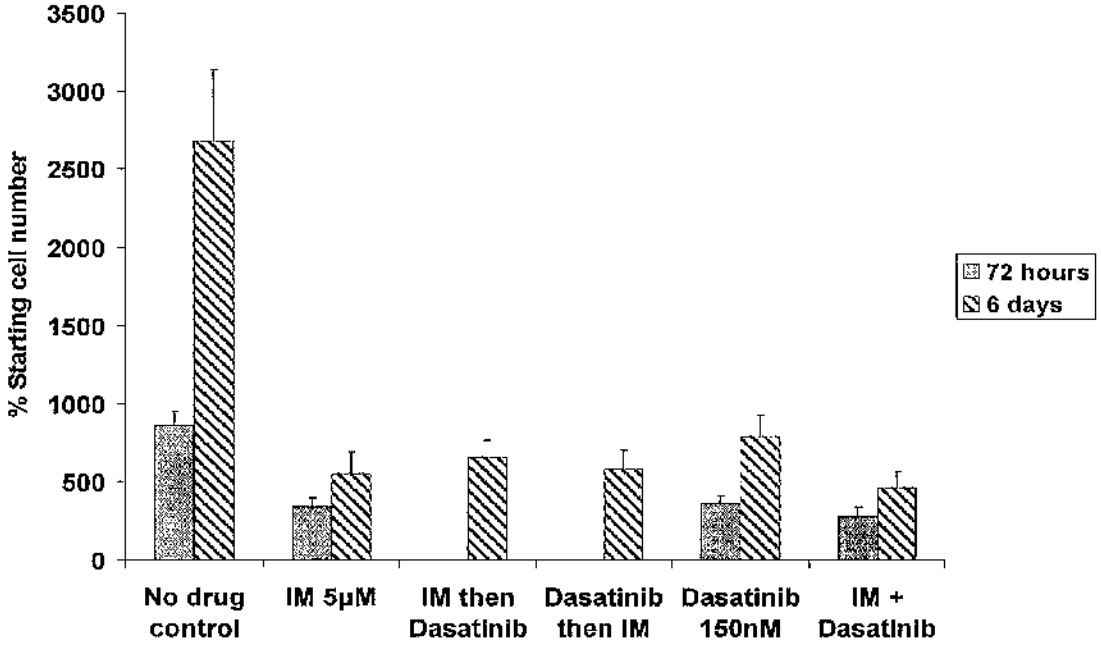


Figure 4-4. Total viable cells after 72 hours and 6 days culture of $CD34^+$ CML cells in SFM + 5GF with the stated treatment conditions. There is expansion of cell numbers due to proliferation in all treatment arms relative to the starting cell number at 3 days with further expansion evident at 6 days. IM then dasatinib and dasatinib then IM have a 6 day time-point only. Results are expressed as percentage starting cell number (± SEM).

There was no significant difference in total cells between the IM and dasatinib arms at either time point (P=0.419 and P=0.118 for 72 hours and 6 days, respectively), although there was a trend for total viable cells to be lower in the IM

arm. The experimental arm which consistently had the lowest total viable cell number was the simultaneous IM and dasatinib arm. At both time points this was significantly lower than the dasatinib only arm (P=0.006 at 72 hours and P=0.007 at 6 days) and significantly lower than either of the sequential treatment arms (IM then dasatinib or dasatinib then IM) at 6 days (P=0.002 for both). There was no significant difference between the IM only and simultaneous IM and dasatinib arms. These results confirm that IM and dasatinib, at high therapeutically achievable concentrations, have equivalent efficacy on primary $CD34^+$ CML cells *in vitro*.

4.1.3.2 The effect of IM versus dasatinib on non-proliferating $CD34^+$ CML progenitor cells

Having assessed total viable cell counts, the effects of IM and dasatinib, either as single agents, as sequential therapy or given simultaneously on the non-proliferating (quiescent) $CD34^+$ cell sub-population after 72 hours and 6 days was studied. As in Chapter 3 (**3.1.5**), these experiments were performed using CFSE to track cell division and, again, a colcemid control was included to identify the position of the non-proliferating $CFSE^{max}$ $CD34^+$ cells during FACS analysis. The absolute number of these non-proliferating cells was calculated and then expressed as a percentage of the starting cell number for each treatment condition at 72 hours and 6 days. The results are shown in **Figure 4-5A** and **B**. Representative FACS profiles for the no drug control, IM and dasatinib arms are shown in **Figure 4-6**.

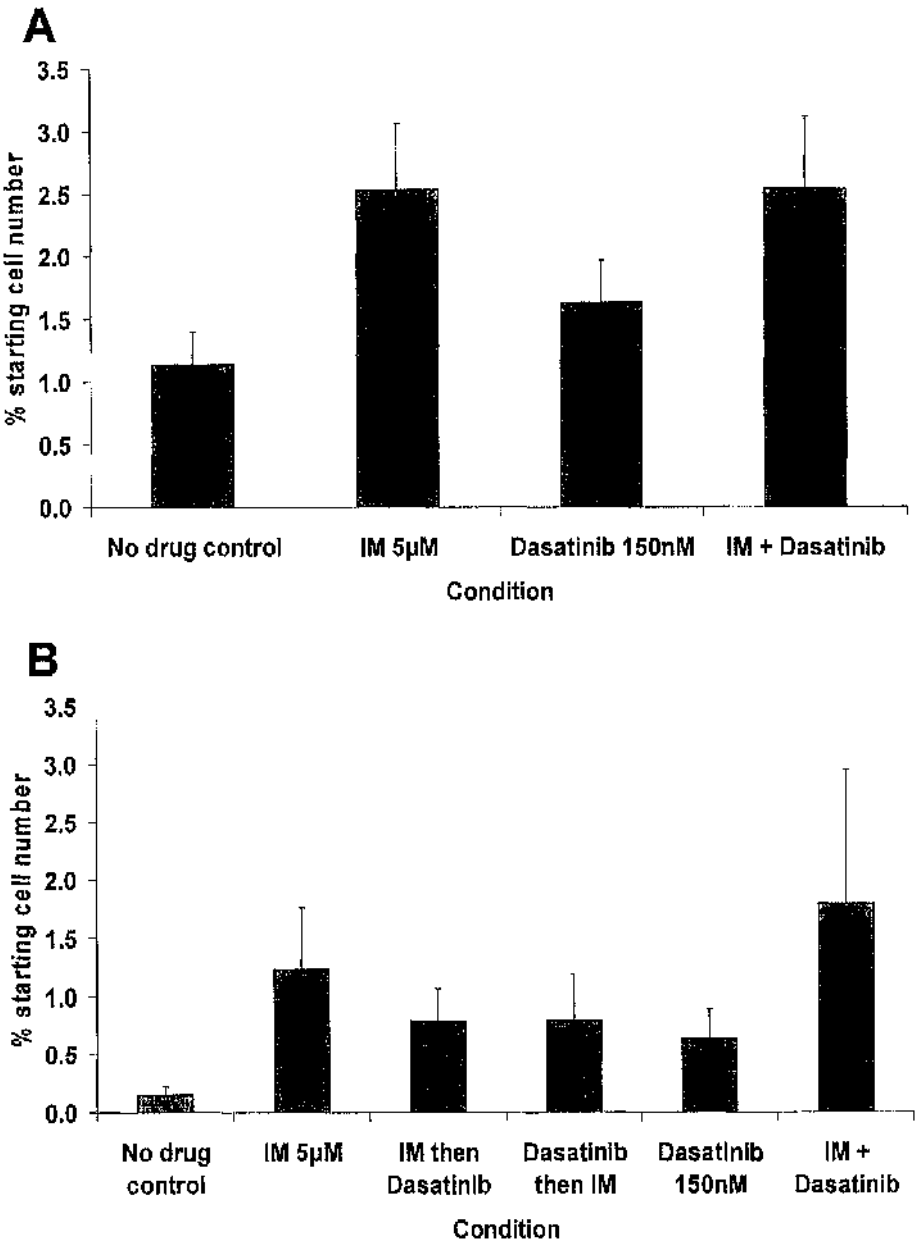


Figure 4-5. Absolute number of non-proliferating CFSE^{max} CD34^{+} cells remaining after 72 hours (A) and 6 days (B) for all experimental conditions. Cells were cultured in SFM + 5GF. Results are expressed as a percentage of the starting cell number (± SEM).

Compared to the no drug control, there was a significant increase in non-proliferating CFSE^{max} CD34^{+} CML cells in the IM and IM plus dasatinib arms at 72 hours (P=0.04 and P=0.05, respectively). However, this increase was not present in the dasatinib arm (P=0.27). After 6 days culture, all treatment arms had increased CFSE^{max} CD34^{+} cells compared to the no drug control. There were no

significant differences between the different treatment arms, although there was a trend for the arms containing continuous IM (IM 5μM and simultaneous IM plus dasatinib arms) to have the largest non-proliferating cell populations (P=0.22).

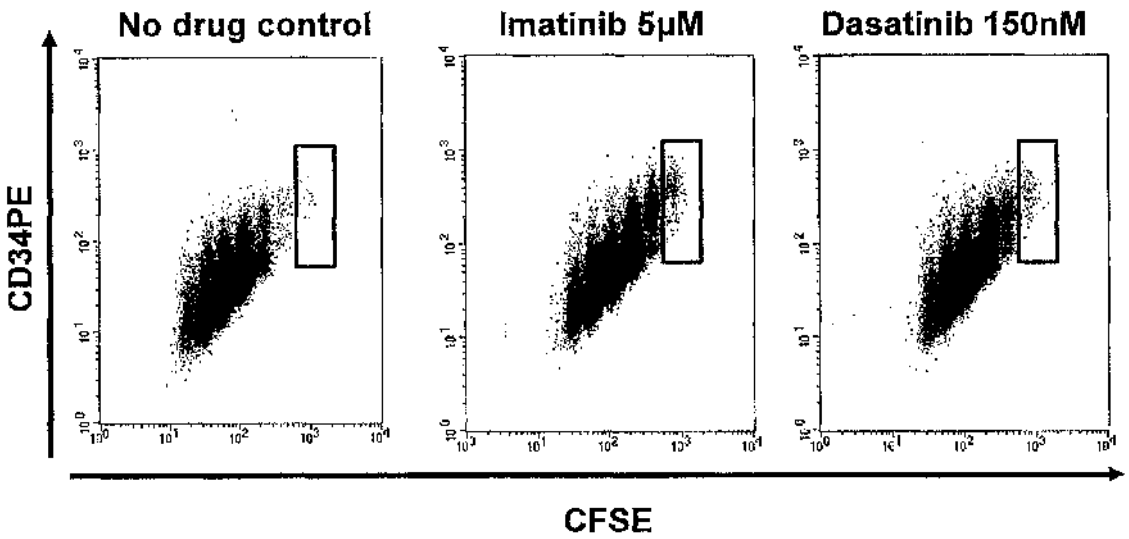


Figure 4-6. Representative FACS dot plots of CML cells after 72 hours culture in the presence of no drug control, IM 5μM or dasatinib 150nM as indicated. The non-proliferating $CFSE^{max}$ $CD34^{+}$ CML cell population is highlighted in the box in each panel. The plots demonstrate the very low number of undivided cells remaining in the no drug control compared to the relatively large population remaining in the IM arm. The intermediate sized $CFSE^{max}$ $CD34^{+}$ population remaining after treatment with dasatinib is also shown.

The relatively large population of non-proliferating $CFSE^{max}$ $CD34^{+}$ CML cells remaining after exposure to IM is due to the accumulation of cells arrested in G_0/G_1 phase of the cell cycle. The intermediate sized non-proliferating $CFSE^{max}$ $CD34^{+}$ CML cell population remaining after dasatinib treatment likely reflects the less profound anti-proliferative effect of dasatinib, resulting in fewer cells accumulating in the $CFSE^{max}$ gate due to reversible G_0/G_1 arrest. This anti-proliferative effect is further demonstrated in **Figure 4-7**.

FISH was performed by Mrs Elaine Allan at baseline and again after 6 days culture in the presence of IM or dasatinib and confirmed that, at both baseline and again

at the end of treatment, >95% of the cells were *BCR-ABL*$^+$ with only single copy *BCR-ABL* being detected.

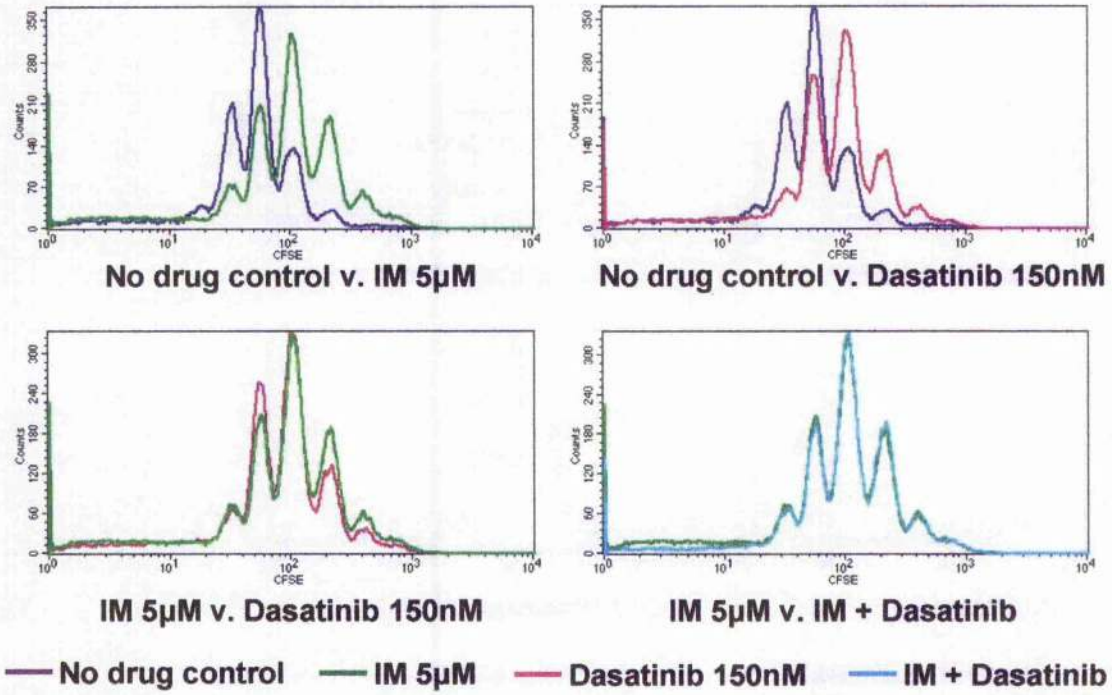


Figure 4-7. Representative FACS histogram plots which demonstrate the anti-proliferative effects of IM and dasatinib. In the upper panels, IM and dasatinib treated cells have progressed through fewer divisions (right shift) compared to the no drug control. In the lower panels, the less anti-proliferative effect of dasatinib compared to IM is seen (left) and the identical profiles for IM and simultaneous IM plus dasatinib administration are demonstrated (right).

As clearly demonstrated here, none of the treatment conditions, including the combined administration of IM and dasatinib, reduced the non-proliferating CFSE^{max} CD34$^+$ CML cell population compared to the no drug control. This suggests that neither IM nor dasatinib targets the most primitive quiescent CML stem cells.

4.1.3.3 Assessment of apoptosis in CD34$^+$ CML cells after exposure to IM or dasatinib

Previous work has shown that the most primitive CML cells do not readily undergo apoptosis after treatment with IM (Graham et al., 2002; Holtz et al., 2002; Bhatia et al., 2003; Jorgensen et al., 2005a), it is the more mature cells which apoptose. Therefore, apoptosis was assessed in CD34$^+$ CML cells (n=4) after treatment with IM 5μM, dasatinib 150nM or a no drug control using an anti-active caspase-3-PE FACS antibody. To enable assessment of apoptosis in the non-proliferating fraction, the cells were stained with CFSE prior to culture as previously. In preliminary experiments, caspase-3 activity was assessed at 48, 72 and 96 hours culture in the presence of different treatment arms. Apoptosis was found to be greatest at 72 hours, therefore in all subsequent experiments, caspase-3 activity was assessed after 72 hours and the results are shown in **Figure 4-8**.

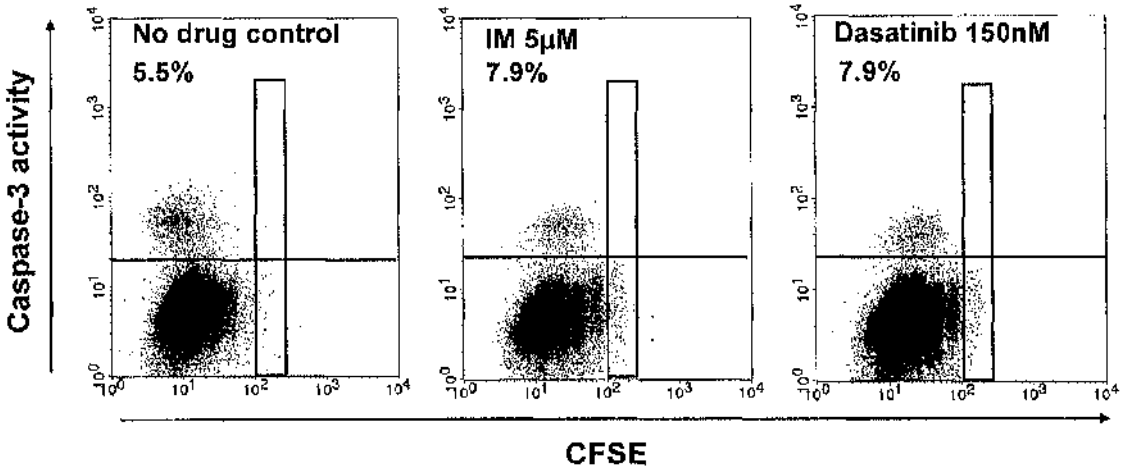


Figure 4-8. Representative FACS dot plots which demonstrate increased caspase-3 activity in the presence of either IM 5μM or dasatinib 150nM compared to no drug control after 72 hours culture. Apoptosis was assessed using anti-active caspase-3 antibody (PE) in CD34$^+$ CML cells. The cells were stained with CFSE prior to culture to allow assessment of apoptosis in the non-proliferating (quiescent; boxed area) fraction. The dot plots illustrate that the majority of apoptosis occurs in the more mature progenitors which have already

undergone several divisions, with the non-proliferating compartment being relatively resistant to apoptosis.

Compared to the no drug control, there was a trend towards increased apoptosis in both the IM and dasatinib arms (5.5% versus 7.9% for both IM and dasatinib) for total cells remaining after 72 hours culture, but these results did not reach statistical significance (P=0.067 and P=0.069 for IM and dasatinib, respectively), due, in part, to inter-patient variability. In the non-proliferating population, there were no significant differences in apoptosis between the 3 arms with very few caspase-3 positive cell events (**Figure 4-8**). Therefore, dasatinib was no more effective than IM at inducing apoptosis in primary $CD34^+$ CML cells, and most particularly, compared to IM, did not increase apoptosis in the non-proliferating $CFSE^{max}$ $CD34^+$ CML cell population. This result also confirms that the trend to fewer cells remaining in the non-proliferating $CFSE^{max}$ $CD34^+$ CML cell gate after treatment with dasatinib, compared to IM, is due to the cells leaving this gate through cell division and not apoptosis.

4.1.3.4 Assessment of CrKL phosphorylation as a marker of BCR-ABL activity in primary $CD34^+$ and more primitive $CD34^+38^-$ CML cells after treatment with IM or dasatinib

To further define the effects of IM and dasatinib on primary CML cells, a flow cytometry method for measuring CrKL phosphorylation has been developed and validated in our lab (Hamilton et al., 2006). This FACS method allows determination of CrKL phosphorylation status on relatively small cell numbers (< $1x10^5$) from which there would have been insufficient protein to perform Western blotting. Our first finding was that CrKL phosphorylation was significantly increased in the more primitive $CD34^+38^-$ population compared to the total $CD34^+$ population (P=0.002; **Figure 4-9A**). Further experiments assessed the effect of IM at a dose

of 5μM on CrKL phosphorylation in total $CD34^+$ and more primitive $CD34^+38^-$ cell populations (**Figure 4-9B**). Results showed that IM inhibited CrKL phosphorylation at 16 hours but not 72 hours in total $CD34^+$ cells, consistent with enrichment of an IM-insensitive population (P=0.01 for 16 hours versus 72 hours). However, at both time-points, in the more primitive $CD34^+38^-$ cell population, IM failed to inhibit CrKL phosphorylation, confirming the inherent insensitivity of these primitive cells to IM. We then went on to compare the effects of either IM 5μM or dasatinib 150nM on the primitive $CD34^+38^-$ cell population at 16 hour and 72 hour time-points (**Figure 4-9C**). At both time points, there was significant inhibition of CrKL phosphorylation compared to IM (P=0.004 for 16 hours and P<0.001 for 72 hours).

These results provide evidence that dasatinib is a more potent inhibitor of BCR-ABL than IM. However, they also suggest that BCR-ABL may be irrelevant in the stem cell compartment as both IM and dasatinib have equivalent potency at clinically achievable concentrations against $CD34^+$ CML cells *in vitro*, despite the superior inhibition of CrKL phosphorylation in the primitive $CD34^+38^-$ compartment demonstrated here. This further suggests that either inhibition of BCR-ABL does not lead to cell death or the enhanced inhibition CrKL phosphorylation demonstrated here with dasatinib is mediated via SRC and not BCR-ABL. An alternative explanation, although unlikely, is that CML cells require more than 72 hours after inhibition of CrKL phosphorylation before undergoing apoptosis.

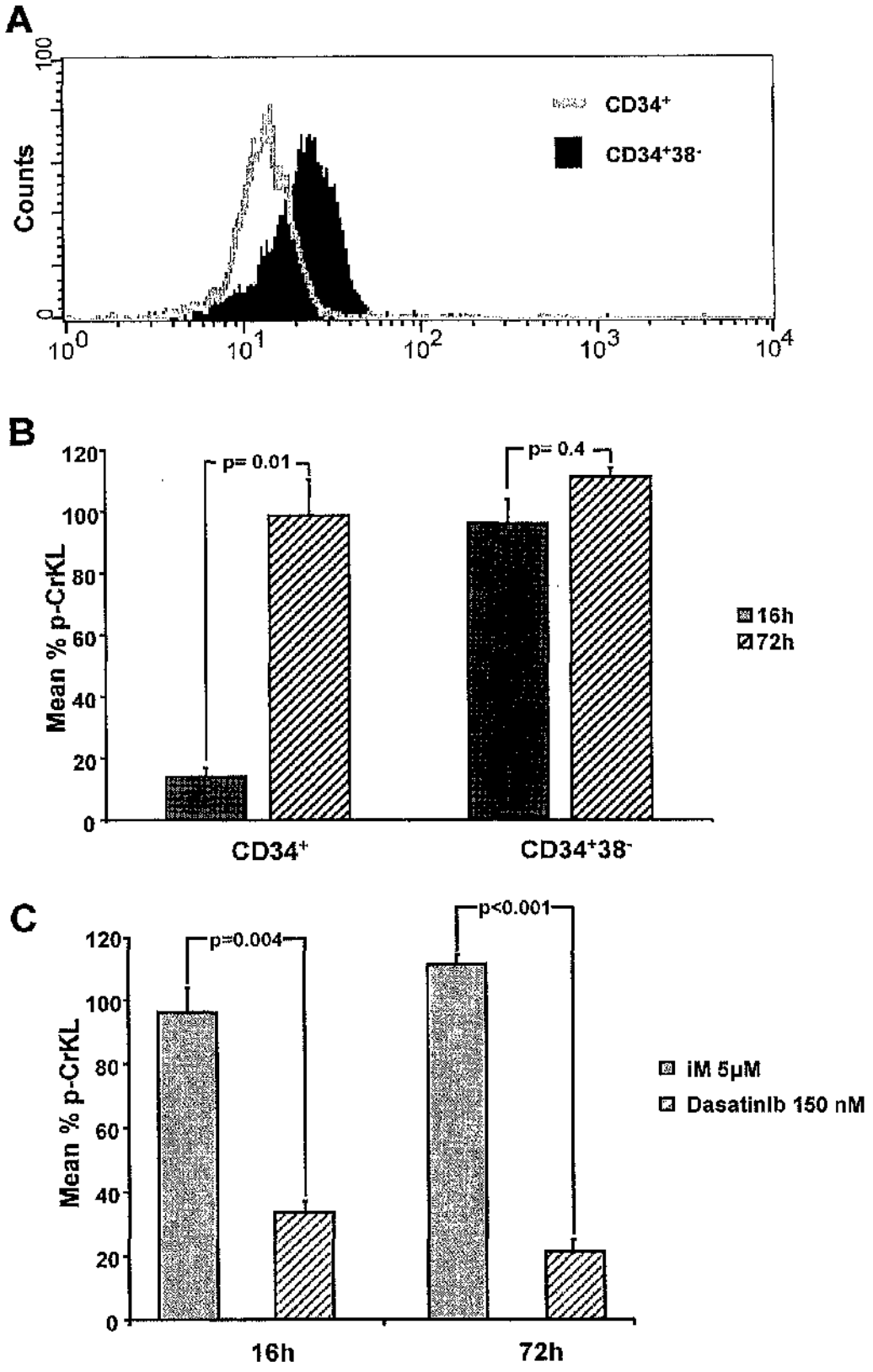


Figure 4-9. Assessment of CrKL phosphorylation (p-CrKL) in total CD34$^+$ and the more primitive CD34$^+$38$^-$ cell populations before and after treatment with IM or dasatinib. (A) Comparison of p-CrKL in total CD34$^+$ and CD34$^+$38$^-$ cells at baseline. CD34$^+$38$^-$ cells exhibit

significantly greater p-CrKL than total CD34$^+$ cells (P=0.002). (B) p-CrKL in total CD34$^+$ and CD34$^+$38$^-$ cells after treatment with IM 5μM for 16 and 72 hours. IM inhibited p-CrKL at 16 hours (P=0.01) but not 72 hours in the total CD34$^+$ cell population, suggesting enrichment of an IM-insensitive population. (C) Comparison of p-CrKL after treatment with IM 5μM or dasatinib 150nM in CD34$^+$38$^-$ cells after 16 and 72 hours. There was significant inhibition of p-CrKL at both time-points with dasatinib (P=0.004 at 16 hours and P<0.001 at 72 hours) compared to IM. h, hours

4.1.4 Determination of the relevance of SRC kinases in CML

Recent research has shown that the SRC kinase LYN is over-expressed and activated in the K562-R cell line (Donato et al., 2003) which is resistant to IM. This cell line was developed by culturing K562 cells with increasing concentrations of IM and then cloning the cells which were resistant to IM by limiting dilution. The surviving cells were then cultured in the absence of IM and then re-assessed for IM sensitivity. The cells which remained IM-resistant were termed K562-R cells. Further experiments showed that inhibition of LYN with the SRC family-specific inhibitor CGP76030 (Novartis) resulted in reduced proliferation and survival of K562-R cells. Samples taken from patients with BC CML who had progressed on IM also showed increased expression of activated LYN similar to the K562-R cell line (Donato et al., 2003). In addition, specimens taken from BC CML patients before and after IM failure suggested that expression of activated LYN and/or HCK occurred during disease progression. The authors concluded from this research that SRC kinases were highly expressed in BC CML and that this increased expression correlated with disease progression or IM resistance. Further studies using a mouse model have also shown that BCR-ABL activated the SRC kinases LYN, HCK and FGR in B-lymphoid cells (Hu et al., 2004) and induced CML, but not ALL, in LYN$^{-/-}$HCK$^{-/-}$FGR$^{-/-}$ mice. The SRC inhibitor CGP76030 impaired proliferation of B-lymphoid cells expressing BCR-ABL *in vitro* and prolonged survival of mice with B-ALL, but not CML. In addition, in B-ALL, the combination of

CGP76030 with IM was superior to IM alone (Hu et al., 2004). These studies suggest that SRC kinases are not required for CML induction but are necessary for Ph^+ ALL and that SRC activation does not contribute to the proliferation or survival of CML cells. Further research in the cell lines K562-R and LAMA-R which exhibit BCR-ABL independent IM resistance has shown that activation of LYN plays a functional role in the up-regulation of the anti-apoptotic protein BCL-2 (Dai et al., 2004). **Figure 4-10** shows the signalling pathways between BCR-ABL, the SRC kinases and BCL-2.

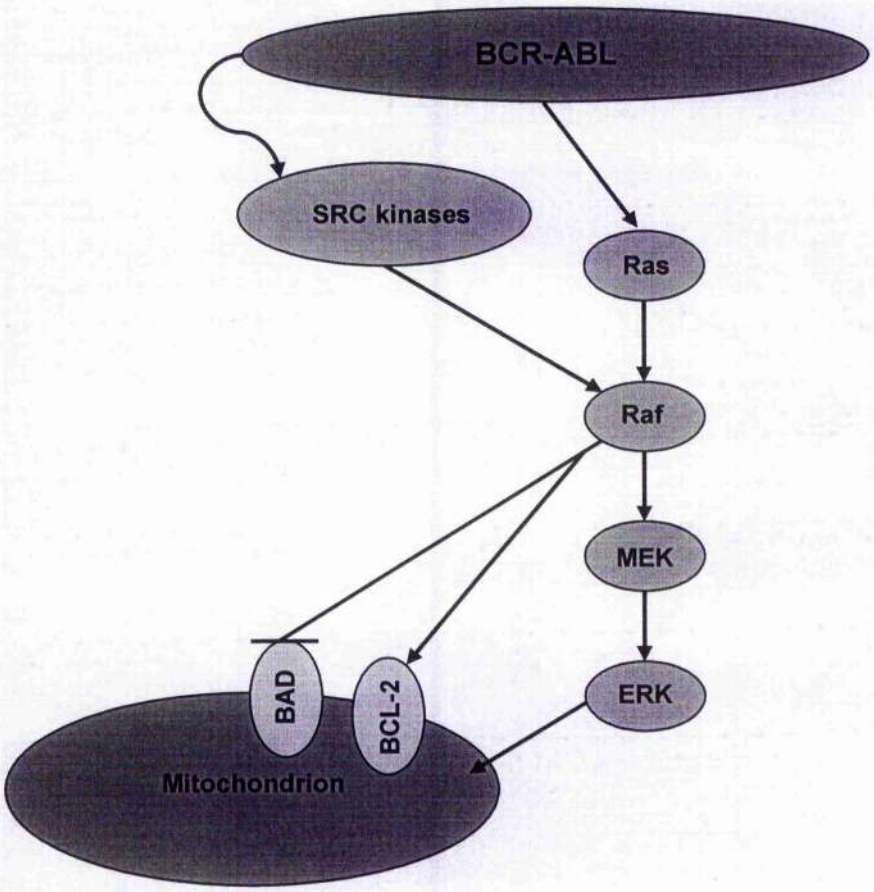


Figure 4-10. Simplified schematic diagram of the signalling pathways showing the indirect relationship between the SRC kinases and the anti-apoptotic protein BCL-2.

In view of the results of these studies which have concentrated on SRC kinase expression in CML cell lines and BC CML, I undertook further research to assess levels of BCL-2, LYN and HCK in primary CML cells before and after treatment

with IM or dasatinib *in vitro* to determine if SRC family kinases are a relevant target in CP CML. I was assisted in some of these experiments by Miss Emma Hamill. The aims of this research section were to answer the following questions:

1. Is p-SRC (used to measure active LYN and HCK) and BCL-2 protein expression increased in $CD34^+$ CML cells at the different disease stages (CP, AP, BC) compared to normal $CD34^+$ cells?

2. Does treatment with TKIs (IM, dasatinib or the SRC kinase inhibitor PP2) alter expression of p-SRC or BCL-2?

3. Is there a difference in p-SRC and BCL-2 expression in the different phases of CML?

4.1.4.1 Expression of p-SRC and BCL-2 in $CD34^+$ cells from the different phases of CML compared to normal $CD34^+$ cells

Protein expression of p-SRC and BCL-2 was assessed in primary $CD34^+$ CML cells from patients in different phases of the disease using intracellular flow cytometry techniques. A p-SRC antibody was used as this was capable of measuring phosphorylation status of all members of the SRC kinase family (SRC, LYN, HCK, FGR etc.) which are all phosphorylated on the same tyrosine residue (Y416). Phosphorylated SRC and BCL-2 were measured in normal $CD34^+$ cells (n=3), CP (n=5) AP (n=2) and BC CML (n=5) $CD34^+$ cells (**Figure 4-11A** and **B**).

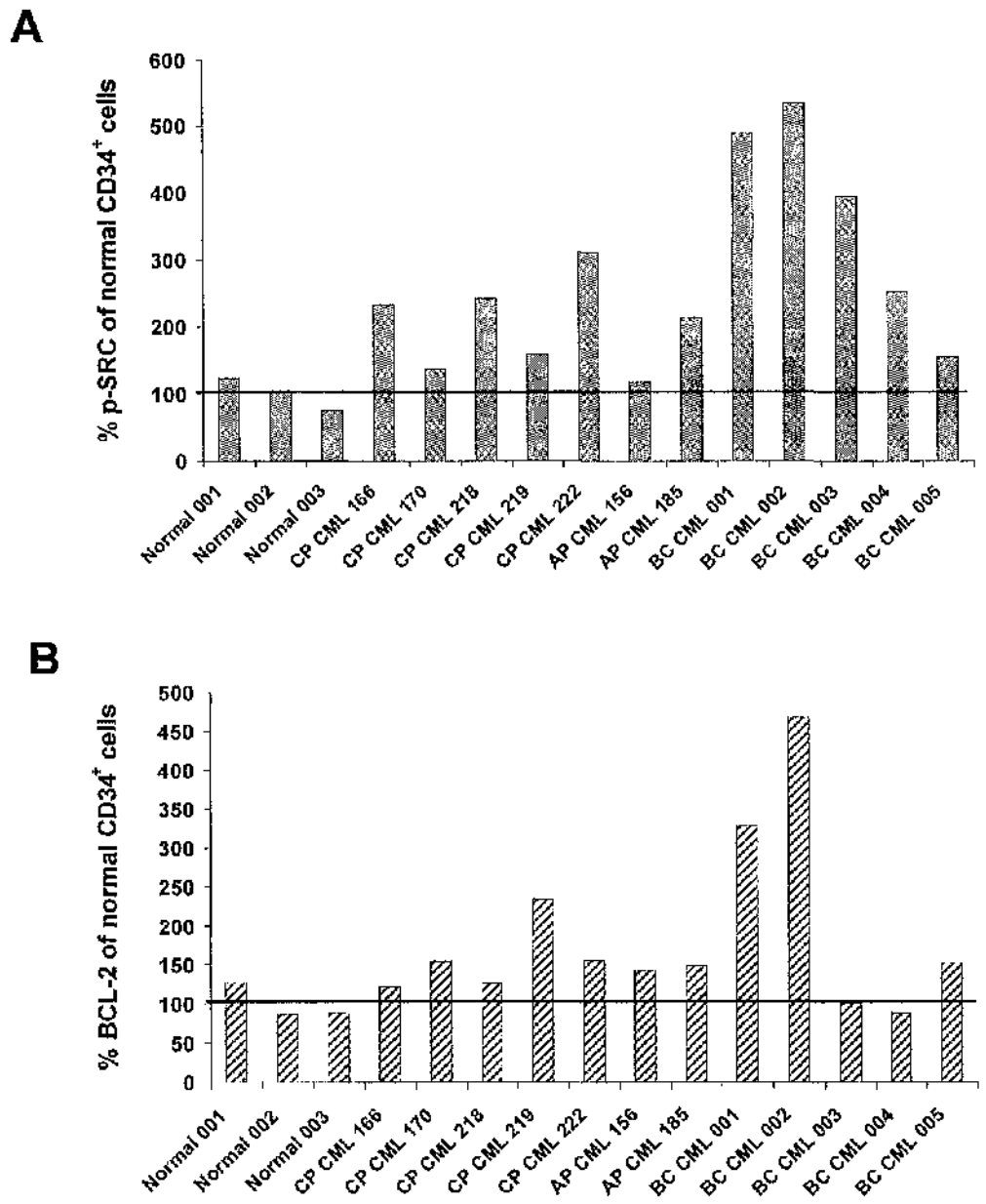


Figure 4-11. Assessment of p-SRC and BCL-2 protein expression in CD34$^+$ cells from normal donors and patients with CP, AP and BC CML. (A) p-SRC expression is increased in CD34$^+$ CML cells compared to normal CD34$^+$ cells (P=0.002 for CML versus normal). (B) BCL-2 expression is increased in the different phases of CML compared to normal CD34$^+$ cells (P=0.029 for CML versus normal). Results are expressed as a percentage of p-SRC or BCL-2 expression in the normal CD34$^+$ cells (average of 3 normal samples).

Compared to normal CD34$^+$ cells, CP and BC CD34$^+$ CML cells showed significantly increased levels of p-SRC (P=0.02 and P=0.022, respectively; **Figure 4-11A**). There was also a trend towards higher p-SRC levels in the BC compared

to CP samples. Due to low sample numbers (n=2), AP CD34$^+$ CML cells failed to show statistical significance, but demonstrated a trend towards increased p-SRC compared to normal CD34$^+$ cells. There was a trend towards increased BCL-2 expression in each of the three phases of CML compared to normal CD34$^+$ cells, however results failed to reach statistical significance. As a group CD34$^+$ CML cells showed increased BCL-2 expression compared to normal CD34$^+$ cells (P=0.029). There was marked inter-patient variability in expression of both p-SRC and BCL-2. **Figure 4-12A** and **B** show FACS histograms for the different phases of CML for p-SRC and BCL-2, respectively.

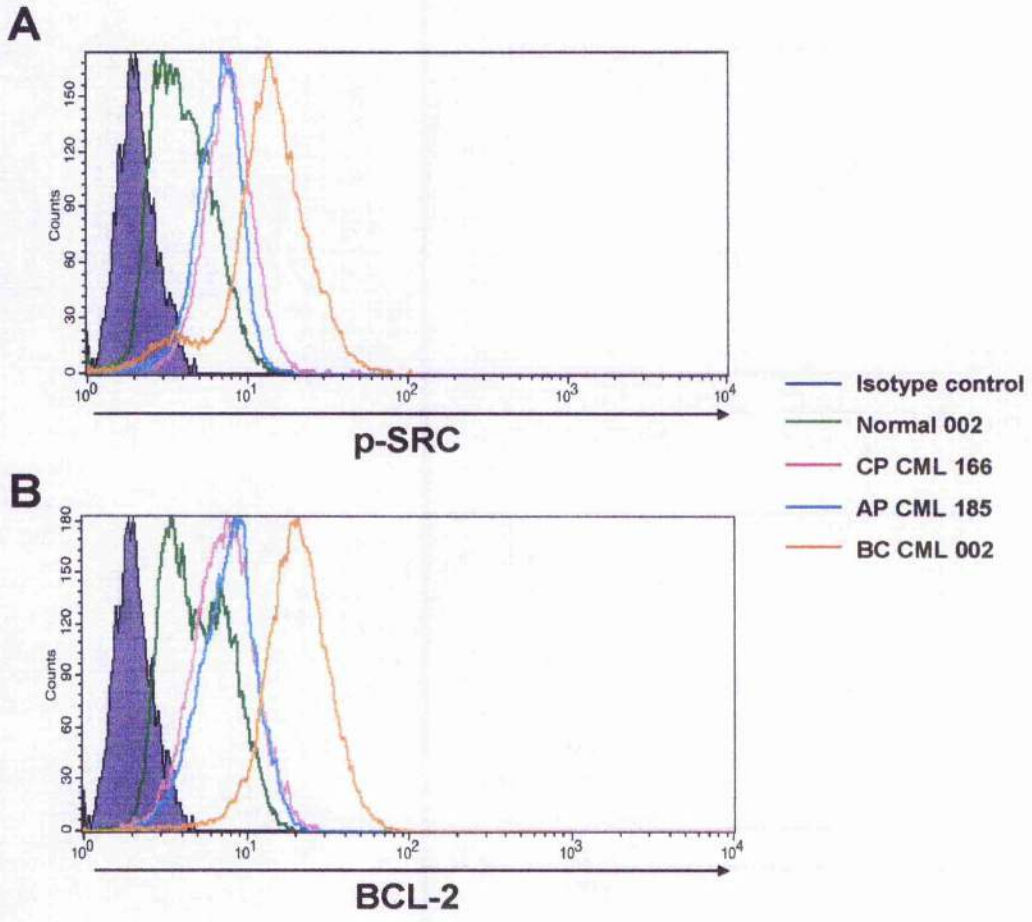


Figure 4-12. Representative FACS histogram plots of (A) p-SRC and (B) BCL-2 in CD34$^+$ cells from normal donors and patients with different phases of CML.

Using three-colour flow cytometry, I went on to assess the expression of p-SRC and BCL-2 in the more primitive CD34$^+$38$^-$ sub-population from CP, AP and BC

CML cells compared to normal $CD34^+38^-$ cells. The results are shown in **Figure 4-13A** and **B.**

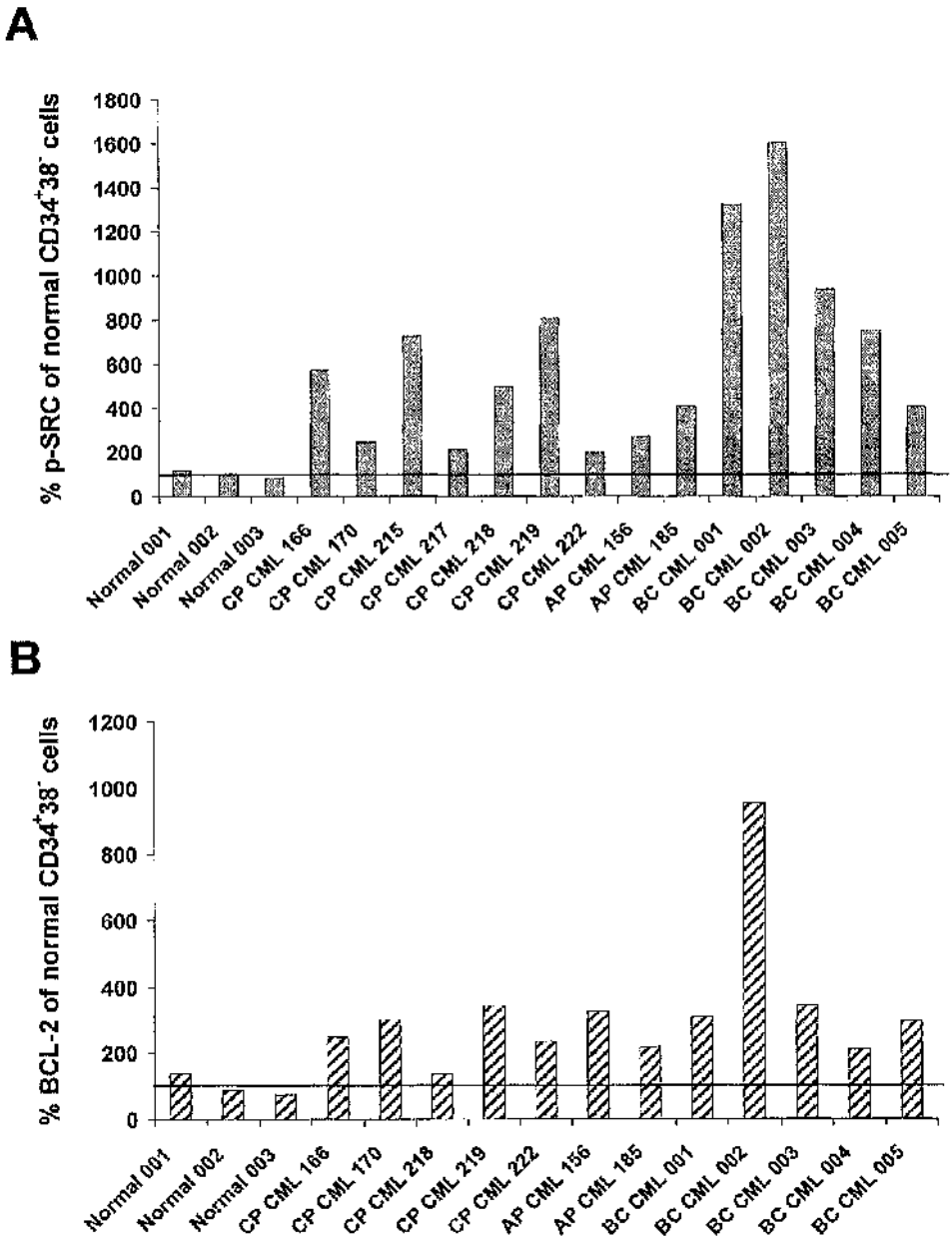


Figure 4-13. Assessment of p-SRC and BCL-2 protein expression in $CD34^+38^-$ cells from normal donors and patients with CP, AP and BC CML. (A) p-SRC expression is increased in $CD34^+38^-$ CML cells compared to normal $CD34^+38^-$ cells (P=0.001 for CML versus normal). (B) BCL-2 expression is increased in $CD34^+38^-$ CML cells compared to normal $CD34^+38^-$ cells (P=0.004 for CML versus normal). Results are expressed as a percentage of p-SRC or BCL-2 expression in the normal $CD34^+38^-$ cells (average of 3 normal samples).

As with total $CD34^+$ cells, CP and BC $CD34^+38^-$ cells CML cells showed significantly increased levels of p-SRC (P=0.032 and P=0.013, respectively; **Figure 4-13A**) in comparison to normal $CD34^+38^-$ cells. Again, there was also a trend towards higher p-SRC levels in the BC compared to CP samples. Due to low sample numbers (n=2), AP $CD34^+38^-$ CML cells failed to show statistical significance, but demonstrated a trend towards increased p-SRC compared to normal $CD34^+38^-$ cells. In the primitive $CD34^+38^-$ sub-population BCL-2 expression was also significantly increased in CP and BC compared to normal $CD34^+38^-$ cells (P=0.012 for both; **Figure 4-13B**). Once more, there was marked inter-patient variability in expression of both p-SRC and BCL-2 in the $CD34^+38^-$ sub-population.

These results show, for the first time, that p-SRC expression is increased in CP CML and may be a relevant target for therapy. However, it is unclear whether this increase in activated SRC is BCR-ABL-dependent or independent because, as well as acting as downstream modulators of BCR-ABL signalling, SRC family kinases can also be directly activated by haemopoietic growth factors via cell surface receptors (Corey and Anderson, 1999). In addition, these results indicate that BCL-2 is up-regulated in CML cells and may be contributing to the anti-apoptotic phenotype of CML.

Interestingly, compared to the total $CD34^+$ cell population, absolute expression levels were lower in the more primitive $CD34^+38^-$ sub-population in all sample groupings (**Figure 4-14A-C**). However, both p-SRC and BCL-2 expression were proportionately much higher in $CD34^+38^-$ CML cells compared to normal $CD34^+38^-$ cells (**Figure 4-13 A** and **B**). Overall, p-SRC was 3-fold and 6.4-fold increased and BCL-2 was 1.7-fold and 2.8-fold increased in $CD34^+$ and $CD34^+38^-$ populations, respectively, compared to normal $CD34^+$ and $CD34^+38^-$ CML populations. A possible explanation for this is that p-SRC and BCL-2 are increased as a direct

result of higher BCR-ABL levels in the primitive $CD34^+38^-$ sub-population (**Figure 4.9**). This relative over-expression of p-SRC and BCL-2 in $CD34^+38^-$ CML cells compared to normal $CD34^+38^-$ cells may contribute to their relative IM resistance.

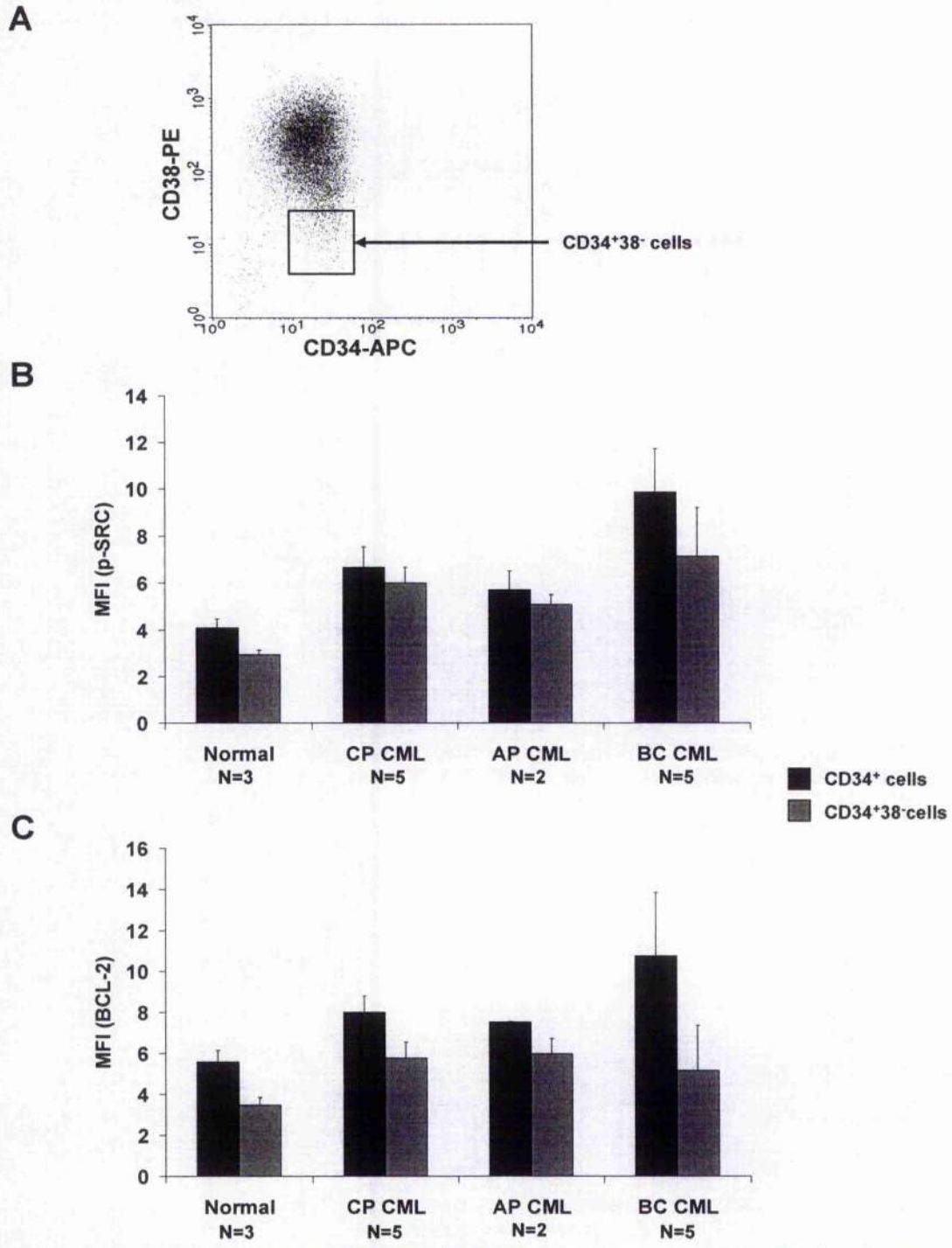


Figure 4-14. Comparison of p-SRC and BCL-2 expression in total $CD34^+$ cells and more primitive $CD34^+38^-$ cells. (A) Representative FACS dot plot highlighting the primitive $CD34^+38^-$ sub-population. (B) and (C) Histograms showing the higher p-SRC (B) and BCL-2 (C) expression in total $CD34^+$ compared to the more primitive $CD34^+38^-$ sub-population. MFI, mean fluorescence intensity.

4.1.4.2 Assessment of the effects of IM, dasatinib or PP2 on p-SRC and BCL-2 expression in CD34$^+$ CML cells in CP and BC CML

Having established that the expression of p-SRC kinases was increased in CML, I went on to determine the effects of IM, dasatinib and the SRC specific kinase inhibitor PP2 on the expression of p-SRC and BCL-2 in CP (n=4) and BC (n=2) CML.

4.1.4.2.1 Assessment of IC_{50} for the SRC kinase inhibitor PP2 in CD34$^+$ CML cells

PP2 has been described as a potent pyrrolo-pyrimidine inhibitor of the SRC family of tyrosine kinases (Hanke et al., 1996). Concentration finding studies to determine the IC_{50} for PP2 in primary CD34$^+$ CML cells (n=3) were performed using ^{3}H thymidine cell proliferation assays and total viable cell counts with assessment after 72 hours in culture in SFM only. PP2 had an IC_{50} of approximately 7μM as demonstrated by ^{3}H thymidine proliferation assay and confirmed by total viable cell counts in primary CD34$^+$ CML cells (**Figure 4-15**). These results are similar to those obtained by another group (Wilson et al., 2002) who reported an IC_{50} of 10μM in the K562 cell line, but did not undertake experiments in primary CML cells.

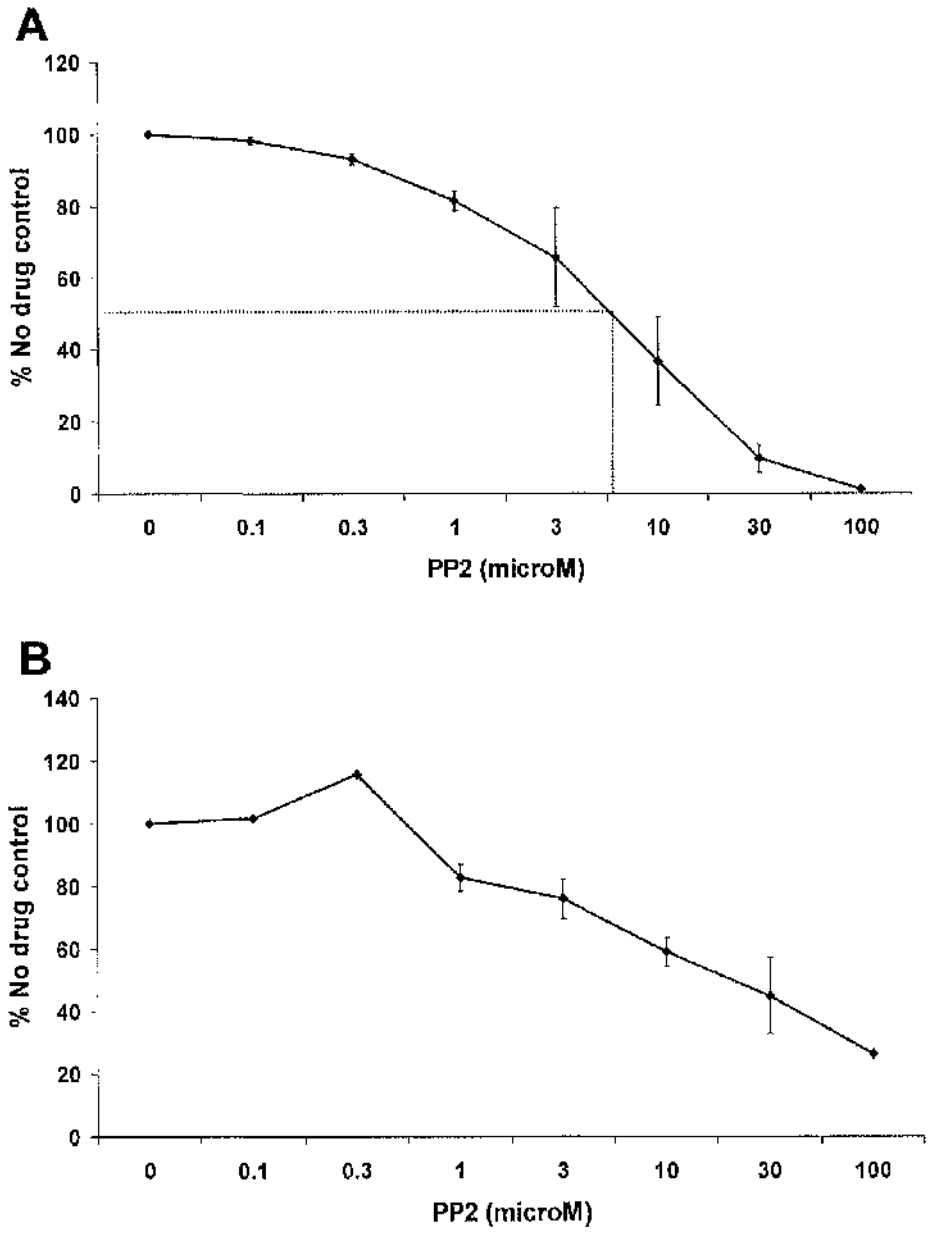


Figure 4-15. Determination of IC_{50} for PP2 using ^{3}H thymidine proliferation assays and total viable cell counts in primary $CD34^{+}$ CP CML cells cultured in SFM only (n=3 experiments). (A) 72 hour ^{3}H proliferation assays demonstrate that PP2 has an IC_{50} of approximately 7μM in primary $CD34^{+}$ CML cells. Five replicates of each condition were performed in each experiment. (B) 72 hour total viable cell counts confirm the proliferation assay results. Results represent the mean ± SEM.

Based on these results, PP2 was used at a concentration of 10μM in subsequent experiments.

4.1.4.2.2 Effect of IM, dasatinib and PP2 on p-SRC and BCL-2 expression in $CD34^+$ and $CD34^+38^-$ CML cells

The effects of IM, dasatinib and PP2 on p-SRC and BCL-2 protein expression were assessed by intracellular flow cytometry after 16 hours and 72 hours culture. Compared to the no drug control (n=6), at 16 hours, both IM and dasatinib significantly reduced p-SRC expression in $CD34^+$ and the more primitive $CD34^+38^-$ CML cells (P=0.001 for no drug control versus IM in both $CD34^+$ and $CD34^+38^-$ populations; and P<0.001 and P=0.003 for no drug control versus dasatinib in $CD34^+$ and $CD34^+38^-$ cells, respectively; **Figure 4-16A**). At 72 hours, there was a trend towards reduced p-SRC in the total $CD34^+$ cells with both IM and dasatinib, however this did not reach statistical significance. In the $CD34^+38^-$ cells at 72 hours, only dasatinib significantly reduced p-SRC compared to no drug control (P=0.001; **Figure 4-16B**). In $CD34^+$ and $CD34^+38^-$ CML cells at both 16 and 72 hours, PP2 had no significant effect on SRC phosphorylation status. This suggests that the majority of activated SRC kinase expression in CML is being mediated via BCR-ABL and is not BCR-ABL-independent. A possible explanation for the inability of the SRC inhibitor PP2 to significantly reduce p-SRC is that, if BCR-ABL is stimulating p-SRC, then in the presence of high levels of BCR-ABL there may simply be too much p-SRC being produced to be inhibited by PP2. In the future it would be important to combine IM and PP2 to determine if the effect of this combination is equivalent to that of the dual SRC/BCR-ABL inhibitor dasatinib on p-SRC and BCL-2 expression in primary CML cells.

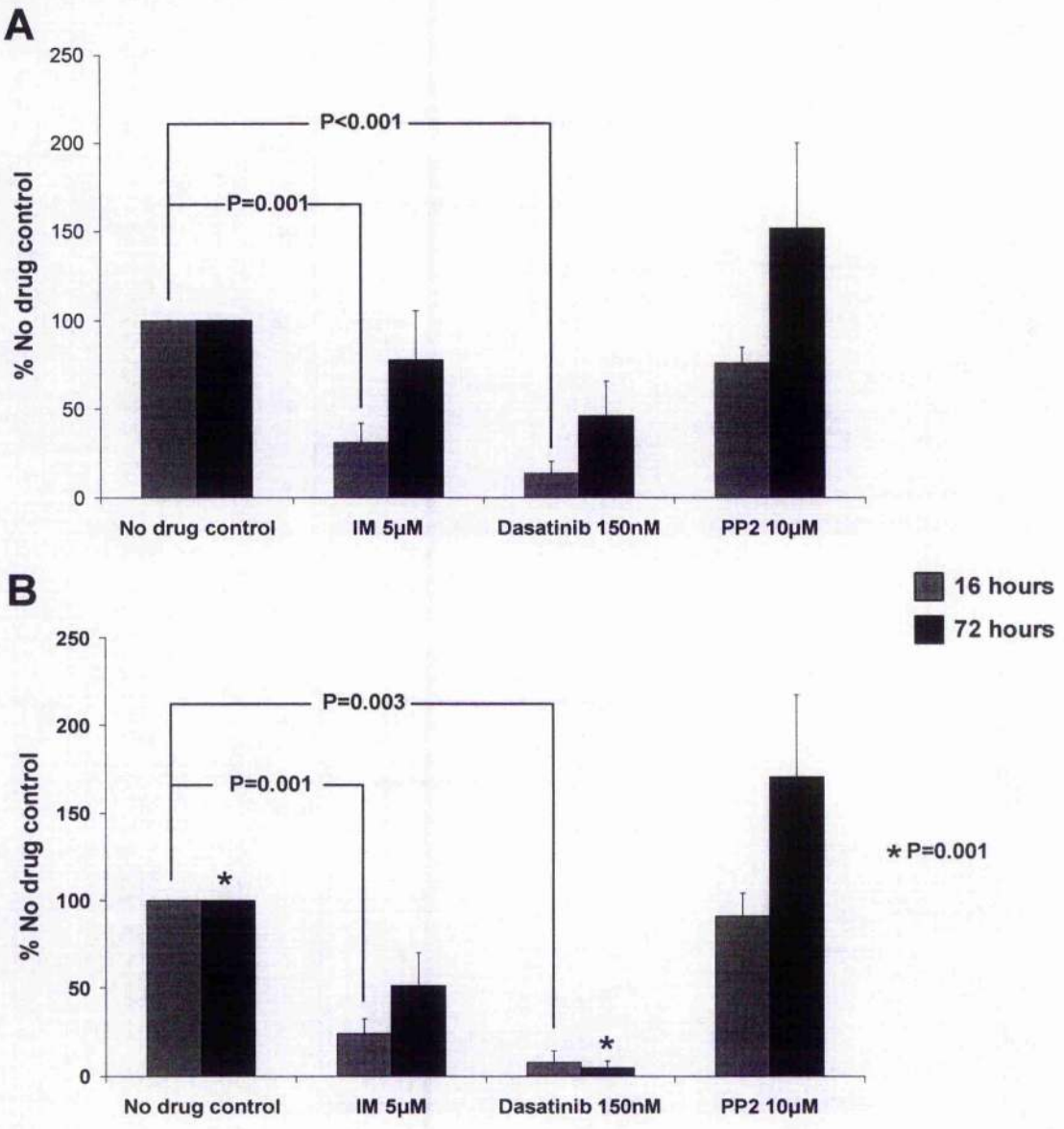


Figure 4-16. The effect of IM, dasatinib and PP2 on p-SRC expression in (A) total $CD34^+$ and (B) more primitive $CD34^+38^-$ CML cells at 16 hours and 72 hours (n=6; 4 CP, 2 BC). Results are expressed as a percentage of the no drug control (± SEM). There was no difference between CP and BC CML in the samples assessed.

At both 16 and 72 hours, compared to no drug control, IM, dasatinib or PP2 did not significantly inhibit expression of BCL-2 in $CD34^+$ CML cells (**Figure 4-17A**). However, in the primitive $CD34^+38^-$ cells, there was a modest reduction in BCL-2 with all treatment arms compared to no drug control (**Figure 4-17B**). This was only significant with dasatinib at 16 hours (P=0.021) but not 72 hours (P=0.061) and

suggests that BCL-2 may be more relevant in the more primitive $CD34^{+}38^{-}$ CML cell population in keeping with the higher BCL-2 expression in these cells relative to normal $CD34^{+}38^{-}$ cells.

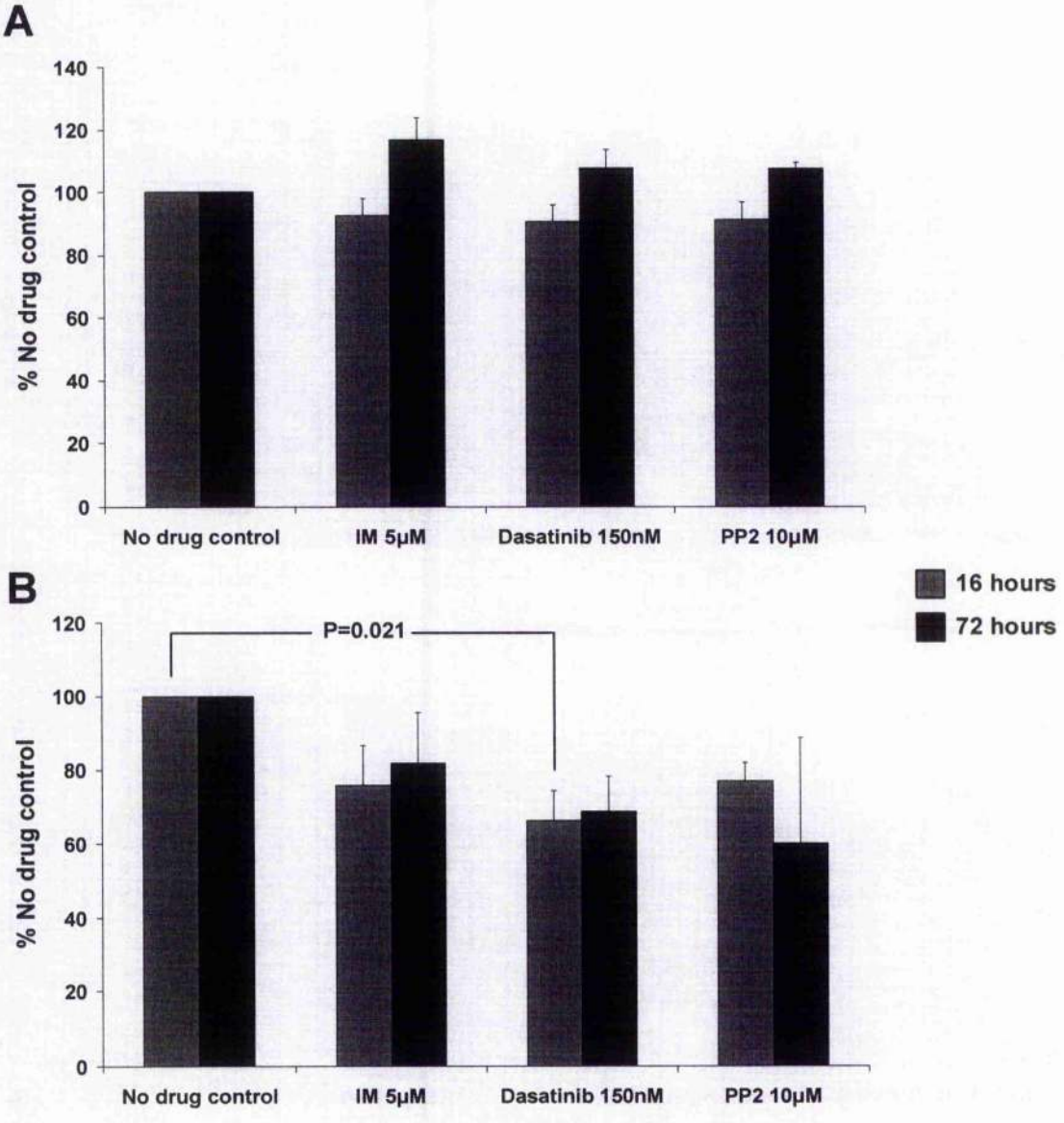


Figure 4-17. The effect of IM, dasatinib and PP2 on BCL-2 expression in (A) total $CD34^{+}$ and (B) more primitive $CD34^{+}38^{-}$ CML cells at 16 hours and 72 hours (n=6; 4 chronic phase, 2 blast crisis). Results are expressed as a percentage of the no drug control (± SEM). In total $CD34^{+}$ cells, there were no significant differences between the experimental arms at either 16 or 72 hours. However in the $CD34^{+}38^{-}$ cells, at 16 hours, any treatment (IM, dasatinib, PP2) versus no treatment significantly reduced BCL-2 expression (P=0.002). No significant differences in BCL-2 expression were present at 72 hours in the $CD34^{+}38^{-}$ population. There was no difference between CP and BC CML in the samples assessed.

The discrepancy between p-SRC and BCL-2 expression after treatment with IM or dasatinib may suggest that, in CML, BCL-2 expression is not mediated by LYN as previously proposed (Dai et al., 2004). Alternatively, BCL-2 is known to have a long half-life (Merino et al., 1994) and may be more stable and not degraded over the culture period compared to p-SRC, which, because of its phosphorylated state has a short half-life. Furthermore, in this study, total BCL-2 and not phosphorylated BCL-2 was assessed. Recent evidence suggests that the anti-apoptotic functions of BCL-2 may be controlled by its phosphorylation (Blagosklonny, 2001), with phosphorylation of BCL-2 appearing to inhibit its anti-apoptotic function (Haldar et al., 1995) The phosphorylation status of BCL-2 can be altered by chemotherapeutic agents and cytokines, but was not assessed here.

4.1.5 Summary and conclusions

The multi-targeted kinase inhibitor, dasatinib has an IC_{50} of ~5nM in $CD34^+$ CML cells *in vitro*. Cell line studies confirm that dasatinib selectively targets BCR-ABL^+ haemopoietic cells.

In vitro, IM and dasatinib have equivalent cytotoxicity on primary $CD34^+$ CML cells at high therapeutically achievable concentrations (5μM versus 150nM, respectively). The simultaneous administration of IM and dasatinib further enhances the cytotoxic effect. However, there is no advantage to sequential treatment with these agents.

Neither IM nor dasatinib, alone or in combination, reduced the non-proliferating $CFSE^{max}$ $CD34^+$ CML cell population compared to the no drug control. However, there was a trend for IM to be more anti-proliferative than dasatinib. Apoptosis studies using caspase-3 activity demonstrated that those cells leaving the undivided $CFSE^{max}$ $CD34^+$ gate did so by cell division and not apoptosis. Further

studies using p-CrKL expression to measure BCR-ABL activity demonstrated that dasatinib was a more potent inhibitor of BCR-ABL than IM.

Activated SRC kinases as measured by p-SRC and BCL-2 expression were significantly increased in $CD34^+$ and $CD34^+38^-$ CML cells compared to normal $CD34^+$ and $CD34^+38^-$ cells. There was a tendency for p-SRC, but not BCL-2 to be higher in BC than CP CML. Imatinib mesylate or dasatinib, but not PP2, reduced p-SRC in CML; however as with p-CrKL expression, the effect was only transient with IM, suggesting that p-SRC expression is mediated by BCR-ABL. Imatinib mesylate, dasatinib and PP2 had only modest effects on BCL-2 expression, suggesting that either BCL-2 expression may be independent of both BCR-ABL and SRC kinases or BCL-2 is a more stable molecule and changes in BCL-2 expression take longer to become evident. In addition, in future studies, it would be useful to assess BCL-2 phosphorylation as well as total BCL-2 levels to determine the amount of active BCL-2 present and the importance of BCL-2 phosphorylation in CML.

However, as shown in Section **4.1.3.2**, the most important finding of this study was that, although dasatinib treatment resulted in a smaller non-proliferating $CFSE^{max}$ $CD34^+$ CML cell population than IM, it did not eradicate this population. Therefore, in overall conclusion, these results suggest that neither BCR-ABL nor SRC kinases are relevant targets in the most primitive stem cell populations in CP CML.

5 Results 3

5.1 The novel FTI BMS-214662 targets quiescent CML stem and progenitor cells and synergises with imatinib or dasatinib *in vitro*

5.1.1 Introduction

Two main strategies to overcome IM resistance have emerged. The first is the development of second generation TKIs such as dasatinib and nilotinib (Shah et al., 2004; O'Hare et al., 2005; Weisberg et al., 2005; Kantarjian et al., 2006; Talpaz et al., 2006) and the second, the use of IM in novel drug combinations (Holtz et al., 2005; Jorgensen et al., 2005a).

The results of Chapter 4 demonstrated that, although dasatinib induced durable inhibition of BCR-ABL tyrosine kinase activity in primitive CML cells as compared to either IM or nilotinib which were significantly less effective (Copland et al., 2006; Jorgensen et al., 2007), neither of these agents targeted the most primitive, quiescent CML stem cell population. A wide range of drug combinations with IM, including arsenic, Ara-C, the heat-shock protein-90 antagonist 17AAG, and the PI3K inhibitor LY294002, have been investigated (Holtz et al., 2005; Jorgensen et al., 2005a) in $CD34^+$ CML progenitor cells. However, the only agent to enhance the activity of IM against the quiescent CML stem cell fraction was the cytostatic FTI lonafarnib (Jorgensen et al., 2005a), but the results failed to reach statistical significance.

As discussed in the introduction (**1.3.3.1**), FTIs are a novel class of small molecules developed to inhibit oncogenic Ras and have entered clinical trials in

both solid tumours and acute leukaemias (Adjei et al., 2000; Cortes et al., 2003; Cortes et al., 2005a; Tabernero et al., 2005). BMS-214662 is a cytotoxic FTI (Hunt et al., 2000; Rose et al., 2001; Manne et al., 2004) that has produced potent tumour regression and curative responses in selected human tumour xenografts and transgenic tumour models, and differs from other cytostatic FTIs such as lonafarnib and R115777 which had non-curative activity (Liu et al., 1998; End et al., 2001). In addition, BMS-214662 has been shown to preferentially kill non-proliferating cells (Lee et al., 2001) and has anti-leukaemic activity in AML (Cortes et al., 2005a).

In view of the cytotoxic effect of BMS-214662 on non-proliferating cells in tumour models (Lee et al., 2001), and the promising results obtained previously with lonafarnib (Peters et al., 2001a; Hoover et al., 2002; Nakajima et al., 2003; Jorgensen et al., 2005a) and tipifarnib (Miyoshi et al., 2005), it was hypothesised that this small molecule inhibitor might target the quiescent CML stem cell population which has been shown to be resistant to IM, dasatinib and nilotinib (Copland et al., 2006; Jorgensen et al., 2007) and, indeed, may synergise with these drugs which induce potent anti-proliferative effects within the stem cell compartment in CML. Therefore, the aims of this study were to determine the effects of BMS-214662 alone and in combination with IM or dasatinib on the primitive quiescent CML stem cell population *in vitro*. In addition, the cytotoxic effects of BMS-214662 in BC CML and cell lines expressing BCR-ABL kinase domain mutations were assessed.

5.1.2 Assessment of IC_{50} for BMS-214662 in primary $CD34^+$ CML cells

Concentration finding studies to determine the IC_{50} for BMS-214662 in primary $CD34^+$ CML cells were performed using ^3H thymidine cell proliferation assays and

counting of total viable cells. Results were assessed after 72 hours culture in SFM alone. BMS-214662 had an IC_{50} of approximately 375nM after 72 hours culture in $CD34^+$ CML cells as determined by ^3H thymidine proliferation assays (n=3; **Figure 5-1A**). Interestingly, cell counting showed a 50% reduction in total viable cells at the lower BMS-214662 concentration of 250nM (**Figure 5.1B**). These results show that BMS-214662, when used as a single agent, is extremely potent against $CD34^+$ CML cells. Based on these results, a dose of 250nM BMS-214662 was chosen for all further experiments in this study. Previous *in vivo* studies have shown that a BMS-214662 plasma concentration of 250nM is readily achievable in patients (Tabernero et al., 2005; Eder et al., 2006).

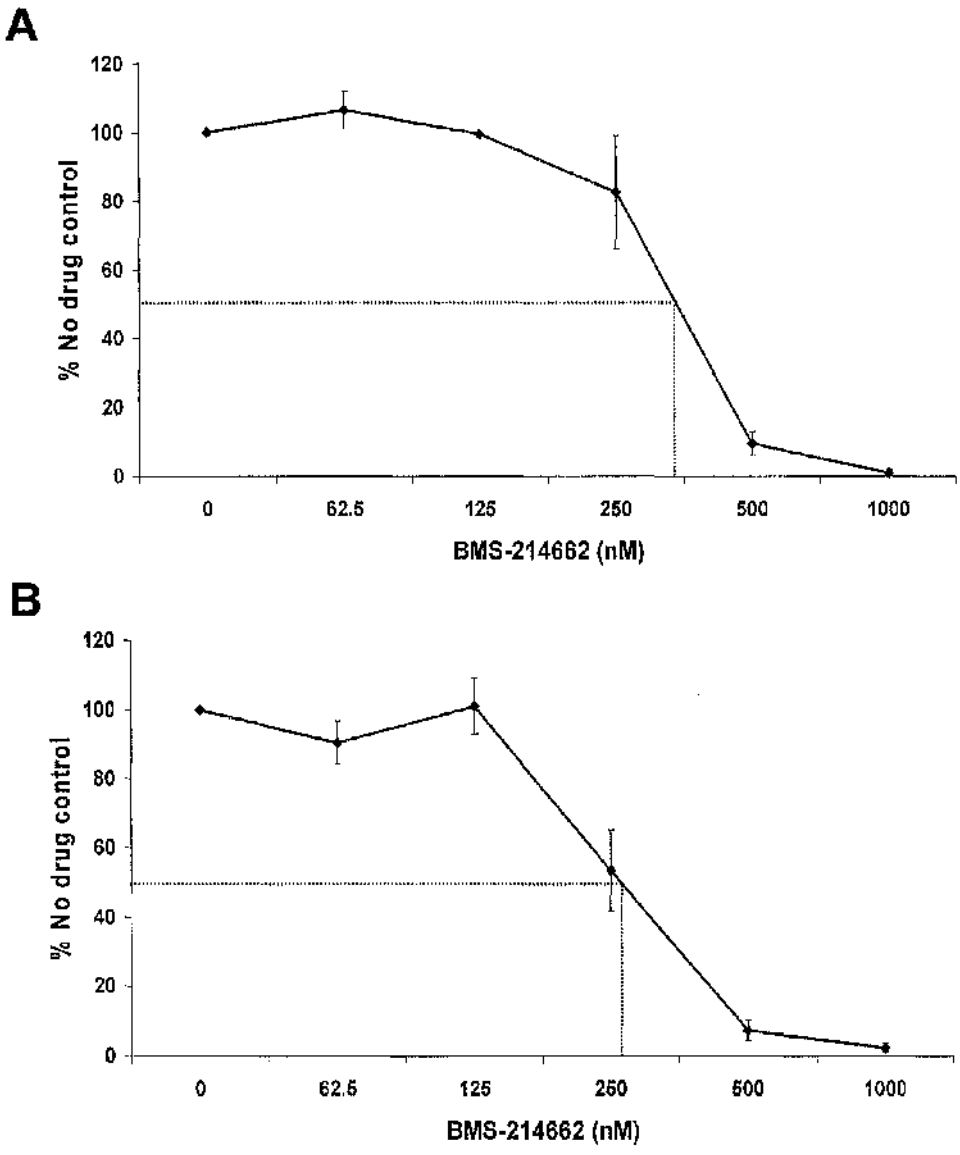


Figure 5-1. Determination of IC_{50} for BMS-214662 by (A) ^{3}H thymidine proliferation assay (n=3) and (B) total viable cell counts performed after 72 hours culture in SFM. ^{3}H thymidine proliferation assays show an IC_{50} of approximately 375nM and total viable cell counts 250nM. Five replicates of each condition were performed in each experiment. Results represent the mean (± SEM).

5.1.3 Determination of the efficacy of BMS-214662 alone or in combination with IM or dasatinib in CD34$^+$ CML cells after short-term culture

5.1.3.1 Assessment of total viable cell counts

To assess the cytotoxic effect of BMS-214662 alone and in combination with IM or dasatinib, primary CD34$^+$ CML cells (n=4) were cultured for 6 days in SFM + 5GF. Once again, the CD34$^+$ CML cells for these experiments were cultured in 5GF to aid identification of the quiescent progenitor population in the CFSE experiments described next (**5.1.3.2**). After 72 hours, an aliquot of cells was removed for counting; the remaining cells were then washed and set up again in culture for a further 72 hours. The conditions studied were: (1) No drug control; (2) BMS-214662 250nM; (3) IM 5μM; (4) BMS-214662 250nM + IM 5μM; (5) dasatinib 150nM and (6) BMS-214662 250nM + dasatinib 150nM. The drug concentrations for BMS-214662, IM and dasatinib were chosen on the basis that these were concentrations which were clinically achievable.

After 3 days culture, there was a significant reduction in total viable cells in the treatment arms compared to the no drug control (P=0.04; **Figure 5-2A**). Also, the combination of BMS-214662 + dasatinib showed a significantly greater cytotoxic effect than dasatinib alone (P=0.026) and the combination of BMS-214662 + IM showed a trend towards increased cytotoxic effect (P=0.055) compared to IM alone. After a total of 6 days in culture, there was a further significant reduction in total viable cells in the treatment arms relative to the no drug control (P=0.001; **Figure 5-2B**). Importantly, in addition, the combinations of BMS-214662 + IM and BMS-214662 + dasatinib showed increased cytotoxic effect over either IM or dasatinib alone (P=0.024 and P=0.034, respectively). Therefore, the addition of

BMS-214662 to either IM or dasatinib significantly enhanced the effect of these drugs *in vitro* by increasing overall cytotoxicity.

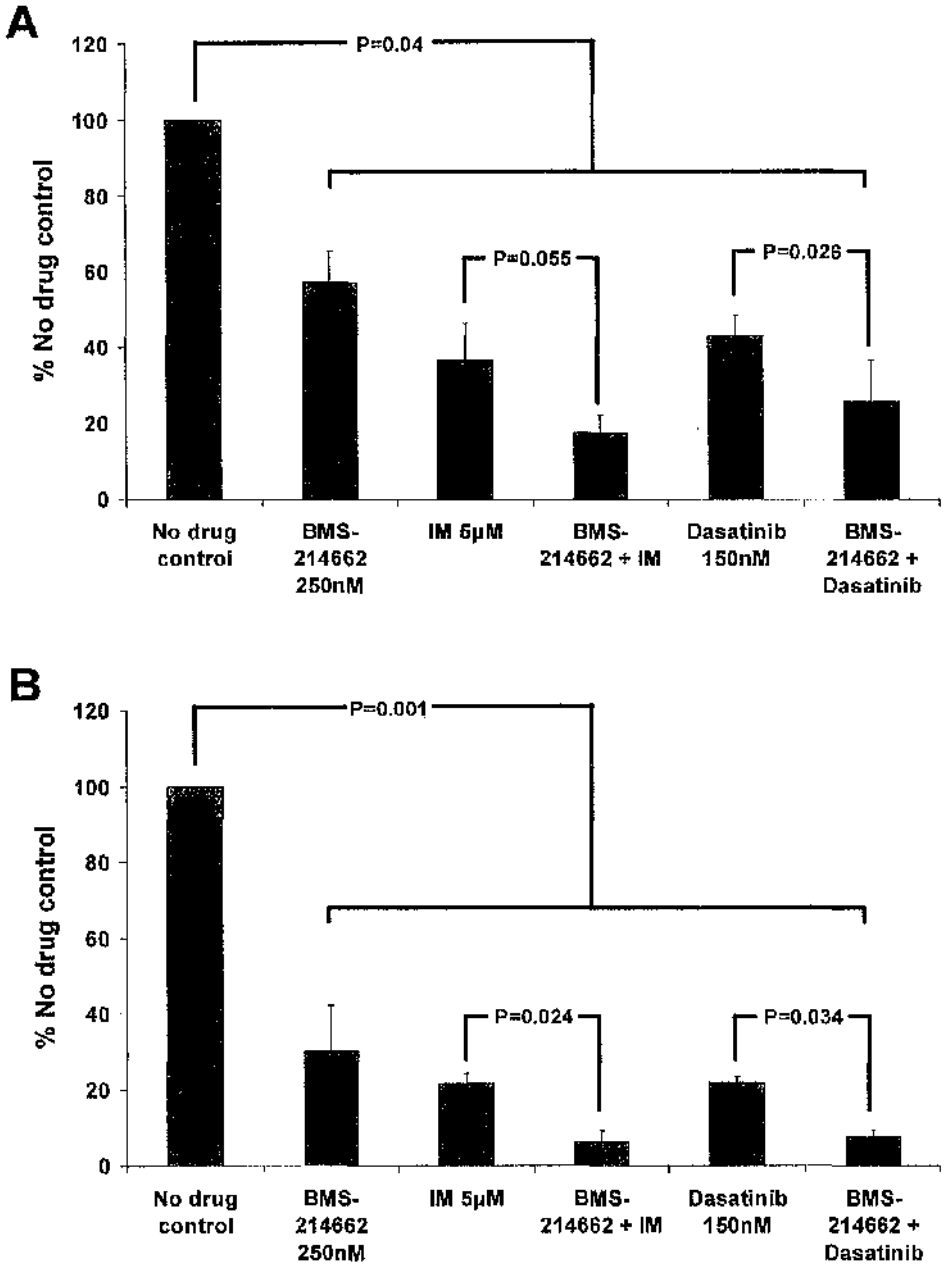


Figure 5-2. Total viable cell counts after (A) 72 hours and (B) 6 days for all experimental conditions (n=4). $CD34^+$ CML cells were cultured in SFM + 5GF. Results are expressed as a percentage of the no drug control (mean ± SEM).

5.1.3.2 The effect of BMS-214662 alone and in combination with IM or dasatinib on non-proliferating CD34$^+$ CML progenitor cells after short-term culture

Previous studies (including those in Chapter 4) have shown that both IM and dasatinib result in increased numbers of non-proliferating CD34$^+$ CML cells remaining after culture (Graham et al., 2002; Copland et al., 2006), in part due to failure to induce apoptosis of CML stem cells and also as a result of their potent anti-proliferative effects leading to reversible G_1 arrest. Therefore, we assessed the efficacy of BMS-214662 alone and in combination with IM or dasatinib in primary CD34$^+$ CML cells *in vitro*, again using CFSE-based flow cytometry to track cell division. After 3 days, there were no significant differences between the arms (**Figure 5-3A**). However, by 6 days, while the IM and dasatinib arms showed significant accumulation of non-proliferating CFSE^{max} CD34$^+$ CML cells over the no drug control (P=0.04 and P=0.023, respectively), the arms containing BMS-214662 with either IM or dasatinib showed a significant reduction in these primitive cells to 40% and 27% of the no drug control (**Figure 5-3B**; P=0.023 and P=0.005) respectively. Despite reducing the non-proliferating CFSE^{max} CD34$^+$ cell population to 48% of the no drug control, results with BMS-214662 alone just failed to reach statistical significance (P=0.06). In addition, the reduction in non-proliferating CFSE^{max} CD34$^+$ CML cells was highly statistically significant when either IM or dasatinib alone was compared to the combination with BMS-214662 (P=0.01 and P=0.043, respectively). Representative FACS plots for each of the experimental arms are shown in **Figure 5-4**.

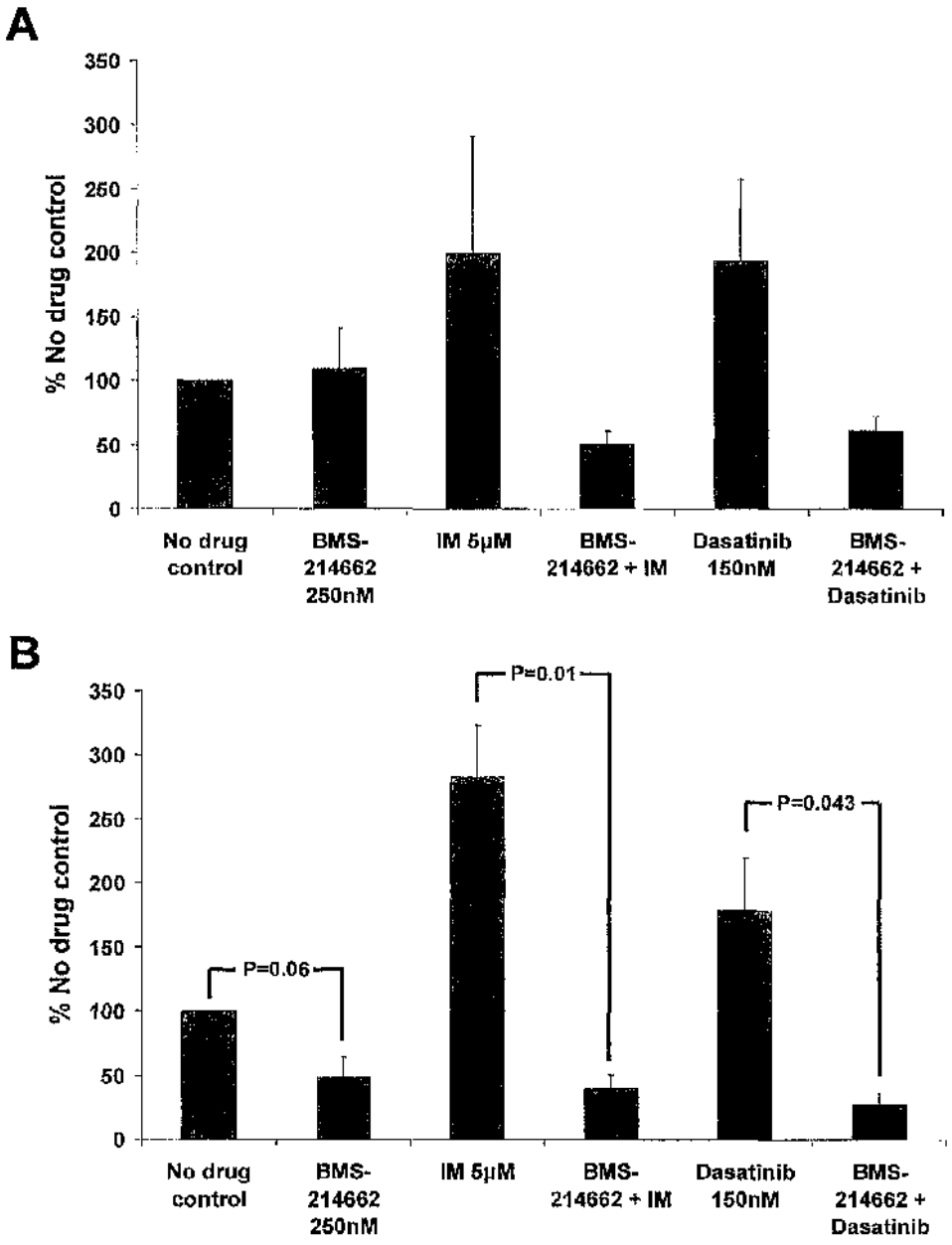


Figure 5-3. Non-proliferating CFSE^{max} CD34^{+} cells remaining after (A) 72 hours and (B) 6 days culture for all experimental conditions. Cells were cultured in SFM + 5GF. (A) There were no significant differences in non-proliferating cell numbers after 72 hours. However, by 6 days (B), compared to either IM or dasatinib alone, the combinations with BMS-214662 showed a significant reduction in non-proliferating cells (P=0.01 and P=0.043, respectively). In addition, compared to no drug control, BMS-214662 + IM and BMS-214662 + dasatinib showed a significant reduction in non-proliferating cells (P=0.023 and P=0.005, respectively). In these experiments, due to inter-patient variability in the size of the non-proliferating CFSE^{max} CD34^{+} cell population, the cells remaining after the culture period were expressed as a percentage of the starting cell number normalised to the no drug control. The absolute number of non-proliferating cell decreased over time; non-

proliferating cells in no drug control = 1.1% at 72 hours and 0.17% at 6 days. Results are expressed as a percentage of the no drug control (mean ± SEM).

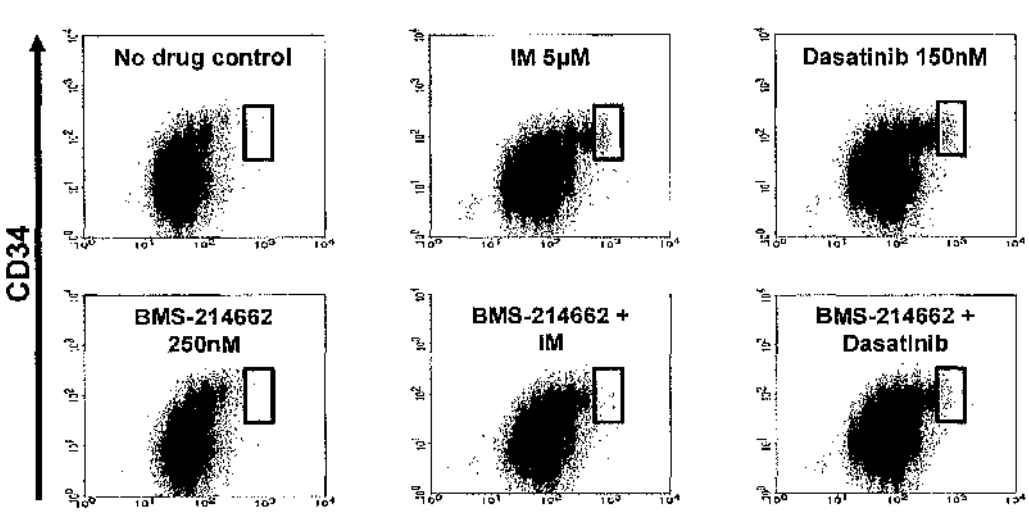


Figure 5-4. Representative FACS dot plots after 6 days culture for each of the experimental conditions. The non-proliferating CFSE^{max} CD34^{+} CML cell population is highlighted in the box in each panel. The plots demonstrate the very low number of undivided cells remaining in the no drug control and BMS-214662-containing arms compared to the relatively large populations remaining in the IM and dasatinib only arms.

The marked anti-proliferative effect of IM or dasatinib due to reversible G_1 arrest is demonstrated in **Figures 5-5A** and **B**, respectively. BMS-214662 had minimal anti-proliferative effect compared to no drug control (**Figure 5-5A** and **B**) and did not overcome the anti-proliferative effect of either IM or dasatinib, indicating that BMS-214662 is able to exert its cytotoxic effect on non-proliferating cells. There were no significant differences in non-proliferating CFSE^{max} CD34^{+} CML cells between the BMS-214662-containing arms. Very significantly, these are the first drug combinations tested using this CFSE-based method, which show a significant reduction in non-proliferating cells remaining after culture to a level below that of the no drug control (Jorgensen et al., 2005a).

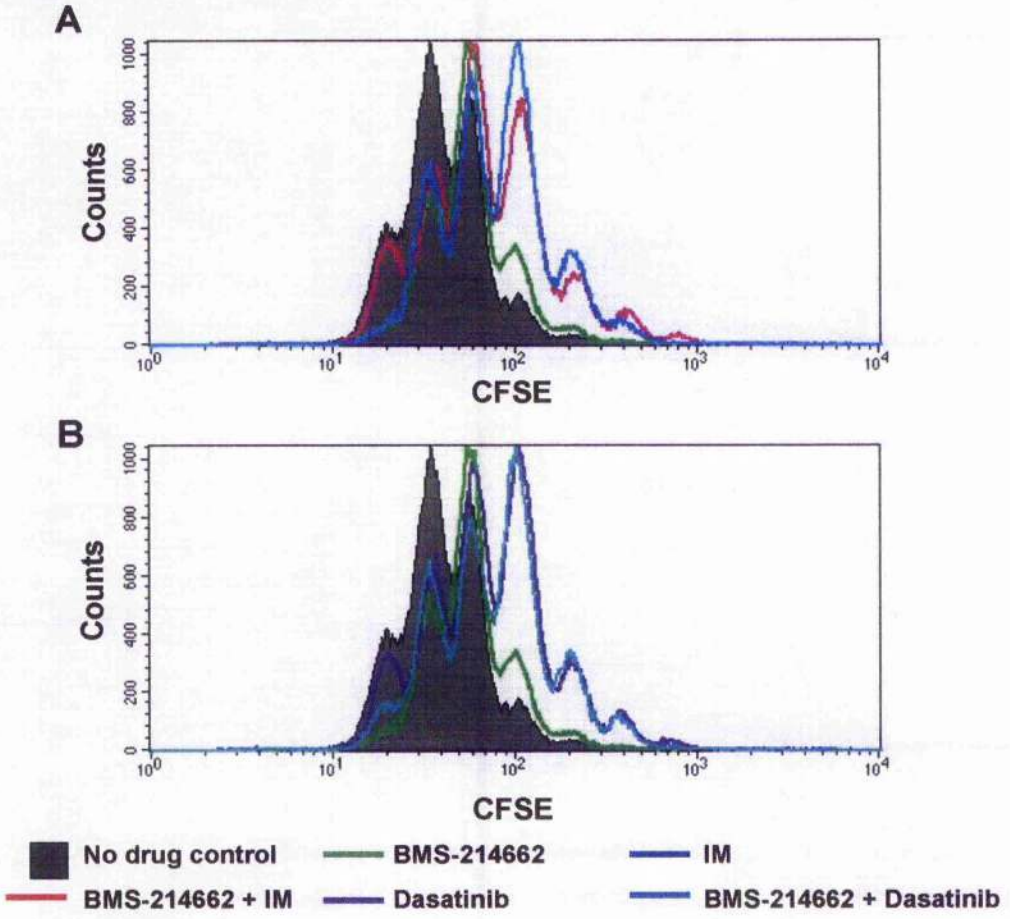


Figure 5-5. Representative overlay flow cytometry histograms demonstrating anti-proliferative effects of IM and dasatinib. (A) No drug control, BMS-214662, IM and BMS-214662 + IM, and (B) no drug control, BMS-214662, dasatinib and BMS-214662 + dasatinib. Compared to the no drug control, the cells treated with IM, BMS-214662 + IM, dasatinib and BMS-214662 + dasatinib have progressed though fewer divisions (right shift) highlighting the anti-proliferative effects of both IM and dasatinib. This anti-proliferative effect was not overcome when either IM or dasatinib was combined with BMS-214662, demonstrating that BMS-214662 exerts its cytotoxic effect on non-proliferating cells. BMS-214662 alone had minimal anti-proliferative effect compared to the no drug control.

5.1.4 Determination of the efficacy of BMS-214662 alone or in combination with IM or dasatinib in CD34$^+$ CML cells after long-term culture using LTC-IC assays

To further investigate the efficacy of BMS-214662 on functional CML stem cells, LTC-IC assays were performed with both CP CML (n=3) and normal (n=3) CD34$^+$ cells to determine drug selectivity. The CD34$^+$ cells were treated for 72 hours under the following conditions: (1) No drug control; (2) BMS-214662 250nM; (3) IM 5μM; (4) BMS-214662 + IM; (5) dasatinib 150nM; (6) BMS-214662 + dasatinib, before LTC-IC assay. Compared to the no drug control, CD34$^+$ CML cells showed increased Ph$^+$ colony formation in the IM and dasatinib arms (191 and 175%, respectively; **Figure 5-6A**; P=0.033 for IM and dasatinib arms versus no drug control), indicating that these drugs exert a protective effect on CML stem cells in culture via their anti-proliferative effect described previously (Graham et al., 2002; Copland et al., 2006). The addition of BMS-214662 to either IM or dasatinib significantly reduced the number of Ph$^+$ colonies compared to either agent alone (P=0.032 and P=0.027, respectively). Furthermore, the BMS-214662 only arm showed a significant reduction in Ph$^+$ colonies compared to either IM or dasatinib alone (P=0.032 and P=0.028, respectively). All three BMS-214662-containing arms showed a virtual elimination of colonies to <1% of the number in the no drug control (P=0.033), with no significant difference between the arms. **Figure 5-7** shows examples of the CFU-GM colonies produced in the committed progenitor cell assays following LTC-IC.

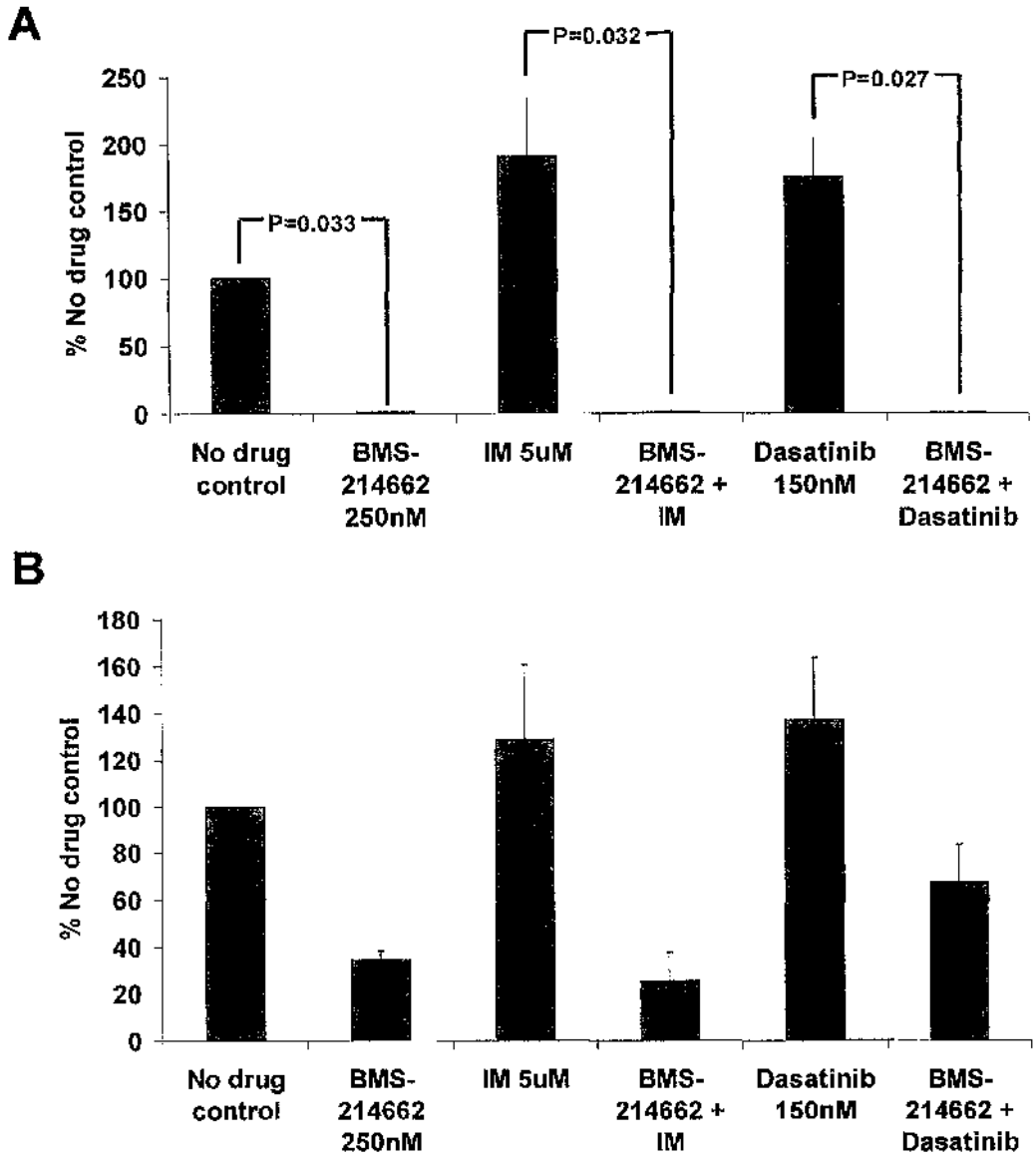


Figure 5-6. Results of LTC-IC assay for (A) $CD34^+$ CML cells and (B) normal donor $CD34^+$ cells treated under the experimental conditions described. (A) The addition of BMS-214662 to either IM or dasatinib significantly reduced the number of Ph^+ colonies produced from $CD34^+$ CML cells compared to either drug alone (P=0.032 and P=0.027, respectively). BMS-214662 alone also significantly reduced the Ph^+ colonies compared to the no drug control (P=0.033) and to either IM or dasatinib alone (P=0.032 and P=0.028, respectively). There were no significant differences between the BMS-214662-contalning arms. (B) Compared to the no drug control, there was a non-significant reduction in colonies produced from the normal donor $CD34^+$ cells in the BMS-214662-contalning arms.

In normal $CD34^+$ cells from healthy donors (n=3), there was a non-significant reduction in LTC-IC in the BMS-214662-containing arms compared to the no drug control (P=0.079-0.29; **Figure 5-6B**), with LTC-IC survival significantly higher for normal compared to Ph^+ $CD34^+$ cells (P=0.001). Neither IM nor dasatinib alone had a significant effect on colony formation from normal $CD34^+$ cells compared to the no drug control. These results confirm that BMS-214662 is targeting primitive CML stem cells and provide evidence of selectivity for CML stem cells over normal HSCs.

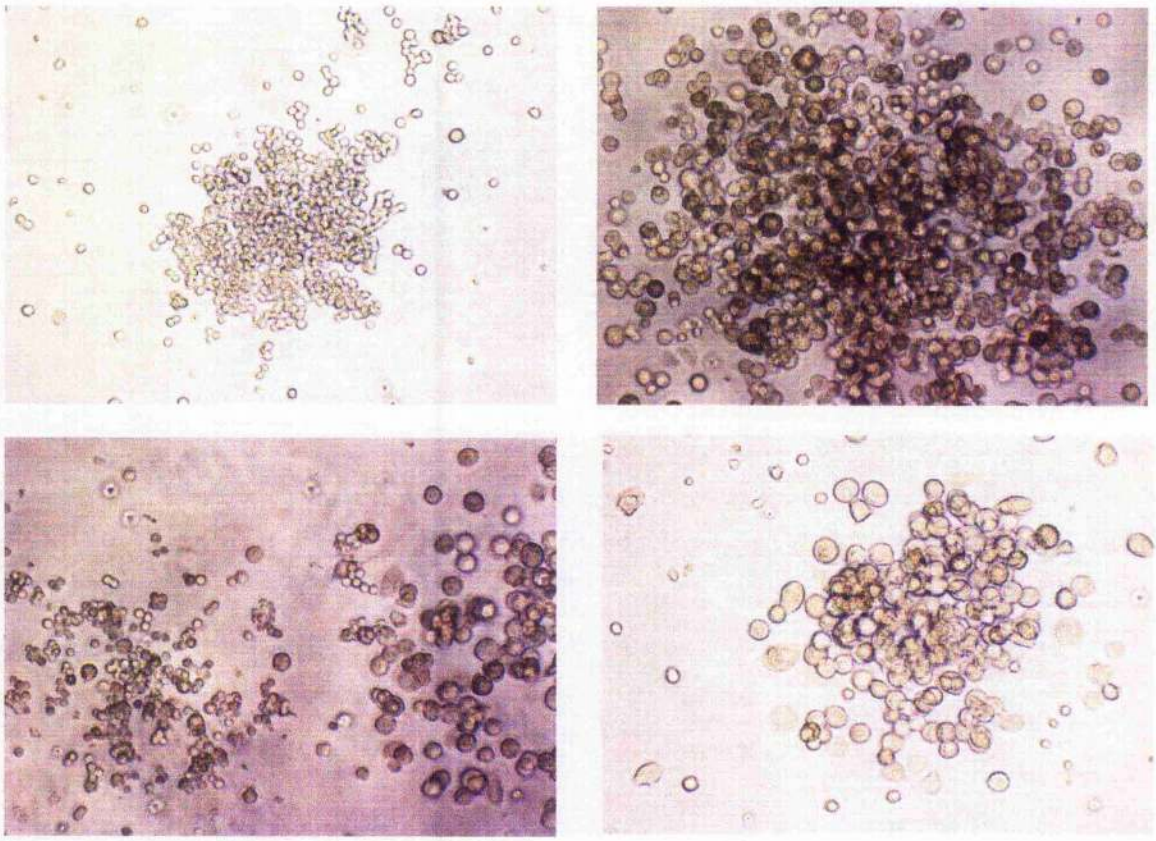


Figure 5-7. Examples of CFU-GM colonies produced in the colony forming assays after LTC-IC.

5.1.5 Fluorescence in situ hybridization results for short-term culture and LTC-IC experiments for CD34$^+$ cells treated with BMS-214662 alone or in combination with IM or dasatinib

FISH was performed at baseline and again after 6 days culture on the cells remaining in selected arms at the end of the CFSE experiments and on the colonies produced following the LTC-IC experiments. In the CFSE experiments, FISH was carried out on selected treatment arms at the end of 6 days culture (**Table 5-1**). All samples tested at either baseline or following culture were >90% Ph$^+$ by FISH confirming that the cells present were from the leukaemic clone, and there was no evident enrichment for normal (Ph$^-$) cells immediately after short term drug treatment.

UPN	BCR-ABL$^+$ / total cells (%)			
	Baseline	Dasatinib	BMS-214662	BMS-214662 + Dasatinib
170	211/218 (97)	70/70 (100)	ND	58/58 (100)
189	932/1003 (93)	217/217 (100)	187/196 (95)	102/104 (98)
215	104/109 (95)	81/83 (98)	115/118 (97)	92/93 (99)
218	233/236 (99)	92/92 (100)	111/111 (100)	131/131 (100)

Table 5-1. FISH results for CFSE experiments with CD34$^+$ CML samples. FISH was performed on the cells remaining after after 6 days culture in selected treatment arms. UPN; unique patient number, ND; not determined.

In the LTC-IC experiments (**Table 5-2**), all samples were >90% Ph$^+$ at baseline. However, after LTC-IC, only 1 of 3 samples (CML 166) remained Ph$^+$ in the BMS-214662-containing arms. This case illustrates the 10-15% of CP CML patients in whom, even at diagnosis, LTC-IC are already exclusively Ph$^+$ (Coulombel et al., 1983b; Petzer et al., 1996a).

UPN	BCR-ABL$^+$ / total cells (%)						
	Baseline	No drug control	IM	Dasatinib	BMS-214662	BMS-214662 + IM	BMS-214662 + Dasatinib
166	439/463 (95)	111/111 (100)	ND	74/74 (100)	97/99 (98)	ND	62/62 (100)
189	932/1003 (93)	51/78 (65)	85/120 (71)	181/291 (62)	12/145 (8)	8/88 (9)	0/335 (0)
215	104/109 (95)	67/71 (94)	40/40 (100)	63/66 (95)	6/134 (4)	ND	ND

Table 5-2. FISH results for LTC-IC experiments with CD34$^+$ CML samples. Colonies were harvested after LTC-IC experiments, pooled for each treatment condition and then FISH was performed on the cells from these colonies. UPN, unique patient number; ND, not determined.

In the remaining samples (CML 189 and 215), following treatment with BMS-214662, more than 90% of cells surviving LTC-IC were Ph$^-$, indicating that these patients had residual normal Ph$^-$ haemopoiesis at the time of leucapheresis (Goto et al., 1982; Coulombel et al., 1983b; Petzer et al., 1996a) and that these Ph$^-$ cells had selectively survived exposure to BMS-214662 *in vitro* as compared to their Ph$^+$ counterparts which were eradicated. This further illustrates the degree of Ph$^+$ versus Ph$^-$ selectivity for BMS-214662, suggesting quiescent cancer stem cells are more susceptible than normal cells to BMS-214662.

5.1.6 Assessment of apoptosis in both proliferating and non-proliferating populations of CD34$^+$ CML progenitor cells in the presence of BMS-214662 alone or in combination with IM or dasatinib

In order to determine the apoptotic effect of BMS-214662, active caspase-3 activity was assessed at 48, 72 and 96 hours culture in CD34$^+$ CML cells (n=4). Compared to the no drug control, apoptosis was increased in all treatment arms at 48, 72 and 96 hours, with maximal apoptosis seen at 72 hours (**Figure 5-8**). The

greatest apoptosis was seen in the BMS-214662-containing arms with the BMS-214662, BMS-214662 + IM and BMS-214662 + dasatinib arms having 14.6, 18.4 and 12.4% caspase-3 positive cells, respectively, compared to 5.5% in the no drug control arm and 7.9% in both the IM and dasatinib only arms. Due to large variations in levels of apoptosis between different primary $CD34^+$ CML samples, the results did not achieve statistical significance.

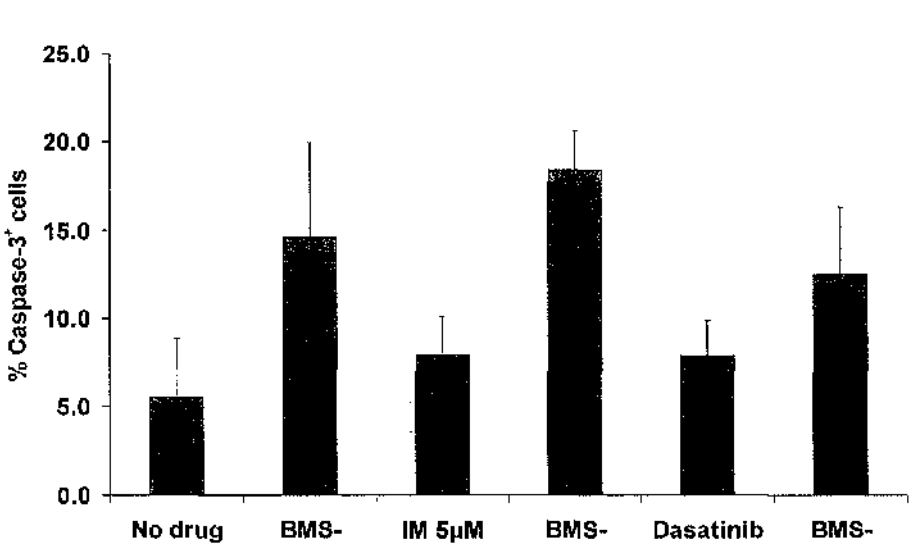


Figure 5-8. Assessment of apoptosis in total $CD34^+$ CML cells (n=4) after 72 hours culture under the experimental conditions shown. Although there was a trend for increased apoptosis in the BMS-214662-containing arms, due to wide variations in the percentage of caspase-3 positive cells between different primary CML samples, the results failed to reach statistical significance (P=0.17). Results are expressed as the percentage of total cells which were caspase-3 positive (mean ± SEM).

Using CFSE stained cells allowed assessment of apoptosis in the quiescent $CFSE^{max}$ fraction (<5% of total $CD34^+$ cells). At 48 and 72 hours there was an increase in caspase-3 positive cells in the quiescent $CFSE^{max}$ population. With IM or dasatinib alone, a modest increase in apoptosis was seen (12.2 and 10.9% $CFSE^{max}$ $CD34^+$ cells caspase-3 positive, respectively) compared to 8.3% in the no drug control; however, the apoptotic effect was increased by the addition of BMS-214662 (**Figure 5-9A** and **B**) with 22.0, 30.8 and 24.0% caspase-3 positive

cells in the BMS-214662, BMS-214662 + IM and BMS-214662 + dasatinib arms, respectively (P=0.045 for BMS-214662 containing versus non-containing arms).

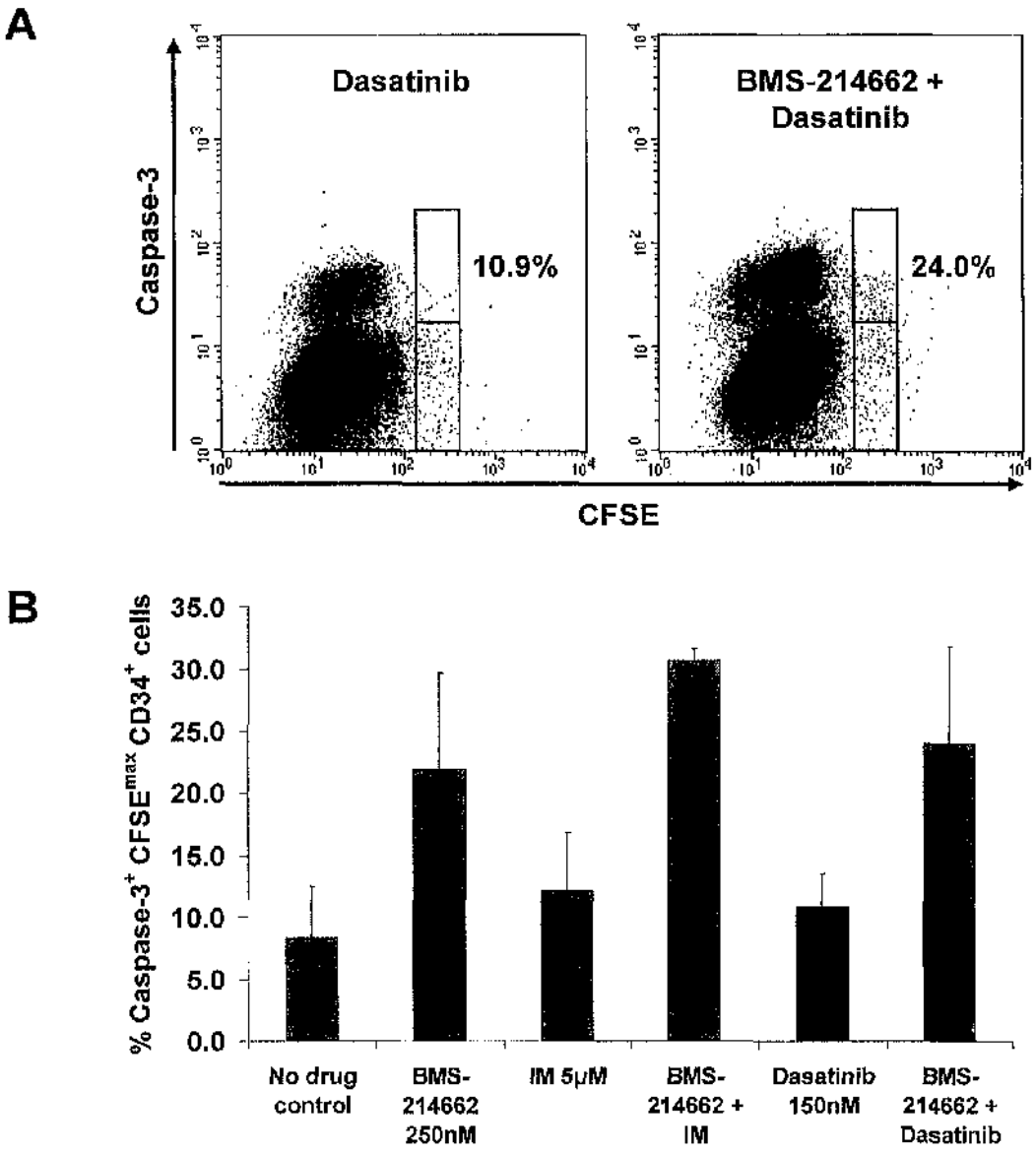


Figure 5-9. Assessment of apoptosis in non-proliferating $CFSE^{max}$ $CD34^+$ CML cells (n=4) after 72 hours culture under the experimental conditions shown. (A) Representative FACS dot plots demonstrating the increased caspase-3 activity in the non-proliferating fraction of cells treated with BMS-214662 + dasatinib compared to dasatinib only. (B) Apoptosis was significantly increased in the BMS-214662-containing arms compared to the IM and dasatinib only arms (P=0.045). Results are expressed as the percentage of total cells which were caspase-3 positive (mean ± SEM).

These results demonstrate that BMS-214662 is causing apoptosis in $CD34^+$ CML cells, in particular in the non-proliferating sub-population, and show that in the

presence of BMS-214662, although cells continue to leave the undivided gate through cell division, increased numbers of non-proliferating cells leave the undivided gate by apoptosis. The data presented here provide further evidence for the selectivity of BMS-214662 for quiescent versus proliferating cancer cells.

5.1.7 Cell cycle analysis of CD34$^+$ CML cells treated with BMS-214662 alone or in combination with IM or dasatinib

To assess the effect of BMS-214662 on the cell cycle in CD34$^+$ CML cells, high resolution cell cycle analysis was performed using Ki-67 and 7-AAD (n=5). After 72 hours culture in the presence of the different treatment conditions, there was a trend towards reduced G_0 cells in the BMS-214662-containing arms (BMS-214662 alone, BMS-214662 + IM and BMS-214662 + dasatinib) compared to the IM and dasatinib only arms (P=0.08). G_0 cells represented 0.65% of total cells in the BMS-214662-containing arms compared to 1.38% in the IM and dasatinib only arms (**Figure 5-10**). G_0 cells represented 1.13% of total cells in the no drug control. These results provide further evidence for the efficacy of BMS-214662 against non-proliferating cells in CML.

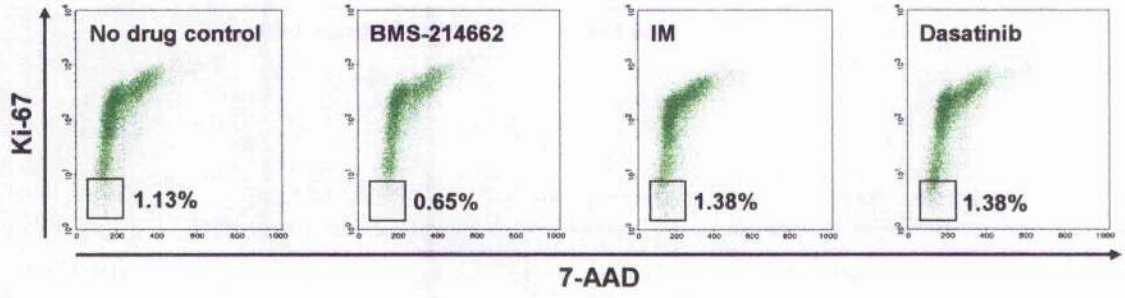


Figure 5-10. Representative FACS plots for high resolution cell cycle analysis in CD34$^+$ CML cells after 72 hours treatment in the stated conditions. There was a trend towards reduced G_0 cells in the BMS-214662-containing arms.

5.1.8 Assessment of CrKL phosphorylation status to determine the effect of BMS-214662 alone or in combination with IM or dasatinib on BCR-ABL activity

To determine the effect of BMS-214662 on BCR-ABL kinase activity in $CD34^+$ CML cells, the CrKL phosphorylation FACS assay described in **Section 2.3.2.2** and used in **Section 4.1.3.4** (Hamilton et al., 2006) was performed after 16 and 72 hours culture under the following experimental conditions (n=6): (1) No drug control; (2) BMS-214662 250nM; (3) dasatinib 150nM; (4) BMS-214662 + dasatinib. As previously, dasatinib inhibited CrKL phosphorylation at both 16 and 72 hours compared to the no drug control (**Figure 5-11**). BMS-214662 alone had no effect on CrKL phosphorylation status and, when used in combination with dasatinib, did not significantly enhance the inhibitory effect of dasatinib on CrKL phosphorylation. Therefore, these results demonstrate that BMS-214662 is not acting through inhibition of BCR-ABL.

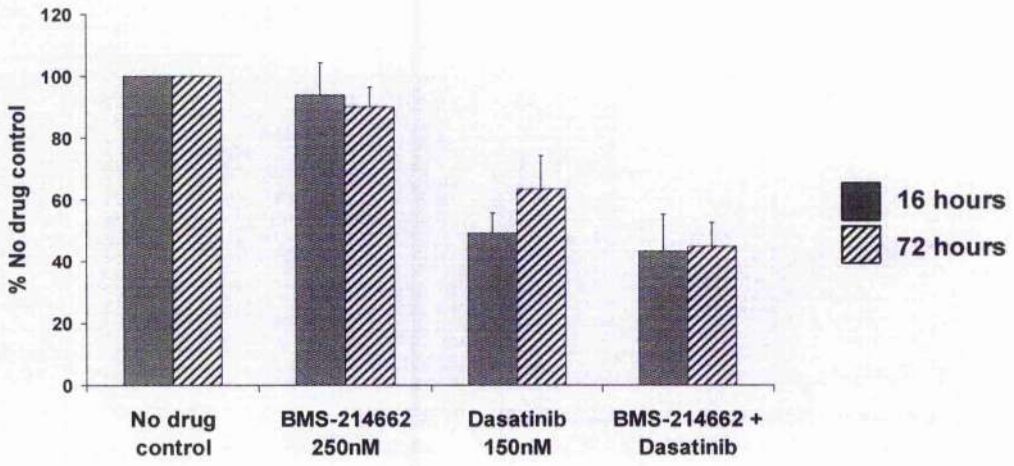


Figure 5-11. Assessment of CrKL phosphorylation status in total $CD34^+$ CML cells after 16 and 72 hours treatment under the described conditions. BMS-214662 alone had no effect on CrKL phosphorylation and did not enhance the effect of dasatinib when the two were used in combination. Results are expressed as a percentage of CrKL phosphorylation in the no drug control (mean ± SEM).

5.1.9 Assessment of efficacy of pulsed BMS-214662 regimens in combination with continuous dasatinib

BMS-214662 has already entered Phase 1 clinical trials in solid tumours and AML (Mackay et al., 2004; Ryan et al., 2004; Cortes et al., 2005a; Dy et al., 2005; Papadimitrakopoulou et al., 2005; Tabernero et al., 2005). In clinical trials, BMS-214662 was given as an intravenous infusion over either 1 hour or 24 hours, which resulted in a plasma concentration of >250nM for at least 8 or 24 hours respectively (Tabernero et al., 2005). In order to mimic the clinical scenario, we set up a multi-armed experiment to look at the effect of treating primary $CD34^+$ CML cells with pulsed BMS-214662 for either 8 or 24 hours (equivalent to a 1 or 24 hour infusion in patients respectively) combined with continuous dasatinib. The test arms assessed in these experiments (n=4) were: (1) No drug control; (2) BMS-214662 250nM for 8 hours; (3) BMS-214662 250nM for 24 hours; (4) dasatinib 150nM; (5) BMS-214662 for 8 hours + dasatinib 150nM; and (6) BMS-214662 for 24 hours + dasatinib 150nM. These experiments consisted of two 96 hour cycles. In the arms containing BMS-214662 for 8 hours, the BMS-214662 was added after 24 hours in culture and in the arms containing BMS-214662 for 24 hours, the BMS-214662 was added after 8 hours. All experimental arms were washed three times in PBS after 32 hours in culture and re-set up in fresh culture medium. Dasatinib was re-added to the dasatinib-containing experimental arms. After 8 days, total viable cells were significantly reduced in all the dasatinib-containing arms compared to the no drug control (P=0.019, P=0.003 and P=0.002 for the dasatinib, BMS-214662 for 8 hours + dasatinib and BMS-214662 for 24 hours + dasatinib arms, respectively; **Figure 5-12A**). There was no significant difference in total viable cells between no drug control and the pulsed BMS-214662 only arms. Undivided $CFSE^{max}$ $CD34^+$ CML cells were significantly increased in the dasatinib arm compared to no drug control (P=0.037; **Figure 5-12B**). There was a trend

towards reduced undivided $CFSE^{max}$ $CD34^+$ CML cells in the pulsed BMS-214662 only arms, but this did not reach statistical significance (P=0.1). The addition of pulsed BMS-214662 to dasatinib reduced the undivided $CFSE^{max}$ $CD34^+$ CML cell population compared to dasatinib alone, but again, this did not reach statistical significance (P=0.1). These results provide useful information for design of a clinical trial for the combination of BMS-214662 with dasatinib in CML.

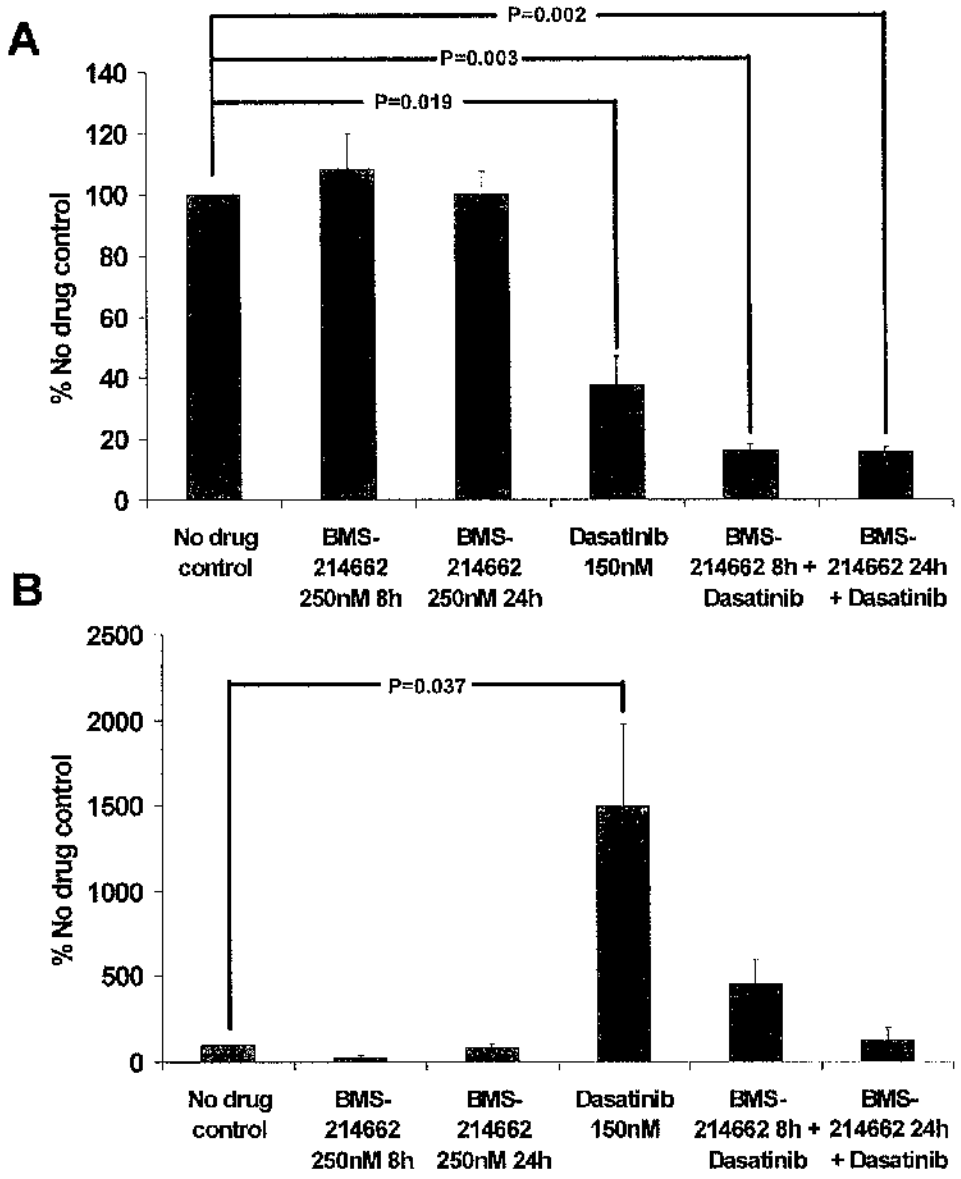


Figure 5-12. Effect of pulsed BMS-214662 with dasatinib on (A) total and (B) non-proliferating CD34$^+$ CML cells (n=4). (A) Total viable cells present after 8 days (two 96 hour cycles) in the presence of different pulsed treatment regimens. There is a significant reduction in total cells in the dasatinib-containing arms. Pulsed BMS-214662 alone had no effect on total viable cells. (B) Non-proliferating CD34$^+$ CML cells present after 8 days culture. As previously, dasatinib alone significantly increased the non-proliferating fraction remaining after culture compared to no drug control. The addition of pulsed BMS-214662 demonstrated a trend towards reducing this non-proliferating fraction, but was less effective than continuous BMS-214662 *in vitro*. Only pulsed BMS-214662 alone reduced the non-proliferating cell fraction to below the level seen in the no drug control, but again was less effective than continuous BMS-214662. Results are expressed as a percentage of the no drug control (mean ± SEM). h, hours.

5.1.10 Comparison of the effects of BMS-214662 and BMS-225975 on CD34$^+$ CML cells

Previous studies have shown that the cytostatic FTI lonafarnib does not significantly reduce the number of quiescent CML stem cells relative to no drug control (Jorgensen et al., 2005a). BMS-225975 is another cytostatic FTI, which is structurally very similar to BMS-214662, the only difference being substitution of a hydrogen ion for a methyl group at position 1. Both these compounds have similar inhibitory effects on FT with BMS-214662 having an IC_{50} of 0.7nM and BMS-225975 0.8nM in HCT-116 tumour xenografts (Frank Lee, personal communication). Therefore, for the following experiments, like BMS-214662, a concentration of 250nM BMS-225975 was chosen.

To determine if the effects of BMS-214662 in CML were due to inhibition of Ras, we directly compared its activity with that of BMS-225975 in primary CD34$^+$ CML cells. CFSE-based flow cytometry was used to track cell division, and caspase-3 activity to measure apoptosis. The treatment conditions studied were: (1) No drug control; (2) BMS-214662 250nM; (3) BMS-225975 250nM; (4) dasatinib 150nM; (5) BMS-214662 + dasatinib; (6) BMS-225975 + dasatinib. After 6 days, total viable cells were significantly reduced in all the treatment arms compared to the no drug control ($P<0.001$; **Figure 5-13A**). BMS-214662 significantly reduced total viable cells compared to BMS-225975 ($P=0.028$). There was no significant difference between the combinations of BMS-214662 + dasatinib and BMS-225975 + dasatinib on total viable cells. As previously, after 6 days culture, not only had BMS-214662 significantly reduced undivided CFSE^{max} CD34$^+$ CML cells compared to the no drug control ($P=0.018$), but also compared to BMS-225975 containing arms ($P=0.024$). Undivided CFSE^{max} CD34$^+$ CML cell numbers were not significantly different in the no drug control and BMS-225975 arms (**Figure 5-13B**).

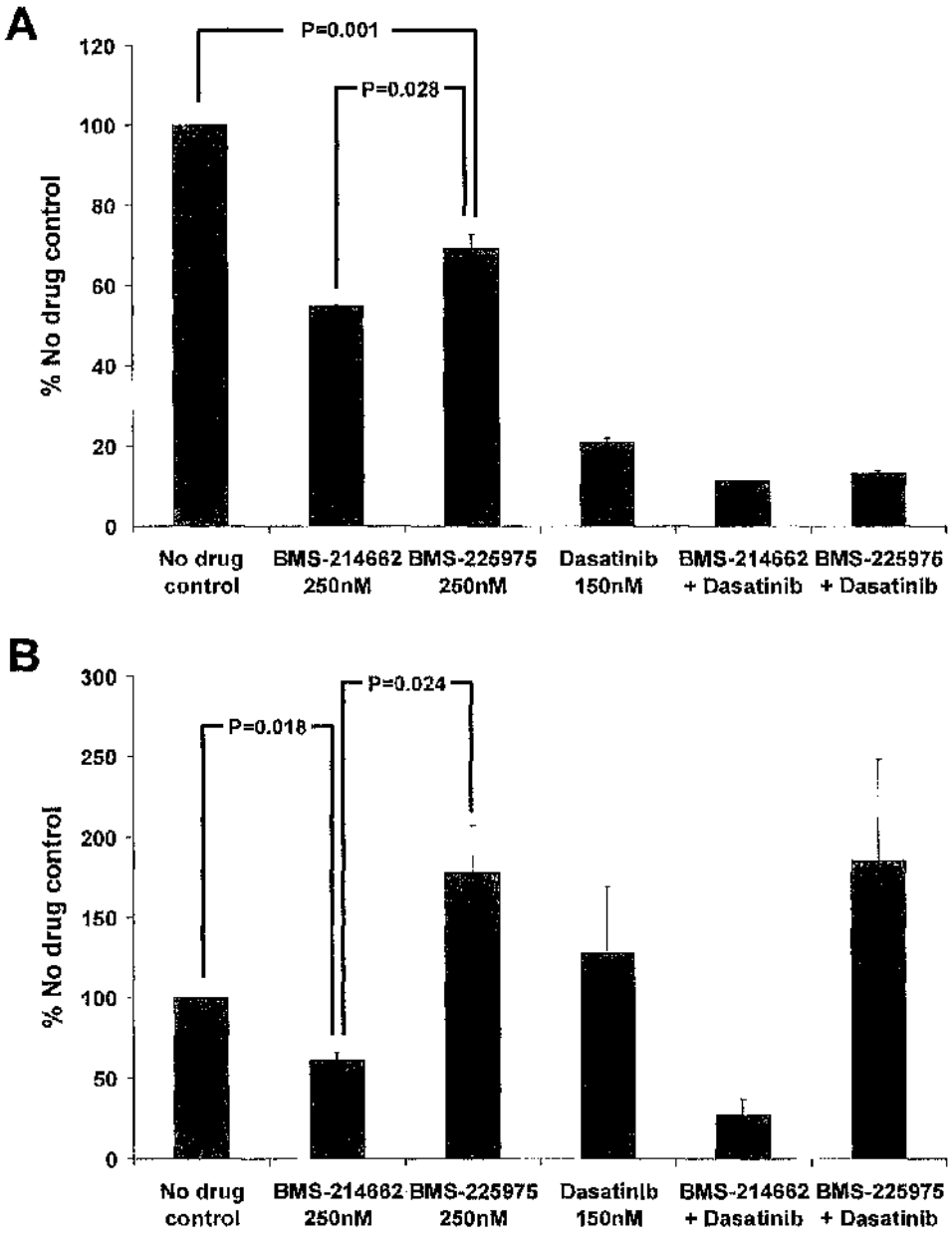


Figure 5-13. Comparison of the effects of BMS-214662 with the cytostatic FTI BMS-225975 on (A) total viable cells and (B) non-proliferating $CD34^+$ CML cells after 6 days culture (n=4). (A) Although BMS-225975 significantly reduced total viable cells compared to no drug control (P=0.001), BMS-214662 had a superior effect (P=0.028 compared to BMS-225975 and P<0.001 compared to no drug control). (B) Once again, BMS-214662 significantly reduced non-proliferating cells compared to no drug control (P=0.018). BMS-225975 did not significantly alter the number of non-proliferating cells remaining after culture compared to the no drug control. However, BMS-214662 significantly reduced non-proliferating cells compared to BMS-225975 (P=0.024). Results are expressed as a percentage of the no drug control (mean ± SEM).

BMS-225975 did not increase caspase-3 activity compared to no drug control in either the BMS-225975 or BMS-225975 + dasatinib arms in total CML cells or the non-proliferating $CD34^+$ fraction (**Figure 5-14A** and **B**). The dissimilar effects of BMS-214662 and BMS-225975 suggest that the efficacy of BMS-214662 in CML may not be via inhibition of the Ras pathway.

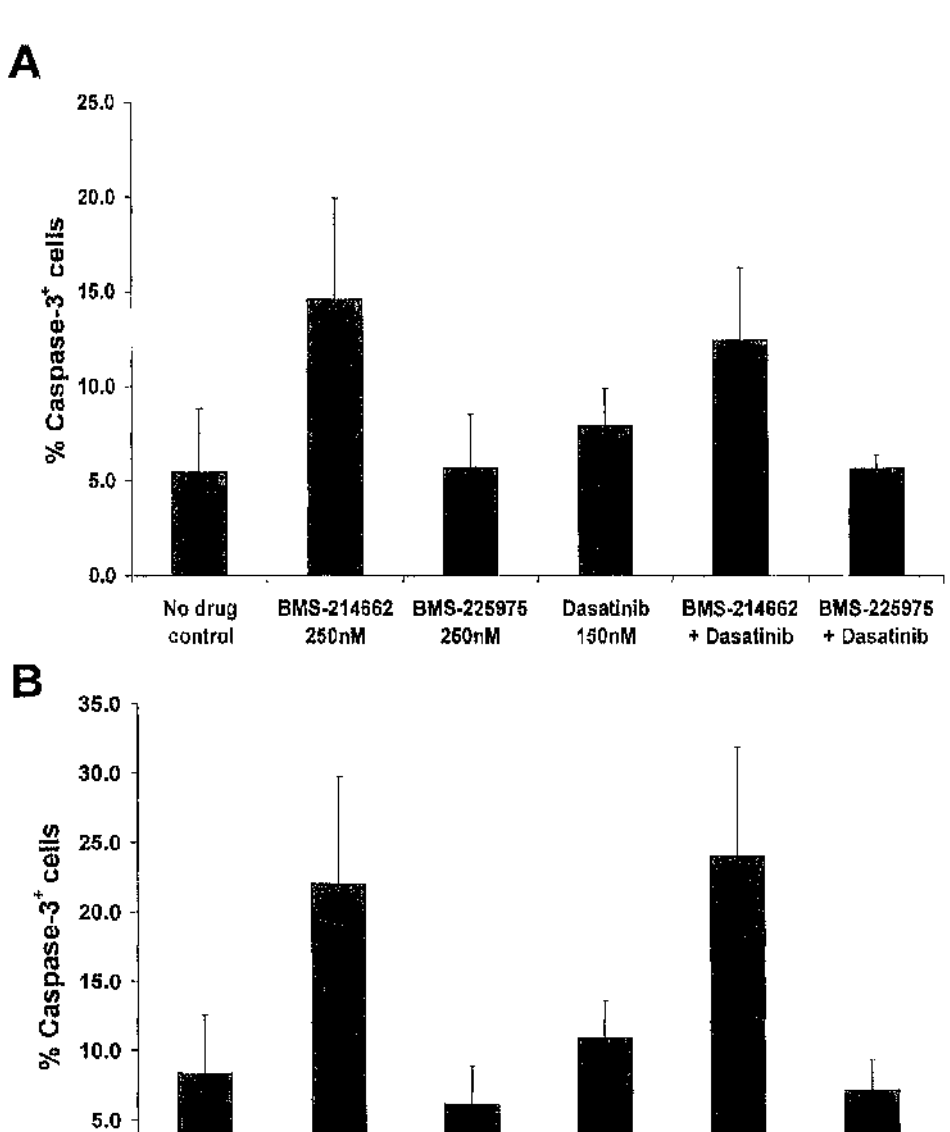


Figure 5-14. Assessment of apoptosis by measuring caspase-3 activity in (A) total cells and (B) non-proliferating $CFSE^{max}$ $CD34^+$ CML cells after 72 hours culture with BMS-214662 or BMS-225975 alone or in combination with dasatinib. BMS-225975 alone or in combination with dasatinib failed to increase apoptosis above the level seen in the no drug control. Results expressed as percentage of cells which were caspase-3 positive (mean ± SEM).

To further confirm that the effects of BMS-214662 are not through inhibition of Ras, H-Ras activity was assessed using Western blotting (n=3) after 72 hours culture in the presence of no drug control, BMS-214662, BMS-225975, IM or dasatinib. The blots showed that there was equivalent inhibition of H-Ras with both BMS-214662 and BMS-225975 with the appearance of an unprenylated band. Imatinib mesylate or dasatinib had no effect on H-Ras activity. A representative blot is shown in **Figure 5-15**. This result further implies that the activity of BMS-214662 in CML is not mediated via Ras.

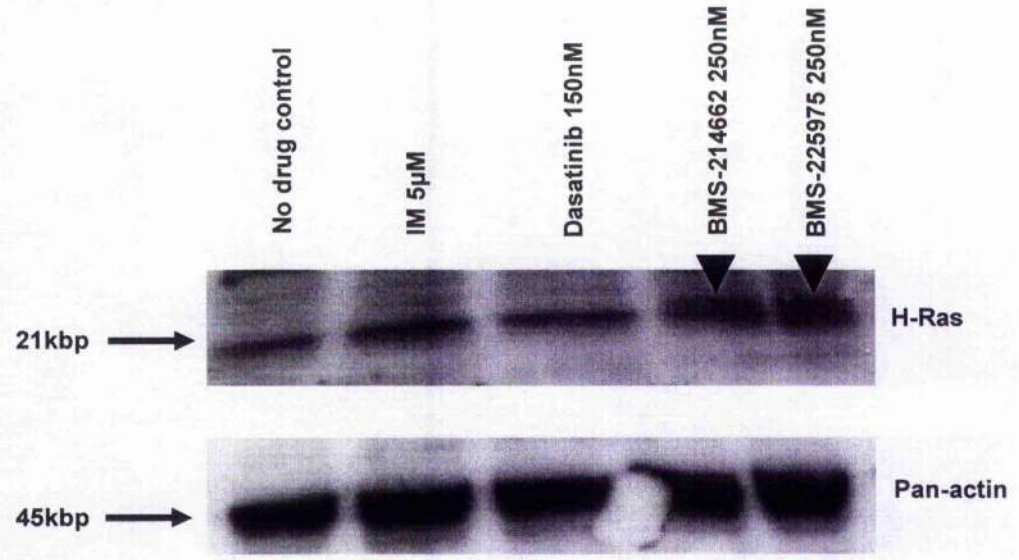


Figure 5-15. Representative Western blot showing that BMS-214662 and BMS-225975 have equivalent activity against H-Ras with similar sized unprenylated Ras bands (arrowhead) appearing after treatment with these compounds in $CD34^+$ CML cells. IM and dasatinib have no direct effect on Ras activity as illustrated here. A pan-actin control confirms equal protein loading.

5.1.11 Assessment of Mcl-1 activity in $CD34^+$ CML cells

Recent *in vitro* studies in B-CLL (Marzo et al., 2004) and myeloma (Aichberger et al., 2005) have identified inhibition of Mcl-1 in association with Bax or Bak activation as potential modes of action for BMS-214662 in these haematological malignancies. However, these conditions have not yet been shown to be stem cell disorders and the mechanism of action may be different in CML. Western blotting

was undertaken after 72 hours culture in the presence of: (1) No drug control; (2) BMS-214662 250nM; (3) BMS-225975 250nM; (4) IM 5μM; (5) Dasatinib 150nM (n=3). The resulting blots showed no evidence for inhibition of Mcl-1 in any of the treatment arms indicating that, in CML, BMS-214662 is not acting via inhibition of Mcl-1 (**Figure 5-16**).

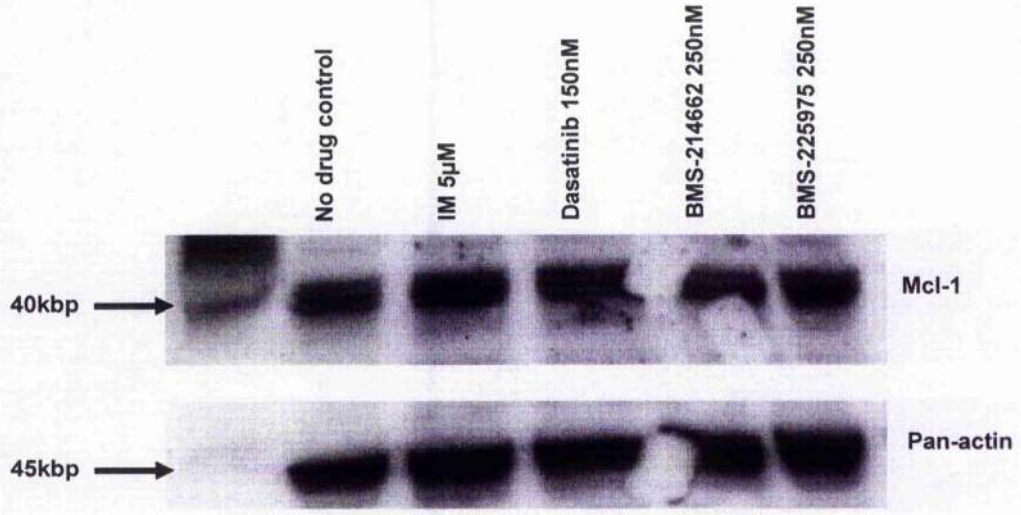


Figure 5-16. Representative Western blot demonstrating that BMS-214662 has no effect on Mcl-1 activity in CD34$^+$ CML cells. A pan-actin control confirms equal protein loading.

5.1.12 Assessment of BMS-214662 activity in BC CML and cells expressing BCR-ABL kinase mutations

Much of this study has focussed on CD34$^+$ CML cells taken from patients with CP CML and the role of the CML stem cell in molecular persistence after treatment with IM or dasatinib. However, it is also very important to assess the effects of novel agents such as BMS-214662 in advanced CML and in cells expressing BCR-ABL kinase domain mutations as it will be those patients with advanced disease or BCR-ABL kinase mutations, who are most likely, in the short-term to benefit from novel therapies. In addition, these are the patients most likely to be recruited to a Phase 1 clinical trial of BMS-214662 in CML.

5.1.12.1 BMS-214662 activity in BC CML

Using CFSE to track cell division, BMS-214662 was assessed alone and in combination with IM or dasatinib in BC CML (n=2; 1 lymphoid and 1 myeloid BC). BMS-214662 had little effect on total viable cells (**Figure 5-17A**) in BC CML when used alone, but when used in combination with either IM or dasatinib, overall cytotoxicity was enhanced. BMS-214662 acted to reduce the number of non-proliferating $CFSE^{max}$ $CD34^{+}$ CML cells (**Figure 5-17B**) compared to the no drug control and acted synergistically with IM or dasatinib to reduce undivided $CFSE^{max}$ $CD34^{+}$ CML cells. These results show than BMS-214662 when used in combination with IM or dasatinib may prolong remission and may prove useful as a single agent if inhibition of tyrosine kinase activity with IM or dasatinib has failed.

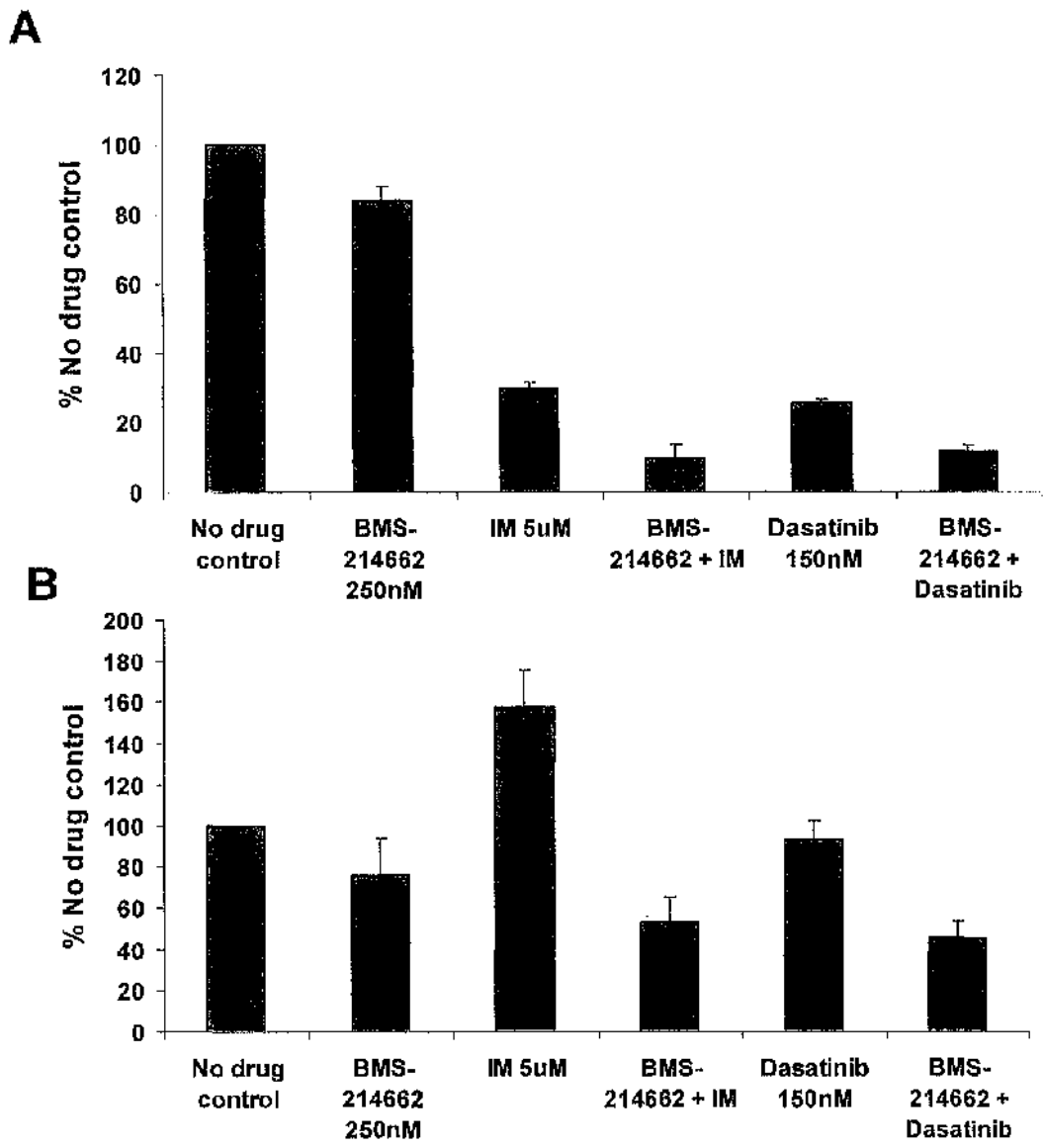


Figure 5-17. Assessment of BMS-214662 activity alone or in combination with IM or dasatinib on BC CML (n=2). (A) Total viable cell counts were performed after 6 days culture. (B) Determination of non-proliferating $CFSE^{max}$ $CD34^{+}$ cells remaining after 6 days culture. Blast crisis CML cells were cultured in SFM + 5GF. The absolute number of non-proliferating cells decreased over time and were 1.62% at 72 hours and 0.7% at 6 days in no drug control. Results are expressed as a percentage of the no drug control (mean ± SEM).

5.1.12.2 BMS-214662 activity in cells expressing BCR-ABL kinase mutations

The efficacy of BMS-214662 in Ba/F3 cells expressing different BCR-ABL kinase mutations (WT BCR-ABL, T315I, M351T and H396P) was assessed using total

viable cell counts and ^{3}H thymidine proliferation assays. It was not possible to use primary $CD34^{+}$ CML cells with kinase domain mutations as a bank of these cells was not available. After 48 hours culture, BMS-214662 was equipotent in both WT BCR-ABL and mutant BCR-ABL kinase expressing cells (**Figure 5-18A** and **B**), indicating that BMS-214662 may be a useful therapeutic option in patients with BCR-ABL kinase domain mutations.

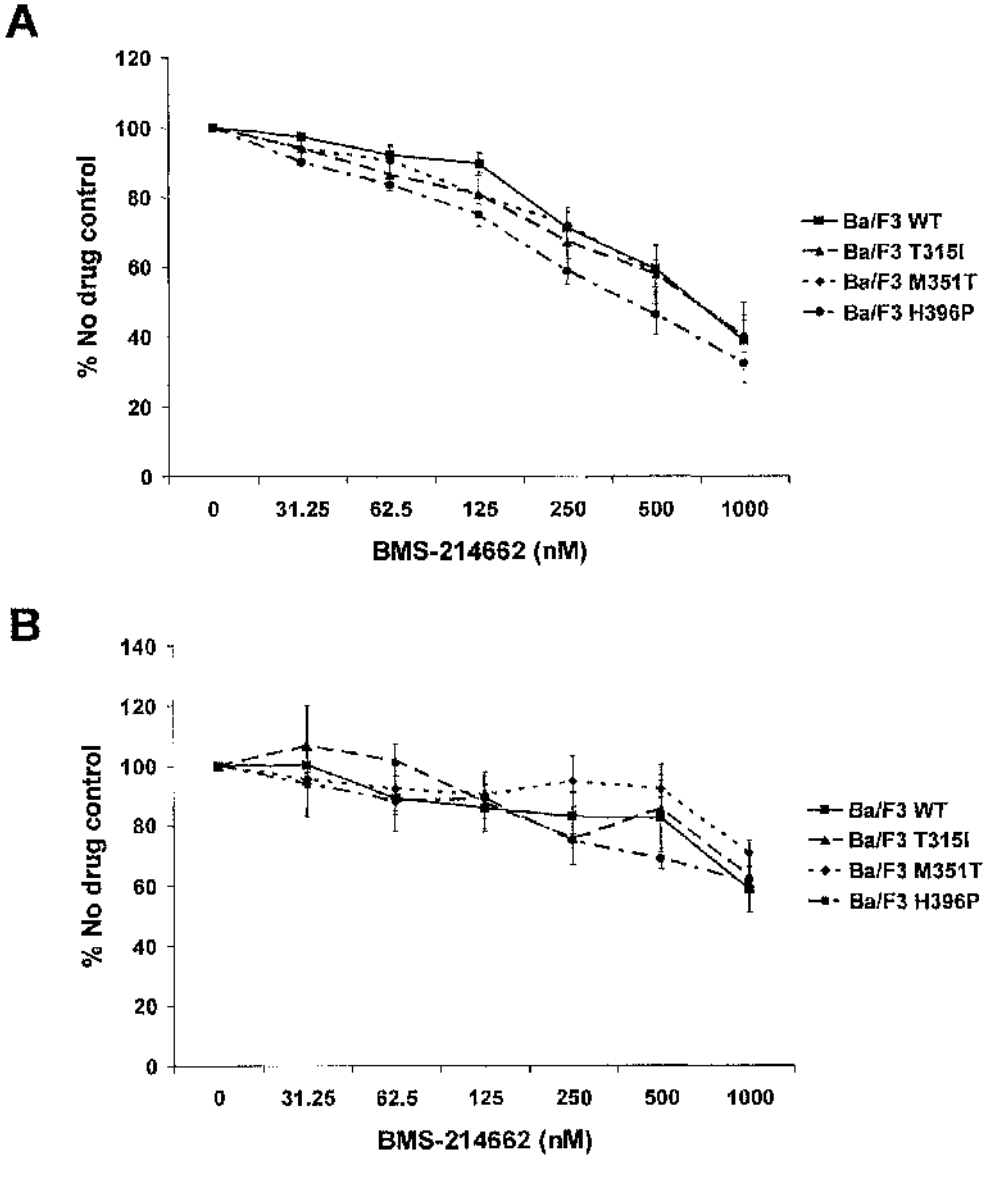


Figure 5-18. Assessment of efficacy of BMS-214662 in Ba/F3 cells expressing different kinase domain mutations (n=3 experiments) by (A) ^{3}H thymidine proliferation assay and (B) total viable cell count after 48 hours culture. Results show that BMS-214662 is equally effective in Ba/F3 cells expressing either wild type or mutant BCR-ABL. Five replicates of each condition were performed in each experiment. Results represent the mean ± SEM.

5.1.13 Summary and conclusions

The novel cytotoxic FTI, BMS-214662 has an IC_{50} of approximately 375nM when assessed by ^{3}H thymidine proliferation assay in $CD34^{+}$ CML cells. However it had a greater inhibitory effect on total viable cell counts with a 50% reduction in total viable cells at a concentration of 250nM. BMS-214662 is very potent against $CD34^{+}$ CML cells at clinically achievable concentrations. When used in combination with IM or dasatinib, BMS-214662 enhances overall cytotoxicity compared to either IM or dasatinib alone, with no significant difference between the combinations of BMS-214662 + IM or BMS-214662 + dasatinib.

BMS-214662 targets non-proliferating cells in short-term culture experiments. In addition, it has minimal anti-proliferative effect and apoptosis studies demonstrate that BMS-214662 increases caspase-3 activity in proliferating and, more importantly, non-proliferating (quiescent) $CD34^{+}$ CML cells. This is the first drug tested using the CFSE method, either alone or in combination, to show a significant reduction in non-proliferating cells compared to the no drug control. In addition, when used in combination with either IM or dasatinib, BMS-214662 overcomes the anti-proliferative effects of these agents to significantly reduce non-proliferating cells remaining after culture. These results are further supported by cell cycle analyses which also shown that non-proliferating cells (G_0) are reduced after treatment with BMS-214662.

Studies using LTC-IC assay of $CD34^{+}$ CML cells confirm that BMS-214662 is targeting primitive CML stem cells and provide evidence of selectivity for CML stem cells versus normal HSCs. These data are further supported by FISH results from the colonies remaining at the end of LTC-IC assay which showed that in 2 of 3 patients, more than 90% of the cells surviving LTC-IC were Ph^{-}. This is further evidence for the selectivity of BMS-214662 for Ph^{+} versus Ph^{-} cells.

The mode of action of BMS-214662 remains elusive. BMS-214662 is not acting via inhibition of BCR-ABL as evidenced by failure to inhibit CrKL phosphorylation. It is unlikely that BMS-214662 is acting solely via inhibition of Ras in CML cells as another FTI, BMS-225975 which is cytostatic and not cytotoxic, inhibits Ras to the same extent, but lacks the efficacy of BMS-214662 against primitive quiescent CML cells. Future studies will be directed at attempting to elucidate a mode of action for BMS-214662 in CML.

Finally, preliminary studies presented here indicate that BMS-214662 is likely to be effective in BC CML and also in patients with BCR-ABL kinase domain mutations, who are resistant to BCR-ABL-directed TKIs. The activity of BMS-214662 against the T315I mutation may prove to be particularly important.

Overall, the results presented here show very promising evidence for the utility of BMS-214662 in CML. Further studies are ongoing to determine its mode of action and design of a Phase 1 clinical trial in CML is underway (**see discussion 7.3**).

6 Results 4

6.1 Assessment of BMS-214662 activity in AML cells *in vitro*

6.1.1 Introduction

The results presented in Chapter 5 demonstrate that BMS-214662 is highly effective against CML stem and progenitor cells *in vitro*. The mode of action for BMS-214662 is not via inhibition of BCR-ABL kinase activity, therefore, BMS-214662 may be effective in other forms of leukaemia, particularly those which have been shown to be stem cell disorders. Thus, the next logical step is to assess BMS-214662 activity in AML. There are a number of reasons for this. Firstly, like CML, AML is a myeloid neoplasm. Secondly, the only Phase 1 clinical trial to date using BMS-214662 in leukaemia was undertaken in patients with AML and myelodysplastic syndrome (Cortes et al., 2005a) and, despite being a Phase 1 study, this trial demonstrated efficacy for BMS-214662 in AML, although the mode of action was uncertain. Thirdly, as with solid malignancies *in vivo* (Mackay et al., 2004; Dy et al., 2005) and as shown here in CML *in vitro*, there is the possibility of combining BMS-214662 with chemotherapeutic agents such as Ara-C in novel therapeutic regimens for AML.

6.1.2 BMS-214662 activity in AML cell line HL60

Concentration finding studies were undertaken to determine the IC_{50} for BMS-214662 in the AML cell line HL60 using ^{3}H thymidine cell proliferation assays and total viable cell counts which were performed after 48 hours culture in the presence of increasing concentrations of BMS-214662 (n=3; **Figure 6-1** and **B**).

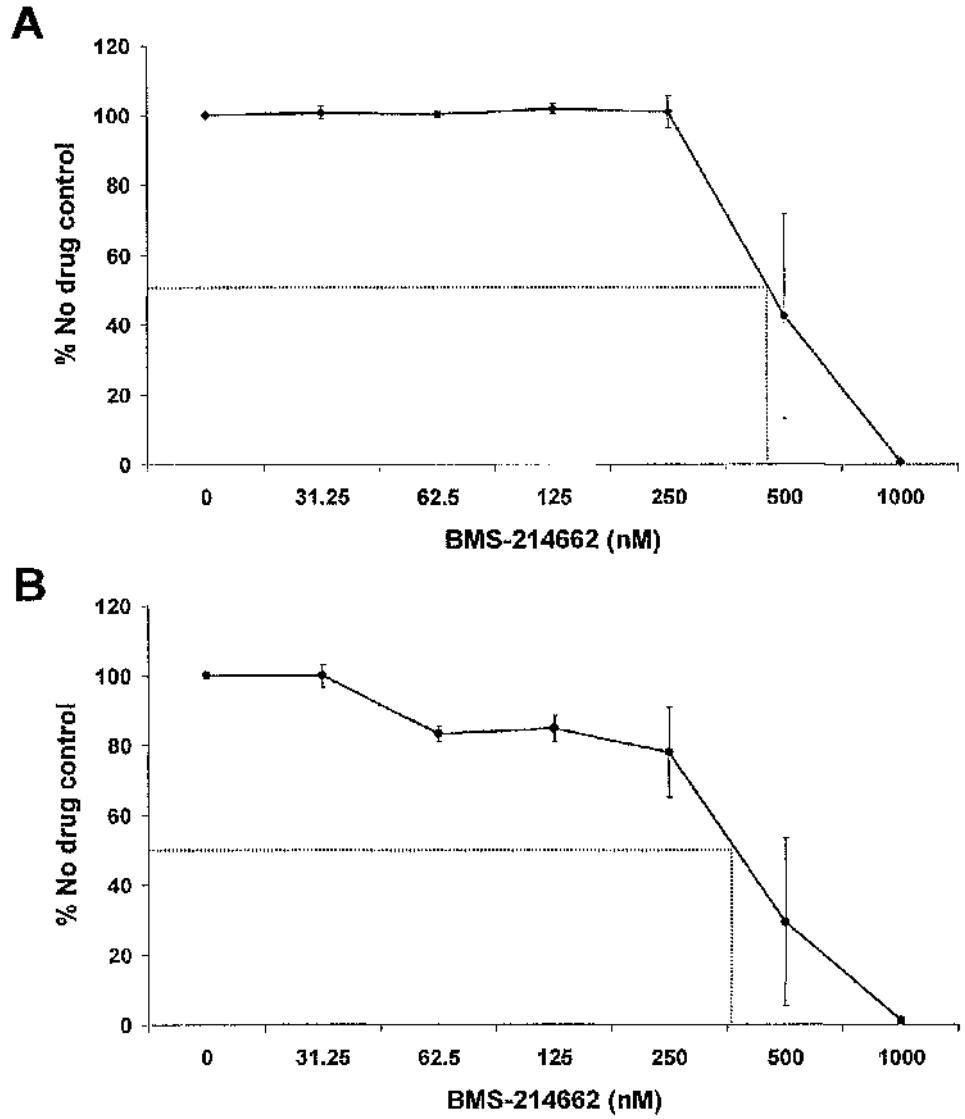


Figure 6-1. Determination of IC_{50} for BMS-214662 in the AML cell line HL60. (A) ^{3}H thymidine proliferation assays (n=3) performed after 48 hours culture showed an IC_{50} of approximately 450nM and (B) total viable cell counts approximately 375nM. Five replicates of each condition were performed in each experiment. Results represent the mean ± SEM.

The IC_{50} for BMS-214662 in HL60 cells was approximately 450nM by proliferation assay. Like CML, BMS-214662 showed a greater reduction in total viable cells than inhibition of proliferation with a 50% reduction in total viable cells at a concentration of 375nM. These results demonstrate that BMS-214662 is effective in the AML cell line HL60 with a similar IC_{50} to that seen in CML cells. Based on these similar results, BMS-214662 was used at a concentration of 250nM in further experiments with primary AML cells.

6.1.3 *Assessment of activity of BMS-214662 in primary AML cells in vitro*

6.1.3.1 Assessment of total viable cell counts in AML

In these experiments (n=2), the effect of BMS-214662 either given as a single agent or in combination with Ara-C on total viable cells counts was assessed in primary AML cells. Ara-C was used at a concentration of 0.5μM as this is a concentration which is achievable in patients (Fleming et al., 1995) and has also been used in similar *in vitro* experiments in CML in our laboratory (Jorgensen et al., 2005a). The cells were cultured in SFM + 5GF (as for $CD34^+$ CML cells) for 72 hours under the following conditions: (1) No drug control; (2) BMS-214662 250nM; (3) Ara-C 0.5μM and (4) BMS-214662 250nM + Ara-C 0.5μM. The total viable cell counts are shown in **Figure 6-2**.

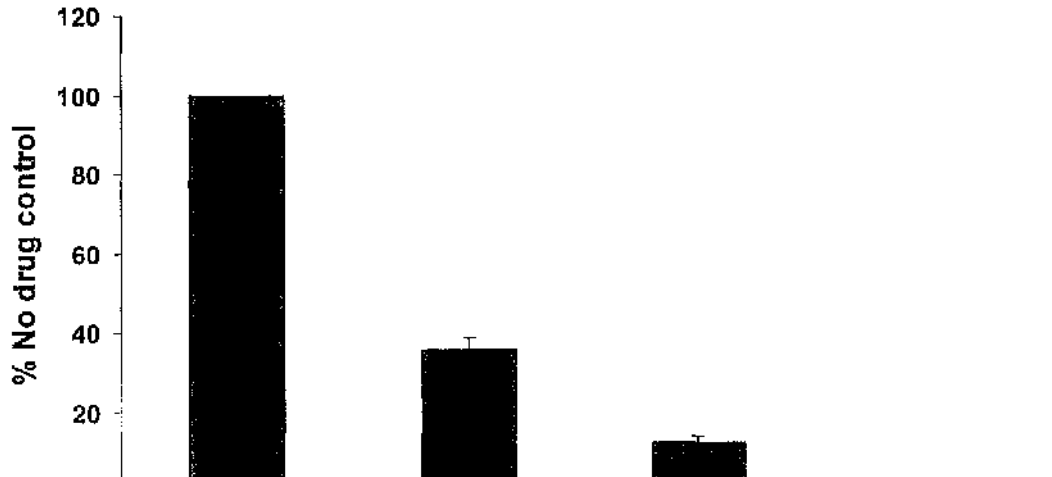


Figure 6-2. Total viable cell counts after 3 days culture for all experimental conditions. Cells were cultured in SFM + 5GF. Results are expressed as a percentage of the no drug control (mean ± SEM).

There was a reduction in total viable cells in all treatment arms compared to the no drug control, with the combination of BMS-214662 + Ara-C having the greatest

effect. However, due to the low number of experiments (n=2) and variation between the two patient samples used, the results did not reach statistical significance. These results suggest that BMS-214662 has a cytotoxic effect in AML and enhances the cytotoxic effect of Ara-C.

6.1.3.2 Effect of BMS-214662 on non-proliferating CFSE^{max} AML progenitor cells

After assessment of total viable cell counts, the effect of BMS-214662 either alone or in combination with Ara-C was assessed on the non-proliferating (quiescent) AML cell sub-population after 72 hours in culture (n=2). Once again, these experiments were performed using CFSE to track cell division, with a colcemid control included to identify the non-proliferating CFSE^{max} AML cell population using flow cytometry. As for the CML experiments, the absolute number of non-proliferating cells was calculated and expressed as a percentage of those present in the no drug control. The results are shown in **Figure 6-3A** with representative FACS profiles for each experimental condition in **Figure 6-3B**. The potency of Ara-C on proliferating AML cells is clearly demonstrated with the majority of cells remaining after culture in the presence of Ara-C being in the non-proliferating population. This population is substantially reduced with the addition of BMS-214662.

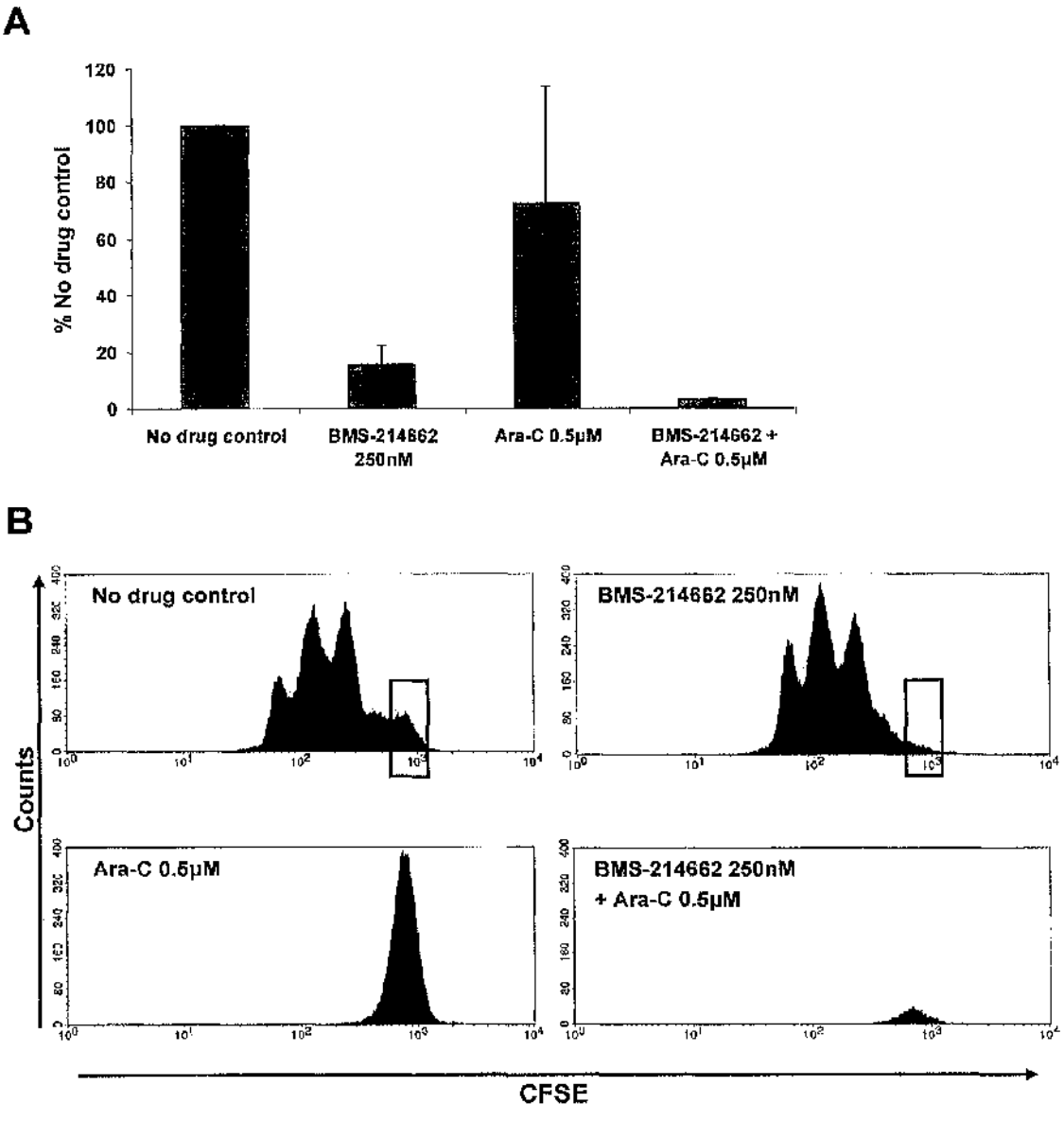


Figure 6-3. Non-proliferating CFSE^{max} primary AML cells (n=2) remaining after 72 hours culture for all experimental conditions in SFM + 5GF. (A) The absolute number of non-proliferating cells is expressed as a percentage of the no drug control (mean ± SEM). There is a substantial reduction in the absolute number of non-proliferating CFSE^{max} cells in the BMS-214662-containing arms compared to the no drug control and Ara-C only arms. (B) Representative FACS histogram plots after 72 hours culture for each of the experimental conditions. The plots demonstrate the very low number of undivided cells remaining (boxed area) in the no drug control and BMS-214662 arms relative to the total cell numbers. The non-proliferating CFSE^{max} AML cell population is the only cell population remaining in the Ara-C arms, with a very much reduced number in the BMS-214662 + Ara-C arm. Any dividing AML cells have been eradicated by Ara-C, however, the majority of CFSE^{max} AML cells

remain undivided in the presence of Ara-C, clearly demonstrating its marked anti-proliferative effect. The addition of BMS-214662 to Ara-C eliminates the majority of these undivided cells.

Non-proliferating $CFSE^{max}$ AML cells were reduced in the arms containing BMS-214662 compared to the arms without BMS-214662 (P=0.039). However, due to the low number of experiments, differences between individual experimental arms did not reach statistical significance. Nonetheless, there was a trend towards reduced non-proliferating cells in the BMS-214662 + Ara-C arm compared to the Ara-C only arm (P=0.062). Despite the limited number of experiments due to sample availability, these results suggest that BMS-214662 targets quiescent AML cells and may be a useful addition to chemotherapy protocols in AML. Further experiments are required to fully explore the potential of BMS-214662 in AML.

6.1.4 Summary and conclusions

BMS-214662 has an IC_{50} of approximately 450nM in the HL60 AML cell line by assessment of ^{3}H thymidine proliferation assays, although, as with CML, this agent had a greater effect on total viable cells. The results presented here indicate that BMS-214662 is cytotoxic to primary AML cells *in vitro*, and enhances the effect of Ara-C. Importantly, BMS-214662 appears to target quiescent AML stem and progenitor cells, resulting in an enhanced effect when BMS-214662 and Ara-C are combined. Overall, these results suggest that BMS-214662 may be a very effective therapy for AML and further investigation of its therapeutic potential in AML is required.

7 Discussion

7.1 Priming primitive CML progenitor cells with rHu-G-CSF improves their eradication by IM *in vitro*

The aims of this chapter were to determine if using an 'IM holiday' (interrupted IM therapy), or priming primitive CML progenitors with rHu-G-CSF would overcome the inherent insensitivity of the most quiescent CML progenitors to IM (Graham et al., 2002). The first experiments in this chapter set out to characterise G-CSF-R protein expression and functionality in $CD34^+$ CML progenitor cells in response to rHu-G-CSF *in vitro*. Previous studies have shown that $CD34^+$ cells from CP CML patients can be maintained in culture in the absence of exogenous growth factors via autocrine production of IL-3 and G-CSF (Jiang et al., 1999). This suggests that proliferating $CD34^+$ cells possess functional G-CSF-Rs. Here, we have shown that G-CSF-R protein expression is increased in proliferating $CD34^+$ CML cells compared to normal $CD34^+$ cells and that this increase is not further augmented by the addition of rHu-G-CSF to culture (**3.1.2**). We then went on to confirm the presence of functional G-CSF-Rs on $CD34^+$ CML cells by demonstrating a concentration-dependent response to rHu-G-CSF in these cells (**Figure 3.4**) which was saturated at a rHu-G-CSF concentration of ~20ng/mL. This G-CSF concentration is readily achievable *in vivo* and equates to a rHu-G-CSF dose of approximately 5µg/kg in the patient (Stute et al., 1992). This is the standard dose of rHu-G-CSF given for peripheral blood stem cell mobilisation (Drummond et al., 2003; Hui et al., 2003), IM-induced neutropenia (Marin et al., 2003b; Sneed et al., 2004) and growth factor priming in combination with cytotoxic chemotherapy in AML (Cannistra et al., 1989; Bhalla et al., 1991; te Boekhorst et al., 1993). We further demonstrated, indirectly, that the quiescent sub-population of $CD34^+$ CML

cells express functional G-CSF-Rs by showing that, in the presence of intermittent rHu-G-CSF (first 24 hours of each 96 hour cycle), this population is significantly reduced due to increased numbers of these cells leaving the undivided (quiescent) gate through cell division.

In the absence of rHu-G-CSF, an 'IM holiday' was less effective than continuous IM therapy at reducing the total viable cell number. However, in the presence of rHu-G-CSF, there was no significant difference between the two strategies, with the combination of intermittent rHu-G-CSF with either interrupted or continuous IM being most effective at reducing total viable cells. Furthermore, in the quiescent sub-population, there was no significant difference in the size of the remaining sub-population between continuous and interrupted IM therapy after 12 days in culture. This suggests that *in vivo*, an 'IM holiday' alone is unlikely to reduce the molecular persistence which occurs with continuous IM therapy. Conversely, the addition of intermittent rHu-G-CSF (G-CSF priming) to either continuous or interrupted IM therapy may be more effective.

The diverging results seen here with intermittent and continuous rHu-G-CSF exposure in the quiescent sub-population suggests that treatment of these primitive cells with intermittent rHu-G-CSF may activate a unique mechanism which is not maintained in later progeny. One possible explanation is that, in the quiescent fraction, initial exposure to rHu-G-CSF up-regulates G-CSF-R protein expression. However, continuous exposure to supra-physiological doses of rHu-G-CSF in this population serves to down-regulate functional G-CSF-R expression removing the drive for these cells to proliferate. Each intermittent exposure to rHu-G-CSF up-regulates the functional G-CSF-Rs and stimulates a proportion of the quiescent cells to proliferate, but because high G-CSF levels are not present

continuously, the cells do not become unresponsive or 'resistant' to the effects of rHu-G-CSF.

The effect of combining different chemotherapeutic agents such as hydroxycarbamide or Ara-C with rHu-G-CSF was not assessed in this study, and these combinations could be equally effective at reducing total viable CML cells as well as the quiescent $CD34^+$ sub-population. However, to date, IM is the only agent which has been shown to prevent progression to more advanced phases of CML (Druker et al., 2006). BCR-ABL increases the production of ROS, inhibits protein tyrosine phosphatases (PTPases) (Sattler et al., 2000), and causes inappropriate regulation of DNA repair pathways which results in unfaithful repair of ROS-dependent DNA double strand breaks (Nowicki et al., 2004). These effects may add to the mutator phenotype shown by $BCR\text{-}ABL^+$ cells and are inhibited by IM *in vitro* (Sattler et al., 2000; Nowicki et al., 2004). It has recently been reported that the production of ROS stimulated by BCR-ABL results in oxidative DNA damage and increases the acquisition of BCR-ABL kinase domain mutations associated with IM resistance *in vitro* (Koptyra 2006). Genotoxic stress such as that induced by agents like Ara-C may further increase ROS and result in additional kinase domain mutations which have previously been shown to exist prior to IM therapy (Roche-Lestienne et al., 2002; Kreuzer et al., 2003). Therefore, these agents may be less suitable than BCR-ABL TKIs for combination therapy in CML. In addition, genomic instability may be related to BCR-ABL expression levels. Therefore, in the primitive $CD34^+38^-$ CML progenitor cells with high levels of BCR-ABL expression (Copland et al., 2006), it is possible that mutations resulting in IM-resistance will occur at the highest frequency. Caution should also be taken if combining haemopoietic growth factors with IM as growth factors including GM-CSF and IL-3 have been shown to increase ROS *in vitro* in haemopoietic cell lines by increasing tyrosine phosphorylation (Sattler et al.,

1999). Thus it would be important to monitor patients for BCR-ABL kinase domain mutations if the BCR-ABL level is rising in a trial combining rHu-G-CSF with IM. The treatment of BCR-ABL$^+$ cells with antioxidants such as N-acetylcysteine or vitamin E reduced accumulation of ROS, reactivated PTPases and resulted in fewer BCR-ABL kinase domain mutations developing in both *in vitro* and *in vivo* models (Sattler et al., 2000; Koptyra et al., 2006). Very interestingly, the addition of IM to an antioxidant had a superior anti-mutagenic effect compared to antioxidant alone (Koptyra et al., 2006) and it will be important to determine if the addition of an antioxidant to IM therapy reduces the incidence of IM-resistant BCR-ABL kinase domain mutations *in vivo*.

As discussed, a further aim of this study was to determine if rHu-G-CSF priming of CD34$^+$ CML cells combined with IM therapy would reverse the anti-proliferative effect of IM alone at the G_0/G_1 interface, allowing these quiescent cells to re-enter the cell cycle. These results confirm that intermittent exposure to rHu-G-CSF does enhance the eradication of quiescent CD34$^+$ CML cells, however, a sub-population of deeply quiescent CML cells does persist, even after combined therapy with intermittent rHu-G-CSF and either continuous or interrupted IM. In agreement with the results of this study, Holtz *et al* have also recently shown that IM in combination with a high concentration growth factor cocktail enhances elimination of quiescent CD34$^+$ CML cells (Holtz et al., 2004).

A number of observational case series have reported that patients with IM-induced neutropenia obtain improved cytogenetic responses when also treated with rHu-G-CSF (Heim et al., 2003; Marin et al., 2003b; Quintas-Cardama et al., 2004). It has been presumed that this is due increased and maintained continuous IM dosing being achieved in the face of a rHu-G-CSF-supported neutrophil count. However, while this may still be true, the results here would also support the alternative

mechanism of action proposed in **3.1.1** – that intermittent exposure to rHu-G-CSF promotes re-entry of quiescent CML cells into the cell cycle, which results in cell division and renders the cells susceptible to the effects of IM in later divisions (Jorgensen et al., 2005b).

We also demonstrated that when non-proliferating $CD34^+$ CML cells treated with IM ± G-CSF leave the undivided gate, they do so by cell division and not apoptosis. This suggests that, in this quiescent CML sub-population, the anti-apoptotic pathways which are activated and maintained by BCR-ABL and, in part, responsible for the dys-regulated cell turnover seen in CML (Bedi et al., 1994), are not inhibited by IM. Conversely, in dividing CML cells, our results and others (Holtz et al., 2005), demonstrate that IM does induce apoptosis, resulting in reduced numbers of total viable cells.

The results of this study have provided a powerful rationale for the design and implementation of a National Cancer Research Network (NCRN; www.ukcrn.org.uk) randomised clinical trial called **G**-CSF with **I**matinib **M**esylate **I**ntermittently (**GIMI**) which compares continuous IM, interrupted IM (21 of every 28 days) and pulsed rHu-G-CSF with interrupted IM (G-CSF on Monday, Wednesday and Friday of the no IM week; **Figure 7.1**). The primary end points of this study are to determine the safety of interrupting IM therapy and giving intermittent rHu-G-CSF in combination with interrupted IM (Mitchell et al., 2006). Secondary endpoints are to compare molecular response using qRT-PCR for *BCR-ABL* and proportion of patients progressing in each of the 3 arms. The interventions (intermittent IM or pulsed rHu-G-CSF plus intermittent IM) will be deemed successful if these arms show no rise in BCR-ABL levels at the end of the study compared to the continuous IM control arm. Patients are required to have achieved a CCR for a minimum of 6 months on IM therapy as demonstrated by

<2% *BCR-ABL* by qRT-PCR prior to study entry. Recruitment has recently been completed in this multi-centre trial based in the UK and France. A total of 45 patients have been recruited, with 15 in each arm. Patients are receiving the randomised intervention for one year with monthly monitoring of qRT-PCR levels for BCR-ABL.

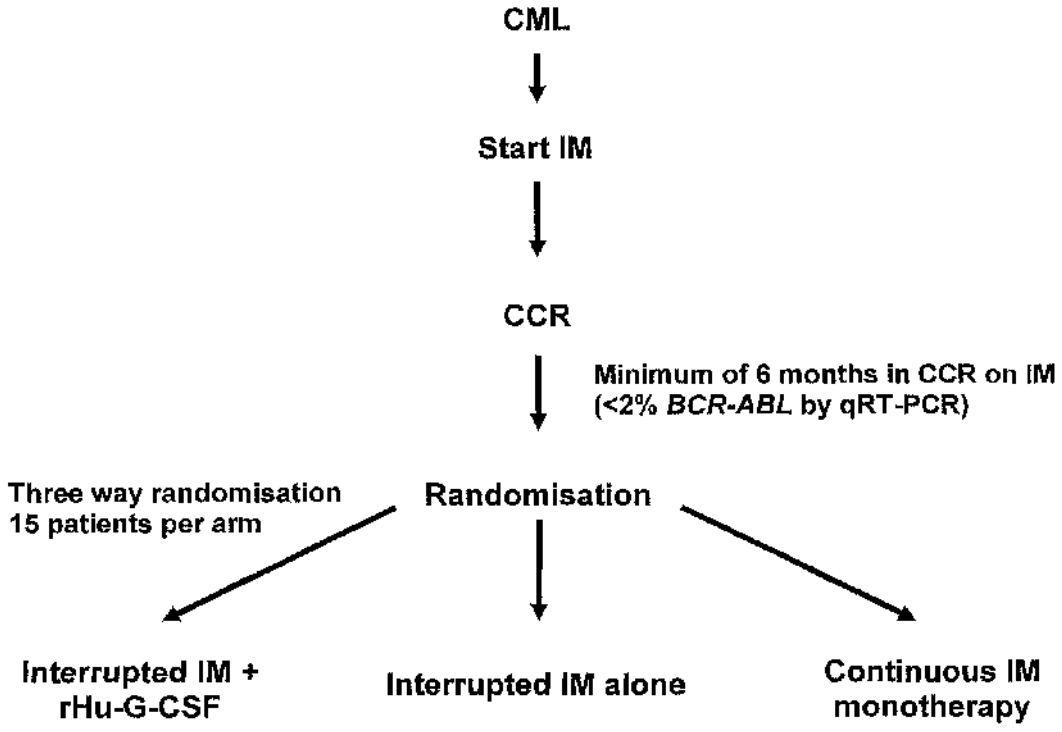


Figure 7-1. Schematic diagram of the protocol for the GIMI trial.

In conclusion, intermittent exposure to rHu-G-CSF enhances the effect of IM on quiescent $CD34^+$ CML cells. However, an 'IM holiday' alone does not reduce the quiescent $CD34^+$ CML cell sub-population, but in combination with intermittent rHu-G-CSF is as effective as continuous IM. This strategy, which is currently in clinical trial, may offer a unique opportunity for improving molecular responses in patients with CP CML by improving eradication of IM-insensitive quiescent CML cells.

7.2 A comparison of the efficacy of dasatinib and IM against quiescent primitive CML progenitor cells *in vitro*

As highlighted in the introduction (**1.2.8**), CML stem cells are relatively resistant to IM (Graham et al., 2002; Holtz et al., 2002; Bhatia et al., 2003; Holtz et al., 2005; Jorgensen et al., 2005a), and it is postulated that this CML stem cell insensitivity to IM results in the molecular persistence seen even in patients responding well to IM (Holtz et al., 2002). The mechanisms underlying the IM resistance seen in CML stem cells, including whether this resistance is BCR-ABL-dependent or independent remain to be determined. Our group has recently demonstrated that primitive IM-resistant CML cells have only single-copy, non-mutated *BCR-ABL*, but express significantly higher levels of *BCR-ABL* transcripts and protein than mature CML cells (Copland et al., 2006), however, like other studies, we were unable to determine whether BCR-ABL was a relevant target in these IM-resistant cells. These results highlight the importance of investigating more potent BCR-ABL kinase inhibitors and determining their effect on the quiescent CML stem cell population. The multi-targeted kinase inhibitor, dasatinib, is one such inhibitor which has been shown to be 325 times more potent than IM at inhibiting wild-type BCR-ABL in cell line studies and also targets mutant BCR-ABL with the exception of the T315I mutation (Shah et al., 2004; O'Hare et al., 2005). Therefore, the aims of this chapter were to determine if dasatinib was more effective than IM in primitive CML progenitor cells and whether dasatinib would eradicate quiescent CML cells *in vitro*. A further aim was to investigate the relevance of SRC kinases and BCL-2 in early CP CML as dasatinib is also a potent inhibitor of SRC kinases (Lombardo et al., 2004) one of which, LYN, is believed to mediate the activity of BCL-2 in CML (Dai et al., 2004).

The data presented in this study demonstrate that, at high therapeutically achievable concentrations (IM 5μM and dasatinib 150nM), IM and dasatinib had equal cytotoxicity in primitive CML progenitor cells. Further studies to assess BCR-ABL kinase activity, using a novel intracellular flow cytometry assay which determined CrKL phosphorylation status after treatment with either IM or dasatinib showed that, after 72 hours treatment with IM, the surviving $CD34^+$ CML cells had fully phosphorylated CrKL, indicating that we had failed to inhibit BCR-ABL. However, after 72 hours culture in the presence of dasatinib, both $CD34^+$ and more primitive $CD34^+38^-$ CML cells had significant inhibition of CrKL phosphorylation, indicating that dasatinib was a more effective inhibitor of BCR-ABL than IM. However, these results do not provide evidence for the relevance of BCR-ABL in the IM-resistant cells.

There are a number of possible explanations for the IM-resistance demonstrated here and also, the superior effect of dasatinib in CML. Firstly, there may be insufficient IM levels within the stem cell population to inhibit BCR-ABL kinase activity. This may be related to gene amplification (Gorre et al., 2001), high transcript levels (Jiang et al., 2003; Jiang et al., 2004), increased kinase activity (Chu et al., 2004) or the balance between drug influx and efflux (Burger et al., 2004; Thomas et al., 2004; Clark et al., 2005) within this primitive quiescent population. The increased potency of dasatinib may overcome the increased transcript levels and kinase activity demonstrated in the stem cell population here. In addition, unlike IM, dasatinib is not a substrate for p-Glycoprotein, and this may be another reason why it reaches further into the stem cell compartment (Lee et al., 2005). A second possible explanation is that cells expressing kinase domain mutations are enriched within the stem cell population (Shah et al., 2002). If this is the case, then the majority of these would be inhibited by dasatinib. However, in this study, mutation analysis demonstrated wild-type BCR-ABL only, although

other studies have detected BCR-ABL kinase mutations at a low level in $CD34^+$ cells from patients both pre- (Jiang et al., 2005) and post treatment with IM (Chu et al., 2005). In this study, if the resistance to IM was due to BCR-ABL kinase domain mutations, then the mutation would need to be present in the majority of the cells to explain the resistance to IM in the $CD34^+38^-$ population. We did not exclude a low level of mutated BCR-ABL in these populations in this study as the sensitivity of direct sequencing is insufficient to detect this. Other groups have shown that BCR-ABL kinase domain mutations can be detected at low level using sensitive techniques in stem cells such as bi-directional sequencing of individually cloned complementary DNA from patient samples (Chu et al., 2005; Jiang et al., 2005). A third explanation is that, in the stem cell compartment, BCR-ABL may tend to remain in its active conformation to which IM cannot bind, thereby reducing the affinity of IM for the ATP binding site of BCR-ABL (Nagar et al., 2002; Burgess et al., 2005). This would also explain the superior inhibition of CrKL phosphorylation seen with dasatinib as this drug is able to bind BCR-ABL in both active and inactive conformations.

Assessment of CrKL phosphorylation status has been accepted as a robust method to determine BCR-ABL activity as it is thought to be a specific marker for BCR-ABL signalling and the CrKL phosphoprotein complex is relatively stable (Hochhaus et al., 2001; White et al., 2005). However, a very recent study indicates that the adaptor molecule CrKL may be a substrate of the SRC kinases (Qiao et al., 2006). If this is the case, then CrKL phosphorylation status is likely to be affected by a SRC kinase inhibitor (i.e. dasatinib). Therefore, the superior inhibition of CrKL phosphorylation seen with dasatinib in $CD34^+$ and $CD34^+38^-$ CML cells may be due to enhanced inhibition of SRC kinases and not BCR-ABL and, thus, IM-resistance in the CML stem cell population may not be BCR-ABL-dependent. In

addition, other BCR-ABL-independent mechanisms of IM resistance may be present in CML stem cells.

It has been postulated that the increased efficacy of dasatinib compared to IM in CML is mediated via BCR-ABL and not SRC kinases. Indeed, previous studies in cell lines and animal models, but not primary CML cells, have failed to demonstrate a role for the SRC kinases, LYN, HCK and FGR, which are most commonly expressed on myeloid cells, in CP CML (Donato et al., 2003; Hu et al., 2004). In this study, I have shown that the expression of activated SRC kinases (p-SRC), is increased in all phases of CML compared to normal $CD34^+$ and $CD34^+38^-$ cells. Of particular interest is the finding that, in the more primitive $CD34^+38^-$ CML sub-population, compared to normal $CD34^+38^-$ cells, expression of p-SRC was relatively higher. However, the action of IM or dasatinib on p-SRC was very similar to that seen with p-CrKL, suggesting that the effect is most likely mediated via BCR-ABL and not direct SRC kinase activation. This was also supported by the fact that the SRC-specific kinase inhibitor PP2 had no significant effect on p-SRC compared to no drug control in $CD34^+$ CML cells. In addition, BCL-2 expression was also significantly increased in all phases of CML. However, I was unable to demonstrate a direct link between p-SRC and BCL-2 expression as IM, dasatinib and PP2 had only modest effects on BCL-2 levels in $CD34^+$ CML cells. This would tend to suggest that expression of BCL-2 may be independent of both BCR-ABL and SRC kinases in CML. Alternatively, because BCL-2 has a longer half-life than activated BCR-ABL or SRC, it may take longer for changes in BCL-2 expression following drug treatment to become apparent.

The use of molecularly targeted therapies such as IM and dasatinib is rapidly increasing in the treatment of malignant disease. As more cancers are being identified as stem cell diseases, researchers are focussing their attention on the

elimination of cancer stem cells as a potentially curative strategy. The importance of LSCs and their resistance to IM has long been recognised in CML (Holyoake et al., 1999; Graham et al., 2002). Therefore, in this study, I used a CFSE-based assay which allows high resolution tracking of cell division and identification of the quiescent (non-proliferating) primitive progenitor population in CML (Nordon et al., 1997; Graham et al., 2002). These experiments demonstrated that, within the stem cell compartment, there was a trend for dasatinib to be less anti-proliferative than IM. Therefore, dasatinib is less likely to induce the reversible G_1 arrest seen *in vitro* and *in vivo* with IM. This is particularly important when combining cytotoxic and/or cytostatic agents, the majority of which tend to cause cell cycle arrest. However, despite its increased potency, dasatinib failed to eradicate the quiescent stem cell population *in vitro*. Although compared to IM, dasatinib showed a trend towards reducing the $CFSE^{max}$ population, it still remained substantially higher than the no drug control and failed to show a statistically significant reduction compared to IM (**Figures 4-5 and 4-6**). This implies that, although dasatinib was able to inhibit CrKL phosphorylation in the primitive $CD34^{+}38^{-}$ population, like IM, it failed to target the truly quiescent CML stem cells in culture.

Overall, these results indicate that dasatinib is a much more potent inhibitor of BCR-ABL than IM and is able to target $BCR\text{-}ABL^{+}$ cells deeper into the stem cell compartment (**Figure 7-2**). Therefore, dasatinib would be expected to produce lower levels of MRD *in vivo* in CML patients. However, it has been demonstrated that quiescent CML stem cells appear intrinsically resistant to IM and dasatinib and thus, dasatinib is unlikely to be curative in CML. The possibility that, in quiescent CML stem cells, neither BCR-ABL nor SRC kinases are relevant targets should be considered. This highlights the need to identify and develop new cancer stem-cell directed therapies to effectively eradicate these primitive cancer initiating cells in patients while sparing the normal stem cell compartment.

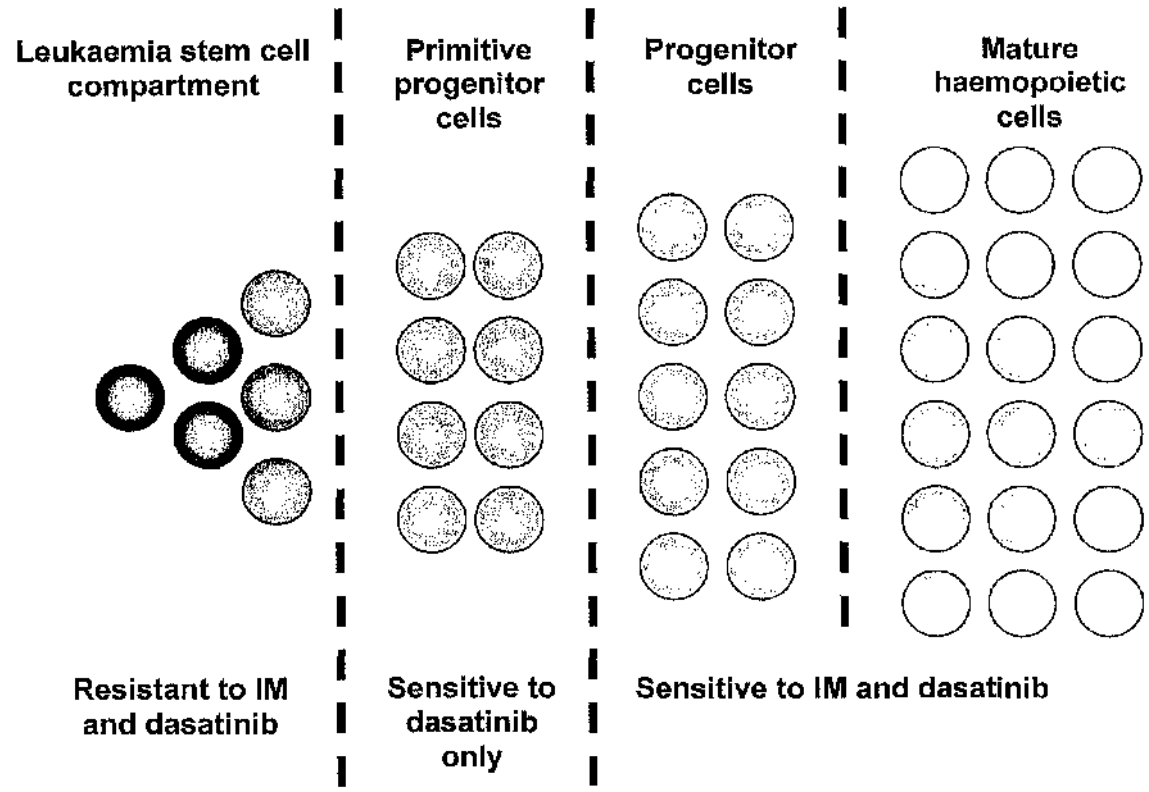


Figure 7-2. Schematic diagram to show the effects of IM and dasatinib on the different progenitor cell populations in CML. Although mature haemopoietic cells and the majority of progenitor cells are sensitive to both IM and dasatinib, primitive CML progenitors ($CD34^{+}38^{-}$) are resistant to IM, and the leukaemia stem cell compartment is resistant to both IM and dasatinib through mechanisms which have still to be fully elucidated.

7.3 Assessment of the efficacy of the novel FTI BMS-214662 against stem and progenitor cells in myeloid leukaemias *in vitro*

There is mounting evidence that strategies to target both quiescent stem cells and proliferating cells are required to eradicate CML (Graham et al., 2002; Holtz et al., 2002; Bhatia et al., 2003; Holtz and Bhatia, 2004; Holtz et al., 2005; Copland et al., 2006). The combination of BMS-214662 with either IM or dasatinib may be one such strategy. The data presented in Chapter 5 show that BMS-214662 is cytotoxic to both quiescent and proliferating primary cells in CP as well as BC

CML, and induces apoptosis, as evidenced by increased caspase-3 activity. Used as a single agent, it has minimal anti-proliferative effect on CML progenitors. In combination with either IM or dasatinib, BMS-214662 does not overcome the anti-proliferative effects of these agents, but kills the quiescent CML stem cells while they remain either in G_0 or arrested in early G_1 phase of the cell cycle. This is demonstrated by greater caspase-3 activity in the undivided $CFSE^{max}$ $CD34^+$ progenitor fraction. LTC-IC assays showed that culture in the presence of BMS-214662 virtually eliminated the ability of CML stem cells to form colonies. This provides further evidence for efficacy of BMS-214662 against quiescent CML stem cells by inhibiting their repopulating activity. BMS-214662 also demonstrated preferential cytotoxicity for leukaemic versus normal stem cells as evidenced by superior inhibition of colony formation in the CML LTC-IC assays compared to normal donor cell LTC-IC assays. In addition, in 2 of 3 CML LTC-IC assays, in which the $CD34^+$ cells used to seed the assay were pre-treated with BMS-214662 for 72 hours, FISH performed on the colonies present at the end of 7 weeks culture showed that the majority of these colonies were $BCR\text{-}ABL^-$. These colonies have come from residual normal haemopoiesis which was present in these patients with early CP CML at the time of leukapheresis (Goto et al., 1982; Coulombel et al., 1983b). In the third patient sample, in which FISH remained $BCR\text{-}ABL^+$ at the end of LTC-IC, it is likely that there was little normal haemopoiesis remaining at the time of leukapheresis. Indeed, after commencing IM, this patient required growth factor support and a reduced dose of IM to manage the cytopenias resulting from IM therapy.

These results demonstrate that, *in vitro*, BMS-214662 is superior to cytostatic FTIs such as lonafarnib (Jorgensen et al., 2005a) and BMS-225975 in eliminating quiescent CML stem cells. The concentration of BMS-214662 used in these experiments (250nM) is a clinically achievable plasma concentration (Tabernero et

al., 2005). Although BMS-214662 has yet to enter clinical trial in CML, phase 1 trials in AML using BMS-214662 as a single agent have shown promising activity (Cortes et al., 2005a). In a future clinical trial, which is currently under development with Bristol-Myers Squibb and the National Cancer Institute (NCI), it is likely that BMS-214662 would be given as a single weekly infusion in combination with continuous therapy with dasatinib. Monitoring of these patients is likely to include qRT-PCR to assess BCR-ABL levels and LTC-IC on bone marrow samples taken pre-treatment and at regular intervals throughout therapy, with FISH on the colonies produced to assess the effect of this combination therapy on the stem cell compartment. In clinical trials in advanced solid tumours, intermittent infusions of BMS-214662 have been successfully combined with cisplatin, carboplatin and paclitaxel (Mackay et al., 2004; Dy et al., 2005). Preliminary experiments done in this study show that, *in vitro*, intermittent exposure to BMS-214662, in combination with either continuous IM or dasatinib, tended to enhance the efficacy of these TKIs in CML with increased caspase-3 activity and a reduction in primitive progenitor cells.

The activity of BMS-214662 in BC CML and cell lines expressing BCR-ABL kinase mutations is also important. Patients with BC CML initially respond to IM but then relapse (Druker et al., 2001a). A proportion of these IM-resistant patients will respond to dasatinib or nilotinib but nearly all relapse within 6 months (Kantarjian et al., 2006; Talpaz et al., 2006). Therefore, BMS-214662 in combination with a TKI may increase the number of patients with BC CML who respond and also the length of remission, possibly allowing time for other therapeutic strategies such as alloSCT to be instituted. For those patients with BCR-ABL kinase mutations, the majority will respond to either dasatinib or nilotinib (Kantarjian et al., 2006; Talpaz et al., 2006), however, patients with the T315I mutation are resistant to these drugs. These patients may benefit from single agent therapy with BMS-214662

which we have shown to be equipotent in both wild type and mutant BCR-ABL expressing cell lines including T315I.

The effect of BMS-214662 on non-proliferating cells was first identified in *in vitro* models of solid tumours (Lee et al., 2001). However, despite extensive investigation, to date, the mode of action of BMS-214662 remains elusive (Rose et al., 2001; Manne et al., 2004). Multiple *in vitro* studies, including this one, have demonstrated its pro-apoptotic action (Rose et al., 2001; Manne et al., 2004; Marzo et al., 2004; Aichberger et al., 2005), but the pathway(s) leading to this has yet to be elucidated. It is unlikely that BMS-214662 is acting via the Ras pathway as it has been shown to be effective in Ras^{null} and Ras mutant cell lines (Rose et al., 2001). In addition, this study demonstrates that the structurally similar cytostatic FTI, BMS-225975, did not induce apoptosis and failed to eliminate CML stem cells. Recent *in vitro* studies in B-cell CLL (Marzo et al., 2004) and myeloma (Aichberger et al., 2005) have identified inhibition of Mcl-1 and activation of Bax and Bak as potential modes of action in these haematological malignancies. However, these conditions have not yet been shown to be stem cell disorders and the mechanism of action may be different. Western blotting showed no evidence of alterations in Mcl-1 levels in $CD34^+$ CML cells after treatment with BMS-214662 either alone or in combination with IM or dasatinib.

Further work has investigated the role of the centromere-associated proteins CENP-E and CENP-F in the activity of FTIs (Ashar et al., 2000). These proteins, which are involved in the mitotic process, stabilise microtubule capture by kinetochores required for complete chromosomal capture during metaphase and are believed to be mediators of the G_2/M checkpoint. Farnesyltransferase inhibitors alter the association between CENP-E and the microtubules resulting in cell cycle arrest with accumulation of cells prior to metaphase. However, this is an

unlikely mode of action in CML as cell cycle analyses performed for this project after treatment with BMS-214662 did not show cell cycle arrest.

Another study (Du and Prendergast, 1999) has focussed on the 21kD G-protein RhoB which regulates receptor trafficking and is believed to be a prenylation target of FTIs. This study demonstrated that RhoB GTPase activity was increased in some human cancer cell lines (predominantly epithelial) and that the accumulation of RhoB GGT increased apoptosis, inhibited tumour cell proliferation, and resulted in cell cycle arrest via multiple mechanisms, including alterations in cell adhesion. In addition, in cancer cells which over-express AKT, FTIs induce apoptosis and inhibit PI3K/AKT-mediated adhesion and growth factor-dependent survival pathways (Jiang et al., 2000a). It is possible that one of these proposed mechanisms of action that alters cell adhesion could explain the dramatic results seen in the LTC-IC assay experiments. The hypothesis would be that after treatment with BMS-214662, the CML stem cells are unable to adhere to the stromal layer, resulting in failure to form colonies. Again, these potential modes of action need further investigation in CML.

An alternative recent study has shown that FTIs directly induce production of ROS resulting in DNA damage in neoplastic cells (Pan et al., 2005; She et al., 2005). However, the pathway by which FTIs induce ROS is not yet fully elucidated, although it has been postulated that ROS are increased primarily by greater generation (Pan and Yeung, 2005). Interestingly, additional recent research has shown that FTIs increase inducible nitric oxide synthase (iNOS), resulting in higher nitric oxide (NO) production and apoptosis in $CD34^+$ CML cells (Selleri et al., 2003). Furthermore, in this study, the Rho inhibitor C3 exoenzyme, significantly increased iNOS expression in CML cells, indicating that FTIs may increase NO production at least partly through FTI-mediated inhibition of Rho. These exciting

studies provide further insights into the potential mechanisms of action through off-target activities of BMS-214662 and highlight the importance of determining a mode of action for BMS-214662 in CML. In future studies, it would be important to measure RhoB and iNOS activity before and after treatment with BMS-214662 in $CD34^+$ CML cells.

A further recent study using a chemical genetics approach in the nemotode *Caenorhabditis elegans* showed that several pro-apoptotic FTIs induced p-53-independent apoptosis (Lackner et al., 2005). In addition, these FTIs also inhibited the eukaryotic protein prenylase RabGGT in mammalian cancer cell lines. Geranylgeranylation by RabGGT is responsible for the membrane localisation of the Rab family of small GTPases which are involved in endosomal to lysosomal trafficking. This study also showed that RabGGT was over-expressed in a sub-set of human solid tumours (ovarian tumours, adenocarcimoma of colon, large cell lung carcinoma and melanoma) and that short interfering RNA (siRNA) against RabGGT induced apoptosis in 3 human cancer cell lines (the lung adenocarcinoma cell line A549, the ovarian cancer cell line A2780, and the p-53 null prostate cancer cell line PC3). This is the first study to suggest a potential role for RabGGT in apoptosis and cancer initiation or progression and identifies a novel pathway for p-53-independent apoptosis and a potential post-translational mode of action for FTIs, including BMS-214662. However, this study did not assess RabGGT in any haematological malignancies, and in further work I would propose determining if RabGGT is over-expressed in myeloid leukaemias. If it is, then I would go on to assess whether RabGGT was inhibited by BMS-214662 in $CD34^+$ CML or AML cells and, if so, whether inhibition of RabGGT with siRNA resulted in increased apoptosis in order to determine if RabGGT is a relevant target in myeloid leukaemias.

Further studies, including gene expression profiling, a kinase screen and detailed investigation of the Ras pathway have been undertaken by us in an attempt to determine the mode of action of BMS-214662 in CML. At present, it is hypothesised that BMS-214662 acts post-translationally on CML cells. In support of this post-translational role for BMS-214662, a comprehensive transcriptional profiling experiment utilising the Affymetrix microarray platform and analysed by Dr Francesca Pellicano showed no evidence for significant up- or down-regulation (by greater than 1.3-fold) of genes in CML $CD34^+$ cells following treatment with BMS-214662 as compared with either BMS-225975 or no drug control. In addition, Bristol Myers Squibb have performed a detailed kinase screen on the CML cell line K562 which was entirely negative and excluded kinase inhibitory activity against BCR-ABL and other kinases. However, one positive finding which is currently under further investigation is that, in association with induction of apoptosis, BMS-214662 resulted in near complete inhibition of phosphorylation of ERK and down-regulation of the inhibitor of apoptosis protein (IAP), whereas BMS-225975 neither induced apoptosis nor inhibited ERK phosphorylation or down-regulated IAP, strongly suggesting that BMS-214662 acts post-translationally.

In addition to its potent effect on CML stem and progenitor cells, very preliminary data, presented here in Chapter 6 indicate that BMS-214662 shows some efficacy when used either alone or in combination with Ara-C in primary AML cells. This is an important finding which requires further investigation. To this end, we are in the process of developing a bank of primary samples taken from AML patients at diagnosis which will allow us to undertake experiments similar to those performed in $CD34^+$ CML cells in this study. A Phase 1 trial of BMS-214662 has already been performed in AML (Cortes et al., 2005a) and demonstrated some efficacy. Further laboratory studies, in particular examining the effect of BMS-214662 alone and in combination with other chemotherapeutic agents on AML stem cells, may provide

a rationale for the development of a Phase 2 trial in AML as have already taken place with tipifarnib (Harousseau et al., 2003; Lancet et al., 2003).

Finally, it will be important to investigate the activity of BMS-214662 on stem cell malignancies such as breast, prostate and brain (Al-Hajj et al., 2003; Singh et al., 2003; Collins et al., 2005). Previous studies in solid tumours have failed to show a significant effect for BMS-214662, but these trials were undertaken in patients with a range of malignancies, not all of which have been demonstrated to be of stem cell origin, who had already received multiple chemotherapy regimens. In addition, in these heavily pre-treated patients, side effects at higher doses of BMS-214662 proved to be a problem with haematological, renal, gastrointestinal and hepatic toxicities being dose-limiting (Mackay et al., 2004; Dy et al., 2005; Papadimitrakopoulou et al., 2005; Eder et al., 2006). These Phase 1 studies were powered to assess safety and tolerability and not efficacy with only one study showing significant clinical benefit from treatment, and in this study (Tabernero et al., 2005), only 5 out of 31 patients had a response. This apparent lack of efficacy led to further trials with BMS-214662 in solid tumours being abandoned. It is only in recent months, based on the results presented here, that further clinical grade BMS-214662 has been manufactured and there has been a resurgence in interest in the potential use of BMS-214662 in stem cell malignancies. It is likely that further research into the use of BMS-214662 in solid tumours will be much more selectively focussed on tumours shown to be derived from stem cells such as breast, prostate and brain.

It is only by understanding the mechanism of action of BMS-214662 that advances can be made in using BMS-214662 in the clinic, resulting in increased efficacy and reduced toxicity. In addition, by using BMS-214662 as a laboratory tool, it may be possible to identify new molecular targets in the treatment of myeloid leukaemias

and other stem cell neoplasms leading to the development of novel therapeutic agents.

7.4 Final discussion and conclusions

The importance of developing targeted cancer therapies is related to the toxicity of conventional chemotherapy regimens to normal cells and the failure of these non-specific agents to target cancer stem cells, resulting in an inability to cure many cancers. The development of molecularly targeted therapies allows specific targeting of cancer cells without affecting normal cells, reducing toxicity and, in most cases, improving patient quality of life. Although the introduction of IM for the treatment of BCR-ABL$^+$ malignancies is widely heralded as the first successful molecularly targeted cancer therapy (Druker et al., 2001b), it was preceded by others. Perhaps the first real targeted approach to cancer therapy was the use of hormonal manipulation in the form of tamoxifen in breast cancer (Early Breast Cancer Trialists' Collaborative Group, 1998). Another was the use of monoclonal antibodies such as the anti-CD20 agent rituximab (Mabthera^{TM}) in B cell disorders (Coiffier et al., 2002). Currently, targeting signal transduction pathways is a major strategy in the development of novel anti-neoplastic agents.

CML represents an excellent model for the study of cancer stem cells because it results from a single genetic mutation (*BCR-ABL*) and is measurable by standard laboratory techniques such as FISH and PCR (Kaeda et al., 2002). This also makes CML an ideal disease in which to identify novel agents which target cancer stem cells.

There have been major advances in the treatment of CML in recent years with the development of IM (Druker et al., 1996; O'Brien et al., 2003) and, more recently,

the oral, multi-targeted kinase inhibitor dasatinib (Talpaz et al., 2006) and the second generation BCR-ABL kinase inhibitor, nilotinib (Kantarjian et al., 2006). These newer compounds target IM-resistant mutations and, in the case of dasatinib, reach further into the stem cell compartment (Copland et al., 2006). However, despite inhibition of BCR-ABL, quiescent CML stem cells remain insensitive to these compounds. Further, as shown here for the first time, in LTC-IC experiments, IM and dasatinib would appear to exert a protective effect on the CML stem cell compartment through their anti-proliferative action, although this may be an *in vitro* culture artefact and has yet to be confirmed *in vivo*. Therefore, strategies are required to target both quiescent and proliferating BCR-ABL$^+$ cells.

The importance of cancer stem cells is a rapidly emerging area of research and the ability of BMS-214662 to selectively target quiescent LSCs is shared by a very few novel anti-cancer agents (Guzman et al., 2002; Guzman et al., 2005). Studies are planned to determine if this property is applicable to other malignancies with quiescent stem cells (breast, brain and prostate cancer etc). However, the study of quiescent primary cancer stem cells is complex as it is difficult to separate this small sub-population of cells from the proliferating majority, making characterisation of these cells difficult. Studies in fibroblasts (Coller et al., 2006), which artificially induced quiescence by different mechanisms (mitogen withdrawal, contact inhibition and loss of adhesion), showed that although there was a cohort of genes universally expressed in quiescent states, the expression of many other genes varied depending on the mechanism inducing quiescence. In addition, it is unclear which of these mechanisms are active in primary cells.

If differences in gene and protein expression between quiescent normal and cancer stem cells can be found, then it is possible that these differences could be exploited to develop cancer-specific therapies which avoid damage to normal

tissues. Although BMS-214662 exhibits some toxicity to normal primitive progenitor cells, it is far more toxic to leukaemia progenitor cells. Thus BMS-214662 may prove to be such a cancer-specific therapy or perhaps a tool for developing other approaches to targeting the cancer stem cell.

Recently, two different dynamic models of CML have been proposed (Michor et al., 2005; Roeder et al., 2006) which arrive at different conclusions. The first model suggests that although IM is a potent inhibitor of differentiated CML cells, it does not reduce the CML stem cell population (Michor et al., 2005). In CML, *BCR-ABL* transcripts exhibit a biphasic decline in patients responding to IM, but even after years of therapy, the majority of patients have persistent disease at the molecular level (Hughes et al., 2003; Branford et al., 2004). The biphasic decline in *BCR-ABL* transcripts consists of an initial rapid decline which represents the death of differentiated CML cells followed by a much slower decrease representing the death of more primitive CML progenitors in response to IM. In support of this first hypothesis, in a proportion of patients that discontinued IM after prolonged treatment and had achieved a CMolR, the number of *BCR-ABL* transcripts rapidly increased over the following three months to at least pre-treatment levels (Cortes et al., 2004b; Mauro et al., 2004). This indicates that IM does not deplete the CML stem cell population which is driving the disease, and supports the hypothesis that CML stem cells are resistant to IM and other agents (Graham et al., 2002; Holtz et al., 2005; Jorgensen et al., 2005a; Copland et al., 2006). This model further proposes that, as CML progresses, the number of LSCs rises and the probability of a patient having a resistance mutation also increases as a result of this larger population of CML stem cells (Michor et al., 2005). In addition, it is suggested that the time to treatment failure as a result of acquired resistance is dependent on the growth rate of the CML stem cells. Therefore, based on the theories put forward in this study, IM is extremely unlikely to cure CML patients, and over time, the

majority of patients will develop acquired resistance as the stem cell population gradually expands. Thus, the development of strategies, such as BMS-214662, to target the LSC population will be vital for the eventual eradication of CML.

The hypothesis proposed in the second study is rather more positive for CML patients (Roeder et al., 2006). It suggests that the clinically observed biphasic pattern of *BCR-ABL* transcript dynamics may be explained by a selective effect of IM on proliferating CML stem cells. This model makes two main assumptions. Firstly, it assumes that IM inhibits proliferative activity and induces death of proliferating CML stem cells and secondly, it assumes that there is a large population of quiescent CML stem cells which are resistant to IM due to their quiescent state as previously demonstrated (Graham et al., 2002). However, these quiescent CML stem cells retain the potential for proliferation and are responsible for the rapid relapses seen after stopping IM (Cortes et al., 2004b; Mauro et al., 2004). This model predicts that, over time, as quiescent CML stem cells gradually enter the cell cycle, they will proliferate and become sensitive to IM. Therefore, levels of MRD will continue to fall over prolonged periods of IM treatment as suggested by clinical data (Branford et al., 2004), and complete disease eradication may be possible if patients do not develop resistance mutations. The model also proposes that promoting quiescent CML stem cells to enter the cell cycle by using additional agents in combination with IM may enhance the eradication of MRD in CML. This hypothesis was tested in Chapter 3 of this thesis which used priming with intermittent exposure to rHu-G-CSF in an attempt to force the quiescent CML stem cells to proliferate with promising results. As discussed, a clinical trial (GIMI trial) to test this potential therapeutic strategy *in vivo* is currently underway (Mitchell et al., 2006).

Although these two different hypothetical models of CML dynamics arrive at different conclusions, they both highlight the importance of developing drug combination strategies with IM or the newer agents (dasatinib, nilotinib and others) to eliminate the quiescent CML stem cell population.

Another hypothesis leading to much debate at present is the concept that CML stem cells may not be dependent on BCR-ABL; therefore BCR-ABL kinase inhibitors may not be appropriate therapy for CML stem cell eradication. There is evidence that CML is a stem cell disorder (Eaves et al., 1998). In addition, there is support for a normal HSC hierarchy in LSCs (Bonnet and Dick, 1997), suggesting that LSCs may retain many of the properties of HSCs. Furthermore, there is increasing evidence that BCR-ABL-targeted therapies such as IM, dasatinib or nilotinib fail to eradicate CML stem cells *in vitro* (Graham et al., 2002; Holtz et al., 2005; Copland et al., 2006; Jorgensen et al., 2007).

Further support for this hypothesis comes from studies which have shown that transduction with either *MOZ-TIF2* or *MLL-ENL* resulted in the development of the capacity for self-renewal in committed non self-renewing myeloid progenitors *in vitro* and rapid induction of leukaemia in murine transplantation models (Cozzio et al., 2003; Huntly et al., 2004), whereas transduction of these same non self-renewing myeloid progenitors by *BCR-ABL* did not (Huntly et al., 2004). The mechanisms underlying this difference in the ability of oncogenes to bestow self-renewal properties on a leukaemia cell remain to be elucidated. Another recent study has shown that, despite inactivation of $p190^{BCR-ABL}$ in a murine model of ALL, there was failure to rescue the malignant phenotype, implying that $p190^{BCR-ABL}$ is not required for disease maintenance in mice (Perez-Caro et al., 2006). It was further hypothesised that $p190^{BCR-ABL}$ induced epigenetic changes or additional mutations, rendering the LSCs insensitive to inactivation of $p190^{BCR-ABL}$.

However, this is controversial as a number of other studies have demonstrated the necessity of BCR-ABL for disease maintenance (Szczylik et al., 1991; Skorski et al., 1994; Zhao et al., 1997; Huettner et al., 2000). In addition, in this murine model (Perez-Caro et al., 2006), the disease did not behave as an acute leukaemia as survival was longer than other acute leukaemia models and also the mice were unresponsive to IM which is not typical of BCR-ABL$^+$ ALL, in which the majority of patients do achieve a remission, albeit short-lived (Druker et al., 2001a). Furthermore, another recent study demonstrated that dasatinib resulted in complete remission of B-ALL with prolonged survival in a murine model (Hu et al., 2006), providing additional evidence of dependence on BCR-ABL for disease maintenance.

In some cells, the ability to self-renew may be due to the acquisition of secondary mutations in committed progenitor cells which allow these cells to take on the properties of a LSC. This has recently been demonstrated in BC CML by Jamieson *et al* (Jamieson et al., 2004) who showed that there was an increased granulocyte-macrophage progenitor population in BC CML compared to earlier stages of disease, and this population had the ability to self-renew *in vitro* in association with activation of β-catenin, a protein of the WNT signalling pathway which is associated with cell differentiation, proliferation and death. Very interestingly, two inhibitors of the WNT signalling pathway have recently been described which show efficacy in myeloid leukaemias (Guzman et al., 2006; Kavalerchik et al., 2006). The first of these, MCC-001, a marine sponge-derived β-catenin antagonist was demonstrated to inhibit the re-plating capacity of CML stem cells, derived from patients with advanced phase CML, at doses which were non-toxic to normal HSCs (Kavalerchik et al., 2006). The second compound, 4-benzyl, 2-methyl 1,2,4-thiadiazolidine 3,5-dione (TDZD-8), a GSK-3β inhibitor, induced rapid cell death in both primary AML and BC CML cells (Guzman et al., 2006).

Further studies in a NOD/SCID mouse xenotransplantation model showed a 93% reduction in engraftment with TDZD-8 for AML samples compared with an 11% reduction for normal cell engraftment. Further studies are currently underway to fully elucidate the mechanisms of action of these two novel compounds and further assess their effect on the LSC compartment.

It should also be considered that *BCR-ABL* may be an oncogenic mutation which is tolerated by HSCs because it has little or no effect on the stem cell in which it arises; rather its effects are expressed in the progeny of the mutated stem cell. It is possible that mutations which increase self-renewal or impair differentiation may be toxic to the HSC and/or trigger apoptotic pathways. *BCR-ABL* may not induce these changes in the stem cell and, therefore may be tolerated. However, there is evidence against this hypothesis from Huntly *et al*, who demonstrated that HSC transduction with *MOZ-TIF2* resulted in a form of AML indistinguishable from other *MOZ-TIF2* associated leukaemias (Huntly et al., 2004). Therefore, in HSCs, this oncogenic mutation was not only tolerated, but was capable of inducing leukaemia without HSC toxicity or apoptosis.

The models of CML disease response dynamics and the hypothesis that BCR-ABL may not be a target in CML stem cells highlight the importance of developing therapies which target the LSC specifically and identifying differences between normal HSCs and LSCs which can be targeted for therapy. A recent study has shown that dependence on the tumour suppressor gene *PTEN* separates HSCs from LSCs (Yilmaz et al., 2006). In an *in vivo* mouse model, conditional deletion of *PTEN* resulted in a myeloproliferative disorder which progressed to acute leukaemia over a number of weeks and also induced acute leukaemia in recipient mice in a mouse transplantation model. In addition, HSCs were not maintained in the absence of *PTEN*, as *PTEN* deletion increased HSC proliferation which

resulted in HSC depletion and the cells were unable to reconstitute irradiated mice, i.e. there was loss of self-renewal capacity. It is likely that these effects were modulated by mTOR as rapamycin not only depleted LSCs but also restored normal HSC function. Further studies in AML have demonstrated that proteasome inhibitors induce apoptosis in AML stem cells in association with inhibition of NF-κB and activation of p-53-related genes (Guzman et al., 2002). Furthermore, the naturally-occurring small molecule inhibitor parthenolide also causes apoptosis in LSC in AML and BC CML, again through inhibition of NF-κB and activation of p-53 and also increased production of ROS (Guzman et al., 2005).

The data presented in this thesis for BMS-214662 in CP CML represents an important step forward in targeting quiescent CML stem cells as this is the first study to demonstrate an agent which selectively kills quiescent CML stem cells, although the mode of action has yet to be elucidated. All these examples highlight the importance of finding agents which specifically target cancer stem cells. It is only by understanding the biology of cancer stem cells and developing novel stem cell-directed therapies that progress will be made in eradicating these diseases.

8 References

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Appendix 1

Patient characteristics for CML samples obtained

UPN	Sex	Disease Stage	WCC at leucapheresis (x 10^9/ L)	Pre-selection % CD34	Post selection % CD34	Type of CD34 selection	%Ph^+ in $CD34^+$ (D-FISH)
23	M	CP	276	16.9	51.2	Stemsep	69.1
31	M	CP	350	14.4	85.3	Isolex	100
81	F	CP	157	17.6	89	Isolex	100
85	M	CP	N/A	42	N/A	Unmanip	88
101	F	CP	67	4.4	49.2	Stemsep	90.9
144	F	CP	234	33.6	98	Isolex	99.7
151	F	CP	270	13.2	98.3	CliniMACS	100
155	F	CP	78	3.6	98.3	CliniMACS	99
160	M	CP	300	1.6	97	CliniMACS	87.4
162	M	CP	173	1.7	98	CliniMACS	97
164	F	CP	147	5.8	99	CliniMACS	100
166	M	CP	206	6.0	99	CliniMACS	94.8
170	M	CP	N/A	N/A	97	CliniMACS	96.8
174	F	CP	221	5.0	98	CliniMACS	99.6
189	F	CP	133	6.0	97	CliniMACS	92.9
198	M	CP	240	4.2	87.3	CliniMACS	98.5
215	M	CP	165	7.7	96	CliniMACS	95.4
217	F	CP	106	0.6	91.1	CliniMACS	92
218	M	CP	68	6.5	97.8	CliniMACS	98.7
219	M	CP	307	4.2	98.7	CliniMACS	98.2
222	M	CP	124	12.7	99.2	CliniMACS	85.8
168	M	AP	330	11.5	98	CliniMACS	100
185	M	AP	139	12.0	95	CliniMACS	100
001	F	LBC	N/A	17.2	N/A	Unmanip	N/A
002	F	MBC	19.1	48.2	N/A	RBC Lysis	N/A
003	F	MBC	N/A	4.1	N/A	RBC Lysis	N/A
004	M	MBC	12.5	3	N/A	Unmanip	N/A
005	M	LBC	N/A	16.8	N/A	RBC Lysis	N/A

UPN, unique patient number; LBC, lymphoid blast crisis; MBC, myeloid blast crisis; Unmanip, unmanipulated sample; RBC Lysis, red blood cell lysis; N/A, not applicable or not available.