

The Role of Cytokines in governing the Expansion of the T315I mutation in Chronic Myeloid Leukaemia

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Abbreviations

7-AAD	7-Aminoactinomycin D
ABCB1	ATP binding cassette, sub-family B1
ABCG2	ATP binding cassette, sub-family G2
<i>ABL1</i>	Abelson murine leukaemia virus human homologue 1 gene
ASK1	Apoptotic signal kinase
Akt	a serine threonine kinase (also known as protein kinase B)
ALL	Acute lymphoblastic leukaemia
Ann V	Annexin V
AP	Accelerated phase
ATP	Adenosine triphosphate
Bad	BCL-2 antagonist of cell death
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated X protein
BC	Blast crisis
Bcl-2	B cell lymphoma 2
Bcl-X _L	B-cell lymphoma-extra large
<i>BCR</i>	Breakpoint cluster region
<i>BCR-ABL1</i>	<i>BCR-ABL1</i> oncogene
BELA trial	Bosutinib efficacy and safety in CML
BH	Bcl-2 homology
Bim	BCL-2 interacting mediator of cell death
BM	Bone marrow
BMSC	Bone marrow stromal cells
bp	Nucleotide base pair

BP	Blastic phase
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
Ca ²⁺	Calcium ion
CaCl	Calcium chloride
CBL	Casitas B-lineage lymphoma proto-oncogene
CCyR	Complete cytogenetic response
CD	Cluster of differentiation
cDNA	Complementary DNA
C/EBP- α	CCAAT enhancer-binding protein α
CFSE	Carboxyfluorescein diacetate succinimidyl ester
Chr	Chromosome
CHR	Complete haematological response
CML	Chronic myeloid leukaemia
CMR	Complete molecular response
CSL	Commonwealth Serum Laboratory
CO ₂	Carbon dioxide
CP	Chronic phase
Crk	CT10 sarcoma oncogene cellular homologue
Crkl	CT10 regulator of kinase-like
CXCL12	C-X-C motif chemokine 12 (also known as Stromal cell-derived factor 1)
CXCR4	C-X-C motif chemokine receptor 4
DAG	Diacylglycerol
das	Dasatinib

DASISION	Dasatinib versus Imatinib Study in Treatment-Naïve CML Patients
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DTT	Dithiothreitol
ECF	Enhanced chemifluorescence substrate
EDTA	Ethylenediaminetetra-acetic acid
eGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ENESTnd	Evaluating Nilotinib Efficacy and Safety in Clinical Trials–Newly Diagnosed Patients
Erk	Extracellular signal related kinase
<i>et al</i>	<i>et alia</i>
<i>ETV6-PDGFRβ</i>	ETS translocation variant 6- Platelet derived growth factor receptor β gene
FACS	Fluorescent activated cell sorting
FBS	Foetal bovine serum
FDA	Federal Drug Authority
Fgr	Gardner-Rasheed feline sarcoma viral oncogene homologue
FGF	Fibroblast growth factor
FGFb	Fibroblast growth factor basic (also known as FGF-2)
FGFR	Fibroblast growth factor receptor
<i>FIP1L1-PDGFRα</i>	Fip 1 like 1- Platelet derived growth factor receptor α gene
FLT3-ITD	FMS-like tyrosine kinase 3- internal tandem domain

c-FMS	Cellular homologue of the feline sarcoma virus, v-FMS
FOXO	Forkhead O transcription factor
FOXO 3A	Forkhead O family of transcription factors 3A
<i>g</i>	Gravitational force (Relative centrifugation force)
Gab1	GRB2-associated-binding protein 1
Gab2	GRB2-associated binding protein 2
GAP	Guanosine triphosphatase-activating protein
G-CSF	Granulocyte colony stimulating factor
GDP	Guanosine diphosphate
GIST	Gastrointestinal stromal tumours
GM-CSF	Granulocyte macrophage colony stimulating factor
Grb2	Growth factor receptor bound protein 2
GTP	Guanosine triphosphate
KD	Kinase domain
Hck	Haematopoietic cell kinase
HGF	Hepatocyte growth factor
hnRNP-E2	Heterogeneous nuclear ribonucleoprotein- E2
HSC	Haematopoietic stem cell
HSCT	Haematopoietic stem cell transplantation
IC50	Concentration of drug required to inhibit Bcr-Abl kinase activity by 50%
IFN α	Interferon- α
I κ B	Inhibitor of NF- κ B
IKK α	Inhibitor of nuclear factor kappa B kinase subunit α
IL-1 α	Interleukin 1 α

IL-1 β	Interleukin 1 β
IL-6	Interleukin 6
IL-8	Interleukin 8
im	Imatinib mesylate
IMDM	Iscoe's modification of Dulbecco's medium
IP3	Inositol triphosphate
IRIS	International randomised study of interferon versus STI571
IS	International standard
Jak	Janus kinase
JNK	c-Jun N-terminal kinase
kb	Kilo base pairs
kDa	kilo Dalton
Lck	Lymphocyte-specific protein tyrosine kinase
LIF	Leukaemia inhibitory factor
LSC	Leukaemic stem cells
Lyn	Lck/Yes-related novel protein
mA	mili (10^{-3}) Ampere
MAPK	Mitogen activated protein kinase
M-bcr	Major breakpoint cluster region
m-bcr	minor breakpoint cluster region
MCyR	Major cytogenetic response
MCP-1	Monocyte chemo attractant protein 1
MDC	Macrophage derived chemokine
MDR1	Multiple drug resistant protein 1
MEK	MAP kinase/Erk kinase

MET	Mesenchymal epithelial transition factor
MFI	Mean fluorescent intensity
μ	micro (10^{-6})
MgCl ₂	Magnesium chloride
MIP-1α	Macrophage inflammatory factor 1α
MIP-1β	Macrophage inflammatory factor 1β
ml	mili (10^{-3}) litre
mM	mili (10^{-3}) Molar
MMR	Major molecular response
mRNA	Messenger Ribonucleic acid
MSC	Mesenchymal stem cells
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mTOR	Mammalian Target of Rapamycin
NaCl	Sodium chloride
NaN ₃	Sodium azide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram (10^{-9} gram)
nil	Nilotinib
nM	nano (10^{-9}) Molar
NSCLC	Non-small cell lung cancer
OA	Organic cation transporter 1 activity
OCT-1	Organic cation transporter 1
PACE	Ponatinib Ph+ ALL and CML Evaluation
PBS	Phosphate buffered saline or Pharmaceutical Benefit Scheme

PCR	Polymerase chain reaction
PDGFR α	Platelet derived growth factor receptor α
PE	Phycoerythrin
pg	pico (10^{-12}) gram
Ph	Philadelphia
PI	Proliferation index
PI3K	Phosphatidylinositol-3 kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PKC	Protein kinase C
PLC γ	Phospholipase C γ
P-loop	Phosphate binding loop
PS	Phosphatidylserine
PUMA	p53 up regulated modulator of apoptosis
PVDF	Polyvinylidene fluoride
r(IL-6)	Recombinant (IL-6)
Rac	Ras-like guanosine triphosphatase
Raf	Rapidly accelerated sarcoma protein kinase
Ras	Rat sarcoma
<i>RET</i>	Rearranged during transfection proto-oncogene
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (media)
RQ-PCR	Real-time quantitative polymerase chain reaction
SA Pathology	South Australia Pathology Services

SAPK	Stress activated protein kinase
SCF	Stem cell factor
SDF-1	Stromal cell derived factor 1
SDM	Serum deprived media
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFK	Src family kinase
SH	Src homology domains
SHC	Src homology 2 domain-containing-transforming protein C1
SKP2	S-phase kinase-associated protein 2
SOS	Son of Sevenless guanine nucleotide exchange factor
STI571	Imatinib
STAT	Signal transducer and activator of transcription family
TAE	Tris-Acetate-EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.1% Tween20
TGF- α/β	Transforming growth factor α/β
TKI	Tyrosine kinase inhibitor
TNF- α	Tumour necrosis factor- α
V	Volts
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
WB	Western blot
WT	Wild-type

Abstract

Since the introduction of imatinib, the outlook for patients with chronic myeloid leukaemia (CML) has dramatically improved. However, approximately 30-40% of patients develop intolerance or resistance to the drug and cease therapy. Among those who develop resistance, the most common cause is the development of point mutations in the kinase domain (KD) of *BCR-ABL1* which impair drug binding and hence result in the loss of kinase inhibition. The T315I mutation (gatekeeper mutation) is of particular significance as it is one of the most common mutations and it is resistant to all 3 tyrosine kinase inhibitors (TKI) currently approved for therapeutic use in Australia.

There is evidence to suggest that KD mutations in *BCR-ABL1* may alter the biological activity of Bcr-Abl. Both *in vitro* and clinical studies have suggested that the T315I mutation results in greater oncogenic potential. Patients harbouring this mutation have poorer prognoses and a significantly higher rate of progression compared to other mutations. There is also ample evidence to suggest that secretion of cytokines and growth factors play a role in rendering *BCR-ABL1* positive cells resistant to TKI.

This thesis focuses on the role of cytokines in the resistance mechanism of cells with the T315I mutation and how this mechanism is achieved. K562-T315I cells were developed in our laboratory by exposing them to increasing levels of dasatinib over several months. HL60 cell lines were virally transduced with the *BCR-ABL1*^{p210} and *BCR-ABL1*^{T315I} constructs. Investigations have identified that several soluble factors are preferentially secreted by cells with the T315I mutation namely, FGF-2, IL-8, MCP-1 and G-CSF. Additionally, the supernatant of K562-T315I cells also contains higher concentrations of GM-CSF and IL-6. These studies also identified that FGF-2 was able to protect K562 naïve cells from TKI-induced cell death and suggests that this occurs via activation of the MAPK and STAT5 pathways. Thus, in addition to acquiring point mutations that result in the inability of TKIs to bind Bcr-Abl, the T315I mutation also results in overexpression of FGF-2 which can confer resistance to non-mutated cells.

Furthermore, in the presence of imatinib, dasatinib and nilotinib, K562-T315I cells proliferate and survive better than in the absence of a TKI. This is due to hyperactivation of the MAPK pathway whereas signalling of other pathways, JAK/STAT5 and PI3K/Akt are not increased.

This phenomenon was demonstrated especially with nilotinib but studies did not indicate a cytokine mediated effect through an autocrine hypersecretion by the K562-T315I cells. Nevertheless, this finding may be one of the reasons why the T315I mutation confers a worse outcome in patients with CML if they remain on imatinib, nilotinib and dasatinib treatment.

Thesis Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Oi-Lin Lee

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Chapter 1

Introduction

Chronic myeloid leukaemia (CML) is a haematopoietic malignancy that originates in a primitive bone marrow stem cell resulting in an increased proliferation of white cells especially of the myeloid series. The expansion of the leukaemic clone is due to excessive proliferation and defective apoptotic (programmed cell death) signalling (1). CML is maintained by a pool of self-renewing malignant cells called leukaemic stem cells (LSCs). It has a worldwide annual incidence of 1-1.5 cases per 100 000 individuals and constitutes approximately 15-20% of all leukaemias in adults (2).

Most patients are diagnosed in chronic phase and if left untreated will progress through accelerated phase and blast crisis which is rapidly fatal (3). Patients with CML have a unique fusion gene, *BCR-ABL1*, which is responsible for the pathogenesis of the disease. Imatinib was designed to inhibit the tyrosine kinase activity of Bcr-Abl and since its introduction, the treatment landscape of the disease has dramatically changed (4). Since then, several other tyrosine kinase inhibitors (TKIs) have been introduced; two of which, nilotinib and dasatinib, are approved for therapeutic use in Australia. However, despite their remarkable success, some patients develop resistance to TKIs. A major cause of treatment failure is the development of mutations in the kinase domain of *BCR-ABL1* (5, 6).

One particular mutation, T315I (where threonine is substituted by isoleucine at the 315 residue of the *ABL1* kinase domain), is especially problematic as it is resistant to all three TKIs (7, 8). Some studies have shown that the development of this mutation is associated with poorer prognosis including overall survival (9, 10).

Not surprisingly, much of CML research is focused on overcoming resistance. This study concentrates on the T315I mutation and in particular how the mutation changes the biological properties of Bcr-Abl and hence affects disease progression.

1.1 Biology of CML

1.1.1 Philadelphia Chromosome (Ph)

CML was the first malignant disease to be associated with a consistent chromosomal abnormality – the Philadelphia (Ph) chromosome (11) (Figure 1-1).

The Ph chromosome was first described in 1960 and is the result of a balanced reciprocal translocation from the fusion of the Abelson kinase (*ABL1*) gene on Chr 9 and the Break point cluster region (*BCR*) gene on Chr 22 - t(9;22)(q34;q11) (12). The *BCR-ABL1* oncogene transcribes a chimeric Bcr-Abl oncoprotein that has constitutive tyrosine kinase activity and was established as essential for the pathogenesis of CML through the use of mouse models (13). Murine bone marrow cells retrovirally-infected with *BCR-ABL1* and transgenic mice models induced a CML-like disease (14-16) confirming the central role *BCR-ABL1* in the pathogenesis of CML (16). These mice developed neutrophilia, splenomegaly, bone marrow myeloid hyperplasia and extramedullary haematopoiesis resembling human CML (16). The Ph chromosome is also found in approximately 25% of adult Acute Lymphoblastic Leukaemia (ALL) and prior to the introduction of TKI confers a poor prognosis in this group (17).

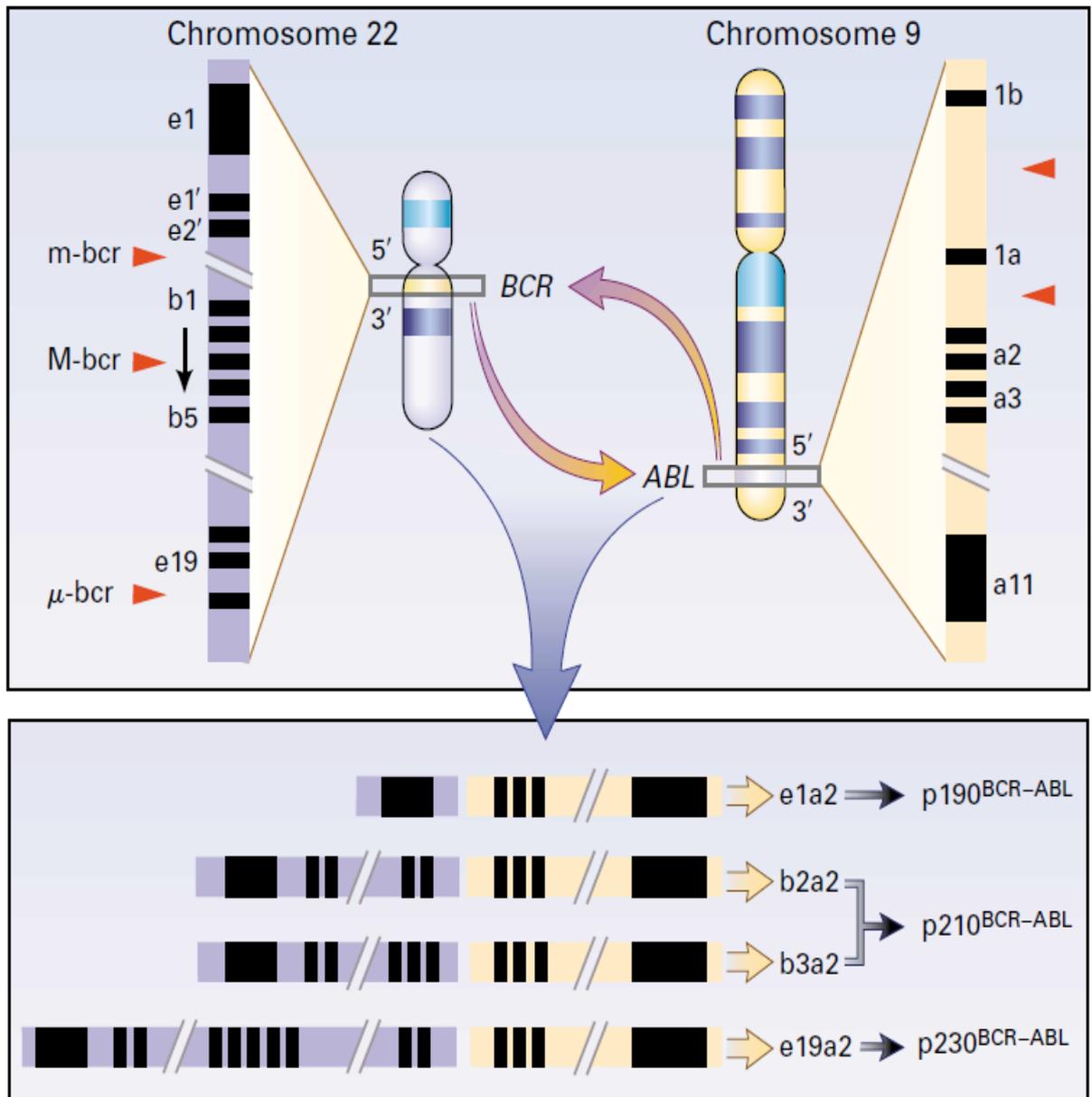
ABL1 is a proto-oncogene that encodes a protein with tyrosine kinase activity which is expressed in most tissues and is normally tightly regulated (18). A tyrosine kinase is an enzyme that transfers phosphate groups from ATP to tyrosine residues on another protein. It functions as an “on-off” switch and is an important mediator of the signalling cascade. Hence, signals can be communicated by protein phosphorylation to regulate cellular activity such as cell division and proliferation (19). Normal Abl protein function has not been totally defined but is thought to be involved in cell cycle regulation (18) and integration of signal transduction from growth factor receptors and adhesion receptors to regulate cytoskeleton structures (20). Two isoforms of *ABL1* exist formed by alternate splicing of the first exon, isoform 1a and 1b. Type 1b is slightly bigger as it has a myristoylation site which anchors it to the plasma membrane (21). At the NH₂ terminal, three Src homology domains (SH) are located (Figure 1-2). SH1 domain carries the tyrosine kinase function and is also known as the kinase domain (KD). SH2 domain binds phosphotyrosine-containing consensus sites and the SH3 domain binds with the SH3 domains of other proteins such as CT10 regulator of kinase-like (Crkl) and growth-factor-receptor bound 2 (GRB2) (22). The C terminal of Abl

Figure 1-1 The Philadelphia chromosome and the resulting fusion oncoproteins

The Ph chromosome results from the translocation of *ABL1* segments on Chr 9 to *BCR* segments on Chr 22. The majority of the translocation breakpoints on the *ABL1* gene are located at the 5' of exon a2. In CML, this mostly occurs between exons 1b and 1a (denoted by red arrows on the right). Different breakpoints are located along the *BCR* gene (denoted by red arrows on the left) and depending on the breakpoint regions, different sized segments of *BCR* are fused with the 3' sequences of the *ABL* gene. This results in the transcription of different fusion mRNA and resultant translation of variant fusion proteins, i.e. p190, p210 and p230 which give rise to different disease phenotypes. m-bcr denotes minor breakpoint region, M-bcr major breakpoint region and μ -bcr is a third breakpoint region that is located between exon e19 and e20.

(Taken from Faderl, S. et al. NEJM 1999)(23)

Figure 1-1



contains the DNA binding domain, nuclear localisation signals and a binding site for actin (24).

The *BCR* gene also transcribes a signalling protein that contains multiple modular domains. It includes a coiled-coil oligomerization domain, a serine/threonine kinase domain and Tyr177 which serves as a docking site for Grb2, Grb10, 14-3-3 and Abl through its SH2 domain (25). The coiled-coil domain is crucial for Bcr-Abl dimerization and hence kinase activity. Two monomers associate in anti-parallel dimers that in turn form a tetramer (26). Mutations in this coiled-coil domain impair oligomerization and thus have been shown to affect kinase activity and reduce transformation (27). The SH2 domain of Grb2 binds to Tyr177 on Bcr, which links it to Ras, a protein which plays a central role in the signalling pathways that regulate cell proliferation and differentiation in CML (28). Phosphorylation of Tyr177 is essential for CML pathogenesis as mutations of Tyr177 (for example Y177F) abolish its ability to bind Grb2 and hence decreases Ras activation (29). Mice with this mutation have greatly reduced ability to develop a myeloproliferative disease which resembles human CML (30). The N-terminal phosphothreonine and phosphoserine residues of Bcr are required for interaction with SH2 proteins including Abl (31).

The breakpoints within the *ABL1* gene take place either upstream of exon 1b, downstream of exon 1a or more frequently, between the 2 exons (Figure 1-1). Major breakpoint cluster region (M-bcr) is found in most patients with CML and a third of patients with Ph positive ALL (5, 13, 15, 32). Breakpoints within the *BCR* gene occur within a 5.8 kb area spanning exons e12-e16. Alternative splicing gives rise to either b2a2 or b3a2 fusion transcripts. Both these transcripts translate into a 210-kDa oncoprotein (p210^{BCR-ABL1}) (33). Prognosis and response to treatment in patients with either b2a2 or b3a2 are similar, however, one large randomised study has shown higher platelet counts in a subset of patients with the b3a2 transcript (34). In rare cases of CML and most patients with Ph positive ALL, the Bcr breakpoint is within a 54.4 kb area known as the minor breakpoint cluster region (m-bcr) which gives rise to an e1a2 transcript that translates to a 190-kDa protein (p190^{BCR-ABL1}) (33). The third breakpoint cluster region (μ -bcr) gives rise to a 230-kDa protein and is associated with chronic neutrophilic leukaemia, a relatively benign myeloproliferative disease (35).

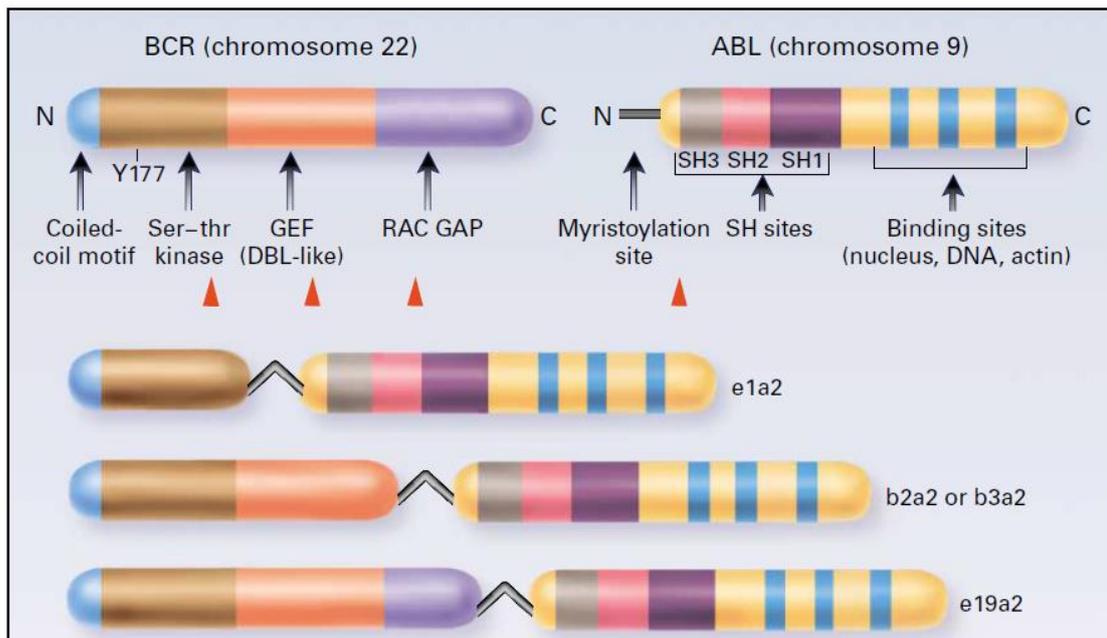


Figure 1-2 Functional domains of Bcr-Abl and different fusion-protein products formed

Bcr contains a coiled-coil oligomerization domain, a serine-threonine kinase domain (ser-thr), a DBL/CDC24 guanine-nucleotide exchange factor (GEF) homology domain, a Ras-like GTPase guanosine triphosphatase-activating function (Rac GAP). It also contains binding sites for Grb2 at tyrosine 177 (Y177) as well as for Grb10, 14-3-3 and Abl through its SH2 domains. Abl also contains the tandem Src homology 3 (SH3), SH2 and SH1 domains which contains the tyrosine kinase activity. .

The arrowheads indicate where the breakpoint regions lie. Depending on which breakpoints involved, different size fusion-proteins are formed. e1a2 translates to p190^{BCR-ABL1}, b2a2 and b2a3 translates to p210^{BCR-ABL1} and e19a2 to p230^{BCR-ABL1}.

N denotes N-terminal amino acid sequence, C: C-terminal amino acid sequence.

(Taken from Faderl, S. et al. NEJM 1999) (23)

1.1.2 Pathophysiology of CML

The constitutively activated tyrosine kinase, Bcr-Abl, results in the activation of multiple signalling pathways. The 4 major mechanisms responsible for the pathophysiology of CML are:

- Increased proliferation signalling
- Altered dependence and response to cytokines
- Inhibition of apoptotic signalling
- Altered adhesion to bone marrow stroma

Increased proliferation signalling

The Bcr-Abl oncoprotein is constitutively activated by dimerization or tetramerization which in turn facilitates autophosphorylation creating docking sites for the binding of SH2 domains of adaptor proteins such as Crkl, p62Dok, paxillin, Cbl, Shc, and Grb2 (1, 15, 29, 36). These interactions in turn activate multiple signalling pathways including Ras, Raf, Erk, JNK, Myc, Jak/STAT, PI3Kinase-Akt, and NF- κ B pathways (Figure 1-3) (1, 29, 37-41).

Grb2 is crucial for connecting Bcr-Abl to Ras via Son of Sevenless (SOS) in which the inactive GDP-bound form of Ras is converted to the active GTP-bound state (25, 42). The Bcr-Abl/Grb2/SOS complex also activates the scaffold adaptor protein, Grb2-associated binding protein 2 (Gab2) (42). Ras can also be activated by Crkl and Shc, both of which are also substrates of Bcr-Abl (43, 44). Ras initiates a signalling cascade via the mitogen-activated protein kinase (MAPK) pathway which leads ultimately to pro-proliferative gene transcription (42).

There are three major MAPK signalling pathways (45). Firstly, p44/42 MAPK, also known as Erk1/2, which are key mediators of proliferation, differentiation and survival. Secondly, c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) and thirdly, p38 MAPK, both of which are activated by environmental stress and inflammatory cytokines which then modulate proteins involved in apoptosis. Erk1/2 are activated by MEK1/2 which are phosphorylated by the Raf family of protein kinases composed of A-Raf, B-Raf and C-Raf (also known as Raf-1) (45, 46). C-Raf also interacts with apoptotic signal kinase (ASK1) to inhibit apoptosis (47). ASK1 appears to interact with JNK and p38 MAPK (46). Studies using

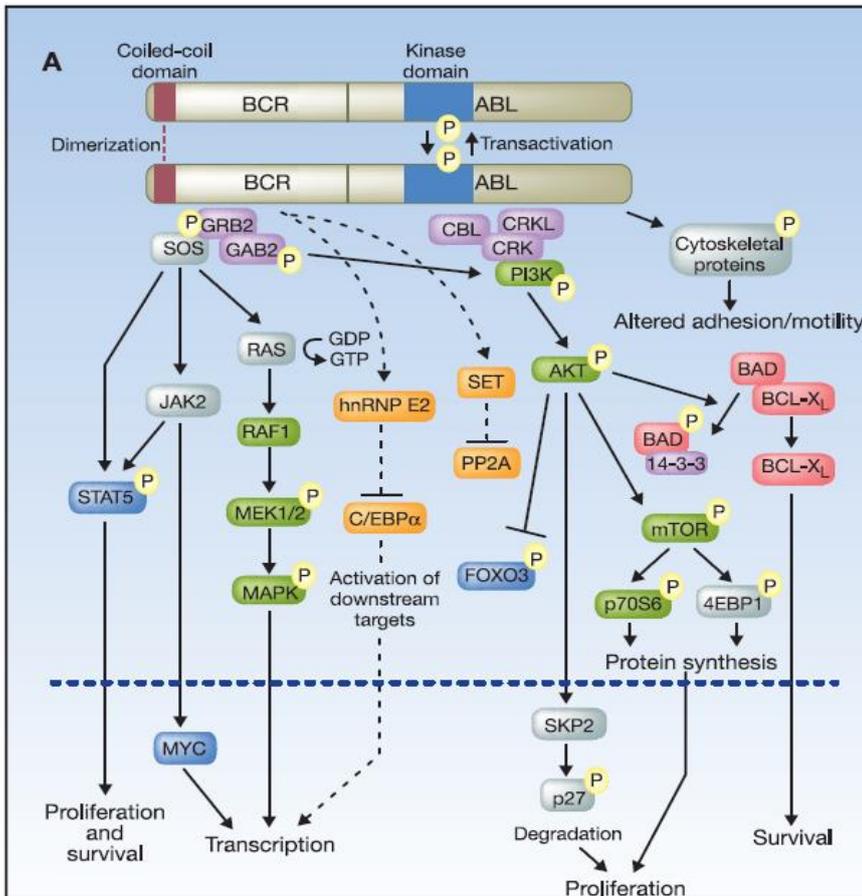


Figure 1-3 Proposed Bcr-Abl signalling network

Bcr-Abl signalling activates multiple downstream pathways that contribute to cell proliferation and survival, inhibition of apoptosis and defective adhesion and cell migration.

(Taken from O'Hare, T et al. Clin Can Res 2011)(48)

CML cell lines indicate that MAPK signalling occurs through Erk1/2 (40) and constitutive activity can render haematopoietic cells growth factor independent (49).

Grb2 when in complex with Gab2 and SOS also recruits Phosphatidylinositol 3-kinase (PI3K) which in turn activates Akt, a serine-threonine kinase (37). Akt has several functions including suppression of the Forkhead O (FOXO) transcription factors promoting survival (50) and upregulation of Skp2 resulting in proteosomal degradation of p27, a tumour suppressor that inhibits cyclin-dependent kinases (51). In turn, this further enhances cell proliferation and activation of Mammalian Target of Rapamycin (mTOR) which also leads to increased transcription and proliferation (52). Akt also has a major role in the control of the apoptosis machinery. Bad, a pro-apoptotic protein is normally in complex with Bcl-X_L (B cell lymphoma-extra large), an anti-apoptotic protein (53). When Akt phosphorylates Bad, it disassociates from the complex promoting survival (54). Akt can also activate the inhibitor of nuclear factor kappa B kinase subunit α (IKK α), which in turn phosphorylates the inhibitor of NF- κ B (I κ B) targeting it for proteosomal degradation (55). Hence, NF- κ B is activated, translocates to the nucleus and promotes transcription of prosurvival genes (55). Bcr-Abl may activate PI3K/Akt through more than one pathway as there is suggestion that Crk/Crkl may also signal through PI3K (56).

The other critical downstream substrate activated by Bcr-Abl is the signal transducer and activator of transcription (STAT) family. Normally, STAT is phosphorylated downstream upon activation of Janus receptor tyrosine kinases (Jak) by cytokine and growth factors (57). In Bcr-Abl-dependent leukaemia, it is thought that it can occur either through direct phosphorylation or indirectly through phosphorylation of Jak2 and the Src family kinases – haematopoietic cell kinase (Hck), Lck/Yes-related novel (Lyn) protein or Gardner-Rasheed feline sarcoma viral oncogene homologue (Fgr) (58, 59). Upon activation, STAT shuttles into the nucleus and regulates transcription of target genes involved in survival, proliferation and differentiation (60, 61). It has been shown that STAT5 is essential for the induction as well as maintenance of CML by using STAT5 knockout mice as these mice were resistant to transformation and leukaemia development (61, 62). This was complemented by lentiviral-mediated RNA interference knockdown of STAT5 which reduced the capacity of primary CML cells to form colonies (63). It has also been demonstrated that a Bcr-Abl positive cell line expressing high STAT5 is resistant to apoptosis induced by TKIs (59). STAT5 activity appears

to contribute to CML leukaemogenesis through increasing transcription of Bcl-X_L leading to inhibition of apoptosis (64, 65).

The proto-oncogene, *MYC* is expressed at a high level in Bcr-Abl positive cells. Myc activation may not depend on the Ras pathway but directly through Bcr-Abl phosphorylation (66). Myc activation results in increased transcription and proliferation of Bcr-Abl cells. Over-expression of *MYC* has been described in CML blast crisis which suggests that it is involved in disease progression (66).

Ultimately, these activated signalling pathways lead to an uncontrolled increase in proliferation and expansion of the leukaemic clone.

Altered dependence and response to cytokines

Cytokines have been implicated in the survival and progression of many malignancies (67-70). Bcr-Abl can promote survival and proliferation of CML cells by activating downstream targets of cytokine receptors. It may also cause over production of certain cytokines and growth factors (71-74). Cytokines such as IL-3, G-CSF, GM-CSF and IL-6 have been implicated in the pathogenesis of CML (15, 71, 72, 75, 76). Jiang *et al* reported that IL-3 and G-CSF are produced by CD34+ CML cells and activate STAT5 signalling (72). It has been found that mice, in which a CML-like disease has been induced, produce excess IL-3 and GM-CSF (75). However, the role of IL-3 and GM-CSF in the pathogenesis of CML remains unclear. Li *et al*, who retrovirally infected murine bone marrow cells with *BCR-ABL1* and transplanted the transduced cells into recipient mice, found that although the recipient mice had increased circulating IL-3 and GM-CSF, knockout mice deficient in both IL-3 and GM-CSF still acquired a CML-like disease (77). They concluded that IL-3 and GM-CSF were not essential for the induction of CML although the knockout mice have lower peripheral white cell count and higher percentage of monocytes/macrophages (77).

The CML bone marrow microenvironment is rich in proinflammatory cytokines and Reynaud *et al* found that IL-6 in particular contributes to the fate of leukaemic stem cells (LSCs) (76). They reported that IL-6 was secreted by myeloid progenitor cells in the *BCR-ABL1* transgenic mice and was responsible for the differentiation and expansion of the myeloid series resulting in a paracrine feedback loop and hence the pathogenesis of CML (76). An earlier study found that patients in advanced stage CML (blast crisis) have higher levels of IL-6 in

their serum compared to those in early phase (chronic phase) (78). This may support the important role IL-6 plays in the development of CML.

There is ample evidence that *BCR-ABL1* positive early progenitor cells are able to proliferate in serum free media (SFM) in the absence of added growth factors (71, 72). Bcr-Abl may render leukaemic cells growth factor and cytokine independent while *BCR-ABL1* positive cells have been shown to produce several cytokines including IL-3, G-CSF and GM-CSF which have an autocrine effect (71, 72, 79, 80). Importantly, excessive growth factors and cytokines have been shown to protect CML progenitor cells from the effects of TKI therapy (80, 81). Primitive CML stem cells are thought to be insensitive to TKI due to the protective effects of the cytokine- rich stem cell niche and importantly this leads to persistence of the disease (minimal residual disease) (82, 83). It has been demonstrated by our group that a cocktail of cytokines consisting of Flt-3 ligand, stem cell factor, IL-3, IL-6 and G-CSF as well as single cytokines GM-CSF and G-CSF, protect CML CD34+ cells from the effects of dasatinib, a second generation TKI (81). When the cytokine signalling pathway was blocked with a Jak inhibitor the sensitivity of these cells to the effects of TKI-induced cell death were re-established (81). Similarly, CML CD34+ progenitor cells exhibit enhanced MAPK activation in the presence of growth factors despite inhibition with imatinib and nilotinib (another second generation Bcr-Abl tyrosine kinase inhibitor) (49). This demonstrates that cytokine signalling can protect CML cells from Bcr-Abl inhibition.

More recently, other cytokines and chemokines have also been proposed to play important roles in the pathophysiology of CML. CXCL12, also known as stromal derived factor 1 (SDF-1), is a chemokine produced by bone marrow stromal cells (BMSCs). It directs migration and retention of haematopoietic stem cells to bone marrow niches through its interaction with CXCR4, which is expressed on stromal cells (84). It has been reported that CML progenitors have reduced homing and retention towards CXCL12 (85-87). This may be due to down regulation of CXCR4 expression or lower expression of CXCL12 in bone marrow stromal cells (86, 87). This results in the early release of immature myeloid cells into the circulation. Moreover, these cells are also not under the regulatory control of the bone marrow microenvironment (88). Zhang *et al* attributed the decreased level of CXCL12 to increased secretion of G-CSF by leukaemic cells, as culturing BMSCs from normal mice with G-CSF reduced CXCL12 mRNA levels which were partially restored by an anti-G-CSF blocking

antibody (87). Other cytokines found to be increased in *BCR-ABL1* positive mice were macrophage inflammatory protein 1 α (MIP-1 α), MIP-1 β , IL-1 α , IL-1 β , IL-6, TNF- α and leukaemia inhibitory factor (LIF) and a similar cytokine profile was also found in newly diagnosed CML patients in CP (87). Zhang *et al* also showed that the altered cytokine expression profile in CML bone marrow resulted in a selective growth advantage for CML stem cells and suppression of normal stem cells (87).

Cytokine production has also been implicated as a means of conferring resistance to TKI in cell lines. Increased secretion of GM-CSF was identified as a causative factor in mediating imatinib and nilotinib resistance in LAMA84 cells (*BCR-ABL1* positive cell line which is imatinib and nilotinib resistant) (89) and this effect was mediated through the pro-survival Jak2/STAT5 pathway (74). Point mutations in the kinase domain of *ABL1* are a common and important cause of resistance to TKIs. There has been some evidence these mutation may confer Bcr-Abl cells with different biological activities including growth factor independence (90, 91). Paracrine secretion of IL-3 by the murine leukaemic cell line, DA1-3b *BCR-ABL1*+ cell lines with the E255K mutation and the composite mutation E255K/T315I, protected non mutated cells from apoptosis by down-regulating Bim, a pro-apoptotic protein and also activation of the pro-proliferative MEK/Erk and Jak2/STAT5 pathways (73). It is currently unknown if cells harbouring the T315I mutation also recruits cytokines as a means of conferring a survival advantage compared to wild-type Bcr-Abl cells especially in certain niches of low concentration of cytokine and growth factors.

Inhibition of apoptosis

Apoptosis is genetically programmed cell death and the survival and growth of any cell clone depends on the balance between cell proliferation and cell death (92). Inhibition of apoptosis is one mechanism by which Bcr-Abl is able to induce expansion of CML cells (93). The Bcl-2 family is critically involved in control of the intrinsic apoptosis pathway (53, 94). Some members of this family reside in the subcellular membranes including the mitochondrial outer-membrane and the integrity of this membrane is governed by the balance between the pro-apoptotic members, Bax and Bak and the anti-apoptotic members, Bcl-2 and Bcl-X_L (53). The activation of this pathway leads to the permeability of the mitochondrial outer-membrane with the resulting release of cytochrome c that initiates apoptosis (53). Most of the Bcl-2 family members are multi-domain proteins, where they

share three to four Bcl-2 homology (BH) domains (53). Another subset of this family, known as the BH3 only members show sequence homology only within the BH3 domain which is the critical death domain required for binding to the multi-domain members. These BH3 members are pro-apoptotic and include Bcl-2 antagonist of cell death (Bad), Bcl-2 interacting mediator of cell death (Bim), BH3 interacting domain death agonist (Bid), Noxa and Puma (92).

In *BCR-ABL1* cells, Bcl-2, the anti-apoptotic protein, is up-regulated by Ras/Erk/MEK and PI3K/Akt signalling, whereas another anti-apoptotic protein, Bcl-X_L, is up-regulated by STAT5 signalling (37, 95). The pro-apoptotic protein Bad normally binds to Bcl-X_L keeping it in the inactive state. However, in the presence of active PI3K/Akt signalling, it becomes phosphorylated and trapped in complex with cytoplasmic 14-3-3 proteins, thus rendering it unable to exert its pro-apoptotic effects (96).

Bcr-Abl expression also down-regulates Bim, a pro-apoptotic protein. Therefore, Bcr-Abl positive cells are able to evade cell death even in a cytokine deprived environment (97). CML cell lines, K562 and KU812 as well as primary CML cells, demonstrate lower expression of Bim compared to normal bone marrow cells (97, 98). Treatment with imatinib has been shown to restore Bim expression (97). Bim expression is also controlled by MAPK and PI3K/Akt signalling (98, 99). The PI3/Akt pathway negatively regulates the Forkhead family of transcription factors 3A (Foxo3A) and is thought to mediate transcriptional up-regulation of Bim (99).

Taken together, these studies indicate that *BCR-ABL1* expressing cells are not governed by the normal physiological control of cell death.

Altered adhesion to bone marrow stroma

CML progenitors do not respond to the normal negative regulatory influences of the bone marrow microenvironment, resulting in their failure to adhere to the bone marrow stroma and extracellular matrix (100). It has been shown that direct contact between normal progenitors and the bone marrow stroma is important for the regulation of proliferation of these cells (101, 102).

Normal haematopoietic progenitors attach to marrow stromal elements by cell surface receptors especially the integrins such as the $\alpha 4\beta 1$ integrin receptor which binds to vascular

cell adhesion molecule (VCAM) on bone marrow stromal cells and $\alpha 5\beta 1$ integrin receptor which binds to fibronectin (103). The α subunit determines ligand specificity while the β subunit initiates signalling after binding to the ligand (104).

Lundell *et al* incubated CML progenitor cells and the CML cell line, K562, with a $\beta 1$ integrin activating antibody which restored adhesion of these cells to fibronectin through $\beta 1$ integrins (105). They also showed that proliferation of these cells was inhibited. Therefore, this suggests that the unresponsiveness of CML progenitors to negative regulatory influences from the bone marrow microenvironment may be caused by the loss of adhesive interactions with the stroma and fibronectin (105). The levels of these $\beta 1$ integrins are normal in CML progenitors so it is proposed that it is the inability to transfer signals that is impaired (103, 106).

The other reason direct contact between progenitor cells and the bone marrow stroma is important for the regulation of progenitor growth is that these cells are anchored within the vicinity of cytokine producing cells (100). Cytokines transmit signals to these cells and depending on which signals they receive, they may survive and proliferate or undergo apoptosis (106). This lack of contact also leads to abnormal release of immature myeloid cells into the peripheral circulation and loss of regulatory signals provided to normal adherent progenitor cells (32).

As mentioned previously, CXCL12 controls haematopoietic stem cell (HSC) homing and quiescence through its receptor, CXCR4, which is expressed on BMSCs (84). It has been shown that CML CD34+ cells have significantly lower expression of CXCR4 compared to normal HSCs (86, 107) and therefore these cells are unable to respond adequately to homing signals. Recently, it has also been shown that CXCL12 level and mRNA expression in CML bone marrow is reduced (87), indicating that there is reduce control of the bone marrow microenvironment on CML progenitor cells.

1.2 Clinical and laboratory features of CML

1.2.1. Clinical features

CML typically evolves over three clinical phases. Most patients are diagnosed in the chronic phase (CP) that may last 5-6 years and if left untreated, progresses through to accelerated

and then blastic phases. Blast phase resembles acute leukaemia and is often rapidly fatal (108). The median age of presentation of CML is 45-55 years but it affects all age groups including children. Over 50% of patients are asymptomatic at diagnosis and only diagnosed incidentally on a full blood count (1). Typical symptoms include fatigue, weight loss, bone pain, sweating and abdominal discomfort due to the presence of splenomegaly (109).

1.2.2. Laboratory features

Laboratory findings include leucocytosis, anaemia and either thrombocytosis or more rarely thrombocytopenia, which is more common in the advanced stages (110). The differential white cell count reveals granulopoiesis at all stages of development and the absolute basophil count is increased (110). In accelerated and blastic phases, the blast percentage is also increased (1, 111).

The hallmark of CML is the Ph chromosome, $t(9:22)(q34;q11)$ (12, 109). Additional chromosomal abnormalities are found in more than 20% of patients at diagnosis and they include Trisomy 8 and deletion Y (112). Some abnormalities like aberrant expression of chromosome 17 are associated with a poor outcome (113). Detection of *BCR-ABL1* transcripts in the blood and bone marrow are routinely performed at diagnosis by real-time quantitative polymerase chain reaction (RQ-PCR) (92, 114). Measurement of *BCR-ABL1* transcript levels is also repeated at regular intervals to monitor patients' response to treatment and to predict outcome (115-117).

1.3 Treatment

1.3.1. Chemotherapy

In the 1980s, CML was considered a fatal disease with a mean survival of 5 years. The first agent to show haematological control for CML was busulphan (118). Hydroxurea is an excellent debulking agent and is still used today to achieve rapid control of blood counts (119). Other agents used in the past include 6-mercaptopurine, 6-thioguanine and cytarabine but none were considered definitive therapy as they only gave temporary control of blood counts and did not change the natural progression of the disease (1, 118). A timeline showing different treatment options is shown in Figure 1-4.

1.3.2 Interferon- α

Interferon- α (IFN α) was introduced in the 1980s and when compared to busulphan or hydroxyurea was associated with longer survival, inducing durable and complete cytogenetic response in 10-20% of patients (120). It is however associated with significant side effects such as fatigue, myalgia, bone pain and depression (121). IFN α has been used in combination with low dose cytarabine and this regime resulted in improved major cytogenetic response and survival compared to either drug alone (122, 123). This combination was considered the standard until imatinib mesylate was introduced in the early 2000s.

1.3.3 Tyrosine kinase inhibitors (TKIs)

At the present time, 3 TKIs are approved for use in Australia, imatinib mesylate (Novartis Pharmaceutical, Basel, Switzerland), dasatinib (BMS-354825, Bristol- Myers Squibb, New York, USA) and nilotinib (AMN107, Novartis, Basel, Switzerland).

Another 2 TKIs, bosutinib (SKI-606, Pfizer, New York City, USA) and ponatinib (AP24534, Ariad Pharmaceuticals, Cambridge, Massachusetts, USA) have also been recently approved in Europe and USA, but not in Australia, for CML patients resistant or intolerant to prior TKI treatment.

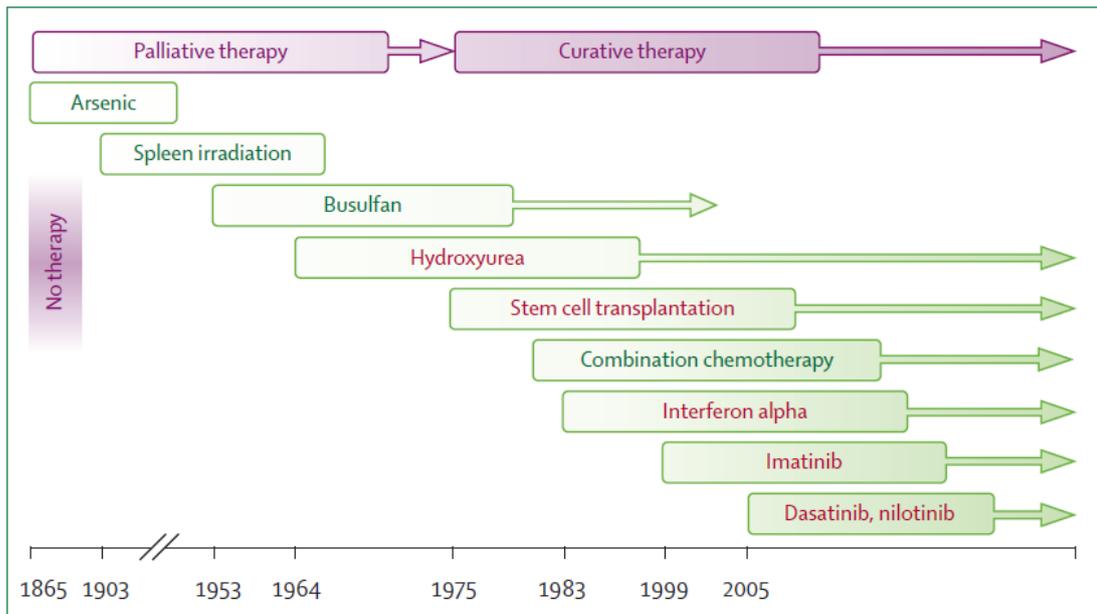


Figure 1-4 Treatment for CML over time.

Treatment in red indicates commonly used treatment for chronic phase disease. Treatment in green can be used for any phase. (Taken from Hehlmann, R et al. *Lancet* 2007)(119)

1.3.3.1 Imatinib

Imatinib mesylate has changed the treatment landscape of CML from an almost invariably fatal disease to one more akin to a chronic disease. Previously known as STI-571, it was the first drug designed to inhibit the aberrant tyrosine kinase encoded by the *BCR-ABL1* fusion gene (4). Protein tyrosine kinases bind at the highly conserved nucleotide-binding pocket of the catalytic domain and imatinib has a very high affinity for the kinase domain of Abl kinase (124). The catalytic activity of the kinase is controlled by the activation loop which switches between the inactive and active states induced by phosphorylation of tyrosine residues (124). Crystallographic analysis revealed that imatinib binds the inactive configuration of the Bcr-Abl oncoprotein thereby preventing a conformational change to the active form (124, 125). It also competitively inhibits the binding of ATP thus blocking the action of Bcr-Abl tyrosine kinase (125). Imatinib also has action against c-KIT (126), Platelet derived growth factor receptor α (PDGFR α) (126) and c-FMS (cellular homologue of the feline sarcoma virus, v-FMS) (127) and has been used successfully in the treatment of other cancers including chronic eosinophilic leukaemia and gastrointestinal stromal tumours (GISTs) (128).

Imatinib has drastically altered the outlook for CML patients who before the imatinib era had a median survival of 5 years (129). A phase III randomised, controlled trial, reported in 2009, the International Randomized Study of Interferon and STI571 (IRIS) showed that approximately 85% of patients have survived 8 years on imatinib (130). For many years, first line treatment for newly diagnosed CML was imatinib and has proven to be very successful in most cases. However, some patients fail to respond or have suboptimal response to imatinib (131). These patients have the ability to switch over to one of the 2nd generation TKIs, nilotinib or dasatinib, in an attempt to improve their therapeutic outcome.

1.3.3.2 Nilotinib

Nilotinib was designed to bind more specifically to Bcr-Abl than imatinib. It is a smaller molecule compared to imatinib and is 30 times more potent, as measured by its ability to block proliferation of *BCR-ABL1* positive cell lines (132). Like imatinib, it binds Bcr-Abl only in the inactive conformation and has activity against c-Kit and PDGFR α receptor (133).

In the Evaluating Nilotinib Efficacy and Safety in Clinical Trials–Newly Diagnosed Patients (ENESTnd) trial, nilotinib was given up-front to *de novo* CML patients and it demonstrated

superiority over imatinib in terms of achievement of therapeutic responses such as complete cytogenetic response (CCyR) and major molecular response (MMR) (See Figure 1-5) (134). Patients taking nilotinib achieved CCyR sooner and at higher rates than in patients taking imatinib (135). At 24 months, MMR rate achieved with nilotinib was 71% compared to 44% with imatinib (134). Based on the ENESTnd trial, nilotinib is now licensed for use as a first line treatment in CML.

Nilotinib is also effective against many *BCR-ABL1* kinase domain (KD) mutations that develop during imatinib treatment (ie. imatinib resistant mutations) and thus is a logical second line treatment in patients who become resistant or intolerant to imatinib (132, 133, 136).

1.3.3.3 Dasatinib

Dasatinib is much more potent than imatinib *in vitro* on non-mutated Bcr-Abl (136). In addition to Bcr-Abl, c-Kit, ephrin and PDGF receptors, it also inhibits Src family kinases (SFKs), suggesting that it may provide additional treatment benefit and/or extra toxicity (137). Recently, it has also been shown that dasatinib has action against other tyrosine kinases and serine/threonine kinases including the Tec family kinases, Btk and Tec, mitogen-activated protein kinases and the discoidin domain receptor 1, a receptor tyrosine kinase (138). Unlike imatinib, it binds both the active and inactive conformation of Bcr-Abl. It is a smaller molecule therefore interactions between the Bcr-Abl ATP-binding site and dasatinib are less critical, making it effective against many mutations that are resistant to imatinib (139). Dasatinib has a half-life ($t_{1/2}$) of 3-6 hours *in vivo* compared to imatinib's 18 hours but despite its short half-life, dasatinib is effective given once a day with less side effects compared to twice daily dosing (7).

Like nilotinib, dasatinib has mostly been used as a second line agent after the failure of imatinib. However, following the recent Dasatinib versus Imatinib Study in Treatment-Naïve CML Patients (DASISION) trial, it has been approved for use in newly diagnosed CML patients (140). Patients randomised to the dasatinib arm of the DASISION trial achieved CCyR and MMR sooner and at a higher rate when compared to those in the imatinib arm. MMR was achieved in 64% at 24 months for patients taking dasatinib versus 46% for those on imatinib (140, 141). Between them, dasatinib and nilotinib are effective against all the KD mutations that confer resistance to imatinib, with the exception of the T315I mutation.

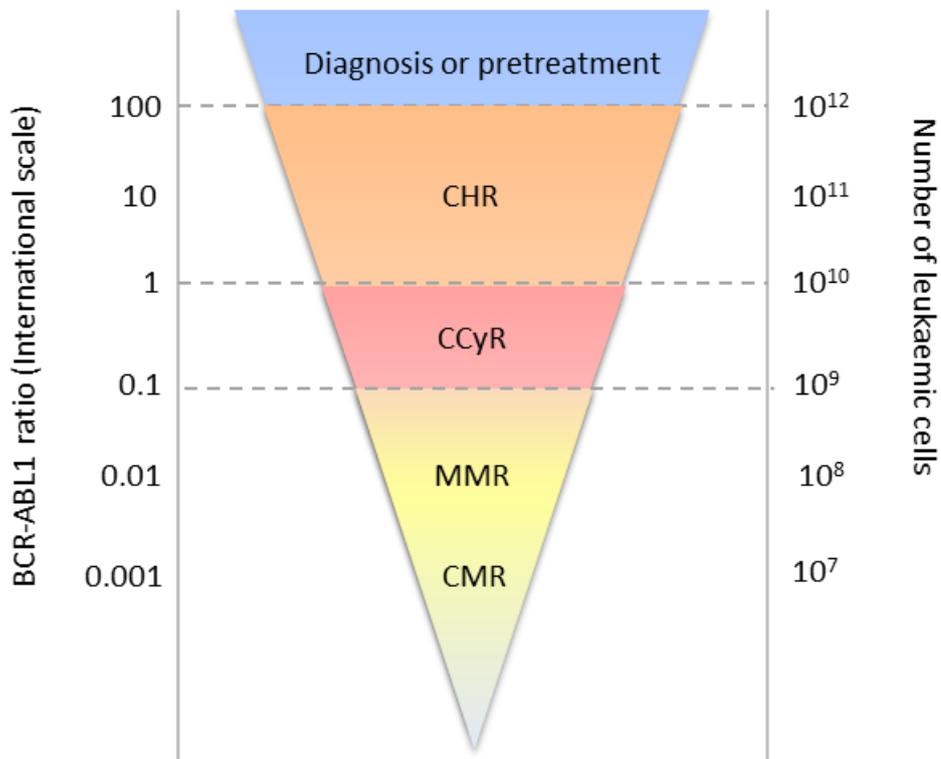


Figure 1-5 Monitoring CML therapeutic response.

Efficacy of TKI treatment is monitored to establish haematological and then cytogenetic response. On achievement of complete cytogenetic response (CCyR), *BCR-ABL1* transcript levels are measured by RQ-PCR and expressed as a percentage ratio of *BCR-ABL1* to *BCR* transcripts. A reduction of *BCR-ABL1* transcript levels of > 3 log reduction (>1000 fold reduction) from a laboratory-specific pre-treatment baseline is defined as major molecular response (MMR). With more sensitive methods of detection, further reduction of *BCR-ABL1* transcript levels may be determined which is termed complete molecular response (CMR).

CHR- Complete haematological response, CCyR- Complete cytogenetic response, MMR- Major molecular response, CMR- Complete molecular response.

(Adapted from O'Hare, T et al. *Nature Reviews Cancer* 2012)(142)

1.3.3.4. Bosutinib

Bosutinib is a dual Src/Abl tyrosine kinase inhibitor like dasatinib but it has only minimal activity against c-Kit and PDGF receptors (143).

In a Phase 1/2 study, where patients in CP-CML who had received imatinib and at least one other second generation TKI, showed a CCyR rate of 24% after a median follow up of 28.5 months. At 2 year follow up, the progression free survival was 73% and overall survival was 83%. It is effective across all KD mutations including those ineffective against dasatinib and nilotinib except for the T315I (144).

However, the Phase 3, Bosutinib Efficacy and Safety in Newly Diagnosed Chronic Myeloid Leukaemia (BELA) trial where newly diagnosed CML patients in CP were randomised to either bosutinib or imatinib failed to demonstrate that bosutinib is more effective than imatinib in achieving CCyR by 12 months. It did however demonstrate superior rate of MMR at 12 months and times to CCyR and MMR were also shorter with bosutinib. There was also a trend towards fewer progressions to more advanced stages of CML (2% in bosutinib vs 4% in imatinib) (145).

As bosutinib did not achieve its primary end point of a more superior rate of CCyR at 12 months compared to imatinib, it is not yet approved for first line treatment but it is approved by the US FDA (Federal Drug Authority) for adult CML patients who are resistant or intolerant to at least one prior TKI treatment.

1.3.3.5 Ponatinib

Ponatinib is the result of a computational and structure-guided design of a drug effective against the T315I mutation (146). As imatinib, nilotinib and dasatinib each form hydrogen bonds with T315 on native Bcr-Abl, this hydrogen bond cannot take place when threonine is replaced by isoleucine. To avoid this, ponatinib was designed to accommodate the T315I side chain by a carbon-carbon triple bond (ethynyl) linkage (147). It is also effective against native Bcr-Abl and all other mutations (146, 147).

Ponatinib has a very broad kinase inhibition profile. In addition to Abl, it also has activity against other targets including c-Kit, PDGFR- α , Flt-3, Ret, Src, Lyn, VEGFR and FGFR- the latter two are not targeted by imatinib, nilotinib or dasatinib (146). This may result in the

effective use of ponatinib against other malignancies with over-expression or mutations involving the aforementioned proteins. For example, there are pre-clinical trials where ponatinib is being investigated as possible treatment of cancers with a variety of abnormalities including *FGFR2* mutations (148, 149), *RET* mutations (150) and *FLT3-ITD* mutations (151, 152)

The 12 month data from the PACE (Ponatinib Ph+ ALL and CML Evaluation) trial showed favourable results achieved by heavily pre-treated patients who are intolerant or resistant to second generation TKIs and/or possess the T315I mutation (153). Major cytogenetic response (MCyR, 35% or less Ph positive metaphases present in the bone marrow) was achieved by 56% of overall CP-CML patients, together with an impressive 70% of the T315I patient cohort achieving MCyR (153, 154). Higher response rate of patients with the T315I mutation was also seen amongst those in accelerated phase and blastic phase (155).

Therefore ponatinib is a promising drug for a cohort of patients highly resistant or intolerant to other TKIs. However, in October 2013, the US FDA suspended marketing of ponatinib because review of clinical trials and post marketing data indicated that ponatinib use was complicated by a range of serious vascular events (156). In the Phase II trial, 24% of patients (median treatment duration- 1.3 years) and Phase I trial, 48% (median treatment duration- 2.7 years) had experienced serious adverse vascular events including myocardial infarctions, worsening of coronary artery disease, narrowing of peripheral blood vessels to extremities and blindness. These events occurred in all types of patients including younger adults and those without cardiovascular risk factors (156, 157). Late December 2013, ARIAD Pharmaceuticals announced that the US FDA has allowed resumption of marketing and distribution of ponatinib. It has revised the prescribing indications and recommended dosing considerations, updated the safety information and included a boxed warning to alert patients and healthcare professionals about the risk of vascular events.

1.3.4 Allogeneic Haematopoietic Stem Cell Transplant (Allogeneic HSCT)

Allogeneic HSCT is still considered the only curative option for CML but the number of patients undergoing allogeneic HSCT has significantly decreased in the imatinib era, and will decrease further with the advent of more effective therapies. However, this mode of treatment still retains a role in patients who are resistant to conventional treatment.

The outcome of allogeneic HSCT is dependent on many factors, the most important being the stage of disease at which transplant takes place. Patients transplanted in chronic phase have a superior outcome compared to those transplanted in accelerated and blastic phases (158). The Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation (EBMT) data showed that the overall survival for patients transplanted in chronic phase is 70% at 2 years compared to those transplanted in accelerated and blastic phases, 47% and 16% respectively (159). The age of the patient is another important factor as older patients have other co-morbidities and are less able to withstand the vigour of the treatment (160). Only a third of patients have a HLA-matched family member to act as a donor and using a matched unrelated donor (MUD) results in poorer outcome in most series especially in older patients (158, 160).

Currently, allogeneic HSCT may be used in patients who fail second generation TKI, those with the T315I mutation and those in accelerated and blastic phases who respond very poorly to conventional treatment (117, 161, 162).

1.4 Mechanisms of Resistance

The failure to respond adequately to treatment and reach landmark milestones is termed primary resistance which, can be further divided into primary haematological resistance or primary cytogenetic resistance (163). Primary haematological resistance in early CP is rare but primary cytogenetic resistance (defined as failure to achieve MCyR in 6 months or CCyR in 12 months) in CP is seen in about 15% (164). Secondary resistance develops after a patient who had initially responded well ultimately relapses. This occurs in 10-15% of cases.

Mechanisms of secondary resistance include:

1. Mutations in the *BCR-ABL1* kinase domain
2. Increased expression of *BCR-ABL1*
3. Cytokine mediated resistance
4. Activation of Bcr-Abl independent signalling pathways
5. Alteration in expression of drug transporters
6. Clonal evolution and expansion

1.4.1 Mutations in the Bcr-Abl kinase domain

Approximately 40% of patients stop imatinib treatment because of development of resistance or intolerance (165). Amongst those who develop resistance to imatinib, approximately 35-45% have KD mutations (5, 6, 166) making this the most common single cause of secondary resistance in imatinib treated patients. Point mutations in the KD may impair drug binding by different mechanisms, for example one group of mutations prevent direct drug binding as the amino acid changes are located at the drug contact site e.g. F311, T315, F317 (167). The second group includes those mutations which occur in the P-loop, also known as the ATP-binding loop, preventing Bcr-Abl from adopting the conformation necessary for drug binding e.g. E255, Y253 (167). The last group of mutations occur in the regulatory motif such as the activation loop which may stabilise the active conformation of Bcr-Abl preventing imatinib binding e.g. H396 (168-170) (Figure 1-6).

KD mutations are found more frequently in patients with accelerated phase or blastic phase disease (9). It is uncertain if mutations contribute to disease progression or are simply a marker of greater genomic instability which is inherently associated with more advanced disease (171, 172). More than 100 point mutations have been described but mutations in 7 amino acids of *BCR-ABL1* account for 66% of all mutations- G250, Y253, E255, T315, M351, F359, and H396 (173) (refer Figure 1-6).

Mutated clones expand as a consequence of selective pressure from a TKI and often deselect upon withdrawal of the TKI, although different mutants have varying kinetics (174). This suggests that non-mutated *BCR-ABL1* cells have faster growth kinetics in the absence of TKI. However, while not without conjecture, there have also been reports of low level KD mutations detected at diagnosis (pre TKI) suggesting that KD mutations exist prior to the selective pressure of TKI therapy (175-177). This may mean that some mutations have an advantage over cells expressing non-mutated *BCR-ABL1*. Additionally, not all mutations are of equal significance. For example, the ATP-binding loop (or P loop) mutations are associated with increasing transformation potential and a worse prognosis regardless of their *in vitro* sensitivity to imatinib in comparison to other mutations such as M351T and F317L (90, 178, 179).

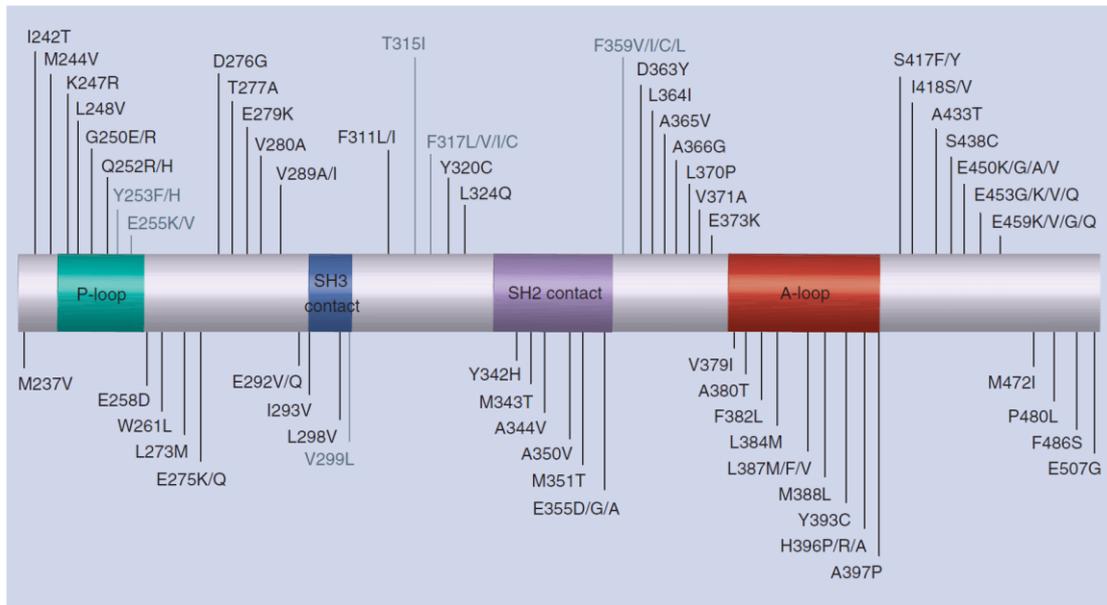


Figure 1-6 Imatinib resistant *BCR-ABL1* kinase domain mutations.

Amino-acid substitution within the *BCR-ABL1* kinase domain reported to be resistant to imatinib. Those highlighted in grey are also insensitive to nilotinib or dasatinib. P-loop- Phosphate binding loop, SH2 and SH3 contact- Contact regions with SH2 and SH3 domain-containing proteins, A loop- Activation loop. (Taken from Soverini, S. et al. *Pharmacogenomics* 2012)(180)

Mutations in the *BCR-ABL1* KD may alter the biological activity of Bcr-Abl with some studies finding that particular mutations increase the transforming potential of Bcr-Abl while others do not (90, 91, 170, 181). Miething *et al* used the BAF/3 cell line and an *in vivo* assay using *BCR-ABL1* transformed cells transplanted into sublethally irradiated mice and found that the T315I and Y253H expressing cells did not show an increase in transformation potency over the cells expressing non-mutated *BCR-ABL1* (170). Conversely, using the modified Whitlock-Witte Pre B cell transformation assay, which has been found to correlate with *in vivo* CML murine models, Skaggs *et al* determined that the T315I mutation had the greatest transformation potential, followed by the E255K mutation (91). According to their findings, the Y253F and E255V mutant clones had similar transformation potential as non-mutated *BCR-ABL1* and the T315A, M351T and F317L mutations had the weakest transformation potentials (91).

Griswold *et al*, using the same assay as Skaggs *et al*, also showed that mutations in the KD influence the transformation potential of *BCR-ABL1* clones (90). However, in contrast, they found that the relative transformation potencies were Y253F > E255K (P-loop) > non-mutated *BCR-ABL1* > T315I (drug binding region) > H396P (activation loop) > M351T (catalytic domain) (90). Both groups found that the kinase activity of the mutant clones correlated with their transformation potential, except for the T315I clone which they postulated had additional factors such as altered substrate specificity and downstream signalling, contributing to its transformation potency (90, 91). In contrast, Yamamoto *et al* found that the kinase activity for E255K and T315I mutations was increased and concluded that it accounts for the increase in their transformation potency. The difference in these results may be attributed to the different methodologies employed, including use of full length *BCR-ABL1* constructs by Griswold *et al* compared to *BCR-ABL1* kinase constructs by Yamamoto *et al* (90, 181).

These studies suggest that the proliferative advantage conferred by some KD mutations is multifactorial and may depend on intrinsic kinase activity, substrate specificity, the target cells used and external factors like cytokines and growth factors (182).

1.4.1.1 The T315I mutation

The substitution of threonine for isoleucine at position 315 in Bcr-Abl is known as the "Gatekeeper Mutation" (183). It is located at the periphery of the nucleotide-binding site of

Abl within the hinge region of the enzymatic cleft. The threonine residue stabilises imatinib binding through hydrogen-bond interactions and allows imatinib access to a deep hydrophobic pocket in the active site (184). This is not able to occur in the presence of the T315I mutation. As one of the more common point mutations in the KD especially in accelerated phase (AP) and blast crisis (BC), it is particularly problematic as it confers resistance to all current TKIs in clinical use (173). This is due to 3 mechanisms. Firstly, all three TKIs form critical hydrogen bonds with the T315 residue (124, 185). Secondly, isoleucine is larger and more hydrophobic and thus causes steric hindrance blocking access of TKIs to the hydrophobic pocket close to the T315 residue (124, 183). Lastly, the T315 participates in a network of highly conserved hydrophobic interactions called the hydrophobic spine which stabilises the active conformation (183). Its mutation to isoleucine, being a more hydrophobic residue further strengthening this interaction and stabilises it further in the active conformation (183, 184, 186).

The clinical behaviour of the T315I appears to be variable. Some studies have found that the development of this mutation is linked to poor prognosis including a high rate of disease progression and poor survival (9, 10, 187). However, others have found that it depends on the disease phase in which the T315I mutation is detected (188). Also, some studies have demonstrated that certain mutations, including T315I, were found in patients even prior to starting TKI therapy (176, 177) Others have shown that the T315I mutation may not have sufficient growth advantage over cells expressing non-mutated *BCR-ABL1*, disallowing the mutated clone to predominate until it is exposed to a TKI (90, 91, 168, 170). This suggests that the behaviour of the T315I mutation may depend on variables such as the experimental conditions and the number of the mutant transcripts in the samples.

Since the T315I mutation is insensitive to first and second generation TKIs, it is anticipated that the incidence of this mutation will rise under the selective pressure of increased TKI therapeutic use. Evidence from the DASISION trial showed that 10 patients in each of the 2 treatment arms (randomized to either dasatinib or imatinib) developed KD mutations (141). In the dasatinib arm, 7 out of 10 developed the T315I mutation whereas in the imatinib arm, no T315I mutation was detected.

1.4.2. Increased expression of *BCR-ABL1*

Amplification of *BCR-ABL1* occurs more commonly in patients with more advanced phases of CML (171). This is due to duplications of the Ph chromosome occurring in mitosis thought to develop at a rate of 10^{-4} per cell division, whereas point mutations occur less frequently at a rate of 10^{-9} per cell division, both which are associated with increased genomic instability (171). However, mutations are more likely to cause clinical resistance than *BCR-ABL1* duplication and this may be explained by the fact that multiple copies of *BCR-ABL1* may actually be detrimental to cell survival (189).

It was recently demonstrated in our laboratory that over expression of *BCR-ABL1* commonly occurs as a preceding event to mutation development (190). There is increasing evidence to support the contention that *BCR-ABL1* itself is directly responsible for acquisition of genetic instability in LSC (191). Bcr-Abl kinase activity drives increased proliferation and a reduction in apoptosis, leading to less accurate DNA repair which over time can lead to accumulation of mutations (192). Bcr-Abl kinase activity also increases DNA damage by generating excessive reactive oxygen species (ROS) which also results in the acquisition of higher rate of mutations and chromosomal aberrations (192, 193).

Cell lines harbouring multiple copies of *BCR-ABL1* have been shown to be less viable when imatinib is withdrawn (194). Cells with high expression of *BCR-ABL1* are less sensitive to imatinib and the cells also take a substantially shorter time to produce a mutant subclone resistant to a TKI than cells with low *BCR-ABL1* expression levels (194).

1.4.3. Cytokine mediated resistance

Cytokine production has also been implicated as a means of conferring TKI-resistance in *BCR-ABL1* positive cell lines. As previously stated, *BCR-ABL1*+ cell lines are able to overproduce IL-3 and GM-CSF which have paracrine or/and autocrine protective effects against TKI-induced cell death (73, 74).

Excessive cytokine production has also been implicated as a means of protecting *BCR-ABL1* positive progenitor cells from TKI-induced cell death, and excessive cytokines and growth factors can rescue cells from the effects of TKI (80, 81). Hiwase *et al* demonstrated that Bcr-Abl inhibition by dasatinib was not able to eradicate CML CD34+ cells in the presence of cytokines, which means that the cytokines provide a Bcr-Abl independent mechanism of

survival (81). However, using a combination of a Jak inhibitor with dasatinib, further reduced cell viability compared to the use of either drug alone (81). Most cytokine signalling is mediated through the Jak/STAT5 pathway but other pathways like the SFK, MAPK and PI3K/Akt may also be involved (72, 195-197). Similarly, Konig *et al* reported that nilotinib and dasatinib reduced phosphorylation of the MAPK, STAT5 and Akt only in the absence of cytokines in CML CD34+ cells (198, 199). Even more intriguing is the finding of Chu *et al* who showed that imatinib treatment actually resulted in an increase in compensatory MAPK signalling which was growth factor dependent (49). This was similar to more recent findings by Konig *et al* using nilotinib (198). Taken together, these results indicate that cytokine mediated downstream signalling may circumvent the need for Bcr-Abl signalling, allowing for survival of CML progenitors.

The bone marrow is a source of signals, for example G-CSF, GM-CSF and stem cell factor (SCF), which support leukaemia cell growth (200, 201). The cytokine-rich bone marrow microenvironment is instrumental in conferring chemo-resistance to primitive LSCs (82, 83, 202). This was demonstrated by Weisberg *et al* who cultured *BCR-ABL1* expressing cells with imatinib in conditioned media either from bone marrow-derived stromal cells rich in cytokines and growth factors or by adding a cocktail of soluble factors found in high concentration in the bone marrow namely, SCF, IL-6, IL-8, IL-11, macrophage stimulating colony factor (M-CSF) and GM-CSF (82, 202). They found that in both situations, cytokines were able to partially rescue *BCR-ABL1* cell lines from imatinib treatment (82). This may partly explain why CML stem cells are so difficult to eradicate and remain a source of persistent residual disease.

1.4.4 Activation of Bcr-Abl Independent Signalling Pathways

Src family kinases (SFK) play an important role in signalling through surface receptors on haematopoietic cells and it has been shown that Bcr-Abl activates Src kinases through direct binding and phosphorylation (203, 204). It has been suggested that imatinib resistance may be mediated via upregulation of SFKs such as Lyn and Hck (205). Imatinib and nilotinib resistant cell lines have been shown to have increased expression of Lyn which confers resistance through overexpression of Bcl-2 (203, 205, 206). Mahon *et al* also showed that *LYN* mRNA expression was increased in peripheral blood cells of patients resistant to

nilotinib (206). Furthermore, patients in CML blast crisis (BC) who progressed on imatinib treatment were also found to have increased expression of Lyn or Hck (205).

Increased expression of STAT5 has been described as a mode of resistance to TKI-induced Bcr-Abl inhibition and is linked to disease progression (59, 74). Warsch *et al* reported that upregulation of STAT5 protein is sufficient to confer resistance to imatinib and is independent of Jak2 signalling (59). High STAT5 expressing murine bone marrow cells, virally transduced with p210^{BCR-ABL1}, were found to be highly resistant to imatinib treatment. Additionally, they found that *STAT5* mRNA expression was increased in AP compared to CP patients and was also increased in imatinib resistant patients (59).

Erk1/2 activation has also been proposed as a possible mechanism for imatinib resistance in certain cell lines (207). Nambu *et al* established a cell line with an IC50 for imatinib 15-fold higher than that of the parental K562 line (207). This cell line did not harbour any *BCR-ABL1* KD mutations or have overexpression of *BCR-ABL1* or Lyn kinase, however, imatinib treatment failed to down-regulate Erk1/2 activation (207). More recently, Hartel *et al* and Packer *et al* postulated that the T315I mutation may also confer resistance by this mechanism (208, 209). Bcr-Abl activity is known to stimulate the Raf/MEK/Erk pathway and when Bcr-Abl activity is blocked by a TKI, this pathway is inhibited (210, 211). Hartel *et al* however, found that the MAPK pathway was paradoxically activated in T315I mutated cells when exposed to imatinib and the effect was even more marked in nilotinib-treated cells (208). They postulated that the T315I mutation may control an intrinsic activation loop that leads to enhanced paradoxical activation of the MAPK pathway. Parker *et al* also demonstrated the same phenomenon (209). They postulated that imatinib, nilotinib and dasatinib are not only ineffective against the T315I mutation, they also have B-Raf inhibitor activity (209). These cells in the presence of TKI show persistent Ras activity but inhibition of B-Raf. This acts to drive formation of B-Raf-C-Raf heterodimers or C-Raf-C-Raf homodimers and thus enhances activation of downstream MEK and Erk (212-214).

This suggests that *BCR-ABL1+* cells may be resistant to treatment by TKI because of overexpression of downstream signal transduction and cells expressing the T315I mutation may actually have enhanced proliferation in the presence of a TKI (208, 209).

1.4.5 Alteration in the expression of drug transporters

The activity of the human organic cation transporter 1 (OCT1) is a significant factor affecting intracellular drug concentrations of imatinib. Patients with high OCT1 activity (OA) have been shown to have the highest probability of achieving MMR, whereas patients with low OA require increasing doses of imatinib (or switch to a second line TKI therapy) to achieve optimal response (215). Single nucleotide polymorphisms in *OCT1* have been associated with cellular imatinib uptake and the prediction of clinical response (216). For example, the M420del is found to be associated with a higher probability of imatinib failure (216). Neither the cellular uptake of dasatinib nor nilotinib appear to be affected by OA (217, 218).

The ATP-binding cassette (ABC) transporter family are efflux pumps involved in the export of TKIs. They include multidrug resistant protein 1 (MDR1) also known as ABCB1, and the breast cancer resistance protein, ABCG2. They contribute to drug resistance by actively transporting drugs out of cells (219, 220). ABCB1 has been found to be over expressed in patients in BC and these patients are generally insensitive to imatinib (221). Drug transporters are highly expressed in haematopoietic stem cells which may contribute to their inherent resistance to TKIs (222). However, there is conjecture in the literature about the role of ABCB1 in imatinib transport (223-226). Some researchers have found that imatinib is a substrate of ABCB1 but others, for example, have demonstrated that cell lines engineered to over express ABCB1 had minimal protection from imatinib-induced apoptosis (227). In support of this finding, another study demonstrated that blocking ABCB1 did not enhance the effect of imatinib (223, 228). Work in our laboratory has suggested that imatinib may in fact inhibit ABCB1 activity (226).

Dasatinib and nilotinib are known to be transported by ABCB1 (206, 217, 229). Dasatinib is also a substrate for ABCG2 transporters, however, there is still speculation in the literature as to whether imatinib and nilotinib are also transported by ABCG2 (217). Recent work in our laboratory suggests that imatinib and nilotinib are not transported by ABCG2 (217, 229).

1.4.6. Clonal Evolution

Advanced phases of CML are associated with clonal evolution where LSCs or progenitors acquire chromosomal abnormalities other than the Ph chromosome. This occurs in up to 80% of patients in BC compared to 10-20% in CP (112). It is unclear if clonal evolution

drives progression or if the chromosomal aberrations have a selective advantage in the BC environment (230). Although it is more common in advanced disease, the presence of clonal evolution is an independent poor prognostic marker in both chronic and more advanced phases of CML (113). The acquisition of additional cytogenetic abnormalities potentially results in a more malignant phenotype and less dependence on Bcr-Abl for survival.

The most common additional cytogenetic abnormalities are trisomy 8 and isochromosome 17q (112). Some abnormalities are more common after certain treatment. For example, Trisomy 8 occurs more frequently after treatment with busulphan compared to hydroxyurea (112). Isochromosome 17q results in the loss of a copy of the p53 gene and is associated with poor response to treatment including imatinib (112, 113, 231). It is a tumour suppressor and plays an important role in DNA repair, cell cycle arrest and apoptosis and hence has a role in the maintenance of genomic stability (232).

However, not all abnormalities are associated with poor outcome particularly if they are not associated with other poor risk factors (113). Cortez *et al* analysed 133 patients in accelerated phase and 51 of these also had clonal evolution. They found that the patients with clonal evolution may not necessarily experience poor outcome, unless other poor prognostic factors were present. These factors include splenomegaly, high percentage of peripheral and bone marrow blasts, basophilia and duration of disease prior to treatment (113).

1.5. Summary, Research hypothesis and Aims

Although the outlook for patients with CML has changed dramatically with the advent of imatinib and now second and third generation TKIs, a significant number of patients will develop resistance to these drugs. Development of KD mutations is the most common cause of secondary resistance comprising approximately 35-45% of all patients who have developed resistance to imatinib. However, it has been reported up to as high as 89%, depending on the methodology used, phase of disease investigated and definition of resistance (173, 178). One of the most common mutations is the T315I (9, 171, 233).

The T315I mutation is especially problematic as it is resistant to all clinically available TKIs. It is sensitive to the third generation TKI, ponatinib, however ponatinib is not yet available through the Pharmaceutical Benefit Scheme (PBS).

KD mutated clones may exhibit different biological properties that may make them more leukaemogenic than the non-mutated clones (90, 91, 181). Some groups have also found that patients harbouring the T315I mutation had clinically worse outcomes compared to those with other mutations or those who developed resistance to imatinib through other mechanisms (9, 187, 234). Moreover, the T315I mutation is often found in patients in BC (9, 235). Taken together, these studies suggest that this mutation may confer a gain-of-function independent of TKI resistance and in this thesis, the aim is to examine if cytokines have a role in contributing to its leukaemogenesis.

KD mutations are unlikely to exist prior to the selection pressure accorded by TKIs. It is believed that TKI treatment suppresses proliferation of the non-mutated *BCR-ABL1* expressing cells allowing the insensitive mutated clones to expand. However, recent findings showed that with TKI treatment, cells harbouring the T315I mutation expand rapidly due to enhanced MAPK signalling (208, 209). The mechanism of this phenomenon is unknown and the aim of this thesis is to ascertain if cytokines play a role.

Hypothesis 1

Cytokines play a role in the leukaemogenesis of the T315I mutation

Aims :

- To ascertain the cytokine profile of *BCR-ABL1*^{T315I} cells in comparison with *BCR-ABL1*^{p210} in conditioned media (CM)
- Assuming a divergent cytokine profile, to determine if *BCR-ABL1*^{T315I} cells proliferate advantageously in serum deprived media
- To determine if the CM of *BCR-ABL1*^{T315I} cells can confer protection to *BCR-ABL1*^{p210} from TKI-induced cell death, and if yes, what cytokine/s is/are responsible for the paracrine protective signalling
- To determine by which signalling pathway this occurs.

Hypothesis 2

In the presence of TKIs, *BCR-ABL1*^{T315I} cells expand rapidly due to hyperactivation of downstream signalling pathways, mediated through increased expression of cytokines.

Aims:

- To ascertain if the cytokine profile of the CM of *BCR-ABL1^{T315I}* cells when cultured with TKI is different to when cultured without TKI
- To determine which signalling pathways are up regulated in the presence of TKI.

Chapter 2

Materials and Methods

2.1 Commonly used reagents and their suppliers

Table 2.1

Reagents	Suppliers	Catalogue number
β -actin antibody	Cell Signaling	4967
Acrylamide/Bis Solution 40% 37.5:1 ratio	BioRad	161-0149
7-Aminoactinomycin D (7-AAD)	Invitrogen	A-1310
Ammonium persulfate 10% (APS)	Sigma-Aldrich	A9164
Annexin V-PE	BD Pharmingen	556421
Anti-Crkl antibody (C20)	Santa Cruz	SC319
Anti-human CD116 PE	BD Pharmingen	551373
Anti-human CD126 PE	BD Pharmingen	551850
Anti-human CD181 APC	BD Pharmingen	551080
Anti-human CD191 AF647	BD Pharmingen	557914
Anti-human CD192 AF647	BD Pharmingen	558406
Anti-human CD194 PE-Cy7	BD Pharmingen	557864
Anti-human CD195 PE	BD Pharmingen	555993
Anti-human FGFR2 APC	R&D Systems	FAB684A
Anti-human IL-1 RI APC	R&D Systems	FAB269A
BD Cytotfix	BD Biosciences	554655
BD Phosphoflow Perm buffer III	BD Biosciences	558050
Benchmark pre-stained protein ladder	Invitrogen	10748-010
BigDye [®] XTerminator [™] Purification kit	Applied Biosystems	4376484
Bovine serum albumin	Sigma-Aldrich	A1470
CellTiter 96 [®] AQueous One Solution Cell Proliferation assay	Promega	G3580
c-Abl antibody	Cell Signaling	2862
Cell trace CFSE proliferation kit	Invitrogen	C34554
Chloroform	Merck	10077613
Colcemid	Invitrogen	15210-040

Criterion™ TGX™ Precast gel 4-15%	Bio-Rad	567-1084
Criterion™ TGX™ Precast gel 10%	Bio-Rad	567-1034
DEPC water	MP Biomedicals	821739
D-Glucose	Sigma	G7528-250G
Dimethyl sulfoxide (DMSO)	Merck	1.02952.2500
DMEM High Glucose + Sodium pyruvate	Sigma-Aldrich	D5671
DMW-500bp DNA ladder	Geneworks	DMW-500
DNA ladder 1kb	New England BioLabs	N3232S
dNTP set (N=A,C,G.T)	GE Healthcare	27-2035-02
ECF attaphos substrate	GE Healthcare	RPN 5785
EXO SAP-IT	Affymetrix	78201
Expand long PCR Template	Roche	11681834001
Foetal calf serum	SAFC Biosciences	12003C-500M
Anti-FGF-2/basic FGF (neutralizing), clone bFM-1 monoclonal antibody	Millipore	05-117
Goat anti-rabbit- IgG-Alkaline phosphatase	Santa Cruz	SC-2007
Hanks balanced salt solution (HBSS) with phenol red, Ca ²⁺ Mg ²⁺ free	Sigma-Aldrich	H9394-500ML
HEPES buffer 1M	Sigma-Aldrich	H0887
Human Albumin	Sigma-Aldrich	A8763
Human cytokine/chemokine magnetic bead panel	Millipore	HCYTOMAG-60K
Isopropanol	Merck	6-10224.2511
Iscove's Modified Dulbecco's Medium (IMDM)	Sigma-Aldrich	13390
Isotype control PE-Cy7 Mouse IgG1k	BD Bioscience	557646
Isotype control PE Mouse IgG2ak	BD Bioscience	555574
Isotype control PE Mouse IgG1k	BD Bioscience	555749
Isotype control AF 647 Mouse IgG2bk	BD Bioscience	557903
Isotype control APC Mouse IgG2bk	BD Bioscience	555745
Isotype control APC Mouse IgG1	R&D Systems Inc	IC002A
Isotype control APC Goat IgG	R&D Systems Inc	IC108A
Kaleidoscope prestained protein standard	Bio-Rad	161-0375
Ki-67 monoclonal antibody	Cell Signaling	12160

L- Glutamine 200 mM	Sigma-Aldrich	G-709
Lymphoprep™	Axis-Shield	1114547
MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)	Life Technologies	G3580
P44/42 MAPK rabbit antibody	Cell signaling	9102S
PD 173074	Merck Millipore	341607
Penicillin G (10 000 U/ml)/Streptomycin sulphate (10 000 µg/ml)	Sigma-Aldrich	P4458
Phosphate Buffer Solution (PBS)	Life Technologies	14190-144
Phospho-Akt (Thr 308) antibody PE	BD Biosciences	558275
Phospho c-Abl (Tyr 245) antibody	Cell Signaling	2861
Phospho-pERK1/2 p44/42 (T202/Y204) antibody	Cell Signaling	9101S
Phospho-ERK1/2 (T202/Y204) antibody PE	BD Biosciences	612566
Phospho-STAT5 (Y694) antibody PE	BD Biosciences	612567
Phospho- STAT5 (Y694) antibody	Cell Signaling	9359S
PVDF membrane	GE Healthcare	PRN303F
Random Hexamers	Geneworks	RP6
Recombinant Human Erythropoetin	RAH Pharmacy	
Recombinant Human FGF-basic	PeproTech	100-18B
Recombinant Human GM-CSF	PeproTech	300-03
Recombinant Human IL-1 α	PeproTech	200-01A
Recombinant Human IL-6	PeproTech	200-06
Recombinant Human IL-8	PeproTech	200-08M
Recombinant Human MCP-1	PeproTech	300-04
Recombinant Human MDC	PeproTech	300-36A
Recombinant Human MIP-1 α	PeproTech	300-08
Recombinant Human MIP-1 β	PeproTech	250-32
RPMI 1640 W/O L-glutamine	Sigma-Aldrich	R0883-500ML
SeaBlue Plus 2 prestained protein standard	Life Technologies	LC5925
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich	L4509
STAT5 rabbit antibody	Cell Signaling	9363S
SuperScript II Reverse Transcriptase	Life Technologies	18064-014

Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	T7024
Trans-Blot® Turbo™ Transfer System RTA Transfer kits LF PVDF Midi	Bio-Rad	170-4275
Transwell collagen coated membrane inserts	Sigma-Aldrich	CLS3493
Tris	Merck	1.08382.0500
TRIzol® RNA Stabilization reagent	Life Technologies	15596-018
Trypan blue solution	Sigma-Aldrich	T-8154
Trypsin 2.5%	Life Technologies	15090-046
β-tubulin antibody	Cell Signaling	2128S
Tween 20	Sigma-Aldrich	P9416
Western blot recycling kit	Alpha Diagnostics International	90101

2.2 Solutions, buffers and media

2.5.1 Cell culture media

Roswell Park Memorial Institute (RPMI) 1640 media supplemented with

10% foetal calf serum (FCS)

50 units/ml Penicillin

50 µg/ml Streptomycin

2 mM L-glutamine

2.2.2 Serum deprived medium (SDM)

Iscove's Modification of Dulbecco's Medium (IMDM) supplemented with

1% bovine serum albumin (BSA)

2 mM L-glutamine

Stored at 4°C, and preheated to 37°C in a water bath prior to use.

2.2.3 Freezing medium

70% Hanks balanced salt solution supplemented with

10 mM HEPES

20% FCS

10% DMSO

Made fresh and chilled on ice or at 4°C prior to use.

2.2.4 Thaw medium for cell lines

RPMI 1640 media supplemented with

10% FCS

2 mM L-glutamine

2.2.5 Flow cytometry fixative (FACS Fixative)

1x PBS

1% w/v Formaldehyde

111 mM D-glucose

0.02% Sodium azide

2.2.6 Reagents required for assessment of cell death by flow cytometry

Binding buffer

2.5 mM CaCl in Hanks balanced salt solution with 10 mM HEPES

Annexin-V-PE working stock

For each sample, mix 1 µl Annexin-V-PE + 9 µl Binding buffer

7AAD working stock

For each sample, mix 0.1 µl 7AAD + 39.9 µl Binding buffer

2.2.7 Reagents required for Western Blotting

1xTBS

20 mmol/L Tris-HCL, pH 7.5

150 mmol/L NaCl

1xTBST

20 mmol/L Tris-HCL, pH 7.5

150 mmol/L NaCl

0.1% Tween20

1x Laemmli's Buffer (modified)

50 mmol/L Tris-HCL, pH 6.8

10% glycerol

2% SDS

5% β -mercaptoethanol

0.1% bromophenol blue

1 mM Sodium Vanadate

10 mM Sodium Fluoride

SDS-Polyacrylamide gel**14% Polyacrylamide Resolving gel****1 gel**

MilliQ H ₂ O	1.9 ml
40% Acrylamide-Bis 37.5:1	1.75 ml
1.5M Tris A, pH 8.8	1.25 ml
10% SDS	50 μ l
10% APS	50 μ l
TEMED	3 μ l

5% Polyacrylamide Stacker gel**1 or 2 gels**

MilliQ H ₂ O	3 ml
40% Acrylamide-Bis 37.5:1	630 μ l

0.5M Tris B, pH 6.8	1.26 ml
10% SDS	50 μ l
10% APS	50 μ l
TEMED	5 μ l

Membrane blocking solutions

2.5% skim milk

Non-fat milk powder	12.5 g
1 x TBS	500 mL

2.5% BSA

BSA	12.5 g
1 x TBST	500 mL

2.2.8 Reagents needed for Phospho-Tyrosine Flow Cytometry of cell lines

BD Cytotfix

BD Phosphoflow Perm Buffer III

Stain/Wash buffer

1x PBS

1% FCS

0.09% Sodium Azide

Antibodies used

Isotype control	Mouse IgG1 PE-conjugated
Anti-Erk 1/2	Mouse anti-Erk1/2 (pT202/pY204) PE-conjugated
Anti-STAT5	Mouse anti-STAT5 (pY694) PE-conjugated
Anti-Akt	Mouse anti-Akt (pT308) PE-conjugated

2.2.9 Tyrosine kinase inhibitors (TKI)

Imatinib mesylate, Mw 589.72

Kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). Stock solutions of imatinib were prepared at 10 mM with distilled water, filter sterilised and stored at -70°C.

Nilotinib, Mw 529.5

Also kindly provided by Novartis Pharmaceuticals. Stock solutions of nilotinib were prepared at 10 mM in DMSO and stored at 4°C.

Dasatinib, Mw 506.2

Kindly provided by Bristol-Myers-Squibb (New Brunswick, USA). Stock solutions of dasatinib were prepared at 10 mM in DMSO and stored at 4°C.

Ponatinib, Mw 532.56

Kindly provided by Ariad Pharmaceuticals (Cambridge, Massachusetts, USA). Stock solutions of ponatinib were prepared at 10 mM in DMSO and stored at 4°C.

2.3 Cell lines

2.3.1 K562 and K562-T315I

The K562 cell line was the first human CML cell line in which the Ph chromosome persisted after prolonged culture *in vitro*. The cells were derived from an erythroleukaemic cell line sourced from the pleural fluid of a female patient in blast crisis (236, 237). The cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

The K562-T315I cell line was established in our laboratory by culturing K562 cells in increasing concentrations of dasatinib (238). Initially, the cells acquired resistance to dasatinib by increasing mRNA transcripts of *BCR-ABL1* but when the concentration of dasatinib increased to 200 nM, the T315I mutation emerged. Thus, the original name given to this cell line was K562 200nM Das (238). The level of T315I transcript reached a maximum of 50-60%. Dasatinib was diluted in DMSO and therefore K562 cells cultured in 0.1% DMSO were performed in parallel and acted as the control cell line. These control cells are referred to as K562 naïve and do not contain any *BCR-ABL1* kinase domain mutations (238).

2.3.2 HL60 parental, HL60 *BCR-ABL1*^{p210} and HL60 *BCR-ABL1*^{T315I}

The HL60 cell line was established in 1977 from the leukopheresis cells of a female patient with acute myeloid leukaemia (239, 240). *BCR-ABL1* HL60 cell line, named HL60 parental was obtained from ATCC.

The HL60 cell lines employed in this study had been previously transduced with *BCR-ABL1* retroviral constructs containing *BCR-ABL1*^{p210} (results in cells expressing *BCR-ABL1*^{WT}) and named HL60 *BCR-ABL1*^{p210} or *BCR-ABL1*^{T315I} (results in cells expressing *BCR-ABL1*^{T315I}) and named HL60 *BCR-ABL1*^{T315I}.

The *BCR-ABL1*^{p210} and *BCR-ABL1*^{T315I} viral constructs were co-expressed with enhanced green fluorescent protein (eGFP) and were fluorescent activated cell sorted (FACS) on an Epics Altra HyperSort using Expo multiComp Software (Beckman Coulter, Miami, Florida, USA) (241).

2.3.3 KU812

The KU812 was established from the peripheral blood of a patient with CML in blast crisis and was initially described as a basophilic cell line (242). The cells are also erythropoietin sensitive and able to differentiate spontaneously towards the erythroid lineage (242). This cell line was obtained from the ATCC.

2.4 General techniques used in the laboratory

2.4.1 Maintenance of cell lines

Cell lines were maintained in pre-warmed cell culture medium (see 2.2.1) in either 25 cm² or 75 cm² tissue culture flasks in a 37°C/5% CO₂ incubator. Cell lines were sub-cultured in Class 2 biosafety cabinets at a cell density between 8 x 10⁴ cells/ml and 1 x 10⁶ cells/ml.

2.4.2 Cell counts and viability assessment by trypan blue exclusion dye

Live cells can exclude trypan blue dye but dead cells cannot and therefore this discriminatory feature of trypan blue dye is exploited to assess cell viability. A mixture of 10 µl of cells and 10 µl of 0.4% trypan blue solution and 10 µl was loaded onto a haemocytometer counting chamber (Neubauer Improved, Assistant, Germany) and under light microscopy live cells and dead (stained) cells were counted and viability and cell density calculated. Experiments

using this technique were repeated at least 3 times and often done in duplicates to ensure reproducibility.

2.4.3 Cryopreservation of cells

Cell-lines were cryopreserved at 1×10^7 cells per cryogenic vial. Under aseptic conditions, the required volume of cell suspension was transferred to a 50 ml tube, centrifuged at 400 *g* for 5 minutes and all media removed by aspiration. The cells were then chilled for 5 minutes at 4°C and then resuspended in Freezing medium (refer section 2.2.3) before being transferred to a pre-labelled, pre-chilled cryogenic vial and controlled-rate frozen in a Nalgene “Mr Frosty” container at -70°C for at least 4 hours before being transferred to the vapour phase of liquid nitrogen (-196°C) for long-term storage.

2.4.4 Thawing of cell lines

Cryopreserved cells were thawed quickly by immersion in the 37°C water bath with gentle agitation and upon thawing, transferred to a 50 ml tube in the Class II Biosafety Cabinet. 10 ml of pre-warmed cell culture media was added drop-wise to the cells and the tube was then centrifuged at 400 *g* for 5 minutes. Media was discarded and the process was repeated. Cells were then resuspended in another 10 ml warm cell culture media, counted and seeded at 2×10^5 cells/ml in a 25cm² flask and cultured in a 37°C/5% CO₂ incubator.

2.4.5 Lymphoprep density gradient centrifugation (Ficoll) of a cell line culture for live cell enrichment

A cell culture with a high proportion of dead or dying cells was transferred under aseptic conditions to a 50 ml tube and pelleted at 400 *g* for 5 minutes. Media was aspirated leaving approximately 8 ml. The cells were resuspended, transferred to a 13 ml polypropylene FACS tube and 5 ml Lymphoprep™ was underlaid below the cell suspension. The tube was then centrifuged at 1000 *g* for 10 minutes with low brake at room temperature. After centrifugation, a cell pellet of dead cells and cell debris was visible at the bottom of the tube and a layer of live cells was visible at the interface between the Lymphoprep™ and media. This cell layer was transferred to a new 50 ml tube and the cells were washed twice with media. After performing the trypan blue count, the cells were re-cultured in a 25 cm² or 75 cm² flask at a concentration of 2×10^5 cells/ml.

2.4.6 Optimal washing of K562-T315I cell line

K562-T315I cell lines were cultured and maintained in 200 nM dasatinib (238). Prior to assay set-up, cells were optimally washed to remove all traces of dasatinib. The cells were first pelleted at 400 *g* for 5 minutes and washed in 5 ml of warm 1x PBS and pelleted again. The supernatant was aspirated, 10 ml of warm culture media (refer section 2.2.1) was added and the cells were incubated at 37°C/5% CO₂ for 1 hour. The process was repeated twice more before a final suspension in the media used in the experiment.

2.5 Special techniques

2.5.1 Flow cytometry to assess cell death by Annexin V and 7AAD

Phosphatidylserine (PS) residues are cell membrane components which are normally localised on the internal surface of the cell membrane but are externalised in early apoptotic cells. Annexin V is a calcium-dependent phospholipid-binding protein which has a high affinity to PS residues and thus can be used to determine apoptotic cells. 7-Amino-actinomycin D (7AAD) is excluded by viable cells but can penetrate the membranes of dead or dying cells where it is intercalated into the double-stranded DNA. Both 7AAD and PE-conjugated Annexin V are detected by flow cytometry (243).

Cells were optimally washed (refer section 2.4.6) and then resuspended in fresh media and 1 ml plated in duplicates or triplicates in a 24-well plate at 1×10^5 cells/ml unless otherwise specified. Reagents were added and the plates placed in sterilised cake-boxes. The cells were incubated at 37°C/5% CO₂ for 3 days and then stained to assess cell death.

For an Annexin V positive control, 1 ml of cells (minimum 1×10^5 cells) was treated with 1 ml DMSO in a 5 ml polypropylene FACS tube and incubated for 10 minutes at room temperature. For the 7AAD positive control, 1 ml of cells (minimum 1×10^5 cells) was treated with 1 ml cold 70% ethanol and incubated on ice for 10 minutes. For the negative control, 1 ml of cells were placed in a 5 ml FACS tube, pelleted at 600 *g* for 2 minutes and then resuspended in 400 µl of binding buffer (refer sections 2.2.6). After incubation, both the positive control tubes were pelleted at 600 *g* for 2 minutes, washed twice in 2 ml 1x PBS and then resuspended in 2 ml binding buffer. 10 µl of Annexin V-PE stock solution (refer

section 2.2.6) was added to the Annexin V positive control and 40 µl of 7AAD stock solution (refer section 2.2.6) was added to the 7AAD positive control.

The experimental samples were transferred into 5 ml polypropylene round-bottomed FACS tubes and pelleted at 600 *g* for 2 minutes and the supernatant removed by aspiration. The cells were washed once in 1 ml binding buffer. 10 µl of Annexin V-PE stock solution (refer section 2.2.6) and 40 µl of 7AAD stock solution (refer section 2.2.6) was added to all the experimental samples. Tubes were mixed by vortexing and incubated for 15 minutes in the dark on ice. After incubation, 400 µl binding buffer was added to all the tubes and kept on ice. Data was acquired on a FC500 flow cytometer (Beckman Coulter, Pasadena, California, USA) within 1 hour and analysed using FCS Express software (DeNovo Software, Los Angeles, California, USA).

2.5.2 Flow cytometry to assess growth factor and cytokine cell surface receptors

Cells (2×10^5) were placed in a FACS tube and made up to 1 ml with staining buffer (RPMI + 10% FCS). Tubes were centrifuged at 600 *g* for 2 minutes to pellet the cells and supernatant removed. The cell pellets were incubated with monoclonal primary antibodies, or their isotype controls, for 30 minutes on ice. After incubation, another 1 ml of staining buffer was added to each tube and the cells were pelleted at 600 *g* for 2 minutes. Cells were then resuspended in 250 µl FACS fixative (refer section 2.2.5) and kept on ice until flow cytometry analysis could be performed. If stimulation of the cell surface receptors was required, the cells were first pre-incubated with recombinant growth factors or cytokines in warm media and incubated at 37°C for 10 or 30 minutes. Following incubation, the cells were pelleted, washed with cold staining buffer and then incubated with antibodies. For both protocol variations, the samples were analysed on a Beckman Coulter FC500 flow cytometer and data analysed using FCS Express software.

2.5.3 Flow cytometry to assess the presence and proportion of intracellular proteins

Treatment of *BCR-ABL1* positive cells with a TKI causes a reduction in tyrosine-phosphorylated protein, such as Crkl, Erk, STAT5 and Akt. The expression of intracellular phospho-tyrosine proteins can be measured by intracellular staining and detection by flow

cytometry. Cells must first be fixed and then permeabilised to allow antibodies directed against the intracellular proteins to penetrate the cell membrane. Refer below for details.

Fixation

At least 1×10^5 cells were used for each sample. The cells were centrifuged at 600 *g* for 2 minutes and the supernatant removed. The cells were then washed in 500 μ l of wash buffer (refer Section 2.2.8) before 250 μ l of BD Cytofix was added into each tube and tubes were incubated on ice for 30 minutes. Post-incubation, the cells were washed again with wash buffer.

Permeabilisation

Ice-cold Phosphoflow Perm Buffer III (250 μ l) was added to each tube of fixed cells while gently vortexing and the cells were incubated again on ice for 30 minutes. The permeabilised cells were then washed with wash buffer.

Staining

Cells were stained with the appropriate fluorochrome-conjugated phospho- antibodies or isotype controls (refer Section 2.2.8) according to the manufacturers' instructions and incubated at room temperature for 1 hour in the dark. Following incubation, the cells were washed and resuspended in 250 μ l wash buffer before analysing on the flow cytometer.

2.5.4 Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling of cells

Carboxyfluorescein succinimidyl ester (CFSE) is used to track proliferation of cells. It passively diffuses into cells and the acetate groups are cleaved by intracellular esterases in actively dividing cells. The resulting fluorescent esters react with intracellular amines and are retained within the cells. Upon cell division, the CFSE fluorescence is progressively halved in the daughter cells and therefore, proliferation can be tracked by the successive halving of the fluorescence intensity.

The cell lines were washed optimally as described in section 2.4.6 and 5×10^6 cells were then resuspended in 2 ml of 1x PBS + 0.1% BSA. Cells were then stained with 10 μ M CFSE, mixed thoroughly by vortexing and incubated for 10 minutes in a 37°C water bath. Five volumes of ice-cold PBS + 10% FCS were added to stop the staining process and tubes were incubated on ice for 5 minutes. This washing step was repeated twice more and the cells were then

resuspended in 2 ml HBSS containing 10 mM HEPES and 1% FCS. The cells were sorted over a narrow range of CFSE fluorescence (40 channels) using the Beckman-Dickinson Aria with FACS Diva Software version 6.1.3 (BD, San Diego, California, USA) after which the cells were “rested” for 1 hour at 37°C in an incubator. The cells were resuspended in either RPMI + 10% FCS or SDM after washing twice in the relevant experimental media and then 1 ml per condition was plated at a concentration of 1×10^5 cells/ml into a 24 well plate with 3 control populations: firstly, unstained cells to detect autofluorescence, secondly, cells stained with CFSE and 10 µg/ml colcemid, which inhibits cell division and therefore detects maximum fluorescence, and thirdly, cells treated with 10 nM dasatinib for 3 days to be stained later with 7AAD to gate for dead cells. After 3 days of culture, the resultant cells were analysed by FC500 flow cytometry using 7AAD to assess cell death. Data was analysed using FCS Express software. Proliferation index (PI) was generated by the software.

2.5.5 Co-culturing *BCR-ABL1*^{WT} cells with *BCR-ABL1*^{T315I} cells

To ascertain if the cytokines and growth factors produced by *BCR-ABL1*^{T315I} cells were able to protect *BCR-ABL1*^{WT} cells from TKI-induced cell death through the paracrine secretion of pro-survival cytokines, *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells were co-cultured separated by Transwell® inserts (Sigma-Aldrich) with membranes that allowed for diffusion of soluble factors.

The cells were optimally washed (refer section 2.4.6), and resuspended in SDM (section 2.2.2). *BCR-ABL1*^{T315I} (either K562-T315I or HL60 *BCR-ABL1*^{T315I}) cells were plated at the bottom of the well and *BCR-ABL1*^{WT} (either K562 naïve or KU812) cells were plated in the Transwell® inserts, both at the concentration of 1×10^5 cells/ml (a total of 1.5×10^5 cells in 1.5 ml for the *BCR-ABL1*^{T315I} cells and 5×10^4 cells in 0.5 ml for the *BCR-ABL1*^{WT} cells). The volume of culture media to add to the plates were recommended by the manufacturer, i.e. 1.5 ml per plate well and 0.5 ml per inside of Transwell insert. Cells were either cultured together with TKI or DMSO as control at 37°C/5% CO₂, and were assessed for viability after 3 days by trypan blue exclusion dye and for cell death using flow cytometric analysis of Annexin V and 7AAD cell staining.

2.5.6 Western blotting analyses

Cell lysates

Cells for the preparation of lysates were optimally washed (refer section 2.4.6), if the cells had been cultured in the presence of a TKI. The number of cells used to prepare a lysate depended on the protein of interest, but generally 1×10^5 to 4×10^5 cells were used. The appropriate TKI was added and the cells were incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 2 hours for pCrkl or at 4°C overnight for pErk, pSTAT5, Erk, STAT5, Bcr-Abl and β -tubulin. Following any given experimental protocol, cells were pelleted at $400 g$ for 5 minutes, washed in 5 ml cold 1x PBS and the wash repeated. The supernatant (PBS) was removed bar approximately 1 ml. The cells were resuspended in the residual PBS and transferred to a 1.7 ml microfuge tube and centrifuged at $3900 g$ for 5 minutes and then pulse spun to $15600 g$. All PBS was then removed and $10 \mu\text{l}$ of Laemmli's buffer (refer section 2.2.7) was used to resuspend the cells. The tubes were vortexed well and then boiled at 100°C for 12 minutes, after which lysates were clarified by microfugation at $15600 g$ for 30 seconds. Lysates were stored at -20°C until required for western blotting analysis.

SDS-PAGE and Western blots

Protein lysates were resolved by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) on manually-poured gels (refer Section 2.2.7) run at 15 mA current for 45 minutes and then 20 mA for approximately 2-2.5 hours to separate the Crkl from pCrkl bands (38 and 39kDa) and pErk (42 and 44 kDa) and pSTAT5 (91 kDa) bands. Lysates can also be resolved on pre-cast Criterion™ TGX™ 4-15% gels or 10% gels at a constant voltage of 100 V for 15 minutes and then 200 V for 70 minutes. The gels were then transferred electrophoretically to low-fluorescence PVDF membrane using a Bio-Rad Trans-Blot® Turbo™ Blotting System following the manufacturer's instructions. After rinsing in 1x TBST (refer section 2.2.7), the membrane was blocked for 1 hour at room temperature with 2.5% blocking solution (refer 2.2.7). After blocking, the membranes were probed with primary antibody (Santa Cruz rabbit anti-Crkl C-20 at room temperature for 2 hours; Cell Signaling Technology anti-pErk or anti-pSTAT5 overnight at 4°C). Following incubation of the primary antibodies, membranes were washed three times for 5 minutes in 1x TBST with constant agitation and then incubated with secondary antibody, alkaline phosphatase

conjugated goat-anti-rabbit immunoglobulin for 1 hour at room temperature with agitation. Following final washes with TBST and TBS (3 x 5 minutes each), bound antibody conjugates were detected with enhanced chemifluorescence (ECF) substrate (Amersham Pharmacia, Piscataway, New Jersey, USA) and analysed on a Typhoon FLA 9000 FluorImager (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK). The results were then quantified with ImageQuantTL software (Molecular Dynamics, GE Healthcare Life Sciences).

Stripping immunoblot membranes

In order to analyse different antibodies on a single western blot membrane, the blot was stripped of previously bound immune-complexes using the Western Blot recycling kit.

All blots were either stripped immediately after use or kept moist in 1x TBS at 4°C. The antibody stripping solution was used at a 1:10 dilution of the 10x stock solution with milliQ water and the blots were incubated in this solution with continual agitation for 10-15 minutes at room temperature. The blots were then blocked twice with 2.5% blocking solution for 5 minutes each before incubation with additional primary antibody (anti-Erk, anti-STAT5 and anti- β tubulin) following the method outlined above.

2.5.7 mRNA isolation

Messenger RNA (mRNA) was isolated using TRIzol® RNA Stabilization reagent. 5×10^5 cells were harvested and centrifuged at 400 *g* for 5 minutes to remove all supernatant. The cells were then lysed in 1 ml of TRIzol® solution, which is a monophasic solution of phenol and guanidine. After incubation at room temperature for 5 minutes, 0.2 ml of chloroform was added and samples incubated for 2 minutes at room temperature. The cells were centrifuged at 12000 *g* for 15 minutes at 4°C and the aqueous layer transferred to a new RNA/DNA free tube. mRNA was precipitated by the addition of 1 μ l (20 μ g) glycogen and 0.5 ml isopropanol with incubation at room temperature for 10 minutes after gentle mixing. mRNA was then pelleted by centrifugation at 12000 *g* for 10 minutes at 4°C, the isopropanol removed by aspiration and the mRNA pellet washed in 75% ethanol. Following removal of the ethanol wash by pipetting, the mRNA was dissolved in 20 μ l of DEPC water at 55°C for 10 minutes.

The concentration of mRNA was measured by the Nanodrop spectrophotometer (Thermo Scientific, Waltham, Massachusetts, USA) and DEPC water was added to give a final mRNA concentration of 1000 ng/ μ l. mRNA stocks were stored at -70°C .

2.5.8 cDNA synthesis

cDNA was synthesized using the Superscript II method where 1000 ng mRNA was added to 250 ng Random hexamers and 9 μ l DEPC water. The mixture was heated at 70°C for 10 minutes and chilled briefly at 4°C on the Mastercycler (Eppendorf, Hamburg, Germany).

Premix was prepared as follows:

Reagent	1 sample (μ l)
5x First strand buffer	4
0.1M DTT	2
5mM dNTP	2
Superscript II	1

The prepared premix was added to the tubes and heated in the Mastercycler with the following programmes:

25°C for 10 minutes

42°C for 50 minutes

70°C for 10 minutes

Kept chilled at 4°C

20 μ l DEPC water added and cDNA was stored at -70°C .

2.5.9 *BCR-ABL1* kinase domain Long PCR amplification and sequencing

BCR-ABL1 kinase domain was detected by the following method. The Mastermix was prepared as follows:

Reagent	1 sample (μ l)
25 mM dNTP	0.75

dH2O	18.325
10X Buffer 3	2.5
25 mM Magnesium chloride	0.75
50 μ M Long "F0810"	0.15
50 μ M Long "R0510"	0.15
Expand enzyme mix	0.375

The prepared Mastermix was added into each PCR tube and 2 μ l of thawed cDNA was added to the appropriate tubes. The samples were run in a Thermacycler (Eppendorf) under following conditions:

94°C for 2 minutes

94°C for 10 seconds, 60°C for 30 seconds, 68°C for 2 minutes x 10 cycles

94°C for 10 seconds, 60°C for 30 seconds, 68°C for 2 minutes - increased by 20 seconds every cycle x 30 cycles

68°C for 7 minutes

Kept chilled at 4°C

After which the PCR products were run on a 2% agarose gel (2 g agarose dissolved by boiling in 100 mL 1x TAE solution (40 mM Tris, pH 8.5, 0.11% acetic acid, 1 mM EDTA) separated by electrophoresis to determine the presence of a product of the correct size (b3a2=1579 base pair (bp)) together with a 500 bp and 1 kb DNA 'markers' The PCR products were purified using ExoSAP-IT. ExoSAP-IT (2 μ l) was added to 5 μ l of PCR product and ran in the Thermocycler under the following conditions:

37°C for 15 minutes

80°C for 15 minutes

Held at 4°C

The purified PCR products were then sequenced using ABLkinaseF:

5' CGC AAC AAG CCC ACT GTC T 3'

Sequencing reaction Mastermix was prepared as follows:

Reagent	1 sample (μ l)
Big Dye v3	1
5x Sequencing buffer	2
50 μ M Forward primer	0.2
DEPC water	5.8

The prepared Mastermix was added into PCR tubes and 1 μ l of 50 ng/ μ l purified PCR product was added to the appropriate tubes. The reaction was then run in the Thermocycler under the following conditions:

96°C for 10 seconds

50°C for 5 seconds

60°C for 4 minutes x 25 cycles

Held at 4°C.

2.5.10 Sequencing reaction Purification

The finished product was cleaned using the BigDye® XTerminator™ Purification kit. Mastermix of 10 μ l XTerminator™ solution and 45 μ l SAM™ solution per sample was prepared and added into a 96-tube plate to which the finished products of the sequencing reaction were also added. The sealed plate was placed on a plate shaker for 30 minutes and then centrifuged at 1000 *g* for 2 minutes. The sequencing was performed by Molecular Pathology in SA Pathology either at Frome Road or Flinders Medical Centre and it was analysed using Mutator Surveyor software (SoftGenetics LLC, Pennsylvania, USA).

2.5.11 Quantification of *BCR-ABL1* mRNA

As the cell lines used in this project contained the B3A2 transcript of *BCR-ABL1*, *BCR* and *B3A2* were amplified by Real-time Quantitative PCR (RQ-PCR). The primers and probes were prepared as previously described (244).

cDNA made previously was thawed and Mastermix was prepared as follows:

BCR	1 sample (μl)
Taqman Universal Mastermix	12.5
dH ₂ O	9.5
50 μM B8	0.1
50 μM B9	0.1
BCR probe	0.3

B3A2	1 sample (μl)
Taqman Universal Mastermix	12.5
dH ₂ O	9.48
50 μM B3	0.1
50 μM 3A2	0.1
B3A2 probe	0.32

The prepared Mastermix (22.5 μl) was added into each well in a 96-well plate and 2.5 μl of standards added to appropriate wells in the PCR cabinet. The standards (copy number 10³-10⁶ for *BCR* and 10-10⁶ for *B3A2*) were prepared “in-house.” Following this, 2.5 μl of sample cDNA or control cDNA (Hela, Low control and High control) was added to the appropriate wells. The samples were then run in an ABI Prism 7500 Sequence Detection System (Applied Biosystems) under the following conditions:

Cycle no x 1 50°C for 2 minutes

Cycle no x 1 95°C for 10 minutes

Cycle no x 45 95°C for 15 minutes and 60°C for 1 minute

Results were analysed using the ABI Prism 7500 software (Applied Biosystems).

2.5.12 MTS assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay measures the conversion of the tetrazolium salt into a soluble formazan compound by dehydrogenase enzymes in metabolically active cells. Therefore, the level of activity of these enzymes is used as a measure of the number of metabolically active cells.

The cells were washed twice with either SDM (refer section 2.2.2) or RPMI + 10% FCS (refer section 2.2.1) and then plated in triplicate into a 96-well plate at a concentration of 2000 cells in 100 µl of appropriate media per well. Media alone was used as a baseline control. Identical plates were set up- Day 0, Day 1 and Day 3 plates. MTS labelling reagent (20 µl) was added into each well of the Day 0 plate and incubated at 37°C/5% CO₂ for 1-4 hours. Following incubation, the cells were assessed using an ELISA plate reader, EL808 Ultra Microplate reader (BioTek, Winooski, Vermont, USA) at 490 nm. The remaining 2 plates were incubated overnight or for 3 days at 37°C/5% CO₂ and were analyzed as done for the Day 0 plate, at the appropriate time points. Average of baseline control absorbance readings from the media only wells was also subtracted from all other absorbance values to give corrected absorbance and Day 1 and day 3 results were normalised to Day 0.

2.5.13 Cytokine profiling of cell culture supernatants

The cells were optimally washed (refer section 2.4.6), then twice again in SDM (section 2.2.2) and resuspended at a concentration of 5×10^5 cells/ml and cultured in SDM overnight. The next day, cells were centrifuged at 400 *g* for 5 minutes and the supernatant decanted into another flask, following which this process was repeated for the decanted supernatant. The resulting clarified supernatant was stored at -70°C prior to analysis. This process was performed at two different time points to obtain two different passage samples for comparison.

The Human Cytokine/Chemokine Magnetic Bead Panel by Millipore (Massachusetts, USA) which utilises Luminex xMAP® technology, was used to quantitate the concentration of growth factors and cytokines within the samples. The beads are internally colour-coded with two fluorescent dyes and through precise concentrations of two dyes, distinct bead sets are created. The beads are also coated with a specific capture antibody.

The assay was performed as per the manufacturer's instructions. Briefly, culture supernatants were added into the wells of a 96-well plate in duplicate and the magnetic beads were introduced with appropriate standards and controls. The plate was then incubated with agitation overnight at 4°C. After washing the plate using a magnetic plate washer (Bio-Plex Pro™ II Wash station, Bio-Rad, Hercules, California, USA) twice the next day, biotinylated detection antibody was added and incubated at room temperature for 1 hour followed by Streptavidin-Phycoerythrin conjugate, the reporter molecule. The plate

was again incubated at room temperature for 30 minutes and then washed twice before reading on the Bioplex200TM analyser (Bio-Rad). Results were analysed using xPONENT software (Invitrogen Life Technologies, California, USA).

2.6 Statistical analysis

Data obtained are from at least 3 independent experiments and reported as the mean plus or minus standard error of the mean (SEM). Significance was determined by Student *t* test analysis and a P value <0.05 was considered statistically significant.

Chapter 3

The *BCR-ABL1*^{T315I} cells protect *BCR-ABL1*^{WT} cells from TKI-induced cell death through a paracrine cytokine mechanism

3.1. Introduction

It is well recognised that oncogenes can alter a cell's requirement for growth factors and enhance its growth potential. For example, oncogenic protein tyrosine kinases such as Bcr-Abl, ETV6-PDGFR β and FIP1L1-PDGFR α can transform the IL-3 dependent murine pro-B cell line, BA/F3, into a factor-independent cell line (245-248). *BCR-ABL1* positive early progenitor cells are able to proliferate in serum free media in the absence of added growth factors and both Jiang *et al* and Holyoake *et al* have demonstrated that these cells produce IL-3 and G-CSF which act as autocrine mediators of proliferation (71, 72). Furthermore, excessive production of growth factors and cytokines act to protect CML progenitor cells from the cytotoxic effects of TKI therapy (80, 81).

It has also been shown that *BCR-ABL1* positive cell lines can acquire resistance through the overproduction of cytokines. *BCR-ABL1* positive and imatinib resistant, LAMA84 cells, mediated imatinib and nilotinib resistance through the activation of JAK2/STAT5 pathway by adaptive secretion of GM-CSF (74). Cell lines harbouring the *BCR-ABL1* E255K mutation and the composite E255K/T315I mutation, protected non-mutated cells from TKI-induced cell death through the paracrine secretion of IL-3 which in turn activated MEK/Erk and JAK2/STAT5 pathways as well as down regulated the pro-apoptotic protein, Bim (73). An IL-3 autocrine loop has also been implicated in the DA1-3b *BCR-ABL1* positive mouse model of tumour dormancy as critical in the development of imatinib resistance (249).

More recently, bone marrow (BM) cells from CP-CML patients were found to have significantly higher mRNA transcript levels of *G-CSF*, *IL-1 α* , *MIP-1 β* and *MIP-2* and lower mRNA expression of *CXCR12* compared to normal BM cells (87). These factors were found to selectively impair expansion of normal progenitor cells while also providing a growth advantage to the CML progenitor cells. Treatment with imatinib reversed some of these

abnormal cytokine mRNA levels but did not normalise them completely (87). This may partly explain the persistence of CML stem cells despite prolonged treatment with TKI.

Therefore, *BCR-ABL1*^{T315I} cells were investigated to ascertain if the cells can survive and proliferate advantageously over *BCR-ABL1*^{WT} cells in serum deprived media (SDM) and if overproduction of cytokines and growth factors by *BCR-ABL1*^{T315I} cells would confer a survival advantage to *BCR-ABL1*^{T315I} cells in low cytokine/growth factor conditions.

3.2. Approach

3.2.1. Characterising the K562 naïve and K562-T315I cell lines

The cells were assessed by RQ-PCR to ascertain the amount of *BCR-ABL1* mRNA transcript in each cell line and also the proportion of T315I mutation.

mRNA was isolated from each cell line using Trizol RNA Stabilization solution (Invitrogen Life Technologies, Carlsbad, California, USA). This method has been mentioned previously (Chapter 2, Section 2.5.7). cDNA was synthesized using the Superscript II (Invitrogen Life Technologies) method (Chapter 2, Section 2.5.8). cDNA was stored at -70°C until needed.

To detect and quantitate *BCR-ABL1* kinase mutations, Long PCR and sequencing was performed. The method has previously been described in detail in Chapter 2, Section 2.5.9. Results were analysed using Mutation Surveyor software (Soft Genetics LLC, Pennsylvania, USA).

3.2.2. Cytokine profiling of cell culture supernatants

Supernatants from K562 and HL60 cell lines cultured in SDM were analysed for the presence of cytokines and growth factors. Cells were washed optimally to ensure that dasatinib in which the K562-T315I cells were resuspended was totally removed (described in Chapter 2, Section 2.4.6). Cell culture supernatant was prepared as detailed in Chapter 2, Section 2.5.13.

The Human Cytokine/Chemokine Magnetic Bead Panel by Millipore (Massachusetts, USA) which utilises Luminex xMAP® technology, was used to quantitate the concentration of growth factors and cytokines within the samples. The beads are internally colour-coded with two fluorescent dyes and through precise concentrations of two dyes, distinct bead sets

are created. The beads are also coated with a specific capture antibody. They were selected to detect FGF-2, G-CSF, GM-CSF, MDC, IL-1 α , IL-1 β , IL-1RA, IL-3, IL-6, IL-8, MCP-1, MIP-1 α , MIP-1 β and TNF- α . The assay was performed as described in Chapter 2, Section 2.5.13.

Data was acquired on the Bioplex200TM analyser (Bio-Rad) and results analysed using xPONENT software (Invitrogen Life Technologies, California, USA).

3.2.3. Proliferation and viability of *BCR-ABL1*^{T315I} cells compared to *BCR-ABL1*^{WT} cells in SDM

Trypan blue exclusion dye

K562 naïve and K562-T315I cell lines were washed optimally. The HL60 cell lines did not need to be washed optimally as the cells were not cultured with TKI. All the cell lines were resuspended in SDM at 1.5×10^5 cells/ml and incubated for 3 days at 37°C/5% CO₂. After which the number of live cells was assessed by trypan blue staining using trypan blue exclusion dye (Sigma-Aldrich, Missouri, USA).

Annexin V and 7AAD cell death assay

This method was previously described in Chapter 2, Section 2.5.1. Phosphatidylserine (PS), a cell membrane component which is normally localised on the internal surface of the cell membrane externalises in early apoptotic cells. Annexin V (BD Pharmingen, San Jose, California, USA) has a high affinity for PS. 7AAD (Invitrogen Life Technologies, Carlsbad, California, USA), penetrates the cell membrane of dead or dying cells where it is intercalated into the double-stranded DNA. Therefore, 7AAD will only stain dead or dying cells. Thus, both of these fluorescent markers, which are detected by flow cytometry can be used as cell viability markers as live cells do not take up the stains (243).

For the experimental samples, a minimum of 1×10^5 cells were used. The data was acquired on flow cytometer (Beckman Coulter Cytomics FC500) using CXP Cytometry List Mode Data Acquisition (Beckman Coulter, Miami, FL, USA) in parallel with positive and negative control samples and analysed using Analysis Software version 2.2 (Beckman Coulter). Viable cells were defined as those negative for both Annexin V and 7AAD.

MTS assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Celltiter96® AQueous One Solution Cell Proliferation assay) (Promega, Wisconsin, USA) was used to assess cell growth rate. This is a colorimetric assay which assesses the conversion of the tetrazolium salt into a soluble formazan compound by dehydrogenase enzymes in metabolically active cells. Therefore, level of activity is a measure of the proportion of actively growing cells. The absorbance at 490 nm was measured on an EL808 Ultra Microplate reader (BioTek, Winooski, Vermont, USA). The method was previously described in Chapter 2, Section 2.5.12. After incubation and addition of reagents, the samples were read using the ELISA plate reader. Media only was used as the baseline control and the average baseline absorbance readings were subtracted from all other absorbance values to give corrected absorbance. Results were normalised to the readings obtained at Day 0.

CFSE cell labelling

CFSE (Carboxyfluorescein succinimidyl ester) cell labelling (Cell Trace, Invitrogen Life Technologies) was also used to compare the proliferation and division of the cell lines. CFSE passively diffuses into cells and the acetate groups are cleaved by intracellular esterases. The resulting fluorescent ester reacts with intracellular amines and is retained within the cells. Upon cell division, the CFSE fluorescence is progressively halved in the daughter cells and therefore, proliferation can be tracked by the successive halving of the fluorescent intensity.

Methodology used is as described in Chapter 2, Section 2.5.4. but briefly, after staining with CFSE, the cells were sorted over a narrow range of CFSE fluorescence (40 channels) using the Beckman-Dickinson Aria with FACS Diva Software version 6.1.3 (BD, San Diego, California, USA). Following this, cells were resuspended in either RPMI + 10% FCS or SDM and 1×10^5 cells were plated into the wells of a 24 well plate with 3 control populations: firstly, the unstained cells to detect autofluorescence, secondly, cells stained with CFSE and colcemid, which inhibits cell division and therefore detects maximum fluorescence and thirdly, cells treated with dasatinib to be stained later with 7AAD to gate for dead cells. After 3 days incubation at 37°C/5% CO₂, the cells were analysed by flow cytometry and the results generated using the De Novo software (Los Angeles, Ca, USA)(250).

3.2.4. Immunophenotyping for growth factor and cytokine receptor expression

Based on previous results obtained from cell line supernatant collected by others in the laboratory and cytokine profiling previously performed by Commonwealth Serum Laboratory (CSL), a number of growth factors and cytokines were deemed to be preferentially expressed in the supernatant of the cells expressing the T315I mutation. They were namely, FGF-2, GM-CSF, MDC common to both the T315I-expressing cell lines, IL-6 and IL-8 specifically from the K562-T315I cells and MCP-1, MIP-1 α and MIP-1 β specifically for the HL60 *BCR-ABL1*^{T315I} cells (refer Table 3-1). The corresponding receptors for these growth factors and cytokines were examined to see if they were expressed by both the wild-type and T315I-expressing cells.

Methodology used was described in Chapter 2, Section 2.5.2 but briefly, 2×10^5 HL60 and K562 cells were stained with either an isotope control antibody or antibody to the cytokine receptor. After 30 minute incubation on ice, the cells were washed and resuspended in fluorescent assisted cell sorting fixative (FACS fix) (Chapter 2, Section 2.2.5) and analysed by flow cytometry. The experiments were performed at least twice. If the reaction was negative, then it was repeated after the cells were cultured overnight in SDM followed by stimulation with the relevant recombinant cytokine at 37°C for either 10 minutes or 30 minutes prior to staining with the antibody. Data was analysed using Analysis Software version 2.2 (Beckman Coulter).

3.2.5. Co-culturing *BCR-ABL1*^{WT} cells with *BCR-ABL1*^{T315I} cells

To ascertain if the cytokines and growth factors produced by *BCR-ABL1*^{T315I} cells were able to protect *BCR-ABL1*^{WT} cells from TKI-induced cell death through the paracrine secretion of pro-survival cytokines, K562 naïve cells and K562-T315I cells were co-cultured, KU812 (a *BCR-ABL1* positive cell line) and K562-T315I cells co-cultured and K562 naïve and HL60 *BCR-ABL1*^{T315I} cells co-cultured separated by Transwell® inserts (Sigma-Aldrich) with membranes that allow for diffusion of soluble factors.

Methodology was previously described (Chapter 2, Section 2.5.5). *BCR-ABL1*^{T315I} cells (1.5×10^5 cells) were plated at the bottom of the wells and *BCR-ABL1*^{WT} cells (5×10^4 cells) in the Transwell® inserts and cultured together with DMSO as control or TKI at 37°C/5% CO₂ and

were assessed for viability after 3 days by trypan blue exclusion dye and flow cytometric analysis of Annexin V and 7AAD cell staining.

3.2.6. Cell rescue by exogenous cytokines

To determine which cytokines were able to rescue K562 naïve cells from TKI-induced cell death, excessive recombinant cytokines were added individually into the culture media.

K562 naïve cells were plated at 1×10^5 cells in 1 ml in a 24-well plate together with 1 μ M imatinib (im), 10 nM dasatinib (das) and 150 nM nilotinib (nil). Recombinant cytokines or growth factors at a concentration of 50 ng/ml diluted in SDM were individually added to the appropriate wells. The concentration was chosen based on titrating experiments performed earlier by others in the laboratory. The cytokines and growth factors selected were the ones indicated by the cytokine profiling performed at CSL to be preferentially secreted by K562-T315I cells over the K562 naïve cells i.e. GM-CSF, FGF-2, MDC, IL-6 and IL-8 (all Peprotech, New Jersey, USA). Erythropoietin (Epo) was used as a positive control as K562 cells are of erythroblastic leukaemia lineage and therefore express Epo receptors (251). The cells were cultured for 3 days at 37°C/5% CO₂ and then analysed by flow cytometric, Annexin V and 7AAD staining. Cells were also assessed for viability using trypan blue exclusion dye analysis.

3.2.7. FGF-2 blocking experiment

To confirm which soluble factor was rescuing K562 naïve cells from TKI-induced cell death, a FGF-2 neutralising antibody, bFM-1, was purchased from Millipore (Billerica, Massachusetts, USA). In order to ascertain the optimal concentration of bFM-1 to use, dilutions of bFM-1 were made in SDM.

K562 naïve and K562-T315I cells were washed optimally and plated into 2 identical 24-well plates at a concentration of 1×10^5 cells/ml in SDM at 1 ml per condition and cultured with 1.5 μ M im, 20 ng/ml recombinant FGF-2 (rFGF-2) with varying concentrations of bFM-1. The cells were incubated at 37°C/5% CO₂ for 3 days after which the media of one of the plates was changed, new reagents added and the plate incubated for a further 2 days. Cell viability was evaluated at Days 3 and 5 by trypan blue exclusion dye and by Annexin V/7AAD cell death assay.

After the optimal concentration of bFM-1 was decided (which was 250 ng/ml), K562 naïve and K562-T315I cells were cultured together in Transwells® with or without 1.5 µM im and 250 ng/ml bFM-1 and analysed at Day 5 by the above methods after changing media and adding new reagents at Day 3.

3.2.8. Intracellular flow cytometry

K562 naïve cells were washed twice in SDM and plated into a 6-well plate with a total of 3.6×10^5 cells in 3 ml. Cells were either left untreated, incubated with 1.5 µM imatinib only, treated with imatinib and 20 ng/ml rFGF-2 or treated with imatinib, rFGF-2 and 250 ng/ml bFM1, as determined by the titration experiment.

After 3 days incubation at 37°C/5% CO₂, the cells were harvested, fixed and permeabilised (described in Chapter 2, Section 2.5.3). Cells were then stained with anti-pErk (BD Biosciences, San Jose, California, USA), anti-pSTAT5 (BD BioSciences) and anti-pAkt (Cell Signaling, Massachusetts, USA) antibodies and analysed by flow cytometry in parallel with the appropriate isotope controls.

3.2.9. Phospho-protein detection by Western blotting analyses

The methodology has been described in Chapter 2, Section 2.5.6. Briefly, following optimal wash, K562 naïve and K562-T315I cells were resuspended in SDM. Cells were then plated at 2×10^5 cells/ml in 1 ml per condition in a 12-well plate, appropriate reagents were added and cultured overnight at 37°C/5% CO₂. Following that, the cells were washed in ice-cold PBS and lysed with 1 x Laemmli's buffer (Chapter 2, Section 2.27) and stored at -20°C until required for analysis. The lysates were either resolved on sodium dodecyl sulphate/polyacrylamide gel (SDS-PAGE) (for pCrkl/Crkl) or loaded into precast Criterion TGX 4-15% gels (Bio-Rad, Hercules, California, USA) for detection of the other proteins. The gels were then transferred electrophoretically onto PVDF membranes (Quantum Scientific, Murarrie, Queensland). Following blocking with 2.5% blocking solution, the membranes were incubated with primary antibodies. Crkl (both phosphorylated and total) (Santa Cruz Biotechnology, Santa Cruz, California, USA) was incubated for 2 hours at room temperature while the rest- Erk (both phosphorylated and total), STAT5 (both phosphorylated and total), Bcr-Abl and β-tubulin (all Cell Signaling) antibodies were incubated overnight at 4°C with agitation. After incubation and washing, the blots were incubated with secondary antibody,

anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Santa Cruz Biotechnology) and immune-complexes were detected using an enhanced chemifluorescence (ECF) substrate (Amersham Pharmacia, Piscataway, New Jersey, USA) and analysed on Typhoon FluorImager (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

3.3. Results

3.3.1. Characterisation of the K562 naïve, K562-T315I, HL60 parental, HL60 *BCR-ABL1*^{p210} and HL60 *BCR-ABL1*^{T315I} cell lines

As mentioned previously in Chapter 2, Section 2.3.1, K562-T315I cells were derived from K562 cells by culturing in increasing concentrations of dasatinib. The T315I mutation was first detected at 1% by MassARRAY when the cells were cultured in 25 nM dasatinib, and the level of T315I mutation rose steadily to approximately 40-50% when the concentration of dasatinib reached 200 nM (238).

The level of T315I mutation in the K562-T315I cell line and the level of *BCR-ABL1* transcripts in all the cell lines were ascertained to ensure that they have not changed. The amount of pCrkl and total Bcr-Abl in the K562-T315I and the K562 naïve cells were also determined. This may have important implication for the results of this study as if the two cell lines behaved differently, it may not be solely due to the presence of the T315I mutation.

mRNA was isolated from both the K562 naïve and K562-T315I cell lines and was used as a template for cDNA synthesis. Long PCR and direct sequencing showed that the *ABL1* sequence of K562 naïve was the same as the reference sequence and did not contain the T315I mutation (Figure 3-1). Conversely, as shown in Figure 3-2, the *ABL1* sequence of the K562-T315I cell line demonstrated that the cytosine peak was partly replaced by the thymine peak (944C>T). It was estimated by the Mutation Surveyor software that the K562-T315I cell line contained approximately 45% of the mutation. Hence, K562-T315I contains both the *BCR-ABL1*^{WT} and the *BCR-ABL1*^{T315I} (238).

Next, the *BCR-ABL1* transcript levels of the K562 naïve, K562-T315I, HL60 parental, HL60 *BCR-ABL1*^{p210} and HL60 *BCR-ABL1*^{T315I} cell lines were determined by RQ-PCR.

K562 naïve cells have a *BCR-ABL1* mRNA expression level of approximately 350% but *BCR-ABL1* mRNA expression in the K562-T315I cells was markedly increased to 2400% in comparison to the naïve cell line (Figure 3-3). It was also confirmed that the HL60 parental cells do not express *BCR-ABL1* and that HL60 *BCR-ABL1*^{p210} and HL60 *BCR-ABL1*^{T315I} have approximately 200% *BCR-ABL1* mRNA.

Next, we looked for Bcr-Abl and Crkl (phosphorylated and non-phosphorylated) expressions in the K562 naïve and K562-T315I cell lines using western blotting analyses.

Western blotting analyses indicated that although expression levels of Crkl and pCrkl were similar for both the K562 naïve and K562-T315I cell lines, Bcr-Abl expression was increased in the K562-T315I cell line (Figure 3-4), reflective of the increased *BCR-ABL1* transcript level in this cell line (refer Figure 3-3).

Although, HL60 cells were transduced with *BCR-ABL1* retroviral constructs containing *BCR-ABL1*^{p210} or *BCR-ABL1*^{T315I}, the cells were derived from a patient with acute myeloid leukaemia (239, 240). Hence, these cells may not exclusively rely on *BCR-ABL1* for survival, but rather be driven by other oncogenes. To determine the sensitivity of the cells to Bcr-Abl kinase inhibition by TKI, the 3 day cell death assay using Annexin V and 7AAD staining and also trypan blue counts to assess viability of the cell lines following TKI treatments were performed.

Not surprisingly, the viability of HL60 parental and HL60 *BCR-ABL1*^{T315I} was not affected by TKI treatment (Figures 3-5). This is in agreement with the fact that HL60 parental cells contain no *BCR-ABL1* and HL60 *BCR-ABL1*^{T315I} cells are resistant to first and second generation TKIs. However, HL60 *BCR-ABL1*^{p210} cells would be expected to be susceptible to Bcr-Abl inhibition, but unexplainably, no effect on cell viability was evident (refer Figure 3-5). HL60 cells as mentioned are an acute myeloid leukaemia cell line and are known to contain amplified *c-MYC* proto-oncogene (240). The cells are therefore not solely dependent on *BCR-ABL1* for survival/oncogenic signalling, which may help explain why the HL60 *BCR-ABL1*^{p210} cells were able to avoid the cytotoxic effects of TKI treatment in this experimental setting.

As a result of this observation, the HL60 cell lines were not employed for further study involving both TKI treatment and cell viability.

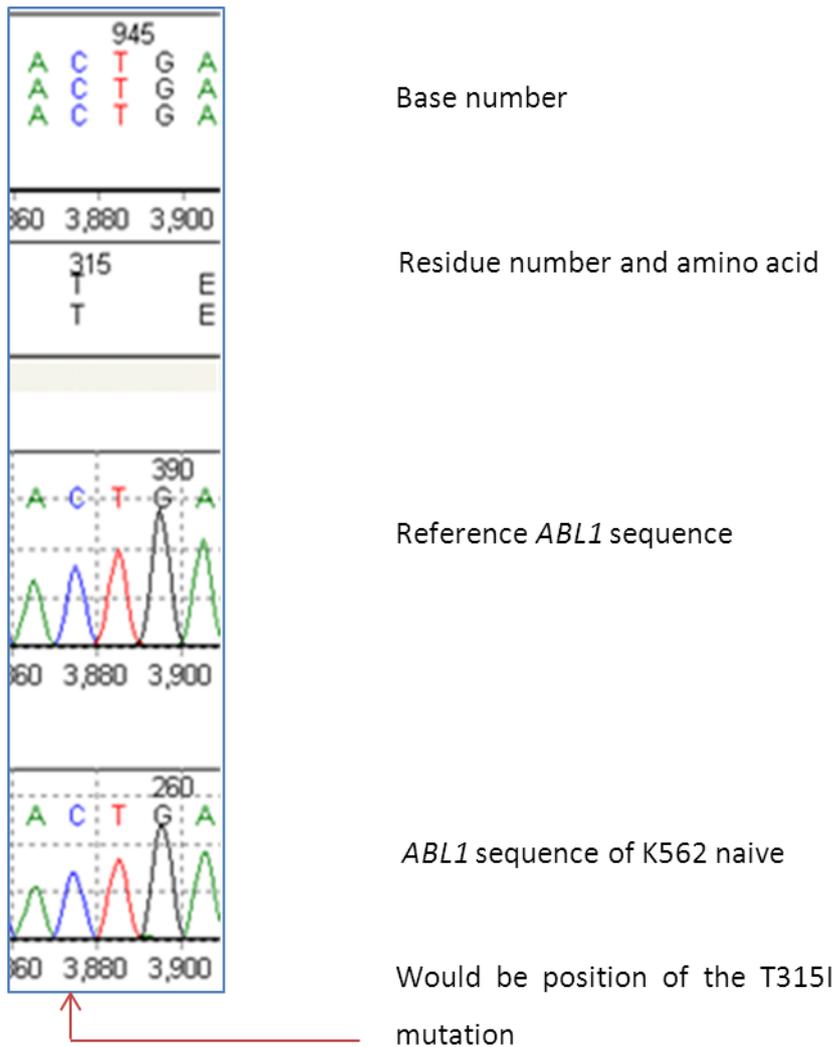


Figure 3-1 K562 naive cells do not carry the T315I kinase domain mutation

3×10^6 K562 naïve cells were harvested, pelleted and resuspended in Trizol reagent. mRNA was extracted, cDNA was prepared and a Long PCR reaction was conducted to amplify the product. After which, Sanger sequencing of the *BCR-ABL1* kinase domain was conducted. This figure shows a portion of the *ABL1* kinase domain sequence of K562 naïve at residue 315 where the T315I mutation is located. As expected, no T315I mutation is evident in the K562 naïve cells.

Each peak represents a DNA base (A = Adenine, C = Cytosine, G = Guanine, T = Thymine)

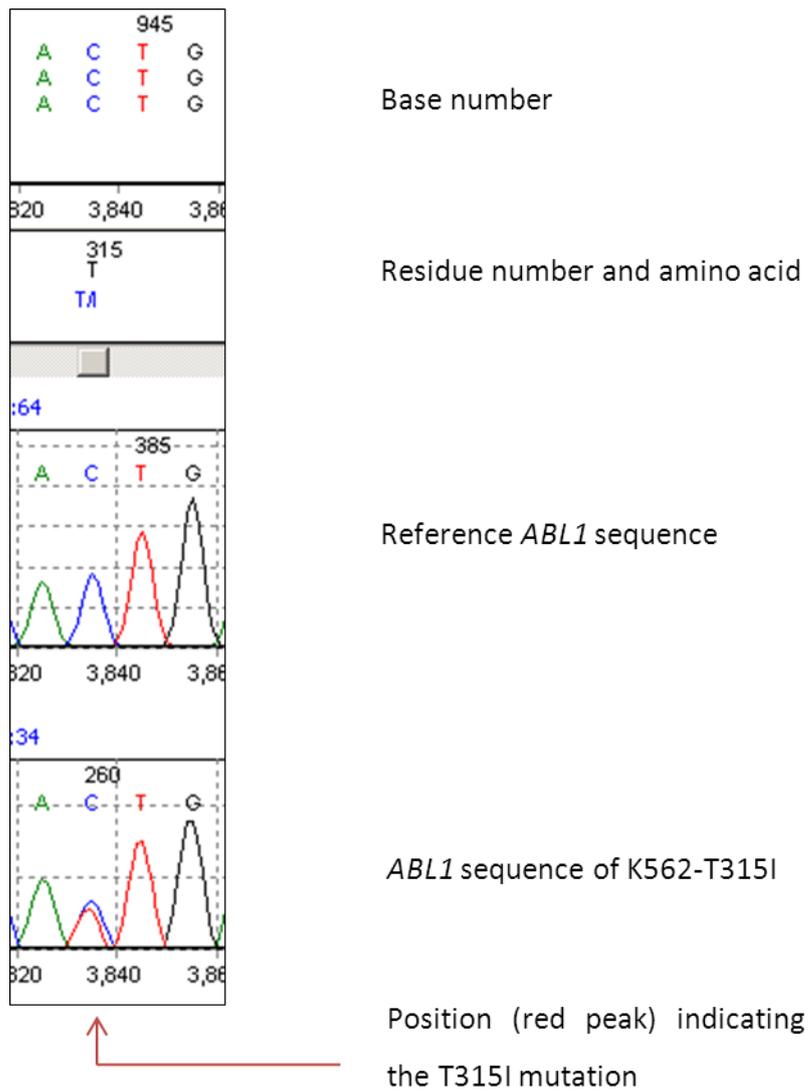


Figure 3-2 K562-T315I cells harbour approximately 45% T315I *BCR-ABL1* kinase domain mutation

3×10^6 K562-T315I cells were harvested, pelleted and resuspended in Trizol reagent. mRNA was extracted, cDNA was prepared and a Long PCR reaction was conducted to amplify the product. After which, Sanger sequencing of the *BCR-ABL1* kinase domain was conducted. This shows the *ABL1* kinase domain sequence of K562-T315I. Note that at residue 315, the cytosine peak was partly replaced by thymine (represented by a red peak) and the amino acid, threonine was partly substituted by isoleucine (T/I). The level of the T315I mutation in this cell line was estimated to be approximately 45%.

Each peak represents a DNA base (A = Adenine, C = Cytosine, G = Guanine, T = Thymine).

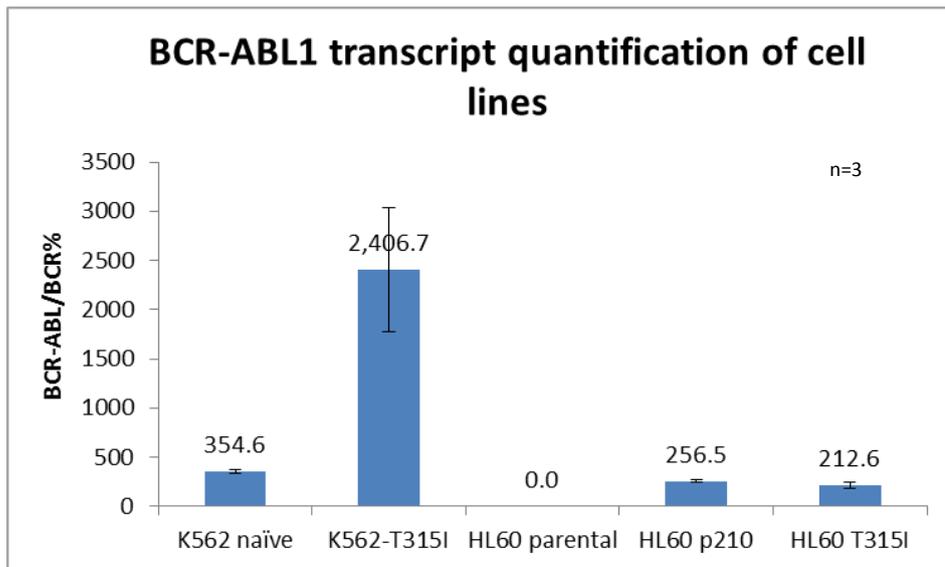


Figure 3-3 K562-T315I have markedly increased *BCR-ABL1* transcript levels

cDNA was synthesized from mRNA extracted from 3×10^6 cells. RQ-PCR was performed using cDNA as the template. *BCR-ABL1* expression was expressed as a ratio of *BCR* expression.

The results show that K562 naïve cells have a *BCR-ABL1* expression of approximately 350% but K562-T315I cells have markedly increased expression at 2400%. As expected, HL60 parental cells do not contain *BCR-ABL1* and both HL60 *BCR-ABL1*^{p210} and HL60 *BCR-ABL1*^{T315I} contained approximately 200% *BCR-ABL1* expression.

Experiments were performed 3 times. Data represent means \pm SEM.

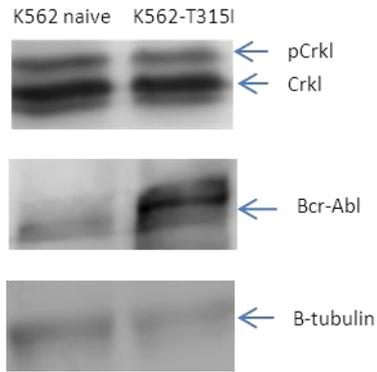


Figure 3-4 Bcr-Abl protein expression in the K562-T315I cells was increased over the K562 naïve cells line

Western blotting analyses of the K562 cell lines for phospho-Crkl (pCrkl), Crkl and Bcr-Abl. β -tubulin was used as the loading control.

Western blots demonstrate that although the p-Crkl and Crkl expressions of K562-T315I cells were similar to K562 naïve cells, Bcr-Abl expression was increased in the K562-T315I cells.

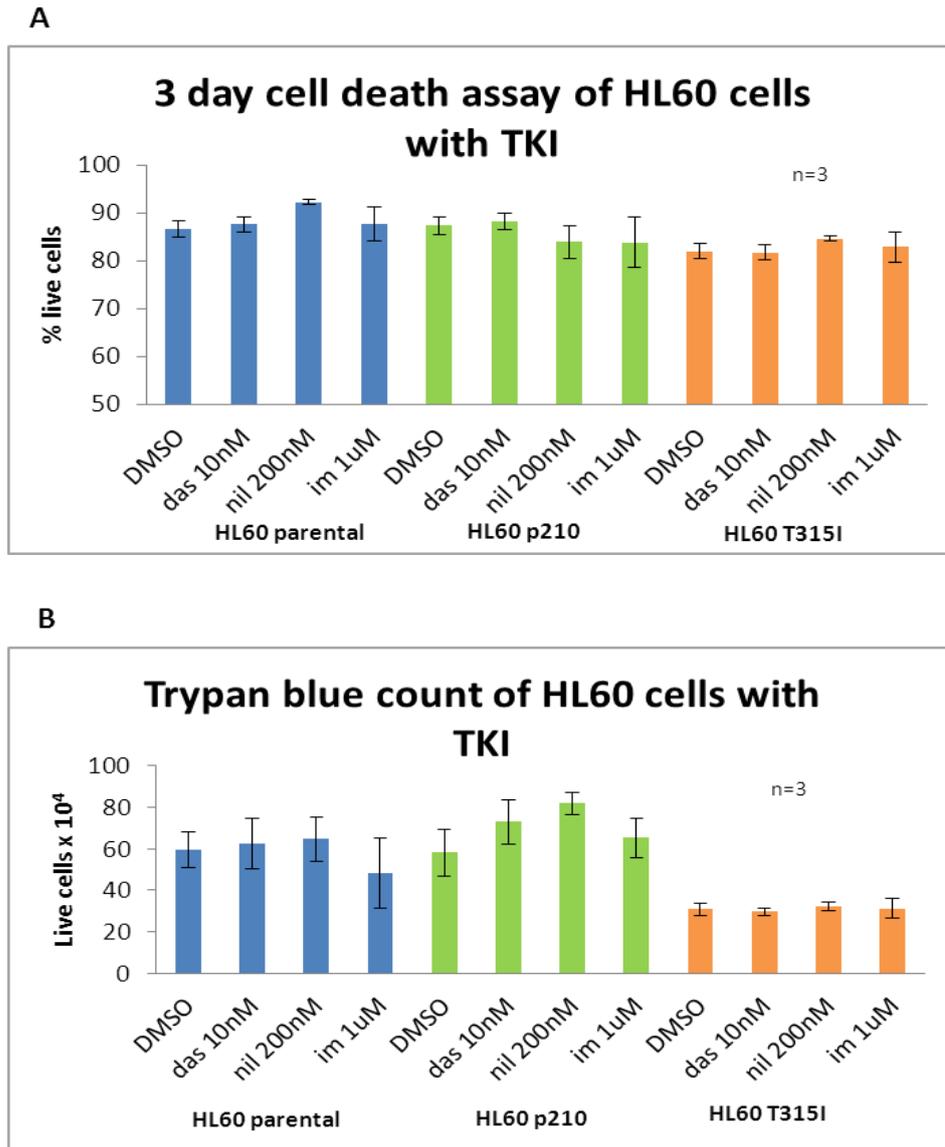


Figure 3-5 Viability of the HL60 cell lines is not affected by TKI treatment

1×10^5 cells were plated in 1 ml culture medium in duplicate wells of a 24-well plate and cultured with the relevant concentrations of TKI for 3 days at $37^\circ\text{C}/5\% \text{CO}_2$. DMSO was used as vehicle control. After 3 days, the cells were analysed by (A) staining for Annexin V and 7AAD and (B) trypan blue exclusion dye analysis.

No significant change in cell death or number of live cells was observed in any condition compared to the control. The experiments were performed 3 times.

Data are means \pm SEM. das=dasatinib, nil=nilotinib, im=imatinib.

3.3.2. Differential cytokine profiling evident in *BCR-ABL1* positive cells harbouring the T315I mutation

Prior to the commencement of these studies, the supernatant from various cell lines (detailed below), collected by others in the laboratory (Drs T. Leclercq and E. Nievergall) were sent to CSL for cytokine profiling. The results demonstrated that both the K562-T315I cells and the HL60 *BCR-ABL1*^{T315I} cells preferentially expressed FGF-2, G-CSF, GM-CSF, IL-6, IL-8, MDC, MCP-1, MIP-1 α and MIP-1 β compared to the K562 naïve cells and the HL60 parental and HL60 *BCR-ABL1*^{p210} cells respectively (refer Table 3-1). Based on these results, 14 cytokines and growth factors were selected for testing in the cell culture supernatants to confirm the earlier results. The cytokines and growth factors chosen were FGF-2, G-CSF, GM-CSF, MDC, IL-1 α , IL-1 β , IL-1RA, IL-3, IL-6, IL-8, MCP-1, MIP-1 α , MIP-1 β and TNF- α . Only the results of the cytokines and growth factors that showed a higher level in the *BCR-ABL1*^{T315I} cells are displayed in Table 3-2.

Cytokine profiling results indicated that both the supernatants of K562-T315I and the HL60 *BCR-ABL1*^{T315I} cells expressed higher concentrations of FGF-2, IL-8, MCP-1 and G-CSF (refer Table 3-2) compared to K562 naïve and HL60 parental and HL60 *BCR-ABL1*^{p210} respectively. The culture supernatants of K562-T315I also had greater concentrations of GM-CSF and IL-6 when compared to K562 naïve cells, while culture supernatants of HL60 *BCR-ABL1*^{T315I} had higher concentrations of MDC, MIP-1 α , MIP-1 β and TNF- α when compared to HL60 *BCR-ABL1*^{p210} and HL60 parental cells (refer Table 3-2). Since these cells have been cultured in SDM (no added cytokines or growth factors), this may mean that the cells preferentially secrete these cytokines and growth factors over the cells without the T315I mutation. The presence of IL-3 was also investigated, but the background fluorescence even when analysed in media only, was always too high to allow interpretation of the experimental results. This was despite repeating with a new kit and following the manufacturers' advice. Results for IL-1 α , IL-1 β and IL-1RA are not shown as they were not found in higher concentration in the cell lines harbouring the T315I mutation.

	FGF-2	GM-CSF	MDC	IL-6	IL-8	MCP-1	MIP-1 α	MIP-1 β	G-CSF	TNF- α
K562 naive	7.70	0.44	0.00	27.58	26.98	28.57	0.00	0.00	0.00	0.00
K562-T315I	23.46	1.00	3.43	337.16	185.07	5.27	0.00	0.00	0.00	0.00
HL60 parental	0.00	11.07	0.00	0.00	614.52	21.11	19.11	0.00	5.48	5.96
HL60 p210	0.00	50.16	0.00	0.00	2908.64	476.01	67.09	28.46	14.28	42.20
HL60 T315I	27.47	67.88	60.50	0.00	517.76	>10000	96.84	117.17	8.82	15.88

Table 3-1 Cytokine profiling results performed by CSL

Cell culture supernatants were prepared by Drs T. Leclercq and E. Nievergall and sent to CSL (Melbourne, Victoria) for analysis. Samples were analysed using the Luminex xMAP® technology. Results highlighted in yellow indicate higher concentrations measured in the cell culture supernatants from the T315I-expressing cells.

The table shows that the supernatant of cell lines harbouring the T315I mutation expressed higher concentrations of FGF-2, GM-CSF and MDC. Compared to K562 naïve cells, K562-T315I cells also secreted more IL-6 and IL-8 whilst *HL60 BCR-ABL1^{T315I}* cells in comparison to HL60 parental and HL60 *BCR-ABL1^{p210}* secreted more MCP-1, MIP-1 α and MIP-1 β . Results are expressed in pg/ml.

	FGF-2	GM-CSF	MDC	IL-6	IL-8	MCP-1	MIP-1 α	MIP-1 β	G-CSF	TNF- α
K562 naive	8.95	4.38	4.09	9.92	76.03	16.1	0.00	0.00	26.71	0.00
K562-T315I	12.33	10.13	3.8	1045.48	1210.47	52.29	0.00	0.00	43.59	0.00
HL60 parental	9.63	0.45	5.16	0.94	1361.52	35.94	15.73	16.88	4.77	9.95
HL60 p210	10.25	0.00	4.64	0.94	1130.81	94.87	17.70	18.43	6.92	9.17
HL60 T315I	14.41	0.90	29.98	1.80	5370.53	218.54	55.93	86.14	119.89	155.23

Table 3-2 'In-house' Cytokine profiling results show that some cytokines and growth factors were over-expressed by the *BCR-ABL1*^{T315I} cells

Cells were resuspended in SDM at a concentration of 5×10^5 cells/ml and cultured overnight in 25 cm² flasks at 37°C/5% CO₂. Cells were centrifuged twice and the resulting clarified supernatant was analysed using the Human Cytokine/Chemokine Magnetic Bead Panel (Millipore) which utilises Luminex xMAP® technology. Samples were assayed on the Bioplex200™ analyser (Bio-Rad) and results analysed using xPONENT software (Life Technologies).

Results highlighted in yellow indicate higher concentrations measured in the cell culture supernatants from the cells harbouring the T315I mutation. Both cell lines harbouring the T315I mutation secreted more FGF-2, IL-8, MCP-1 and G-CSF compared to the WT. K562-T315I cells also overproduced GM-CSF and IL-6 compared to the K562 naïve cells whilst HL60 *BCR-ABL1*^{T315I} in comparison to HL60 parental and HL60 *BCR-ABL1*^{p210} also overproduced MDC, MIP-1 α , MIP-1 β and TNF- α .

The experiment was performed in duplicate. Results are expressed as an average of the internal duplicates in pg/ml.

3.3.3 Immunophenotyping of the cytokine and growth factor receptors revealed no significant difference between the cell lines

Results so far confirmed that cells harbouring the T315I mutation preferentially expressed certain cytokines. The cognate receptors were also determined to ascertain if they are also preferentially expressed on *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells which may give an indication that the cytokines have either an autocrine or paracrine pro-survival effect. The receptors chosen for study were based on the cytokine profiling results obtained by CSL as this experiment was performed prior to repeating the cytokine profiling 'in-house'. Hence, receptors for G-CSF and TNF- α were not studied.

Results for surface expression of receptors for FGF-2 (CD332), GM-CSF (CD116), MDC (CD194), IL-6 (CD126), IL-8 (CD181) and MIP-1 α (CD191) are shown as the means of at least 2 experiments for HL60 parental, HL60 *BCR-ABL1*^{p210}, HL60 *BCR-ABL1*^{T315I}, K562 naïve and K562-T315I cells in Figures 3-6 to 3-11. Histograms of the results together with the positive controls for the receptor expressions are shown in the Appendix.

Results for MCP-1 (CD192) and MIP-1 β (CD195) are not shown as the antibodies did not give positive expression with positive control cells (monocytes for CD192 and T-lymphocytes for CD195) despite communication with the manufacturer and following their instructions.

CD181 (receptor for IL-8) was not expressed on the HL60 *BCR-ABL1*^{p210} and HL60 *BCR-ABL1*^{T315I} cells despite stimulation with recombinant IL-8 (rIL-8). This is surprising given the high concentration of IL-8 found in the supernatant of these cells (refer Table 3-2). CD191 (receptor for MIP-1 α) was not expressed on the K562 naïve cells even after pre-incubation with rMIP-1 α and this cytokine was not found to be expressed in the supernatant of either the K562 naïve or K562-T315I cells (refer Table 3-2). Although stimulation with recombinant cytokines were performed for 10 or 30 minutes, receptor expressions may be transient and analysis at those time points may not have "captured" the receptor expressions. Hence, non-detection of surface receptors may not mean that the receptors are not expressed by that cell line. Overall, results of this study indicate that surface cognate receptors were not significantly over-expressed on one cell line compared to the other; however, autocrine or paracrine pro-survival effect of a cytokine on a cell line may not be reflected by detection of surface receptor over-expression.

Receptor for FGF-2 (CD332)

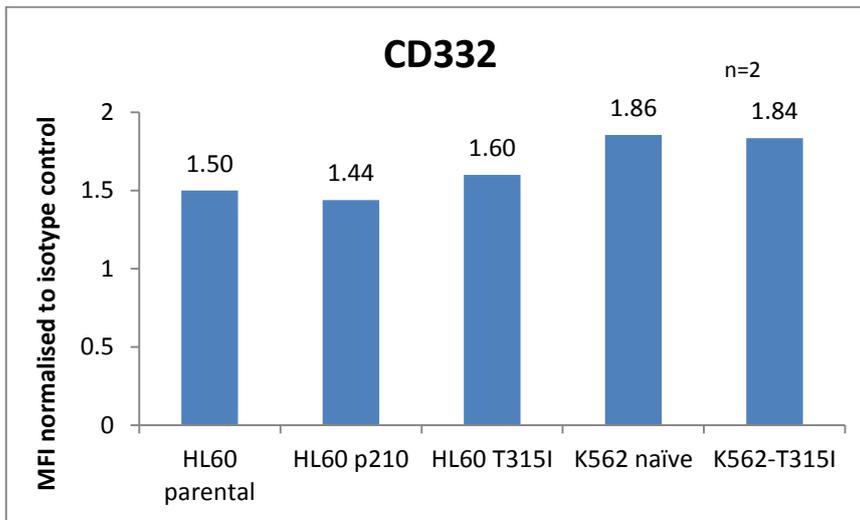


Figure 3-6 HL60 cells and K562 cell lines expressed the FGF-2 receptor

2×10^5 cells were incubated with APC-conjugated CD332 antibody (R & D Systems, Minneapolis, Minnesota, USA) or the appropriate isotype control for 30 minutes on ice. The assay was analysed by flow cytometry. The results are expressed as mean fluorescent intensity (MFI) in comparison to the isotype control.

The figure shows that all the cell lines tested expressed the FGF-2 receptor.

The experiment was performed twice and the data are means \pm SEM.

Figure 3-7 HL60 and K562 cells expression of the GM-CSF receptor A) without pre-incubation and B) K562 cells with pre-incubation with rGM-CSF

A) 2×10^5 cells were incubated with PE-conjugated CD116 antibody (BD Pharmingen, San Diego, Ca, USA) or the appropriate isotype control and the samples analysed by flow cytometry. B) As the K562 cell lines did not show much receptor expression, cells were cultured overnight in SDM. The next day, cells were pre-incubated with 50 ng/ml rGM-CSF (PeproTech) for 10 minutes or 30 minutes at 37°C/5% CO₂. At the end of the incubation period, the cells were washed with cold media and the experiment continued as above. The results are expressed as MFI in comparison to the isotype control.

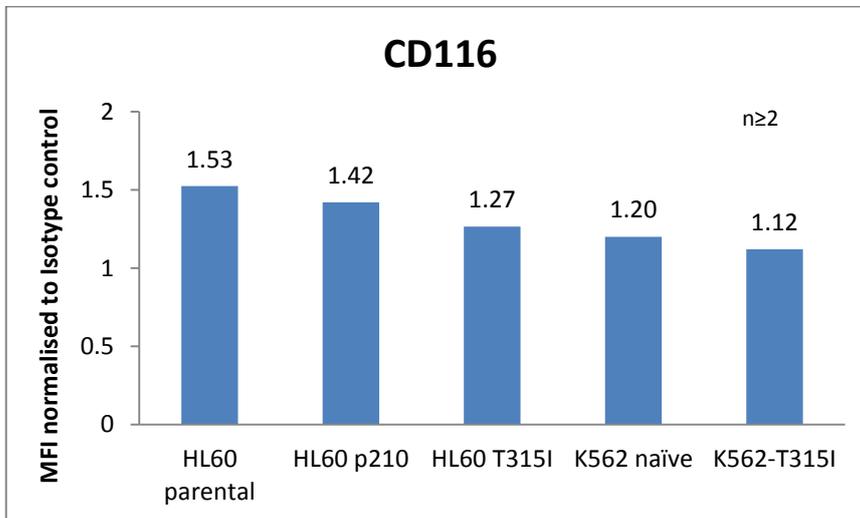
The HL60 cells lines expressed the GM-CSF receptor but both K562 naïve and K562-T315I needed to be stimulated with rGM-CSF. Expression on K562 naïve cells was weak even after stimulation.

The experiments were performed 2 times each and the data are means \pm SEM.

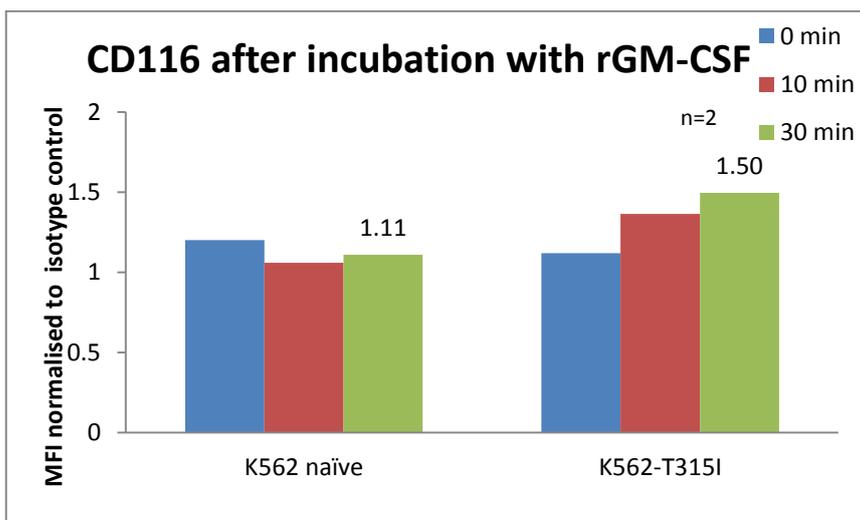
Figure 3-7

Receptor for GM-CSF (CD116)

A



B



Receptor for MDC (CD194)

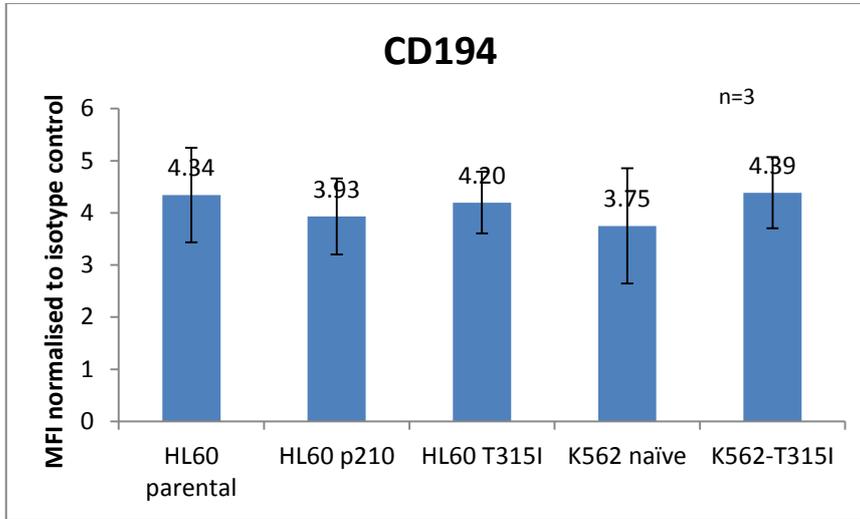


Figure 3-8 HL60 and K562 cells expressed the receptor for MDC

2×10^5 cells were incubated with PEcy7-conjugated CD194 antibody (BD Pharmingen) or the appropriate isotype control for 30 minutes on ice and analysed by flow cytometry. The results are expressed as MFI in comparison to the isotype control.

This indicates that all cell lines strongly expressed the receptor for MDC and there is no significant difference in receptor expression of 1 cell line over the other.

The experiment was performed 3 times and the data are means \pm SEM. p was not significant.

Figure 3-9 HL60 and K562 cells expression of the IL-6 receptor A) without pre-incubation B) with pre-incubation with rIL-6

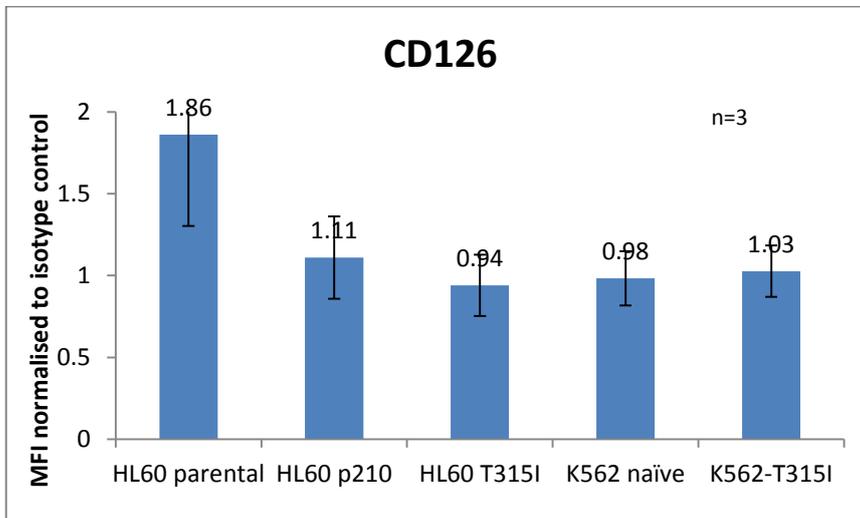
A) 2×10^5 cells were incubated with PE-conjugated CD126 antibody (BD Pharmingen) or the appropriate isotype control and analysed on flow cytometry. B) After overnight culture in SDM the cells were pre-incubated with 50 ng/ml rIL-6 (PeproTech) for 10 minutes or 30 minutes at 37°C/5% CO₂. At the end of the incubation period, the cells were washed with cold media and the experiment continued as above. The results are expressed as MFI in comparison to the isotype control.

The receptor for IL-6, CD126 was expressed strongly by HL60 parental cells but the other cell lines only showed expression after pre-incubation with rIL-6.

Figure 3-9

Receptor for IL-6 (CD126)

A



B

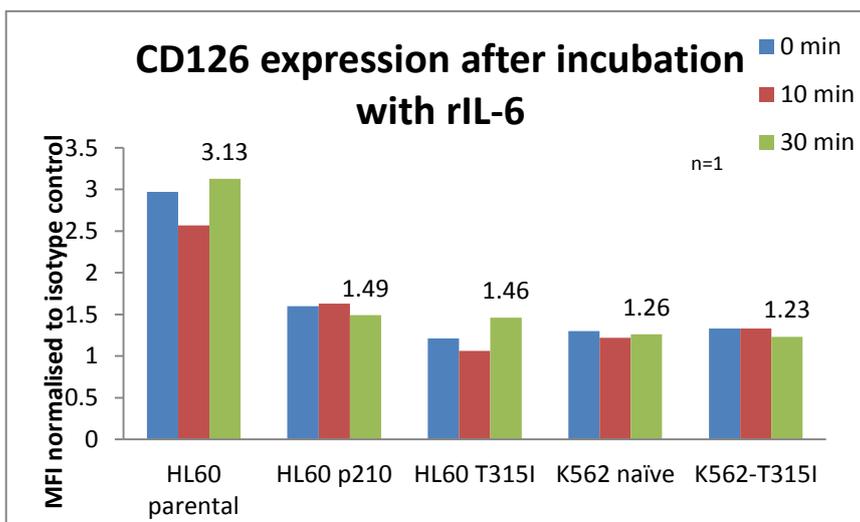


Figure 3-10 HL60 and K562 cells expression of the IL-8 receptor A) without pre-incubation and B) with pre-incubation with rIL-8

A) 2×10^5 cells were incubated with APC-conjugated CD181 antibody (BD Pharmingen) or the appropriate isotype control and analysed on flow cytometry. B) After overnight culture in SDM, cells were pre-incubated with 50 ng/ml rIL-8 (PeproTech) for 10 minutes or 30 minutes respectively at 37°C/5%CO₂. After incubation, the cells were washed with cold media and the experiment was continued as above. The results are expressed as MFI in comparison to the isotype control.

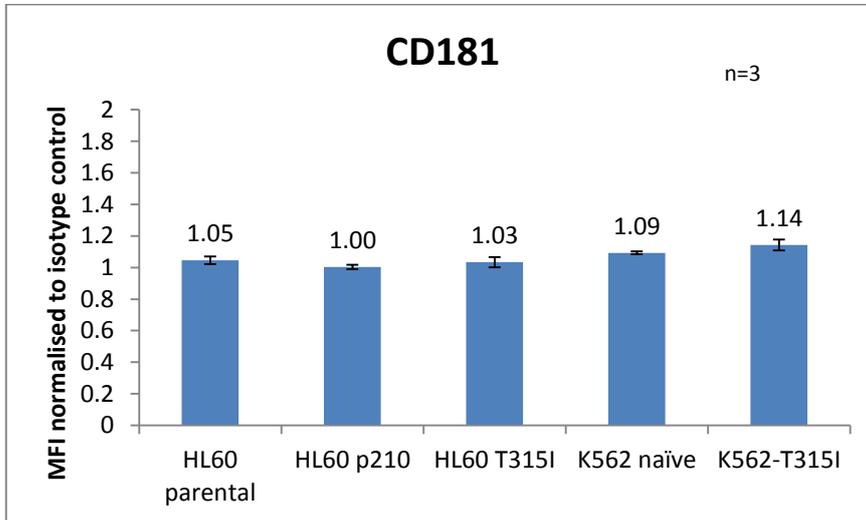
The receptor for IL-8 was only weakly expressed by HL60 parental, K562 naïve and K562-T315I and only after 30 minutes stimulation with rIL-8. Both HL60 *BCR-ABL1*^{p210} and HL60 *BCR-ABL1*^{T315I} did not appear to express this receptor.

The experiments were performed 3 times without pre-incubation with rIL-8 and twice with pre-incubation. The data are means \pm SEM. p was not significant.

Figure 3-10

Receptor for IL-8 (CD181)

A



B

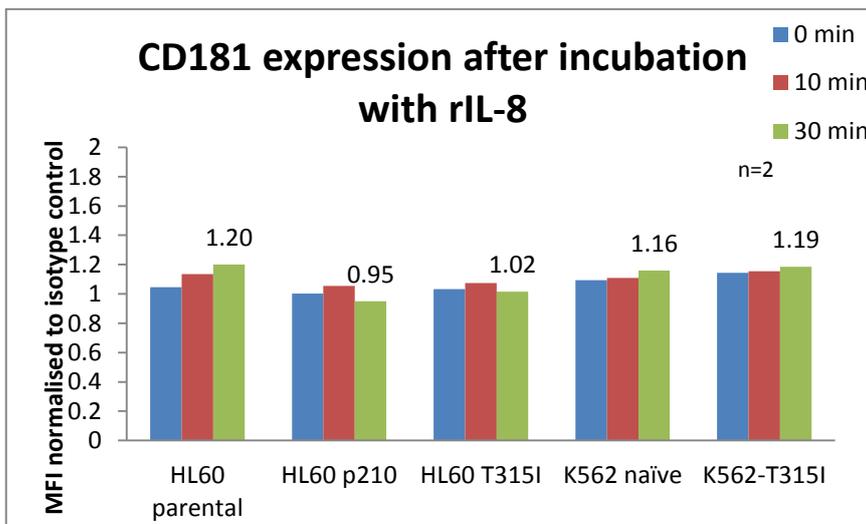


Figure 3-11 HL60 and K562 cells expression of the receptor for MIP-1 α A) without pre-incubation and B) with pre-incubation with rMIP-1 α

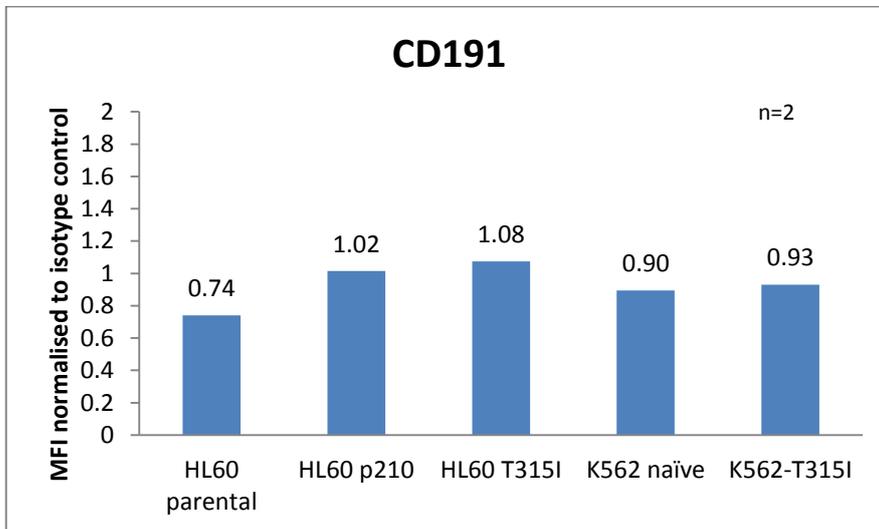
A) 2×10^5 cells were incubated with Alexa F647-conjugated CD191 antibody (BD Pharmingen) or the appropriate isotype control and analysed by flow cytometry. B) After overnight culture in SDM the cells were pre-incubated with 50 ng/ml MIP-1 α (PeproTech) for 10 minutes or 30 minutes respectively at 37°C/5% CO₂. After incubation, the cells were washed with cold media and the experiment continued as above. The results are expressed as MFI in comparison to the isotype control.

The results indicate that the receptor for MIP-1 α was not expressed by the cell lines tested without pre-incubation. After 10 or 30 minute pre-incubation with rMIP-1 α , it was only expressed weakly but not by the K562 naïve cells.

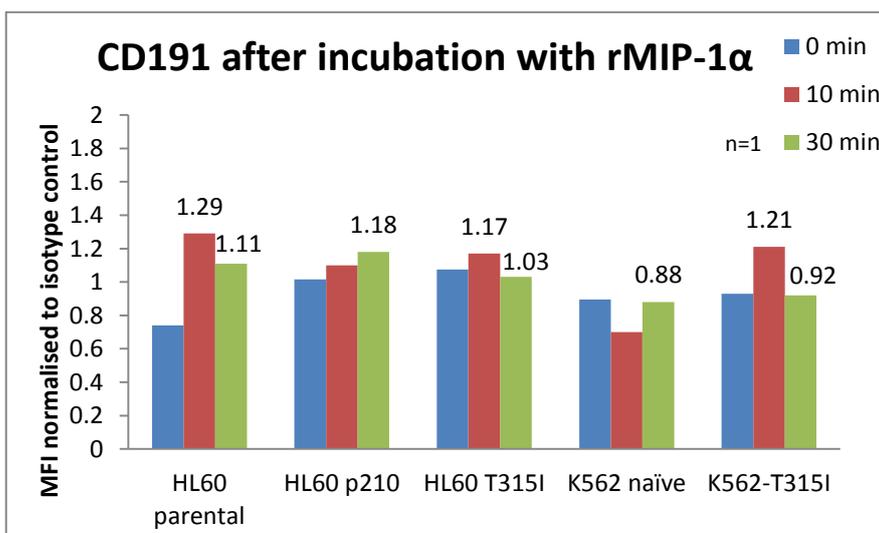
Figure 3-11

Receptor for MIP-1 α (CD191)

A



B



3.3.4. Cells harbouring the T315I mutation do not have a proliferative or survival advantage in SDM

Since the cells harbouring the T315I mutation expressed higher concentration of some cytokines and growth factors, to ascertain if these cytokines and growth factors conferred an autocrine survival effect on these cells all 5 cell lines were cultured in SDM to compare their growth rates and also their viability. The MTS assay, trypan blue exclusion dye and also CFSE labelling to track the number of generations arising from a pioneering generation were performed. CFSE labelling was not performed on the HL60 cell lines as HL60 *BCR-ABL1*^{p210} and HL60 *BCR-ABL1*^{T315I} were labelled with eGFP which like CFSE is detected within the same fluorescence spectrum on the flow cytometer.

The MTS assay results (Figure 3-12) suggest that K562-T315I cells have a higher rate of proliferation compared to the K562 naïve cells. Normally, a higher absorbance reading in an MTS assay would indicate a higher number of cells. However, direct cells counts with trypan blue dye did not support this (Figure 3-13).

Proliferation using CFSE was not significantly different between K562 naïve and K562-T315I cells. Both cell lines divided into 4 generations over 3 days although there were less undivided K562-T315I cells (shown as green peaks in proliferation histograms in Figures 3-14) and more K562-T315I cells divided into Generation 4 (pink peaks in Figures 3-14). Proliferation index (PI) which is a measure of the number of proliferating cells is useful for comparison between two or more population of cells under the same conditions. It is calculated by the sum of all the cells in all generations divided by the calculated number of original parent cells and is generated by the De Novo software (Chapter 2, Section 2.5.4). As indicated in Figure 3-15, PI was not significantly different between K562 naïve and K562-T315I cells. This is in support of the data obtained by trypan blue counts.

Similarly, HL60 *BCR-ABL1*^{T315I} cells did not show increase proliferation using the MTS assay compared to HL60 parental or HL60 *BCR-ABL1*^{p210} cells (Figure 3-16) and direct counts using trypan blue exclusion dye actually demonstrated that HL60 *BCR-ABL1*^{T315I} cells were less viable in SDM compared to HL60 parental and HL60 *BCR-ABL1*^{p210} (Figure 3-17).

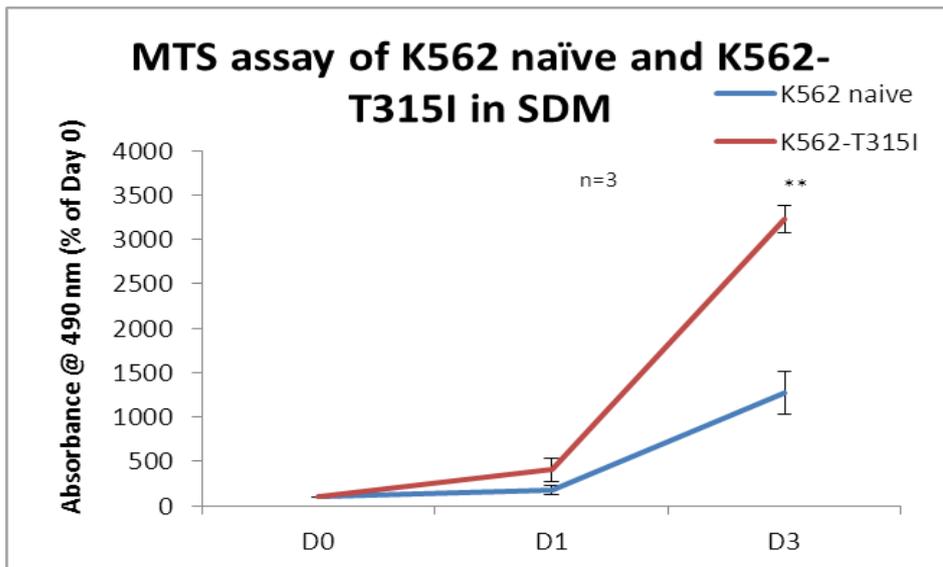


Figure 3-12 K562-T315I cells have increased metabolic activity in serum deprived media

2000 cells in 100 μ l of SDM were plated in a 96-well plate in triplicates. Media only was used as the control. 3 identical plates were set up for analysis at Days 0, 1 and 3. After an appropriate period of incubation at 37°C/5% CO₂, MTS reagent was added and after further incubation of 1-2 hours, the plates were read with an ELISA plate reader. Results of each experiment are the average of the triplicates, normalised to the controls and expressed as a percentage of Day 0 (where Day 0 is 100%).

The results suggest that the K562-T315I cells have an increased proliferation over the K562 naïve cells at Day 3 in the absence of exogenous cytokines because K562-T315I cells showed increased metabolic activity.

The experiment was performed 3 times and significant value ** indicates $p < 0.01$ compared to K562 naïve cells at Day 3. Data are the means \pm SEM.

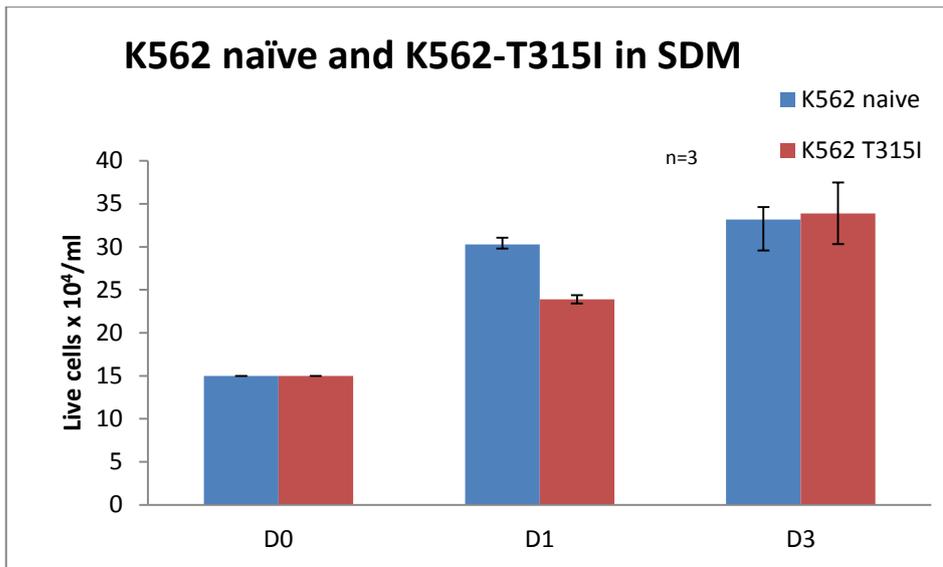


Figure 3-13 K562-T315I cells do not have increase viability in serum deprived media

1.5 x 10⁵ cells/ml were cultured in 5 ml SDM in T25 flasks at 37°C/5% CO₂. At Days 1 and 3, trypan blue counts were performed.

This assay shows that both K562 naïve and K562-T315I were of similar viability when cultured in the absence of exogenous cytokines at both Days 1 and 3.

The assay was performed 3 times and data are means ± SEM. p was not significant.

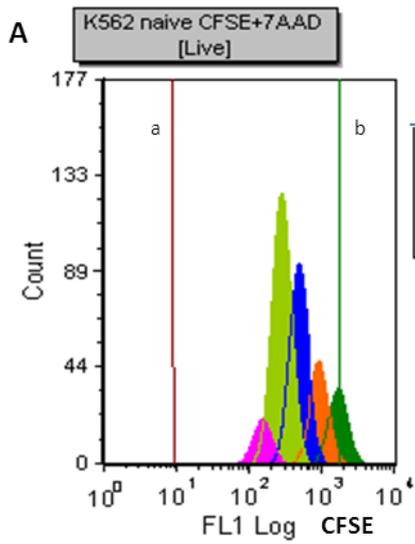
Figure 3-14 K562-T315I cells do not demonstrate increased proliferation over K562 naïve cells in serum deprived media

K562-T315I cells were stained with CFSE, sorted over a narrow range of CFSE fluorescence and cultured for 72 hours at 37°C/5% CO₂. The cells were analysed by flow cytometry. The red line (a) represents background fluorescence of the unstained cells (the unstained control population) and green line (b) indicates the CFSE fluorescence of the cells treated with colcemid and therefore represents the CFSE fluorescence of undivided cells.

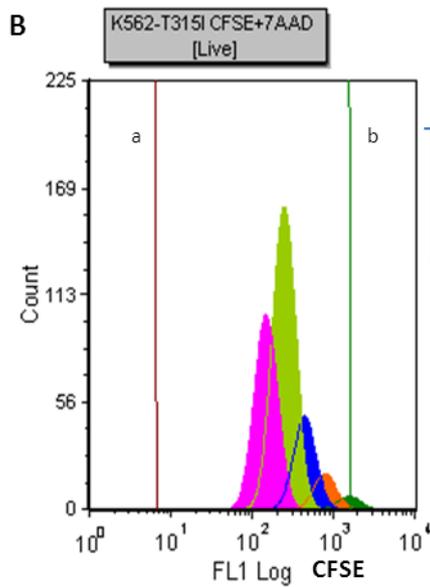
(A) K562 naïve cells have divided into 4 generations in SDM over a 3 day period. The percentage of undivided cells was 10.72% and the percentage of cells that have divided into 4 generations was 6.02%. (B) K562-T315I cells also divided into 4 generations over 3 days in SDM. However, compared to K562 naïve cells, there were less undivided cells (1.93%) and more cells that have divided into 4 generations (30.21%).

The proliferation histograms shown are representative of three similar experiments.

Figure 3-14



Generation	# of Cells	% of Orig Cells
Undivided	2876.00	10.72
Generation 1	3879.00	14.46
Generation 2	7725.00	28.80
Generation 3	10492.00	39.12
Generation 4	1615.00	6.02



Generation	# of Cells	% of Orig Cells
Undivided	588.00	1.93
Generation 1	1607.00	5.27
Generation 2	4385.00	14.39
Generation 3	14421.00	47.33
Generation 4	9204.00	30.21

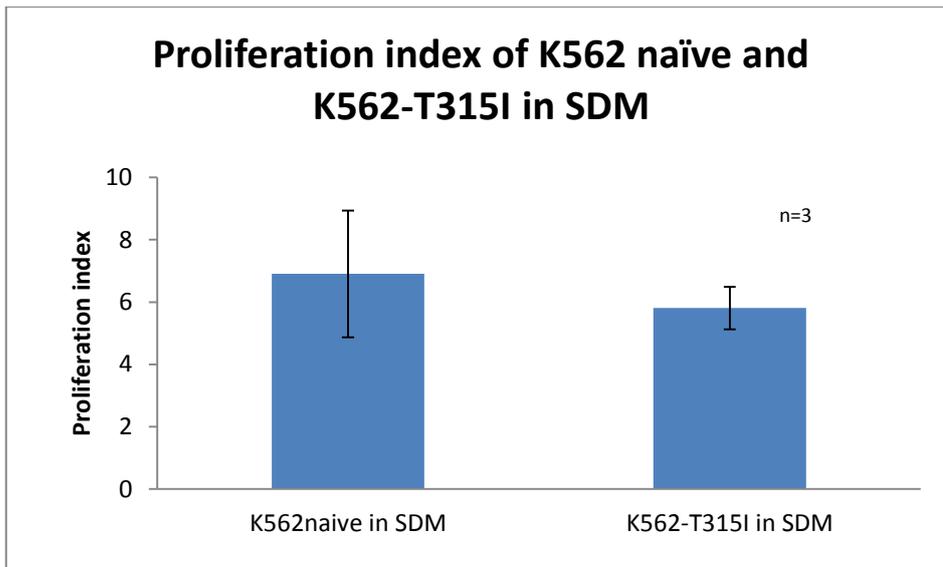


Figure 3-15 K562-315I cells do not have an increased proliferation index (PI) when cultured in serum deprived media

PI is a measure of the average number of cells that an initial parent cell became. It is calculated by the sum of all the cells in all generations divided by the calculated number of original parent cells. The results are generated based on the proliferation histograms, one of which of each cell line is represented in Figure 3-14 and calculated by the DeNovo software (Chapter 2, Section 2.5.4).

PI of the K562-T315I cells was not significantly increased over the K562 naïve cells when cultured over 3 days in serum deprived conditions. The experiments were performed 3 times and the data are the means \pm SEM. p was not significant.

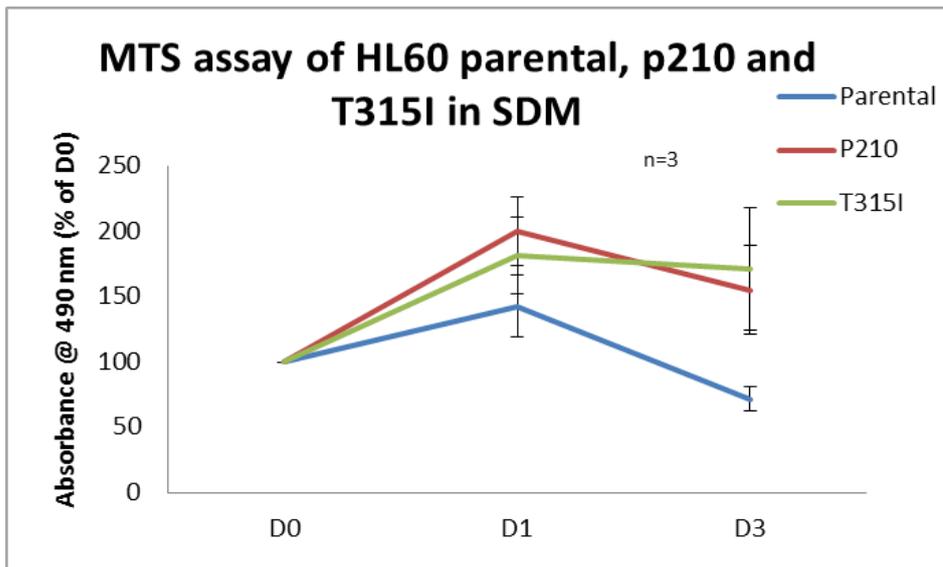


Figure 3-16 HL60 *BCR-ABL1*^{T315I} do not demonstrate increased proliferation in serum deprived media compared to HL60 parental and HL60 *BCR-ABL1*^{p210} cells

2000 cells in 100 µl of SDM were plated in a 96-well plate in triplicate. 3 identical plates were set up for analysis at Days 0, 1 and 3. After an appropriate period of incubation at 37°C/5% CO₂, MTS reagent was added and after further incubation, the plates were read with an ELISA plate reader. Media only was used as the baseline control. Results of each experiment are an average of the triplicates (internal replicates), normalised to the controls and expressed as a percentage of Day 0 (where Day 0 is a 100%).

HL60 *BCR-ABL1*^{T315I} cells in comparison with the HL60 parental and HL60 *BCR-ABL1*^{p210} cells did not exhibit increase proliferation without exogenous cytokines.

The experiment was performed 3 times. Data are the means ± SEM. p was not significant.

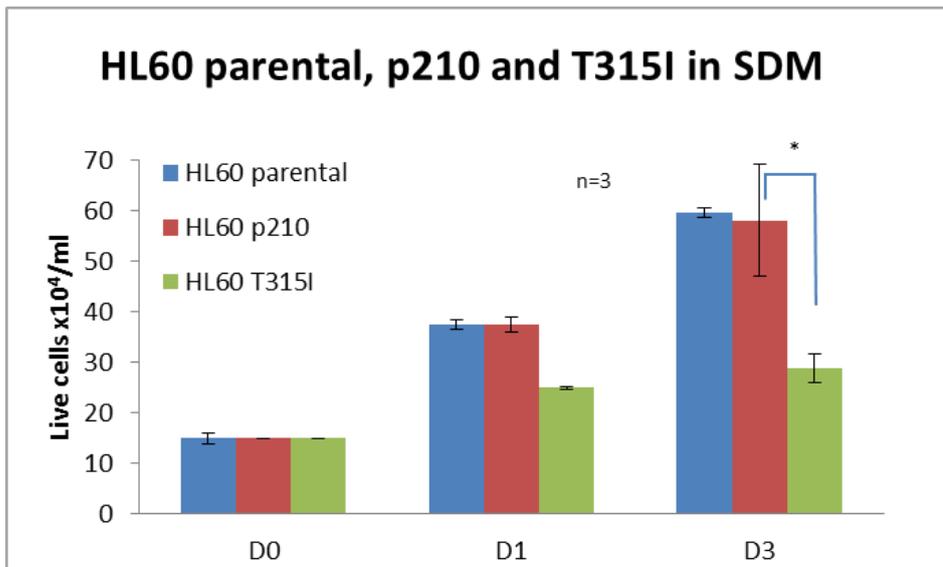


Figure 3-17 HL60 *BCR-ABL1*^{T315I} cells have decreased viability in serum deprived media

1.5 x 10⁵ cells/ml were cultured in 5 ml SDM in T25 flasks and cultured at 37°C/5% CO₂. At Days 1 and 3, trypan blue counts were performed.

The results demonstrate that HL60 *BCR-ABL1*^{T315I} do not have increase viability and in fact, showed decreased survival over 3 days in comparison with HL60 parental and HL60 *BCR-ABL1*^{p210} cells when cultured in the absence of exogenous cytokines.

The assay was performed 3 times and data represent means ± SEM. * p<0.05 was calculated using the Student's t-test.

Taken together, this data suggests that cells harbouring the T315I mutation did not have a survival or proliferative advantage over *BCR-ABL1*^{WT} cells when cultured without the addition of exogenous cytokines or growth factors. This suggests that despite a different cytokine profile, the measured cytokines and growth factors did not seem to provide an autocrine survival effect for the cells harbouring the T315I mutation.

Next, the cytokines and growth factors preferentially expressed by the *BCR-ABL1*^{T315I} cells were investigated to ascertain if they could be having a protective paracrine effect on the *BCR-ABL1*^{WT} cells.

3.3.5. Culturing *BCR-ABL1*^{WT} cells together with *BCR-ABL1*^{T315I} cells reduced TKI-induced cell death in *BCR-ABL1*^{WT} cells

BCR-ABL1^{WT} cells were co-cultured with *BCR-ABL1*^{T315I} cells in the presence of TKI in Transwell® plates (Sigma-Aldrich) separated by inserts containing membranes with a pore size of 0.4 µm that allowed for diffusion of soluble factors. The experiment was performed using K562 naïve cells co-cultured with K562-T315I cells (K562 naïve/K562-T315I). It was repeated using KU812, another *BCR-ABL1* positive cell line containing *BCR-ABL1*^{WT} together with K562-T315I cells (KU812/K562-T315I) and K562 naïve together with HL60 *BCR-ABL1*^{T315I} cells (K562 naïve/ HL60 *BCR-ABL1*^{T315I}). KU812 was used to ascertain whether another *BCR-ABL1*^{WT} cell line would also give similar results as the K562 naïve cells and that it is not a cell line effect alone. The concentrations of TKI chosen for K562 and KU812 cell lines were determined by earlier titration experiments. For K562 naïve cells, 5 nM dasatinib, 150 nM nilotinib and 1-1.5 µM imatinib were used and for KU812 cells, dasatinib 0.5 nM and imatinib 500 nM were used. As mentioned previously, HL60 *BCR-ABL1*^{p210} cell lines are not susceptible to the effects of Bcr-Abl inhibition by TKI and used in this experiment to determine only if the cells over-produced certain cytokines or growth factors in the presence of a TKI. Results for the co-culture experiments (Figures 3-18 to 3-20) are shown as the means of at least 3 experiments. Representative flow cytometry plots from which the data were derived from are shown in the Appendix.

BCR-ABL1^{WT} cells, when co-cultured with *BCR-ABL1*^{T315I} were demonstrated to have increased survival compared to *BCR-ABL1*^{WT} cells cultured alone, in the presence of a TKI (Figures 3-18 to 3-20). As the Transwell® insert membranes separated the two cell lines

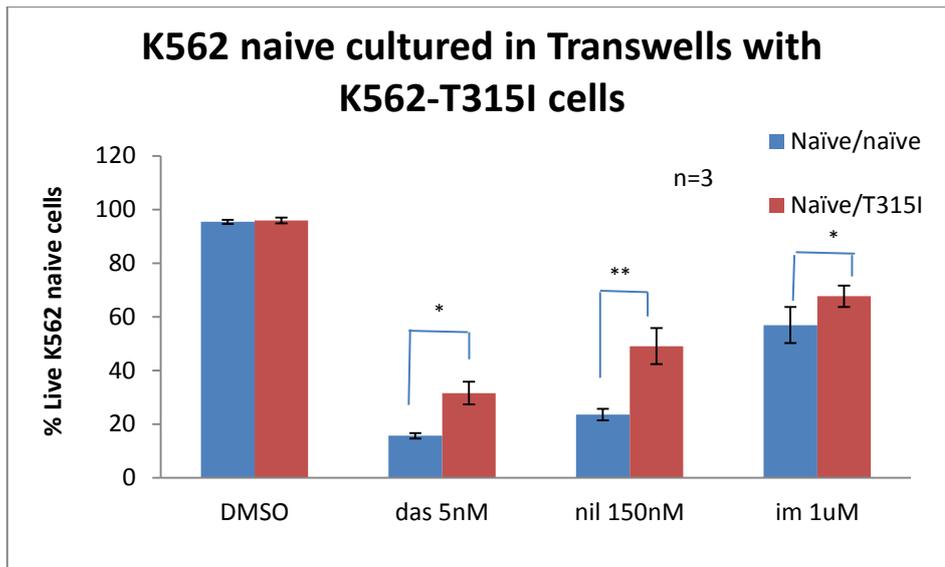


Figure 3-18 K562 naïve cells co-cultured with K562-T315I cells in the presence of TKI, demonstrate improved survival

The cells were washed optimally and resuspended in SDM at a concentration of 1×10^5 cells/ml. K562 naïve or K562-T315I cells were plated at the bottoms of the Transwell® plates at 1.5 ml and 0.5 ml of K562 naïve cells in the Transwell® inserts. Appropriate concentrations of TKI were added with DMSO used as the vehicle control. After incubation at 37°C/5% CO₂ for 3 days, K562 naïve cells from the inserts were assessed for cell viability using Annexin V and 7AAD staining.

K562 naïve co-cultured with K562-T315I cells for 3 days with TKI showed improved viability compared to K562 naïve cells cultured alone (dasatinib- approximately 30% viable vs 15%, nilotinib- approximately 50% vs 25% and imatinib- approximately 65% vs 55%).

The experiments were performed 3 times. Data are means \pm SEM. * $p < 0.05$ and ** $p < 0.01$ compared to K562 naïve cells cultured alone was calculated using the Student's t-test.

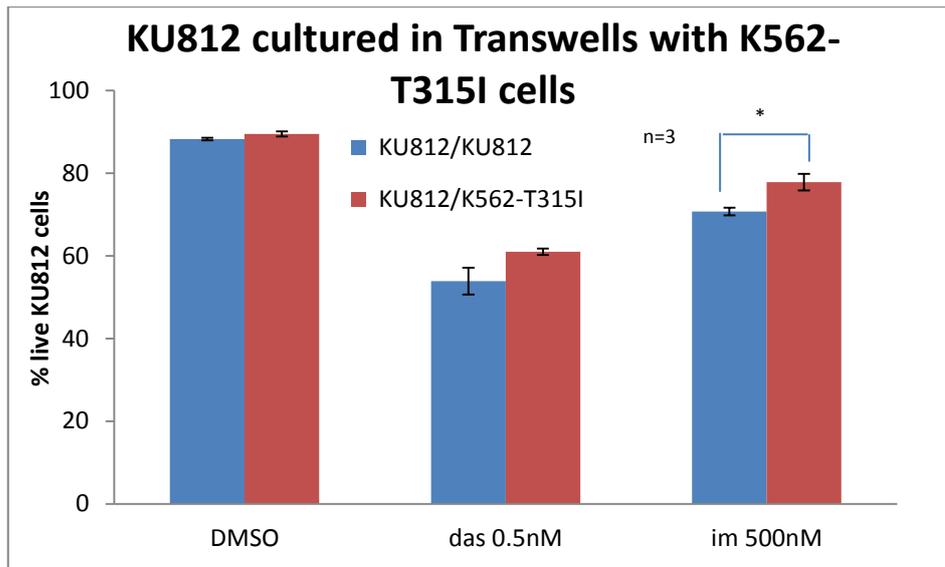


Figure 3-19 KU812 cells when co-cultured with K562-T315I cells in the presence of TKI demonstrate improved survival

After washing, both cell lines were resuspended in SDM at a concentration of 1×10^5 cells/ml. 1.5 ml of either KU812 or K562-T315I cells were plated at the bottoms of the Transwell® plates and 0.5 ml of KU812 cells on the Transwell® inserts and the experiment was performed in duplicates. Appropriate concentrations of TKI were added with DMSO used as the vehicle control. After incubation at 37°C/5% CO₂ for 3 days, KU812 cells from the inserts were assessed for cell viability using Annexin V and 7AAD death assay.

Statistical significant results were only obtained with KU812 cells co-cultured with K562-T315I cells in the presence of imatinib compared to the cells cultured alone (approximately 80% viability compared to 70%) whilst cells cultured in dasatinib only showed a trend towards increased survival (60% vs 55%).

The experiments were performed 3 times. Data represent means \pm SEM, significant values * represent $p < 0.05$ and was calculated using the Student's t-test.

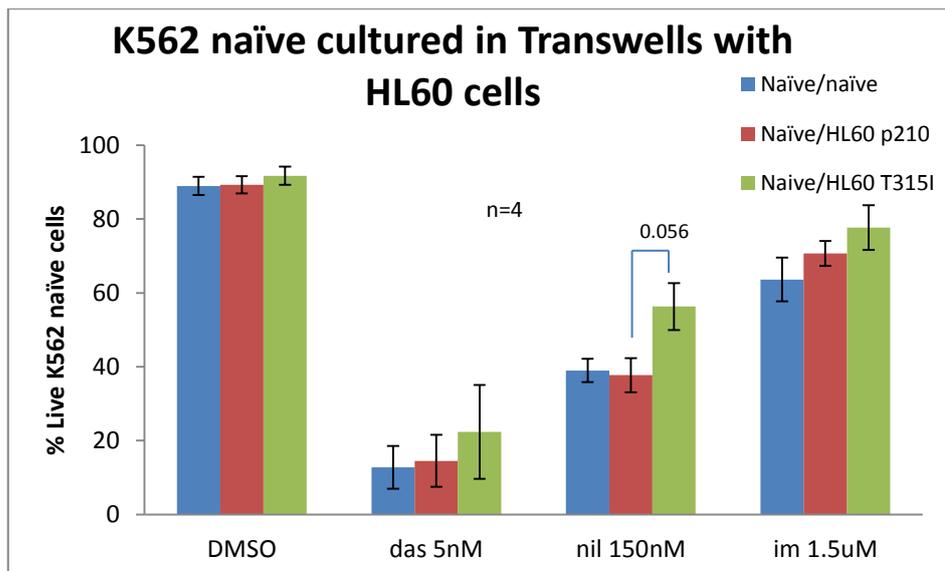


Figure 3-20 K562 naïve cells when co-cultured with HL60 *BCR-ABL1*^{T315I} cells demonstrated a trend towards improved survival in the presence of nilotinib

Cells were washed and resuspended in SDM at a concentration of 1×10^5 cells/ml or 1.2×10^5 cells/ml for HL60 *BCR-ABL1*^{T315I} cells as the cells expanded more slowly (refer Figure 3-5 and 3-17). 1.5 ml of either K562 naïve, HL60 *BCR-ABL1*^{p210} or HL60 *BCR-ABL1*^{T315I} cells were plated at the bottoms of the Transwell® plates and 0.5 ml of K562 naïve cells on the Transwell® inserts. The appropriate concentrations of TKI were added with DMSO used as the vehicle control. After incubation for 3 days at 37°C/5% CO₂ K562 cells from the inserts were assessed for cell viability using Annexin V and 7AAD death assay.

The results show a trend towards increased survival of K562 naïve cells when co-cultured with HL60 *BCR-ABL1*^{T315I} cells with a TKI although results did not reached statistical significance. K562 naïve co-cultured with HL60 *BCR-ABL1*^{T315I} cells compared to cells cultured alone with dasatinib showed an improve viability of approximately 20% from 10%, with nilotinib 55% from 38% and imatinib 75% from 65%.

Experiments were performed at 4 times and data are means \pm SEM.

but allowed for free diffusion of soluble factors, one or more of the cytokines or growth factors preferentially expressed by *BCR-ABL1*^{T315I} (refer Tables 3-1 and 3-2) may be conferring this survival effect to the *BCR-ABL1*^{WT} cells in the setting of TKI treatment.

The results were best demonstrated with the K562 naïve/K562-T315I cells (Figure 3-18) as treatment with all 3 TKI showed statistical significant “rescue” effect of K562 naïve cells co-cultured with K562-T315I cells over those cultured alone. For example, when K562 naïve cells were cultured alone with nilotinib, only approximately 25% of the cells were viable but when co-cultured with K562-T315I cells, about 50% of the cells survived which is a 50% increase in survival. The same experiment using KU812/K562-T315I cells treated with TKI also showed a trend towards survival of KU812 cells co-cultured with K562-T315I cells over those cultured alone but significance was only achieved through the use of imatinib (from approximately 70% viable to 80%) (Figure 3-19). Using K562 naïve/HL60 *BCR-ABL1*^{T315I} cells, the results again showed a trend towards increased survival in TKI (Figure 3-20) but a statistical significant difference between those cells co-cultured with *BCR-ABL1*^{T315I} or *BCR-ABL1*^{WT} cells was not demonstrated. For example, K562 naïve co-cultured with HL60 *BCR-ABL1*^{T315I} cells showed that after 3 days of culture in nilotinib, approximately 55% of the cells were viable compared to 38% when cultured alone with nilotinib. This may mean that the “rescue” effect observed with the K562 and the KU812 cell lines was not demonstrated with the HL60 cell lines.

Next, cytokine/cytokines responsible for the paracrine protective effect from cell death for the K562 naïve and KU812 cells was investigated.

3.3.6. Exogenous FGF-2 was able to rescue K562 naïve cells from TKI induced cell death

Recombinant cytokines and growth factors were introduced one at a time to the culture media of K562 naïve cells treated with TKI. The cytokines chosen were based on the results of the cytokine profiling performed on the cell culture supernatant of K562-T315I cells as determined by CSL (refer Table 3-1). Accordingly, 5 cytokines were chosen, namely, GM-CSF, MDC, IL-6, IL-8 and FGF-2.

Figure 3-21 shows the effects of adding exogenous cytokines and growth factors to K562 naïve cells treated with 10 nM dasatinib, 200 nM nilotinib and 1 uM imatinib respectively. Epo rescued the cell line from the effects of TKI as expected. FGF-2 rescued K562 naïve cells from nilotinib (22.8% viable cells compared to 17.1% with nilotinib alone) and imatinib-induced cell death (69.7% from 48.2%) but in dasatinib treated cells, the results did not reach statistical significance. This may have been due to the more potent concentration of dasatinib chosen, however there was a trend towards increased viability in the presence of FGF-2 (13.2% from 9.8%).

Next, all the cytokines together (GM-CSF, IL-6, IL-8, MDC and FGF-2) were introduced to determine if it would result in an additive rescue effect from TKI-induced cell death. It resulted in the rescue of K562 naïve cells from cell death induced by all three TKIs but did not have an incremental effect over FGF-2 alone (Figure 3-22).

In summary, the above results suggest that the K562 naïve cells were able to be rescued from cell death induced by TKI by the addition of Epo or FGF-2 but not by other cytokines or growth factors tested. A combination of cytokines and growth factors also did not have an additive effect over FGF-2 alone as it did not rescue beyond that demonstrated by FGF-2. This was performed as there is evidence that cytokines and growth factors interact and may synergize with one another to amplify a cellular response (252).

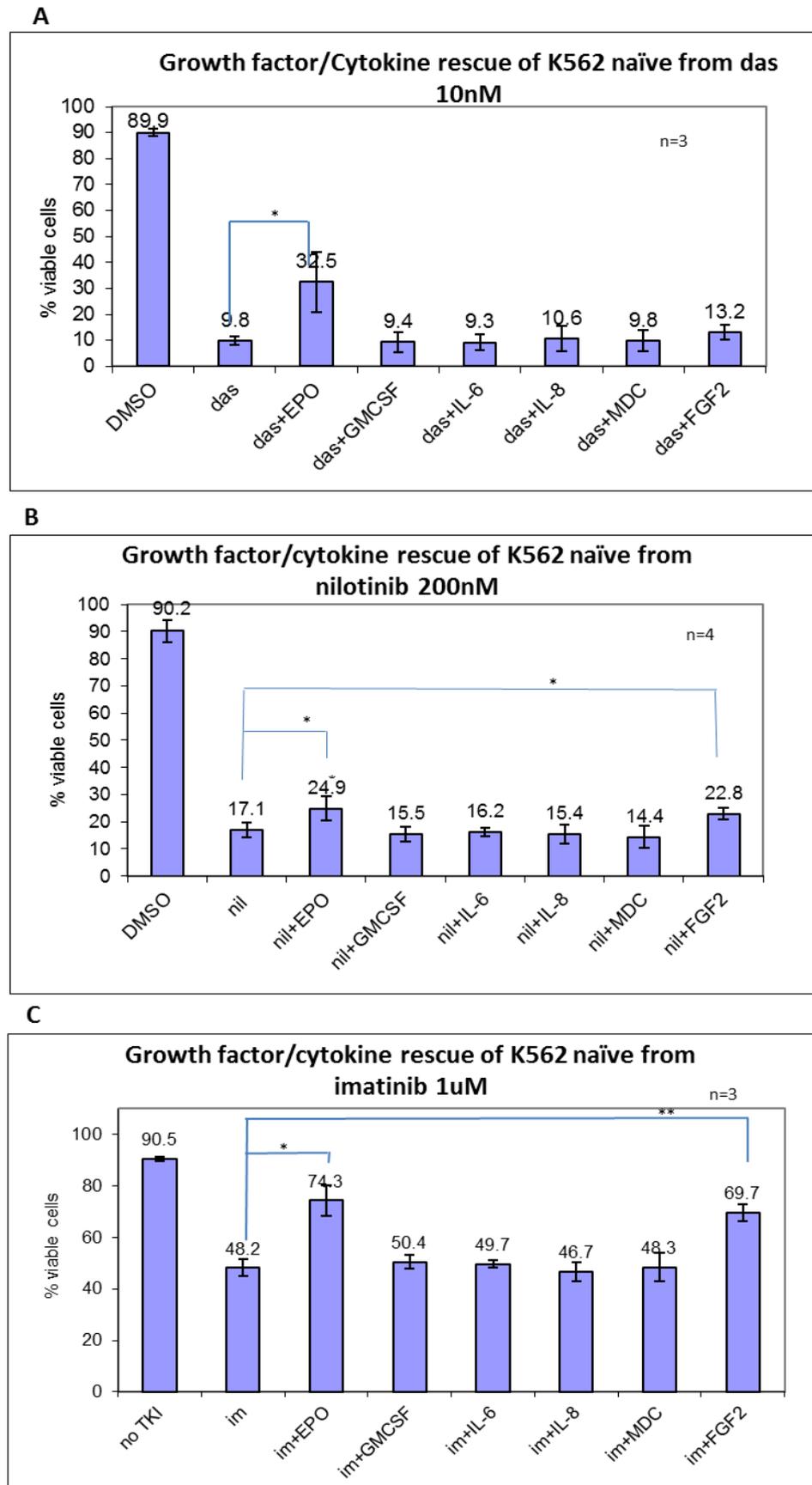
From the experiments performed so far, it was hypothesized that *BCR-ABL1*^{WT} cells co-cultured in Transwells® with *BCR-ABL1*^{T315I} cells were protected from TKI-induced cell death due to the paracrine actions of the secreted FGF-2 from the *BCR-ABL1*^{T315I} cells.

Figure 3-21 FGF-2 is able to rescue K562 naïve cells from nilotinib and imatinib-induced cell death

1×10^5 cell/ml resuspended in 0.5 ml SDM was plated in duplicate in a 24-well plate with either no TKI or DMSO (as control), A) dasatinib (das), B) nilotinib (nil) or C) imatinib (im). Recombinant cytokines 50 ng/ml (all PreproTech), were added to the appropriate wells. Erythropoietin (Epo) 4 U/ml was used as the positive control. After 3 days incubation at 37°C/5% CO₂, the cells were analysed by Annexin V and 7AAD staining on flow cytometry. Compared to the control, in a 3 day assay, K562 naïve cells were inhibited by A) dasatinib, B) nilotinib and C) imatinib, but rescued by Epo. FGF-2 was also able to rescue K562 naïve cells from nilotinib and imatinib induced cell death. Results for dasatinib did not reach significance compared to dasatinib alone.

Experiments were performed at least 3 times. * p<0.05 and ** p<0.01 compared to TKI only treated cells. It was derived using Student's t-test. Data are means ± SEM.

Figure 3-21



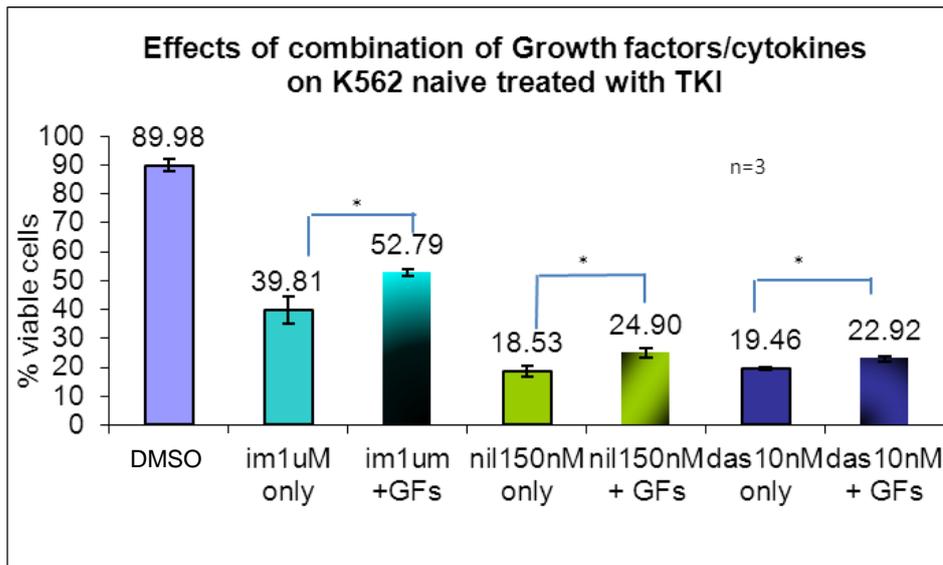


Figure 3-22 A combination of GM-CSF, MDC, IL-6, IL-8 and FGF-2 did not result in additional effect on TKI treated K562 naïve cells over FGF-2 alone

Cells were washed as for the prior experiments and resuspended in SDM at a concentration of 1×10^5 cells/ml. K562 naïve cells were plated into a 24-well plate in duplicates at 0.5 ml and the above TKI and growth factors were added to the appropriate wells. DMSO was used as control. The cells were incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 3 days and then assessed for viability by Annexin V and 7AAD death assay.

As shown, the addition of a combination of all 5 cytokines and growth factors did not appear to have an additional effect to that obtained with FGF-2 alone (refer Figure 3-21).

Experiments were performed 4 times. * $p < 0.05$ compared to TKI only treated cells and was derived using the Student's test. Data are means \pm SEM. GFs- Growth factors.

3.3.7. Inhibiting FGF-2 by bFM-1, a FGF-2 neutralizing antibody

To ascertain if cells harbouring the T315I mutation were conferring protection to *BCR-ABL1^{WT}* cells from TKI-induced cell death by secretion of FGF-2, the action of FGF-2 was blocked by using bFM-1 (a FGF-2 neutralizing antibody; Millipore, Massachusetts, USA). A dose titration for bFM-1 was initially performed to determine the optimal dose for blocking FGF-2 which was 250ng/ml as detailed below.

As shown in Figure 3-23 by the blue bars, imatinib resulted in cell death at Day 3 (40% viability from 85%) and rFGF-2 partially reversed this effect (60% viability). The addition of bFM-1 did not overcome the rescue effect of rFGF-2 at Day 3 up to a concentration of 500 ng/ml although there was a trend towards it (from 60% with no bFM-1 to 50% viability with bFM-1 500 ng/ml). However, at Day 5 (as shown by the red bars in Figure 3-23), bFM-1 negated the protective effects of FGF-2 from imatinib-induced cell death at the concentration of 250 ng/ml or higher (from 25% with no bFM-1 to 10% with 250 ng/ml bFM-1).

Ponatinib, a third generation TKI is the only currently available TKI that has activity against the T315I mutation. The ability of rFGF-2 to overcome the *BCR-ABL1^{T315I}* inhibitory effects of ponatinib was investigated. Ponatinib is known, in addition to activity against Bcr-Abl kinase, to have an inhibitory activity against the FGF receptor (FGFR) (146).

K562 naïve cells treated with ponatinib could also be rescued by recombinant FGF-2 (Figure 3-24). The minimum concentration of bFM-1 that was able to overcome the protective effect of FGF-2 against ponatinib was determined to be 250 ng/ml but this became apparent by Day 3 (shown by the blue bars in Figure 3-24). Cells treated with ponatinib and rFGF-2 were approximately 55% viable but with the addition of 250 ng/ml bFM-1, this was reduced to approximately 41% by Day 3. Therefore, future experiments involving the use of imatinib and bFM-1, cells were analysed after culturing for 5 days with media changed and fresh reagents added at Day 3, whilst those performed using ponatinib and bFM-1, were analysed at Day 3.

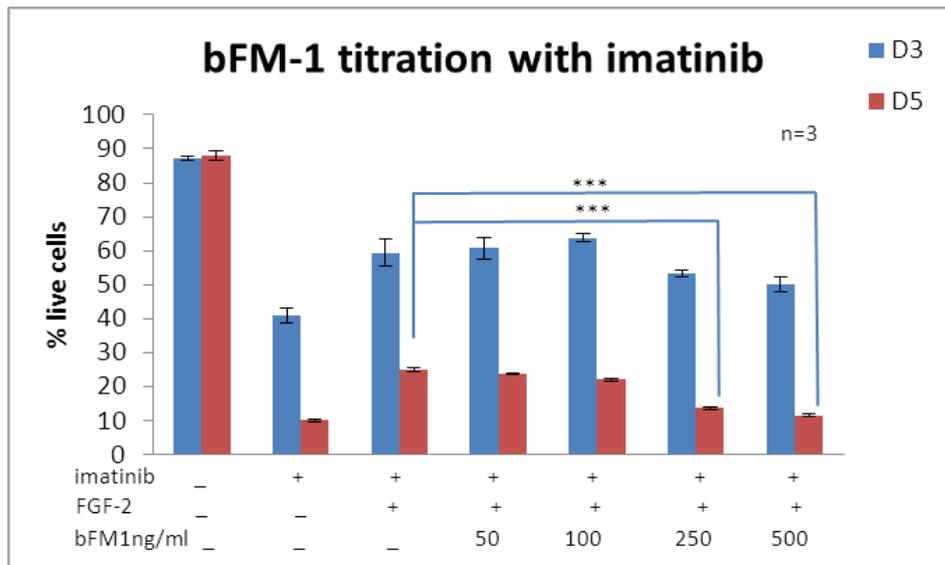


Figure 3-23 The minimum concentration of bFM-1 which is able to overcome the protective effects of rFGF-2 from imatinib-induced cell death by Day 5 was 250 ng/ml

K562 naïve cells resuspended in SDM at a concentration of 1×10^5 cells/ml were plated at 1 ml in a 24-well plate in duplicates and 2 identical plates were set up. The cells were incubated for 3 days at 37°C/5% CO₂ either untreated, treated with 1.5 μM imatinib, 20 ng/ml rFGF-2 and different concentrations of bFM-1 as shown. At Day 3, the cells from one plate were harvested and analysed by Annexin V and 7AAD staining. For the second plate, at Day 3, the media was changed, fresh reagents added and it was further incubated for an additional 2 days. The cells were then analysed at Day 5 by the same methods.

The results show that at Day 3 (blue bars), bFM-1 did not have an effect on neutralizing the effect of FGF-2 but by D5 (red bars), bFM-1 250 ng/ml and 500 ng/ml were able to negate the effect of FGF-2.

The experiments were performed 3 times. Significant value *** $p < 0.001$ compared to the imatinib and FGF-2 treated cells at Day 5. Significance is derived using Student's t-test. Data are means \pm SEM.

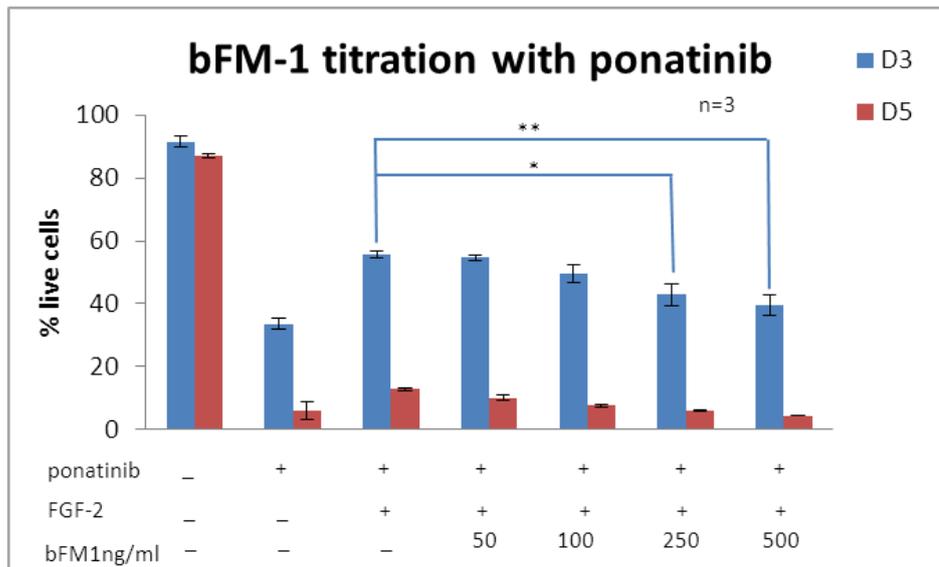


Figure 3- 24 bFM-1 at 250 ng/ml is able to overcome the protective effects of rFGF-2 from ponatinib-induced cell death by Day 3

K562 naïve cells (1×10^5 cells/ml) in SDM were plated at 1 ml in duplicates in a 24 well plate and 2 identical plates were set up. Reagents were added to the appropriate wells- 5 nM ponatinib, 20 ng/ml FGF-2 and bFM-1 at different concentrations as indicated. After incubation at $37^\circ\text{C}/5\% \text{CO}_2$ for 3 days, the cells in one of the plates were analysed by Annexin V and 7AAD staining. Media was changed and fresh reagent added for the other plate. After further incubation for another 2 days, the cells were harvested and analysed by the same method.

bFM-1 at 250 ng/ml showed a statistical significant reduction in cell viability compared to cells untreated with bFM-1 and this effect was significant even at Day 3 (blue bars).

Experiments were performed 3 times. Significant values * represent $p < 0.05$, ** $p < 0.01$ compared to cells treated only with ponatinib and FGF-2 at day 3. Data are means \pm SEM.

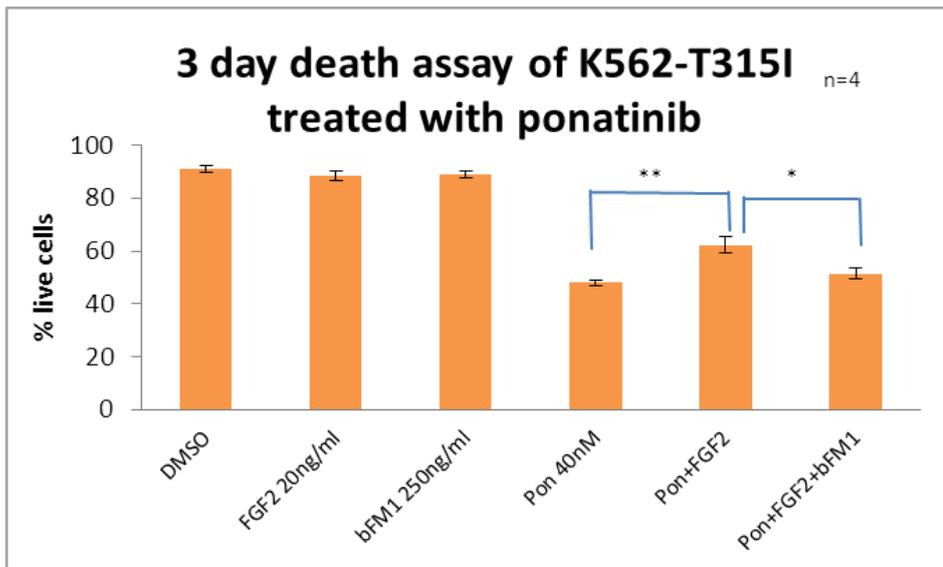


Figure 3-25 Recombinant FGF-2 is able to overcome Bcr-Abl kinase inhibition in ponatinib treated K562-T315I cells

After optimal wash, K562-T315I cells were resuspended in SDM at a concentration of 1×10^5 cells/ml and plated in duplicates in a 24 well plate. Appropriate reagents were added- 20 ng/ml FGF-2, 250 ng/ml bFM-1 or 40 nM ponatinib. DMSO was used as vehicle control. After 3 days incubation at $37^\circ\text{C}/5\% \text{CO}_2$, the cells were analysed by Annexin V and 7AAD staining.

This shows that ponatinib efficiently induced K562-T315I cell death but the cells were partially protected by rFGF-2. bFM-1 was successful in overcoming this protective effect.

Experiments were performed 4 times. Significant values * $p < 0.05$ and ** $p < 0.01$ was calculated using the Student's t-test. Data are mean \pm SEM. Representative flow cytometry plots from which this data was derived from are shown in the Appendix.

To determine if FGF-2 was also able to rescue K562-T315I cells from ponatinib-induced cytotoxicity, a 3 day cell death assay was performed. K562-T315I cells were incubated with ponatinib and rFGF-2 was added. As shown in Figure 3-25, like the K562 naïve cells, K562-T315I cells surprisingly, can also be partially rescued from TKI-induced cell death by the addition of rFGF-2 (45% viability with ponatinib only and 60% with ponatinib and rFGF-2). As expected, bFM-1 was able to reverse this protective effect (from 60% back down to 50% viability) (refer Figure 3-25). This demonstrates that additional exogenous FGF-2 can overcome the cytotoxic effects of ponatinib even though it is known to have activity against the FGF receptor (146).

3.3.8. K562-T315I cells protect K562 naïve cells from imatinib-induced cell death by secretion of FGF-2 when cultured together in SDM

To further confirm that the cytokine produced by *BCR-ABL1*^{T315I} cells, which protected *BCR-ABL1*^{WT} cells from TKI-induced cell death was FGF-2, *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells were cultured in Transwells® and treated with imatinib. It was predicted that *BCR-ABL1*^{WT} would be protected from TKI-induced cell death when co-cultured with *BCR-ABL1*^{T315I} cells due to the paracrine actions of FGF-2. To test this hypothesis, bFM-1 was used to negate the effect of FGF-2 to determine if this would overcome the predicted protective effects of FGF-2.

Similar to previously shown (refer figures 3-18 and 3-20), K562 naïve cells, when co-cultured with K562-T315I cells demonstrated increased survival in the presence of imatinib. However this time, the cells were cultured over 5 days with media changed at Day 3. K562 naïve cells treated with imatinib showed that approximately 30% were alive after 5 days and when co-cultured with K562-T315I cells, viability increased to 36% (Figure 3-26). This is despite the fact the some of the cytokines produced by the K562-T315I cells have been removed by media change. On addition of bFM-1, viability dropped back to 30%. The protective trend of co-culturing K562 naïve cells with HL60 *BCR-ABL1*^{T315I} cells which was reversed upon the addition of bFM-1 was also demonstrated (Figures 3-27). This strongly suggests that the soluble factor protecting the K562 naïve cells from TKI-induced cell death was indeed FGF-2. Therefore, *BCR-ABL1*^{T315I} cells were able to protect *BCR-ABL1*^{WT} cell from TKI-induced cell death through the paracrine actions of secreted FGF-2.

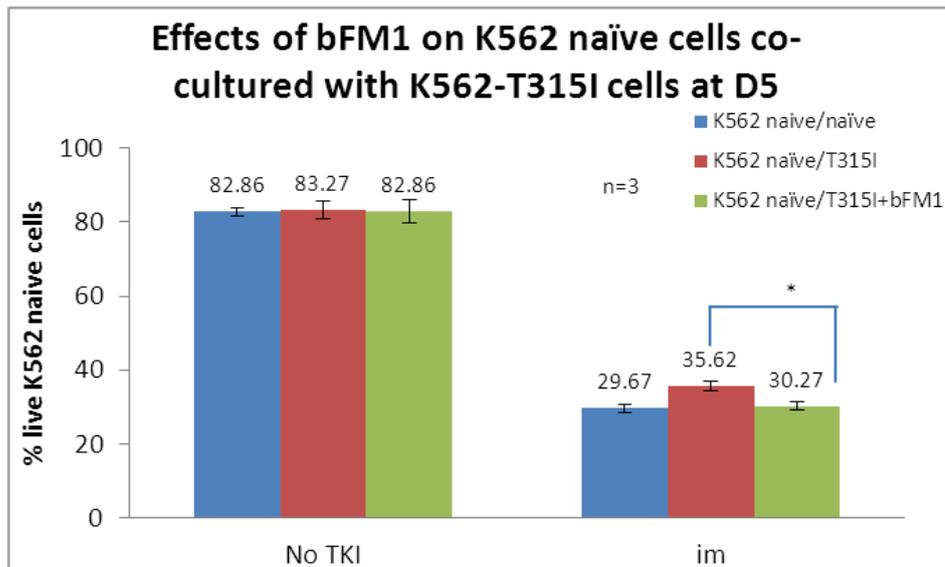


Figure 3-26 K562 naïve cells co-cultured with K562-T315I cells with imatinib are partially protected from cell death but this can be overcome by the addition of bFM-1

After optimal wash, cells were resuspended in SDM at a concentration of 1×10^5 cells/ml. K562 naïve or K562-T315I cells were plated at 1.5 ml at the bottom wells of the Transwell® plates and 0.5 ml of K562 naïve cells at the inserts. The cells were either left untreated or with 1.5 μ M imatinib and 250 ng/ml bFM-1 added into the appropriate wells. After 3 days of incubation at 37°C/5% CO₂, the cells from the bottom wells were pelleted, media changed and new reagents added. After further incubation for 2 days, the cells were harvested and analysed by the death assay performed by staining for Annexin V and 7AAD.

Compared to K562 naïve cells alone, those co-cultured with K562-T315I cells demonstrated a trend to increase survival in imatinib (from 29.7% to 35.6%). Following co-treatment with bFM-1, the viability of the K562 naïve cells was decreased nearly back to the same level as the naïve cells cultured alone.

Experiments were performed 3 times. * $p < 0.05$ is calculated using the Student's t-test. Data are means \pm SEM.

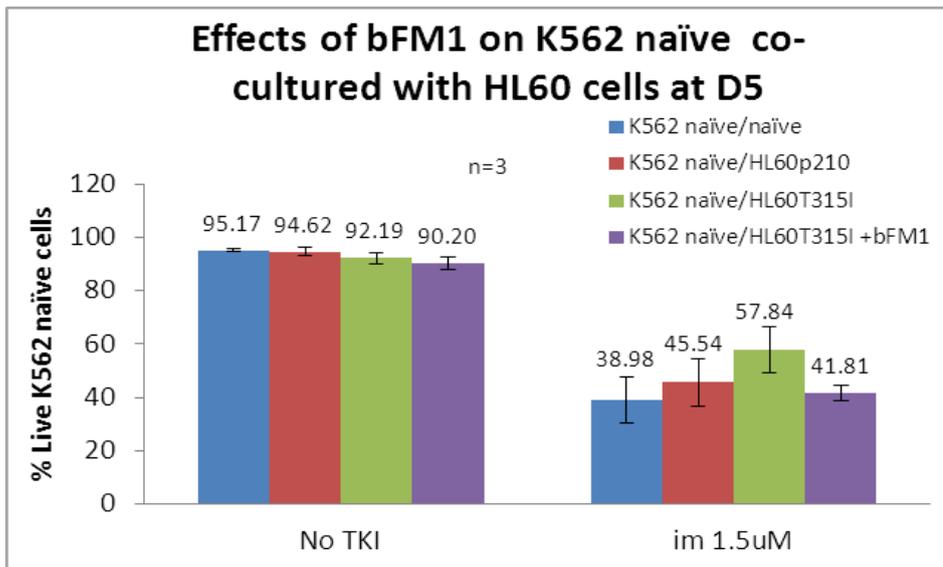


Figure 3-27 K562 naïve cells co-cultured with HL60 *BCR-ABL1*^{T315I} cells are partially protected from imatinib-induced cell death but this effect can be overcome with bFM-1

Cells were resuspended in SDM at a concentration of 1×10^5 cells/ml except HL60 *BCR-ABL1*^{T315I} cells which were resuspended at 1.2×10^5 cells/ml. 1.5 ml of either K562 naïve, HL60 *BCR-ABL1*^{p210} or HL60 *BCR-ABL1*^{T315I} cells were plated at the bottom wells of the Transwell® plates and 0.5 ml of K562 naïve cells at the inserts. The cells were either left untreated or with 1.5 μ M imatinib. 250 ng/ml bFM-1 were added into the appropriate wells. After 3 days of incubation at 37°C/5% CO₂, the cells from the bottom wells were pelleted, media changed and new reagents added. After further incubation of 2 days, the cells at the inserts were harvested and analysed by staining for Annexin V and 7AAD.

K562 naïve co-cultured with HL60 *BCR-ABL1*^{T315I} cells demonstrated a trend to survival from imatinib but this protection can be overcome by bFM-1.

Experiments were performed 3 times. Data are means \pm SEM.

3.3.9. FGF-2 rescues K562 naïve cells from cell death via reactivation of pErk and pSTAT5

The mechanism by which FGF-2 was able to rescue K562 naïve cells from TKI-induced cell death was postulated to be through reactivation of the signalling pathways downstream of Bcr-Abl. FGF receptor (FGFR) when stimulated by its ligand results in upregulation of the MAP kinase, PI3K-Akt pathways and phospholipase C γ (PLC γ) (253, 254) (Figure 3-28). STAT, p38 and JNK pathways are also activated but the predominant pathway upregulated by FGFR is the MAP kinase pathway (255, 256). Activation of this pathway results in promotion of cell entry into the S phase of the cell cycle and thus increases in cell proliferation (257).

Therefore, Erk, STAT5 and Akt reactivation was studied in K562 naïve cells treated with imatinib and FGF-2 by performing phosphoflow and western blotting analyses.

In K562 naïve cells, imatinib treatment reduced the phosphorylation of Erk and more markedly STAT5 as shown by both phosphoflow and western blotting. Phosphorylation of Erk in the untreated cells was normalised to 100% and imatinib reduced it to 14% (Figure 3-29A). The addition of FGF-2 to the imatinib-treated cells partially restored the phosphorylation of Erk back to 37% but when the cells were incubated with bFM-1, phosphorylation of Erk was reduced again to 17.2%, which was nearly the level of phosphorylation evident following treatment with imatinib (Figures 3-29A). Similarly, STAT5 phosphorylation was reduced to 9.7% with imatinib, partially restored to 24.6% with FGF-2 and reduced back down to 11.2% with the addition of bFM-1 (Figure 3-29B). The same trend could be seen with phosphorylation of Akt but as demonstrated with phosphoflow, it was not as pronounced (Figure 3-29C). Comparably, this was also shown by western blotting and analysis of the densitometry of pErk and pSTAT5 expressions (Figures 3-30 to 3-32). Similar to the phosphoflow results, pErk was reduced with imatinib, restored with FGF-2 and reduced again with bFM-1 (Figure 3-30). pSTAT5 was markedly reduced with imatinib and only partially restored with FGF-2 but reduced again with bFM-1 (Figure 3-32). Unfortunately, Akt expression by Western blotting was not successfully demonstrated. This data suggests that FGF-2 when over-secreted by *BCR-ABL1*^{T315I} cells rescues *BCR-ABL1*^{WT} cells from TKI-induced cell death by re-activating pErk and pSTAT5 pathways. The Akt pathway may also contribute but does not appear critical to the survival of *BCR-ABL1*^{WT} cells.

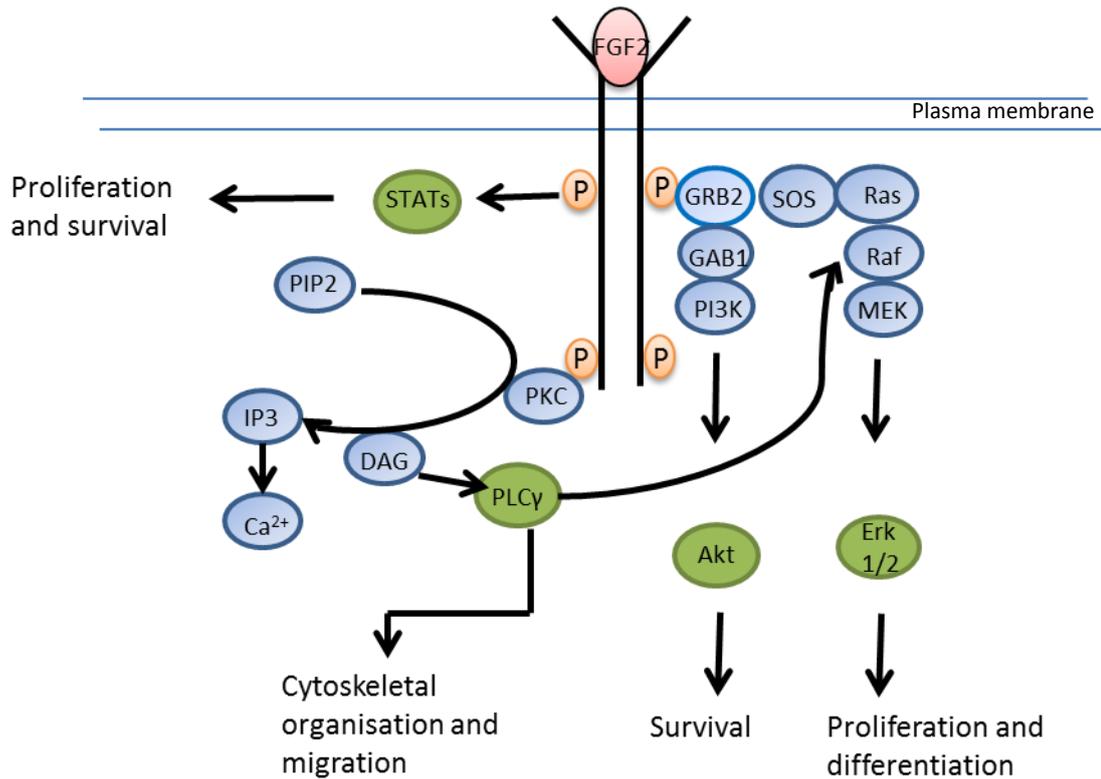


Figure 3-28 Fibroblast growth factor receptor signalling network

Following ligand binding and receptor dimerization, autophosphorylation events activate kinases and create docking sites for adapter proteins and activation of the 4 key downstream pathways: the Ras-MEK-Erk1/2, PI3K-Akt, STAT and PLCγ. FGFR-dependent signalling facilitates activation of multiple downstream pathways that results in increased proliferation, enhanced survival, inhibition of apoptosis and affects cell adhesion and migration. DAG, diacylglycerol; GAB1, GRB2-associated-binding protein 1; GRB2- growth factor receptor- bound 2; IP3, inositol triphosphate; P, phosphorylation; PIP2, phosphatidylinositol-4,5-biphosphate; PKC, protein kinase C ; PLCγ, phospholipase Cγ; SOS, son of sevenless.

(Adapted from Turner et al) (253).

Figure 3-29 In K562 naïve cells, A) pErk, B) pSTAT5 and C) pAkt expression is reduced when treated with imatinib, partially restored with FGF-2 and the effect of FGF-2 negated by the addition of bFM-1

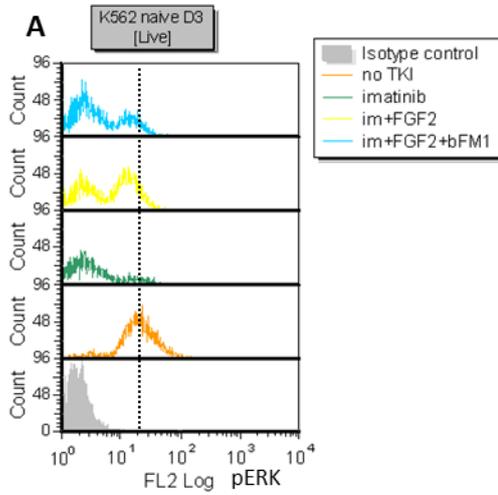
3 ml each of K562 naïve cells resuspended in SDM at a concentration of 1×10^5 cells/ml were plated in a 12-well plate and incubated for 3 days at 37°C/5% CO₂. The cells were either untreated or treated with the appropriate reagents (1.5 µM imatinib, 20 ng/ml FGF-2 and 250 ng/ml bFM-1). After incubation, the cells were fixed, permeabilized and 1 ml of cells each was stained with either A) pErk, B) pSTAT5, C) pAkt antibodies or the appropriate isotype controls and analysed by flow cytometry.

Results are displayed as stacked histograms with signal peak traces of isotype control, untreated cells and cells treated with reagents as stated. Vertical black dashed line represents peak signal in untreated K562 naïve cells. Results are expressed as MFI normalised to the untreated cells.

Compared to untreated cells (shown by orange histograms), K562 naïve cells treated with imatinib for 3 days (green histograms) showed markedly reduced expression of pErk and pSTAT5, with pAkt expression also decreased but not as markedly. The addition of FGF-2 (yellow histograms) partially restored phosphoprotein expression and treatment with bFM-1 (light blue histograms) brought this expression back down to the level demonstrated following imatinib treatment.

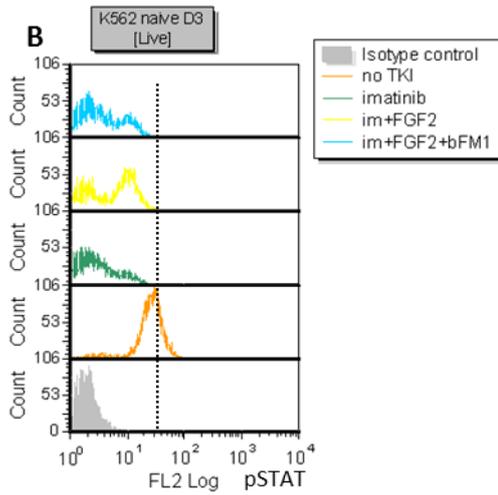
Figures are representative of three similar experiments.

Figure 3-29



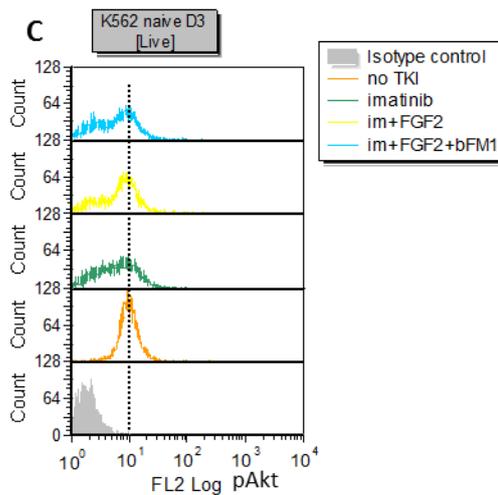
MFI pErk

No TKI	10.86 (100%)
Imatinib	1.52 (14%)
Im +FGF2	4.02 (37%)
Im+FGF2+bFM1	1.86 (17.2%)



MFI pStat5

No TKI	14.23 (100%)
Imatinib	1.39 (9.7%)
Im +FGF2	3.5 (24.6%)
Im+FGF2+bFM1	1.6 (11.2%)



MFI pAkt

No TKI	5.24 (100%)
Imatinib	3.34 (63.7%)
Im +FGF2	4.08 (77.8%)
Im+FGF2+bFM1	3.5 (66.8%)

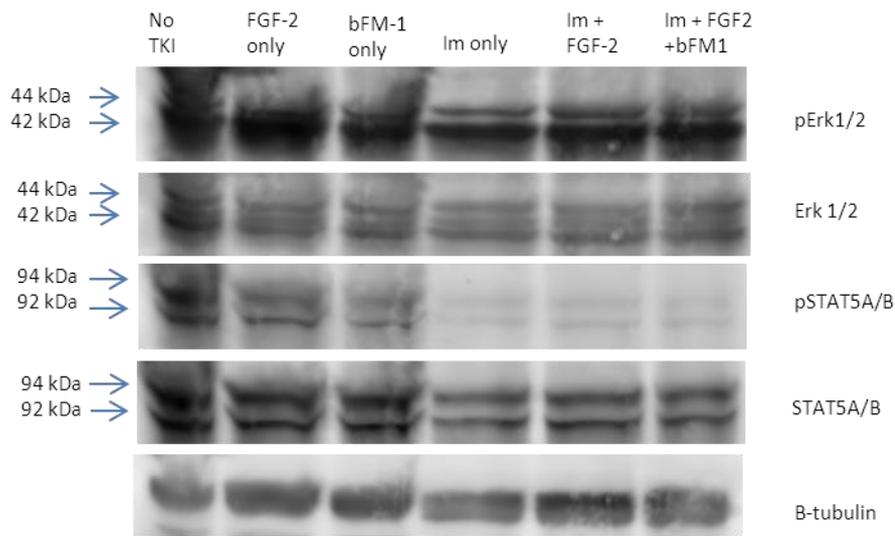


Figure 3-30 Western blots of K562 naïve cells with imatinib, FGF-2 and bFM-1

K562 naïve cells were incubated overnight at a concentration of 2×10^5 cells/ml in SDM either untreated, with 20 ng/ml FGF-2, 250 ng/ml bFM-1, 1.5 μ M imatinib as stated. pErk (T202/Y204), Erk, pSTAT5 (Y694), STAT5 and β tubulin (all from Cell Signalling), used as the loading control were assessed by Western blotting.

Western blots show reduction of pErk and marked reduction of pSTAT5 with imatinib. Phosphorylation of Erk and STAT5 was restored partially with the addition of FGF-2 but reduced again when bFM-1 was added.

kDa- kilodalton.

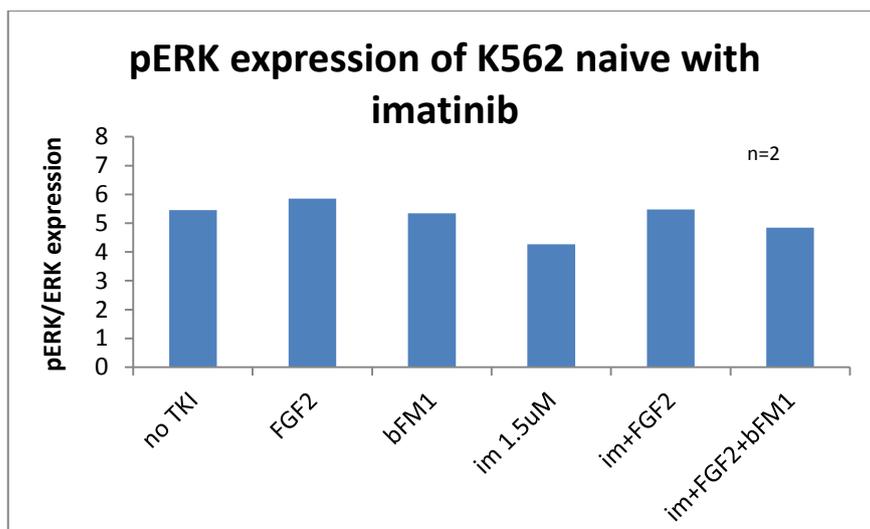


Figure 3-31 Densitometry analysis of pErk expression of Western blots in Figure 3-30

Analysis was performed by ImageQuaint software (GE Healthcare Life Sciences). The experiment was performed twice and the graph is presented as the average of both.

Incubation of K562 naïve cells with imatinib reduced pErk expression but it was increased to approximately back to baseline with the addition of FGF-2. The addition of bFM-1 reduced pErk expression to back to almost the level of cells treated with imatinib only.

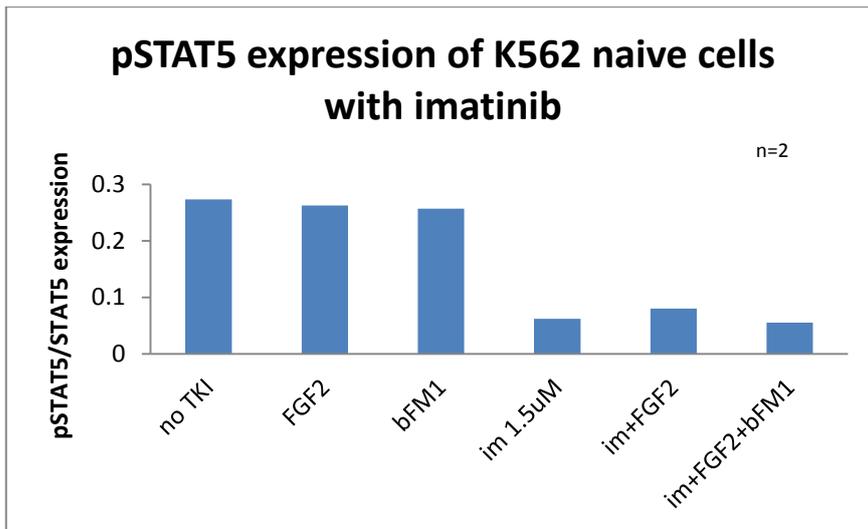


Figure 3-32 Densitometry analysis of pSTAT5 expression of Western blot in Figure 3-30

Analysis was performed by ImageQuaint software (GE Healthcare Life Sciences). The experiment was performed twice and the graph is the average of both.

Compared to cells untreated with imatinib, incubation with imatinib markedly reduced pSTAT5 expression. Densitometry analysis indicated a slight increase in pSTAT5 expression when FGF-2 was added to the cells which were reversed on the addition of bFM-1.

3.3.10. FGF-2 does not confer a survival advantage to K562-T315I cells through an autocrine action

So far, this study has demonstrated that *BCR-ABL1*^{T315I} cells are able to confer protection to *BCR-ABL1*^{WT} cells through the paracrine actions of FGF-2. However, over-expression of growth factors and cytokines do not appear to have a pro-survival autocrine effect on the *BCR-ABL1*^{T315I} cells themselves. To investigate this observation further, the actions of FGF-2 were inhibited to ascertain if inhibition would have any effect on the viability of K562-T315I cells.

K562-T315I cells were treated with imatinib and bFM-1 to ascertain if neutralising FGF-2, would affect the cells' viability with imatinib and also if activation of signalling pathways downstream of Bcr-Abl would be affected by studying phosphorylation of Erk, STAT5 and Akt.

K562-T315I cells did not show decreased survival with imatinib as expected and the addition of bFM-1 also did not affect viability of the cells (Figure 3-33). Phosphorylated Erk and Akt were also not affected by imatinib nor did the addition of bFM-1 significantly reduce phosphorylation of Erk or Akt (Figures 3-34A and 3-34C). Taken together, this data suggests that that FGF-2 does not have an autocrine role in the survival or survival signalling of K562-T315I cells.

However, phosphorylation of pSTAT5 (Figure 3-34B) was reduced to a small degree when cultured with imatinib and may have further reduced with the addition of bFM-1 (Figure 3-34B). pSTAT5 was reduced to 82% in the imatinib-treated cells (from 100% in the untreated cells) and was further reduced to 73.7% when bFM-1 was added. K562-T315I cells are as mentioned previously contained approximately 45% of the T315I mutation. It is uncertain if only 45% of the cells in the cell line have the mutation or if the level of the mutation varies between cells. If the former were true, then not all the cells in this cell line have the same resistance to TKI and pSTAT5 is a very sensitive marker of Bcr-Abl inhibition by TKI.

Figure 3-33 Viability of K562-T315I cells is not affected by blocking the activity of FGF-2

After optimal wash, K562-T315I cells were resuspended in SDM and 1×10^5 cells per condition was plated in a 24 well plate. 2 identical sets were set up. Cells were either left untreated, with 250ng/ml bFM-1, 3 $\mu\text{g}/\text{ml}$ imatinib or imatinib + bFM-1. The first set was cultured for 3 days and analysed by A) staining for Annexin V and 7AAD and B) trypan blue exclusion dye. The media for the second set was changed at day 3 with fresh reagents added, cultured for 2 additional days and assayed at Day 5 as with the first set.

K562-T315I cells were not affected by imatinib as shown by both the death assay and trypan blue counts and also showed no decrease in survival when bFM-1 was added. This was seen at both Day 3 and Day 5.

The experiment was performed 4 times. Data represent means \pm SEM and ns indicates not significant.

Figure 3-33

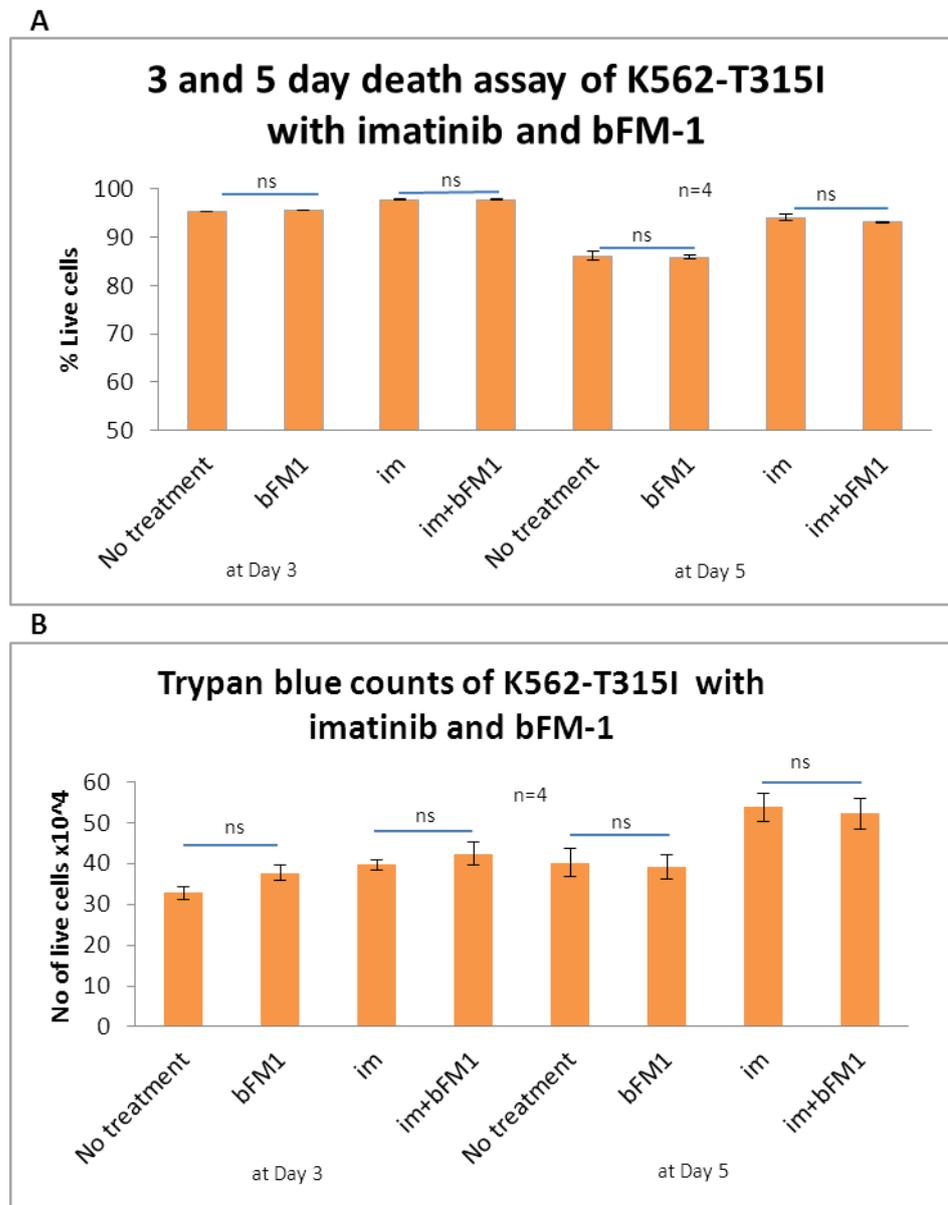
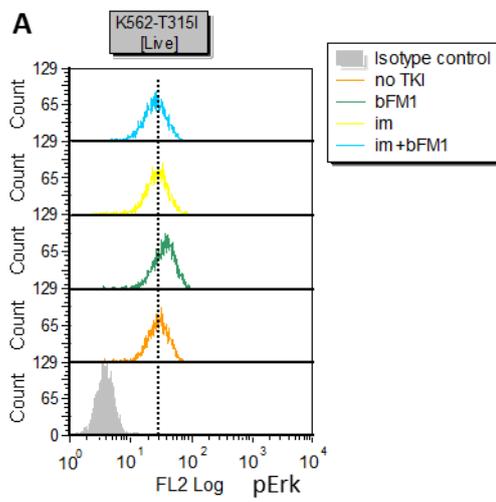


Figure 3-34 In K562-T315I cells, A) pErk and C) pAkt expression is not significantly changed when treated with imatinib and bFM-1 but B) pSTAT5 expression is reduced

After optimal wash, K562-T315I cells were resuspended in SDM at a concentration of 1×10^5 cells/ml and 3 ml each were plated in a 12-well plate. The cells were incubated for 3 days at 37°C/5% CO₂ either untreated or with the appropriate reagents (3 μM imatinib and 250 ng/ml bFM-1) and then fixed, permeabilized and incubated with either pErk, pSTAT5, pAkt antibodies or the appropriate isotype controls before flow cytometry analysis. Results are displayed as stacked histograms of signal peak traces of isotype control, untreated cells and cells treated with reagents as stated. Vertical black dashed line represents peak signal in untreated K562-T315I cells. Results are expressed as MFI normalised to the untreated cells. The results show that in K562-T315I cells, compared to the untreated cells (represented by the orange histograms), phosphorylation of A) pErk, and C) pAkt was not significantly affected by imatinib (yellow histograms) or imatinib + bFM-1 (light blue histograms). However, B) pSTAT5 expression showed a minor degree of inhibition by imatinib and may be further reduced by the addition of bFM-1.

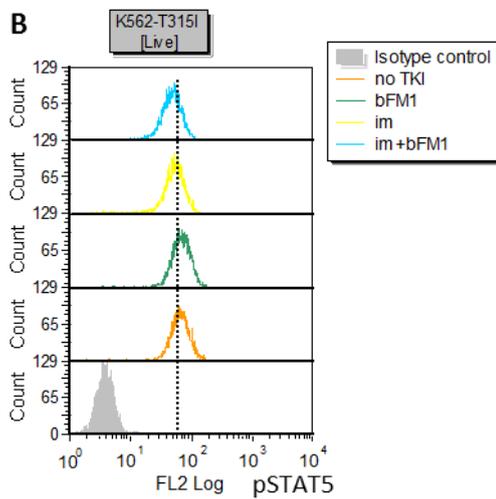
Experiments were performed 3 times and figures are representative of the 3.

Figure 3-34



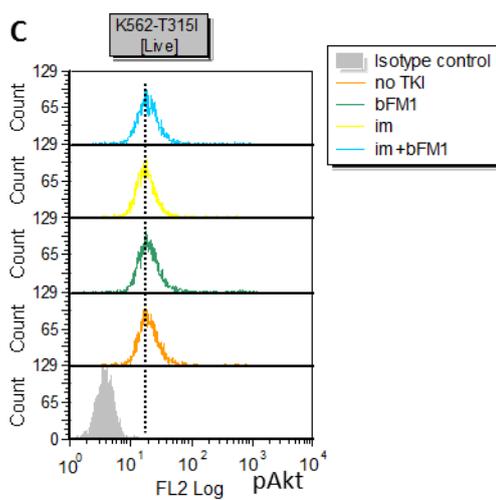
MFI pERK

No TKI	7.5 (100%)
bFM1	9.5 (127%)
Im	7.8 (104.3%)
Im+bFM1	6.9 (92.2%)



MFI pSTAT5

No TKI	17 (100%)
bFM1	17.8 (104.7%)
Im	14 (82.4%)
Im+bFM1	12.5 (73.7%)



MFI pAkt

No TKI	5.1 (100%)
bFM1	5.15 (100.9%)
Im	4.8 (94.2%)
Im+bFM1	5.1 (100%)

3.4. Discussion

It has been contentious whether some *BCR-ABL1* mutations confer a biological advantage (gain of function). Some studies have found that some mutations including the T315I have greater oncogenic potential, increase phosphorylation and different substrates specificity that target downstream proteins compared to *BCR-ABL1*^{WT} cells but not others (90, 91, 170, 181, 183, 258). Clinically, patients harbouring certain mutations have been found to have worse outcomes in some studies that examined the P loop and the T315I mutations (9, 10, 178, 187, 259) and these mutations are also more common in the advanced stages of CML (171).

BCR-ABL1 positive cells have been known to utilize cytokines and growth factors to their advantage. Early progenitor *BCR-ABL1* positive cells are able to survive in serum free conditions by autocrine secretion of IL-3, G-CSF and GM-CSF (71, 72, 80). Recently, more cytokines have been implicated in the pathogenesis of CML. Reynauld *et al* found that IL-6 secreted by CML myeloid progenitors control a paracrine loop which enhances myeloid differentiation of the multipotent progenitor cells. This amplifies a positive feedback loop promoting CML development (76). TNF- α plasma levels were found to be elevated in CML patients as well as *TNF- α* mRNA levels in LSC (260). Gallipolli *et al* suggested that elevation of this cytokine results in survival and proliferative signals in CML stem cells via activation of NF- κ B and increased expression of GM-CSF common β chain receptor (260).

Wang *et al* and Liu *et al* showed that certain resistant cell lines secreted GM-CSF and IL3 and confer paracrine protection of *BCR-ABL1* positive non-mutated cell lines (73, 74). Moreover, excessive growth factors and cytokines can also protect CML progenitor cells from the effects of TKI treatment (80, 81). Various cytokines used by the authors were IL-3 (80) G-CSF, GM-CSF and six growth factors (FLT-3 ligand, stem cell factor, IL-3, IL-6, G-CSF and thrombopoietin) (81). CML progenitor cells have been shown to have increased MAPK activation with imatinib, nilotinib and dasatinib treatment in the presence of cytokines (49, 198, 199). This mechanism is thought to contribute to their resistance to inhibition by TKI in the cytokine-rich microenvironment of the bone marrow niche.

This study aimed to investigate if excessive cytokine production is also contributing to resistance to TKI in *BCR-ABL1*^{T315I} through an autocrine loop which will then lead to its ability

to survive cytokine-poor niches that may partly contribute to the poor clinical outcome of patients harbouring this mutation. At the same time, to understand if these cytokines can also protect *BCR-ABL1*^{WT} cells from TKI-induced Bcr-Abl inhibition via a paracrine mechanism. In this study, the supernatant of cells with T315I mutation was found to have a different cytokine profile when cultured in SDM which suggested that the cells overproduced some cytokines and growth factors. K562-T315I secreted more FGF-2, GM-CSF, G-CSF, IL-6, IL-8 and MCP-1 while HL60 *BCR-ABL1* T315I secreted more FGF-2, G-CSF, IL-8, MCP-1, MDC, MIP-1 α , MIP-1 β and TNF- α which was similar but not identical to the results obtained earlier performed in CSL. Although it was performed on the same platform (Luminex xMAP[®] technology), different results may be due different preparation of the supernatant. The supernatant was prepared by culturing cells in RPMI + 10% FCS while for the cell supernatant analysed “in-house”, cells were cultured in SDM, as it was reasoned that no added cytokines and growth factors in the media may stimulate cytokine production by the cells. Other factors that may account for the differences seen include different cell concentrations used and different cell passage numbers.

Other authors have studied the cytokine profile of CML patients. Zhang *et al* analysed cytokine expression of newly diagnosed CP-CML patients and compared it to patients treated with imatinib and in CCyR with normal controls (87). The authors studied the cytokine mRNA expression in the bone marrow mononuclear cells and observed that compared to normal controls, newly diagnosed CP-CML patients had decreased CXCL12 and increased G-CSF, IL-1 α , MIP-1 β and MIP-2. In patients treated with imatinib who achieved CCyR, CXCL12 expression was increased over the newly diagnosed patients but remained significantly lower than observed in the normal bone marrow. Conversely, in this setting, G-CSF, TNF- α and MIP-2 were decreased back to the levels seen in the normal bone marrow (87). However, no published studies have looked at the cytokine profile of CML patients harbouring KD mutations compared to patients without mutations.

Studies have demonstrated the importance of the interaction between stromal cells with leukaemic cells and the role of the microenvironment in the expansion, terminal differentiation and the protection of leukaemia cells from TKI-induced cell death (82, 87, 200). Studies have also observed that specific cytokines produced by leukaemic cells or stromal cells within the bone marrow microenvironment confer a growth advantage to CML

stem cells and protection from TKI treatment (82, 87).

This study was limited in using a completely *in-vitro* system which may not be totally extrapolated to the *in-vivo* system where leukaemic stem cells interact within the bone marrow microenvironment. Additionally, K562 which is a CML blast crisis cell line (237) may give a totally different cytokine profile compared to CP-CML patients and HL60 cells which although transduced to express *BCR-ABL1* is actually an acute myeloid leukaemia cell line (240). K562-T315I cells also have a much higher *BCR-ABL1* transcript level compared to K562 naïve cells and may affect cytokine production. Nevertheless, the aim of this study was to compare cytokine levels of *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells and a different cytokine profile was observed. This suggested that cytokines would either confer an autocrine or paracrine protection in the setting of TKI treatment to the leukaemia cells.

Immunophenotyping of the relevant cytokine and growth factor receptors indicated that all the cell lines tested appeared to express most of the cognate receptors although some needed to be stimulated first by culturing the cells overnight in SDM and exposing them to the relevant cytokines. It was anticipated that if the cell line overexpressed the receptors, the relevant cytokines or growth factors would more likely have an autocrine or paracrine role on that cell line. However, as FGF-2 appeared to be the only cytokine conferring a protective effect on *BCR-ABL1*^{WT} cell lines and that FGFR was not found to be over-expressed in K562 naïve or HL60 *BCR-ABL1*^{p210} cells, surface receptor expression may not be the only factor which determines how the cells respond to cytokines. Similarly, Hantchel *et al* demonstrated that cell supernatant of K562 cells expressed increased IL-8 compared to U937 cells (a non *BCR-ABL1* cell line) which was reduced on dasatinib or nilotinib treatment. They did not however demonstrate IL-8 receptor expression on K562 cells (261). Nonetheless, a recent publication suggests that the receptor most important for FGF-2 binding and receptor stimulation in CML is FGFR3 as siRNA targeting *FGFR3* was the only one able to overcome the protective effects of FGF-2 (262). In this present study, only the level of expression of CD332, which is FGFR2 has been determined so far and thus the expression of this receptor in the cell lines tested may not be as informative. Conversely, surface receptors may be only transiently expressed and although stimulation of receptor expressions was attempted, expressions may not have been “captured”. mRNA levels of *FGFR1-4* in the white cells of CML patients are currently being studied in our laboratory.

Surprisingly, the survival and expansion of the cell lines carrying the T315I mutation did not appear to be increased compared to the *BCR-ABL1*^{WT} cell lines when cultured without additional cytokines and growth factors despite having a different cytokine profile. This contrasted with Griswold *et al* who found that all BA/F3 cells expressing KD mutations including the T315I proliferated more rapidly in serum poor conditions compared to the native *BCR-ABL1* cells with the exception of the M351T mutation (90). However, when the authors repeated the experiment using primary murine myeloid cells, colony formation of cells transduced with the T315I was significantly reduced compared to native *BCR-ABL1* cells only in the absence of cytokines (90). Similarly, this present study has also demonstrated that overproduction of cytokines and growth factors do not appear to have an autocrine role in the proliferation and expansion of *BCR-ABL1*^{T315I} cells.

To investigate if the cytokines and growth factors have a role in paracrine protection of *BCR-ABL1*^{WT} cells from TKI treatment, *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells were cultured together in Transwells® separated by permeable membranes that allow diffusion of soluble factors. This study demonstrated that *BCR-ABL1*^{WT} cells when cultured together with *BCR-ABL1*^{T315I} cells were protected from TKI-induced cell death. This suggests that one or more factors secreted by *BCR-ABL1*^{T315I} cells were able to rescue *BCR-ABL1*^{WT} cells from the cytotoxic effects of TKI. Although, there was a trend towards rescue of K562 naïve cells from TKI treatment by HL60 *BCR-ABL1*^{T315I} cells compared to HL60 *BCR-ABL1*^{p210}, it did not achieve statistical significance. Results between repeats of experiments were variable which affected the standard deviation. This may mean that the essential cytokines/growth factors secreted by the 2 HL60 cell lines may be overlapping so that the differences in rescue of K562 naïve cells were inconsistent. The other explanation could be the property of the HL60 cell line itself. This will be addressed later.

Nevertheless, to ascertain which soluble factor was responsible for protecting K562 naïve and KU812 cells from TKI-induced cell death, excessive exogenous cytokines which were indicated by cytokine profiling were added to K562 naïve cells and showed that out of the cytokines tested, only FGF-2 was able to protect these cells from the effects of TKI. This is in keeping with the findings of Traer *et al* who also demonstrated that FGF-2 protected K562 cells from imatinib (262). This suggested cells with the T315I mutation secreted FGF-2 which protected *BCR-ABL1*^{WT} cells from cell death induced by TKI. In order to prove this, *BCR-*

ABL1^{WT} and *BCR-ABL1*^{T315I} cells were co-cultured in Transwells®, with imatinib and a FGF-2 neutralizing antibody, bFM-1. It was demonstrated that bFM-1 was able to negate the rescuing effect which supports the earlier observation that FGF-2 was instrumental in reversing the effects of Bcr-Abl inhibition by imatinib.

K562-T315I cells which are sensitive to the third generation TKI, ponatinib, were also shown in this study to be rescued from ponatinib-induced Bcr-Abl inhibition by excessive FGF-2. This is despite the fact that ponatinib is also known to have activity against FGFR.

This study also demonstrated that FGF-2 protected *BCR-ABL1*^{WT} cells from TKI-induced cell death by reactivating the MAP kinase and the STAT5 pathways. The Akt pathway may also contribute but in this study, it is not a critical pathway activated by FGF-2. It has also been shown by others that FGFR upon ligand binding results in up-regulation of predominantly the MAP kinase pathway although among others, PI3-Akt, STAT and JNK pathways are also involved (253, 255, 256).

Conversely, FGF-2 does not seem to have an autocrine effect on K562-T315I cells as blocking the action of FGF-2 with bFM-1 did not result in increased cell death, nor did it affect pErk or pAkt expressions. This indicates that the mutation itself is enough to confer resistance to imatinib and this is in agreement with the findings of others (73). This confirms earlier observation that *BCR-ABL1*^{T315I} did not proliferate and expand over *BCR-ABL1*^{WT} in SDM despite increased secretion of certain cytokines and growth factors. It is difficult to speculate why some cytokines appear to be produced in excess by the cells harbouring the T315I mutation if no autocrine effect is apparent. Cytokines produced by leukaemia cells or stromal cells within the CML bone marrow microenvironment have complex interactions and have been demonstrated to confer a growth advantage to the leukaemic cells (87). Additionally, cytokines may also have effects on stromal cells and hence indirectly affect the leukaemic cells (263). Therefore, to further answer this question, *in-vivo* studies using primary cells or co-culture of *BCR-ABL1*^{T315I} with human mesenchymal stromal cells have to be performed.

In summary, K562-T315I and HL60 *BCR-ABL1*^{T315I} cell lines have different cytokine profiles compared to the *BCR-ABL1*^{WT} cell lines and the mutated cells are able to spread resistance to the *BCR-ABL1*^{WT} cells by increased secretion of FGF-2. The paracrine effect is not accompanied by an additional autocrine protective effect on cells harbouring the T315I

mutation which suggests that the mutation itself is sufficient to confer resistance to first and second generation TKIs. However, as shown, excessive FGF-2 could rescue these cells from effects of ponatinib. This may suggest that ponatinib-induced cell death can be overcome by excessive FGF-2 by bypassing Bcr-Abl inhibition and upregulating signalling pathways downstream of Bcr-Abl like the MAP kinase pathway. Increased FGF-2 or overexpression of the FGFR may be possible resistant mechanisms by which cells harbouring the T315I mutation could escape Bcr-Abl inhibition by ponatinib.

Increased FGF-2 or overexpression of the FGFR may also be a factor contributing to resistance to other *BCR-ABL1* positive cells including those without any detectable kinase domain mutations. A recent publication demonstrated increased FGF-2 expression in the bone marrow of patients without *BCR-ABL1* mutations who responded to ponatinib (262). FGF-2 in the bone marrow of these patients decreased after treatment with ponatinib. Work in our laboratory have shown that patients with low human organic cation inhibitor 1 (OCT1) have poorer response to imatinib and less likely to reach MMR (215). In this group of patients, mRNA extracted from white cells showed increased *FGFR2* expression compared to those with high OCT1 activity (personal communication from Dr C. Kok). *BCR-ABL1* positive cells with other *BCR-ABL1* mutations may also use overexpression of cytokines as an additional mechanism of resistance. Therefore, in patients with known mutations not responding to appropriate treatment, it may be worth checking their cytokine profile.

The limitations of this study are the use of the cell lines. As mentioned previously, the K562-T315I cell line only contains up to 45% T315I mutation and also differs from the K562 naïve cell line not only because of the mutation but it also contains higher amount of *BCR-ABL1* transcript (refer Figure 3-3). As not all the cells in the K562-T315I cell line contains the mutation, some of the cells may be affected by TKI as shown by decreased expression of pSTAT5 in these cells when treated with imatinib and may make interpretation of some of the results difficult.

Unexpectedly, the HL60 *BCR-ABL1*^{p210} was found to be not susceptible to TKI-induced cell death. The HL60 cell line as previously mentioned was derived from a patient with acute myeloid leukaemia (239, 240) and is known to contain amplified *c-MYC* proto-oncogene (240). Therefore, these cells are not solely dependent on *BCR-ABL1* for survival and oncogenic signalling and this cell line could not be used in some of the experiments.

Additionally, the HL60 cell line may spontaneously differentiate to more mature cells and the addition of DMSO and other agents such as retinoic acid markedly increases this spontaneous differentiation to granulocytes which also reduces their proliferative capacity (264). DMSO is used as a diluent for dasatinib, nilotinib and ponatinib and as a control for the experiments in this study which may have a bearing on some of the results. Differentiation to more mature haematopoietic cells may also affect their functional, enzymatic and surface antigen expression characteristics (264) and may also affect their production of cytokines and growth factors. This may be partly accounted for the variation demonstrated with the co-culture experiments using K562 naïve/HL60 cells. This factor may also account for the differences in the cytokine profile of the cell supernatant obtained by CSL compared to the one performed “in-house”.

As a result of findings in this chapter, further work is being planned. This includes the use of different cell lines for example the murine pro-B cell line, BA/F3, transformed by retroviral transduction with *BCR-ABL1*^{p210} and *BCR-ABL1*^{T315I} in the cytokine profiling, cytokine rescue and co-culture experiments. The use of this cell line would hopefully address some of the limitations outlined above. It would also provide more confidence that overproduction of FGF-2 by the K562-T315I and HL60 *BCR-ABL1*^{T315I} cells is not specific to these cell lines alone but applies to all cells harbouring the T315I mutation. As the receptor expression of FGFR3 was not examined in this study, immunophenotyping or mRNA expression of both *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells and primary CD34+ cells for FGFR1-FGFR4 should be carried out. Primary CD34+ progenitor cells in the cytokine rescue experiment will be used to determine if FGF-2 will also rescue these cells from TKI-induced cell death. Lastly, cytokine levels in particularly FGF-2 in the plasma and bone marrow of patients harbouring the T315I mutation in comparison to those without the mutation will be determined.

This study has demonstrated that cells harbouring the T315I mutation have a different cytokine profile compared to *BCR-ABL1*^{WT} cells and FGF-2 in particular was shown to confer paracrine protection to *BCR-ABL1*^{WT} cells from TKI-induced Bcr-Abl inhibition. Although cytokines have not been shown to confer cells harbouring the T315I mutation with an autocrine pro-survival effect, it does contribute to the evidence that KD mutations besides affecting TKI-binding affinity, can also modulate biological properties that may negatively impact clinical outcome.

Chapter 4

In *BCR-ABL1*^{T315I} expressing cells, the Mitogen activated protein kinase pathway is hyperactivated in the presence of tyrosine kinase inhibitors

4.1. Introduction

In *BCR-ABL1* positive leukaemia, the T315I mutation is unique in that it causes insensitivity to all the commonly used tyrosine kinase inhibitors (TKI). Imatinib, nilotinib, and dasatinib all form critical hydrogen bonds with threonine at position 315 of *BCR-ABL1* (124, 185). Therefore, if threonine is substituted, the formation of this critical hydrogen bond cannot occur. Isoleucine also causes steric hindrance as it is a larger hydrophobic molecule and blocks access of TKI to the hydrophobic pocket located close to T315 residue (132, 185, 186). Moreover, T315 participates in a network of highly conserved interactions named the hydrophobic spine which stabilises Bcr-Abl in the active conformation. Isoleucine, being a bulkier hydrophobic residue further strengthens this spine and further stabilises the active conformation (183). Hence, the T315I mutation results in complete insensitivity to TKI.

The T315I is one of the most common mutations to occur in the setting of secondary resistance to TKI (13, 233). On treatment with a TKI, *BCR-ABL1*^{WT} diminishes allowing mutant T315I clones to expand. Once TKI treatment is stopped, *BCR-ABL1*^{WT} usually re-expands. This presumably occurs because *BCR-ABL1*^{WT} has a proliferative advantage in the absence of TKI treatment (170, 174, 265). However, an alternative explanation may be that cells harbouring the T315I mutation have a proliferative advantage in the presence of TKI compared to in its absence. Even more intriguing, is what the mechanism involved may be.

Cytokines and growth factors have been shown to play a part in mediating resistance to TKI and *BCR-ABL1* positive cells, including those with kinase domain mutations (72-74, 87). Chu *et al* demonstrated that CML CD34+ cells had a compensatory increase in mitogen activated protein kinase (MAP kinase) expression when treated with imatinib only in the presence of cytokines (49). Similar findings were seen with nilotinib and dasatinib treated CML CD34+ cells (198, 199). It is thought that cytokine-mediated downstream signalling may bypass Bcr-

Abl inhibition of CML progenitor cells. More importantly, Hartel *et al* have reported that cells harbouring the T315I mutation show activated MAP kinase signalling with nilotinib and imatinib, even in the absence of cytokines, in this case, IL-3 (208).

The objective of this project is to ascertain if cells with the T315I mutation survive and have superior expansion in the presence of TKI compared to in the absence. If so, to determine which downstream signalling pathways are preferentially activated and if these cells over-express certain growth factors or cytokines which may explain these findings.

4.2. Approach

4.2.1. Viability and proliferation of K562-T315I cells cultured in SDM with TKI

K562-T315I cells were washed optimally as the cells were cultured with dasatinib (as previously described in Chapter 2, Section 2.4.6.). The cells were cultured at concentration of 1.5×10^5 cells/ml with 200 nM dasatinib, 200 nM nilotinib, 1 μ M nilotinib, 200 nM imatinib, 1 μ M imatinib and 5 μ M imatinib and DMSO (as vehicle control) for 3 days. Cells were assessed for viability by trypan blue exclusion dye and the 3 day Annexin V and 7AAD death assay (described in Chapter 2, Sections 2.4.2. and 2.5.1.) The death assay was performed on flow cytometer, Beckman Coulter Cytomics FC500 (Beckman Coulter, Miami, Florida, USA) and analysed with Analysis Software version 2.2 (Beckman Coulter). Live cells were defined as those negative for both Annexin V and 7AAD stains.

Cell proliferation was assessed by labelling the cell with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen Life Technologies, Carlsbad, Ca, USA) to ascertain the rate of cell division and hence used to track proliferation of cells. The assay was performed as per manufacturer's recommendation and the methodology was described in detail in Chapter 2 (Section 2.5.4). Data acquisition was by Beckman Coulter Cytomics FC500 and analysed with FCS Express 4 software (DeNovo Software, Los Angeles, Ca, USA).

Cell proliferation was also assessed by the detection of human nuclear nonhistone protein, Ki-67, which is only present in dividing cells and absent in quiescent cells. This method was performed by labelling the cells with Ki-67 antibody and detecting by flow cytometry. K562 naïve and K562-T315I cells were washed optimally and resuspended in SDM at 1×10^5 cells/ml and 3 ml for each condition were plated in a 12-well plate. TKI was introduced at the following concentrations, for K562 naïve cells -DMSO (control), 10 nM dasatinib, 150 nM

nilotinib, 1.5 μM imatinib and 5 nM ponatinib and for K562-T315I cells- DMSO (control), 200 nM, 1 μM , 5 μM dasatinib, 200 nM, 1 μM , 5 μM nilotinib, 200nM, 1 μM , 5 μM imatinib and 10 nM and 40 nM ponatinib. The concentration of TKI chosen for K562 naïve cells were based on earlier titration experiments (data not shown). For K562-T315I cells, it was based on two factors. Firstly, peak plasma concentrations of imatinib, nilotinib and dasatinib are 5 μM , 3 μM and 90 nM respectively (266). Secondly, K562-T315I cells were generated in increasing concentration of dasatinib and reached a peak of approximately 45% mutant transcripts at a concentration of 200 nM dasatinib. The cells were maintained at this concentration to prevent the level of mutational load from deteriorating (238).

The cells were incubated for 3 days at 37°C/5% CO₂, harvested into FACS tubes, pelleted, fixed and permeabilised as described in Chapter 2, Section 2.5.3. The cells were then incubated with Ki-67-PE conjugate monoclonal antibody (Cell Signaling, Danvers, Massachusetts, USA) for 1 hour. Cells were washed, resuspended in wash buffer and analysed on flow cytometer, Beckman Coulter Cytomics FC500 with further analysis by FCS Express 4 software.

4.2.2. Phosphoprotein detection of pErk and pSTAT5 by western blotting

The methodology was previously described in Chapter 2, Section 2.5.6. K562 naïve and K562-T315I cells were washed optimally and resuspended in SDM at 2×10^5 cells per condition, plated at 2 ml into a 24-well plate and incubated overnight with TKI or DMSO as control at 37°C/5% CO₂. K562 naïve were cultured with 10 nM dasatinib, 200 nM nilotinib or 5 μM imatinib. K562-T315I cells were cultured with 200 nM dasatinib, 1 μM dasatinib, 5 μM dasatinib, 200 nM nilotinib, 1 μM nilotinib, 5 μM nilotinib, 200 nM imatinib, 1 μM imatinib and 5 μM imatinib. The next day, the cells were washed in cold PBS, resuspended in Laemmli's buffer, boiled and stored at -20°C until needed.

The lysates were separated by SDS-PAGE gel electrophoresis using the Criterion™ TGX™ precast gels (Bio-Rad, Hercules, California, USA) together with pre-stained protein ladders and transferred onto PVDF membranes. The blots were blocked with 2.5% BSA and then incubated with 1° antibody overnight for p-Erk or pSTAT5 at 4°C (both Cell Signaling). After washing, the blots were then incubated with 2° antibody, α -Rabbit-IgG-Alkaline Phosphatase (Santa-Cruz, Dallas, Texas, USA) at room temperature for 1 hour and washed. Bound

antibodies were detected by enhanced chemifluorescence substrate (ECF) and scanned on Typhoon FluorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The blots were stripped and re-probed for Erk, β -tubulin and STAT5 (all Cell Signaling Technology). The resulting blots were analysed using ImageQuant software (GE Healthcare).

4.2.3. Phosphoprotein detection of pAkt by intracellular flow cytometry

The protein expression of phospho-tyrosine can be measured by staining intracellular proteins and is detected by flow cytometry. This method had been described previously (Chapter 2, Section 2.5.3). In brief, after optimal washing, 2×10^5 /ml cells were resuspended in 3 ml of SDM each and plated into a 12-well plate with DMSO (control) or with various concentrations of TKI as for western blot. The next day, the cells were fixed, permeabilised and stained with phospho-Akt (BD Biosciences) or the appropriate isotype control. After incubation at room temperature for 1 hour, the cells were washed and resuspended in wash buffer. Data was acquired by flow cytometer (Beckman Coulter Cytomics FC500) and results were analysed using Analysis Software version 2.2 (Beckman Coulter).

4.2.4. Cytokine profile of K562-T315I cells cultured overnight with different TKI in SDM

Cell line supernatant was prepared as described previously (Chapter 2, Section 2.5.13). After optimal wash, the cells were resuspended at a concentration of 5×10^5 cells/ml in SDM together with DMSO or TKI (10 nM and 200 nM dasatinib, 200 nM, 1 μ M, 5 μ M nilotinib, 200 nM, 1 μ M and 5 μ M imatinib) overnight. The next day, the cells were pelleted twice and the resulting clarified culture supernatant stored at -70°C prior to analysis. This was performed at 2 different time points to obtain 2 different passage samples for comparison.

The Human Cytokine/Chemokine Magnetic Bead Panel, a multiplex bead array by Millipore (Billerica, Massachusetts, USA) which uses the Luminex xMAP[®] technology was used for detection of cytokines and used according to the manufacturer's instructions (Chapter 2, Section 2.5.13). The beads were selected to detect FGF-2, G-CSF, GM-CSF, MDC, IL-1 α , IL-1 β , IL-1RA, IL-3, IL-6, IL-8, MCP-1, MIP-1 α , MIP-1 β and TNF- α based on previous cytokine profiling performed by others (described in Chapter 3, Section 3.3.2). The samples were read on the Bioplex200[™] analyser (Bio-Rad, Hercules, California, USA) and results analysed with xPONENT software (Invitrogen Life Technologies).

4.3. Results

4.3.1. K562-T315I cells are more viable in the presence of TKI

To initiate this project, the hypothesis that cells with the T315I mutation survived and proliferated better in the presence compared to the absence of TKI was first tested. K562-T315I cells were cultured with and without TKI and their viability assessed by using the trypan blue exclusion assay and also by 3 day Annexin V and 7AAD death assay.

K562-T315I cells were more viable in TKI compared to without TKI as shown by Figure 4-1. K562 naïve cells were actually more viable compared to K562-T315I cells when cultured without TKI. However, as expected K562 naïve cells were inhibited by TKI treatment (results not shown). K562-T315I cells were more viable in all doses of TKI compared to those without TKI but some did not reach statistical significance. Cells cultured with 200 nM dasatinib would be expected to be more viable due to the fact that the cells were maintained in 200 nM dasatinib so as to prevent the mutational load from deteriorating. Although the cells showed significantly improved survival with direct visualisation using trypan blue exclusion dye (the cells have expanded to 9×10^5 cells/ml after 3 days of culture compared to only 6×10^5 cells/ml for the untreated cells) (Figure 4.1A), unexpectedly, results with the death assay did not reach statistical significance (Figure 4-1B). Cells cultured with 200 nM imatinib as assessed by trypan blue dye (Figure 4-1A) did not show a significant difference compared to cells cultured without TKI. This is not unexpected as the peak therapeutic dose of imatinib is much higher and 200 nM may not be of optimal potency (266). However, at this dose, it did show a significant trend to improve survival with the death assay (Figure 4-1B). Normally, the two assays complement each other and often demonstrate the same trend, but results are not identical and interpretation should take into account both assays.

Next, to corroborate these findings the proliferation index of K562-T315I cells cultured with TKI by staining with CFSE and Ki-67 were performed.

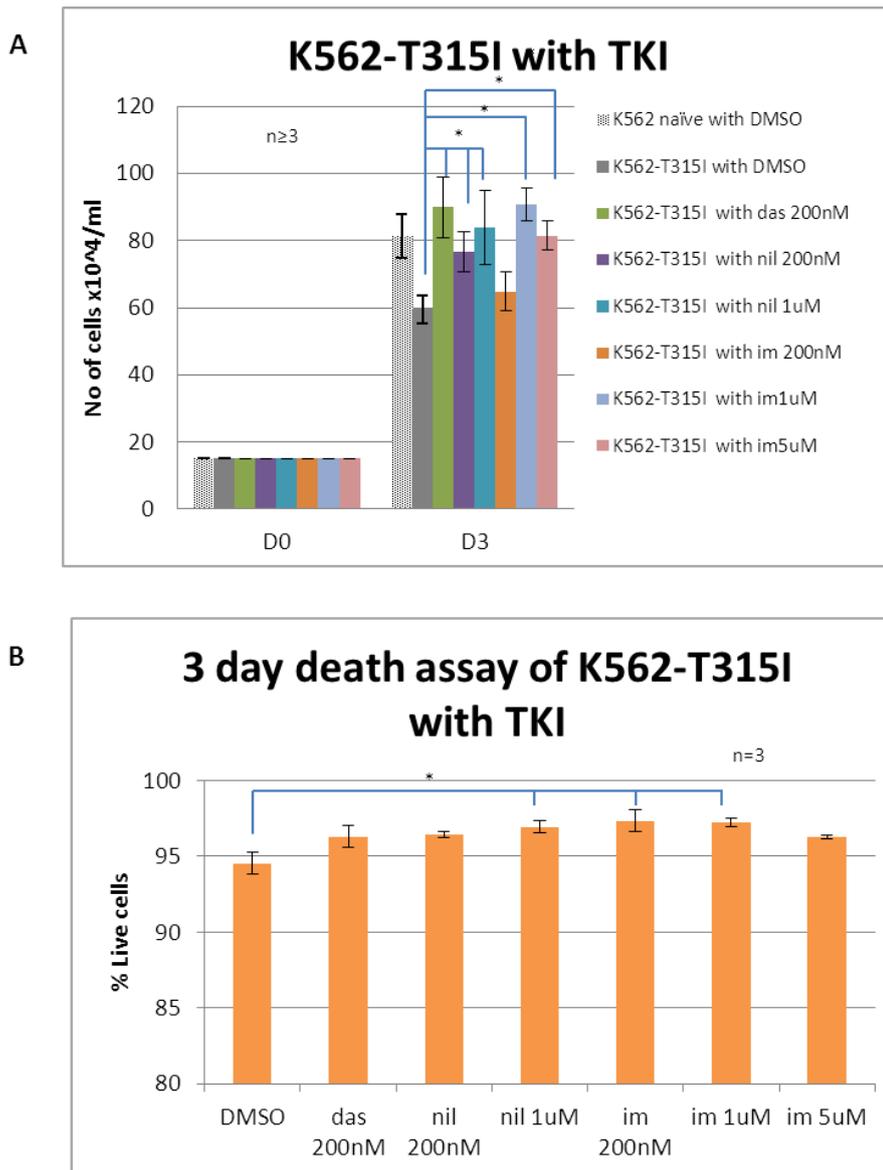


Figure 4-1 K562-T315I cells are more viable when cultured with TKI

After optimal wash, 1.5×10^5 cells/ml were resuspended in SDM and 5 ml were cultured per condition in T25 flasks. The cells were treated with the relevant concentrations of TKI and were incubated at $37^\circ\text{C}/5\% \text{CO}_2$. At Day 3, the cells were harvested and assessed by (A) trypan blue exclusion dye and (B) staining for Annexin V and 7AAD. Cells negative for both stains were considered to be live cells. Compared to untreated cells, K562-T315I cells showed increased viability after 3 days when cultured with TKI in serum poor condition. The experiments were performed at least 3 times and the data are the means \pm standard error of the mean (SEM). Significance values * $p < 0.05$ were calculated using the Student's t test.

4.3.2. K562-T315I cells do not appear to have a greater proliferation index when cultured with TKI when assessed with CFSE staining

CFSE (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen) is used as a cell-tracing fluorescent reagent. It passively diffuses into cells and is retained in the cells throughout cell development. On cell division, the daughter cells inherit half the dye each and thus with subsequent division, the dye is further diminished and allows the number of cell divisions to be tracked. CFSE is initially non-fluorescent but on entering the cells, the acetate groups are rapidly removed by esterases found in the cells to yield highly fluorescent compound which forms covalent bonds with amino groups and can be detected by flow cytometry.

K562-T315I cells both untreated and those treated with dasatinib, nilotinib and imatinib have divided over 4 generations over 3 days as shown in the proliferation histograms (Figure 4-2). However, in the untreated cells (Figure 4-2A), some of the cells have not undergone division (undivided cells represented by the green peak) whereas all the TKI treated cells have. This may account for the lower number of K562-T315I cells when not cultured in TKI as detected on trypan blue assay. In the dasatinib treated cells (Figure 4-2B), more cells have proliferated to Generation 4 compared to the untreated cells, whereas for cells treated with nilotinib and imatinib (Figures 4-2C and 4-2D), although all the cells have divided, most have only proliferated to Generation 3 (light green peaks).

Proliferation index (PI) is the average number of cells that an initial cell generated and is a useful value to compare from sample to sample as it only considers the fraction of the responding cells (267). PI was generated from the proliferation histograms by the DeNovo software and the means of 3 experiments represented in Figure 4-3 shows that there was no significant difference between PI of K562-T315I cells untreated or treated with TKI.

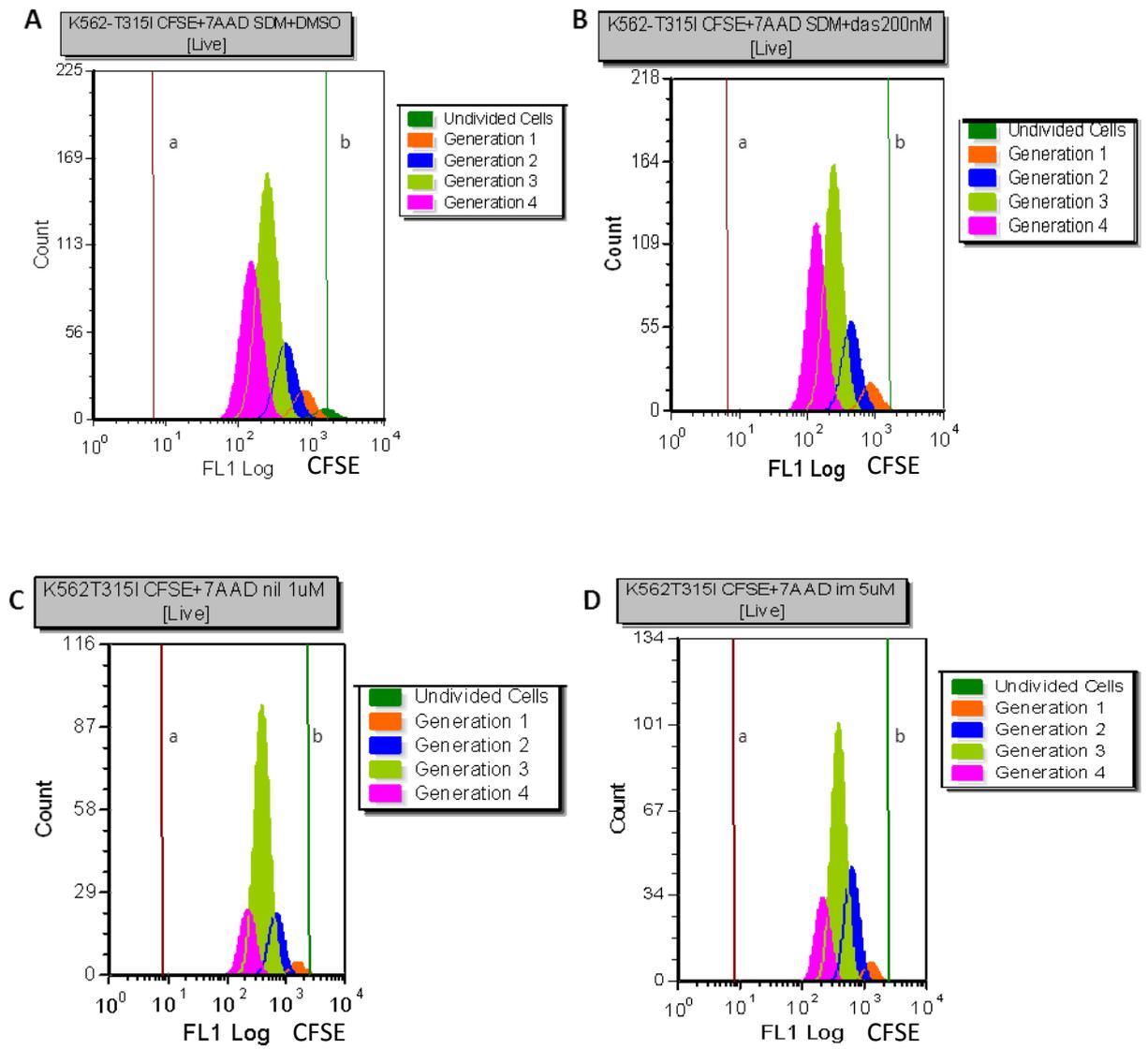
Figure 4-2 K562-T315I cells cultured in SDM over 3 days in the presence of TKI continue to divide.

K562-T315I cells were stained with CFSE, sorted through a narrow range of CFSE fluorescence and then plated at a concentration of 1×10^5 cell/ml and cultured in SDM over 3 days at 37°C/5% CO₂ with A) DMSO (control) B) 200 nM dasatinib, C) 1 μM nilotinib and D) 5 μM imatinib. The red line a) represents background fluorescence of control unstained cells and the green line b) CFSE expression of K562-T315I cells stained and cultured in colcemid which inhibits cell division and represents the undivided cells. Only live cells were selected for analysis by excluding 7AAD stained cells (not shown).

K562-T315I cells both untreated and treated with TKI have divided into 4 generations after 3 days of culture in SDM, indicating that by this method, K562-T315I cells did not have increased proliferation in the presence of TKI.

The experiment was performed at least three times and the plots are representative of the three experiments.

Figure 4-2



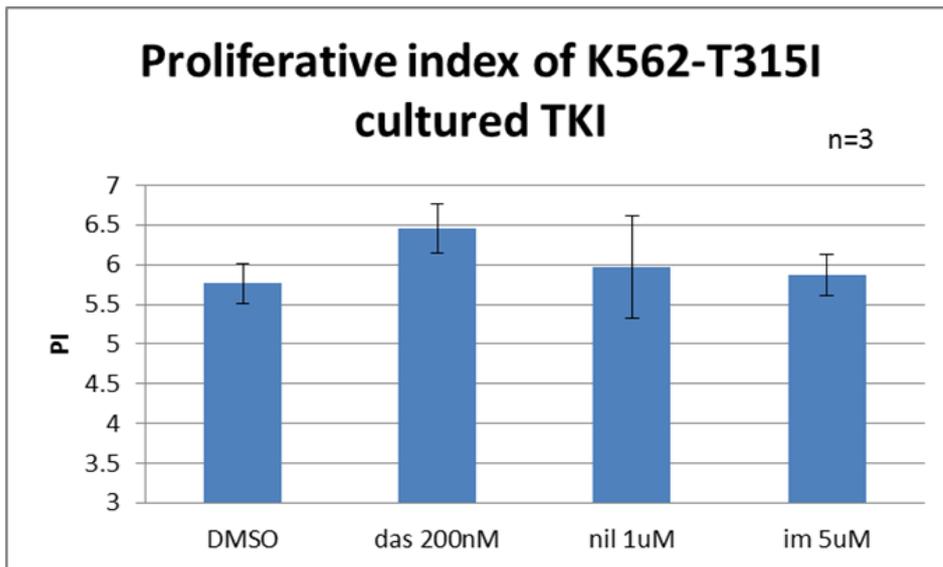


Figure 4-3 Proliferation index (PI) of K562-T315I cells untreated or cultured in TKI is not significantly different

Graphical representation of PI as generated by proliferation histograms and calculated using the FC Express 4 software (DeNovo Software). Only live cells were selected for analysis by excluding 7AAD stained cells.

Proliferation index is defined as the average number of cells that an initial cell generated and thus shows that it was not significantly different in K562-T315I cells cultured for 3 days in SDM with or without TKI.

The experiment was performed 3 times and the data are means \pm SEM.

4.3.3. Ki-67 expression may be increased in K562-T315I cells when incubated with TKI

Ki-67 is a nuclear non-histone protein which varies in intensity throughout the cell cycle. The level is low in early G1 and S phase and increases to a maximum during mitosis. Therefore, it can be used as a marker of cell proliferation (268, 269).

Ki-67 expression was decreased in K562 naïve cells when treated with TKI for 3 days (Figures 4-4). As summarised in Figure 4-4B, compared to the untreated cells, in the presence of TKI, Ki-67 expression in K562 naïve cells was decreased to approximately 40% of the untreated cells except for imatinib. This may be a reflection of the concentrations of TKI used. However, K562-T315I when exposed to TKI showed either similar or even increased expression of Ki-67 compared to the untreated cells (Figures 4-5A to 4-5D) except for ponatinib which is known to be an effective kinase inhibitor against *BCR-ABL1*^{T315I} cells. 40 nM ponatinib reduced Ki-67 expression to 60% compared to the untreated K562-T315I cells but 10 nM ponatinib was too low a concentration to affect cellular proliferation (Figure 4-5D). This is summarised in Figure 4-5E. The histograms also show two peaks for the expression of Ki-67 which may indicate the heterogeneous nature of the cell line. This will be discussed later.

Next, the rationale for the increase in survival and proliferation of cells harbouring the T315I mutation when exposed to TKI was explored. Hartel *et al* and Packer *et al* demonstrated that it is through activation of the MAP kinase pathway (208, 209). Other signalling pathways that could also be involved were explored in this study.

Figure 4-4 Ki-67 expression of K562 naïve cells is reduced when treated with TKI

K562 naïve cells (3 ml) resuspended in SDM at a concentration of 1×10^5 cells/ml was plated into each well per condition in a 12-well plate and incubated for 3 days with either 10 nM dasatinib, 150 nM nilotinib, 1.5 μ M imatinib, 5 nM ponatinib or DMSO (as vehicle control). After the incubation period, the cells were then fixed, permeated and stained with Ki-67-PE antibody (Cell Signaling) or the appropriate isotype control. The cells were analysed on flow cytometer.

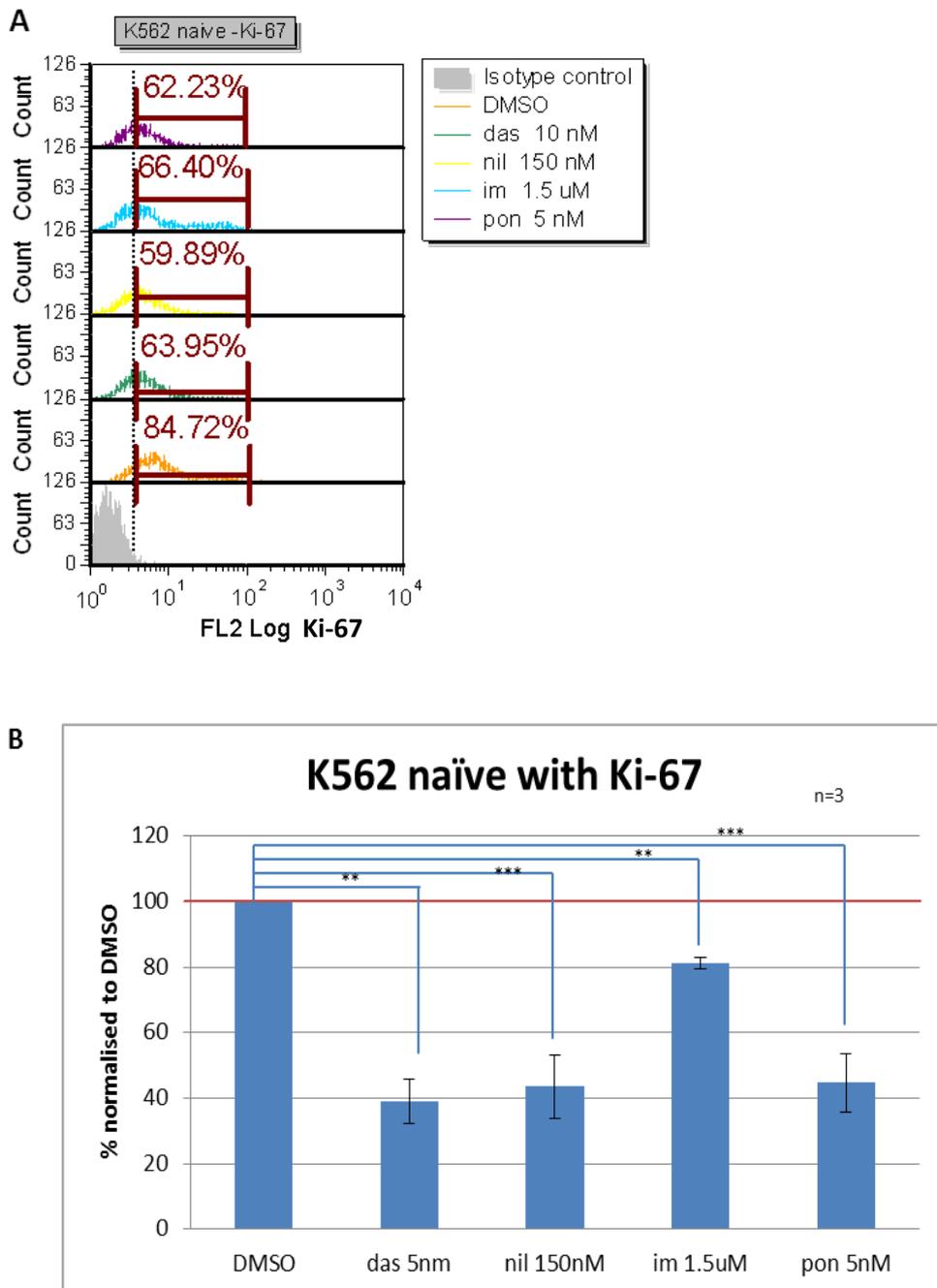
A) Stacked histograms of Ki-67 expression in K562 naïve cells. The red markers indicate expression of the fluorochrome (PE) as measured from the expression of the isotype control cells (as shown by the vertical dashed line). The experiment was performed 3 times and the histograms are a representative example of the 3 experiments.

B) Graphical representation of Ki-67 expression in K562 naïve cells. The red line represents the expression of Ki-67 on cells cultured with DMSO.

Compared to untreated cells, K562 naïve cells treated with dasatinib, nilotinib, imatinib and ponatinib showed significantly reduced Ki-67 expression.

The experiment was performed 3 times and the results shown are means \pm SEM. Significance values * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ were calculated using the Student's t test.

Figure 4-4



Figures 4-5 Proliferation of K562-T315I may be increased when incubated with dasatinib, nilotinib and imatinib but not with ponatinib as shown by Ki-67 expression.

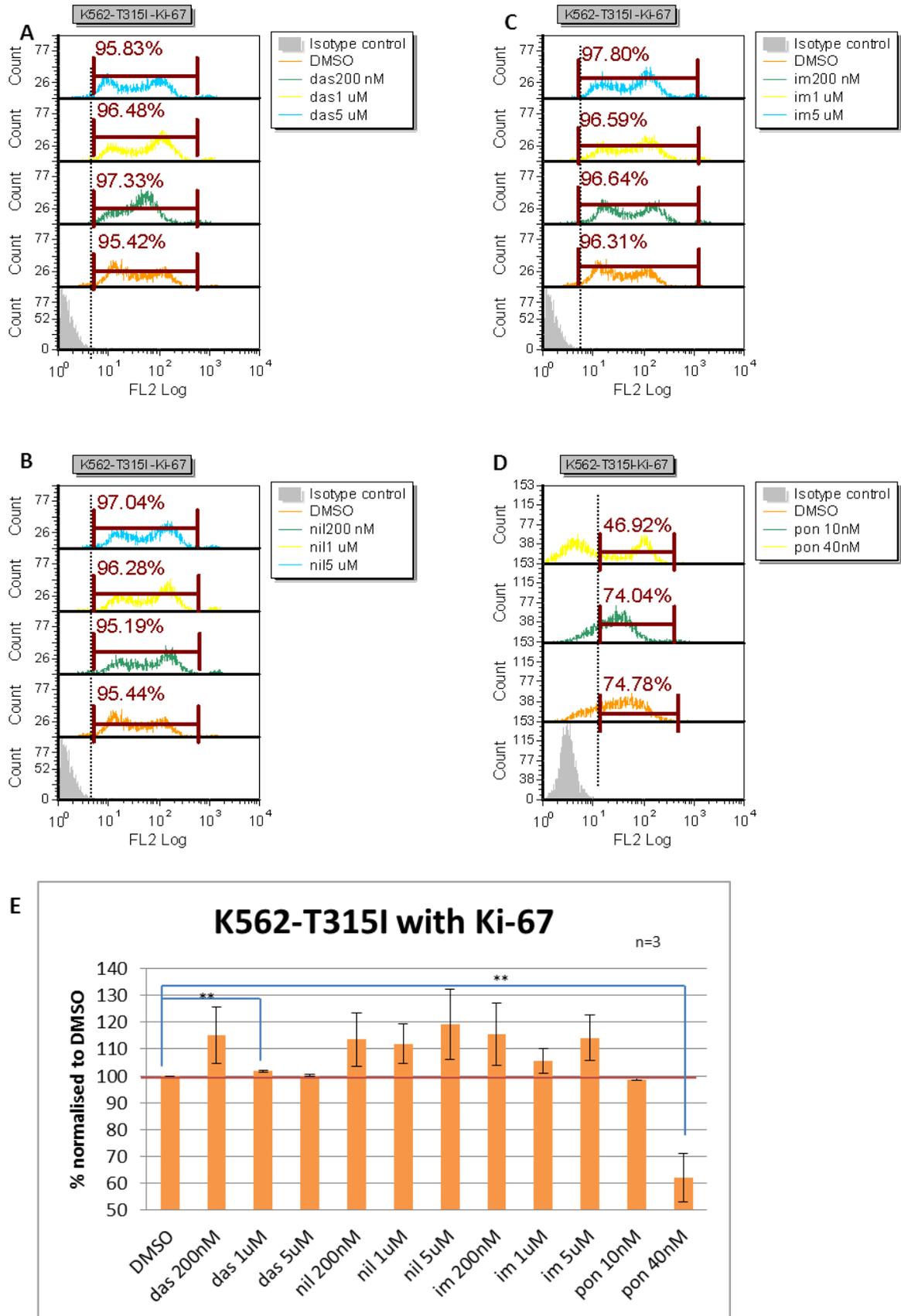
K562-T315I cells were optimally washed and resuspended in SDM at 1×10^5 cells/ml and 3 ml per condition were plated into a 12 well plate. The cells were cultured for 3 days at 37°C/5% CO₂ with A) 200 nM, 1 µM, 5 µM dasatinib, B) 200 nM, 1 µM, 5 µM nilotinib, C) 200 nM, 1 µM, 5 µM imatinib D) 10 nM, 40nM ponatinib or DMSO (control). After the period of incubation, the cells were fixed, permeated and then stained with Ki-67 antibody or appropriate isotype control. (A-D) The red markers indicate expression of the fluorochrome (PE) as measured from the expression of the isotype control cells (as shown by the dashed line). The experiment was performed 3 times and the histograms are a representative example.

E) Graphical representation of Ki-67 expression in K562-T315I cells. The red line represents the expression of Ki-67 in K562-T315I cells treated with DMSO as the control.

Compared to untreated cells, K562-T315I cells showed a similar or even increased expression of Ki-67 after 3 days of culture with dasatinib, nilotinib and imatinib but demonstrated reduction of expression when treated with ponatinib. Ki-67 expression was significantly decreased with 40 nM ponatinib.

The experiments were performed 3 times and the data shown are the means of the 3 experiments. Significance value ** $p < 0.01$ was calculated using the Student's t test.

Figure 4-5



4.3.4. pErk signalling is increased in K562-T315I cells when cultured with TKI compared to untreated

The major MAP kinase signalling pathway is through p44/42 MAPK, also known as Erk1/2 which are the key mediators of proliferation, differentiation and survival. Erk1/2 are activated by MEK1/2 which are phosphorylated by the Raf family of protein kinases composed of A-Raf, B-Raf and C-Raf (45). Therefore, phosphorylation of Erk1/2 is used as the surrogate of MAP kinase activation.

K562 naïve and K562-T315I cells were incubated overnight with TKI in SDM and western blotting was performed the next day. As shown in Figure 4-6, imatinib, nilotinib and dasatinib blocked pErk signalling in K562 naïve cells. However, compared to the untreated cells, pErk expression in K562-T315I cells exposed to dasatinib, nilotinib and imatinib showed increased expression (Figure 4-6) and hence indicates that pErk signalling in these cells in the presence of TKI was hyperactivated. This phenomenon was best shown with nilotinib. This suggests that increased proliferation of K562-T315I cells in the presence of TKI may be mediated through enhanced signalling of Erk. Interestingly, untreated K562-T315I cells showed weaker pErk expression compared to untreated K562 naïve cells (Figure 4-6A) and this was also observed by others (209).

4.3.5. pSTAT5 expression is not increased in K562-T315I compared to K562 naïve cells when cultured with TKI

To determine if other pathways downstream of Bcr-Abl may also be activated, phosphorylation of STAT5 was also investigated by western blotting. Figures 4-7 indicate that imatinib, nilotinib and dasatinib block STAT5 signalling in K562 naïve cells. K562-T315I cells are resistant to TKI treatment and therefore expected to show full expression of pSTAT5 even when incubated with TKI. However, as shown in Figures 4-7, pSTAT5 expression was actually reduced with TKI. This can be explained by the heterogeneous nature of the K562-T315I cell line. It contained only approximately 45% of the mutant transcripts (refer Figure 3-2) which means either 55% of the cell line does not have the mutation or some cells have more copy of the mutant transcripts.

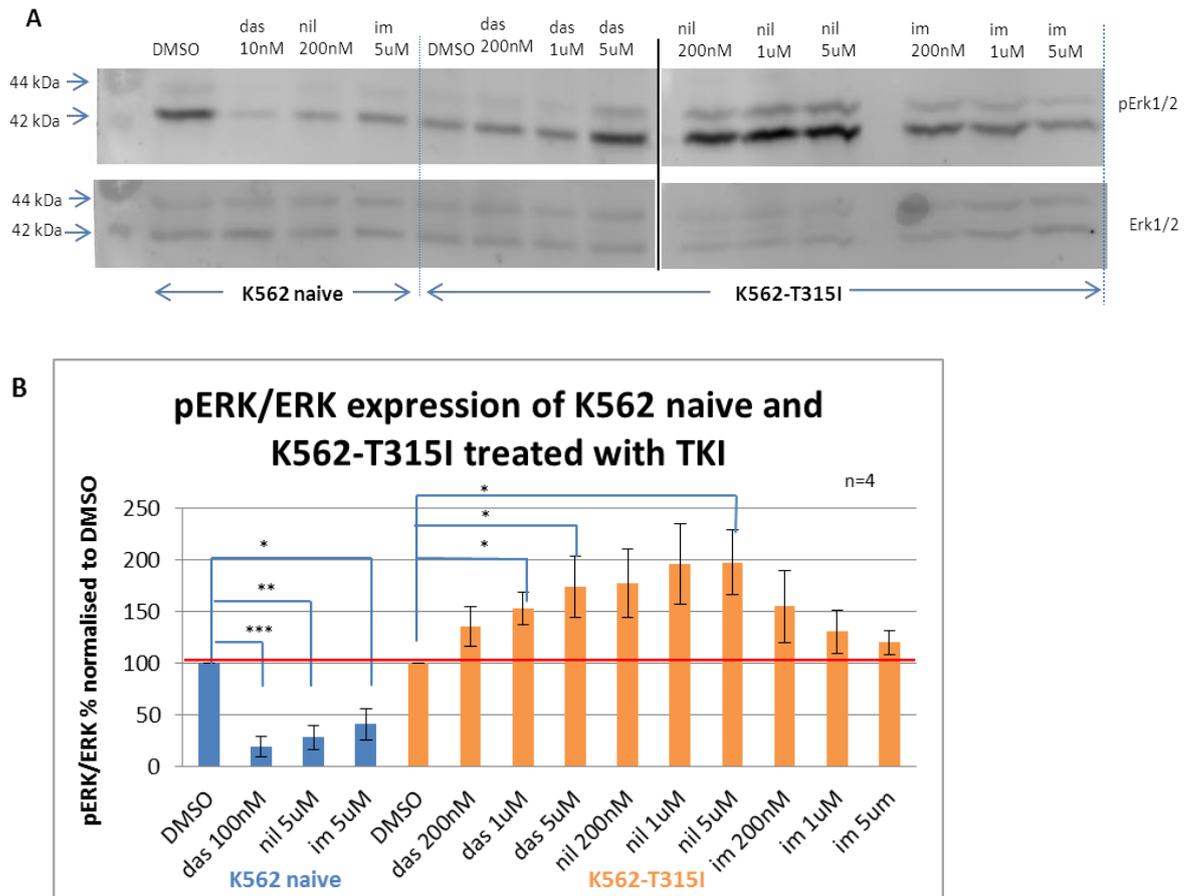


Figure 4-6 pErk expression is reduced in K562 naïve but paradoxically increased in K562-T315I cells when incubated with TKI

After optimal wash, cells were resuspended at 2×10^5 cells/ml in SDM and plated at 2 ml per condition in a 24-well plate with overnight incubation at $37^\circ\text{C}/5\% \text{CO}_2$ with DMSO (control), dasatinib, nilotinib or imatinib at the concentrations shown. A) pErk (T202/T204) and Erk expression of K562 naïve and K562-T315I was assessed by western blot analysis. kDa-kilo Dalton. Bold black vertical line denotes the joining of 2 separate blots.

B) Graphical representation of mean densitometry measurements of pErk/Erk expression normalised to DMSO. The red line denotes % pErk/Erk expression of K562 naïve and K562-T315I cells cultured overnight with DMSO and normalised to 100%.

Compared to untreated K562 naïve cells, TKI treatment significantly reduced pErk expression but in K562-T315I cells, pErk expression was increased with TKI treatment.

Significant values * represent $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as calculated by Student's t-test. This is representative of 4 experiments and data are means \pm SEM.

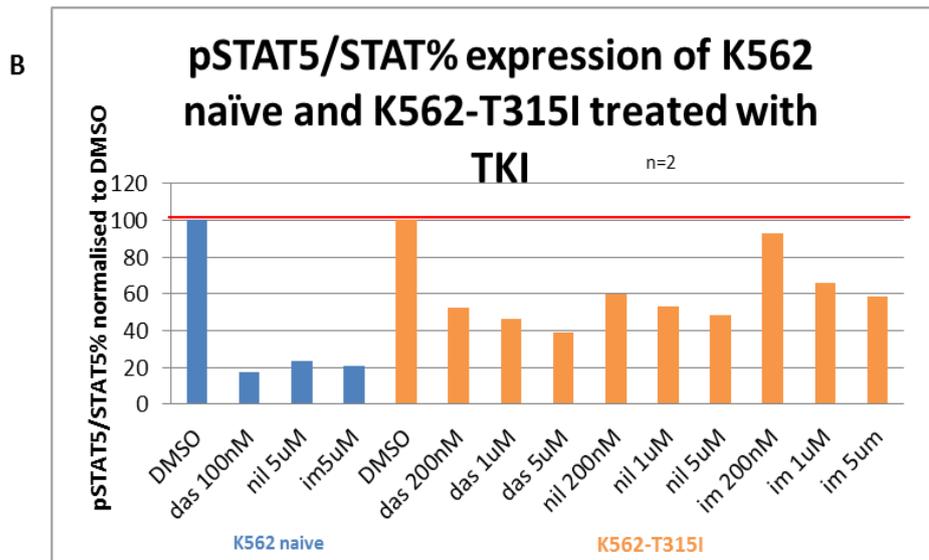
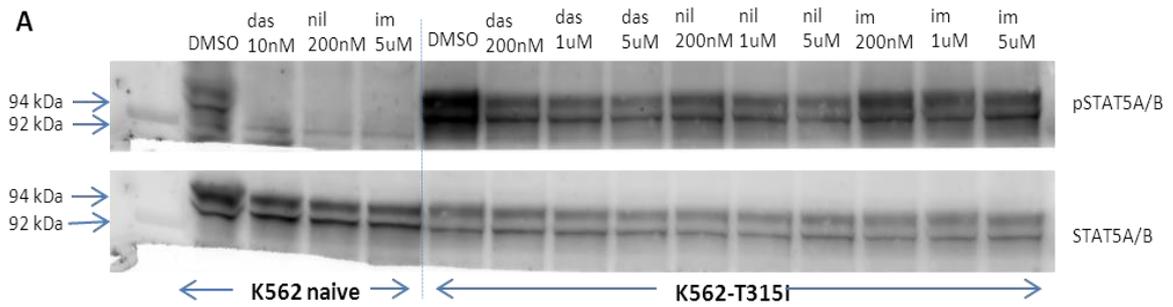


Figure 4-7 pSTAT5 expression is reduced in both K562 naïve and K562-T315I cells when incubated with TKI

After optimal wash, cells were resuspended at 2×10^5 cells/ml and plated at 2 ml per condition in a 24-well plate with DMSO (control), dasatinib, nilotinib or imatinib at the concentrations as shown and incubated overnight at $37^\circ\text{C}/5\%\text{CO}_2$. A) pSTAT5 (T202/T204) and STAT5 (Y694) expression of K562 naïve and K562-T315I was assessed by western blot analysis. kDa- kiloDalton.

B) Graphical representation of mean densitometry measurements of pSTAT5/STAT5 normalised to DMSO. The red line denotes % pSTAT5/STAT5 expression of K562 naïve and K562-T315I cells cultured overnight with DMSO and normalised to 100%.

Both K562 naïve and K562-T315I cells showed decrease pSTAT5 expression with overnight TKI treatment compared to the untreated cells. K562 naïve cells were more sensitive to the effects as a similar concentration of TKI resulted in a greater reduction in pSTAT5 expression. This data are the means of 2 experiments.

This means that not all cells in this cell line have the same resistance to TKI. Hence overall, pSTAT5 was partly reduced with TKI except with 200 nM imatinib which showed nearly equal pSTAT5 expression compared to the untreated cells. This is because 200 nM imatinib is below the peak therapeutic plasma level, which is 5 µM (266). However, compared to the K562 naïve cells, TKI treatment resulted in less inhibition of STAT5 signalling in K562-T315I cells as similar concentrations of TKI resulted in less reduction in pSTAT5.

4.3.6. pAkt expression is decreased in K562-T315I when cultured in TKI compared to untreated

Attempts to investigate the effects of TKI on pAKT expression in K562 naïve and K562-T315I cells by western blotting were unsuccessful. Therefore, intracellular flow cytometry was used to determine the expression of this protein under the same conditions as previously described. As shown in Figure 4-8A, pAkt expression was reduced compared to control in K562 naïve cells when treated with TKI. Cells treated with TKI showed a reduction of pAkt expression of 57.3%, 58.3%, 78.7% and 64.5% respectively for dasatinib, nilotinib, imatinib and ponatinib compared to the control cells. Like pSTAT5, the pAkt expression was also predicted to be unchanged in K562-T315I cells with TKI compared to the control, but instead it appeared to be reduced to between 77%-90% relative to the untreated cells (Figure 4-9B to 4-9D). This may also be explained by fact that the K562-T315I cells do not harbour 100% of the mutation.

4.3.7. Cytokine profile of the supernatant of cells harbouring the T315I mutation when cultured in SDM with TKI

So far, K562-T315I cells were determined to have increase survival and proliferation in TKI and that this may be mediated through the MAP kinase pathway as Erk signalling was increased in these cells when cultured with TKI. Next, cytokine and growth factor levels were investigated to determine if an increased production of these factors may play a role.

K562-T315I and HL60 *BCR-ABL1*^{T315I} cells were cultured in various concentrations of TKI, the cell supernatant collected and the concentrations of various growth factors and cytokines determined. The growth factors and cytokines chosen were based on an earlier experiment carried out by others in the laboratory prior to the start of this project as described in Chapter 3. The HL60 cell line as previously described in Chapter 2, Section 2.3.2, had been

previously transduced with *BCR-ABL1* retroviral constructs containing *BCR-ABL1*^{p210} (results in cells expressing *BCR-ABL1*^{WT}) and named HL60 *BCR-ABL1*^{p210} or *BCR-ABL1*^{T315I} (results in cells expressing *BCR-ABL1*^{T315I}) and named HL60 *BCR-ABL1*^{T315I}. Hence, in order to be more confident of the results of cytokine profiling, two cells lines with *BCR-ABL1*^{T315I} were utilized.

The supernatant of K562-T315I (Table 4-1A) and HL60 *BCR-ABL1*^{T315I} (Table 4-1B) cells cultured in various concentrations of TKI did not consistently show different concentration of a cytokine/cytokines over the supernatant of the cells cultured without TKI as measured by Luminex xMAP® technology.

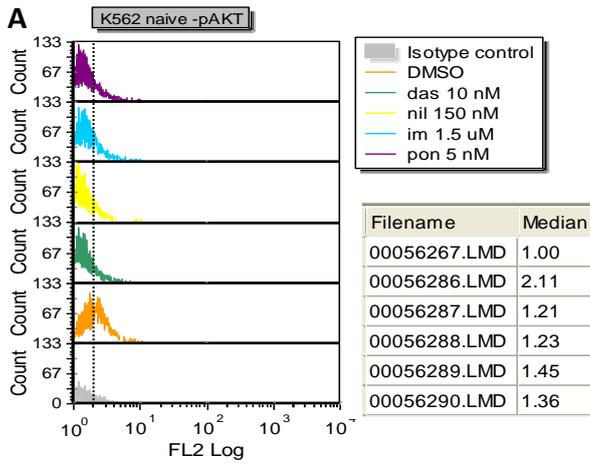
For example, MIP-1α was found in highest concentration in the supernatant of K562-T315I cells incubated with 5 μM imatinib but the supernatant of the cells incubated with 200 nM or 1 μM imatinib did not show a similar increase over the supernatant of the untreated cells (Table 4-1A). More importantly, the supernatant of HL60 *BCR-ABL1*^{T315I} cells cultured in 5 μM imatinib did not demonstrate any expression of MIP-1α (Table 4-1B). Likewise, IL-6 was highly expressed by HL60 *BCR-ABL1*^{T315I} cells when cultured with 5 μM imatinib (Table 4-1B). However, it was found expressed highest in the supernatant of K562-T315I cells in DMSO (Table 4-1A). Similarly, GM-CSF which was increased in the supernatant of HL60 *BCR-ABL1*^{T315I} cells cultured in 5 μM imatinib, did not demonstrate a similar change in the supernatant of K562-T315I cells. Interestingly, concentrations of several cytokines and growth factors appeared to be highest in either the supernatant of 5 μM imatinib-treated or the untreated *BCR-ABL1*^{T315I} cells but none of factors appear to overlap between the 2 cell lines. For example, FGF-2, GM-CSF and IL-6 were produced in excess by the 5 μM imatinib-treated HL60 *BCR-ABL1*^{T315I} (refer Table 4-1B) whereas the K562-T315I under the same condition overproduced IL-8, MCP-1, MIP-1α, MIP-1β, G-CSF and TNF-α (refer Table 4-1A). More disappointingly, no consistent change was observed with FGF-2. The concentration of this growth factor was seemingly increased in the supernatant of HL60 *BCR-ABL1*^{T315I} cells cultured in 5 μM imatinib but no significant change was observed in the supernatant of K562-T315I cells untreated or treated with TKI. FGF-2 was found in Chapter 3 to be the only growth factor that protected *BCR-ABL1*^{WT} from TKI-induced cell death. Therefore, there does not appear to be a consistent pattern to support the hypothesis that *BCR-ABL1*^{T315I} cells secrete more of the selected cytokines/growth factors when cultured with a TKI.

Figure 4-8 pAkt expression is reduced in K562 naïve and may be reduced in K562-T315I cells with TKI

After optimal wash, 1×10^5 cells/ml were resuspended in SDM and 3 ml each were plated in a 12 well plate. The cells were incubated overnight at 37°C/5% CO₂ either untreated or with the appropriate reagents- A) K562 naïve cells with DMSO, 10 nM dasatinib, 150 nM nilotinib, 1.5 µM imatinib and 5 nM ponatinib B) K562-T315I cells with DMSO, 200 nM, 1 µM and 5 µM dasatinib, C) K562-T315I cells with 200 nM, 1 µM and 5 µM nilotinib and D) K562-T315I cells with 200 nM, 1 µM and 5 µM imatinib. The next day, the cells were fixed, permeabilized and stained with pAkt antibodies (Cell Signaling) or the appropriate isotype controls and analysed by flow cytometry. Results are displayed as overlaid signal peak traces of isotype control and cells treated with TKI as stated. Black dashed line represents peak signal in control cells. Results are expressed as MFI normalised to the control cells.

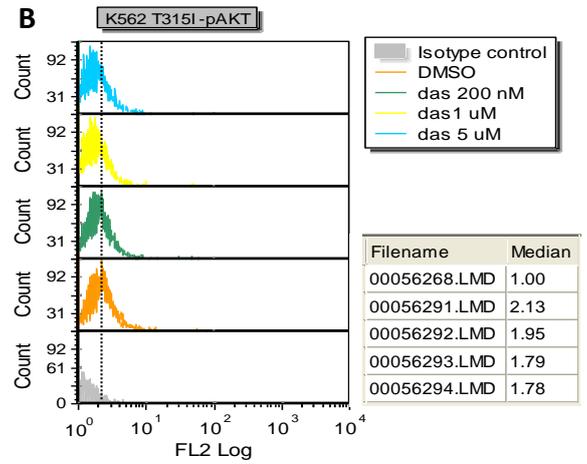
Compared to the untreated cells, the pAkt expression in the K562 naïve cells was reduced when treated with TKI. pAkt may also be reduced in the K562-T315I cells, although it was not inhibited to the same extent. Experiments were performed 2 times and histograms are a representative example.

Figure 4-8



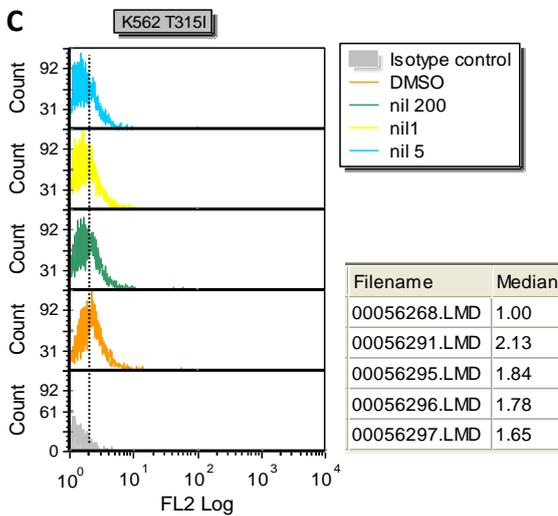
K562 naïve

MFI	pAkt
DMSO	2.11 (100%)
das 10 nM	1.21 (57.3%)
nil 150 nM	1.23 (58.3%)
im 1.5 μ M	1.45 (78.7%)
pon 5 nM	1.36 (64.5%)



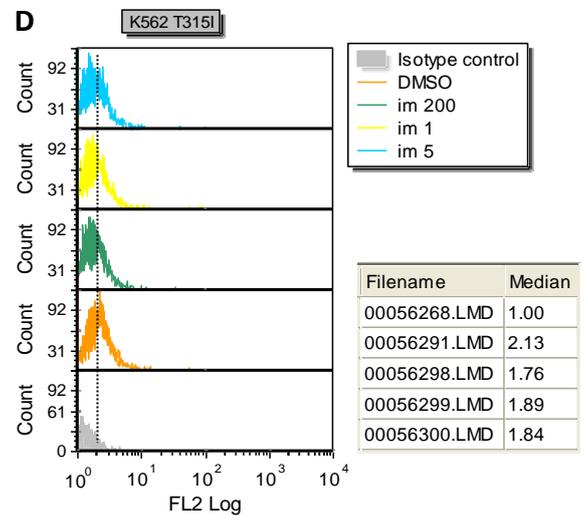
K562-T315I

MFI	pAkt
DMSO	2.13 (100%)
das 200 nM	1.95 (91.5%)
das 1 μ M	1.79 (84%)
das 5 μ M	1.78 (83.6%)



K562-T315I

MFI	pAkt
DMSO	2.13 (100%)
nil 200 nM	1.84 (86.4%)
nil 1 μ M	1.78 (83.6%)
nil 5 μ M	1.65 (77.5%)



K562-T315I

MFI	pAkt
DMSO	2.13 (100%)
im 200 nM	1.76 (82.6%)
im 1 μ M	1.89 (88.7%)
im 5 μ M	1.84 (86.4%)

Table 4-1 Cytokine profile of supernatant of cells with the T315I mutation are not consistent when cultured with or without TKI

After optimal wash, 5×10^5 cells/ml were cultured overnight in SDM with either DMSO as control or relevant concentrations of TKI. After overnight incubation, the cells were centrifuged twice and the resulting clarified supernatant was analysed in duplicate using Luminex xMAP® technology. Data was acquired on Bioplex200™ (BioRad) results were analysed with xPONENT software (Life Technologies) and expressed as the average of the duplicates in pg/ml for the A) K562-T315I and B) HL60 *BCR-ABL1*^{T315I} cells. Areas highlighted in yellow show the highest concentration within the cell line.

Table 4-1**A**

K562-T315I	FGF-2	GM-CSF	MDC	IL-6	IL-8	MCP-1	MIP-1 α	MIP-1 β	G-CSF	TNF- α
DMSO	20.17	7.8	3.8	1025.29	1147.02	65.04	0	3.75	43.59	0
Das10nM	21.2	3.51	3.17	267.38	158.31	21.22	0	0	29.48	0
Das200nM	14.78	2.55	3.8	152.21	258.86	13.33	0	0	26.18	0
Nil200nM	17.43	5.36	2.05	348.71	236.8	17.71	0	0	32.57	0
Nil1 μ M	23.5	3.92	3.17	341.19	205.72	19.18	0	0	35.91	0
Im200nM	16.81	6.35	4.09	862.01	787.6	36.33	0	4.49	33.6	0
Im1 μ M	16.49	3.74	2.05	710.18	465.32	23.19	0	0	28.59	0
Im5 μ M	13.21	1.04	4.37	681.42	2606.5	118.46	45.11	61.86	109.9	118.08

B

HL60 T315I	FGF-2	GM-CSF	MDC	IL-6	IL-8	MCP-1	MIP-1 α	MIP-1 β	G-CSF	TNF- α
DMSO	14.41	0.9	5.16	1.8	5370.53	218.54	55.93	86.14	119.89	155.23
Das10nM	8.95	0	3.49	0.55	4378.35	154.38	38.33	52.44	105.28	91.72
Das200nM	6.16	0	3.8	0.44	4017.23	172.04	36.28	55.18	98.1	92.74
Nil200nM	12.33	1.01	4.9	1.49	4450	203.94	45.02	66.34	111.79	132.82
Nil1 μ M	9.63	0	4.37	1.4	4134.77	221.19	39.33	69.77	112.67	76.18
Im200nM	8.18	0	3.49	0.94	4831.04	192.11	41.95	60.49	139.13	98.37
Im1 μ M	8.18	0	2.46	0.33	4937.13	194.6	43.14	57.54	113.74	101.81
Im5 μ M	27.73	7.31	3.8	552.5	448.2	33.06	0	6.08	48.33	0

4.4. Discussion

The T315I mutation is thought to be a gain-of-function mutation as it has been shown *in-vitro* by some to have greater transformation potential and kinase activity compared to *BCR-ABL1^{WT}* (90, 91, 181). These observations are supported clinically by several series. A French study of imatinib resistant patients showed that the median overall survival of patients in all phases of CML with the T315I mutation was significantly shorter compared to those with other mutations (270). Another study confirmed the findings in which matched pairs of patients resistant to imatinib in chronic phase (CP-CML) with or without the T315I mutation were compared (187). The patients were matched for age at diagnosis, interval between diagnosis and commencement of imatinib and duration of treatment with imatinib and showed that patients with the T315I mutation had significantly poorer overall survival and failure free survival (187). Moreover, the T315I mutation is also the most commonly found mutation in patients who have progressed to blast crisis (BC), both in lymphoid BC and myeloid BC (235).

It has been shown that cells harbouring the T315I mutation have markedly increased MAP kinase activation when exposed to a TKI (208, 209). The mechanism of this phenomenon is unknown. Packer *et al* postulated that imatinib, nilotinib and dasatinib possess weak B-Raf inhibitory activity (209). The B-Raf inhibitor, vemurafenib, results in dramatic response in melanoma patients with the *BRAF* mutation, V600E but not in patients with *BRAF^{WT}* (271). However, vemurafenib revealed an unexpected paradox if it were to be given to a patient with both *BRAF^{WT}* and mutated *RAS*. It paradoxically enhanced signalling via MEK and Erk worsening tumour development and results in secondary cutaneous squamous cell carcinoma development (212-214, 272).

The mechanism of enhanced MAP kinase signalling is incompletely understood but it is thought to be driven by formation of B-Raf-C-Raf heterodimers and signalling through C-Raf (213, 214). There are 2 main models for this phenomenon. When Ras is activated, B-Raf shuttles to the cell membrane and is phosphorylated. It can either form B-Raf-B-Raf homodimers or heterodimers with C-Raf (273). MEK and Erk are activated downstream of Ras and Raf promoting survival and proliferation in tumours (274). Mutated *BRAF*, V600E, preferentially causes homodimerization therefore vemurafenib inhibits mutant B-Raf activity (273). The first model proposed by Polikakos *et al* suggests that in tumours with *RAS*

mutation, *BRAF*^{WT} forms heterodimers with C-Raf (214). Although the B-Raf half of the heterodimer is inhibited by a B-Raf inhibitor, transactivation still occurs through C-Raf and hence MEK and Erk phosphorylation (214). The second model suggests that in *RAS* mutated cells, B-Raf is normally inhibited (213). However, in the presence of a B-Raf inhibitor, it escapes this auto-inhibited state and translocates to the plasma membrane and forms a complex with C-Raf. This results in C-Raf hyperactivation and hence downstream MEK and Erk signalling. Using a pan-Raf inhibitor, sorafenib, which blocks both B-Raf and C-Raf activity, the authors showed that Erk phosphorylation can be blocked in *RAS* mutated cells proving that signalling through C-Raf is crucial (213).

Compensatory activation of the MAP kinase pathway is reported in TKI treated CML CD34+ cells which is cytokine dependent (49, 198). It is thought that cytokine-mediated downstream signalling may bypass Bcr-Abl inhibition of CML progenitor cells (49, 81). Conversely, cells harbouring the T315I mutation have markedly increased MAP kinase activation when exposed to TKI even in the absence of added cytokines (208, 275).

MAP kinase activation is also thought to be important for the pathogenesis of the blastic phase of CML (BP-CML). The molecular mechanisms underlying progression to BP-CML is still unknown. BP-CML is associated with high levels of *BCR-ABL1* which results in the constitutive activation of factors with known mitogenic, anti-apoptotic and anti-differentiation activity such as MAP kinase, STAT5, Myc, Bcl-2 and heterogeneous nuclear ribonucleoprotein E2 (hnRNP-E2) (276). Expression of hnRNP-E2, a transcriptional-inducer and translational regulator, is high in BP-CML CD34+ progenitor cells (277). It is known to inhibit CCAAT enhancer-binding protein α (C/EBP α) which leads to inhibition of differentiation of myeloid progenitors (277, 278). The ability of hnRNP-E2 to inhibit C/EBP α depends on the constitutive activation of the MAP kinase pathway which stabilised hnRNP-E2 (279, 280). Constitutive activation of MAP kinase is observed in BP-CML CD34+ cells but not in CP-CML or normal bone marrow CD34+ cells (280). Thus, hyperactivation of the MAP kinase pathway is not only essential for transduction of survival signals but also promotes activation of differentiation arrests seen in BP-CML.

This study sought to investigate if this phenomenon of TKI-induced MAP kinase activation may be due to increased expression of certain cytokines or growth factors by T315I cells when exposed to TKI. This study also aimed to interrogate if other signalling transduction

pathways, namely STAT5 and Akt, are also hyperactivated and contribute to T315I cells survival and expansion in the presence of TKI.

K562-T315I cells were more viable when cultured in TKI by trypan blue exclusion assay and Annexin V and 7AAD death assay and showed a trend towards increased proliferation as assessed by Ki-67 nuclear protein expression. However, with CFSE staining, K562-T315I cells cultured for 3 days in the presence of TKI did not show increased number of daughter generations. Proliferation index (PI) as generated by CFSE was also not significantly increased over untreated cells although in the untreated population, fewer cells have divided. This may be due to the property of the cell line that was used.

The K562-T315I cell line was developed in our laboratory by exposing K562 naïve cells in increasing concentrations of dasatinib over several months (as described in Chapter 2, Section 2.3.1) (238). On Sanger sequencing, the K562-T315I cells contained approximately 45% of the mutated transcripts (Figure 3-2) and was unclear if some cells have no mutated transcripts and expressed other forms of resistance to dasatinib (for example, increase *BCR-ABL1* expression) or if all cells possessed the T315I mutation but at different levels in each cell. If the former is true, then the cell line was heterogeneous and may not respond to TKI treatment uniformly. This was indicated by the double peaks seen when the cells were assessed for Ki-67 expression (Figure 4-5) and may have affected the results of some experiments including the PI of K562-T315I cells cultured with TKI as assessed by CFSE staining (Figure 4-3) and reduction of pSTAT5 and pAkt expression when treated with TKI (Figures 4-7 and 4-8).

Additionally, CFSE labelled cell-tracing is used mainly to track lymphocytes and progenitor cells and is known to be difficult to use in cell lines. Therefore, the use of this method to track proliferation of the K562-T315I cells is not the best assay to use as cell lines are known to have a heterogeneous protein content and are known to give broad levels of CFSE staining making resolution of the divisions difficult (281). To address this, Ki-67 expression was used to determine cell proliferation rate of K562-T315I cells cultured in TKI.

High proliferation rate of a tumour has been linked to tumour aggressiveness and to poor disease outcome (282). Ki-67 is a nuclear protein involved in regulation of cell proliferation and is expressed throughout the cell cycle in proliferating cells but not in quiescent cells (269). High Ki-67 expression is associated with poor prognosis in breast cancer (268) as well

as other malignancies including mantle cell lymphoma (283) and non-small cell lung cancer (284). In this study as expected, K562 naïve cells when exposed to TKI showed less Ki-67 expression compared to the untreated cells as the proliferation of the cells were inhibited by TKI. K562-T315I cells however, had similar or slightly higher Ki-67 expression when treated with TKI compared to untreated cells demonstrating an equal or even higher proliferation rate when exposed to TKI. This would be consistent with the higher number of viable cells visualised on direct counting with trypan blue exclusion.

To ascertain the mechanism by which K562-T315I cells have higher viability and proliferation in TKI, the signalling pathways downstream of Bcr-Abl were studied. Erk, STAT5 and Akt signalling were selected for study as they are the three critical downstream substrates activated by Bcr-Abl (37, 40, 58). Results obtained for western blots (WB) for phospho-protein signalling was variable in that the expression level of phospho-protein showed wide fluctuations when performed over time. This may be due to the heterogeneous nature of the cell line. As previously mentioned, the K562-T315I cell line only contained 45% T315I mutation. If cell lysates used contained a lower level of the T315I mutation, phospho-proteins were able to be inhibited by TKI. Despite this, pErk expression in T315I cells as detected by WB was consistently increased when the cells were exposed to TKI.

pSTAT5 expression should not be inhibited in cell lines harbouring the T315I mutation as it is insensitive to Bcr-Abl inhibition by TKI (285). However, in the K562-T315I cells when treated with TKI, pSTAT5 expression was decreased. WB was also used to determine if pAkt expression of K562-T315I cells was increased when cultured with TKI but was not successful. As a substitute, using phosphoflow, this study did not demonstrate increased expression but instead unchanged or even inhibition of phosphorylation of Akt. As with pSTAT5 expression, Akt phosphorylation was expected to be unchanged in the T315I-expressing cells with TKI (286). Again, this can be explained by the heterogenous nature of the K562-T315I cells. Conversely, Tseng *et al* studied the effect of combining phosphoinositide-dependent kinase 1 inhibitor with imatinib in overcoming imatinib resistance in *BCR-ABL1* positive cells. They demonstrated that in their cell line, the BA/F3p210^{T315I} which carried the T315I mutation, pAkt expression was inhibited by imatinib but large doses were necessary (287). The authors showed that 10 µM imatinib showed a trend towards reduction of pAkt compared to no treatment, 1 µM and 5 µM imatinib by western blotting. However, 20 µM imatinib was

able to reduce pAkt expression down to 20% compared to the untreated cells (287). Although the levels used were far in excess of the clinical peak therapeutic plasma levels of imatinib (5 μ M), this lends support to the findings of this study that pAkt is not increased in *BCR-ABL1*^{T315I} cells when exposed to TKI. Therefore, despite using a cell line that was not ideal, this study still confirmed that pErk expression was increased in K562-T315I cells when cultured with TKI especially with nilotinib. This suggests that in the presence of TKI, cells harbouring the T315I mutation have enhanced signalling through Erk and the MAPK pathway which may explain their increased survival and expansion.

The findings of this study confirm and extend the work of others (208, 209). Both Hartel *et al* and Packer *et al* also found excessive TKI-dependent MAP kinase activation in T315I cells especially with nilotinib and is mediated in a dose dependent manner (208, 209). Both groups showed that dasatinib did not hyperactivate the MAP kinase pathway with therapeutic relevant concentrations (208, 209). Peak plasma level of dasatinib is approximately 90 nM (266). In this study, higher concentrations of dasatinib (200 nM and above) were used as the K562-T315I cell line was developed in 200 nM dasatinib and showed that at higher concentrations (1 μ M and 5 μ M), there was a significant and dose dependent increased phosphorylation of Erk over the untreated cells. Packer *et al* treated cells with the T315I mutation, BAF/3 *BCR-ABL1*^{T315I} cells with 100 nM, 1 μ M and 10 μ M dasatinib and demonstrated that pMEK and pErk were hyperactivated only at 10 μ M dasatinib (209).

Hartel *et al* also used a CML human myeloid cell line, KBM5 which contains 30% of the T315I mutation and found that although in this cell line, MAP kinase activation was increased with imatinib and nilotinib, the results did not reach statistical significance (208). This is in agreement with the data in this study as the K562-T315I cell line used contained approximately 45% mutant and statistical significance was not reached with some of the results.

Packer *et al*, found that cell lines expressing the *BCR-ABL1*^{T315I} similar to those with mutated *NRAS*, demonstrated activated MEK and Erk phosphorylation with imatinib, nilotinib and dasatinib (209). They also determined that imatinib, nilotinib and dasatinib act as weak B-Raf inhibitors which drive B-Raf-C-Raf dimerization in the presence of activated Ras and hence hyperactivation of the MAP kinase pathway (209). Using the Bcr-Abl allosteric

inhibitor, GNF-2, however, they found no evidence of B-Raf inhibition or increase B-Raf-C-Raf binding proving that Bcr-Abl inhibitors that do not inhibit B-Raf activity do not hyperactivate MAP kinase. (209). Nilotinib was determined to have the strongest B-Raf inhibitory activity whilst dasatinib the weakest (209). This may explain why MAP kinase activity in *BCR-ABL1^{T315I}* cells was the strongest with nilotinib as demonstrated by this study and others (208, 209).

Cytokine expression by cells harbouring the T315I mutation in the presence of TKI was investigated because of the known cytokine dependence of MAP kinase activation in *BCR-ABL1* positive CD34+ progenitors (49, 198). Autocrine cytokine or growth factor secretion by the T315I-expressing cells was postulated to be induced by TKI treatment which may be a mechanistic explanation for the hyperactivation of MAP kinase. The cytokines and growth factors selected to test pertained from an earlier experiment performed and described in Chapter 3, Sections 3.2.2 and 3.3.2. Out of a panel of 39 cytokines and growth factors, 10 were preferentially found in the supernatant of *BCR-ABL1^{T315I}* cells (refer Table 3-2) and this study concentrated on these same 10 cytokines for the analysis of cell supernatant incubated with TKI. The 10 cytokines were FGF-2, GM-CSF, MDC, IL-6, IL-8, MCP-1, MIP-1 α , MIP-1 β , G-CSF and TNF- α . No evidence was found that the supernatant of *BCR-ABL1^{T315I}* cells cultured overnight with no additional exogenous cytokines in the presence of different TKI showed a consistent change in cytokine expression. Disappointingly, FGF-2 which was the only cytokine which protected *BCR-ABL1^{WT}* cell from TKI-induced cell death as described in Chapter 3, also did not show convincingly higher concentrations with TKI. Similarly, Hartel *et al* analysed supernatants of BA/F3 cells transduced with *BCR-ABL1^{T315I}* cultured with nilotinib for IL-3 but found no evidence of increased concentration of the cytokine. They chose to look for IL-3 because they have ascertained that *BCR-ABL1^{WT}* cells are able to activate MAP kinase despite Bcr-Abl inhibition by imatinib and nilotinib only in the presence of IL-3 (208). The negative findings of this study may be due to several factors. The “right” cytokine may not have been chosen for study. Equally likely the cell lines studied in isolation may not directly be reflective of the behaviour of primary cells in a tumour microenvironment as cytokine signalling is mediated through interaction between stromal cells and leukaemia cells (82, 87, 200, 288).

Therefore, the fact that the T315I mutation is the most commonly found in BP-CML and the dismal prognosis of patients harbouring this mutation as shown by some studies may be due in part to the increase in MAP kinase signalling.

As a result of these findings, further studies are planned to investigate other pro-survival pathways such as the NF- κ B pathway that may also be hyperactivated in the cells harbouring the T315I mutation in the presence of TKI. The levels of anti and pro-apoptotic proteins may also be increasingly imbalanced in this setting and this may also help explain the increase viability and proliferation of the mutated cells in TKI. Primary cells of patients with the T315I mutation will be used to confirm some of the findings of this study.

In summary, this study has confirmed that the MAP kinase pathway is increased in cells with the T315I mutation when exposed to TKI especially with nilotinib. STAT5 and Akt pathways are not similarly hyperactivated. This anomaly is shown to be not mediated through increased expression of selected cytokines or growth factors. The clinical observation that patients with the T315I mutation have shorter median overall survival with imatinib and that this mutation is the most frequently detected in the BP-CML can partly be explained by the increase in MAP kinase signalling especially when TKI are continued.

Chapter 5

Discussion

5.1. Introduction

Imatinib was the first drug designed to act against a critical genetic lesion, the *BCR-ABL1* fusion gene, which is responsible for the pathogenesis of CML (127). Since its introduction in early 2000, imatinib has dramatically changed the outlook of patients with the malignancy where the median survival used to be 5 years. Now, the majority of CML patients on imatinib achieve complete cytogenetic remission (CCyR) and approximately 85% of patients are still alive after 8 years (130). However, about 30%-40% of patients who receive imatinib develop resistance or intolerance to the drug and as a result have to switch to alternate therapy (164, 165). The most common cause of resistance to imatinib is the development of point mutations in the kinase domain (KD) of *BCR-ABL1* (5, 6). The development of second generation tyrosine kinase inhibitors (TKIs), nilotinib, dasatinib and bosutinib have somewhat abrogated this problem but they are inactive against the gatekeeper mutation, T315I.

The T315I mutation was the first mutation discovered in patients who developed resistance while on imatinib (186). Gorre *et al* reported that 6 out of 9 patients who relapsed after successful response to imatinib had the T315I mutation (186). Although, more than 100 KD mutations have now been described, T315I remains one of the most common comprising 15-20% of all mutations (233). It is also the most frequent mutation to emerge after transient response to second generation TKIs (168) and the most prevalent in advanced stages of CML including the blastic phase (235).

Mutations in the KD of *BCR-ABL1* impart resistance due to reduced binding affinity to TKI (289). In addition, some mutations have increased kinase activity and transformation potential (90, 91) which suggests that mutations may modify the biological functions of *BCR-ABL1* positive cells contributing to their leukaemogenic potential and influencing disease progression. There have been reports of the T315I mutation detected in the plasma of patients prior to therapy with TKI (175-177) and clinically this has been linked to poor overall survival and prognosis in patients harbouring this mutation (10, 187). Cells harbouring the

T315I mutation have been shown by some *in-vitro* studies to exhibit increased proliferation in serum poor conditions relative to *BCR-ABL1*^{WT} (90), increased transformation potential (91), increase kinase activity (181, 183) and have altered phosphor-substrate signature (91). This phenomenon has also been demonstrated in mutations in other cancers. For example, the T790M mutation of the epidermal growth factor receptor (EGFR) gene in non-small cell lung cancer (NSCLC) is analogous to the *BCR-ABL1*^{T315I}, as it also arises at a critical “gatekeeper” residue and when combined with other activating mutations in the kinase domain results in dramatic enhancement of EGFR activity (290). Moreover, this mutation, like T315I, has been detected in patients with NSCLC prior to treatment with EGFR inhibitors and also in the germ-line of families with inherited susceptibility to certain subtypes of lung cancer (291, 292). This suggests that the *EGFR*^{T790M} mutation also might confer a growth advantage to the ensuing tumour. Mesenchymal epithelial transition factor (*MET*) proto-oncogene encodes a tyrosine kinase receptor for hepatocyte growth factor (HGF) which controls cell proliferation, invasiveness and protection from apoptosis. It is over-expressed in many human cancers (293-295) and is known to be mutated in both inherited and sporadic papillary renal cell carcinoma (296, 297), as well as in lung (298) and gastric cancers (299). Different *MET* mutations have been shown to result in the acquisition of distinct biological properties in the transfected cells; some mutations result in increased kinase activity and transforming potency, while others provide protection from apoptosis, anchorage-independent growth and increased invasiveness (296, 297).

Aberrant cytokine signalling is a feature in many malignancies and may contribute to disease pathogenesis, resistance to treatment and overall prognosis (300, 301). Different cytokines are thought to be involved in the pathogenesis of CML and this study aims to study cytokines that may be important in the pathogenesis of the T315I mutation.

This study hypothesizes that the altered biological properties of the cells harbouring the T315I mutation are mediated through increased production of certain cytokines which would then confer an autocrine pro-survival effect. Cytokines may also provide paracrine protective effect to *BCR-ABL1*^{WT} cells from TKI-induced cytotoxicity. Initially, cytokine profiling was performed on the supernatant of *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells cultured in conditioned media to determine if *BCR-ABL1*^{T315I} overproduced certain cytokines or growth factors. Then the corresponding cognate receptors were investigated to determine if

they were overexpressed in either the *BCR-ABL1*^{WT} or *BCR-ABL1*^{T315I} cells which may provide an indication if the expressed cytokines accord pro-survival effect to the relevant cells. To further investigate if overproduction of cytokines have an autocrine pro-survival effect, the proliferation and viability of *BCR-ABL1*^{T315I} cells cultured in SDM which has no added cytokines or growth factors were compared with the *BCR-ABL1*^{WT} cells. Both *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells were next co-cultured in Transwells® plates in the presence of TKI to ascertain if the cytokines secreted by *BCR-ABL1*^{T315I} would provide paracrine protection to the *BCR-ABL1*^{WT}. The cytokines responsible for the protective action were determined and the mechanism by which this occurred elucidated.

The significance of these studies is a better understanding of how KD mutations may additionally impart resistance to TKI other than reduction of TKI binding affinity. It may also help us understand why in some series, patients harbouring the T315I mutation have a particularly poor outcome compared to those who have acquired imatinib resistance through other mechanisms.

5.1.1. *BCR-ABL1*^{T315I} cells have a different cytokine profile compared to *BCR-ABL1*^{WT} cells

In the current study, the cell lines harbouring the T315I mutation, K562-T315I and the retrovirally transduced HL60 *BCR-ABL1*^{T315I} demonstrated different cytokine profiles compared to their WT counterparts. The culture supernatants of both the K562-T315I and HL60 *BCR-ABL1*^{T315I} cell lines contained higher concentrations of FGF-2, IL-8, MCP-1 and G-CSF (refer Table 3-2). The supernatant of the K562-T315I cells also demonstrated higher concentrations of GM-CSF and IL-6 compared to K562 naïve cells, whilst HL60 *BCR-ABL1*^{T315I} had higher concentrations of MDC, MIP-1 α , MIP-1 β and TNF- α compared to HL60 *BCR-ABL1*^{b210} and HL60 parental cells (refer Table 3-2). From these results, differential cytokine expression was postulated to accord cells harbouring the T315I mutation with growth advantages in the setting of a factor deprived environment.

IL-3, G-CSF and GM-CSF were the earliest cytokines implicated in the pathogenesis of CML (71, 72, 75). These cytokines have been demonstrated to be secreted by *BCR-ABL1* positive progenitor cells and results in autocrine pro-survival effects (71, 72). Other cytokines implicated includes IL-6 and TNF- α (76, 78, 87, 260). Increased vascularity has been reported

in the bone marrows of haematological malignancies including CML (302, 303) and there is evidence to suggest that angiogenesis is important for the pathogenesis and progression of these disorders (302-304). Levels of cytokines that regulate angiogenesis such as VEGF, FGF-2, TNF- α , transforming growth factor (TGF- α), TGF- β , IL-8 and hepatocyte growth factor (HGF) (302, 305) have been correlated with poor prognosis in solid tumours as they are associated with growth and metastasis (306, 307). Similarly, increased levels of some of these cytokines in particularly VEGF and FGF-2 have also been reported in haematological malignancies including CML (302, 303, 308, 309) and may have prognostic significance (302, 310).

Gene expression of IL-8 has been shown to be upregulated on Bcr-Abl expression and downregulated with Bcr-Abl inhibition (261). Hantchel *et al* also demonstrated that cell lines with *BCR-ABL1* secreted higher levels of IL-8 compared to those without and that treatment with dasatinib and nilotinib markedly reduced IL-8 level in *BCR-ABL1* cell line supernatant. This was confirmed on comparing serum IL-8 of newly diagnosed CML patients with normal controls and the authors showed that the levels reduced on treatment with imatinib (261).

Patients with ALL and AML have been reported to have higher bone marrow concentrations of MCP-1 (311, 312). In CML, MCP-1 was shown by Cashman *et al* to inhibit cell cycle progression of normal progenitor cells in long term marrow culture system but not primitive CML cells (313). It is intriguing to speculate that *BCR-ABL1*^{T315I} cells may also secrete more MCP-1 to inhibit cell cycle progression of normal progenitors.

Few published studies have looked at the cytokine profile of *BCR-ABL1* resistant cells including those with KD mutations (73, 74). Comparatively, others have demonstrated, using cell lines harbouring the T315I mutation, overproduction of different cytokines (73). In the model of resistance employed by Liu *et al*, murine DA1-3b cells were transduced with *BCR-ABL1*^{p210} and exposed to progressively higher concentrations of imatinib until the E255K mutation emerged (73). When DA1-3b cells were cultured in increasing concentrations of dasatinib, 2 clones emerged- 1 bearing the compound mutations E255K and T315I and the other with 3 mutations, E255K, T315I and V299L. The authors observed that the cell lines harbouring the mutations produced higher levels of IL-3 compared to the non-mutated cells and were able to protect naïve cells from imatinib-induced cell death (73). Hence, the use of a different cell line, from the ones employed in the studies presented here, resulted in an

entirely different cytokine profile. Wang *et al* employed a different *BCR-ABL1* positive cell line, LAMA84, which was resistant to imatinib, and found that the cells protected non-imatinib resistant LAMA cells and CML CD34+ progenitor cells from imatinib and nilotinib-induced cell death by secretion of GM-CSF via activation of the JAK2/STAT5 pathway (74). Therefore, the differences in secreted cytokines which confer paracrine protection to non-mutated *BCR-ABL1* positive cells may be due to different modes of resistant mechanisms such as activation of different signalling pathways or increased expression of *BCR-ABL1* and the different cell lines used, although this study has demonstrated similar cytokine profiles from two distinct cell lines. As mentioned in Chapters 3 and 4, there were limitations in the choice of cell lines but nevertheless, as with other published studies, this study has also demonstrated that resistance can develop via secretion of cytokines and growth factors, and as discussed later, the paracrine protection conferring resistance to non-mutated cells.

5.1.2. Cytokine receptor expression on *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells

Immunophenotyping of the cognate receptors for the cytokines demonstrated to be preferentially overproduced by the *BCR-ABL1*^{T315I} was not very informative. When a ligand (cytokine) binds to its specific receptor on the cell surface, this induces a conformational change in the receptor and results in phosphorylation of downstream targets which leads to a specific cellular response such as regulation of cell proliferation, differentiation or survival (211, 314). Hence, it was predicted that if the receptors were overexpressed in *BCR-ABL1*^{WT} or *BCR-ABL1*^{T315I} cells, it would give an indication if the cytokines would have an autocrine or paracrine pro-survival effect. However, the surface cytokine receptors tested were expressed on most of the cells but not significantly overexpressed over the other cell lines (refer Figures 3-6 to 3-11). After demonstrating that FGF-2 alone had a paracrine protective effect on *BCR-ABL1*^{WT} cells, the FGFR was expected to be highly expressed on the same cells. However, recent studies have indicated that FGFR3 was the important receptor for FGF-2 signalling in CML (262) and not FGFR2 which is further supported by earlier studies performed by another group (315, 316). This group has demonstrated that both FGFR3 protein and mRNA levels were increased in the *BCR-ABL1* positive CD34+ cells isolated from the peripheral blood from CML patients, which subsequently decreased following treatment with bone marrow transplantation or imatinib (315, 316). Moreover, surface receptor expression may be transient and receptor expression may not be “captured” at time of

analysis. The result of this study suggests that the autocrine or paracrine effects of cytokines may not be reliably demonstrated by the presence or absence of surface receptor expression.

5.1.3. *BCR-ABL1*^{T315I} do not proliferate more rapidly compared to *BCR-ABL1*^{WT} in SDM

Surprisingly, when cultured in SDM, cells harbouring the T315I mutation did not demonstrate increased proliferation over *BCR-ABL1*^{WT} cells (refer Figures 3-13 to 3-15 and 3-17). This suggests that despite a different cytokine profile, the expressed cytokines and growth factors did not provide an autocrine pro-proliferative effect on the cells expressing T315I. This is supported by Miething *et al* who showed that murine bone marrow cells expressing *BCR-ABL1*^{T315I} did not have a growth advantage over *BCR-ABL1*^{WT} cells in the absence of imatinib (170). However, in contrast, Griswold *et al* demonstrated that BA/F3 cells expressing *BCR-ABL1* KD mutations including the T315I mutation proliferated more rapidly in serum poor conditions compared to the *BCR-ABL1*^{WT} cells except for the M351T mutation (90). In the study presented here, the cells harbouring the T315I mutation did not have a growth advantage in SDM; however this was not unexpected as the *BCR-ABL1*^{T315I} cells did not have overexpression of the receptors for the relevant expressed cytokines and hence, did not to show an autocrine effect.

5.1.4. *BCR-ABL1*^{T315I} cells over-secrete FGF-2 which protects *BCR-ABL1*^{WT} cells from TKI-induced cytotoxicity

This study has also shown that one or more soluble factors produced by cells harbouring the T315I mutation were able to protect *BCR-ABL1*^{WT} cells from TKI-induced cell death (refer Figures 3-18 to 3-20). To determine which factors were mediating this paracrine protective effect, recombinant cytokines and growth factors (indicated to be preferentially overproduced by the cells harbouring the T315I mutation), were added to *BCR-ABL1*^{WT} cells co-treated with TKI. The only cytokine which “rescued” K562 naïve (*BCR-ABL1*^{WT}) cells from TKI-induced cell death was FGF-2 (refer Figure 3-21).

In order to confirm that FGF-2 was responsible for the observed protective effect, bFM-1 (Millipore), a FGF-2 neutralizing antibody was employed. bFM-1 was able to abrogate the protective effect previously elicited to TKI treated *BCR-ABL1*^{WT} cells when co-cultured with

cells harbouring the T315I mutation (refer Figures 3-26 and 3-27). This data strongly suggests that the factor produced by cells harbouring the T315I mutation that confers resistance to *BCR-ABL1*^{WT} cells is FGF-2. This is supported by a recent study that detailed FGF-2 as the most protective growth factor for the K562 cell line from the effects of imatinib (262). The authors also showed that patients without detectable KD mutations, but with resistance to first and second generation TKI, have increased FGF-2 in their bone marrow (262). In these patients, FGF-2 decreased on treatment with ponatinib (262), a third generation TKI with activity against Bcr-Abl and FGFR. This indicates that overproduction of FGF-2 may be an important means of acquiring resistance to TKI treatment in *BCR-ABL1* positive cells including cells harbouring the T315I mutation. In support of this, the study presented here has demonstrated that the addition of exogenous FGF-2 was able to “rescue” K562-T315I cells from ponatinib-induced cell death (refer Figure 3-25) suggesting that production of FGF-2 is a potential mechanism by which cells harbouring the T315I mutation may develop resistance to ponatinib. Mutant cells may also develop FGFR over-expression and/or activating mutations in the *FGFR* may lead to ponatinib resistance. In our laboratory, ponatinib-resistant cell lines are being developed and there are plans to screen these cell lines for FGF-2 over-production, FGFR over-expression and *FGFR* mutations.

FGF-2 has angiogenic activity (303, 305) and increased levels of FGF-2 (309) have been described in the plasma as well as increased vascularity demonstrated in bone marrows of some CML patients (302, 303, 305, 317). Kjeci *et al* postulated that the source was likely from bone marrow stromal fibroblasts (309). The authors demonstrated that white cells from 3 out of 4 CML patients proliferated more advantageously with supplemented FGF-2. Furthermore, as mentioned previously, FGFR3 levels were increased in the *BCR-ABL1* positive CD34+ cells from patients, which subsequently decreased following treatment with bone marrow transplantation or imatinib (315, 316). The authors postulated that treatment resulted in the decrease of *BCR-ABL1* positive cells which overexpressed *FGFR3* and allowed the expansion of normal CD34+ cells (316). This further lends support that FGF-2 plays a role in the pathogenesis of CML.

This paracrine effect which contributes to resistance may play a more important part *in vivo* where primary CML cells reside and interact within a bone marrow microenvironment rich in cytokines and growth factors. This has been supported clinically where patients with very

low levels of mutations demonstrate poor responses to second generation TKIs, nilotinib and dasatinib (318). A small proportion of mutated cells are able to protect the non-mutated cells from the effects of TKI. In support of this, as mentioned previously, Liu *et al* established an imatinib resistant cell line that carried the E255K mutation and a cell line with the composite mutation E255K/T315I that was resistant to both imatinib and dasatinib (73). The resistant cells secreted significantly higher levels of IL-3 which protected the non-mutated cells from imatinib-induced cytotoxicity (73). The authors then co-cultured the non-mutated cells with the E255K/T315I cells at different ratios and found that co-culture at a ratio of as low as 16:1 non-mutated to E255K/T315I mutants was enough to confer protection to the non-mutated cells from imatinib (73). Therefore, this lends support that a small proportion of resistant cells amongst sensitive cells may show complete resistance to TKI.

5.1.5. FGF-2 rescues *BCR-ABL1*^{WT} cells from TKI-induced cell death by reactivation of Erk and STAT5 signalling

Further to this finding, this study has demonstrated that FGF-2 'rescued' *BCR-ABL1*^{WT} cells exhibit reactivation of Erk and STAT5 signalling (refer Figures 3-29 to 3-32). This may imply that FGF-2 re-activates these survival pathways and by-passes Bcr-Abl inhibitions by TKI. The predominant signalling pathway activated downstream of FGFR is the MAP kinase pathway although other pathways may also be activated and they include PI3K-Akt, PLC γ , p38 MAP kinase, JUNK and the STAT (253, 255, 256). Trauer *et al* have revealed that when FGF-2 binds to its receptor, MAP kinase was activated to promote survival of *BCR-ABL1* positive cells in the presence of imatinib (262). Activation of the MAP kinase was also demonstrated by Kjeci *et al* using white blood cells from CML patients and exposing the cells to exogenous FGF-2 (309). However, it was not revealed if either of the authors also looked for other signalling pathways which may also be activated by FGF-2. In this present study, Akt signalling was also investigated and found that it may possibly be reactivated in imatinib-treated *BCR-ABL1*^{WT} cells by FGF-2 (refer Figure 3-29C). It is possible that other pathways may also be involved including NF- κ B and the apoptotic pathways such as Bcl-2, Bcl-xL and Bim. Liu *et al* demonstrated that their cell lines bearing the compound mutations E255K/T315I overexpressed IL-3 and protected naïve cells through reactivation of MEK/Erk, JAK/STAT pathways and down-regulation of Bim, an inducer of apoptosis (73).

5.1.6. K562-T315I cells are more viable and proliferate more rapidly when exposed to TKI and this is mediated through hyperactivation of the MAP kinase pathway

Non-mutated *BCR-ABL1* cells are suppressed on treatment with TKI allowing insensitive mutated cells to expand and on stopping treatment the non-mutated cells to re-expand, presumably because the non-mutated cells have a proliferative advantage without TKI (173, 174). However, an alternate explanation is that *BCR-ABL1*^{T315I} cells survive and expand more advantageously in the presence of TKI. Continuing treatment with TKI has been found to be associated with increased oncogenic fitness of cells harbouring the T315I mutation (319). Recent studies have demonstrated that when exposed to a TKI, cells harbouring the T315I mutation expand rapidly due to enhanced MAP kinase signalling (208, 209).

These studies were first confirmed by determining if K562-T315I cells have an advantageous proliferation and survival when incubated with a TKI and secondly, if the MAP kinase pathway is hyperactivated in K562-T315I cells in the presence of TKI. Other major signalling pathways were also investigated to determine if they are similarly hyperactivated which could explain the cells' pro-survival propensity in TKI. Lastly, as cytokines have been shown to play a part in mediating resistance in *BCR-ABL1* positive cells including those with KD mutations (73, 74), cytokine profiling was performed on the supernatant from *BCR-ABL1*^{T315I} cells cultured in conditioned media with and without the presence of TKI to ascertain if cytokines play a role in this phenomenon.

This study demonstrates that K562-T315I cells exhibited increased proliferation and survival in the presence of TKI, compared to without (refer Figures 4-1 and 4-5), and this appears to be mediated via enhanced signalling through the MAP kinase pathway, particularly in the presence of nilotinib (refer Figure 4-6). This has also been confirmed by others (208, 209). Both Packer *et al* and Hartel *et al* have demonstrated that cells harbouring the T315I mutation showed hyperactivation of MEK/Erk signalling when exposed to nilotinib at therapeutically relevant concentrations (208, 209). The mechanism by which this occurs is unknown but Packer *et al* has postulated that it may occur through inhibition of B-Raf and enhancement of C-Raf signalling (209). Imatinib, nilotinib and to a lesser extent dasatinib, act as weak B-Raf inhibitors. *BCR-ABL1*^{T315I} cells exhibit persistent Ras-dependent activity

and in the presence of B-Raf inhibitors, drive formation of B-Raf and C-Raf heterodimers and this results in enhanced MAP kinase activation (209).

5.1.7. Hyperactivation of MAP kinase signalling does not appear to be cytokine-mediated

In this study, other signalling cascades were also investigated to ascertain if they may also be hyperactivated in cells harbouring the T315I mutation when exposed to a TKI. Erk, STAT5 and Akt signalling were chosen as they are the three key downstream substrates activated by Bcr-Abl which mediates proliferation and inhibits apoptosis (37, 40, 58). If these pathways were hyperactivated, it would result in increased expansion and enhanced survival of the cells harbouring the T315I mutation. However neither STAT5 nor Akt signalling was demonstrated to be increased (refer Figures 4-7 and 4-8). Additionally, the observed enhanced MAP kinase signalling in cells harbouring the T315I mutation in the presence of TKI, was determined if it could be due to a cytokine-mediated effect through an autocrine loop. However, no evidence was found that the cytokine profile of cells harbouring the T315I mutation when cultured with TKI was different from those cultured without (refer Table 4-1).

Presently, no peer-reviewed publications have investigated if other potential signalling pathways are also activated in the cells harbouring the T315I mutation or the link between increased survival and proliferation in the setting of resistant cells and TKI therapy. However, a few studies have investigated the effect of TKI treatment on cytokine signalling in resistant cells (208, 320). Hartel *et al* has studied the levels of IL-3 in the cell supernatant of the T315I expressing cell line, BA/F3 p210^{T315I} in the presence of nilotinib but did not demonstrate increased levels (208). IL-3 was chosen as the authors have earlier determined that *BCR-ABL1*^{WT} cells were able to activate MAP kinase despite imatinib and nilotinib treatment when supplemented with IL-3. They had postulated that the resistant cells may over-secrete this cytokine as a means to overcome the effects of nilotinib (208).

In the study presented here, 14 cytokines and growth factors were selected for analysis however, Park *et al* investigated protein expression profiles using a mass spectrometry approach on a spectrum of 118 proteins on K562 naïve and imatinib-resistant, K562R cells with or without imatinib (320). The K562R cells had increased *BCR-ABL1* gene amplification

(320). The authors reported that 46 proteins were differentially expressed in the K562R cells with and without imatinib and the proteins studied included those involved in protein synthesis, signal transduction, regulation and apoptosis (320). Therefore, even though it was not demonstrated by the study presented here, the findings by Park *et al* show that it is still possible that cytokine expressions may change in resistant cells when exposed to a TKI. This would then result in increased survival and expansion by hyperactivating downstream signalling cascades.

5.2. Future directions

As a result of the findings presented in this thesis, the following studies will be undertaken to further interrogate the role of cytokines in resistant cells including those that harbour the T315I mutation:

- The use of the murine pre-B, BA/F3 cell line transformed by retroviral transduction with *BCR-ABL1*^{p210} and *BCR-ABL1*^{T315I} in cytokine profiling, cytokine rescue and co-culture experiments to provide more confidence that the over-expression of certain cytokines is not pertaining to specific cell lines only.
- Interrogation of other signalling pathways that may be different in the cells harbouring the T315I mutation including the NF-κB pathway.
- Seek *in vivo* confirmation of findings including the determination of cytokine levels, particularly FGF-2, in the plasma/bone marrow of patients harbouring the T315I mutation compared to those without.
- Use primary CD34+ *BCR-ABL1* cells in cytokine rescue experiments as determined by cytokine profile found above.
- To determine the protein expression and mRNA levels of FGFR3 in both *BCR-ABL1*^{WT} and *BCR-ABL1*^{T315I} cells.

5.3. Summary

In summary, *BCR-ABL1*^{T315I} cells have been shown to have a different cytokine profile compared to *BCR-ABL1*^{WT} cells but increased cytokine production did not result in an autocrine pro-survival effect. However, *BCR-ABL1*^{T315I} cells were able to protect *BCR-ABL1*^{WT} cells from the effects of imatinib via a paracrine mechanism by an overproduction of FGF-2 and this is mediated through reactivation of pErk and pSTAT5. *BCR-ABL1*^{T315I} cells also

demonstrated increased survival and proliferation in the presence of imatinib, nilotinib and dasatinib relative to their proliferation in the absence of TKI and this is through enhanced signalling through the MAP kinase pathway. STAT5 and Akt are not similarly hyperactivated and no evidence to suggest the involvement of cytokines in this setting was demonstrated. These findings support and extend the work of others that the *BCR-ABL1*^{T315I} mutation confers the cells added oncogenic fitness in the presence of TKI (91, 181, 183). It also supports the clinical data where patients who had acquired imatinib resistance through the T315I mutation had a worse clinical outcome compared to those acquired through different mechanisms especially when continued on TKI therapy (9, 10, 187, 270).

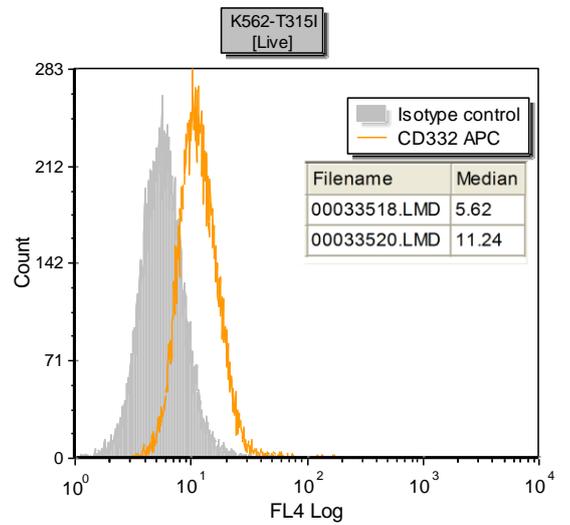
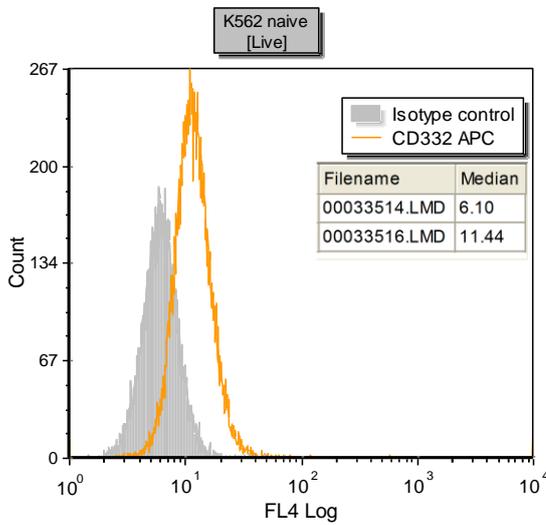
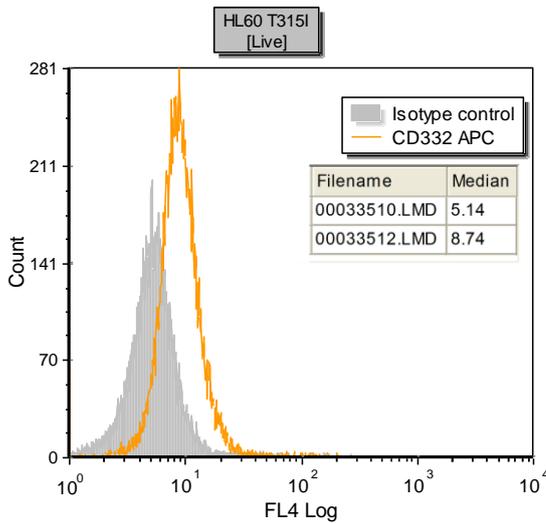
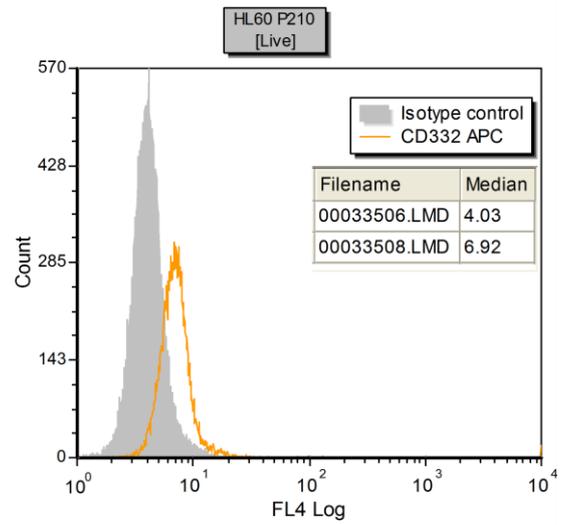
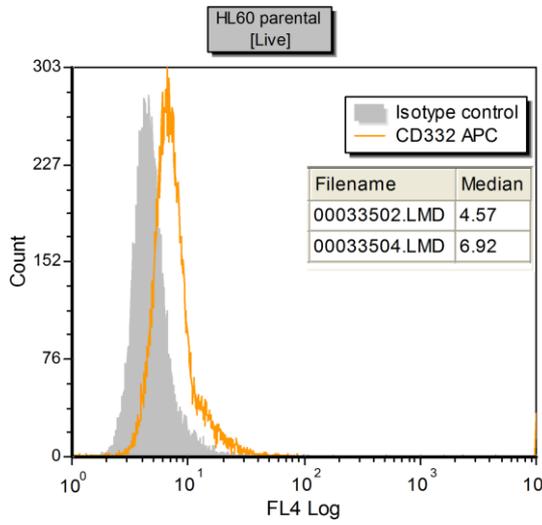
Appendix

Supplementary Figures

S-1: Histograms for Figure 3-6: HL60 and K562 cells expression of the FGF-2 receptor

2×10^5 cells were harvested and were made up to 1 ml with culture media, pelleted and supernatant aspirated. The cells were incubated with APC-conjugated CD332 antibody (R & D Systems, Minneapolis, Minnesota, USA) or the appropriate isotype control for 30 minutes on ice and resuspended in FACS fix after washing. The assay was analysed by flow cytometry. The results are expressed as mean fluorescent intensity (MFI) in comparison to the isotype control. The experiment was performed twice and figures are representative.

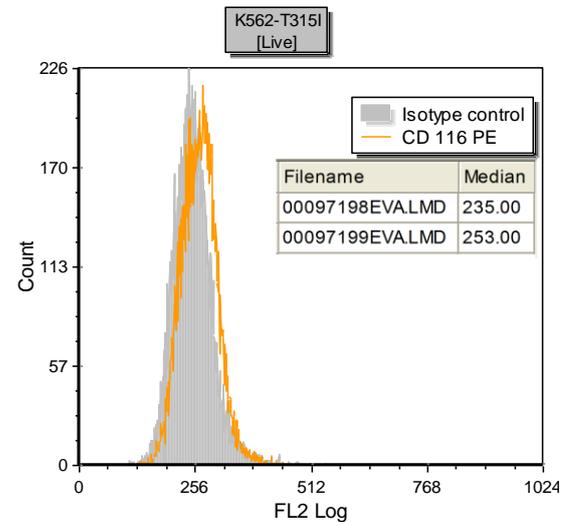
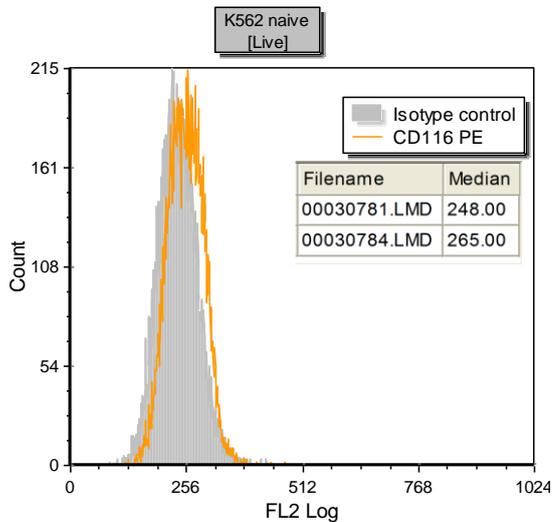
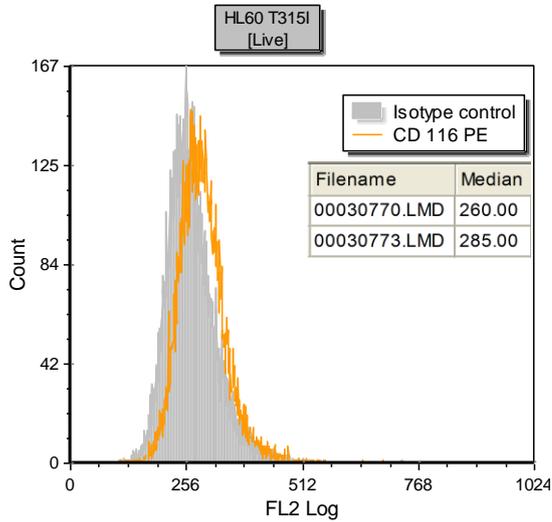
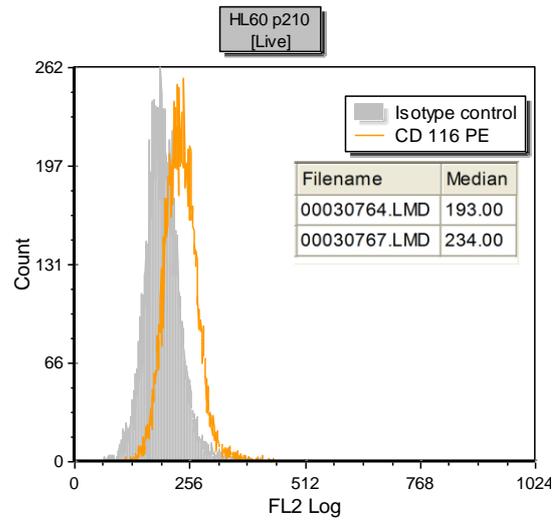
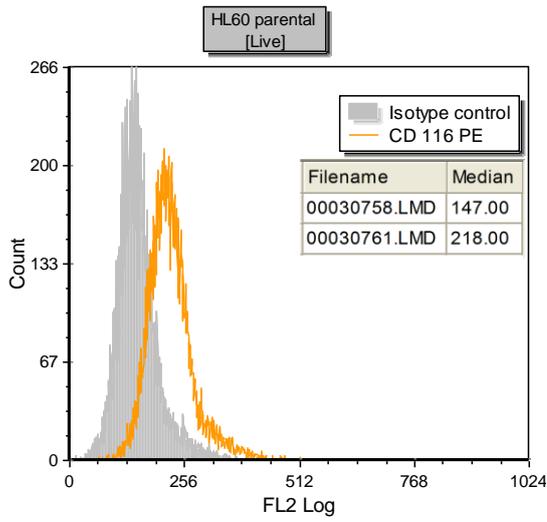
Figure S-1 CD332



S-2: Histograms for Figure 3-7A: HL60 and K562 expression of the GM-CSF receptor without pre-incubation with GM-CSF

2×10^5 cells were harvested and were made up to 1 ml with culture media, pelleted and supernatant aspirated. The cells were incubated with PE-conjugated CD116 antibody (BD Pharmingen, San Diego, Ca, USA) or the appropriate isotype control for 30 minutes on ice, resuspended in FACS fix after washing and the samples analysed by flow cytometry. The results are expressed as mean fluorescent intensity (MFI) in comparison to the isotype control. The experiment was performed twice and figures are representative.

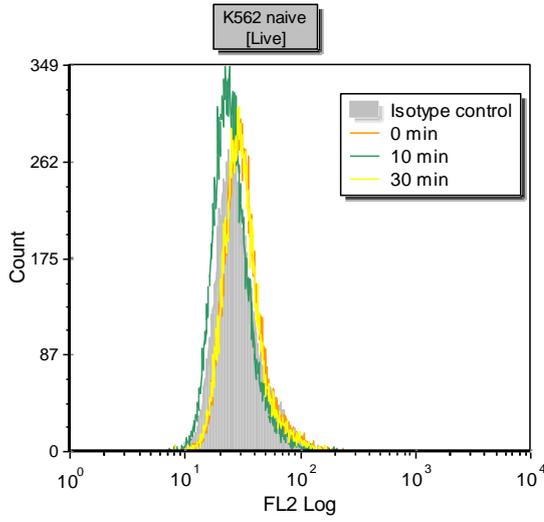
Figure S-2 CD116



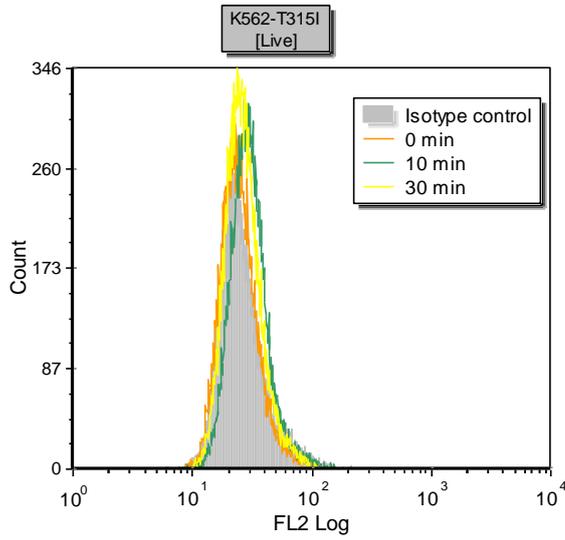
S-3: Histograms for Figure 3-7B: K562 expression of the GM-CSF receptor after pre-incubation with rGM-CSF

As the K562 cell lines did not show much receptor expression, they were cultured overnight in SDM. Additionally, the next day, cells were harvested and pre-incubated with 50 ng/ml rGM-CSF (PeproTech) for 10 minutes or 30 minutes at 37°C/5% CO₂. At the end of the incubation period, the cells were washed with cold media and the experiment continued as above. The results are expressed as MFI in comparison to the isotype control. The experiment was performed twice.

Figure S-3 CD116 +stimulation with GM-CSF



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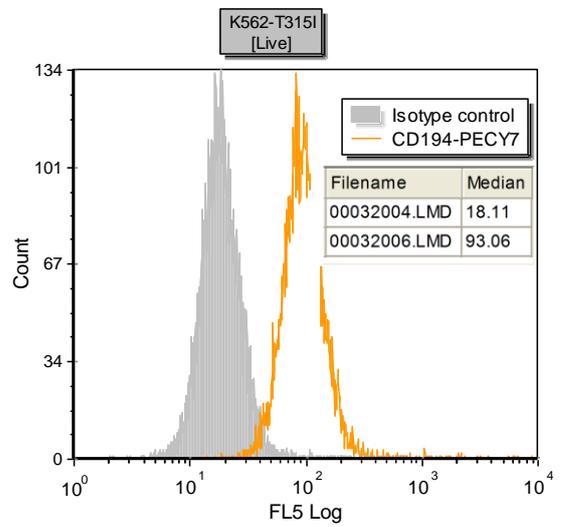
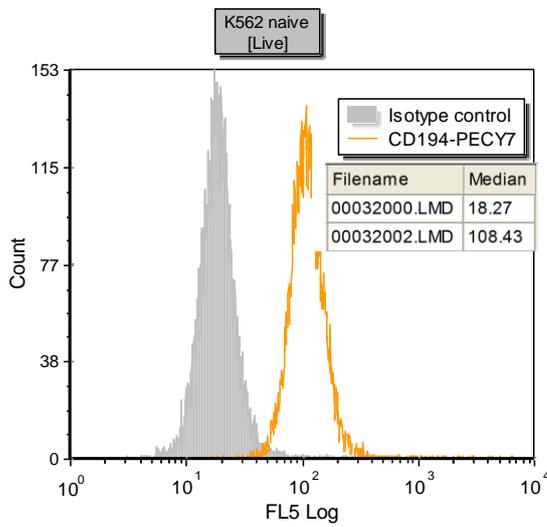
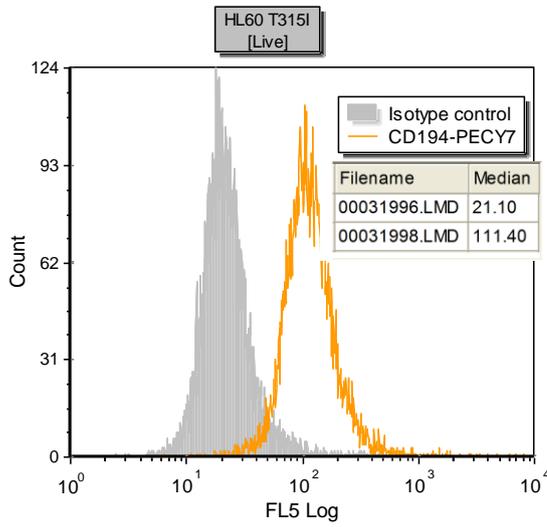
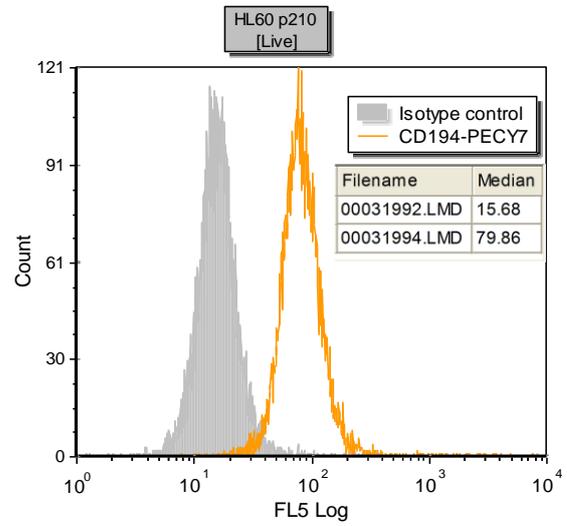
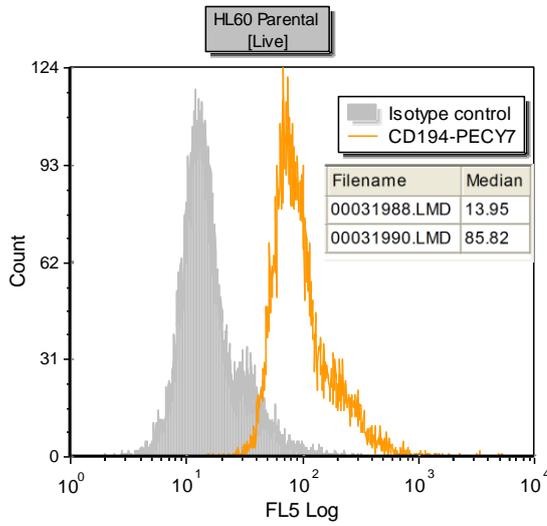


Filename	Median
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00039990.LMD	28.90
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S-4: Histograms for Figure 3-8; HL60 and K562 expression of the MDC receptor

2×10^5 cells were harvested and were made up to 1 ml with culture media, pelleted and supernatant aspirated. The cells were incubated with PEcy7-conjugated CD194 antibody (BD Pharmingen) or the appropriate isotype control for 30 minutes on ice and resuspended in FACS fix after washing. The assay was analysed by flow cytometry. The results are expressed as MFI in comparison to the isotype control. The experiment was performed 3 times and figures are representative.

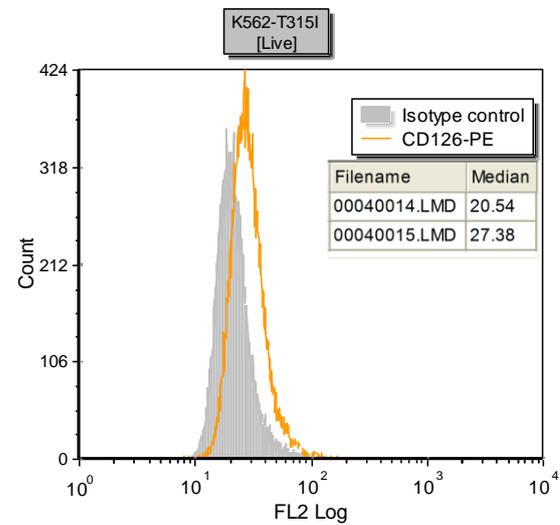
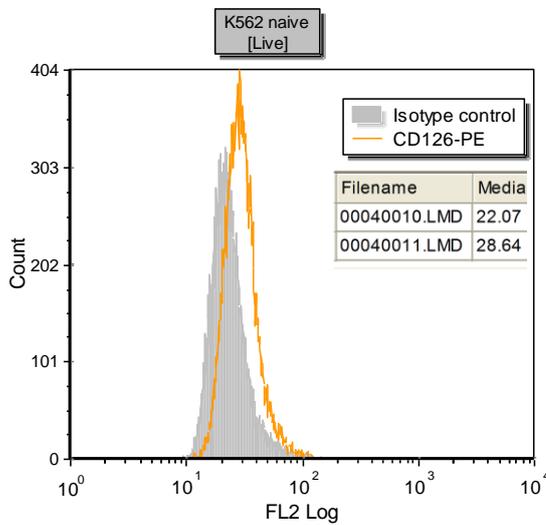
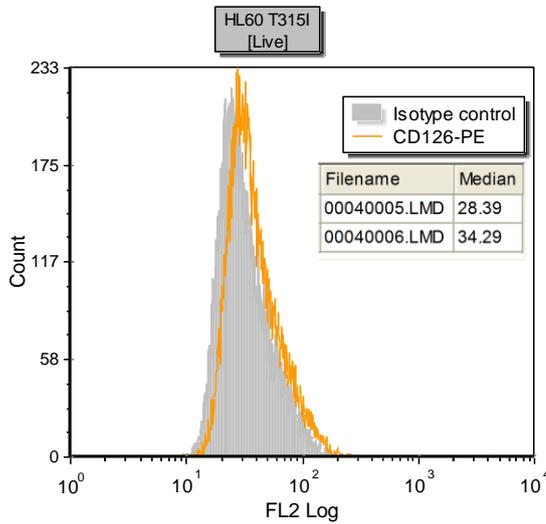
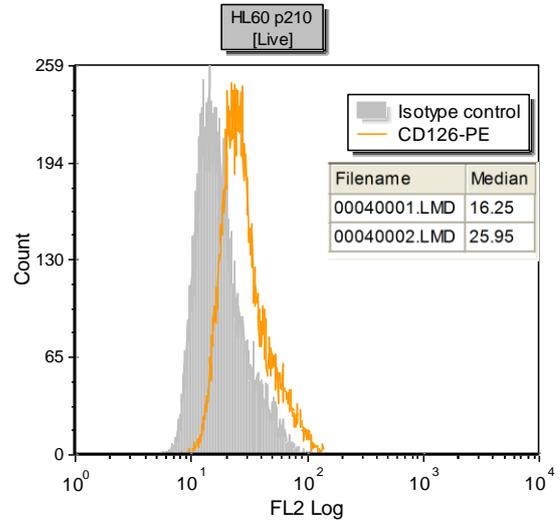
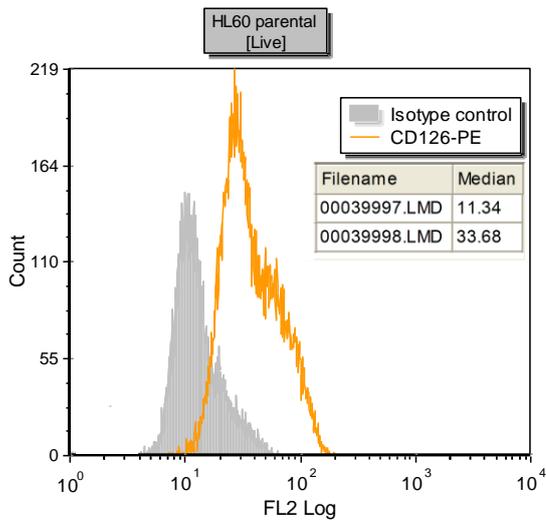
Figure S-4 CD194



S-5: Histograms for Figure 3-9A: HL60 and K562 expression of the IL-6 receptor without pre-incubation with rIL-6

2×10^5 cells were harvested and were made up to 1 ml with culture media, pelleted and supernatant aspirated. The cells were incubated with PE-conjugated CD126 antibody (BD Pharmingen) or the appropriate isotype control for 30 minutes on ice, resuspended in FACS fix after washing and analysed on flow cytometry. The results are expressed as MFI in comparison to the isotype control. Experiment was performed 3 times.

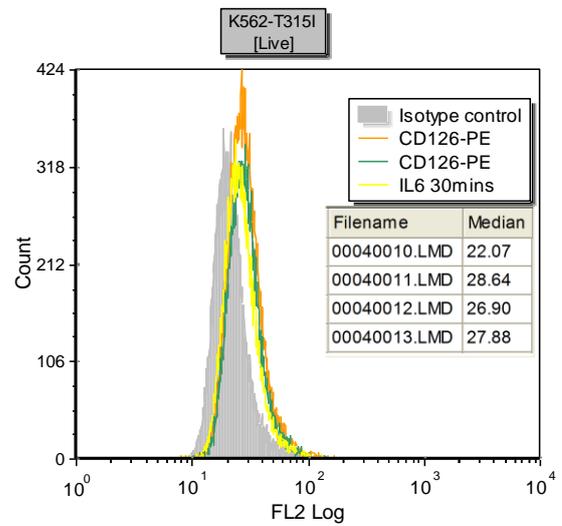
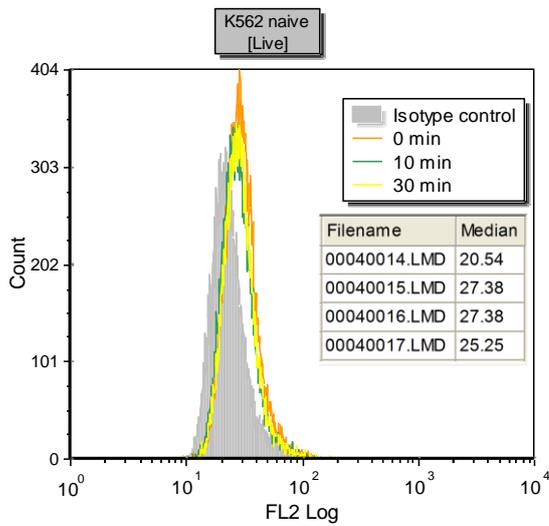
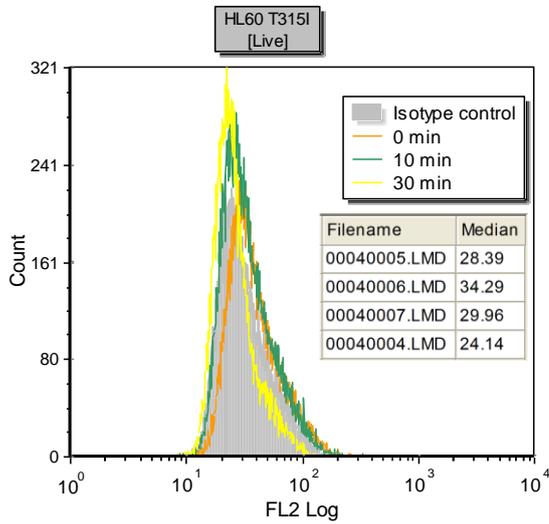
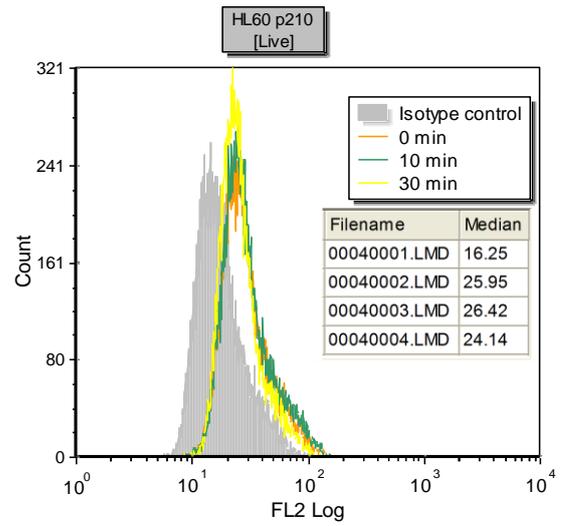
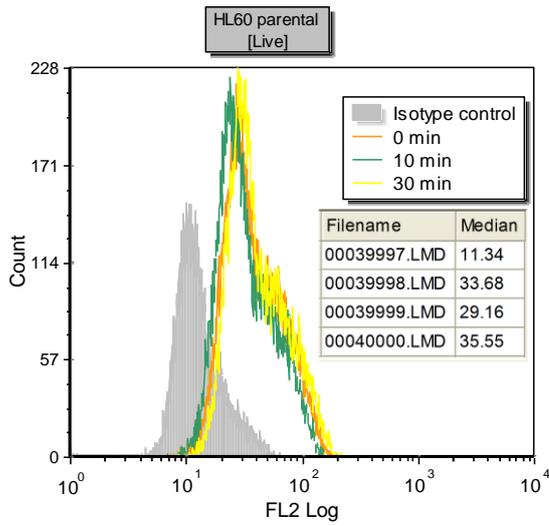
Figure S-5 CD126



S-6: Histograms for Figure 3-9B: HL60 and K562 expression of receptor for IL-6 after pre-incubation with rIL-6

The cells were cultured overnight in SDM and additionally, the next day, the cells were harvested and pre-incubated with 50 ng/ml rIL-6 (PeproTech) for 10 minutes or 30 minutes at 37°C/5% CO₂. At the end of the incubation period, the cells were washed with cold media and the experiment continued as above. The results are expressed as MFI in comparison to the isotype control.

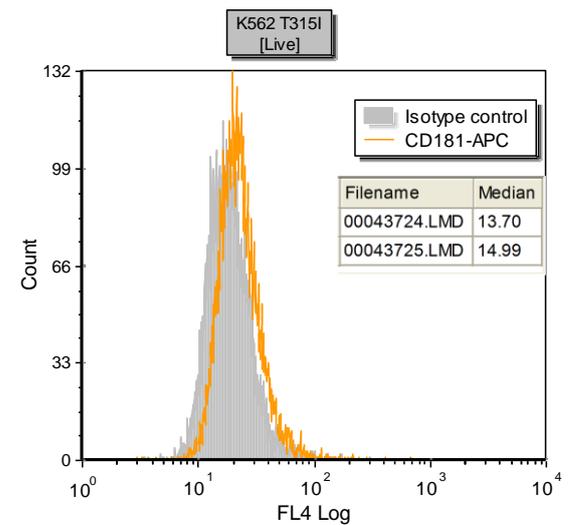
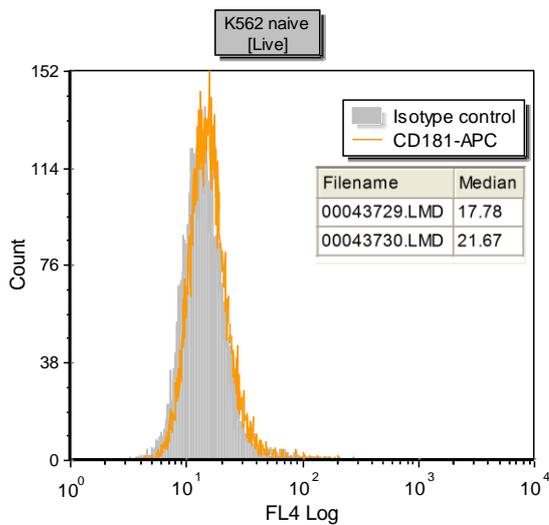
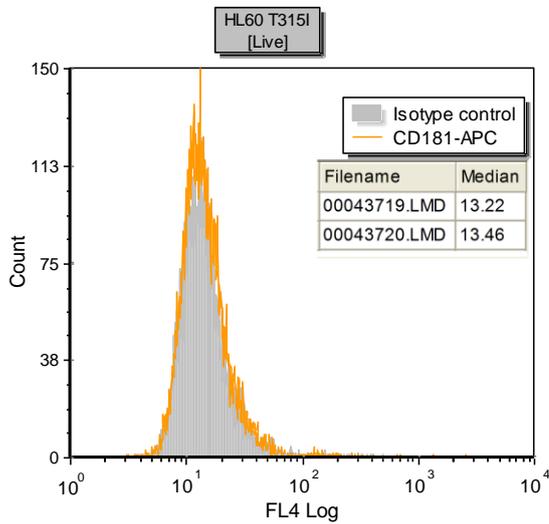
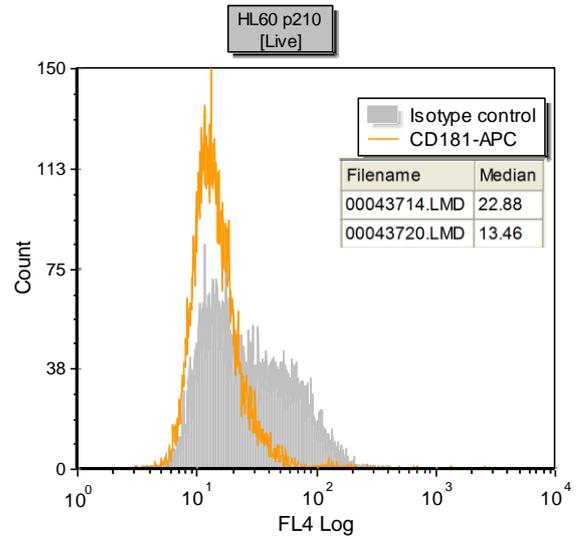
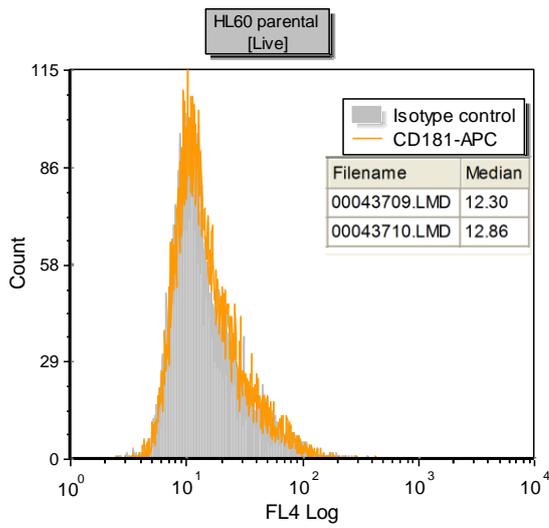
Figure S-6 CD126 + stimulation



S-7: Histograms for Figure 3-10A: HL60 and K562 expression of receptor for IL-8 without pre-incubation with rIL-8

2×10^5 cells were harvested and were made up to 1 ml with culture media, pelleted and supernatant aspirated. The cells were incubated with APC-conjugated CD181 antibody (BD Pharmingen) or the appropriate isotype control for 30 minutes on ice and resuspended in FACS fix after washing. The assay was analysed on flow cytometry. The results are expressed as MFI in comparison to the isotype control. Experiment was performed 3 times.

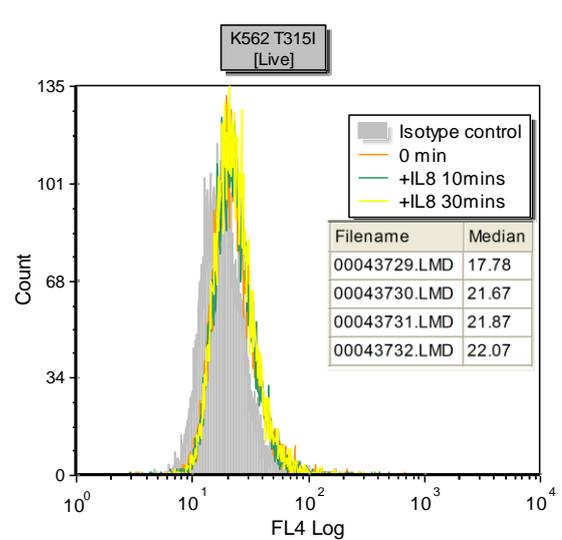
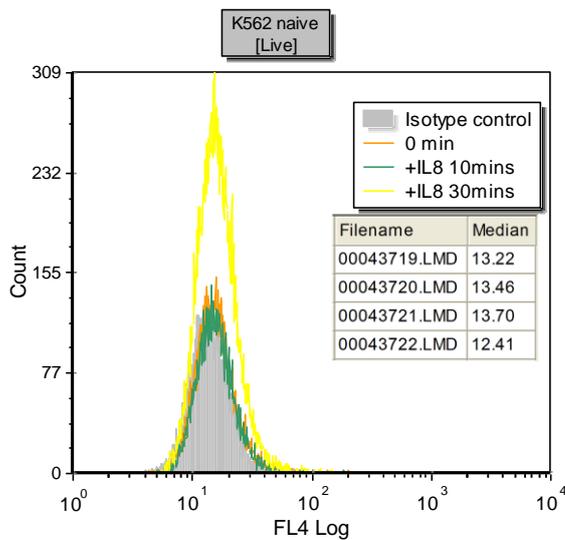
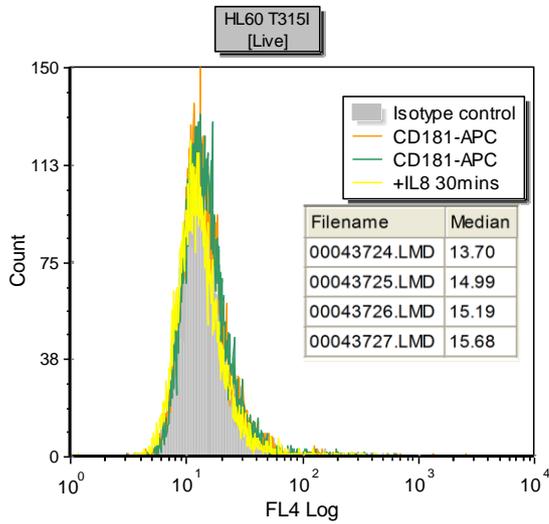
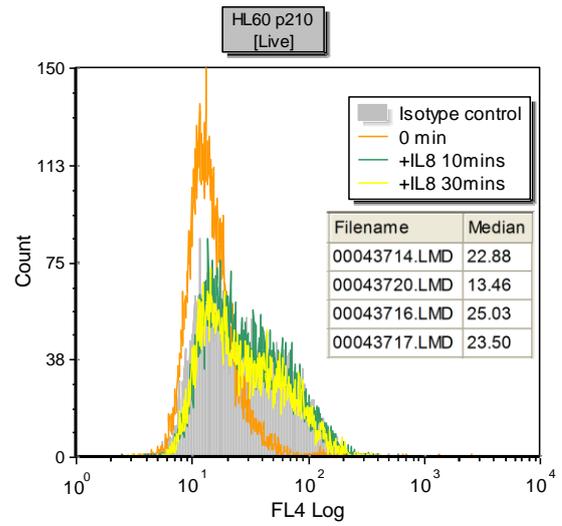
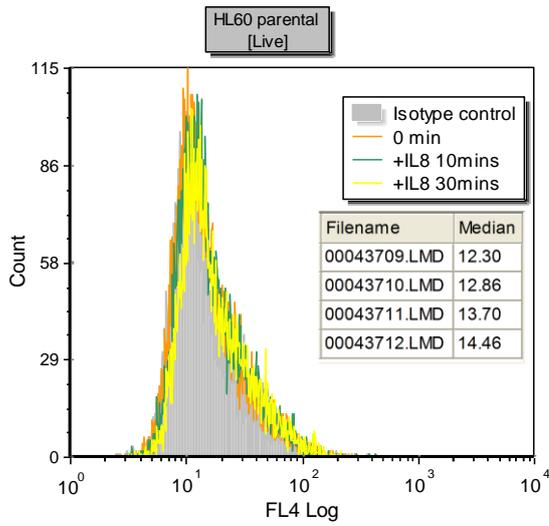
Figure S-7 CD181



S-8: Histograms for Figure 3-10B: HL60 and K562 cells expression of the IL-8 receptor after pre-incubation with r-IL-8

The cells were cultured overnight in SDM and the next day, additionally, cells were harvested and pre-incubated with 50 ng/ml rIL-8 (PeproTech) for 10 minutes or 30 minutes respectively at 37°C/5%CO₂. At the end of the incubation period, the cells were washed with cold media, pelleted and the supernatant aspirated. The experiment was continued as above. The results are expressed as MFI in comparison to the isotype control. The experiment was performed twice.

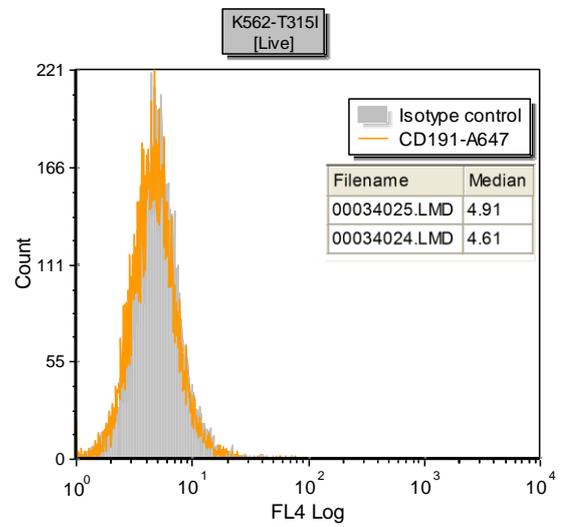
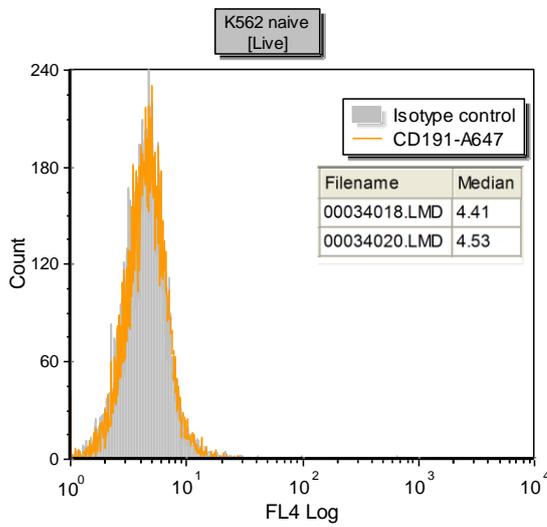
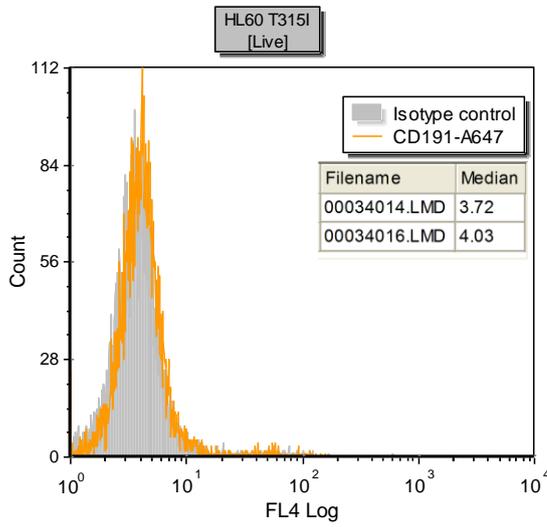
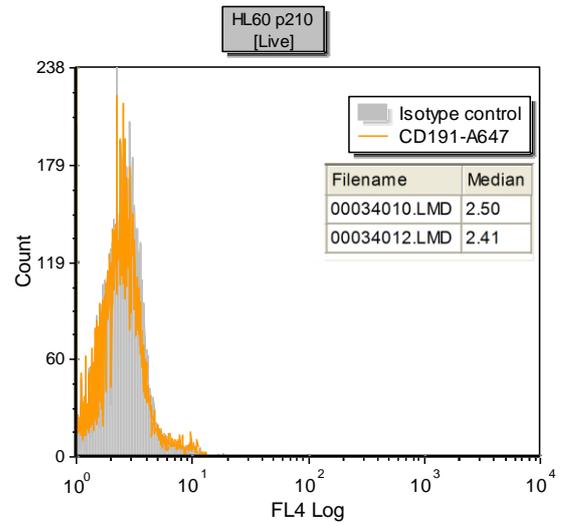
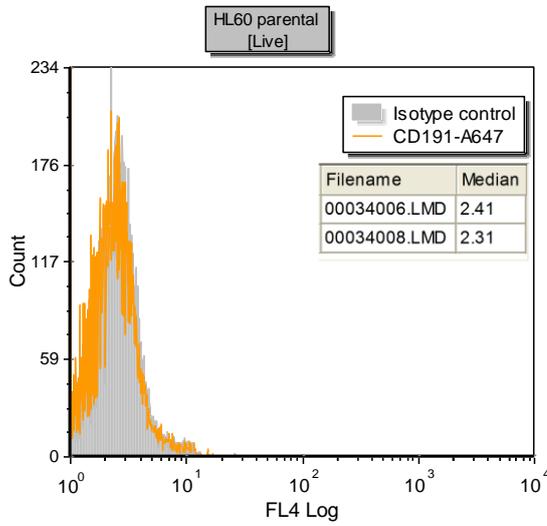
Figure S-8 CD181 + stimulation



S-9: Histograms for Figure 3-11A: HL60 and K562 expression of the MIP-1 α receptor without pre-incubation with rMIP-1 α

2×10^5 cells were harvested and were made up to 1ml with culture media, pelleted and supernatant aspirated. The cells were incubated with Alexa F647-conjugated CD191 antibody (BD Pharmingen) or the appropriate isotype control for 30 minutes on ice and resuspended in FACS fix after washing. The assay was analysed by flow cytometry. The results are expressed as MFI in comparison to the isotype control. The experiment was performed twice.

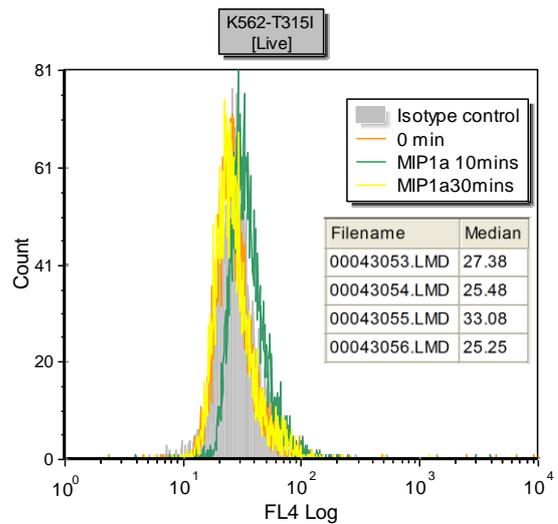
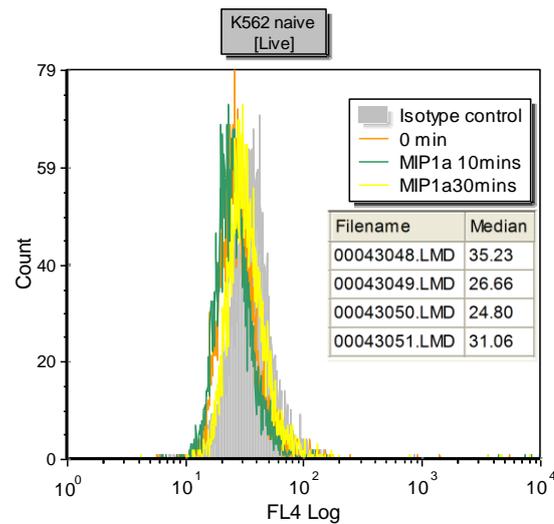
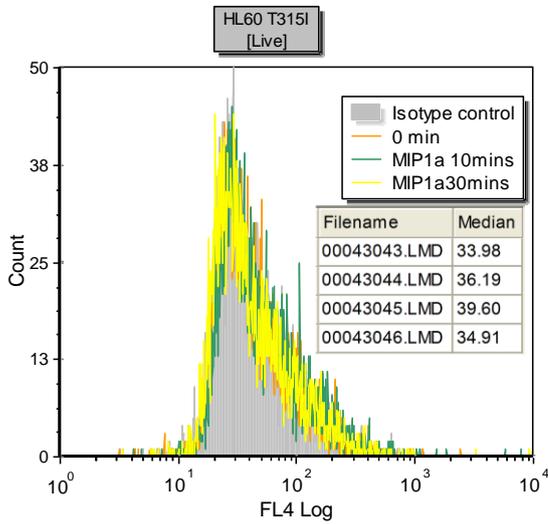
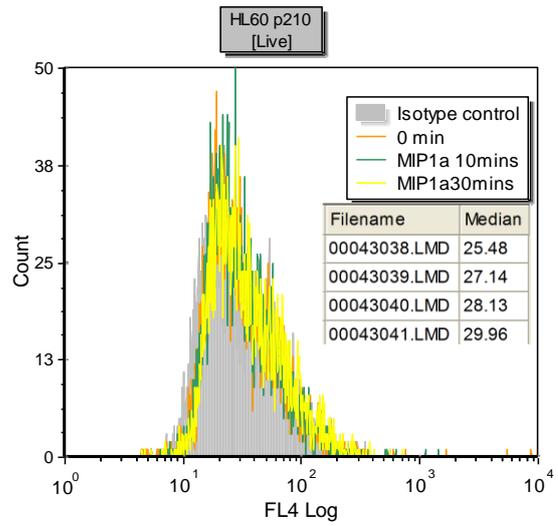
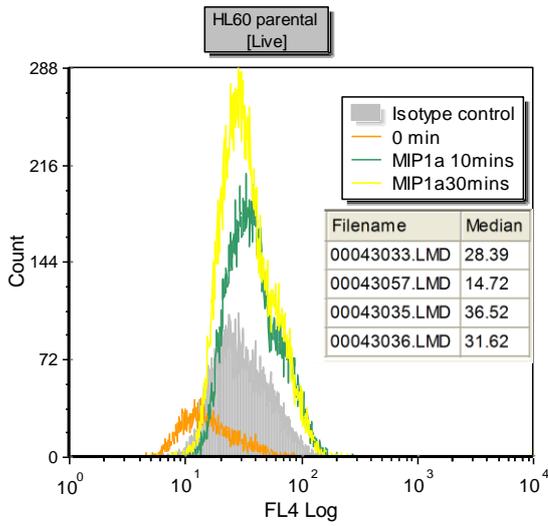
Figure S-9 CD191



S-10: Histograms for Figure 3-11B: HL60 and K562 expression of MIP-1 α receptor with pre-incubation with rMIP-1 α

The cells were cultured overnight in SDM. The next day, cells were harvested and pre-incubated with 50 ng/ml MIP-1 α (PeproTech) for 10 minutes or 30 minutes respectively at 37°C/5% CO₂. At the end of the incubation period, the cells were washed with cold media and the experiment continued as above. The results are expressed as MFI in comparison to the isotype control.

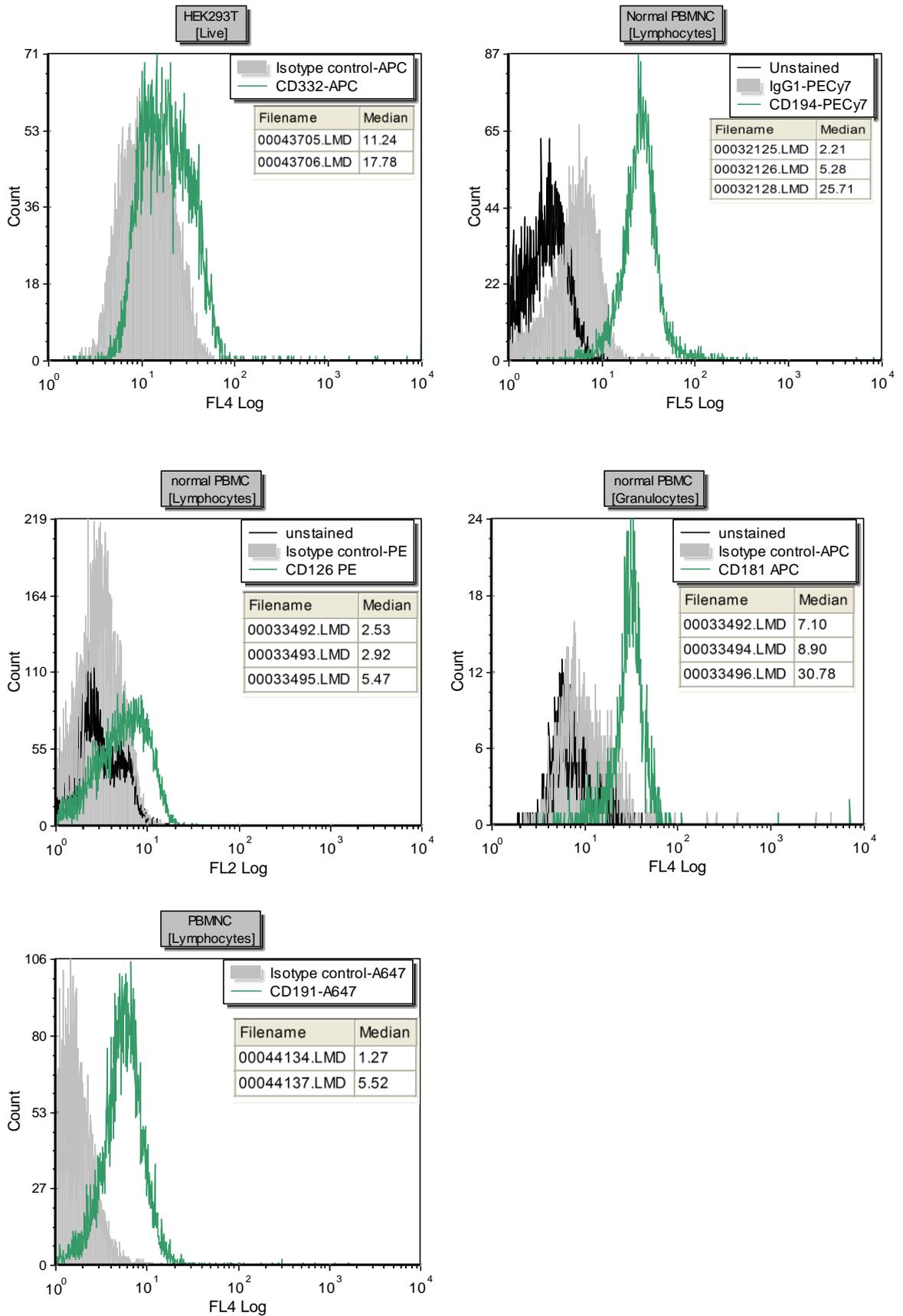
Figure S-10 CD191 + stimulation



S-11: Positive control histograms for figures 3-6 to 3-11

Approximately 5×10^5 cells of appropriate cell type were harvested (as indicated in the histogram title) and aliquot into appropriate FACS tubes. Appropriate reagents were added and incubated for 30 minutes on ice in the dark. After incubation, the cells were resuspended in FACS fix after washing. The assay was analysed by flow cytometry. The results are expressed as MFI in comparison to the isotype control.

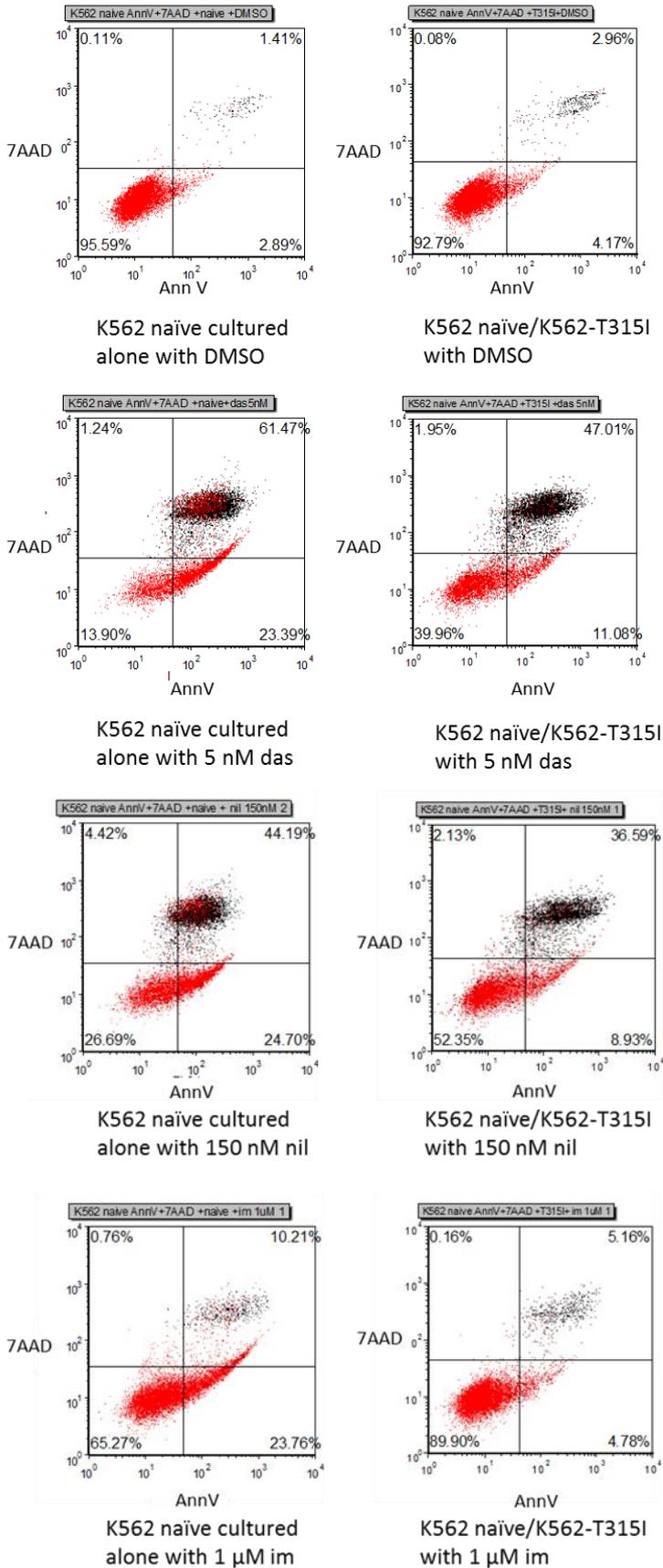
Figure S-11 Positive control histograms for figures 3-6 to 3-11



S-12: Representative flow cytometry plots for Figure 3-18: Annexin V and 7AAD expression on K562 naïve cells either cultured alone or co-cultured with K562-T315I cells (K562 naïve/K562-T315I) with TKI

After optimal wash and resuspension in SDM at a concentration of 1×10^5 cells/ml, K562 naïve or K562-T315I cells were plated at the bottoms of the Transwell[®] plates at 1.5 ml and K562 naïve cells at 0.5 ml in the Transwell[®] inserts. Appropriate concentrations of TKI were added with DMSO used as the vehicle control. After incubation at 37°C/5% CO₂ for 3 days, K562 naïve cells from the inserts were assessed for cell viability using Annexin V and 7AAD staining.

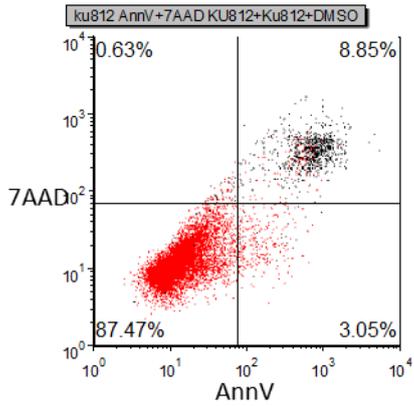
Figure S-12



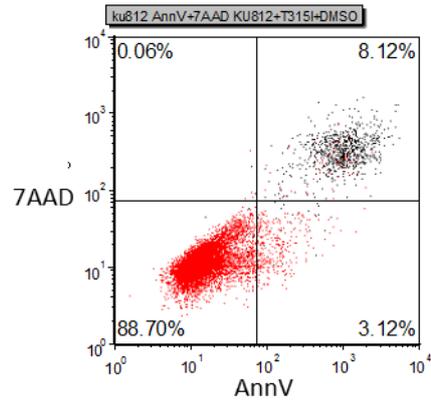
S-13: Representative flow cytometry plots for Figure 3-19: Annexin V and 7AAD expression on KU812 cells either cultured alone or co-cultured with K562-T315I cells (KU812/K562-T315I) with TKI

After washing, both cell lines were resuspended in SDM at a concentration of 1×10^5 cells/ml. 1.5 ml of either KU812 or K562-T315I cells were plated at the bottoms of the Transwell® plates and 0.5 ml of KU812 cells on the Transwell® inserts. Appropriate concentrations of TKI were added with DMSO used as the vehicle control. After incubation at 37°C/5% CO₂ for 3 days, KU812 cells from the inserts were assessed for cell viability using Annexin V and 7AAD death assay.

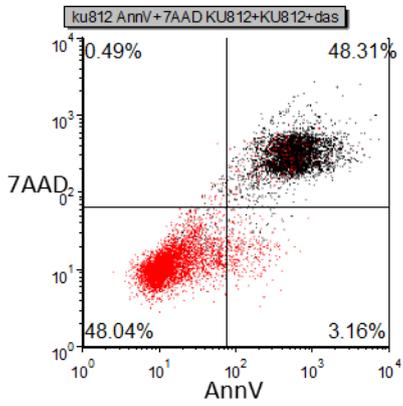
Figure 3-13



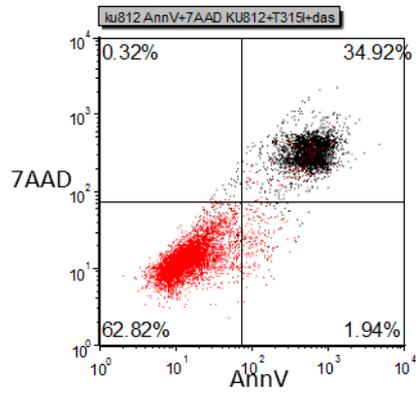
KU812 cultured alone with DMSO



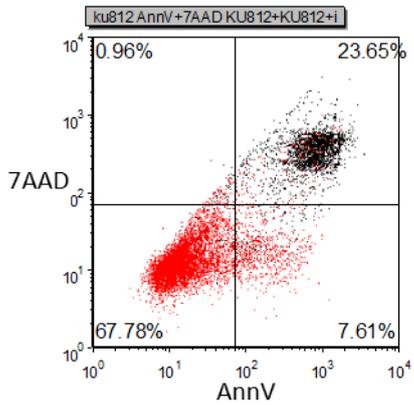
KU812/K562-T315I with DMSO



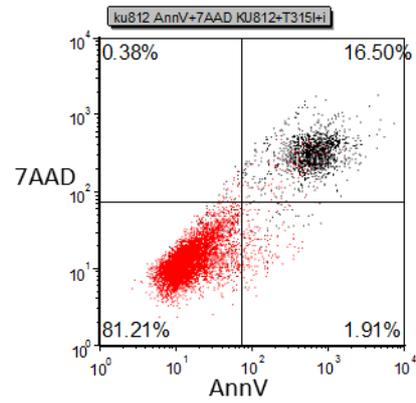
KU812 cultured alone with 0.5 nM das



KU812/K562-T315I with 0.5 nM das



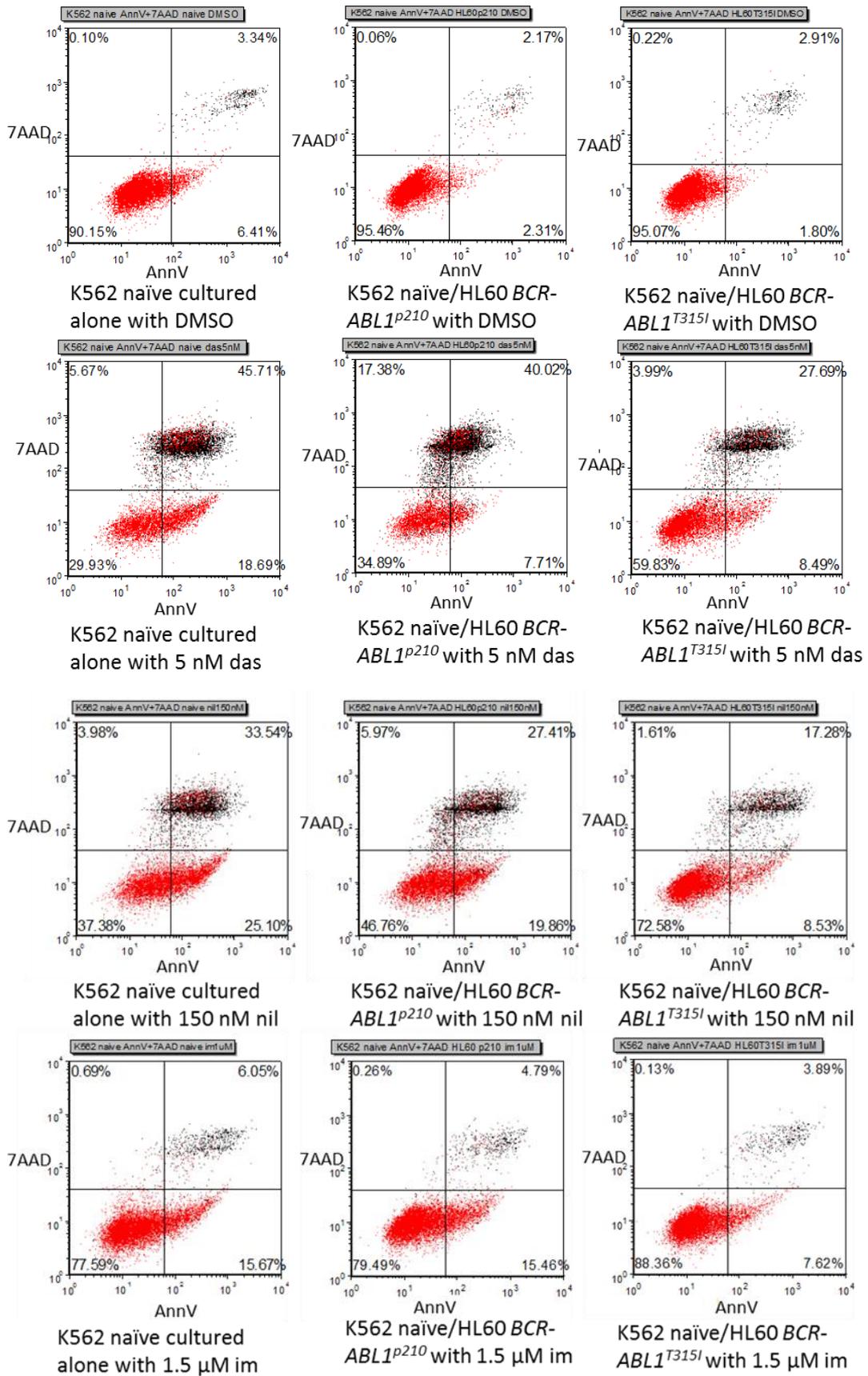
KU812 cultured alone with 500 nM im



KU812/K562-T315I with 500 nM im

S-14: Representative flow cytometry plots for Figure 3-20: Annexin V and 7AAD expression on K562 naïve cells either cultured alone, co-cultured with HL60 *BCR-ABL1*^{p210} (K562 naïve/HL60 *BCR-ABL1*^{p210}) or with HL60 *BCR-ABL1*^{T315I} (K562 naïve/HL60 *BCR-ABL1*^{T315I}) Cells were washed and resuspended in SDM at a concentration of 1×10^5 cells/ml or 1.2×10^5 cells/ml for HL60 *BCR-ABL1*^{T315I} cells as the cells expanded more slowly (refer Figure 3-5 and 3-17). 1.5 ml of either K562 naïve, HL60 *BCR-ABL1*^{p210} or HL60 *BCR-ABL1*^{T315I} cells were plated at the bottoms of the Transwell® plates and 0.5 ml of K562 naïve cells at the Transwell® inserts. The appropriate concentrations of TKI were added with DMSO used as the vehicle control. After incubation for 3 days at 37°C/5% CO₂, K562 cells from the inserts were assessed for cell viability using Annexin V and 7AAD death assay.

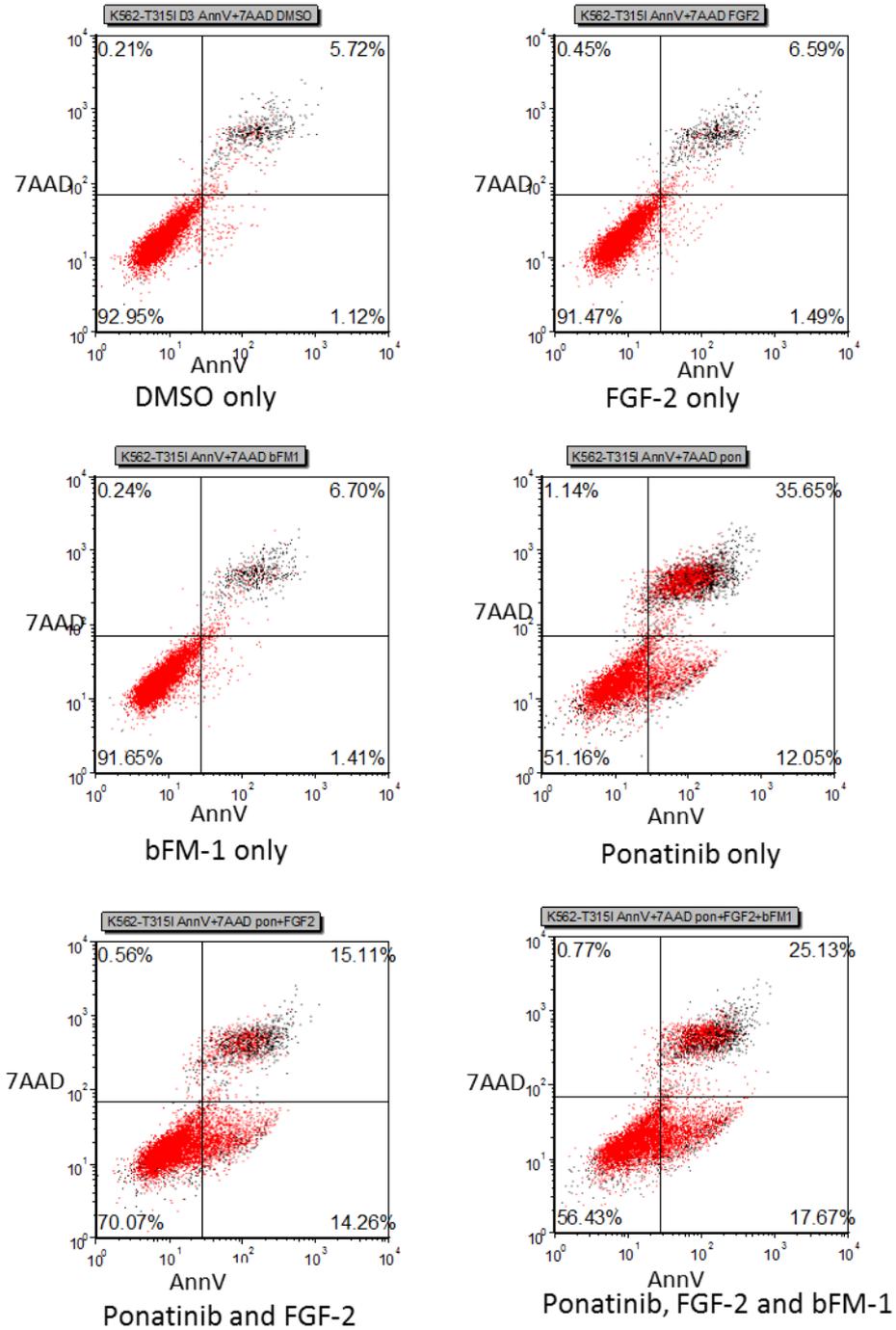
Figure S-14



S-15: Representative flow cytometry plots for Figure 3-25: Annexin V and 7AAD expression on K562-T315I cells when cultured with ponatinib

After optimal wash, K562-T315I cells were resuspended in SDM at a concentration of 1×10^5 cells/ml and plated in a 24 well plate. Appropriate reagents were added- 20 ng/ml FGF-2, 250 ng/ml bFM-1 or 40 nM ponatinib. DMSO was used as vehicle control. After 3 days incubation at 37°C/5% CO₂, the cells were analysed by Annexin V and 7AAD staining.

Figure S-15



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