CELL LINEAGE, CELL MATURITY AND BCR-ABL: FACTORS WHICH INFLUENCE IMATINIB UPTAKE IN CHRONIC MYELOID LEUKAEMIA

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candor dat viribus alas

(Sincerity gives wings to strength)

ipsa scientia potestas est

(Knowledge itself is power)

prefer et obdura; dolor hic tibi proderit olim

(Be patient and tough; some day this pain will be useful to you)

aut viam inveniam aut faciam

(I'll either find a way or make one)

per aspera ad astra

(Through adversities to the stars!)

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Jane Engler 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.

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Manuscripts

JR Engler, TP Hughes & DL White. OCT-1 as a determinant of response to anti-leukemic treatment. *Clinical Pharmacology and Therapeutics.* 2011. Feb 23; Epub ahead of print. (Impact Factor: 7.586)

JR Engler, AC Zannettino, C Bailey, J Rasko, TP Hughes & DL White. OCT-1 function varies with cell lineage but is not influenced by BCR-ABL. *Haematologica.* 2011. Feb;96(2):213-20. (Impact Factor: 6.416)

JR Engler, A Frede, V Saunders, AC Zannettino, DL White & TP Hughes. The poor response to imatinib observed in CML patients with low OCT-1 activity is not attributable to lower uptake of imatinib into their CD34+ cells. *Blood.* 2010. Oct;116(15):2776-8. (Impact Factor: 10.555)

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JR Engler, A Frede, V Saunders, AC Zannettino, DL White & TP Hughes. OCT-1 activity in CML CD34+ cells is not predictive of molecular response to imatinib treatment in CP-CML patients, despite the strong predictive value of MNC OCT-1 activity. *American Society of Hematology Annual Meeting*, December 2009. New Orleans, USA. Poster Presentation.

JR Engler, A Frede, V Saunders, AC Zannettino, R D'Andrea, TP Hughes & DL White. CML CD34+ cells have reduced uptake of imatinib due to uniformly low OCT-1 activity, which can be increased with diclofenac treatment. *Centre for Stem Cell Research Annual Meeting*, November 2009. Adelaide, Australia. Poster Presentation.

JR Engler, A Frede, V Saunders, AC Zannettino, DL White & TP Hughes. OCT-1 activity in CML CD34+ cells is not predictive of molecular response to imatinib treatment in CP-CML patients. *Haematology Society of Australia and New Zealand Annual Meeting*, October 2009. Adelaide, Australia. Oral Presentation.

JR Engler, A Frede, AC Zannettino, DL White & TP Hughes. Reduced activity of the OCT-1 protein in primitive CML cells: A likely determinant of stem cell resistance in imatinib treated CML patients. *American Society of Haematology Annual Meeting*, December 2008. San Francisco, USA. Oral Presentation.

JR Engler, A Frede, S Quinn, AC Zannettino, TP Hughes & DL White. Reduced activity of the OCT-1 protein in primitive CML cells may be a key to stem cell resistance in imatinib treated CML patients. *New Directions in Leukaemia Research*, March 2008. Sunshine Coast, Australia. Poster Presentation.

SCHOLARSHIPS & AWARDS

Poster Prize, Centre for Stem Cell Research Annual Meeting. 2009.

For the abstract entitled “CML CD34+ cells have reduced uptake of imatinib due to uniformly low OCT-1 activity, which can be increased with diclofenac treatment”, Adelaide, November 2009.

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To provide support for the educational and professional development of researchers and other professionals undertaking a PhD. The award is to support basic, applied and translational research in Australia into the causes, treatment and care of people with leukaemia, lymphoma, myeloma and related blood disorders.

ABBREVIATIONS

ABL	Abelson kinase
ACD	Anticoagulent Citrate Dextrose Solution Formula A
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
AP	Accelerated phase
ATP	Adenosine triphosphate
BC	Blast crisis
BCR	Breakpoint cluster region
BM	Bone marrow
BSA	Bovine serum albumin
C	Celcius
CCR	Complete cytogenetic response
cDNA	Complementary deoxyribonucleic acid
CFSE	5-6-carboxyfluorescein diacetate, succinimidyl ester
CML	Chronic myeloid leukaemia
CMR	Complete molecular response
CP	Chronic phase
CPM	Counts per minute
Crkl	Crk-like protein
p-Crkl	Phosphorylated Crk-like protein
CV	Control vector
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetate
eGFP	Enhanced green fluorescence protein
FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
FCS	Foetal calf serum
Hanks	Hanks Balanced Salt Solution
HSC	Haematopoietic stem cell
IC50	50% inhibitory concentration
IFN-α	Interferon alpha
IM	Imatinib (STI571)
IRIS	International randomised study of interferon versus STI571
IUR	Intracellular uptake and retention
kD	Kilo Dalton
L	Litre
M	Molar
mA	Milli Amp (10^{-3} Amp)
MACS	Magnetically activated cell sorting
MCR	Major cytogenetic response
MMR	Major molecular response
mM	Milli Molar (10^{-3} Molar)
MNC	Mononuclear cells
mRNA	Messenger ribonucleic acid
µM	Micro Molar (10^{-6} Molar)
µg	Micro gram (10^{-6} gram)

ng	Nano gram (10^{-9} gram)
Nil	Nilotinib (AMN107)
OA	OCT-1 activity
OCT-1	Organic cation transporter 1
PB	Peripheral blood
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Ph	Philadelphia chromosome
PI	Propidium Iodide
PMA	Phorbol-12-myristate-13-acetate
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute (media)
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RQ-PCR	Real time quantitative polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
S/N	Supernatant
STI571	Signal transduction inhibitor 571 (imatinib)
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.1% Tween20
TKI	Tyrosine kinase inhibitor

U	Units
UV	Ultraviolet
v/v	Volume per volume
WBC	White blood cells
WCC	White cell count
w/v	Weight per unit volume

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ABSTRACT

Despite the excellent responses observed in patients with chronic phase (CP) chronic myeloid leukaemia (CML) on imatinib therapy, approximately 25% display primary resistance or sub-optimal response. The organic cation transporter 1 (OCT-1) is the major active influx pump for imatinib in CML cells. The functional OCT-1 activity in mononuclear cells (MNC) is highly variable between patients and significantly correlates with a patient's molecular response to imatinib treatment and overall survival. Given the strong predictive value of OCT-1 activity, the present study was aimed at identifying factors responsible for the variation in OCT-1 activity seen in patients.

Pure populations of neutrophils, monocytes and lymphocytes were isolated from the peripheral blood of CML patients at diagnosis. The OCT-1 activity and OCT-1 mRNA expression was found to be the highest in the neutrophil population, followed by monocytes then lymphocytes. When the surface expression of the granulocytic antigens CD15 and CD16 were examined, a significant correlation was observed between MNC OCT-1 activity and the proportion of immature myeloid cells expressing CD15+16-. Interestingly, the neutrophil OCT-1 activity was found to be similar when recovered from CML patients at diagnosis, CML patients in cytogenetic remission and normal donors, implying that BCR-ABL expression is unlikely to influence OCT-1 activity. This hypothesis was confirmed in a cell line model, in which ectopic BCR-ABL expression was not found to directly affect OCT-1 expression or function, but stimulated myeloid differentiation which, in turn, led to increased OCT-1 activity. These data suggest that the predictive MNC OCT-1 activity is most strongly related to cell lineage, particularly the proportion of immature myeloid cells, but is not directly related to BCR-ABL.

CML early progenitor cells are less sensitive to imatinib induced apoptosis and are likely contributors to disease persistence. It was found that the OCT-1 activity and OCT-1 mRNA expression was significantly lower in primitive CD34+ cells compared with mature CD34- cells recovered from CML patients. These results indicate that low imatinib accumulation in primitive CML cells may be a critical determinant of long-term disease persistence. Studies to investigate whether the MNC OCT-1 activity provides a surrogate indicator of effective targeting of the more immature CD34+ cells failed to identify a relationship between high CD34+ OCT-1 activity and the achievement of major molecular response. This is despite the confirmation of previous findings that high MNC OCT-1 activity is significantly associated with the achievement of major molecular response to imatinib treatment. These important findings suggest that kinase inhibition in these mature cells, and not the CD34+ cells, may be the key determinant of response in CML.

In conclusion, the studies outlined in this thesis have identified cell lineage as a key contributor to MNC OCT-1 activity and hence response to imatinib treatment. While primitive CD34+ cells demonstrate low OCT-1 activity, which may contribute to their persistence despite imatinib therapy, the OCT-1 activity in these cells does not correlate with patient response to treatment. Therefore, direct targeting of this primitive population may not be essential for achievement of early and deep molecular responses.

1 INTRODUCTION

1.1 Haematopoiesis

1.1.1 The hierarchy of haematopoiesis

Formation of haematopoietic (blood) cells occurs in a structured and hierarchical fashion (Figure 1.1) [1]. Haematopoietic stem cells (HSC's), at the top of the hierarchy, are very rare and found in the stem cell niche in the bone marrow. HSC's are defined as having the capacity to self-renew and to give rise to more differentiated cells which are able to fully reconstitute the haematopoietic system [2]. The majority of HSC's typically exist in a quiescent state with only a small number of cells actively supplying the haematopoietic system at a time. Under certain stimuli, however, these quiescent HSC's are able to rapidly enter cell cycle and contribute to bone marrow reconstitution. The first step of HSC differentiation is a loss of the cell's self-renewal capacity, which is then followed by a commitment to either the myeloid or lymphoid lineage. Following a continuum of various committed progenitors, the hierarchy results in terminally differentiated mature cells. The myeloid lineage gives rise to platelets, erythrocytes, monocytes and granulocytes (neutrophils, eosinophils and basophils). The lymphoid branch gives rise to B-lymphocytes, T-lymphocytes and natural killer (NK) cells.

1.1.2 Regulation of haematopoiesis

HSC's are pluripotent and have the potential to give rise to all haematopoietic lineages [3]. HSC's are regulated by intrinsic and external (bone marrow microenvironment) factors. The fate options for HSC's are to maintain quiescence, or undergo self-renewal (proliferation), differentiation, apoptosis or migration out of the bone marrow (Figure 1.2). The major intrinsic factors contributing to stem cell fate are transcription factors [4]. For example, the transcription factors PU.1 and GATA-1 have been found to have a concentration-dependent effect on differentiation and lineage choice of HSC's [5] and Hox-B4 has been found to increase the self-renewal potential of HSC's [6]. The major external factors in the bone marrow microenvironment that regulate HSC's are cytokines, chemokines, the extracellular matrix and

Figure 1.1 The hierarchy of haematopoiesis

Haematopoietic stem cells give rise to multipotent progenitors from which the lineage specific common myeloid and lymphoid progenitors arise. Following a chain of committed progenitors, terminally differentiated mature cells are formed.

MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage progenitor; CFU, colony forming unit.

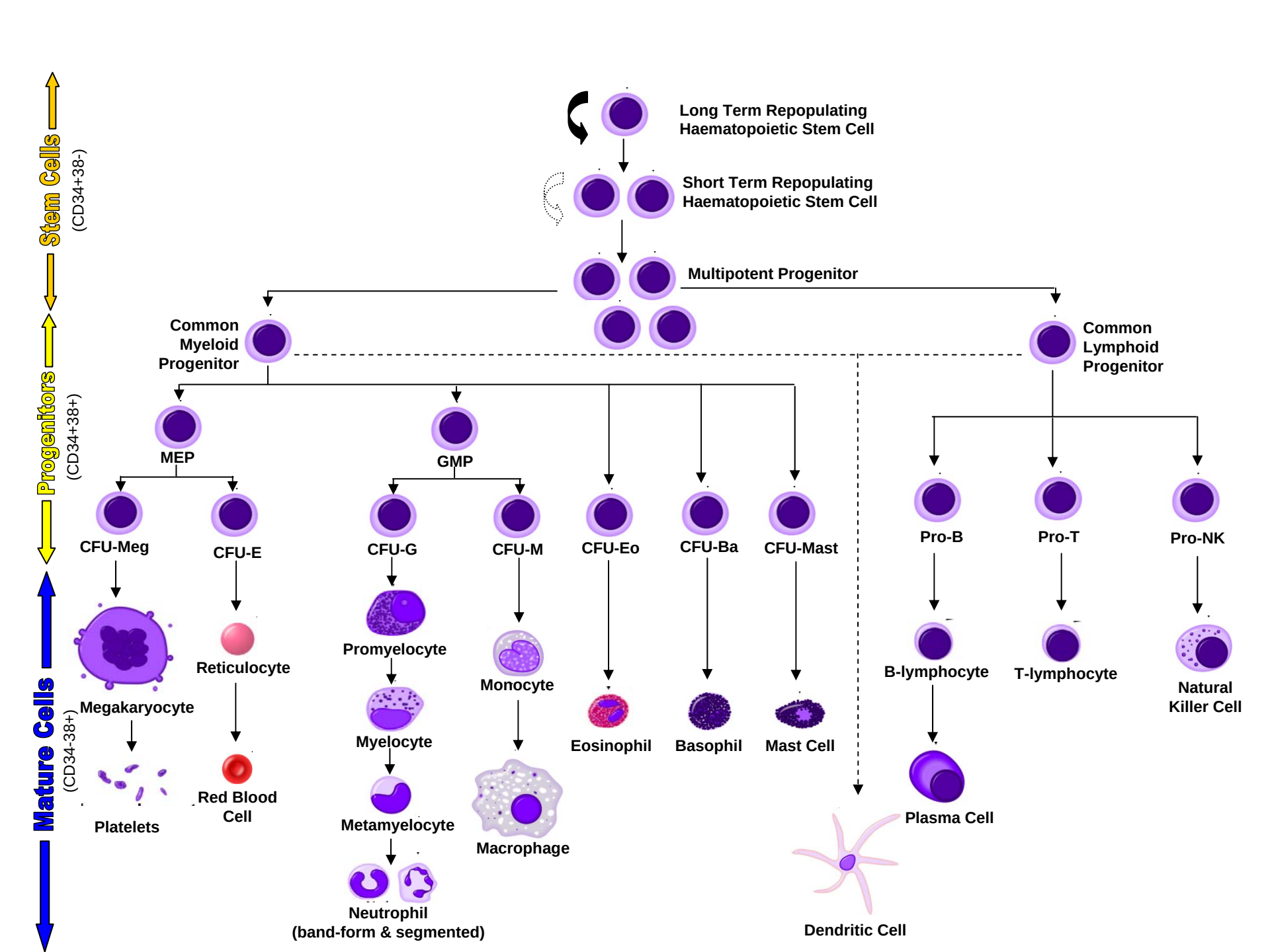

Stem Cells
(CD34+38-)
Progenitors
(CD34+38+)
Mature Cells
(CD34-38+)
Long Term Repopulating Haematopoietic Stem Cell
Short Term Repopulating Haematopoietic Stem Cell
Multipotent Progenitor
Common Myeloid Progenitor
Common Lymphoid Progenitor
MEP
GMP
CFU-Meg
CFU-E
CFU-G
CFU-M
CFU-Eo
CFU-Ba
CFU-Mast
Pro-B
Pro-T
Pro-NK
Megakaryocyte
Reticulocyte
Promyelocyte
Monocyte
Myelocyte
Eosinophil
Basophil
Mast Cell
B-lymphocyte
T-lymphocyte
Natural Killer Cell
Platelets
Red Blood Cell
Metamyelocyte
Macrophage
Plasma Cell
Neutrophil (band-form & segmented)
Dendritic Cell

Figure 1.2 Fate of haematopoietic stem cells

Signals from internal and/or external factors determine if HSC's proliferate, differentiate, migrate, undergo apoptosis, are maintained in quiescence or develop a malignant clone that multiply in an uncontrolled manner. Figure from Larsson and Karlsson (2005).

interactions with the bone marrow stroma. The growth factor thrombopoietin and its receptor c-mpl along with stem cell factor and its receptor c-Kit have been shown to be expressed on HSC's and promote survival of progenitor cells [7, 8]. Both interferfon-γ and tumour necrosis factor-α inhibit self-renewal of HSC's, thereby acting as negative regulators [9, 10]. Bone marrow stromal cells express a variety of extracellular matrix molecules, including collagen and fibronectin, and cellular adhesion molecules. These molecules are essential for maintenance of HSC localisation and mobility [11, 12].

1.1.3 Haematopoietic malignancies

Leukaemias are a group of cancers that occur within the haematopoietic system. Leukaemias are a result of aberrations in intracellular signalling processes which ultimately alters the proliferation, survival and differentiation of affected cells. As a consequence, there is uncontrolled expansion of progenitor and mature haematopoietic cells, with preference to a specific lineage. Leukaemogenesis has generally been attributed to alterations in intrinsic factors that influence stem cell fate. Genetic modifications, such as chromosomal translocations and mutations, which result in altered signalling and/or abnormal transcription factor expression, have been implicated in causing leukaemia [2, 4]. In many cases it is likely to be a combination of factors that bring about the leukaemic phenotype.

1.2 Chronic Myeloid Leukaemia

1.2.1 Clinical features

Chronic Myeloid Leukaemia (CML) is a clonal malignancy that results in a massive expansion of mature myeloid cells [13]. It was first reported in 1845 [14] and encompasses approximately 15% of all leukaemia's, and affects 0.001% of the population worldwide [15, 16]. Classically the disease is considered triphasic. The majority of patients are diagnosed within the first phase of the disease, chronic phase (CP). CP-CML is a relatively indolent disease, which is characterized by an excess of myeloid cells (particularly those of the neutrophil lineage) that

retain normal function [16]. When treated with cytoreductive agents, chronic phase generally lasts between 4-6 years. The disease then transforms to a more aggressive accelerated phase (AP) and then progresses to blast crisis (BC). Blast crisis is more representative of an acute leukaemia with the presence of >30% blasts in the peripheral blood and once entered is invariably fatal [13, 17]. Disease progression is most likely a result of an accumulation of chromosomal and molecular abnormalities, including trisomy 8, and mutations of p53, c-MYC and Ras [17].

1.2.2 Molecular pathogenesis of CML

The cytogenetic hallmark of CML is the Philadelphia Chromosome (Ph) [18], which is the product of a reciprocal translocation between chromosomes 9 and 22 (Figure 1.3) [19]. This translocation results in the fusion of the Abelson kinase gene (ABL) on chromosome 9 and the breakpoint cluster region gene (BCR) on chromosome 22, resulting in the BCR-ABL fusion gene [20, 21]. This fusion gene encodes for the constitutively active tyrosine kinase, BCR-ABL. The activity of this kinase significantly alters cell signalling processes within Ph+ cells, and mediates an increase in cell proliferation, a suppression of apoptosis, altered cell adhesion and genetic instability (Figure 1.4) [22-24]. These altered signalling pathways give BCR-ABL-expressing cells a proliferative and survival advantage over normal cells, and as such results in the pathogenesis of CML. BCR-ABL alone has been demonstrated to be essential and sufficient for inducing a CML phenotype in both *in vitro* and *in vivo* studies [25, 26]. Transduction of human cord blood CD34+ cells with a BCR-ABL-containing retroviral vector resulted in reduced cellular adhesion, increased migration, enhanced survival in cytokine depleted media and increased numbers of granulocyte-macrophage colony-forming units (GM-CFU) [26]. Mice transplanted with murine haematopoietic cells engineered to over-express BCR-ABL, developed symptoms resembling CML [25]. Including elevated leukocyte count, splenomegaly, and an accumulation of granulocytes and immature myeloid cells in the spleen, liver and bone marrow [25].

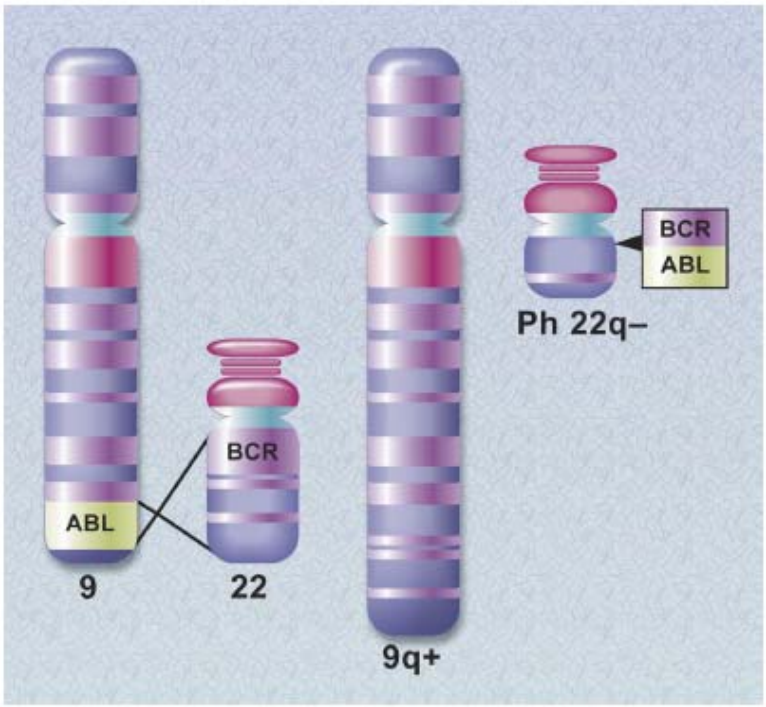


Figure 1.3 The translocation that creates the Philadelphia chromosome

The reciprocal translocation between the long arms of chromosomes 9 and 22 cause the fusion of the BCR and Abl genes on the derivative chromosome 22, the Philadelphia chromosome. Figure from Druker 2008 (ASH 50th anniversary invited reviews).

Figure 1.4 The BCR-ABL gene disrupts normal cell signal pathways

The formation of the BCR-ABL gene results in constitutive activation of signal transduction pathways critical for normal haematopoiesis, which are normally under tight regulation. Importantly proliferation, adherence and apoptosis are affected. Figure from Goldman and Melo (2003).

1.2.2.1 BCR-ABL kinase activity

Under normal conditions Abl kinase activity is highly regulated and resides in the nucleus [27]. BCR-ABL however, is located predominantly in the cytoplasm and its kinase activity is constitutively activated due to the disruption of the SH3 domain on Abl, which has been shown to have a repressive function [28]. Additionally, dimerisation and autophosphorylation of BCR-ABL enhances its activity [28]. The action of BCR-ABL significantly alters cell signalling processes, including the activation of the Ras, Jak/Stat and PI3-kinase pathways (Figure 1.5) [29-31]. Activation of these pathways is a result of phosphorylation of a number of tyrosine residues on adaptor proteins such as Crkl, Grb-2, Shc and Dok [32, 33]. The primary consequences of these cellular pathways are increased proliferation, reduced apoptosis and altered cellular adhesion. The proliferative advantage of BCR-ABL-expressing cells is due to the formation of multimeric complexes between BCR-ABL and PI3-kinase, Cbl, Crk and Crkl, all activating the PI3-kinase pathway [34, 35]. PI3-kinase activation has been shown to down regulate cell cycle checkpoint proteins such as p27 [36]. Furthermore, activation of Akt (the major effector protein of PI3-kinase) promotes β-catenin translocation to the nucleus where it induces the expression of several genes, including cyclin D1 to induce cell cycle progression [30]. Activation of PI3-kinase in BCR-ABL cells results in Akt phosphorylating the pro-apoptotic protein Bad. Bad becomes inactive when phosphorylated, thereby inhibiting apoptosis in these cells [37]. Bad has also been shown to be phosphorylated by Raf-1, a direct downstream protein of Ras [37]. Furthermore, Stat-5 phosphorylation in BCR-ABL-expressing cells results in the transcriptional activation of the anti-apoptotic protein Bcl-xL [31]. Reduced stromal adherence of CML progenitor cells results in the premature release of these cells into the blood [38]. This may be due to CML cells expressing an adhesion-inhibitory variant of β1 integrin, which is not seen in normal cells (Zhao, RC, 1997, Blood [abstract]). Furthermore, the adaptor protein Crkl, which is highly phosphorylated in CML cells, has been shown to be involved in the regulation of integrin-mediated cell adhesion [39].

Figure 1.5 BCR-ABL signal transduction pathways

This schematic outlines the major signal transduction pathways activated by BCR-ABL. Including the PI3-kinase, Ras/Raf and Jak/Stat pathways. Figure from Goldman and Melo (2003).

1.2.3 Historical therapies for CML

From the late 1800's to mid 1900 treatment options for CML patients were limited to arsenic, radiotherapy and splenectomy [40]. In 1953 the first clinical trials were initiated for the chemotherapeutic agent, busulfan [41]. Busulfan was found to have a relatively selective action on haematopoietic cells, particularly those of the granulocytic lineage. In comparison to radiotherapy, busulfan treatment resulted in more efficient control of disease and longer survival times. Hence, it became the primary treatment for CML until hydroxyurea was found to increase survival in patients with reduced toxicity [42]. The introduction of interferon-α for the treatment of CML provoked great interest as it was found to significantly improve survival compared to busulfan or hydroxyurea [43] and it induced complete cytogenetic remissions (CCR, Ph negativity) in some patients [44]. The combination of interferon-α and cytarabine (arabinosylcytosine, Ara-C) has been reported to improve cytogenetic responses and survival rates in patients compared with interferon-α alone, however this is also associated with greater toxicity [45]. To date, the only consistent proven curative treatment for CML is total-body irradiation or myeloablative chemotherapy followed by syngeneic or allogeneic bone transplantation [15]. The largest clinical study found that CP patients who underwent this procedure had a 70% survival rate at 10 years [46]. In recent years, more accurate and reliable techniques of detecting minimal residual disease (ie: FISH, RQ-PCR) have found many patients to remain BCR-ABL negative many years following transplant [47] and even some patients who do display persistent low levels of BCR-ABL transcripts remain in remission and do not relapse [48]. Despite these durable responses, transplantation is associated with high rates of morbidity and mortality and this option is only available to approximately 30% of patients due to age and donor availability.

1.3 Tyrosine Kinase Inhibitors

The identification that BCR-ABL was the key molecular mechanism of cellular transformation to CML has provided a unique opportunity to develop targeted molecular therapy. High-

throughput screening of chemical libraries, in search of kinase inhibitory compounds, revealed the 2-phenylaminopyrimidine class of kinase inhibitors as having inhibitory potential against ABL [49]. Further testing identified compound STI571 (imatinib mesylate, Glivec®; Novartis Pharmaceuticals) as having the greatest specificity at killing CML cells [50].

1.3.1 Imatinib

Imatinib (Figure 1.6) was the first rationally designed tyrosine kinase inhibitor (TKI) to target BCR-ABL. Imatinib inhibits all ABL kinases (c-ABL, BCR-ABL and Tel/ABL), and in addition also targets the macrophage colony stimulating factor receptor, c-fms [51], PDGF receptors [49], ARG [52] and c-Kit [49, 50]. Imatinib inhibits the ABL kinase by binding to amino acid residues in the ATP-binding site. This binding of imatinib alters the conformation of the BCR-ABL activation loop, locking the kinase domain in the inactive form. This formation prevents the binding and hence phosphorylation of substrates by BCR-ABL [53]. Therefore, imatinib significantly reduces the kinase activity of BCR-ABL and the signal transduction pathways are not activated (Figure 1.7).

1.3.1.1 Efficacy

Imatinib rapidly progressed through *in vitro* and *in vivo* studies to phase I and II clinical trials and was approved by the US Food and Drug Administration (FDA) in 2001 [54]. The International Randomised Study of Interferon versus imatinib STI571 (IRIS) trial was a multi-centre, international, phase III randomized study [55]. 1106 CP-CML patients at diagnosis were recruited, and patients were randomized to receive either 400mg of imatinib daily or a regimen of interferon-α and low dose Ara-C (IFN+Ara-C). After a follow-up of 18 months it was clearly shown that treatment with imatinib resulted in exceptionally superior haematologic and cytogenetic responses in comparison to IFN+Ara-C (Figure 1.8) [55]. It was found that 76% of patients treated with imatinib achieved CCR by 18 months in comparison to 14% of IFN+Ara-C treated patients. Furthermore, compared with IFN+Ara-C, significantly less patients treated with imatinib discontinued therapy due to intolerance (3% vs. 31%) or progressed to AP or BC (3%

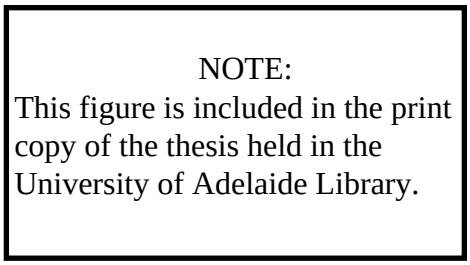


Figure 1.6 The chemical structure of imatinib mesylate

The molecular formula of imatinib is C29H31N7O●CH4SO3 and its molecular weight is 589.7. Figure kindly provided by Novartis Pharmaceuticals.

Figure 1.7 Action of imatinib

Imatinib competitively binds into the ATP binding pocket in the BCR-ABL protein. This binding prevents ATP from binding and phosphorylating adaptor proteins, thereby inhibiting the downstream signalling pathways. Figure from Goldman and Melo (2001).

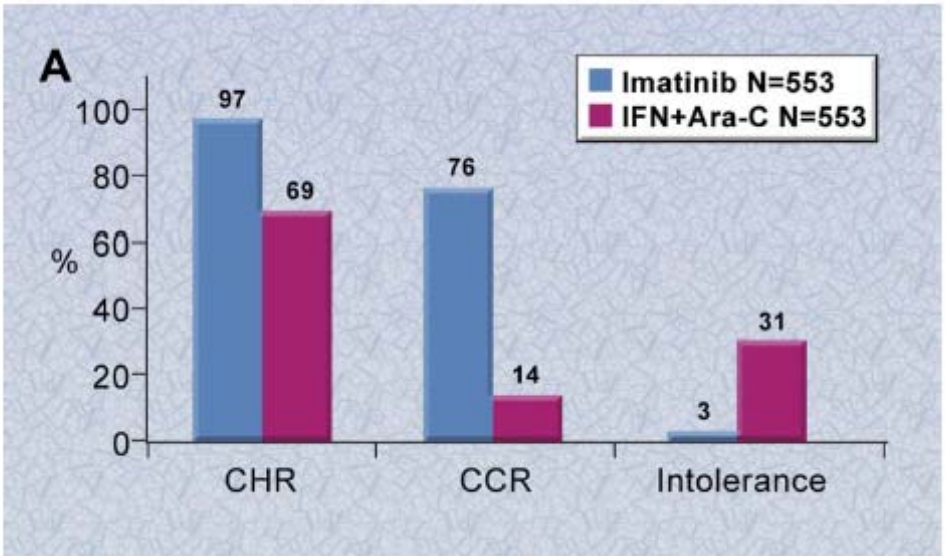


Figure 1.8 Efficacy of imatinib versus interferon-α+cytarabine for CP-CML

Percentage of patients achieving complete haematological response (CHR) and complete cytogenetic response (CCR) and patients showing intolerance to imatinib treatment following imatinib therapy. Median follow-up of 18 months. Figure from Druker 2008 (ASH 50th anniversary invited reviews).

vs. 8%). The obvious superiority of imatinib therapy resulted in the majority of IFN+Ara-C patients being crossed over to the imatinib arm and as such the study became a long-term study on imatinib efficacy as a frontline therapy.

At five years of follow-up, imatinib treated patients were shown to have durable and sustained responses [56]. 87% of patients treated with imatinib achieved CCR after 5 years of treatment (Figure 1.9A). Furthermore, 93% of patients had not progressed to AP or BC and overall survival was 89% (Figure 1.9B). These favourable cytogenetic responses were mimicked by significant molecular responses as seen by reductions in the level of BCR-ABL transcripts following imatinib treatment [57]. Of those patients who had achieved CCR at 12 months of imatinib treatment, 57% had a 3-log reduction in their BCR-ABL transcript levels (major molecular response, MMR). This achievement of MMR at 12 months was associated with a complete absence of progression to AP/BC at 24 months. The most recent IRIS update, found that 86% of patients had achieved MMR at 8 years of imatinib treatment, and none of these patients had progressed to AP/BC [58]. The rates of event free and overall survival at 8 years were 81% and 85%, respectively and a total of 92% of patients remained free from progression to AP/BC. However, approximately 40% of patients discontinued therapy and came off study, and were not followed for progression.

1.3.1.2 Resistance

Despite the excellent clinical responses seen with imatinib treatment, a number of patients exhibit sub-optimal response to therapy as a result of primary or secondary resistance to imatinib. Primary resistance is defined as an initial poor response to imatinib treatment, most likely due to either factors intrinsic to the patient, or representative of their disease. Secondary resistance is defined as a loss of response after a period of good response to imatinib. Point mutations within the ATP binding region of BCR-ABL have been found to be the major cause of

Figure 1.9 Rates of response and survival following 5 years of imatinib therapy

(A) Percentage of patients achieving complete haematologic response, major cytogenetic response (<35% Ph+) and complete cytogenetic response (0% Ph+) after 5 years of imatinib therapy. (B) 5 year survival curve of CP-CML patients treated with imatinib. Figure from Druker at al (2006).

secondary resistance to imatinib [59-61]. An additional mechanism of secondary resistance may be BCR-ABL amplification [62].

1.3.2 Second Generation Tyrosine Kinase Inhibitors

In an attempt to combat imatinib-resistant disease and as an option for imatinib-intolerant patients, a number of second generation BCR-ABL inhibitors such as nilotinib (AMN107, Tasigna®; Novartis Pharmaceuticals) and dasatinib (BMS-354825, Sprycel®; Bristol-Myers Squibb) have been developed.

1.3.2.1 Nilotinib

Based on crystal structures of the imatinib-ABL complex the second generation TKI, nilotinib (Figure 1.10), was rationally designed to increase drug interaction with the ABL kinase domain [53, 63, 64]. This approach resulted in the generation of a drug that was found to be 10-50 times more potent than imatinib in inhibiting the proliferation of BCR-ABL expressing cell lines [65, 66]. Importantly, nilotinib has been shown to be active against most of the BCR-ABL mutants, except for the T315I, Y253H, E255K/V and F359V/C mutations [65-67]. Nilotinib has been found to be effective in inducing cytogenetic response in CP and AP patients following failure of imatinib therapy [68]. Furthermore, as a frontline therapy in CP-CML, nilotinib has been shown to be superior to imatinib at producing cytogenetic and molecular responses [69]. Following 12 months of therapy, the rates of MMR in nilotinib treated patients was 44% compared with 22% for imatinib treated patients.

1.3.2.2 Dasatinib

Dasatinib (Figure 1.10) is a dual SRC/ABL kinase inhibitor and differs from both imatinib and nilotinib, in that it is able bind to both the active and inactive confirmations of BCR-ABL [70]. Dasatinib has been found to be up to 300 times more potent, at nanomolar concentrations, than imatinib in inhibiting BCR-ABL. In addition, dasatinib is active against 14 of 15 imatinib-resistant mutations tested to date [65, 71]. Dasatinib has been shown to be effective in inducing

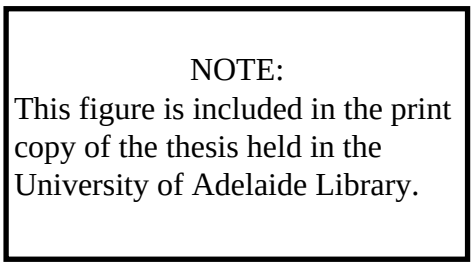


Figure 1.10 The chemical structure of nilotinib and dasatinib

Nilotinib has a molecular weight of 529.516. The molecular formula for dasatinib is C22H26C1N7O2S●H2O and its molecular weight is 506.02. Figures kindly provided by Novartis Pharmaceuticals and Bristol-Myer Squibb.

cytogenetic responses in the majority of imatinib-resistant or intolerant patients [72]. Additionally, in comparison to imatinib, dasatinib frontline therapy was found to produce significantly higher and faster rates of CCR and MMR in CP-CML [73].

1.4 Primary Resistance to Imatinib

Overall, clinical responses to imatinib by far exceed those achieved by previous therapies and many patients are expected to survive 20+ years [56]. However, there is a significant group of patients who do not respond adequately to imatinib treatment. In a trial of 400mg of imatinib up-front, at 12 months of therapy 42% of patients had failed to achieve CCR [74]. By 5 years of treatment, 17% failed to achieve CCR and 50% failed to achieve MMR. Furthermore, at 5 years, 25% of patients had discontinued imatinib treatment due to unsatisfactory response and/or intolerance. A more recent study demonstrated that 27% of patients treated with imatinib failed to achieve CCR by 12 months (defined as sub-optimal response) and 33% failed to achieve CCR by 18 months (defined as failure) [75]. Of importance, these patients were found to have significantly reduced overall survival, were more likely to progress to AP/BC and had a reduced probability of achieving CCR by 5 years. Therefore, approximately one third of patients treated with imatinib exhibit primary resistance. As such, it is essential to understand the reasons behind this resistance to therapy and to identify these patients prior to commencement of therapy.

1.4.1 Inadequate BCR-ABL kinase inhibition

The most likely explanation for poor response to imatinib is inadequate inhibition of the kinase activity of BCR-ABL. Studies in our laboratory have developed and optimized an assay to measure how well imatinib is able to inhibit BCR-ABL kinase activity within a patient's MNC *in vitro* [76]. This assay utilizes the phosphorylation status of the adaptor protein Crkl, as a direct correlation has been demonstrated between p-Crkl and the presence of BCR-ABL [77]. The dose required to reduce p-Crkl by 50% (IC50) is determined for each patient and this value has

been shown to be highly predictive of a patient's subsequent molecular response (Figure 1.11A). Patients with poor kinase inhibition (high IC50) were found to have a significantly lower probability of achieving cytogenetic and molecular remissions by 12 months compared with patients with good kinase inhibition (low IC50). Further to this, *in vivo* measurement of p-Crkl in a patient's PB-MNC shows that all of those patients who achieved a 50% reduction in p-Crkl by day 28 of imatinib treatment went on to achieve a MMR by 24 months [78]. Therefore, the degree of BCR-ABL kinase inhibition achieved in patients is highly variable and is a critical determinant of response to imatinib therapy.

1.4.2 Intracellular concentration of imatinib

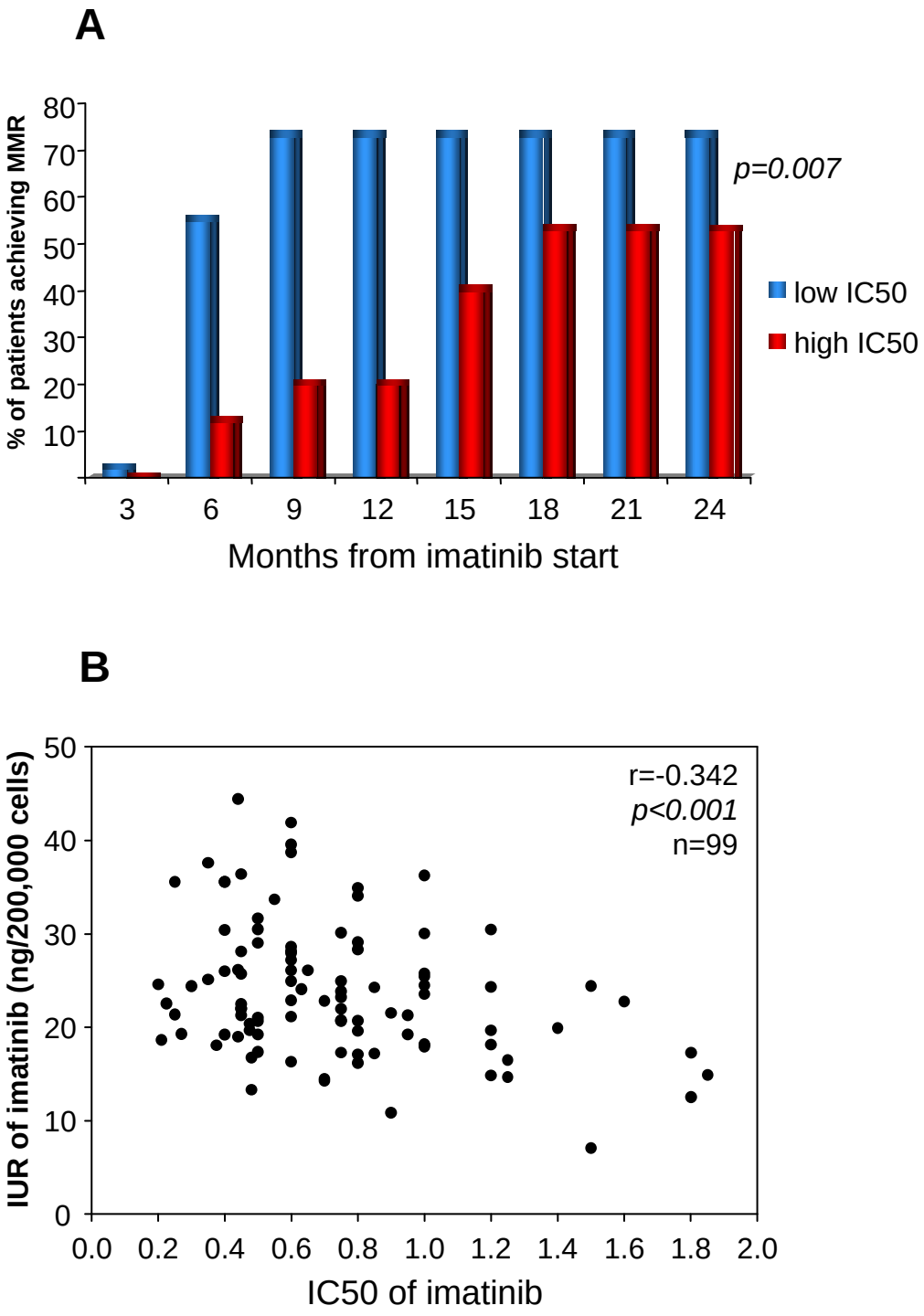
The reasons behind patient variability in imatinib-induced BCR-ABL kinase inhibition can be, in part, explained by a variation in the amount of drug that reaches the intracellular target. An assay to measure the intracellular concentration of imatinib achieved in patient's cells *in vitro* has been developed in our laboratory [79]. PB-MNC isolated from de novo patients are cultured with [^{14}C]-labelled imatinib, and the intracellular concentration of imatinib is measured using scintillation counting. The intracellular uptake and retention (IUR) of imatinib is determined, and a patient's IUR at 2μM has been found to significantly correlate with their IC50 (Figure 1.11B). Therefore, patients with poor kinase inhibition (high IC50) possess a significantly lower intracellular concentration of imatinib (low IUR) compared to those with good kinase inhibition (low IC50). This implies that the intracellular concentrations of imatinib achieved in patients is a major cause of their level of BCR-ABL kinase inhibition and hence response to treatment.

There are a number of factors which may influence the intracellular concentration of imatinib achieved in patient's leukaemic cells *in vivo*. The efficiency of absorption of imatinib by the gastrointestinal tract, the metabolism of imatinib in the liver and plasma protein binding may all play a role in the amount of drug available to the target cell [80, 81]. However, these factors do not explain the variability in BCR-ABL kinase inhibition in the *in vitro* assay, which so far is the

Figure 1.11 The relationship between BCR-ABL kinase inhibition, intracellular concentration of imatinib and molecular response

(A) Patients exhibiting good BCR-ABL kinase inhibition in vitro (low IC50) achieve significantly better and faster rates of major molecular response to imatinib treatment compared to patients with poor BCR-ABL kinase inhibition (high IC50). (B) There is a significant correlation between BCR-ABL kinase inhibition (IC50) and intracellular concentrations of imatinib (IUR) achieved in vitro. Patients with high IUR tend to have low IC50 and vice versa.

Figures adapted from White *et al* (2005) and White *et al* (2006) respectively.


A
% of patients achieving MMR
80
70
60
50
40
30
20
10
3
6
9
12
15
18
21
24
Months from imatinib start
p=0.007
low IC50
high IC50
B
IUR of imatinib (ng/200,000 cells)
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
IC50 of imatinib
r=-0.342
p<0.001
n=99

best predictor of response to imatinib treatment. More direct factors influencing the intracellular concentration of imatinib and hence kinase inhibition may be related to the expression and function of influx and efflux transporters of imatinib in leukaemic cells. Thomas *et al* found imatinib transport to be a predominantly active process and as such the role of efflux and influx transporters may be of significance in determining patient response to imatinib [82].

1.5 Efflux Transporters

Efflux mechanisms have long been implicated as a cause of drug resistance in many malignancies, including the leukaemia's [83]. Imatinib has been suggested to be a substrate for both ABCB1 (MDR1, p-glycoprotein) and ABCG2 (breast cancer resistance protein, BCRP) proteins belonging to the ATP-binding cassette (ABC) transporter family [82, 84-88].

1.5.1 ABCB1

ABCB1 has been shown to mediate multi-drug resistance and has been implicated as a mechanism for resistance to imatinib in CML. A number of studies have utilized ABCB1 over-expressing cell lines to determine if it is a mode of resistance to imatinib treatment. It has been demonstrated that the presence of ABCB1 results in reduced levels of apoptosis compared to control cells following treatment with imatinib. However, response to imatinib-induced apoptosis is restored in these cells following co-treatment with ABCB1 inhibitors such as verapamil and cyclosporin A [84, 85]. In addition, cell lines over-expressing ABCB1 have been shown to have a reduced intracellular accumulation of imatinib compared to controls [82, 85, 86], indicating that ABCB1 is a transporter for imatinib and may be a cause of resistance to imatinib. However, this notion has been disputed in other cell line and mouse models [89, 90]. Ferrao *et al* demonstrated that over-expression of ABCB1 in cell lines had no significant effect on growth or apoptosis following culture with imatinib [89]. An additional study compared mice expressing normal levels of ABCB1 in their bone marrow with those with ABCB1-null bone marrow. A CML-like disease was induced in these mice via transduction with a BCR-ABL retroviral vector.

Mice were subsequently treating with imatinib and it was found that the incidence and latency of disease did not differ between the two ABCB1 expressing and non-expressing mice, thus indicating that ABCB1 expression in haematopoietic cells is not a cause for imatinib resistance [90]. In primary patient samples, our laboratory has found that co-incubation of imatinib with the ABCB1 inhibitor PSC833 had no effect on the intracellular concentration of imatinib achieved in patient PB-MNC [79]. Therefore, while imatinib may be a substrate of ABCB1 in cell line systems expressing high levels of ABCB1, this may not be clinically relevant within patients as these high levels may not exist in CP.

1.5.2 ABCG2

In addition to ABCB1, imatinib has been shown to interact with the efflux transporter ABCG2 [86-88]. However, there is much debate regarding how imatinib interacts with ABCG2, whether imatinib is acting as a substrate [88, 91]}, an inhibitor [92, 93] or both. Studies from our laboratory, showed that the ABCG2 inhibitor, fumitremorgin C, had no significant effect on the intracellular concentration of imatinib in patient's PB-MNC [79]. Therefore, as with ABCB1, the clinical relevance of efflux via ABCG2 in CP-CML patients remains under debate.

1.5.3 ABCB1 and ABCG2 transcript levels

A number of studies have investigated the relationship between patient mRNA expression of ABCB1 and ABCG2 and response to imatinib treatment. In a study of 76 CP-CML patients it was found that mRNA expression of ABCG2 and ABCB1 at diagnosis was significantly correlated with the achievement of MMR at 6 and 12 months, respectively [94]. In addition, another study found that patients who failed to achieve CCR by 12 months had significantly higher transcript levels of ABCG2 at diagnosis, compared with patients who achieved MMR by 12 months [95]. However, these results have been contradicted by 2 other studies. Crossman *et al* found no difference in the pre-imatinib mRNA expression of ABCB1 or ABCG2 between patients who did and did not achieve CCR by 12 months of treatment [96]. Furthermore, Wang

et al found no relationship between ABCB1 and ABCG2 transcript levels and rates of CCR, overall and progression free survival [97].

Of interest, studies from our laboratory have found that a number of patients who develop kinase domain mutations, following imatinib therapy, exhibit an increased level of ABCB1 mRNA expression at diagnosis. These findings may indicate a role for high expression of efflux transporters in ensuing suboptimal dosing of imatinib and hence setting an ideal scenario for mutation development [98].

1.5.3.1 ABCB1 and ABCG2 polymorphisms

Single-nucleotide polymorphisms (SNPs) in the ABCB1 and ABCG2 genes have the potential to alter protein function and have been shown to affect the efficiency of absorption or elimination of imatinib [99]. As such, a number of studies have investigated the relationship between SNPs in ABCB1 or ABCG2 and patient response to imatinib therapy. Dulucq *et al* demonstrated increased rates of MMR by 12 months in patients who had the ABCB1 SNPs, C1236T SNP or G2677T/A [100]. Conversely, Kim *et al* found that the ABCB1 SNP, I1145I was significantly associated with reduced overall survival [101]. In addition it was found that the presence of the ABCG2 SNPs V12M and Q141K had adverse impacts on the achievement of CCR and MMR, respectively. However, while the frequency of these SNPs ranged from 8-49%, the majority of these SNPs were only present in a small number of patients. Therefore, this predictive analysis is not applicable for the majority of patients.

1.6 Influx Transporters

In humans, the solute carrier superfamily (SLC) contains the major influx transporters. The organic cation transporters (OCT) belong to the SLC22 family and mediate the transport of endogenous and exogenous substrates which exist as cations at physiologic pH [102, 103]. The OCT family along with the organic anion transporters (OAT) are responsible for the

absorption, elimination and distribution of drugs and other xenobiotics *in vivo* [104], particularly in the kidneys and the gastrointestinal tract. The OCT family has four known members, OCT-1, OCT-2, OCT-3 and OCT-6. The work of Thomas *et al* used a number of OCT inhibitors to determine the role of these OCT's in the intracellular concentration of imatinib [82]. It was found that the inhibitors prazosin (OCT-1&3), procainamide (OCT-1&2), verapamil (OCT-1 & ABCB1) and amantadine (OCT-1&2) all significantly reduced the intracellular concentration of imatinib achieved in a cell line model. The specific OCT-2 inhibitor (N-methylnicotinamide) and OCT-3 inhibitor (corticosterone) were found to have little effect on the uptake of imatinib. Therefore, the active uptake of imatinib is most likely mediated via the OCT-1 protein. While there are very few published reports on OCT-6, studies suggest that unlike the other three OCT's, OCT-6 is highly expressed in haematopoietic cells [105]. As such, it is possible that OCT-6 is also involved in imatinib transport. However, as there are no current inhibitors for this protein this hypothesis is difficult to address.

1.6.1 Organic cation transporter 1 (OCT-1)

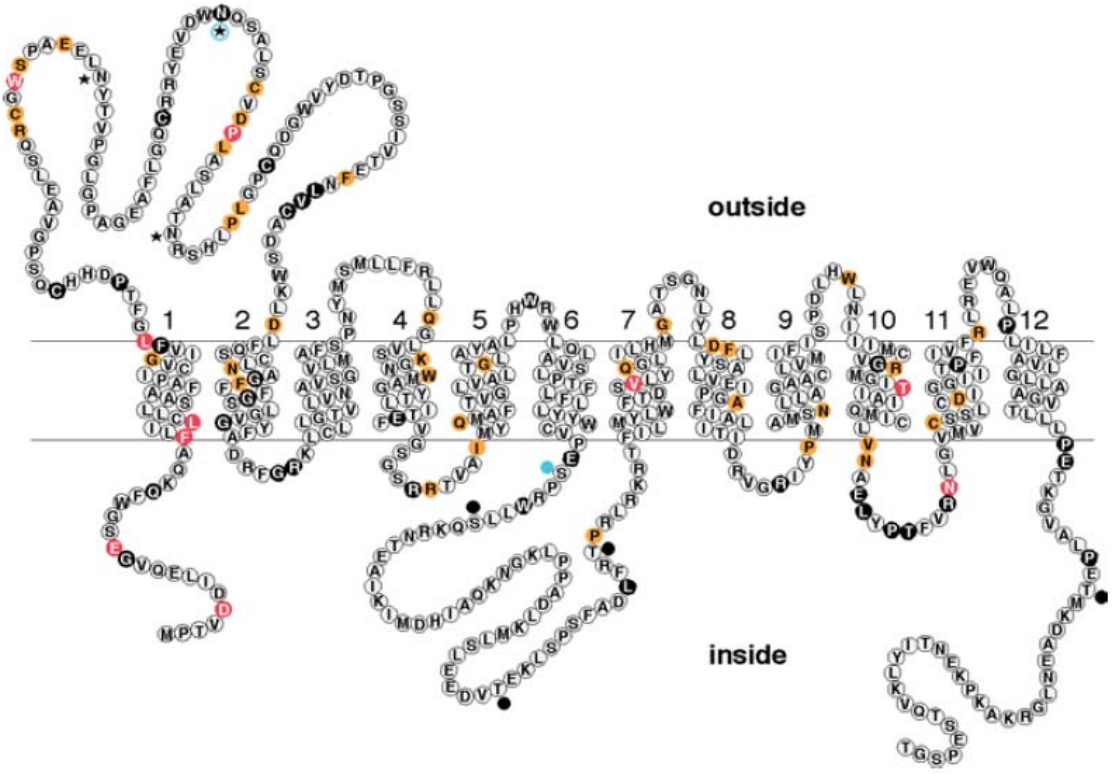
OCT-1 is a polyspecific transporter first identified in 1994 by expression cloning [106], and is primarily expressed in the liver [107]. The predicted structure of OCT-1 (Figure 1.12A) comprises 12 α–helical transmembrane domains (TMDs), a large glycosylated extracellular loop between TMDs 1 and 2, a large intracellular loop containing phosphorylation sites between TMDs 6 and 7 and an intracellular N and C-terminus [103]. Our laboratory has confirmed the results of Thomas *et al* [82], in demonstrating that imatinib is indeed a substrate of OCT-1 in patient cells [79]. It was found that both prazosin and procainamide reduced the intracellular uptake and retention (IUR) of imatinib achieved in patient's PB-MNC *in vitro*. Furthermore, the addition of prazosin reduced the IUR of imatinib to the same level seen when conducting the assay at 4°C (ie: removing the effect of all ATP dependent transporters). Therefore, not only is OCT-1 a transporter of imatinib, it appears to be the major active influx pump for imatinib in patient PB-MNC. In addition to this, studies by Wang *et al*, transfected the

Figure 1.12 Protein structure and regulation of the organic cation transporter 1

(A) The amino acid sequence and current model of membrane topology for OCT-1. The protein has 12 transmembrane domains and a large intracellular and extracellular loop. Black dots show protein kinase C phosphorylation sites. Stars show N-glycosylation sites. Coloured dots show conserved regions of the sequence with other SLC22 family members. (B) This figure represents known pathways that affect the post-transcriptional regulation of OCT-1. Protein kinase C is an inhibitor and calmodulin and p56lck are activators.

Figures from Koepsell *et al* (2003) and Ciarimboli and Schlatter (2005), respectively.

A


outside
inside
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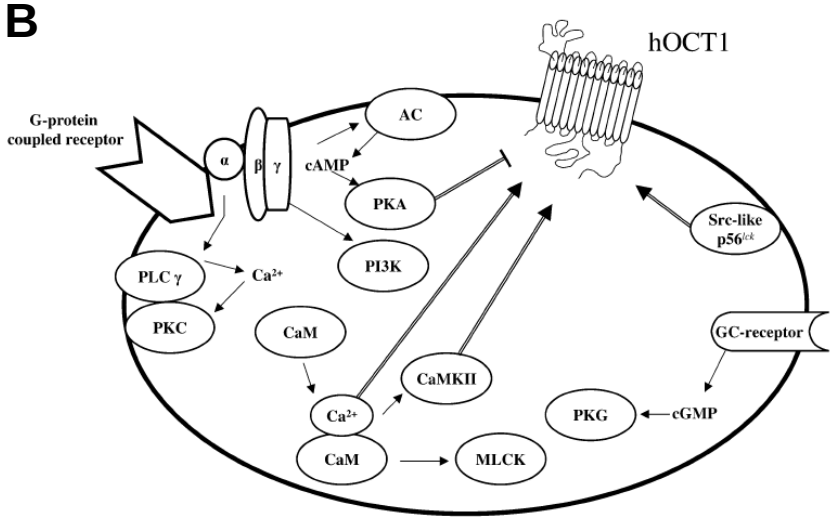
B


hOCT1
G-protein coupled receptor
α
β
γ
AC
cAMP
PKA
PI3K
PLC γ
Ca2+
PKC
CaM
CaMKII
Ca2+
CaM
MLCK
Src-like p56lck
GC-receptor
PKG
cGMP

CML cell line KCL22 with pcDNA-OCT-1 plasmid or control plasmid [97]. It was found that the uptake of imatinib and the specific OCT-1 substrate ASP (4-[4-(dimethylamino)-styryl]-N-methylpyridinium) was significantly higher in the OCT-1 over-expressing cells compared with the control vector or parental line. This demonstrates that OCT-1 mRNA expression is related to OCT-1 function and imatinib uptake.

1.6.1.1 OCT-1 transcript levels

A number of studies have investigated the relationship between OCT-1 mRNA transcript levels in CML patients and their contribution to outcome on imatinib therapy. The first study to investigate this demonstrated that CML patients who had achieved CCR by 12 months had significantly higher OCT-1 mRNA expression compared with those who did not [96]. Secondly, Wang *et al* found that those patients with high OCT-1 mRNA expression had significantly better rates of overall and progression free survival compared to those with low OCT-1 mRNA levels [97]. However, both of these studies were inconsistent with their patient cohorts, and used patients who had undergone pre-treatment or were in more advanced phases of disease. Most recently, Marin *et al* investigated OCT-1 transcript levels in the PB of diagnosis CP patients [108]. Using multivariate analysis, it was identified that patients with high OCT-1 transcript levels had significantly better rates of achievement of MMR and complete molecular response (CMR, undetectable BCR-ABL by RQ-PCR) by 6 years of imatinib treatment. Furthermore, Labussiere *et al* found a significant correlation between diagnosis OCT-1 transcript levels and the achievement of MMR by 24 months [94]. However, this data is contradicted by Zhang *et al*, who were unable to discern a difference in diagnosis OCT-1 transcript levels between patients who did and did not achieve CCR by 12 months of imatinib therapy [109]. Furthermore, our laboratory has consistently found that patients with high OCT-1 mRNA expression at diagnosis do not achieve superior molecular responses to those with low OCT-1 mRNA expression [110, 111]. The discrepancies between these studies are likely to be due to the cell populations

analysed and differing RQ-PCR techniques. Therefore, analysis of OCT-1 transcript levels has not been established to be a reliable method to determine patient response to therapy.

1.6.1.2 OCT-1 polymorphisms

A number of non-synonymous SNPs have been described for OCT-1, and these SNPs have been shown to affect the transport of the model OCT-1 substrates MPP and TEA [112-114]. A number of studies have investigated the association between OCT-1 SNPs and patient response to imatinib treatment. Kim *et al* found a significant relationship between the L160F SNP and loss of response or treatment failure on imatinib [101]. In addition, Bazeos *et al* demonstrated that the G401S SNP was significantly associated with a higher rate of MMR [115]. However, the frequency of these SNPs were low at 18% and 4.5%, respectively. Therefore, OCT-1 SNP analysis is unlikely to provide response predictions for the majority of patients. Furthermore, studies from our laboratory, found no association between the presence of OCT-1 SNPs and molecular response to imatinib when measured in 136 CP-CML patients [116].

1.6.1.3 OCT-1 activity

In addition to polymorphisms, it has been shown that the OCT-1 protein has a high level of post-transcriptional regulation (Figure 1.12B). Protein kinase A has been shown to inhibit OCT-1 and OCT-1 has been shown to be endogenously activated by calmodulin and the $p56^{lck}$ tyrosine kinase [102, 117]. Therefore, as opposed to OCT-1 transcript levels and polymorphisms, an assay which measures the functional activity of OCT-1 in relation to imatinib transport may be a better measure of patient response to treatment.

As mentioned previously, prazosin is a potent inhibitor of OCT-1 and to a lesser extent OCT-3 (IC50 values 1.8 µM and 13µM respectively) [118]. In an adaptation of our intracellular uptake and retention (IUR) assay, patient diagnosis PB-MNC are cultured in 2µM [^{14}C]-labelled imatinib with and without prazosin. In almost all cases, the addition of prazosin reduces the IUR

of imatinib in patient cells. The specific “OCT-1 activity” in a patient’s MNC is then calculated as the difference between the IUR in the absence of prazosin and the IUR in the presence of prazosin [110]. This *in vitro* test of patient OCT-1 activity demonstrates a large heterogeneity in patients OCT-1 function and correlates significantly with a patients level of BCR-ABL kinase inhibition (IC50) [110]. Grouping patients into high and low OCT-1 activity groups (above or below the median), demonstrated that those patients with high OCT-1 activity achieved significantly better molecular responses to imatinib by 24 months of therapy than those with low [110]. A study with a follow-up of 5 years found that high OCT-1 activity patients achieved significantly better rates of MMR (Figure 1.13A) and CMR [111]. Furthermore, these patients had significantly improved overall and event free survival (Figure 1.13B) and none of these patients transformed to AP/BC. Therefore, OCT-1 activity is a highly successful prognostic test to determine how well a specific patient will respond to long-term imatinib therapy.

1.7 Disease Persistence

In addition to primary and secondary imatinib resistance, another potential downfall of imatinib therapy is the existence of disease persistence. It has been shown in a number of case studies that approximately 50% of patients who exhibit long-term CMR on imatinib therapy, relapse following imatinib cessation [119-122]. This indicates that imatinib treatment may not be targeting all of the leukaemic cells within a patient. A number of studies have shown that leukaemic Ph+ cells persist in the primitive CD34+ cellular compartment in the bone marrow of patients who have achieved stable CCR [123, 124]. Therefore, imatinib may not be efficiently targeting and eliminating CML progenitor cells, allowing for the re-population of Ph+ cells upon the discontinuation of imatinib.

1.7.1 Leukaemic stem cells

A number of studies have clearly identified that CML progenitor cells are resistant to imatinib-induced apoptosis. *In vitro* studies have shown that at the clinically relevant concentrations of

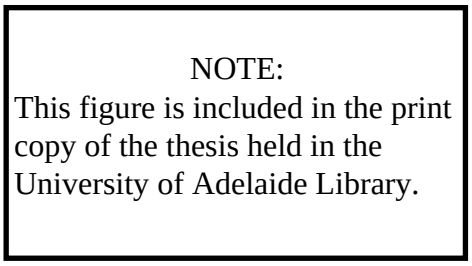


Figure 1.13 Relationship between OCT-1 activity and response to imatinib therapy

(A) 89% of patients with high OCT-1 activity achieve MMR by 5 years of treatment in comparison to 55% of patients with low OCT-1 activity. (B) Percentages of event free survival at 5 years of imatinib treatment. Patients with high OCT-1 activity have significantly higher rates of event free survival compared to patients with low OCT-1 activity.

Figures adapted from White *et al* (2010).

1-2µM, imatinib inhibits proliferation and colony formation (CFC and LTC-IC) in primitive CD34+ cells isolated from CP-CML patients [125]. However, treatment with imatinib was not found to induce apoptosis within these CD34+ cells. In agreement, other studies have found that treatment with imatinib (1-5µM) resulted in a significant reduction in proliferation over a 3-12 day culture [126, 127]. Most recently, while treatment of CD34+ cells with 1µM imatinib was found to significantly reduce colony formation (CFC and LTC-IC), it did not completely eliminate Ph+ colonies [128]. Furthermore, a minimal effect on apoptosis was seen in the CD34+ and the more primitive CD34+38- cells with imatinib treatment, thereby indicating that imatinib exhibits anti-proliferative, but not pro-apoptotic effects on primitive CML cells. Of significance, Holyoake *et al* has identified a rare non-cycling pool of Ph+ quiescent CD34+ cells from CML patients [129]. These quiescent cells are transient, able to spontaneously re-enter cycle and are particularly resistant to imatinib treatment [126, 128]. Further to this, treatment with imatinib appears to increase the proportion of these quiescent progenitor cells [126], hence selecting for resistant cells. Therefore, CML progenitor cells have been implicated as a major source of disease persistence and relapse in imatinib treated patients.

1.7.2 Methods of resistance

A number of studies have investigated the characteristics of CML progenitor cells to determine their method of resistance to imatinib. Three major features have been identified as possible resistance mechanisms in CML progenitors: 1) autocrine production of growth factors, 2) increased transcript and protein levels of BCR-ABL and 3) increased ABCB1 and ABCG2 and/or reduced OCT-1 transcript levels. It has been shown that CML CD34+ and CD34+38- cells express significantly higher transcript levels of the growth factors IL-3 and G-CSF than mature CML cells [130, 131]. This production of cytokines may activate BCR-ABL-independent survival signalling, resulting in resistance to imatinib. It has also been demonstrated that both CD34+ and CD34+38- cells have significantly increased BCR-ABL transcript levels compared with mature CML cells [131, 132]. Furthermore, these primitive populations were found to

express significantly higher levels of BCR-ABL protein and p-Crkl [131, 132l]. Treatment with imatinib (1-5μM) was only found to partially inhibit BCR-ABL kinase activity (as measured by p-Crkl) in CD34+ cells and no significant effect on p-Crkl was seen in CD34+38- cells [127, 128, 132]. Therefore, as with primary resistance, stem cell resistance to imatinib treatment may also be due to inadequate kinase inhibition. A small number of studies have identified that CML progenitor cells express significantly higher transcript levels of ABCB1 and ABCG2 compared to mature CML cells [93, 131]. In addition, it was identified that CD34+ and CD34+38- cells had a significantly lower expression of OCT-1 mRNA compared to their mature counterparts [131]. Therefore, influx and efflux proteins may play a significant role in the resistance of CML progenitors to imatinib treatment.

1.8 Summary and Project Aims

Despite the excellent clinical responses seen with imatinib therapy for the majority of CP-CML patients, primary and secondary resistance to imatinib and disease persistence remain clinical challenges in this era. The function of the influx protein OCT-1 (OCT-1 activity) has been shown to be highly variable between CP-CML patients and is a strong predictor of molecular response to imatinib therapy and of overall and event free survival. It is therefore important to understand the reasons underlying this variability in OCT-1 function between patients, and to address the role of OCT-1 function in stem cell resistance to imatinib.

This thesis therefore addressed the following aims:

- To evaluate the relationship between cell lineage and OCT-1 function.
- To assess granulocytic surface markers as a surrogate for OCT-1 activity.
- To determine the effect of BCR-ABL and imatinib on OCT-1 function.
- To evaluate the relationship between cell maturity and OCT-1 function.
- To assess the relationship between CD34+ OCT-1 activity and response to imatinib.

2 MATERIALS AND METHODS

2.1 Commonly Used Reagents

Table 2.1: Suppliers and catalogue numbers of commonly used reagents

Reagent	Supplier	Catalogue Number
Bovine Serum Albumin (BSA) powder	Sigma	49418
CD3 PE/FITC antibodies	BD Biosciences	347347 / 340542
CD11b PE antibodies	BD Biosciences	555388
CD14 PE/FITC antibodies	BD Biosciences	347497 / 347493
CD15 PE/FITC antibodies	BD Biosciences	555402 / 555401
CD16 PE antibodies	BD Biosciences	555407
CD34 PE/FITC antibodies	BD Biosciences	348057 / 348053
CD38 PE antibodies	BD Biosciences	347687
Chloroform	Merck	100776B
DEPC H_2O	MP Biomedicals Inc.	821739
Dimethyl sulphoxide (DMSO)	Merck	K39661852
dNTP set (N = A, C, G, T)	Amersham	27-2035-02
Ethylenediaminetetraacetic acid (EDTA)	APS	180-500G
Ethanol	Merck	4.10230.2511
Foetal Calf Serum (FCS)	JRH Biosciences	12003-500M
Glycogen	Roche	901393
Hanks Buffered Saline Solution (Hanks)	Sigma	H9394
HEPES 1M	Sigma	H0887
IgG PE/FITC antibodies	Dako Cytomation	X0928 / X0927
Isopropanol	Ajax Finechem	425-2.5L PL
L-glutamine 200mM	SAFC Biosciences	59202C-100ML
Lymphoprep	Axis Shield	1114547
MACS beads CD3	Miltenyi Biotech	130-050-101
MACS beads CD14	Miltenyi Biotech	130-050-201
MACS beads CD15	Miltenyi Biotech	130-046-601
MACS beads CD16	Miltenyi Biotech	130-045-701
MACS beads CD34	Miltenyi Biotech	130-046-703
Methanol	Chem Supply	MA004-P
MICROSCINT-20 scintillation fluid	Perkin Elmer	6013621
Phosphate Buffered Saline (PBS)	SAFC Biosciences	59331C
Penicillin 5000U/mL Streptomycin 5000µg/mL	Sigma	P4458
Percoll (ρ = 0.99g/mL)	GE Healthcare	17-0891-02
Phorbol-12-myristate-13-acetate (PMA)	Sigma	P8139
Prazosin Hydrochloride (MW = 419.9)	Sigma	P7791
Random Hexamer Primer	Geneworks	RP-6
RPMI-1640 Medium w/o L-glutamine	Sigma	R0883
Superscript II Reverse Transcriptase	Invitrogen	18064-014
SYBR Green Supermix	SA Biosciences	PA-012-24
Taqman Master Mix	Applied Biosystems	4318157
Trizol Reagent	Invitrogen	15596-018
Trypan Blue Solution (0.4%)	Sigma	T8154
Trypsin	SAFC Biosciences	59417C

2.2 Solutions, Buffers & Media

2.2.1 Blocking buffer

Hanks - 100mL

HEPES - 2mL

1% BSA powder - 1.0g

5% Normal human serum - 5mL

5% FCS - 5mL

The BSA was dissolved slowly in Hanks using a rolling mixer. HEPES, normal human serum and FCS were then added to the Hanks/BSA solution. The solution was sterilised with a 0.2µM bottle top filter and stored at 4°C.

2.2.2 Cell culture media

RPMI medium - 500mL

1% L-Glutamine (200mM) - 5mL

Penicillin/Streptomycin - 5mL

10% FCS - 50mL

Store at 4°C, and preheat to 37°C in a water bath prior to use.

2.2.3 dNTP set (N = A, C, G, T)

25mM stock = 40µL of each dNTP

Working stock: 5mM = 20µL of 25mM stock in 80µL DEPC water

2.2.4 Flow cytometry fixative (FACS Fix)

1xPBS - 500mL

40% w/v Formaldehyde - 5mL

D-glucose - 10g

NaN_3 - 0.1g

The solution can be stored indefinitely at 4°C.

2.2.5 Freeze mix

70% Hanks

20% FCS

10% DMSO

This is made up fresh for each batch of samples to be cryopreserved.

2.2.6 Hanks Balanced Salt Solution (Hanks)

Ca^{++} and Mg^{++} Free. Add 10mM HEPES 1M prior to use.

2.2.7 Imatinib mesylate, MW = 589.72

Imatinib mesylate (imatinib; Glivec; formerly STI-571) was provided by Novartis Pharmaceuticals (Basel, Switzerland). Stock solutions of this compound were prepared at 10mM with distilled water, sterile filtered and stored at -70°C.

2.2.8 50% [^{14}C]- Imatinib (100µM)

[^{14}C]-Imatinib (1695.72µM) - 29.5µL

10mM imatinib - 5µL

RPMI medium - 966µL

2.2.9 Ko143 – inhibits ABCG2

Analogue of fumitremorgin C was kindly provided by Dr John Allen, Centenary Institute, Sydney, Australia. Used at 10μM from 1mM stock in sterile water

2.2.10 Laemmli's buffer

50 mM Tris-HCL (pH 6.8)

10% glycerol

2% SDS

5% β-mercaptoethanol

0.1% bromophenol blue

1mM NaVanadate

10mM NaFluoride

2.2.11 MACS buffer

1xPBS - 500mL

BSA - 2.5g

2mM EDTA - 0.37g

BSA was dissolved slowly in PBS using a rolling mixer. The EDTA was added to the buffer, and the solution sterilised using a 0.2μm bottle top filter. The buffer was stored at 4°C.

2.2.12 2.5% Membrane blocking solution

Membrane Blocking agent (GE Healthcare) – 2.5g

1xTBS – 100mL

Solution was dissolved on a rolling mixer and stored at 4°C.

2.2.13 Nilotinib, MW = 529.5

Nilotinib (formerly AMN107) was provided by Novartis Pharmaceuticals. Stock solutions were prepared at 10mM in DMSO and stored at 4°C.

2.2.14 50% [^{14}C]-Nilotinib

[^{14}C]-Nilotinib (1882µM) - 2.66µL

1mM nilotinib - 5µL

RPMI medium - 992.3µL

2.2.15 Percoll (ρ = 0.99g/mL)

Percoll (ρ = 1.130g/mL) - 40mL

10x PBS - 6.6mL

The Percoll and PBS were combined and the pH adjusted to 7.0 with 0.1M HCl. The solution was sterilised using a 0.2µm single use syringe filter, and stored at 4°C for a maximum of 2 months.

2.2.16 Prazosin hydrochloride - inhibits OCT-1

Used at 100µM from 10mM stock

10mM stock = 4.2mg in 1mL of methanol

2.2.17 PSC833 – inhibits ABCB1

(Kindly provided by Novartis Pharmaceuticals)

Used at 10µM from 8.23mM stock

8.23mM stock = 10 mg/mL = 10mg / 500µL 9:1 Ethanol: Tween20, + 500µL water

2.2.18 Random Hexamer Primer (100mg stock)

Working stock: 250ng/mL = 100mg in 400µL DEPC water

2.2.19 SDS-Polyacrylamide Gel

	Resolving gel (12%)	Stacking gel (5%)
H_2O	12.9mL	6mL
40% Acrylamide	9mL	1.26mL
1.5M Tris-HCL	7.5ml	2.52mL
10% SDS	300µL	100µL
10% APS	300µL	100µL
TEMED	8µL	10µL

2.2.20 1 x TBS

20mM Tris-HCL (pH 7.5)

150mM NaCl

2.2.21 1 x TBST

20mM Tris-HCL (pH 7.5)

150mM NaCl

0.1% Tween20

2.2.22 Thaw solution

Hanks - 500mL

5% FCS - 25mL

5% ACD - 25mL

HEPES 1M - 5mL

The solution (without ACD) was stored at 4°C. ACD was added and the solution was heated to 37°C in a water bath prior to use.

2.2.23 White cell fluid

Glacial acetic acid - 2mL

Milli-Q® Water - 98mL

Methyl Violet - few crystals

Acetic acid was added slowly to Milli-Q® water. The methyl violet (Gurr®, BDH) was then added, and the solution mixed well to ensure that the crystals completely dissolved. The solution was filtered using a 0.2µm bottle top filter, and stored at room temperature.

2.3 General Techniques.

2.3.1 Lymphoprep isolation of mononuclear cells (MNC)

40-60mL of peripheral blood (PB) from patients with CML or normal donors was collected into Lithium Heparin tubes. All samples were collected with informed consent in accordance with the Institutional Ethics approved protocols and with reference to the Declaration of Helsinki. A white cell count was performed using white cell fluid and a maximum of $1x10^8$ cells (maximum of 15mL of blood) were transferred into a 50mL polypropylene conical tube (Falcon tube). The blood volume was brought to 35mL using Hanks and underlain with 15mL of lymphoprep. Tubes were then centrifuged at 1,200rpm for 30 minutes with no brake. The interface containing the mononuclear cells (MNC) was then transferred to another 50mL Falcon tube and washed once in Hanks buffer.

2.3.2 Percoll isolation of monocytes

MNC were enriched for monocytes using a Percoll continuous gradient. A maximum of $1x10^9$ MNC were re-suspended in 3.5mL RPMI in a 15mL round bottom tube (Falcon). 6.7mL of 0.99g/mL Percoll solution was added to the cells and the suspension was mixed well. The tube was centrifuged in a fixed angle rotor (Beckman Avanti-J-25I centrifuge with JA-25.50 rotor) at

400g for 25 minutes at room temperature. The surface layer of cells (~1cm) was removed using a transfer pipette. These cells were then MACS sorted (CD14) for high purity monocytes.

2.3.3 Magnetic Cell Sorting (MACS)

MicroBeads for CD3 (lymphocytes), CD14 (monocytes), CD15 & CD16 (granulocytes) and CD34 (progenitor cells) were obtained from Miltenyi Biotech.

2.3.3.1 CD3, CD14, CD15 & CD16 Staining

Cells were suspended in 500µL of blocking buffer and incubated on ice for 30 minutes. Cells were washed once in MACS buffer and re-suspended in MACS buffer and then MicroBeads were added to magnetically label the cells, according to Table 2.2. The suspension was incubated at 4°C with continuous mixing for the times indicated in Table 2.2.

2.3.3.2 CD34 Staining

Cells were suspended in MACS buffer, MicroBeads and FcR Blocking Reagent and incubated at 4°C with continuous mixing, as indicated in Table 2.2.

Table 2.2: Volumes and conditions for MACS staining

MicroBeads	Cell Number	MACS Buffer Volume (minimum)	MicroBead Volume (minimum)	Incubation Time
CD3	$1x10^7$	80µL	20µL	15 mins
CD14	$1x10^7$	80µL	20µL	15 mins
CD15	$1x10^7$	80µL	20µL	15 mins
CD16	$5x10^7$	50µL	50µL	30 mins
CD34	$1x10^8$	300µL	100µL + 100µL FcR blocking reagent	30 mins

2.3.3.3 Magnetic Separation

A pre-cooled MACS LS column was placed in a midi-MACS separator magnet and rinsed with 3mL of degassed MACS buffer. The magnetically labelled cell suspension was then placed onto the column, and the effluent collected as the negative fraction. The column was washed

thrice with 3mL of buffer, then removed from the magnet and flushed with 5mL of buffer to collect the positive fraction. In some cases the positive fraction was passed over a new freshly prepared column to increase the purity of positively labelled cells. The purity of cells following the isolation procedure was measured by staining with immunofluorescent antibodies, as described in section 2.3.5.

2.3.4 Fluorescence Activated Cell Sorting (FACS)

2.3.4.1 For CD15/CD16 CML cells

CML MNC were aliquoted into 5mL polystyrene round bottom tubes (Falcon, FACS tubes) as described in Table 2.3. Test cells were sorted for CD15+16+, CD15+16- and CD15-16- fractions on a Becton Dickinson Aria, using FACS Diva Software version 6.1.3 (BD Biosciences).

Table 2.3: Antibody staining for FACS sorting

	Cell Number	Antibody 1	Volume	Antibody 2	Volume
Control	$1x10^6$ cells	IgG1 PE	10µL	IgG1 FITC	10µL
Control	$1x10^6$ cells	IgG1 PE	10µL	CD15 FITC	20µL
Control	$1x10^6$ cells	CD16 PE	20µL	IgG1 FITC	10µL
Test	$2x10^7$ cells	CD16 PE	100µL	CD15 FITC	100µL

2.3.4.2 For CD34+/CD38- CML cells

MACS sorted CML CD34+ cells were aliquoted into 5mL FACS tubes as described in Table 2.4. Test cells were sorted for CD34+38+ and CD34+38- fractions on a Becton Dickinson Aria, using FACS Diva Software version 6.1.3 (BD Biosciences, San Diego, CA). The CD34+38- fraction was routinely <5% of the total CD34+ population.

Table 2.4: Antibody staining for FACS sorting

	Cell Number	Antibody 1	Volume	Antibody 2	Volume
Control	$1x10^6$ cells	IgG1 PE	10µL	IgG1 FITC	10µL
Control	$1x10^6$ cells	IgG1 PE	10µL	CD34 FITC	20µL
Control	$1x10^6$ cells	CD38 PE	20µL	IgG1 FITC	10µL
Test	$2x10^7$ cells	CD38 PE	100µL	CD34 FITC	100µL

2.3.5 Antibody staining for Flow Cytometric analysis

$1x10^5$-$1x10^6$ cells were transferred to 5mL FACS tubes and suspended in approximately 500µL of Hanks. Phycoerythrin (PE) or Fluorescein isothiocyanate (FITC) conjugated antibodies were added to tubes at a concentration of 10µL/$1x10^6$ cells, unless otherwise stated (see Table 2.5), and incubated for a period of 40 minutes on ice in the dark. Cells were then washed twice with Hanks and re-suspended in FACS fixative. Samples were stored at 4°C in the dark until analysis was performed. Control tubes were stained with isotype control IgG PE or FITC antibodies and these were used to define the gates for positive and negative expression. The fluorescence intensity of cell suspensions was examined using an Epics XL-MCL flow cytometer and CXP Analysis Version 2.2 Software (Beckman Coulter). Cell populations were analysed based on their forward and side light scattering properties (indicative of cell size and granularity, respectively) and the fluorescence intensity of PE or FITC fluorochromes.

Table 2.5: Antibodies used for immunophenotyping

Antibody	Volume	Specificity
IgG1 PE/FITC	5µL (for $1x10^5$ cells)	Isotype control
CD3 PE/FITC	10µL (for up to $1x10^6$ cells)	Lymphocytes
CD11b PE	10µL (for up to $1x10^6$ cells)	Activated lymphocytes, monocytes, granulocytes & NK cells
CD14 PE/FITC	10µL (for up to $1x10^6$ cells)	Monocytes, macrophages & granulocytes (weak)
CD15 PE/FITC	10µL (for up to $1x10^6$ cells)	Granulocytes & monocytes (weak)
CD16 PE	10µL (for up to $1x10^6$ cells)	Granulocytes, NK cells & macrophages
CD34 PE/FITC	10µL (for up to $1x10^6$ cells)	Haemopoietic progenitor cells

2.3.6 Cryopreservation of cells

Cells were pelleted at the desired concentration, re-suspended in 1ml of Freeze Mix per $1x10^7$ cells and quickly transferred to cryo-ampoules (Nalgene). The cells were then frozen to -80°C

using a Controlled Rate Freezer (Planer KRYO10 Series II) at a rate of 1°C per minute. The samples were stored for up to 10 years in liquid nitrogen (-196°C).

2.3.7 Thawing of cells

Cells were removed from liquid nitrogen and thawed rapidly in a 37°C water bath. The cell suspension was quickly transferred to a 50mL Falcon tube, and approximately 20mL of thaw solution (warmed to 37°C) was added drop-wise with constant mixing. The sample volume was then increased to 30mL with thaw solution and the cells pelleted by centrifugation at 1,200rpm for 10 minutes. The supernatant was aspirated and the procedure was repeated to remove all residual DMSO.

2.3.8 Cell counts and viability

Cell concentration was determined by diluting the cell suspension in white cell fluid (WCF) and cell viability was assessed by diluting samples with trypan blue solution. 10µL of these suspensions were transferred to a haemocytometer counting chamber (Neubauer Improved, Assistant, Germany) and cell concentration and viability calculated accordingly.

2.3.9 Maintenance of cell lines

All appropriate tissue culture techniques were performed in a Class two “biohazard” laminar flow hood (Gelman Sciences). Suspension cell lines were maintained at a cell density between $1x10^5$-$1x10^6$ cells/mL in 25cm^2, 75cm^2 or 150cm^2 tissue culture flasks (Greiner). Media was pre-warmed to 37°C prior to use. Cultures were incubated in a 37°C/5%CO_2 incubator. Cell cultures were checked every second day for contamination, counted and re-plated at the above concentrations.

2.4 Specialised Techniques

2.4.1 Imatinib/nilotinib Intracellular Uptake and Retention (IUR) assay

The IUR and OCT-1 activity assay were performed as previously described by White *et al* [110]. All assay points were performed in triplicate. $2x10^5$ viable cells were incubated in 2mL of RPMI + 10% FCS media in the presence or absence of 2μM [^{14}C]-labelled imatinib or nilotinib. The influx and efflux inhibitors (prazosin, PSC833 and KO143) were also added as required. Cells and reagents were incubated for 2 hours at 37°C/5% CO_2. Following this time tubes were centrifuged at 6,800rpm for 5 minutes, then pulse spun to 13,000rpm, for 30 seconds. A 20μL aliquot of supernatant (S/N) from each tube was then added to 100μL of Microscint-20 in 96-well flat bottomed plate. The remaining supernatant was then aspirated from the tubes and 50μL of Microscint-20 was added. The tube was vortexed well, and pulse spun for 15 seconds at 13,000rpm. The lysed cells were then transferred to a 96 well plate with wells containing 50μL of Microscint. The plate was covered with an adhesive plastic seal and was then counted on a Top Count scintillation counter (Perkin Elmer) as counts per minute (Cpm).

The ng/200,000 cells of [^{14}C]-labelled imatinib/nilotinib was then calculated using the formula below:

$$\frac{\text{(Cpm cells – background)}}{\text{[(Cpm cells – background) + (Cpm S/N – background)]}} \times \text{ng of [}^{14}\text{C]-TKI added}$$

2.4.1.1 OCT-1 activity

The OCT-1 inhibitor prazosin was used in the IUR assay at 100μM. The OCT-1 activity was calculated as the difference between the IUR in the absence of prazosin and the IUR in the presence of prazosin. Where IUR values in the presence of prazosin were equal or higher than the values in the absence of prazosin, these patients were scored as having negligible (0 ng/200,000 cells) OCT-1 activity.

2.4.2 Real Time Quantitative PCR (RQ-PCR)

2.4.2.1 RNA extraction

$1x10^6$-$1x10^7$ cells were lysed in 1mL of Trizol reagent and incubated at room temperature for 5 minutes. 200µL of chloroform was then added, tubes were shaken vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. Following this time the tubes were centrifuged at 12,000g for 15 minutes at 4°C. The aqueous phase (top layer) was transferred to a fresh RNase/DNase free 1.5mL tube. RNA was precipitated by the addition of 1µL glycogen (20µg) and 500µL isopropanol. The samples were gently mixed and incubated at room temperature for 10 minutes. RNA was pelleted by centrifugation at 12,000g for 10 minutes at 4°C. The supernatant was removed from the RNA pellet, was washed in 1mL of 75% ethanol and was centrifuged at 7,500g for 5 minutes at 4°C. Supernatant was removed and tubes centrifuged briefly to remove any excess ethanol. The RNA pellet was briefly dried then dissolved in 20-80µL DEPC water (depending on size of pellet) at 55°C for 10 minutes. RNA was quantified using a NanoDrop Spectrophotometer (Thermo scientific) and samples were stored at -70°C.

2.4.2.2 cDNA synthesis

1µg of RNA was added to 1µL of random hexamers (250ng/µL) and total volume taken to 11µL with DEPC water. The mixture was heated to 70°C for 10 minutes and chilled briefly at 4°C. The first strand synthesis reagent mix was prepared as described below in Table 2.6.

Table 2.6: First Strand Synthesis Reagent Mix

Reagent/concentration	Volume (Per Sample)
5x First strand buffer	4µL
0.1M DTT	2µL
5mM dNTPs	2µL
Superscript II	1µL

9µL of the reagent mix was then added to each tube and contents gently mixed, and placed in Mastercycler (Eppendorf). Incubation conditions are as follows: 25°C for 10 minutes

42°C for 50 minutes

70°C for 10 minutes

4°C ∞

At the completion of this cycle, 20µL DEPC water was added to each tube, and the cDNA stored at - 20°C.

2.4.2.3 RQ-PCR for transporter mRNA expression

RQ-PCR for transporter mRNA expression was performed as previously described by White *et al* [110]. Primers (see Table 2.7) were diluted to a working stock of 100ng/µL in DEPC water. PCR master mix was prepared as per Table 2.8 for each primer set to be tested.

Table 2.7: Primer sequences for transporter PCR

Primer Name	**Sequence 5' to 3'**
Genes of interest	
hOCT-1 Forward	CTG AGC TGT ACC CCA CAT TCG
hOCT-1 Reverse	CCA ACA CCG CAA ACA AAA TGA
ABCB1 Forward	AGA CAT GAC CAG GTA TGC CTA T
ABCB1 Reverse	AGC CTA TCT CCT GTC GCA TTA
Standard	
hRANKL-D Forward	TCA GCC TTT TGC TCA TCT CAC TAT
hRANKL-G Reverse	CCA CCC CCG ATC ATG GT
House-keeping genes	
BCR Forward	CCT GCG ATG GCG TTC AC
BCR Reverse	CCT TCG ACG TCA ATA ACA AGG AT

Table 2.8: Preparation of PCR Master Mix

Reagent	**Volume (per sample)**
SYBR Green	5µL
Forward Primer (100ng/µL)	0.5µL
Reverse Primer (100ng/µL)	0.5µL
DEPC H_2O	2µL

8µL of appropriate Master Mix was added to each tube. 1µL of RankL standard cDNA was added to RANKL tubes and 2µL of unknown or cell line control cDNA was added to the allocated tubes. Tubes were then placed into a 72 well Rotor-Gene (Corbett Research). Results were analysed using Rotor-Gene 6000 Series software (Corbett Research) and transporter expression was calculated as a percentage of BCR expression.

Profile settings on the Rotor-Gene are as follows:

Hold 1 @ 50°C, 2 minutes

Hold 2 @ 95°C, 15 minutes

Cycling (48 repeats) Step 1 @ 95°C, hold 15 seconds

Step 2 @ 60°C, hold 26 seconds

Step 3 @ 72°C, hold 10 seconds, acquiring to Cycling A (Sybr)

Hold 3 @ 72°C, 30 seconds

2.4.2.4 RQ-PCR for BCR-ABL mRNA expression

RQ-PCR for BCR-ABL mRNA expression was performed as previously described by Branford *et al* [133]. Primers (see Table 2.9) were ordered at a concentration of 50µM. Taqman probes were diluted to a working stock of 10µM in DEPC water. PCR master mix was prepared as per Table 2.10 for each primer set to be tested.

Table 2.9: Primer sequences for BCR-ABL PCR

Primer Name	**Sequence 5' to 3'**
Genes of interest	
B3A2 Forward	GGG CTC TAT GGG TTT CTG AAT G
B3A2 Reverse	CGC TGA AGG GCT TTT GAA CT
B3A2 Probe	CAT CGT CCA CTC AGC CAC TGG ATT TAA GC
B2A2 Forward	ATC CGT GGA GCT GCA GAT G
B2A2 Reverse	CGC TGA AGG GCT TCT TCC TT
B2A2 Probe	CCA ACT CGT GTG TGA AAC TCC AGA CTG TCC
House-keeping genes	
BCR Forward	CCT TCG ACG TCA ATA ACA AGG AT
BCR Reverse	CCT GCG ATG GCG TTC AC
BCR Probe	TCC ATC TCG CTC ATC ATC ACC GAG A

Table 2.10: Preparation of PCR Master Mix

Reagent	Volume (per sample)
Taqman Master Mix	12.5µL
Forward Primer (50µM)	0.1µL
Reverse Primer (50µM)	0.1µL
Taqman Probe (10µM)	0.25µL
DEPC H_2O	9.55µL

22.5µL of appropriate Master Mix was added to each tube. 2.5µL of standard, cell line control or unknown cDNA was added to the appropriate tubes. Lids were then added to all tubes, and they were placed into a 7500 ABI Sequence Detector instrument (Applied Biosystems). Results were analysed using 7500 System SDS software (Applied Biosystems) and BCR-ABL expression was calculated as a percentage of BCR expression.

Profile settings on the ABI Sequence Detector are as follows:

Hold 1 @ 50°C, 2 minutes

Hold 2 @ 95°C, 10 minutes

Cycling (40 repeats) Step 1 @ 95°C, hold 15 seconds

Step 2 @ 60°C, hold 60 seconds

2.4.3 Western Blot for phosphorylated Crkl (p-Crkl)

Western blotting for p-Crkl was performed as previously described by White *et al* [76]. $2x10^5$ cells (cell lines) were placed into a 1.5mL eppendorf tube, and pelleted by centrifugation for 5 minutes at 6,500rpm. All supernatant was removed and cells were lysed in 20µL of Laemmli's buffer, by boiling in a 100°C heat block for 12 minutes. Cell lysates were stored at -20°C. 10µL of protein lysate (corresponding to $1x10^5$ cells) was resolved on an SDS 12% polyacrylamide gel, and the protein was electrophoretically transferred to a PVDF membrane (GE Healthcare). The membrane was incubated for 1 hour at room temperature with 2.5% membrane blocking agent (GE Healthcare). The membrane was then probed for 2 hours at room temperature with

1:500 anti-Crkl antibody (Santa Cruz) in 2.5% membrane blocking solution. Following this, the membrane was rinsed twice in 1xTBST buffer, and then washed for 3x5 minutes with 1xTBST buffer. The membrane was then incubated with 1:2000 alkaline-phosphatase conjugated anti-rabbit immunoglobulin (SantaCruz) in 2.5% blocking solution for 1 hour at room temperature. The membrane was then washed twice in 1x TBST buffer, 3x5 minutes in 1xTBST buffer and then 2x5 minutes in 1xTBS buffer. Bound antibodies were detected with ECF substrate (Amersham Biosciences) by FluorImager analysis (Molecular Dynamics). Phosphorylated and non-phophorylated Crkl bands were then quantified using ImageQuant software (Molecular Dynamics) and phosphorylated Crkl (p-Crkl) was determined as a % of the total Crkl protein.

2.4.4 BCRABL gene transduction in HL60 cell line

Lentivirus particles comprised of either control vector or BCR-ABL co-expressed with Green Fluorescence Protein were kindly provided by Professor John Rasko and Dr Charles Bailey (Centenary Institute, Sydney, Australia).

2.4.4.1 Viral vector construction

(These methods were kindly provided by Dr Charles Bailey.)

The pHIV-1SDm-based lentiviral vector and four accessory plasmids were used for Tat-dependent lentiviral vector production [134]. The SV40 promoter was exchanged for a CMV promoter and the woodchuck post-transcriptional regulatory element was added to generate pHIV-1SDmCMV.wpre. A control vector (CV) containing enhanced green fluorescent protein (eGFP) was generated by cloning in a BamHI/NotI Klenow-blunted fragment from peGFP-1 (BD Biosciences). The 6.1 kb BCR-ABL fusion gene cDNA representing the p210 variant was PCR-amplified from pSIN-p210 [135] and fused to eGFP using the porcine teschovirus-1 2A peptide sequence (P2A) represented as the amino acids (SGSGATNFSLLKQAGDVEENPGP). This system allows stoichiometric expression of separate eGFP and BCR-ABL proteins resulting from a ribosomal skipping mechanism [136]. Lentiviral particles comprised of either control vector or BCR-ABL were generated as previously described [137].

2.4.4.2 Viral transduction of the HL60 cell line

$2x10^5$ fresh HL60 cells were suspended in 500µL of culture media in a 5mL FACS tube. Polybrene (8 µg/ml) and concentrated viral particles (10µL of control vector or 50µL of BCR-ABL vector) were added to the cells and centrifuged for 3 hours at 1,500rpm. Following this time, cells were washed twice in Hanks and cultured in 1mL of culture media in a 24-well plate. Cells were then grown between 4-12 days to a sufficient number for FACS sorting.

2.4.4.3 FACS sorting for GFP+

Cells were washed twice in Hanks and suspended at $1.5x10^7$ cells per FACS tube in Hanks. eGFP+ cells were sorted into FACS tubes containing HL60 media on an Epics Altra HyperSort, using Expo MultiComp Software version 1.2B (Beckman Coulter, Miama, FL). If possible the top 30% of eGFP positive cells were selected for sorting. Cells were re-sorted up to 4 times to improve purity.

2.5 Statistical Analyses

Figures were constructed using GraphPad Prism 5.01 © software (GraphPad Software Inc.). Column graphs represent the mean plus the standard error of the mean (SEM). Box plots display the median value, the upper 25th and lower 75th percentiles and whiskers encompass the 10th and 90th percentiles. All statistical analyses were performed using SigmaStat 3.0 © software (SPSS Inc.). Normality tests were performed on each data set using a Kolmogorov-Smirnov test. The Levene Median Test was used to assess for equal variance when appropriate. The Mann-Whitney Rank Sum or the Student's T-test were used to determine differences between experimental groups. Where the data sets passed the normality and equal variance tests a T-test was applied, where the data failed either test a Mann-Whitney Rank Sum was used. Correlations were performed using the Pearson Product Moment or Spearman Rank Order as appropriate, depending on the data distribution. Log-Rank Survival analysis and the Fisher Exact test were used to evaluate the probability of achievement of major molecular

response within the first year of imatinib therapy. Differences were considered to be statistically significant when the probability value (p value) was <0.05.

3 THE RELATIONSHIP BETWEEN CELL LINEAGE AND OCT-1 ACTIVITY

3.1 Introduction

The majority of chronic phase (CP) CML patients treated with imatinib as frontline therapy achieve excellent cytogenetic and molecular responses [138]. Despite this, patient response is varied, with 25% to 35% of patients displaying primary resistance or sub-optimal response [75]. Identification of both the underlying causes of poor response to imatinib and prognostic indicators of response may enable changes to therapy for those patients at high risk of treatment failure.

In the pre-imatinib era, the two strongest predictors of survival were the Sokal [139] and Hasford scores [140]. The Sokal score emerged from a study of 813 Ph+ CML patients during the period where busulfan was the most common treatment. The study found spleen size, platelet count, blast cell percentage and patient age to be the parameters that best differentiated good and poor prognosis. Combining these parameters a Hazard Ratio can be determined, and patients can be grouped into low, intermediate and high risk groups. The Hasford score was developed in response to interferon-α becoming the more commonly used treatment. The Hasford score incorporates all parameters of the Sokal score but also measures eosinophil and basophil percentages. In patients treated with imatinib for 5 years there is a significant relationship between Sokal score and achievement of complete cytogenetic response (CCR) [56]. The rate of CCR in low risk patients was 89%, the rate in intermediate risk patients was 82% and the rate in high risk patients was 69%. Furthermore, rates of progression were significantly higher in high risk patients at 17% as opposed to 8% and 3% in the intermediate risk and low risk patients, respectively. The Sokal score is also predictive of molecular response, where 50% of low risk patients achieve MMR by 12 months in comparison to 30% in intermediate risk patients and 19% in high risk patients [57]. However, these data show that even patients with low risk Sokal scores exhibit variable molecular responses.

Much work has focused on the early achievement of cytogenetic and molecular responses as predictors of long term survival to imatinib treatment. The most recent update of the IRIS trial grouped patients into cytogenetic response status following 6 months of treatment [138]. The event free survival rates at 6 years for patients with no cytogenetic response (>95% Ph+), minor cytogenetic response (35%-95% Ph+), partial cytogenetic response (>35% Ph+) and CCR (0% Ph+) were 59%, 58%, 85% and 91%, respectively. Molecular analysis performed at 3 months following imatinib treatment revealed that 100% of patients who achieved a >2-log reduction in BCR-ABL went on to achieve MMR by 24 months of treatment and do not exhibit resistance to therapy [141]. In contrast, only 13% of patients who achieve a 0-1 log reduction in BCR-ABL by 3 months go on to achieve MMR and 83% of these patients display a primary or acquired resistance to imatinib. However, this analysis only identifies patients at risk after exposure to imatinib treatment and cannot be used as a diagnosis screen to tailor specific frontline therapies.

In recent years significant focus has been placed on biological parameters at diagnosis which can facilitate prediction of response to imatinib. To this end, a number of groups have performed gene expression profiling analysis and have identified putative gene sets that identify good and poor response patients. Genes that are differentiated between cytogenetic non-responsers and responders include those associated with adhesion, angiogenesis and drug metabolism [109, 142, 143]. However, most of these studies have only assessed cytogenetic responses not molecular responses. Furthermore, many of these gene sets do not overlap and lack rigorous validation. The most promising study developed a gene “classifier” using primitive CD34+ cells from CML patients [144]. Patients were classed as cytogenetic responder or non-responders and microarray gene profiling was performed. 885 genes were found to be differentially expressed between the two groups. The “classifier” was developed from this data to minimize classification errors and includes 75 genes. In an independent

validation cohort the "classifier" correctly predicted cytogenetic response at 12 months of imatinib therapy in 20 of 23 patients tested (86.9%). However, complete analysis was only successful in 63% of patients due to difficulties in CD34+ cell isolation and the quality of RNA extracts. Therefore, this method is not suitable for widespread use for response prediction.

A number of studies have identified a relationship between OCT-1 mRNA expression and response to imatinib treatment [96, 97, 108]. In two studies, OCT-1 expression was found to be significantly greater in patients who had achieved CCR at both 6 and 12 months of imatinib treatment compared to cytogenetic non-responders [96, 97]. However, both of these studies compared inconsistent groups of patients who were at different disease phases or had been pre-treated. Other studies have analysed OCT-1 expression in chronic phase patients at diagnosis prior to the start of therapy [108, 109, 111]. However, only one of these studies found a relationship between high OCT-1 mRNA expression and favourable molecular responses to imatinib, using multivariate analysis [108]. The inconsistent findings in these studies may relate to different cell populations analysed and varying RQ-PCR techniques. As seen with BCR-ABL RQ-PCR monitoring, global standardization to measure gene expression is very difficult to achieve [145].

Like most membrane transporters, OCT-1 is a complex protein with the ability to be rapidly up-regulated in response to certain substrates and toxins. Post-transcriptional regulation, membrane localisation and polymorphisms have all been reported to modulate OCT-1 function [102, 103, 113]. Therefore, as opposed to mRNA expression, a more informative assay is one which measures the functional activity of OCT-1 in the transport of imatinib. Our laboratory has developed the OCT-1 activity assay which measures the OCT-1 function and the amount of imatinib actively influxed via OCT-1 in patients [146]. OCT-1 activity measured in mononuclear cells (MNC) from untreated de novo CP-CML patients, while highly variable, is significantly

associated with their molecular response to imatinib treatment at both 24 months [110] and at 5 years [111]. For patients with a higher than median OCT-1 activity the rates of achieving MMR and CMR by 5 years were 89% and 59% respectively. Whereas patients with a lower than median OCT-1 activity, the MMR and CMR rates were significantly lower at 55% and 31%, respectively [111]. In addition, OCT-1 activity was found to be a strong predictor of overall, event free and transformation free survival following 5 years of imatinib treatment [111]. Event free survival in patients with high OCT-1 activity was 74% compared with low OCT-1 activity patients at 48%.

While OCT-1 activity has been demonstrated to be a strong predictor of response to imatinib treatment, the reasons behind the high interpatient variability in OCT-1 activity between patients is unclear. It has recently been suggested that OCT-1 mRNA expression is increased in polymorphonuclear cells compared to MNC in normal PB [115, 147], indicating that different cell subsets express varying levels of OCT-1. This chapter aimed to investigate the potential role that cell lineage plays on OCT-1 activity and expression.

3.1.1 Approach

MNC, neutrophils, monocytes and lymphocytes were isolated from the PB of de-novo CP-CML patients, CML patients in complete cytogenetic remission (CCR) and normal individuals. CML patients in CCR were treated with 600mg of imatinib and entered CCR following 3-9 months of treatment. MNC cell isolations were performed using a lymphoprep density gradient (section 2.3.1). Neutrophil, monocyte and lymphocyte isolations were performed using magnetic cell sorting (MACS; section 2.3.3). Isolated populations were assessed for purity by morphology and immunophenotyping for CD15+ granulocytes, CD15+16+ neutrophils, CD14+ monocytes and CD3+ lymphocytes (section 2.3.5). The intracellular uptake and retention of 2µM imatinib (IUR) and OCT-1 activity were measured in all selected populations using [^{14}C]-labelled imatinib and the OCT-1 inhibitor prazosin. The IUR and OCT-1 activity are expressed as ng of

imatinib per 200,000 cells (section 2.4.1). In addition, mRNA expression of OCT-1, ABCB1 and BCR-ABL were measured in all populations using RQ-PCR and are expressed as a % of BCR expression (section 2.4.2). This analysis was used to determine whether OCT-1 expression and function varied in different cell populations and through comparisons between diagnosis CML and remission CML or normal individuals, if leukaemic state affects OCT-1 activity. All assays performed on each individual CML patient and normal individual are shown in Tables 3.1, 3.2 and 3.3.

Table 3.1: Assays performed on CML patients at diagnosis

Patient Number	MNC						Neutrophils						Monocytes						Lymphocytes					
	M	I	OA	O	A	B	M	I	OA	O	A	B	M	I	OA	O	A	B	M	I	OA	O	A	B
1			■	■	■									■	■	■	■							
2		■	■	■	■									■	■	■	■			■	■			
3		■	■	■	■									■	■	■	■			■	■			
4		■	■											■	■					■	■			
5	■	■	■										■	■	■				■					
6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■					
7	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	□	■	■	■
8	■	■	■	■	■		■	■	■	■	■		■	■	■	■	■		■	■	■	■	■	
9	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■			
10	■	■	■	■	■	■	■						■	■	■	■	■	■	■	■	■			
11	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■				■	■	■			
12	■	■	■	■	■	■	■	■	■	■	■	■	■						■	■	■			
13	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■
14	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■				■					
15	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■			
16	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■				■	■	■			

M = morphology, I = immunophenotyping, OA = IUR and OCT-1 Activity, O = OCT-1 mRNA, A = ABCB1 mRNA, B = BCRABL mRNA

■ Assay performed □ Assay discarded due to poor controls

Table 3.2: Assays performed on CML patients in cytogenetic remission

Patient Number	MNC					Neutrophils					Monocytes					Lymphocytes				
	M	I	OA	O	A	M	I	OA	O	A	M	I	OA	O	A	M	I	OA	O	A
6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
9	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
10	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■
11	■	■	■			■	■	■			■	■	■			■	■	■		
12	■	■	■			■	■	■			■	■	■			■	■	■		
13	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
14	■	■	■	■	■	■	■	■	■	■	■	■	■			■	■	■		

M = morphology, I = immunophenotyping, OA = IUR and OCT-1 Activity, O = OCT-1 mRNA, A = ABCB1 mRNA

■ Assay performed □ Assay discarded due to poor controls

Table 3.3: Assays performed on normal individuals

Sample Number	MNC					Neutrophils					Monocytes					Lymphocytes				
	M	I	OA	O	A	M	I	OA	O	A	M	I	OA	O	A	M	I	OA	O	A
1		■	■	■	■		■	■	■	■		■	■	■	■		■	■	■	■
2		■	■	■	■		■	■	■	■		■	■	■	■		■	■	■	■
3		■	■	■	■		■	■	■	■		■	■				■	■		
4		■	■	■	■		■	■	■	■		■	■				■	■		
5	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
6	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
7	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
8	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
9	■	■	■	■	■	■	■	■			■	■	■	■	■	■	■	■	■	■
10	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
11				■	■				■	■				■	■				■	■
12	■			■	■	■			■	■	■			■	■	■			■	■

M = morphology, I = immunophenotyping, OA = IUR and OCT-1 Activity, O = OCT-1 mRNA, A = ABCB1 mRNA

■ Assay performed □ Assay discarded due to poor controls

3.2 Results

3.2.1 Imatinib intracellular uptake and retention (IUR) and OCT-1 activity in CML and normal MNC

The OCT-1 activity in CML patients MNC at diagnosis is highly variable and is correlated with patient response to imatinib treatment [110, 111]. To determine if this variation is a characteristic of the leukaemia or is patient specific, MNC were isolated from 16 CML patients at diagnosis, 7 of these patients following complete cytogenetic remission and 10 normal individuals. For CP-CML patients at diagnosis the average IUR of imatinib in their MNC population was 28.3 ng/200,000 cells (range 13.8-41.1). For patients in cytogenetic remission, the average IUR of imatinib in their MNC was 23.2 ng/200,000 cells (range 15.8-36.8). Normal donors had the lowest uptake of imatinib with an average IUR of 16.3 ng/200,000 cells (range 10.4-28.0). The IUR in normal donors was significantly lower than that of both CML patients at diagnosis and in remission ($p=0.001$ and $p=0.028$ respectively, Figure 3.1A). The addition of prazosin to the IUR assay inhibits the influx of imatinib via OCT-1 [79]. In the presence of prazosin, the IUR of imatinib was reduced in CML patients at diagnosis, in remission and normal donors (average IUR 15.5, 17.8 and 13.1 ng/200,000 cells respectively, Figure 3.1A). As observed with IUR, OCT-1 activity was the highest in diagnosis CML MNC with an average of 12.8 ng/200,000 cells (range 0.5-28.3). Remission patients had a lower OCT-1 activity with an average of 6.2 ng/200,000 cells (range 0.0-19.6) and normal donors expressed the lowest OCT-1 activity (average 3.4 ng/200,000 cells; range 0.0-11.1), which was significantly lower than CML patients at diagnosis ($p=0.005$, Figure 3.1B).

3.2.2 Immunophenotyping and morphological analysis of the MNC population

There are two possible factors contributing to the low imatinib IUR and MNC OCT-1 activity in normal individuals and patients in remission compared to CML patients at diagnosis: 1) there may be differences in the cell types comprising the MNC population between these groups or 2) the leukaemic phenotype in CML patients at diagnosis (ie: BCR-ABL signalling) may affect OCT-1 activity. To investigate the first possibility, the MNC population for each group was

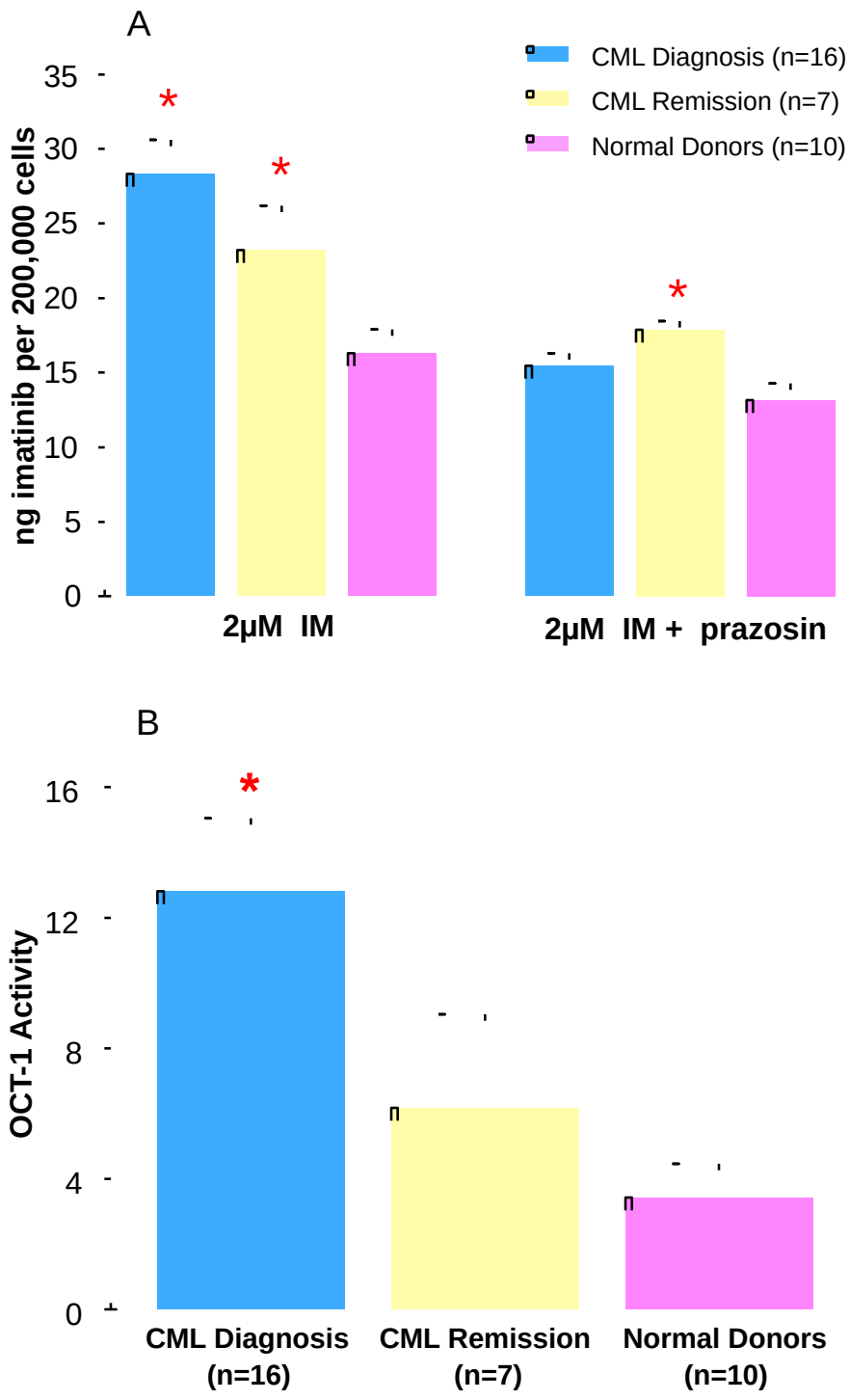
Figure 3.1 Intracellular uptake and retention of imatinib and OCT-1 activity in CML and normal MNC

MNC were isolated from the PB of CML patients at diagnosis (blue columns), CML patients in complete cytogenetic remission (yellow columns) and normal donors (purple columns). The IUR of 2μM imatinib in the presence and absence of prazosin (A) and OCT-1 activity (B) was measured. The IUR and OCT-1 activity was greatest in CML patients at diagnosis, reduced in CML remission patients and lowest in normal healthy donors.

Columns represent the mean plus standard error of the mean.

* denotes $p < 0.05$ in comparison to normal MNC

(A) p=0.001, p=0.028 and p=0.005 respectively (B) p=0.005


A
CML Diagnosis (n=16)
CML Remission (n=7)
Normal Donors (n=10)
ng imatinib per 200,000 cells
35
30
25
20
15
10
5
0
*
*
*
2μM IM
2μM IM + prazosin
B
OCT-1 Activity
16
12
8
4
0
*
CML Diagnosis (n=16)
CML Remission (n=7)
Normal Donors (n=10)

assessed by immunophenotyping and morphology (Figure 3.2). Typically, the MNC cell layer should comprise monocytes and lymphocytes due to the specific gravity of these cells. However, it was found that at diagnosis, CML MNC primarily consists of maturing and mature granulocytes (with an average of 71% CD15+, range 20%-92%). Of interest, only 27% of the cells were positive for both CD15 and CD16 (markers present on mature neutrophils) demonstrating a significant contribution from immature granulocytes. Morphology confirmed these analyses with diagnosis CML MNC exhibiting predominantly band-form or segmented neutrophils, with the presence of more immature myelocytes and metamyelocytes. In contrast, for remission CML MNC and normal MNC, the majority population was found to be comprised of lymphoid cells (with and average of 21% and 50% CD3+ lymphocytes) as anticipated for a post-ficoll MNC layer. Morphological analyses showed that CML remission MNC were comprised predominantly of lymphocytes and a few scattered segmented neutrophils. In normal donors the MNC population was predominantly lymphocytic, with some monocytes and no observable granulocytes. These data suggest that the intrinsic differences between myeloid and lymphoid cells may account for the differences in imatinib uptake and hence OCT-1 activity in the post-ficoll “MNC compartment” of the various sample types.

3.2.3 Isolation of neutrophils, monocytes and lymphocytes from CML patients and normal donors

To explore the possibility that different cell types have variable OCT-1 function and hence imatinib uptake, pure populations of neutrophils, monocytes and lymphocytes were isolated from these CML patients and normal donors as described in section 2.3.3 and shown in Figure 3.3. The granulocyte population present in normal and remission blood are mature neutrophils which express both CD15 and CD16 surface markers. Given diagnosis CML blood contains some more immature granulocytes (myelocytes, etc) CD16 MACS beads were used to isolate the mature neutrophil population to ensure that the populations being compared between CML and normal were consistent. Monocytes were isolated using CD14 MACS beads and lymphocytes using CD3 MACS beads. The purity of all isolated populations were assessed

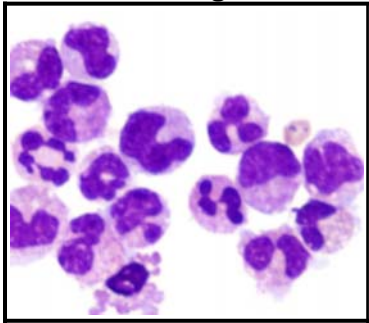
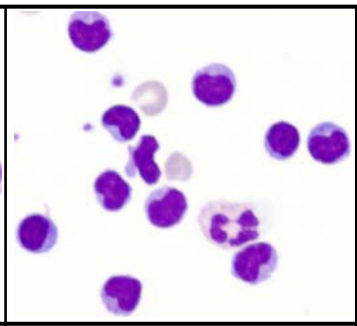
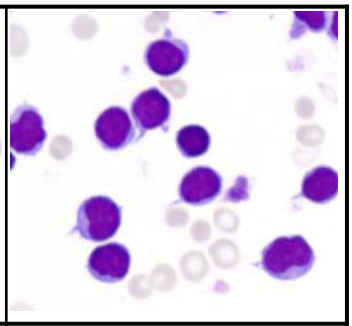
CML Diagnosis	CML Remission	Normal Donors
		
CD15+16+ Neutrophils **27%**	CD15+16+ Neutrophils **4%**	CD15+16+ Neutrophils **NT**
CD15+ Granulocytes **71%**	CD15+ Granulocytes **5%**	CD15+ Granulocytes **3%**
CD14+ Monocytes **5%**	CD14+ Monocytes **7%**	CD14+ Monocytes **4%**
CD3+ Lymphocytes **1%**	CD3+ Lymphocytes **21%**	CD3+ Lymphocytes **50%**

Figure 3.2 Morphology and immunophenotype of the MNC population

MNC were isolated from the PB of diagnosis CML patients, remission CML patients and normal donors. Cytospin preparations of MNC were stained with Wrights for morphological analysis. A representative photo was taken from one individual in each group. CD16, CD15, CD14 and CD3 antibodies were used to assess surface marker expression. Percentages represent the average from all individuals tested. Diagnosis CML MNC primarily consist of mature and immature granulocytes, whereas remission CML and normal donor MNC primarily consist of lymphocytes. NT= not tested.

Figure 3.3 Isolation procedures for neutrophils, monocytes and lymphocytes

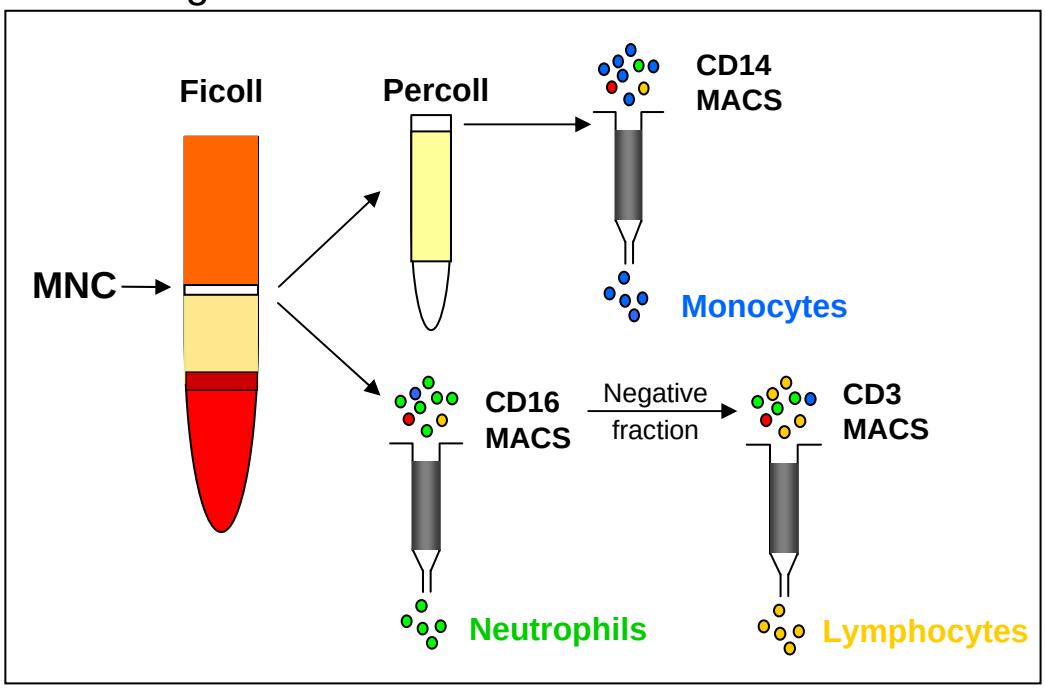
MNC were isolated from PB by ficoll density gradient centrifugation.

For CML diagnosis samples: MNC were enriched for monocytes using a Percoll continuous gradient and pure monocytes were then isolated using CD14 microbeads (MACS). Pure neutrophils and lymphocytes were also isolated from the MNC compartment using CD16 and CD3 microbeads respectively (MACS).

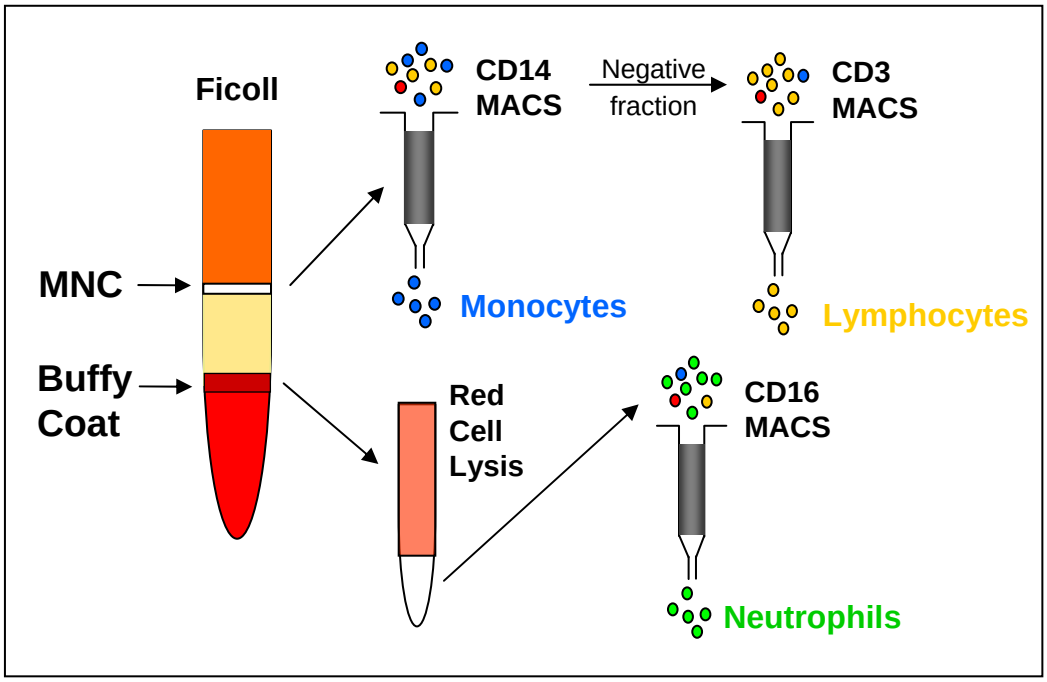
For CML remission samples and normal donors: Pure monocytes and lymphocytes were isolated directly from MNC using CD14 and CD3 microbeads respectively (MACS). Granulocytes were obtained from the red blood cell layer of the ficoll and were washed twice in red blood cell lysis buffer. Pure neutrophils were then isolated using CD16 microbeads (MACS).

The purity of all MACS sorted populations were assessed using immunophenotyping and morphological analysis as shown in Figure 3.4.

CML Diagnosis PB



Normal Donors and CML Remission PB



using immunophenotyping and morphology as shown in Figure 3.4. Populations isolated from normal individuals and CML remission patients were highly pure, and exceeded 75% purity as assessed by immunophenotyping (range 75%-99%). In CML patients at diagnosis, the isolated neutrophil population was very pure (average 94%, range 74%-99%), however the monocyte and lymphocyte populations were not (average 70% and 60% respectively). Given that monocyte and lymphocyte populations constitute only a small proportion of the blood in CML patients at diagnosis, it was difficult to isolate pure populations. Of note, the granulocytes present in the lymphocyte-enriched population appeared to be more promyelocytic in appearance.

3.2.4 Imatinib intracellular uptake and retention (IUR) and OCT-1 activity in diagnosis CML cell populations

As indicated in Figure 3.5A, the IUR of imatinib in CML patients at diagnosis was highest in the neutrophil population with an average of 24.5 ng/200,000 cells (range 20.3-30.1). The IUR of imatinib in monocytes and lymphocytes were significantly lower than that of neutrophils with an average of 19.8 ng/200,000 cells (range 8.0-30.3, $p=0.047$) and 17.2 ng/200,000 cells (range 12.7-23.8, $p<0.001$), respectively. The addition of prazosin reduced the IUR to the same level for all cell subsets (average neutrophils 15.3, monocytes 14.9 and lymphocytes 14.7 ng/200,000 cells). Assessment of OCT-1 activity showed similar trends to IUR (Figure 3.5B), where neutrophils had the highest OCT-1 activity with an average of 9.2 ng/200,000 cells (range 2.2-16.8). The average OCT-1 activity in monocytes was lower at 5.8 ng/200,000 cells (range 0.0-15.4) and the average OCT-1 activity in lymphocytes of 2.8 ng/200,000 cells (range 0.0-7.3) was significantly lower than that of neutrophils ($p<0.001$). Therefore, the high MNC OCT-1 activity in CML patients at diagnosis is likely to be due to the strong presence of granulocytes in the MNC population.

Figure 3.4 Purity of isolated neutrophils, monocytes and lymphocytes

The average percentage of surface markers as assessed by flow cytometry are expressed for each isolated population and a representative picture of Wright stained cells. Isolated populations in CML remission and normal individuals were highly pure with a minimum of 83% purity by immunophenotyping. For CML diagnosis patients, poorer purity was achieved for the monocyte and lymphocyte populations with 30% and 40% contaminating granulocytes respectively.

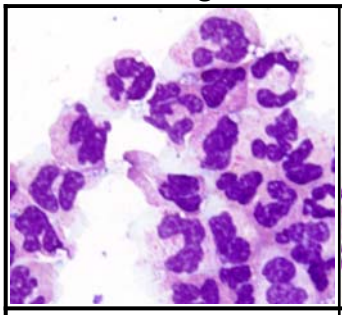
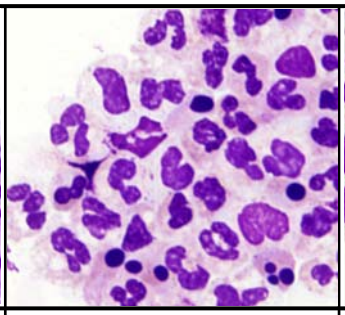
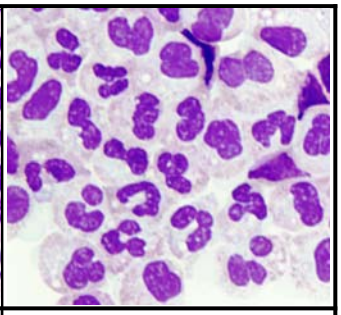
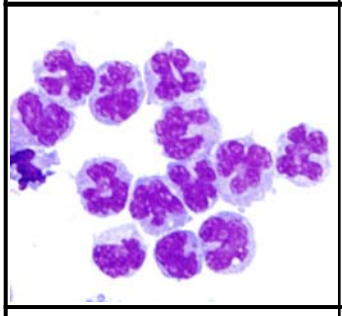
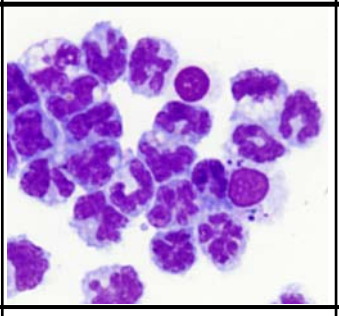
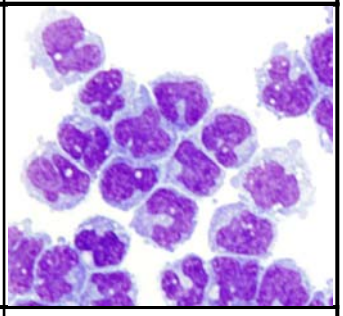
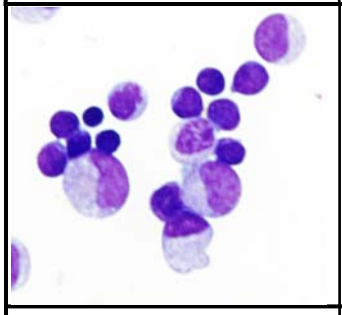
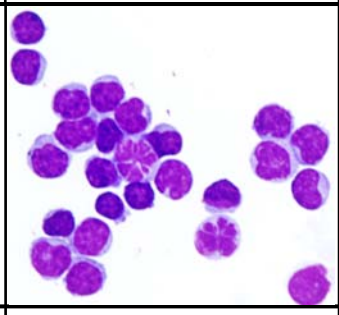
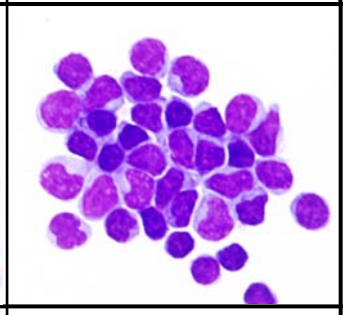
	CML Diagnosis	CML Remission	Normal Donors
Neutrophils			
	CD15+16+ Neutrophils **94%**	CD15+16+ Neutrophils **93%**	CD15+16+ Neutrophils **88%**
	CD14+ Monocytes **0%**	CD14+ Monocytes **1%**	CD14+ Monocytes **2%**
	CD3+ Lymphocytes **0%**	CD3+ Lymphocytes **1%**	CD3+ Lymphocytes **6%**
Monocytes			
	CD15+ Granulocytes **30%**	CD15+ Granulocytes **9%**	CD15+ Granulocytes **0%**
	CD14+ Monocytes **70%**	CD14+ Monocytes **83%**	CD14+ Monocytes **92%**
	CD3+ Lymphocytes **0%**	CD3+ Lymphocytes **6%**	CD3+ Lymphocytes **2%**
Lymphocytes			
	CD15+ Granulocytes **40%**	CD15+ Granulocytes **4%**	CD15+ Granulocytes **0%**
	CD14+ Monocytes **0%**	CD14+ Monocytes **3%**	CD14+ Monocytes **2%**
	CD3+ Lymphocytes **60%**	CD3+ Lymphocytes **92%**	CD3+ Lymphocytes **94%**

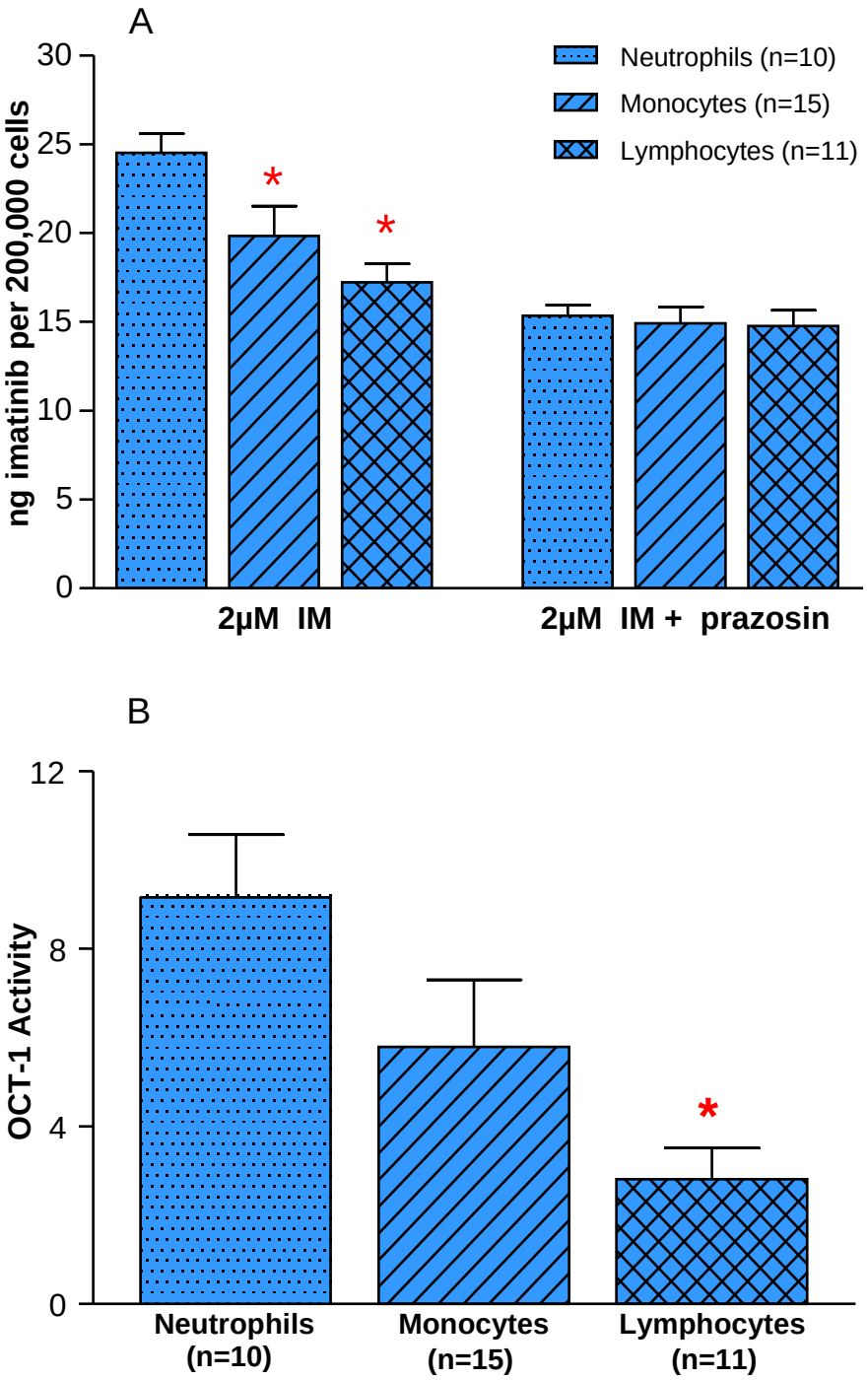
Figure 3.5 Intracellular uptake of imatinib and OCT-1 activity in diagnosis CML cell populations

Neutrophils, monocytes and lymphocytes were isolated from the PB of CML patients at diagnosis. The IUR of 2μM imatinib (A) and OCT-1 activity (B) was measured.

Columns represent the mean plus standard error of the mean.

* denotes $p < 0.05$ in comparison to neutrophils

(A) $p=0.047$, $p<0.001$ (B) $p<0.001$


A
30
25
20
15
10
5
0
ng imatinib per 200,000 cells
Neutrophils (n=10)
Monocytes (n=15)
Lymphocytes (n=11)
*
*
2μM IM
2μM IM + prazosin
B
12
8
4
0
OCT-1 Activity
*
Neutrophils (n=10)
Monocytes (n=15)
Lymphocytes (n=11)

3.2.5 BCR-ABL mRNA expression in diagnosis CML cell populations

To examine whether the differences in OCT-1 activity observed was related to BCR-ABL expression, BCR-ABL mRNA was measured in all isolated cell populations from CML patients at diagnosis. As seen in Figure 3.6 BCR-ABL expression was not significantly different between MNC, neutrophils and monocytes in these patients, with average expression of 78%, 88% and 68%, respectively. As anticipated, the BCR-ABL expression was found to be substantially lower in the lymphocyte population (average 15%) consistent with previous studies which suggest CP-CML is solely a disease of the myeloid lineage [148]. The measurable amounts of BCR-ABL mRNA in this population are most likely due to the 40% contamination with granulocytes. The variability in BCR-ABL mRNA expression in MNC (range 33%-88%) and neutrophils (range 23%-172%) from patients at diagnosis was then compared with their OCT-1 activity. No significant correlation was observed between BCR-ABL expression and OCT-1 activity in the MNC compartment ($r=-0.582$, $p=0.100$) or in the neutrophil population ($r=-0.306$, $p=0.423$, Figure 3.7).

3.2.6 Imatinib intracellular uptake and retention (IUR) and OCT-1 activity in remission CML cell populations

The IUR of imatinib and OCT-1 activity was then measured in neutrophils, monocytes and lymphocytes recovered from the PB of 7 CML patients in complete cytogenetic remission. Consistent with CML samples at diagnosis the IUR of imatinib in CML remission patients was the highest in the neutrophil population and lower in the monocyte and lymphocyte populations ($p=0.049$ and $p=0.014$ respectively, Figure 3.8A). The respective average IURs for neutrophils, monocytes and lymphocytes are 36.7 ng/200,000 cells (range 20.8-60.9), 22.2 ng/200,000 cells (range 16.1-31.6) and 18.5 ng/200,000 cells (range 12.9-23.8). In the presence of prazosin, the IUR was reduced to the same level in all cell subsets (average neutrophils 15.7, monocytes 17.8 and lymphocytes 18.0 ng/200,000 cells). Neutrophils also displayed the highest OCT-1 activity (average 19.9 ng/200,000 cells, range 2.5-42.9) and lower in monocyte and lymphocyte populations with average OCT-1 activity of 5.2 ng/200,000 cells (range 0.0-14.9, $p=0.047$) and

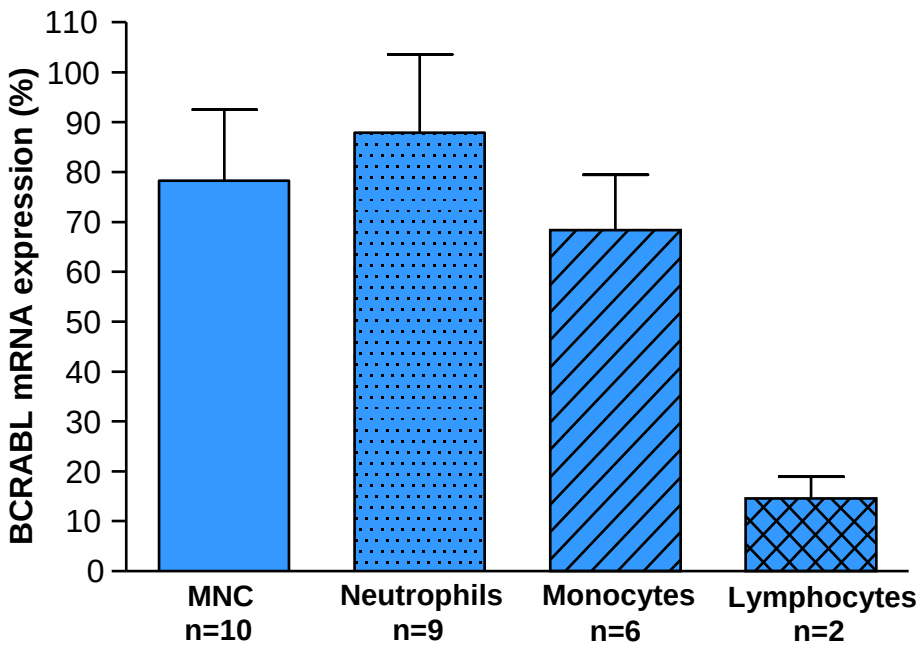


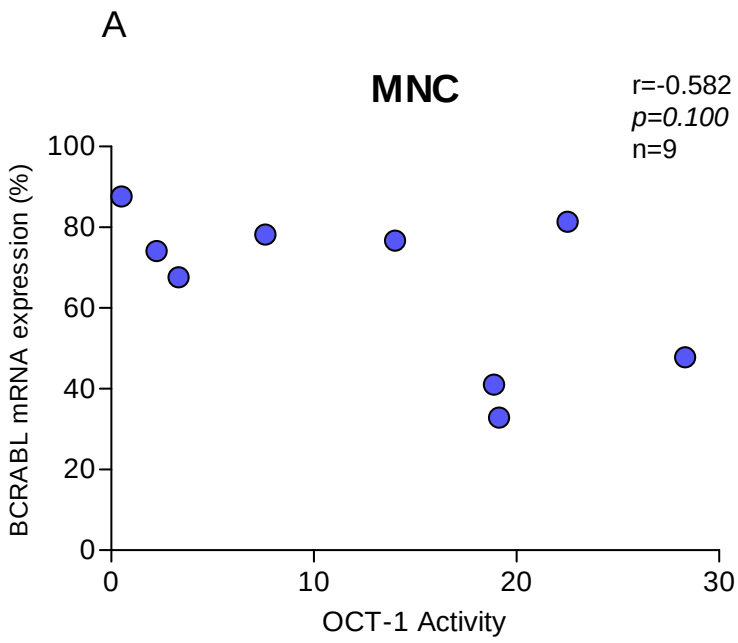
Figure 3.6 BCR-ABL mRNA expression in diagnosis CML cell populations

Neutrophils, monocytes and lymphocytes were isolated from the PB of CML patients at diagnosis. BCR-ABL mRNA expression was measured using RQ-PCR. Columns represent the mean plus standard error of the mean.

Figure 3.7 Relationship between BCR-ABL mRNA expression and OCT-1 activity

BCR-ABL mRNA expression and OCT-1 activity was assessed in (A) MNC (B) and neutrophils recovered from CML patients at diagnosis. No significant correlation was observed between OCT-1 activity and BCR-ABL expression.

The pearson product moment was used to assess the correlation.


A
MNC
r=-0.582
p=0.100
n=9
BCRABL mRNA expression (%)
100
80
60
40
20
0
0
10
20
30
OCT-1 Activity

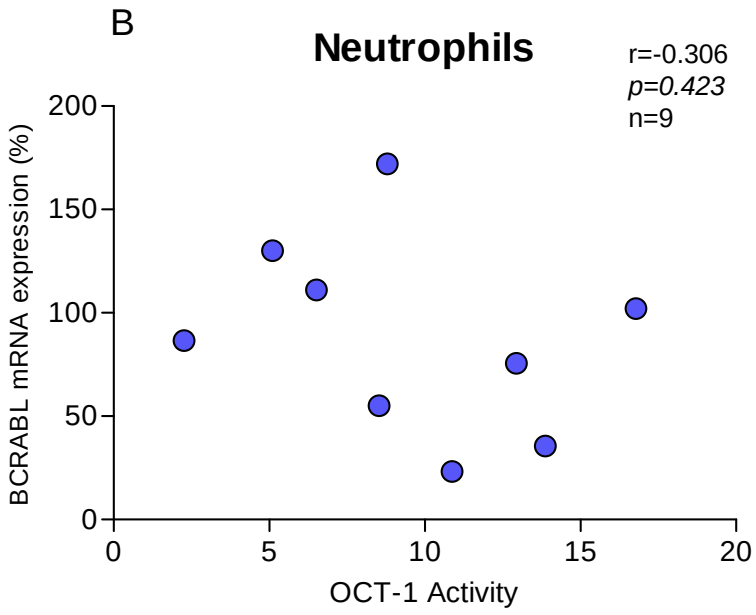

B
Neutrophils
r=-0.306
p=0.423
n=9
BCRABL mRNA expression (%)
200
150
100
50
0
0
5
10
15
20
OCT-1 Activity

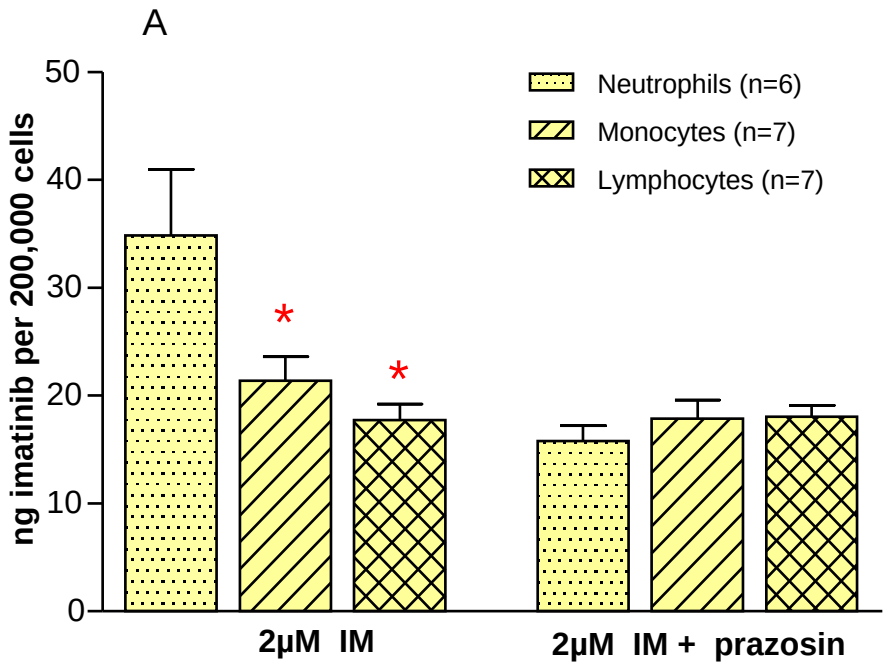
Figure 3.8 Intracellular uptake of imatinib and OCT-1 activity in remission CML cell populations

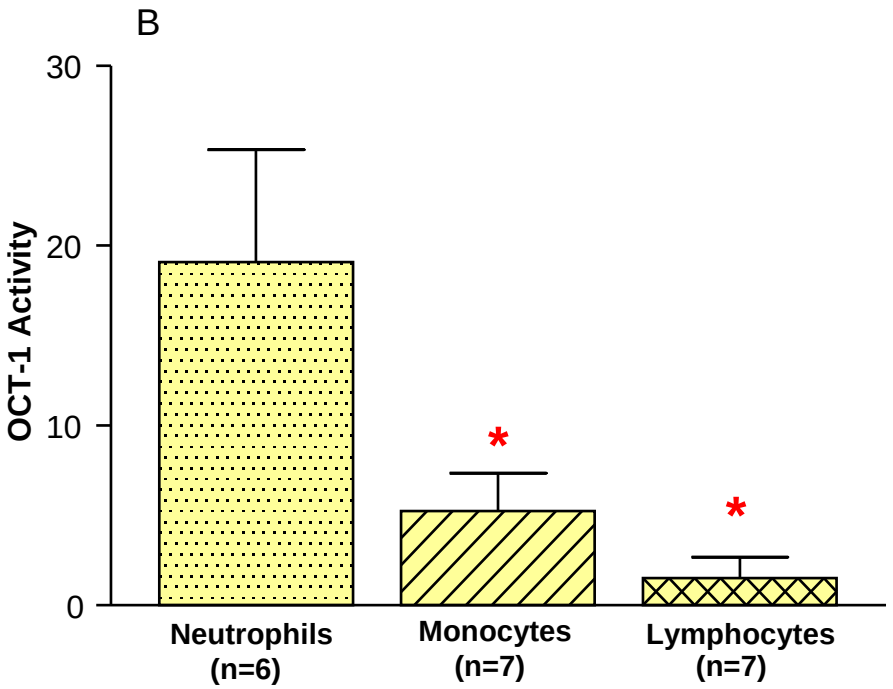
Neutrophils, monocytes and lymphocytes were isolated from the PB of remission CML patients. The IUR of 2μM imatinib (A) and OCT-1 activity (B) was measured.

Columns represent the mean plus standard error of the mean.

* denotes $p < 0.05$ in comparison to neutrophils

(A) p=0.049, p=0.014 (B) p=0.047, p=0.012


A
50
40
30
20
10
0
ng imatinib per 200,000 cells
Neutrophils (n=6)
Monocytes (n=7)
Lymphocytes (n=7)
*
*
2μM IM
2μM IM + prazosin


B
30
20
10
0
OCT-1 Activity
*
*
Neutrophils
(n=6)
Monocytes
(n=7)
Lymphocytes
(n=7)

1.5 ng/200,000 cells (range 0.0-8.1, p=0.012), respectively (Figure 3.8B). These data suggest that the low MNC OCT-1 activity in CML remission patients is likely to be due to the high lymphocyte population in their MNC.

3.2.7 Imatinib intracellular uptake and retention (IUR) and OCT-1 activity in normal cell populations

The IUR of imatinib and OCT-1 activity was then measured in neutrophils, monocytes and lymphocytes recovered from the PB of 10 normal healthy individuals. The same trends were observed in these normal donors as with both CML patients at diagnosis and in remission. The IUR and OCT-1 activity was again highest in the neutrophil population and lowest in the lymphocyte population (Figure 3.9). The average IUR and OCT-1 activity in neutrophils was 28.9 ng/200,000 cells (range 19.2-36.9) and 13.5 ng/200,000 cells (range 4.0-19.6) respectively. The IUR and OCT-1 activity in monocytes was significantly lower than that of neutrophils with an average of 20.4 ng/200,000 cells (range 15.7-24.3, $p<0.001$) and 8.9 ng/200,000 cells (range 3.8-13.7, $p=0.026$) respectively. In lymphocytes the IUR and OCT-1 activity was significantly lower than that of both monocytes and neutrophils with an average IUR of 12.9 ng/200,000 cells (range 10.0-19.5, $p<0.001$) and an average OCT-1 activity of 1.9 ng/200,000 cells (range 0.0-4.5, $p<0.001$). Therefore, the low MNC OCT-1 activity in normal individuals is consistent with the high proportion of lymphocytes in their MNC.

3.2.8 Relationship between OCT-1 activity in MNC and in neutrophils, monocytes and lymphocytes

The relationship between MNC OCT-1 activity and OCT-1 activity in neutrophils, monocytes and lymphocytes was then examined. A significant correlation between MNC OCT-1 activity and neutrophil OCT-1 activity was observed in CML patients at diagnosis and normal individuals ($r=0.765$, $p=0.016$ and $r=0.762$, $p=0.016$ respectively, Figure 3.10A and B). A significant correlation was also found between CML diagnosis MNC OCT-1 activity and monocyte OCT-1 activity ($r=0.617$, $p=0.014$, Figure 3.10C). No relationship was observed between the OCT-1 activity in lymphocytes and MNC (Figure 3.10E and F).

Figure 3.9 Intracellular uptake of imatinib and OCT-1 activity in normal cell populations

Neutrophils, monocytes and lymphocytes were isolated from the PB of normal donors. The IUR of 2μM imatinib (A) and OCT-1 activity (B) was measured.

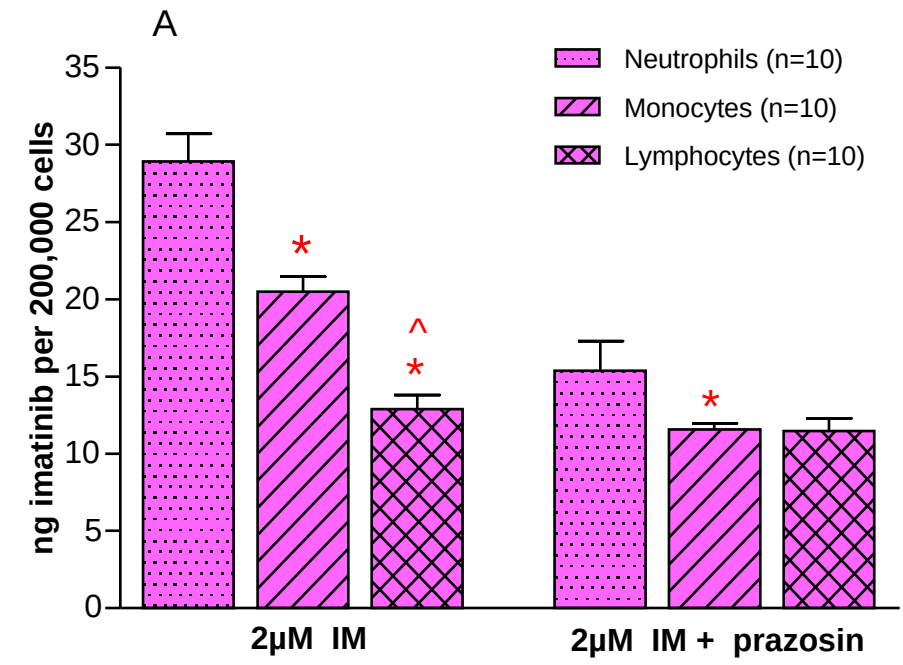
Columns represent the mean plus standard error of the mean.

* denotes $p < 0.05$ in comparison to neutrophils

(A) $p<0.001$, $p<0.001$, $p=0.038$ (B) $p=0.026$, $p<0.001$

^ denotes $p < 0.05$ in comparison to monocytes

(A) $p<0.001$ (B) $p<0.001$


A
Neutrophils (n=10)
Monocytes (n=10)
Lymphocytes (n=10)
ng imatinib per 200,000 cells
35
30
25
20
15
10
5
0
*
^
*
*
2μM IM
2μM IM + prazosin

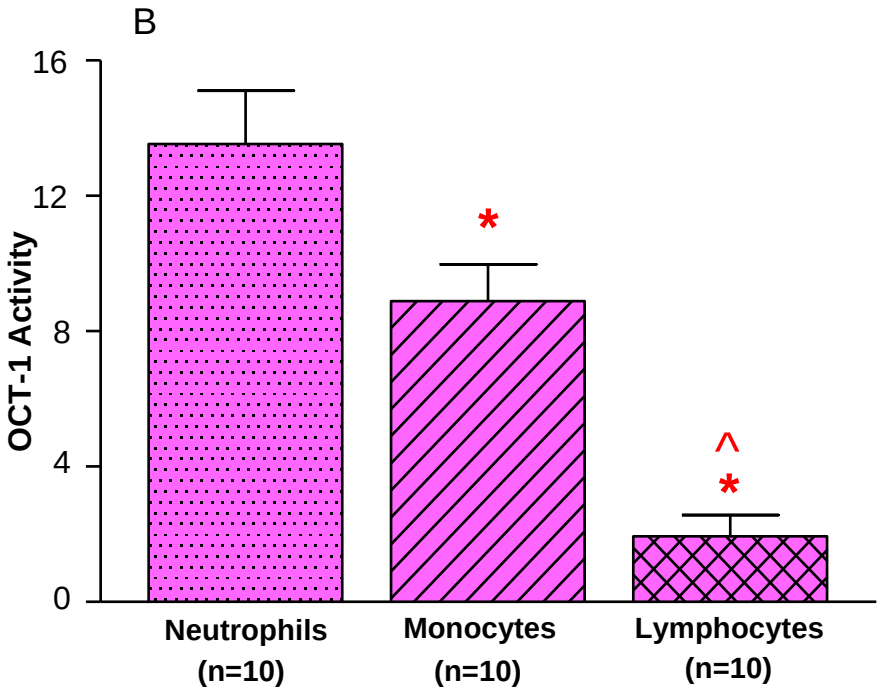
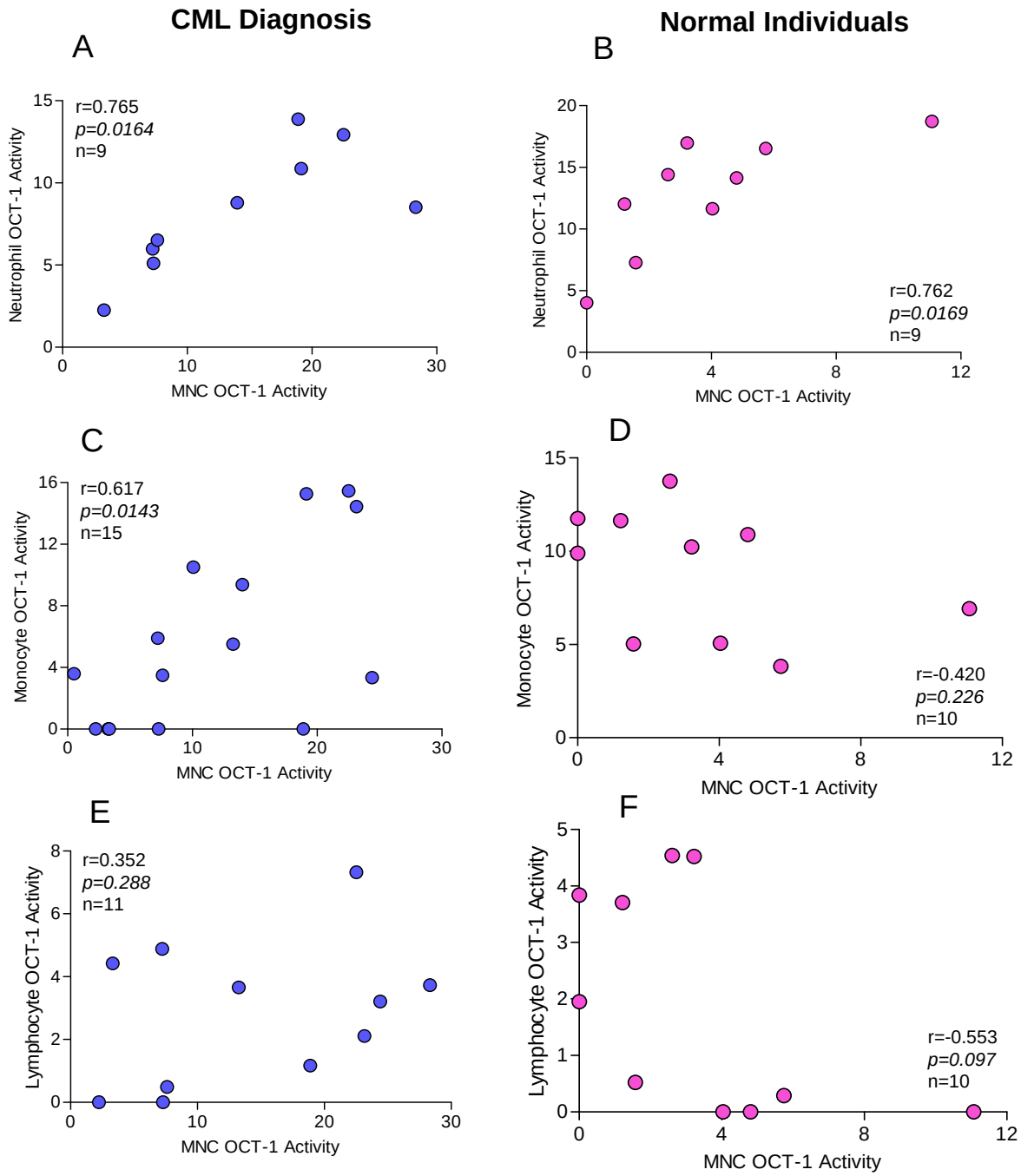

B
OCT-1 Activity
16
12
8
4
0
*
^
*
Neutrophils
(n=10)
Monocytes
(n=10)
Lymphocytes
(n=10)

Figure 3.10 Relationship between MNC OCT-1 activity and neutrophil, monocyte and lymphocyte OCT-1 activity

In CML diagnosis patients (A, C & E) and normal donors (B, D & F) the OCT-1 activity measured in individuals MNC was associated with the OCT-1 activity in their neutrophil (A & B), monocyte (C & D) and lymphocyte (E & F) populations. A significant correlation was seen between neutrophil OCT-1 activity and MNC OCT-1 activity in both CML diagnosis patients and normal donors. A significant correlation was also found between monocyte OCT-1 activity and MNC OCT-1 activity in diagnosis patients.

The pearson product moment was used to assess the correlation.


CML Diagnosis
Normal Individuals
A
B
C
D
E
F
r=0.765
p=0.0164
n=9
Neutrophil OCT-1 Activity
MNC OCT-1 Activity
r=0.762
p=0.0169
n=9
r=0.617
p=0.0143
n=15
Monocyte OCT-1 Activity
r=-0.420
p=0.226
n=10
r=0.352
p=0.288
n=11
Lymphocyte OCT-1 Activity
r=-0.553
p=0.097
n=10

3.2.9 Imatinib intracellular uptake and retention (IUR) and OCT-1 activity between CML diagnosis, CML remission and normal individuals

To assess the potential role of the leukaemic phenotype and BCR-ABL in regulating OCT-1 activity, the IUR and OCT-1 activity were compared between the neutrophils, monocytes and lymphocytes isolated from CML patients at diagnosis, in cytogenetic remission and in normal individuals (Figure 3.11). When the neutrophil IUR and OCT-1 activity was compared between normal donors, CML patients at diagnosis and in remission no significant difference between all three groups was observed. This finding was also consistent in monocytes and lymphocytes. Therefore, the presence of BCR-ABL in CML patients at diagnosis is unlikely to be contributing to differences in IUR or OCT-1 activity.

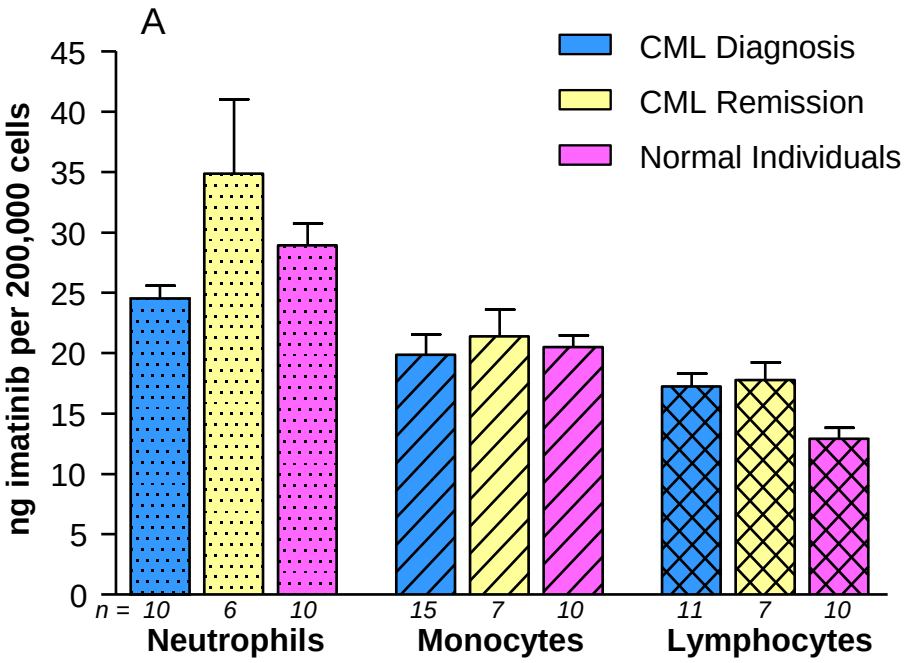
3.2.10 OCT-1 mRNA expression in CML and normal cell populations

The differences in OCT-1 activity between cell types may be due to differences in OCT-1 transcript expression in these populations. OCT-1 mRNA expression was therefore assessed in all cell populations in CML patients at diagnosis, remission and in normal donors. As seen in Table 3.4 the highest level of OCT-1 mRNA expression was seen in the neutrophil population (average diagnosis 54%, remission 198% and normal 76%), while the lowest expression was observed in lymphocytes (average diagnosis 3%, remission 1.2% and normal 0.5%, $p<0.05$ for all groups). In keeping with our observations regarding OCT-1 activity, there was no difference in the neutrophil OCT-1 mRNA expression between CML patients at diagnosis, CML patients in remission and normal individuals. However, the OCT-1 mRNA expression in monocytes and lymphocytes from CML patients at diagnosis and remission was significantly higher than that in normal individuals. This is unlikely to be due to an effect of BCR-ABL as there is no difference between CML patients at diagnosis and in remission. It was then examined if a relationship exists between OCT-1 mRNA expression and OCT-1 activity. In CML patients at diagnosis and normal individuals no significant relationship was found between OCT-1 activity and OCT-1 mRNA when examined in the MNC, neutrophil, monocyte or lymphocyte populations (Figures 3.12 and 3.13).

Figure 3.11 Intracellular uptake of imatinib and OCT-1 activity in neutrophils, monocytes and lymphocytes in CML patients and normal donors

Neutrophils, monocytes and lymphocytes were isolated from the PB of CML patients at diagnosis (blue columns), CML patients in cytogenetic remission (yellow columns) and normal healthy donors (purple columns). The IUR of 2μM imatinib (A) and OCT-1 activity (B) was measured. No difference in IUR or OCT-1 activity was observed between CML diagnosis patients, CML remission patients and normal donors when specific cell types were analysed.

Columns represent the mean plus standard error of the mean.


A
CML Diagnosis
CML Remission
Normal Individuals
ng imatinib per 200,000 cells
45
40
35
30
25
20
15
10
5
0
n = 10 6 10 15 7 10 11 7 10
Neutrophils
Monocytes
Lymphocytes

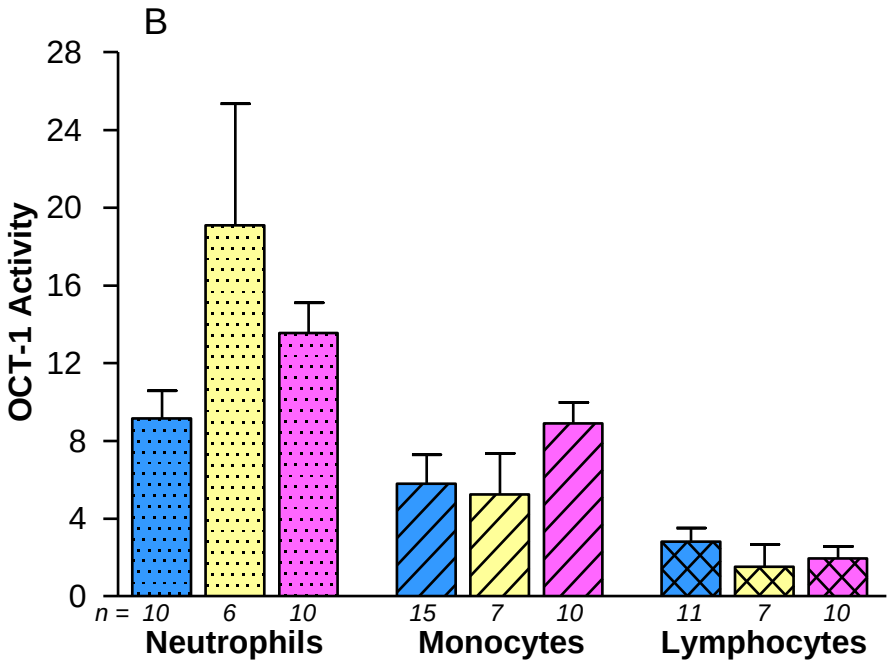

B
OCT-1 Activity
28
24
20
16
12
8
4
0
n = 10 6 10 15 7 10 11 7 10
Neutrophils
Monocytes
Lymphocytes

Table 3.4: OCT-1 mRNA expression between cell types

	MNC	Neutrophils	Monocytes	Lymphocytes
CML Diagnosis	**10%** ± 10% (n=14) *	**54%** ± 33% (n=10)	**13%** ± 12% (n=10) *	**3%** ± 2.2% (n=3) *
CML Remission	**5.5%** ± 1.2% (n=5) *	**198%** ± 163% (n=5)	**11%** ± 9% (n=4) *	**1.2%** ± 1.2% (n=4) *
Normal Donors	**1.2%** ± 1% (n=12)	**76%** ± 121% (n=11)	**2%** ± 1.2% (n=10)	**0.5%** ±0.5% (n=9)

Average OCT-1 mRNA ± Standard Deviation
(number of patients assessed)

* Represents $p<0.05$ compared to normal donors

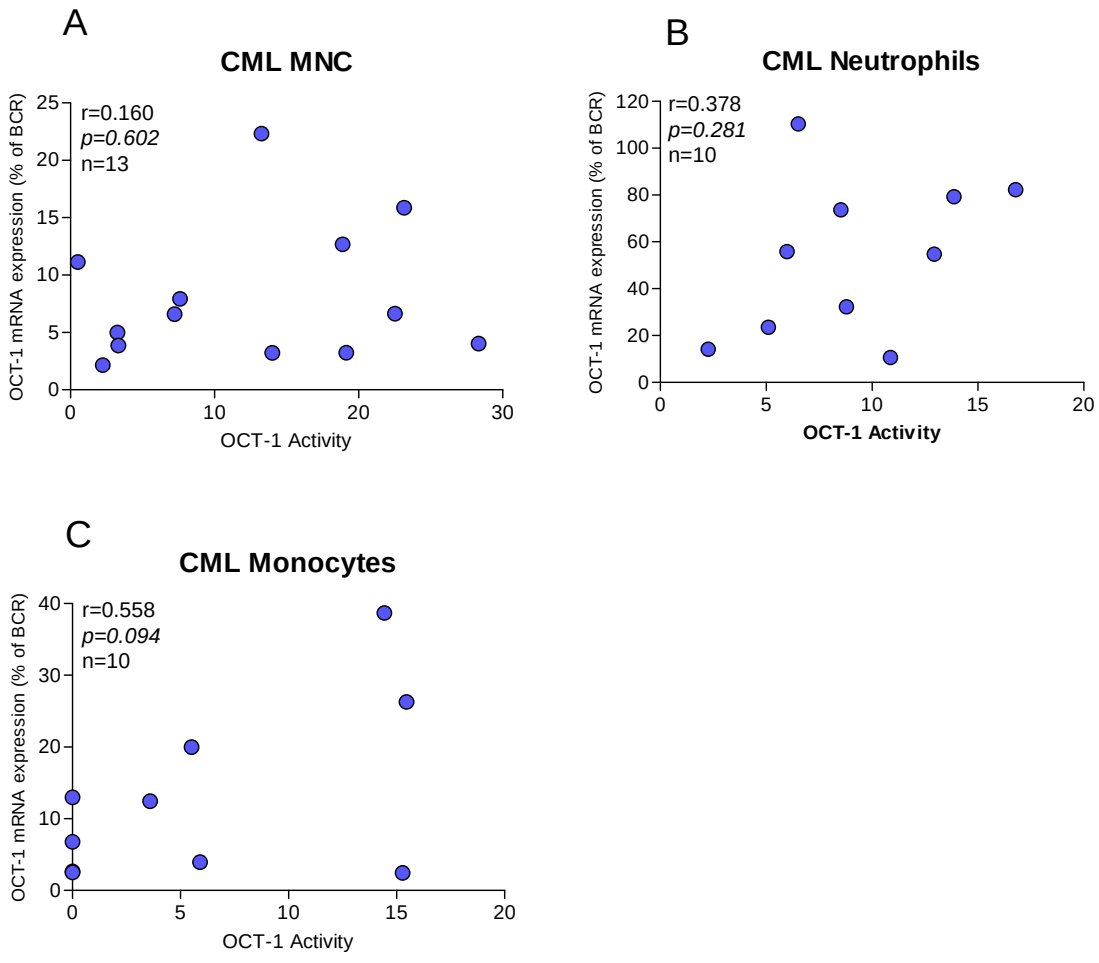


Figure 3.12 Relationship between OCT-1 activity and OCT-1 mRNA in CML diagnosis patients

OCT-1 activity and OCT-1 mRNA expression was measured in MNC (A), neutrophils (B) and monocytes (C) recovered from CML diagnosis patients. No significant relationship was observed between OCT-1 activity and OCT-1 mRNA expression for any cell population.

The pearson product moment was used to assess the correlation.

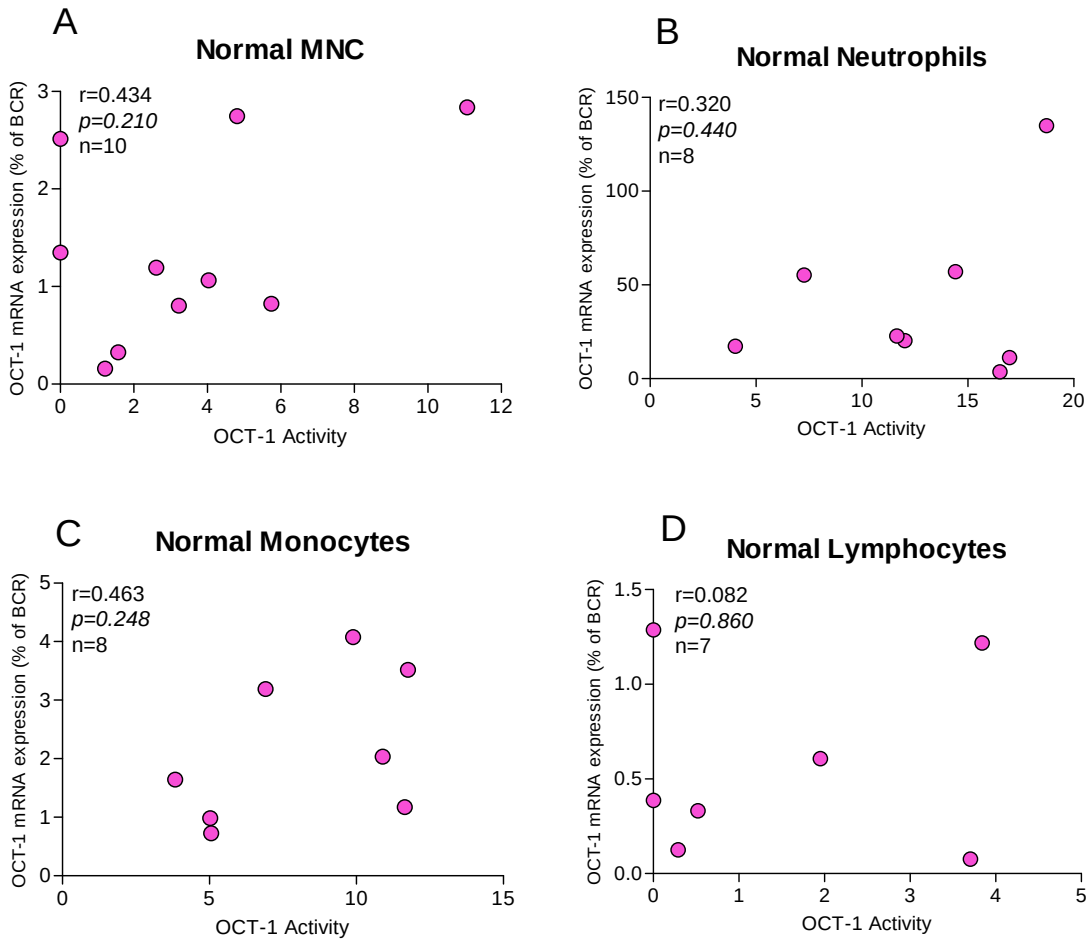


Figure 3.13 Relationship between OA and OCT-1 mRNA in normal donors

OCT-1 activity and OCT-1 mRNA expression was measured in MNC (A), neutrophils (B), monocytes (C) and lymphocytes (D) recovered from normal individuals. No significant relationship was observed between OCT-1 activity and OCT-1 mRNA expression for any cell population.

The pearson product moment was used to assess the correlation.

3.2.11 ABCB1 mRNA expression in CML and normal cell populations

While OCT-1 function appears to be the main factor impacting upon imatinib IUR, there is also a potential role for the efflux protein ABCB1. The mRNA expression of ABCB1 was assessed in all the isolated cell populations in CML and normal donors (Table 3.5). In MNC the ABCB1 mRNA expression was highest in CML remission patients (average 224%) compared with both CML patients at diagnosis (average 15%, p=0.003) and normal donors (average 30%, p=0.013). Similar trends were seen for neutrophils, monocyte and lymphocytes, where in all cases remission patients expressed the highest ABCB1 mRNA expression. These remission patients have on average received 12 months of imatinib therapy by the time of this testing, therefore it is possible that long-term treatment with imatinib has caused these cells to up-regulate ABCB1.

Table 3.5: ABCB1 mRNA expression between cell types

	MNC	Neutrophils	Monocytes	Lymphocytes
CML Diagnosis	**15%** ± 30% (n=14) #	**13%** ± 19% (n=9) #	**1.3%** ± 1.1% (n=10)	**10%** ± 7.7% (n=3)
CML Remission	**224%** ± 170% (n=5) *	**49%** ± 60% (n=5)	**13%** ± 23% (n=4)	**75%** ± 48% (n=4) *
Normal Donors	**30%** ± 43% (n=12)	**16%** ± 25% (n=11)	**0.5%** ± 0.6% (n=10)	**22%** ± 21% (n=9)

Average ABCB1 mRNA ± Standard Deviation
(number of patients assessed)

* Represents $p<0.05$ compared to normal donors
# Represents $p<0.005$ compared to CML remission

3.3 Discussion

OCT-1 activity, measured in MNC at diagnosis, is a strong predictive test to identify patients who will respond well to treatment, and more importantly, to identify patients who are most likely to fail treatment. An understanding of the biology of OCT-1 activity is central to identifying those patients who are likely to be poor responders to imatinib therapy and in developing new strategies to better target these patients at risk.

The findings described in this chapter suggest that the intracellular uptake and retention (IUR) of imatinib and OCT-1 activity in the MNC compartment was reduced in both normal individuals and CML patients in cytogenetic remission compared to CML patients at diagnosis. To assess whether the low MNC OCT-1 activity in normal healthy volunteers was due to either differences in the cellular composition of the MNC population, or related to the absence of BCR-ABL signalling, MNC populations for each group were examined by immunophenotyping and morphological assessment. It was found that the MNC compartment in diagnostic CML patients is comprised predominantly of a mixture of granulocytes at varying stages of differentiation. In contrast, the major populations present within remission CML and normal MNC were found to be lymphoid in nature. These data suggest that the intrinsic differences between myeloid and lymphoid cells may account for the differences in imatinib uptake and hence OCT-1 activity in the MNC compartment.

The intrinsic OCT-1 activity of lineage marker-defined cell populations was examined following the isolation of pure populations of CD15+16+ neutrophils, CD14+ monocytes and CD3+ lymphocytes from CML patients and normal donors. For all groups, neutrophils were found to express the highest IUR and OCT-1 activity, while lymphocytes exhibited the lowest IUR and OCT-1 activity. These data suggest that high MNC OCT-1 activity in CML patients at diagnosis may be due to the predominance of granulocytes in the MNC population. Furthermore, the low

MNC OCT-1 activity in CML remission and normal donors may reflect the presence of greater numbers of lymphocytes. A recent study by Hatziieremia *et al* suggested that differences in imatinib uptake between patient cells and cell lines may be associated with cell volume [149]. Given the considerable size differences between neutrophils and lymphocytes it is possible that reduced cell volume may contribute to the lower OCT-1 activity seen in lymphocytes. When MNC OCT-1 activity was correlated with OCT-1 activity in neutrophils, monocytes and lymphocytes the strongest significant relationship was observed between MNC OCT-1 activity and neutrophil OCT-1 activity. This was found in both CML patients at diagnosis and normal individuals. As a result, individuals with high MNC OCT-1 activity, tend to have high OCT-1 activity in their neutrophils but not necessarily in their monocytes or lymphocytes. Therefore, OCT-1 activity in the granulocyte lineage is likely to be a key contributor to MNC OCT-1 activity in CML patients at diagnosis.

Variation in OCT-1 activity may be a result of variation in OCT-1 mRNA expression. Studies in this chapter show that in keeping with OCT-1 activity, OCT-1 mRNA expression is highest in the neutrophil population and lowest in the lymphocyte population. This was observed in CML patients at diagnosis, patients in remission and normal donors. These findings are in agreement with two recent studies which showed that polymorphonuclear cells (ie: neutrophils) had a higher OCT-1 mRNA expression compared with MNC (lymphocytes) in normal PB [115, 147]. However, despite this agreement between OCT-1 expression and activity, no direct correlation was observed between OCT-1 activity and OCT-1 mRNA expression in CML diagnosis patients and normal donors. Therefore, while OCT-1 mRNA is one factor influencing OCT-1 activity, it is not likely to be the key contributor to OCT-1 function.

A recent study by Bazeos *et al* examined OCT-1 mRNA expression in total white blood cells (WBC) between CML patients at diagnosis, in remission and in normal donors [115]. This group found that OCT-1 mRNA expression was increased in CML remission patients and normal

individuals compared to CML patients at diagnosis, and they attributed this finding to an inhibitory effect of BCR-ABL on OCT-1 expression. However, it is important to note that between these groups, the total WBC population may be comprised of different cell populations. As shown in this chapter, and by others [147], PB from CML patients at diagnosis contains more immature forms of granulocytes (ie: myelocytes and metamyelocytes) which are not seen in normal PB. These differences in the total WBC population may be influencing the results of Bazeos *et al*. To overcome this, studies in this chapter have compared OCT-1 activity and OCT-1 mRNA expression in the mature neutrophil population recovered from CML patients at diagnosis, in remission and in normal donors and no significant difference in OCT-1 activity or expression between groups was observed. This indicates that the leukaemic phenotype and BCR-ABL are unlikely to be affecting OCT-1 activity or expression. In support of this, no relationship was observed between OCT-1 activity in CML patients at diagnosis and their BCR-ABL mRNA expression.

Interestingly, the studies presented here show that there is increased OCT-1 activity and expression in the neutrophil population recovered from CML remission patients compared to both CML patients at diagnosis and normal individuals. While not reaching statistical significance, possibly due to insufficient numbers, it is tempting to speculate that long-term imatinib treatment may have an effect on OCT-1 expression and/or function.

In conclusion, the findings presented in this chapter illustrate that OCT-1 activity and expression is strongly related to cell lineage. Neutrophils were found to have the highest OCT-1 activity and expression and lymphocytes the lowest. Furthermore, neutrophil OCT-1 activity correlated strongly with MNC OCT-1 activity. Interestingly, OCT-1 activity was similar in lineage-defined cell populations recovered from CML patients at diagnosis, CML patients in cytogenetic remission and normal donors, implying that BCR-ABL expression is unlikely to affect OCT-1 activity. Therefore, variation in MNC OCT-1 activity and hence response to

imatinib therapy in CML patients may be related to factors associated with the “majority cell population” at diagnosis.

4 THE ROLE OF CELL COMPOSITION IN MNC ON A PATIENT'S OCT-1 ACTIVITY

4.1 Introduction

Immunological phenotyping is important for classification and lineage determination of acute leukaemias. Both acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) are a heterogeneous group of diseases with variable responses to therapies [150, 151]. Immunophenotyping has been critical for diagnosis, prognosis and detection of minimal residual disease in AML and ALL. For AML, an association has been described between the expression of myeloid markers and improved responses to therapy [152]. The expression of myeloid markers: MPO, CD13, CD33, CDw65 and CD117 has been shown to be associated with a good prognosis with complete remission rates of 80% in contrast to 48% for patients who do not express all of these markers ($p<0.001$). Disease free survival and overall survival was also significantly higher in patients expressing all of these markers ($p=0.02$ and $p=0.008$, respectively). Expression of the primitive cell marker CD34 has been associated with poorer responses to chemotherapy in AML [153]. The rates of complete remission for CD34 negative patients were 79% in contrast to 58% for CD34 positive patients ($p=0.0003$). Furthermore, expression of CD56 (natural killer cell marker) was associated with shorter overall survival ($p=0.026$). For T-lineage ALL, an association has been described between the expression of T-lineage markers and more favourable outcomes [154]. 100% of patients expressing 6-7 of the T-lineage markers CD1, CD2, CD3, CD4, CD5, CD7 and CD8 achieved complete remissions, compared with 90% for the 4-5 marker group and 60% for the 1-3 marker group ($p=0.02$). Similar findings were also seen for disease free survival and overall survival. For B-lineage ALL, CD34 positivity was associated with reduced overall survival and disease free survival [154].

While CML is a disease arising from a single genetic alteration, the Philadelphia chromosome (t 9;22) there remains substantial heterogeneity in disease progression and outcome to therapies among patients. Although immunophenotyping has been used to define AML and ALL, few

studies have investigated the use of immunophenotyping to define subgroups in CML. Notably, immunological phenotyping has been used to identify the origin of blast cells from blast crisis (BC) CML patients [155]. This was used to classify blasts which cannot be determined by conventional morphology and cytochemistry, thereby allowing classification of BC as lymphoid, myeloid or megakaryoblastic. It has been reported that patients with lymphoid BC exhibit increased survival rates, with a median of 48.3 weeks compared to 15.5 weeks for non-lymphoid BC ($p<0.025$). More recently, Hirose *et al* [156] investigated lineage markers on MNC isolated from BC-CML patients and found that surface expression of the T and NK-lymphocyte marker CD7 was seen in the majority of myeloid blast crisis cases and this resulted in poorer outcomes to chemotherapy. The median survival duration in CD7+ BC patients was found to be 2.6 months in comparison to 11.8 months for CD7- patients ($p=0.0046$).

For CP-CML, Normann *et al* [157] examined difference in lineage markers between BM CD34+ cells from CML patients and normal individuals. They found increased CD7, CD33, CD61 and CD71 expression and reduced CD133, CD10, CD19, CD62L expression in CML patients compared to normal. Again, CD7 was found to be associated with a poor prognosis. 0% of patients with low CD7 expression exhibited disease progression, whereas 70% of patients with high CD7 expression showed some sign of disease progression. However, these studies were performed on both treated (hydroxyurea) and untreated patients. Most recently, Yong *et al* [143] investigated differences between CP-CML patients who developed aggressive versus indolent forms of disease as defined by the length of time in CP before progression to BC. It was found that high surface expression of CD7 on primitive CD34+ cells was associated with an aggressive form of disease and poorer outcomes on hydroxyurea or interferon-α treatment ($p=0.032$).

Therefore, all previous lineage studies in CML have been performed in the setting of blast crisis or only on primitive CD34+ cells and not in relation to imatinib therapy. Prognostic evaluation

using CD34+ cells is not ideal due to the difficulties in isolating adequate cell numbers. As such, prognostic measurements using whole blood or MNC would be preferable and would enable easy clinical translation. As it has been demonstrated, variations in the functional activity of OCT-1 in the MNC predicts for a patients response to imatinib therapy [110, 111]. Data presented in Chapter 3 showed that OCT-1 activity varies greatly between cell lineages and is not affected by BCR-ABL expression. Therefore, it is possible that the specific cell composition within individual patients blood may underlie their specific OCT-1 activity and hence their response to imatinib therapy. Chapter 3 also showed that the MNC compartment in CML patients at diagnosis is predominantly comprised of immature and mature granulocytes, as shown by the surface expression of CD15 and CD16.

CD15 antibodies recognize a 3-fucosyl-N-acetyllactosamine carbohydrate which is involved in phagocytosis, bactericidal activity and chemotaxis. The CD15 antigen is expressed on most granulocytes including neutrophils and eosinophils, but not on basophils or lymphocytes [158-160]. CD16 antibodies recognize the IgG Fc receptor III which has been reported to be involved in signal transduction and NK cell activation. The CD16 antigen is expressed on NK cells, neutrophils and macrophages [161, 162].

This chapter presents studies which investigate the variations in the expression of the granulocytic surface markers CD15 and CD16 in the MNC population. The OCT-1 expression and function is examined in these CD15 and CD16 expressing populations and the expression of these markers are related to patients MNC OCT-1 activity.

4.1.1 Approach

MNC were isolated from the PB of CP-CML patients at diagnosis using lymphoprep density gradient separation (section 2.3.1). The expression of the granulocytic surface markers CD15 and CD16 were assessed using FITC and PE conjugated antibodies respectively and

measured using flow cytometry (section 2.3.5). CD15+16+, CD15+16- and CD15-16- populations were isolated from the MNC using either magnetic cell sorting (MACS; section 2.3.3) or fluorescence activated cell sorting (FACS; section 2.3.4) dependent upon the availability of the FACS Aria cell sorter. Morphological assessment of isolated populations was performed on Wrights stained cytospins. The intracellular uptake and retention of 2µM imatinib (IUR) and OCT-1 activity were measured in all isolated populations using [^{14}C]-labelled imatinib and the OCT-1 inhibitor prazosin, and are expressed as ng of imatinib per 200,000 cells (section 2.4.1). In addition, mRNA expression of OCT-1, ABCB1 and BCR-ABL were measured in all populations using RQ-PCR and expressed as a % of BCR expression (section 2.4.2). This analysis was used to determine whether OCT-1 activity varied depending on these two surface markers and if the percentages of cells expressing these markers correlated with OCT-1 activity. All assays performed on each individual CML patient are shown in Table 4.1.

Table 4.1: Assays performed on CML patients at diagnosis

Patient Number	Fresh MNC						CD15+16+						CD15+16-						CD15-16-						Frozen MNC	
	M	I	OA	O	A	B	M	I	OA	O	A	B	M	I	OA	O	A	B	M	I	OA	O	A	B	I	OA
2		■	■																							■
3		■	■																							■
4		■	■																							■
5		■	■																							
6		■	■																						■	■
7		■	■																						■	■
8		■	■																						■	■
9		■	■																						■	■
10		■	■																						■	■
11	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
12	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
13	■	■	■	■	■	■	■		■	■		■	■		■	■		■	■		■					
14	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■					
15	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
16	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		
17			■																							■
18			■																							■
19			■																							■
20			■																							■

M = morphology, I = immunophenotyping, OA = IUR and OCT-1 Activity, O = OCT-1 mRNA, A = ABCB1 mRNA, B = BCRABL mRNA

■ Assay performed

Table 4.1 continued

Patient Number	Fresh MNC						CD15+16+						CD15+16-						CD15-16-						Frozen MNC	
	M	I	OA	O	A	B	M	I	OA	O	A	B	M	I	OA	O	A	B	M	I	OA	O	A	B	I	OA
21			■																							■
22			■																							■
23			■																							■
24			■																							■
25			■																							■
26			■																							■
27			■																							■
28			■																							■
29			■																							■
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31			■																							■
32			■																							■
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34			■																							■
35			■																							■
36			■																							■
37			■																							■
38			■																							■
39			■																							■

M = morphology, I = immunophenotyping, OA = IUR and OCT-1 Activity, O = OCT-1 mRNA, A = ABCB1 mRNA, B = BCRABL mRNA

■ Assay performed

4.2 Results

4.2.1 Expression of CD15 and CD16 on MNC from CML patients

As described in Chapter 3 the MNC compartment in CML patients at diagnosis consists predominantly of granulocytes at various stages of maturation. This was assessed using antibodies to the CD15 and CD16 cell surface antigens. CD15 is a marker expressed on all granulocytes at different stages of differentiation and CD16 is expressed on mature neutrophils. Using dual colour immunofluorescence and flow cytometry the MNC in CML patients fall into three quadrants: CD15+16+, CD15+16- and CD15-16-. The percentages of cells in each quadrant varies substantially from patient to patient, with an average of 27% CD15+16+ cells (range 3-64%), 44% CD15+16- cells (range 16-69%) and 28% CD15-16- cells (range 8-80%). Figure 4.1 demonstrates this variability in CD15/CD16 expression in 6 different CML patients MNC.

The cells present in each of these quadrants were isolated from fresh samples recovered from 6 CML patients using either a MACS (n=4) or FACS (n=2) protocol. As seen in Figure 4.2 the CD15+16+ population is a highly homogenous population of mature band-form and segmented neutrophils. In contrast, the CD15+16- populations is a heterogeneous population of mature and immature neutrophils, with the presence of myelocytes, metamyelocytes and even promyelocytes (Figure 4.3). Similarly, the CD15-16- population is heterogeneous and comprised of lymphocytes, monocytes, eosinophils, blasts and some red blood cells (Figure 4.4).

4.2.2 Intracellular uptake and retention (IUR) and OCT-1 activity in CD15/CD16 isolated populations in CML patients

The IUR of 2µM imatinib was performed in each of these populations and the total MNC population. MNC and CD15+16- cells were found to have the highest IUR compared with CD15+16+ and CD15-16- cells (mean IUR MNC: 33, CD15+16+: 24, CD15+16-: 36, CD15-16-:

Figure 4.1 Surface expression of CD15 and CD16 in MNC from diagnosis CML patients

MNC were isolated from the PB of diagnosis CML patients. CD15 and CD16 antibodies were used to assess surface marker expression. Expression falls into 3 quadrants: CD15+16+, CD15+16- and CD15-16-. FACS plots show the variability in expression between six CML patients.

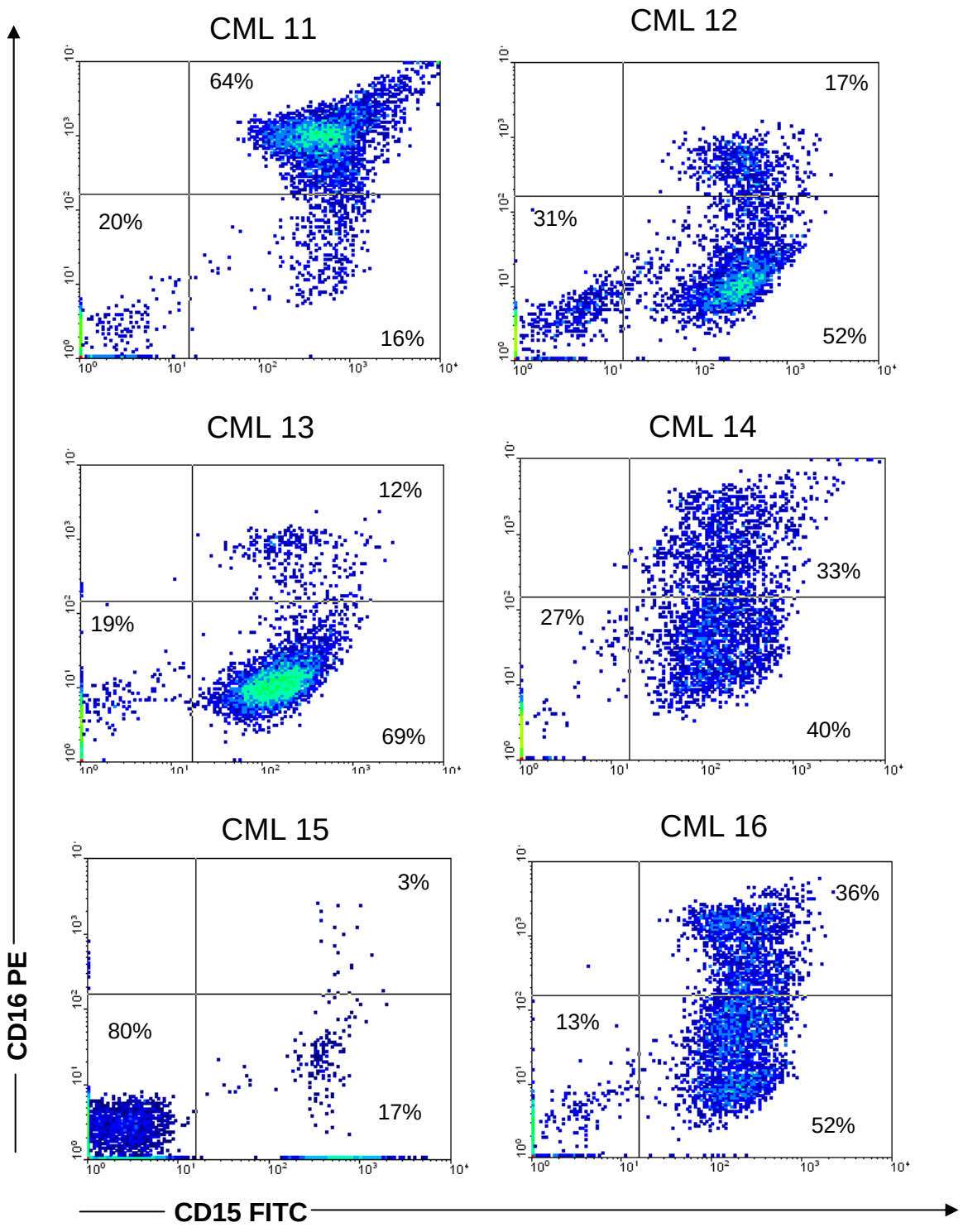
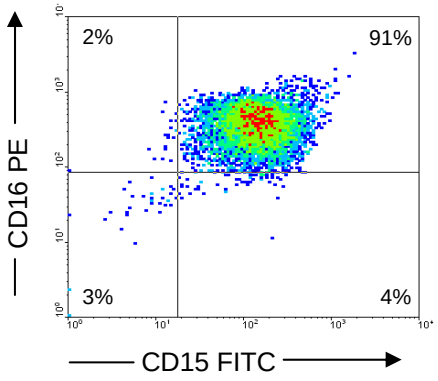

CML 11
64%
20%
16%
CML 12
17%
31%
52%
CML 13
12%
19%
69%
CML 14
33%
27%
40%
CML 15
3%
80%
17%
CML 16
36%
13%
52%
CD16 PE
CD15 FITC

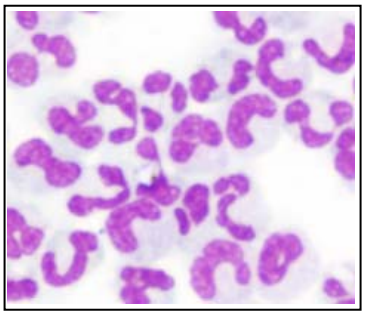
Figure 4.2 Morphology and of isolated CD15+16+ population

MNC were isolated from the PB of diagnosis CML patients. CD15+16+ cells were isolated from the MNC of six patients using either a MACS (n=4) or FACS (n=2) protocol. Cytospin preparations of populations were stained with Wrights for morphological analysis. The figure shows a representative FACS plot of the isolated population from patient #12. Representative photos of the CD15+16+ population is shown from all 6 patients. The CD15+16+ cells consist mainly of mature neutrophils.

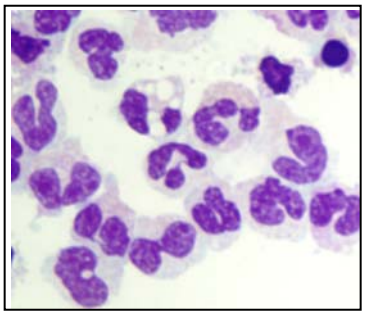
CD15+16+



CML 11



CML 12



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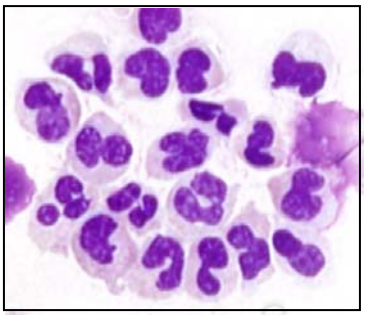

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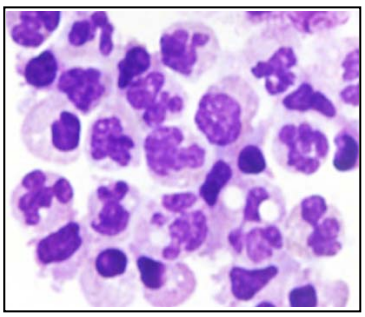
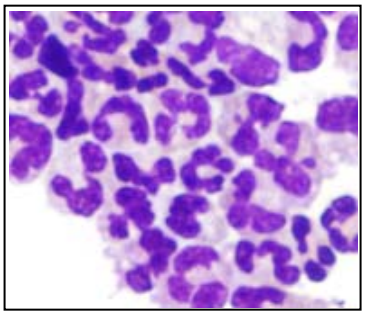


CML 15



CML 16

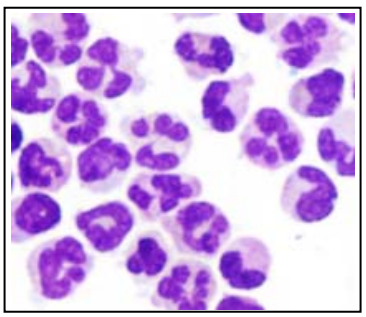
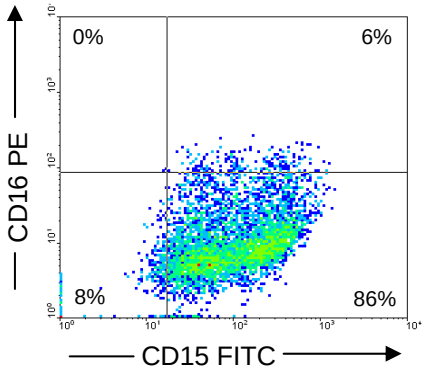


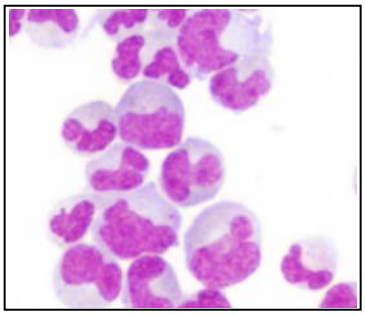
Figure 4.3 Morphology and of isolated CD15+16- population

MNC were isolated from the PB of diagnosis CML patients. CD15+16- cells were isolated from the MNC of six patients using either a MACS (n=4) or FACS (n=2) protocol. Cytospin preparations of populations were stained with Wrights for morphological analysis. The figure shows a representative FACS plot of the isolated population from patient #12. Representative photos of the CD15+16- population is shown from all 6 patients. The CD15+16- cells is a heterogeneous population containing a mix of myelocytes, metamyelocytes and neutrophils.

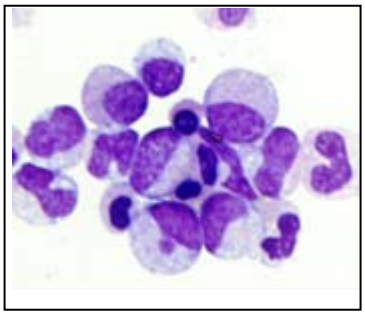
CD15+16-



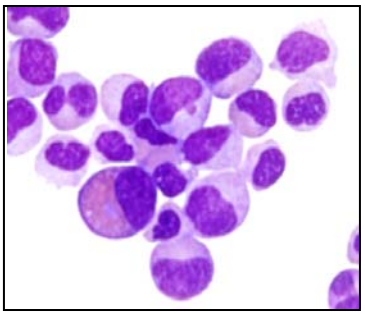
CML 11



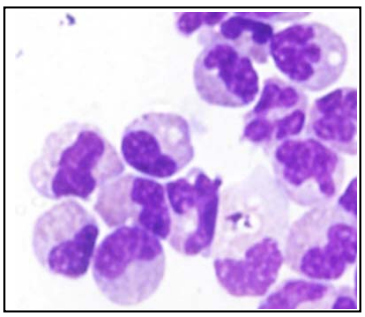
CML 12



CML 13



CML 14



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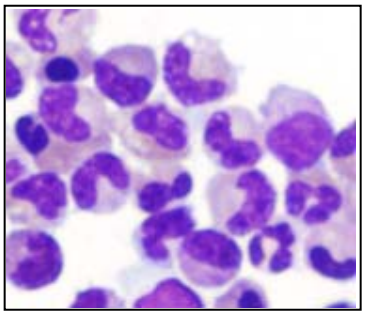

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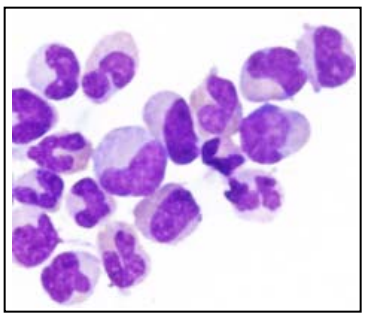
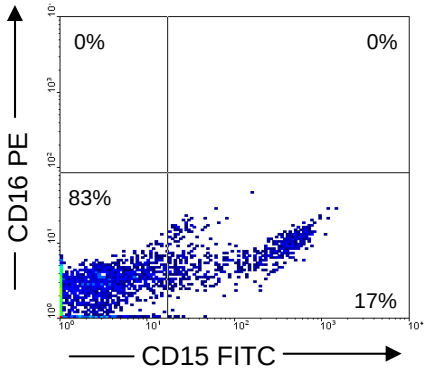


Figure 4.4 Morphology and of isolated CD15-16- population

MNC were isolated from the PB of diagnosis CML patients. CD15-16- cells were isolated from the MNC of six patients using either a MACS (n=4) or FACS (n=2) protocol. Cytospin preparations of populations were stained with Wrights for morphological analysis. The figure shows a representative FACS plot of the isolated population from patient #12. Representative photos of the CD15-16- population is shown from all 6 patients. The CD15-16- cells is a heterogeneous population containing a mix of lymphocytes, monocytes, blasts and red blood cells.

CD15-16-



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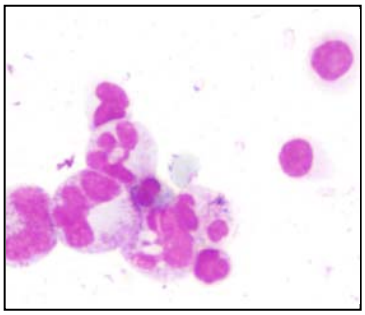

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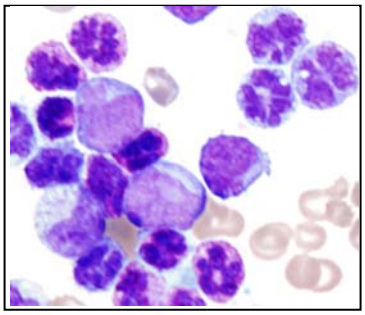
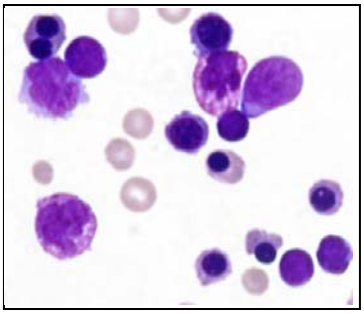


CML 13



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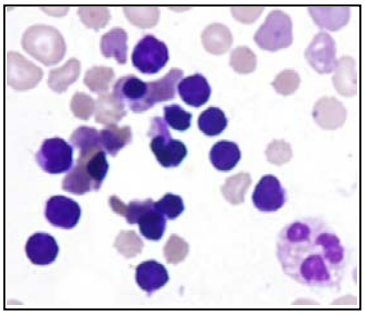
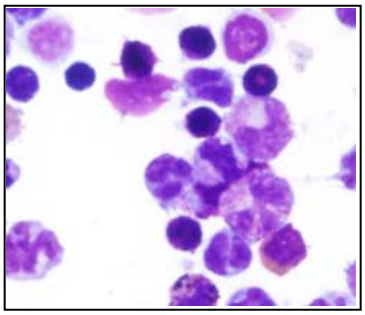
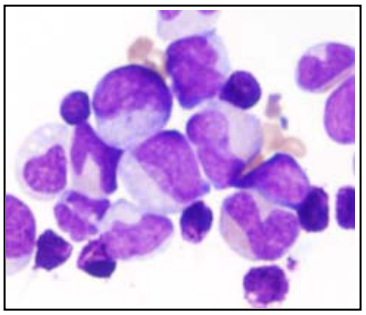


CML 15



CML 16



21 ng/200,000 cells, Figure 4.5A). The OCT-1 activity was also found to be highest in the MNC and CD15+16- populations and lowest in the CD15-16- population (mean OCT-1 activity MNC: 16, CD15+16+: 9, CD15+16-: 17, CD15-16-: 4 ng/200,000 cells, Figure 4.5B). Of note, the MNC OCT-1 activity most closely mimicked the "immature" CD15+16- population.

4.2.3 OCT-1, ABCB1 and BCR-ABL mRNA expression in CD15/CD16 isolated populations in CML patients

OCT-1 mRNA expression was measured in the CD15/CD16 selected populations. In contrast to OCT-1 activity, the highest OCT-1 mRNA expression was seen in the "mature" CD15+16+ population and was significantly greater than all other groups ($p<0.05$). The lowest OCT-1 mRNA expression was seen in the CD15+16- and CD15-16- populations. The mean OCT-1 expression for MNC, CD15+16+, CD15+16- and CD15-16- populations were 12.5%, 62.2%, 2% and 2%, respectively (Figure 4.6A). This data is in agreement with that presented in Chapter 3 where no direct correlation between OCT-1 mRNA expression and OCT-1 activity was observed. Measurement of the ABCB1 efflux mRNA expression showed that the "immature" CD15+16- population possessed very low expression of ABCB1 in comparison to all other groups ($p<0.05$). The mean expression for MNC, CD15+16+, CD15+16- and CD15-16- populations were 28%, 14%, 1% and 40%, respectively (Figure 4.6B). Finally, the level of BCR-ABL mRNA expression was similar between MNC, CD15+16+ and CD15+16- groups (87%, 97% and 94% respectively, see Figure 4.6C). The BCR-ABL mRNA expression was low in the CD15-16- population (average 38%), which is likely to be a reflection of the predominance of lymphocytes in this group.

4.2.4 Relationship between CD15/CD16 expression and MNC OCT-1 activity

A significant relationship between MNC OCT-1 activity and the OCT-1 activity in the mature CD15+16+ neutrophil population was reported in Chapter 3. The patients with high MNC OCT-1 activity also exhibited high neutrophil OCT-1 activity. In addition, it is demonstrated in Figure 4.1 that the percentage of cells in each CD15/CD16 quadrant is highly variable between

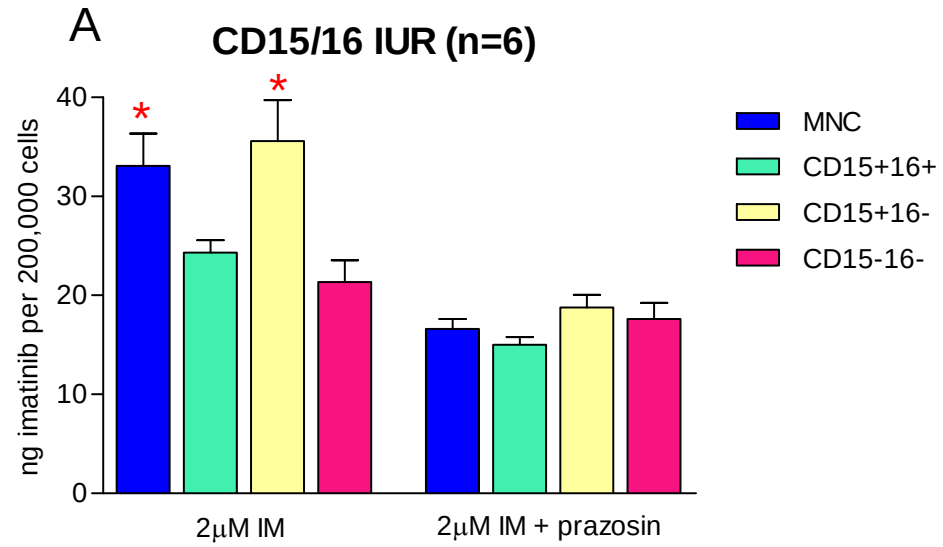
Figure 4.5 Intracellular uptake of imatinib and OCT-1 activity in diagnosis CML CD15/CD16 cell populations

MNC, CD15+16+, CD15+16- and CD15-16- cells were isolated from the PB of six diagnosis CML patients. The IUR of 2μM imatinib (A) and OCT-1 activity (B) was measured.

Columns represent the mean plus standard error of the mean.

* denotes $p < 0.05$ in comparison to CD15-16-

(A) p=0.014, p=0.013 (B) p=0.002, p=0.02, p=0.008


A
CD15/16 IUR (n=6)
ng imatinib per 200,000 cells
40
30
20
10
0
*
*
2μM IM
2μM IM + prazosin
MNC
CD15+16+
CD15+16-
CD15-16-

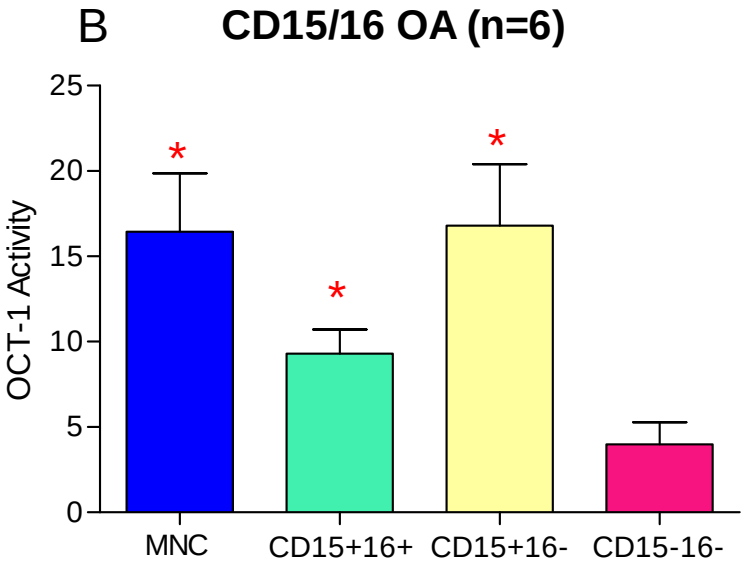

B
CD15/16 OA (n=6)
OCT-1 Activity
25
20
15
10
5
0
*
*
*
MNC
CD15+16+
CD15+16-
CD15-16-

Figure 4.6 OCT-1, ABCB1 and BCR-ABL mRNA expression in diagnosis CML CD15/CD16 cell populations

MNC, CD15+16+, CD15+16- and CD15-16- cells were isolated from the PB of six diagnosis CML patients. The mRNA of OCT-1 (A), ABCB1 (B) and BCR-ABL (C) were measured in all populations.

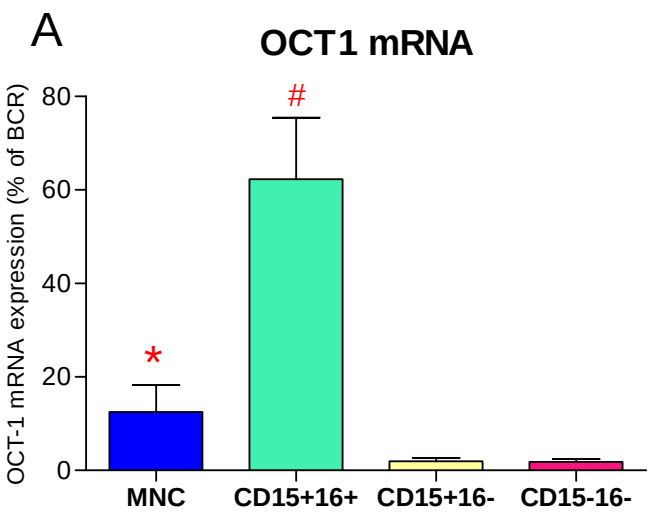
Columns represent the mean plus standard error of the mean.

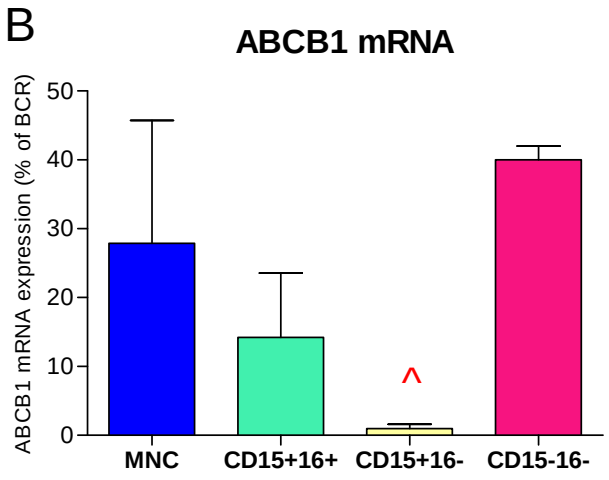
* denotes $p<0.05$ in comparison to CD15+16- and CD15-16- ($p=0.009$ & $p=0.010$)

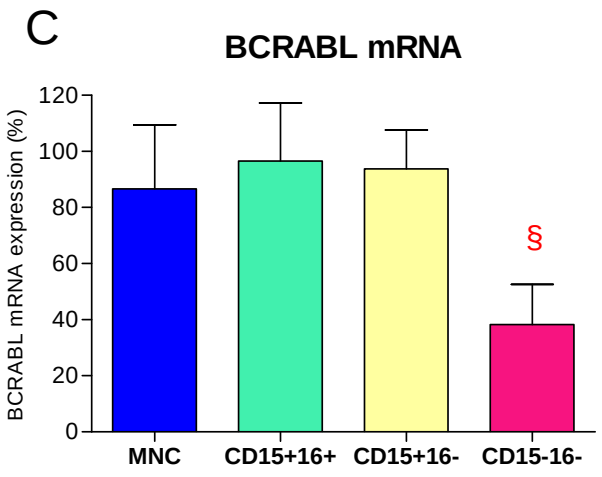
# denotes $p<0.05$ in comparison to MNC, CD15+16- and CD15-16- ($p=0.006$, $p=0.002$ & $p=0.010$)

^ denotes $p<0.05$ in comparison to MNC, CD15+16+ and CD15-16- ($p=0.009$, $p=0.016$ & $p=0.016$)

§ denotes $p<0.05$ in comparison to CD15+16- ($p=0.028$)


A
OCT1 mRNA
OCT-1 mRNA expression (% of BCR)
80
60
40
20
0
*
#
MNC
CD15+16+
CD15+16-
CD15-16-


B
ABCB1 mRNA
ABCB1 mRNA expression (% of BCR)
50
40
30
20
10
0
^
MNC
CD15+16+
CD15+16-
CD15-16-


C
BCRABL mRNA
BCRABL mRNA expression (%)
120
100
80
60
40
20
0
§
MNC
CD15+16+
CD15+16-
CD15-16-

patients and the OCT-1 activity in the "immature" CD15+16- population most closely matches that of MNC (Figure 4.5). As such, it was then examined whether the percentage of CD15+16+, CD15+16- or CD15-16- cells in a patient's MNC compartment was related with their MNC OCT-1 activity. As shown in Figure 4.7, in the 15 patients who were assessed, there was no significant correlation between their MNC OCT-1 activity and the percentage of cells in the CD15+16+ quadrant (r=-0.350, p=0.20), CD15+16- quadrant (r=0.074, p=0.793) or the CD15-16- quadrant (r=0.257, p=0.356).

4.2.5 OCT-1 activity in fresh and thawed MNC

The studies presented in Chapters 3 and 4 were conducted on cells freshly isolated from the PB of CML patients, within a day or two of collection. This was essential as attempting to isolate populations of cells from thawed MNC resulted in poor yields and purity. However, the original studies that identified MNC OCT-1 activity as a strong predictor of response to imatinib were conducted on thawed MNC following a period of cryopreservation in liquid nitrogen. This raises the possibility that the thawing process alters the cellular composition of the MNC compartment, resulting in the selective or preferential loss of one population. Therefore, in 31 CP-CML patients, the OCT-1 activity was measured in fresh MNC and thawed MNC. Using the Spearman Rank Order no correlation was observed between the OCT-1 activity measured in fresh and thawed MNC (r=0.049, p=0.791, Figure 4.8). The mean OCT-1 activity measured in MNC following thawing was 4.0 ng/200,000 cells (range 0-8.8 ng/200,000 cells) and was significantly lower than MNC pre-freezing of 12 ng/200,000 cells (range: 1.25-35.5 ng/200,000 cells, $p<0.001$, Figure 4.8B). Therefore, the freezing and thawing process appears to significantly lower the OCT-1 activity in these cells, potentially by changing the cell composition.

4.2.6 CD15/CD16 expression in fresh and thawed MNC

In 7 CP-CML patients the MNC compartment was assessed for cell surface expression of CD15, CD16, CD14 and CD3 before and after freeze/thawing. In this cohort of 7 patients, the

Figure 4.7 Relationship between CD15/CD16 expression and OCT-1 activity

Surface expression of CD15 and CD16 was assessed in the MNC of 15 diagnosis CML patients. Percentages of cells in the three quadrants: CD15+16+ (A), CD15+16- (B) and CD15-16- (C) were correlated with patients MNC OCT-1 activity. No significant correlation was observed between OCT-1 activity and surface expression of CD15 and CD16.

The pearson product moment was used to assess the correlation.

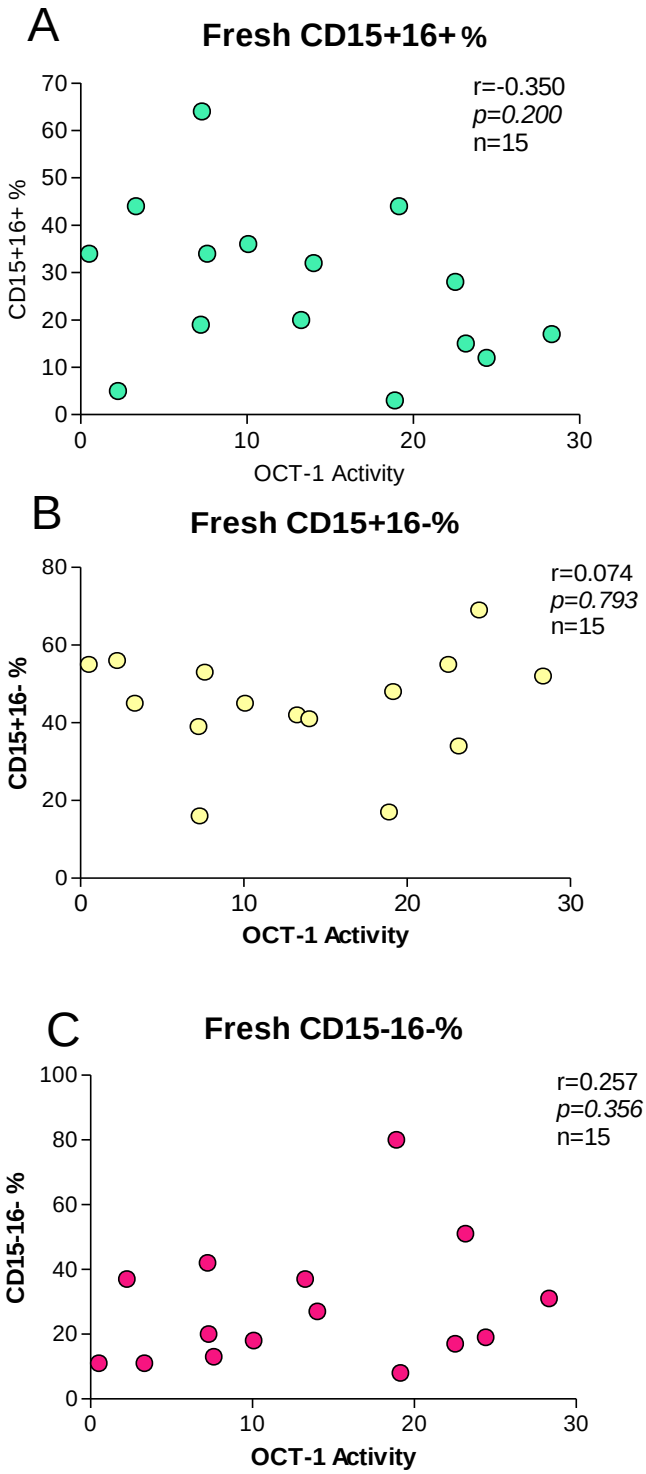
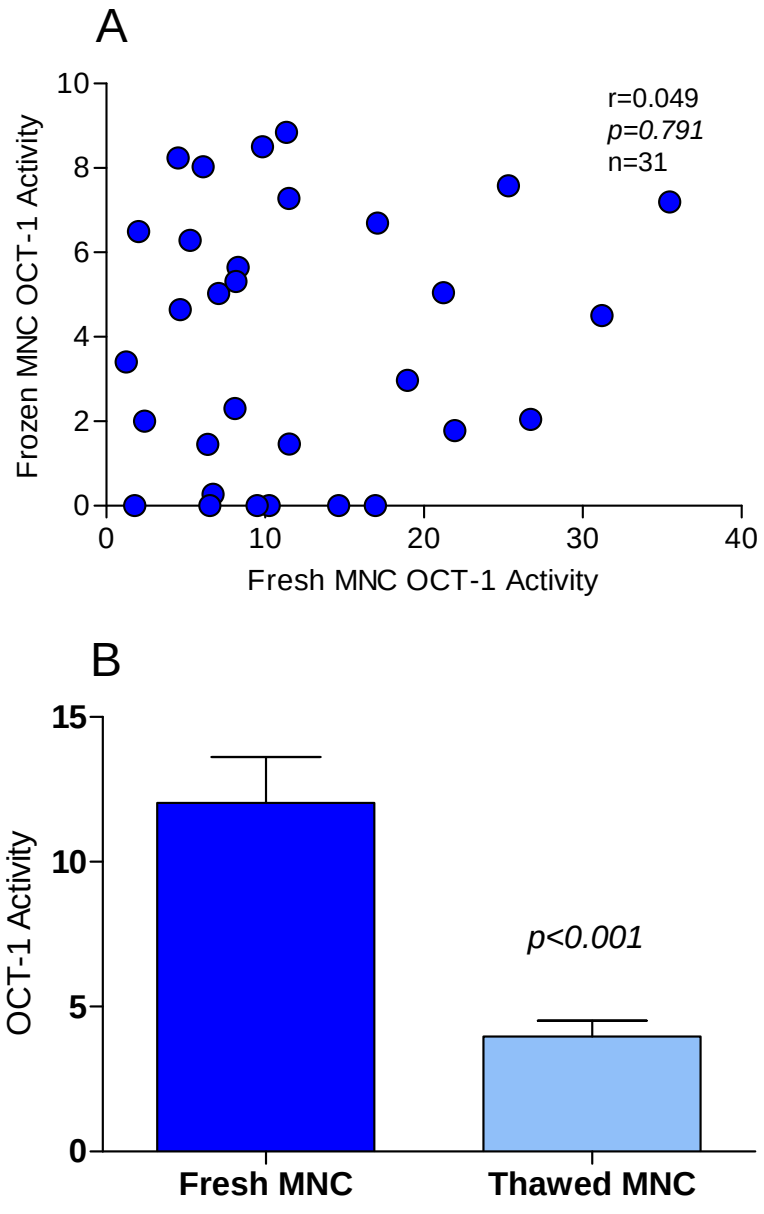

A
Fresh CD15+16+%
r=-0.350
p=0.200
n=15
CD15+16+ %
OCT-1 Activity
B
Fresh CD15+16-%
r=0.074
p=0.793
n=15
CD15+16- %
OCT-1 Activity
C
Fresh CD15-16-%
r=0.257
p=0.356
n=15
CD15-16- %
OCT-1 Activity

Figure 4.8 Comparison of fresh and thawed MNC OCT-1 activity

MNC were isolated from the PB of 31 diagnosis CML patients. The OCT-1 activity was measured on freshly isolated cells and on cells after a freeze/thaw process. (A) Demonstrates a lack of association between the OCT-1 activities measured at these two times. (B) Demonstrates the significant reduction in OCT-1 activity following the freeze/thaw process.

The spearman rank order was used to assess the correlation.

Columns represent the mean plus standard error of the mean.


A
r=0.049
p=0.791
n=31
Frozen MNC OCT-1 Activity
Fresh MNC OCT-1 Activity
0
2
4
6
8
10
0
10
20
30
40
B
OCT-1 Activity
0
5
10
15
p<0.001
Fresh MNC
Thawed MNC

average fresh OCT-1 activity was 9.7 ng/200,000 cells. In contrast in these samples post freeze/thawing the average OCT-1 activity was reduced to 4.8 ng/200,000 cells (p=0.236, Figure 4.9A). No differences were observed between fresh and thawed MNC with regard to the percentages of mature CD15+16+ cells (average 32% and 34%, respectively, Figure 4.9B). Notably, a significant reduction in the number of immature CD15+16- cells from an average of 44% to 28% was seen after thawing (p=0.017). In addition, increases, though not significant, were seen in thawed compared to fresh MNC in the number of CD15-16- cells (average 23% to 35%, p=0.383), CD14+ cells (average 5% to 8.5%, p=0.306) and CD3+ cells (average 3% to 5%, p=0.096). The individual immunological and OCT-1 activity changes for each of these 7 patients are shown in Table 4.2. It is therefore a possibility that these compositional changes combined may be causing the reduced OCT-1 activity in the thawed MNC.

4.2.7 Relationship between CD15/CD16 expression and thawed MNC OCT-1 activity

Given the changes in the composition and OCT-1 activity in the MNC population after freeze/thaw, the relationship between CD15/CD16 expression and OCT-1 activity was assessed in the thawed setting. In 7 patients, CD15+16+, CD15+16- and CD15-16- percentages were assessed in thawed MNC and compared with their OCT-1 activity. Using the Pearson Product Moment, no correlation was observed between CD15+16+% or CD15-16- % and OCT-1 activity (r=-0.240, p=0.605 and r=0.044, p=0.926, respectively, Figure 4.10A and C). However, a significant correlation was found between CD15+16-% and OCT-1 activity (r=0.848, p=0.016, Figure 4.10B). Therefore, the number of immature myelocytes and metamyelocytes in a patient's MNC after thawing is significantly associated with their OCT-1 activity. Therefore, patients with a high OCT-1 activity have greater numbers of these immature cells.

Figure 4.9 Comparison of fresh and thawed MNC immunophenotype

MNC were isolated from the PB of 7 diagnosis CML patients. The OCT-1 activity and immunophenotype was measured on freshly isolated cells and on cells after a freeze/thaw process. The reduction in OCT-1 activity (A) and the alterations in immunophenotype (B) following the freeze/thaw process is shown.

Columns represent the mean plus standard error of the mean.

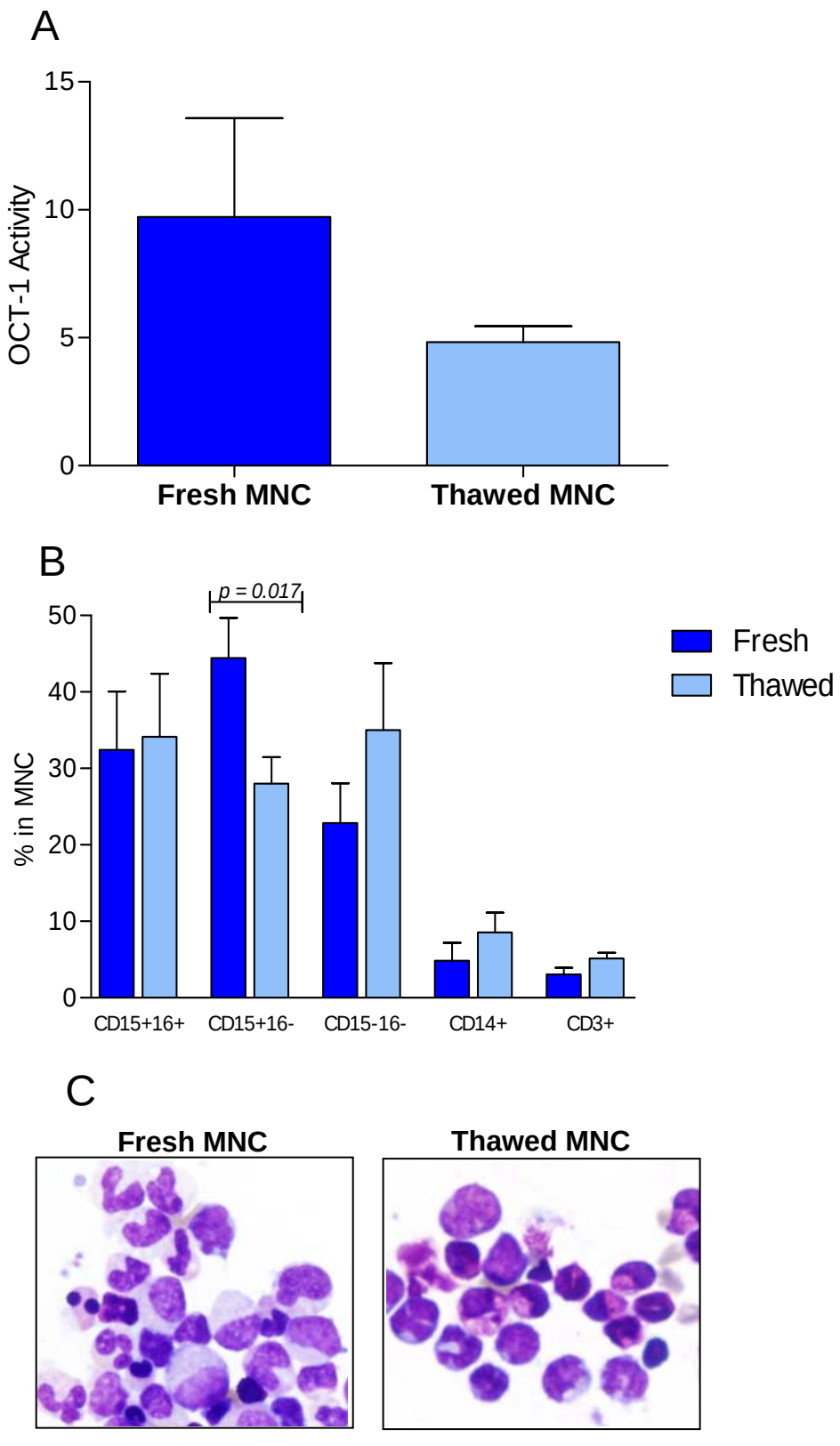

A
15
10
5
0
OCT-1 Activity
Fresh MNC
Thawed MNC
B
p = 0.017
50
40
30
20
10
0
% in MNC
Fresh
Thawed
CD15+16+
CD15+16-
CD15-16-
CD14+
CD3+
C
Fresh MNC
Thawed MNC

Table 4.2: OCT-1 activity and immunophenotype of fresh and thawed MNC

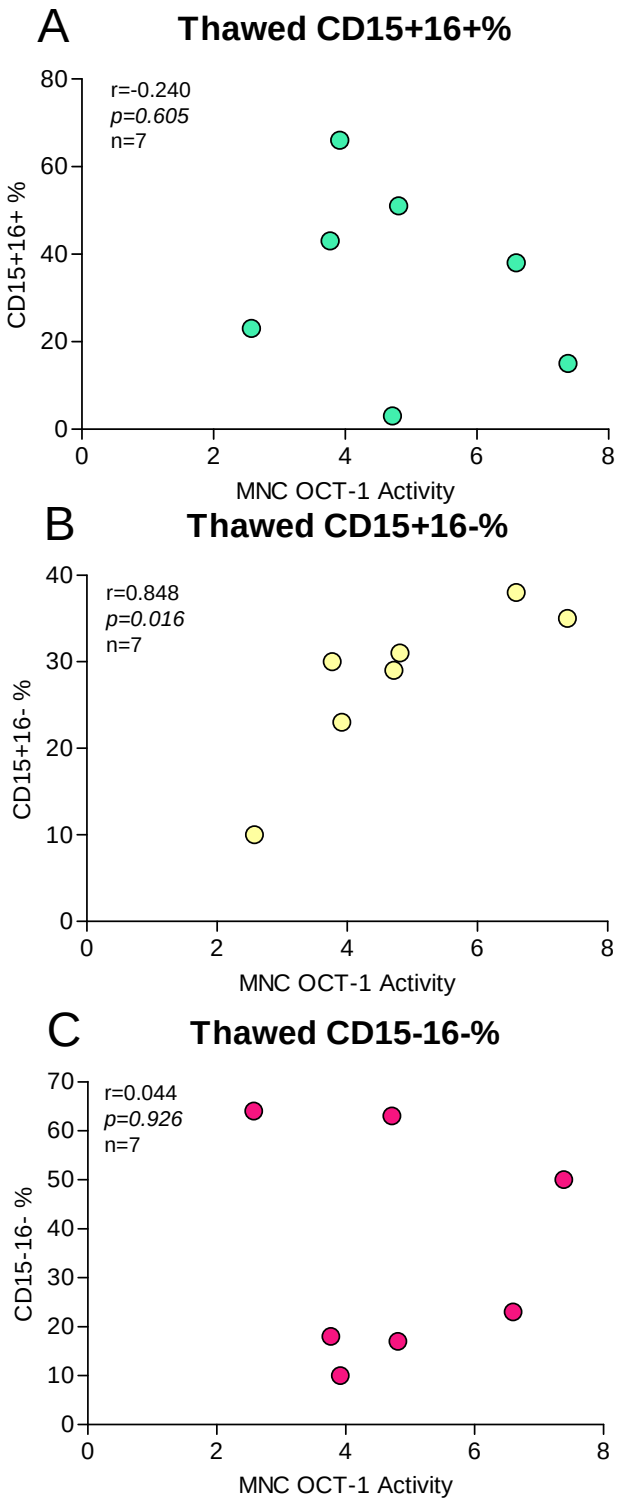
Patient Number	MNC Fresh						MNC Thawed					
	OA	CD15+16+	CD15+16-	CD15-16-	CD14+	CD3+	OA	CD15+16+	CD15+16-	CD15-16-	CD14+	CD3+
6	0.5	34%	55%	11%	4%	3%	2.6	23%	10%	64%	5%	7%
7	19.1	44%	48%	8%	3%	1.5%	4.8	51%	31%	17%	6%	4%
8	7.2	19%	39%	42%	15%	3%	6.6	38%	38%	23%	21%	3%
9	3.3	44%	45%	11%	0%	0%	3.8	43%	30%	18%	5%	6%
10	2.2	5%	56%	37%	12%	7%	7.4	15%	35%	50%	11%	8%
11	7.3	64%	16%	20%	0%	2%	3.9	66%	23%	10%	0%	3%
12	28.3	17%	52%	31%	0%	5%	4.7	3%	29%	63%	12%	5%

OA = OCT-1 activity

Figure 4.10 Relationship between CD15/CD16 expression and OCT-1 activity in thawed MNC

Surface expression of CD15 and CD16 was assessed in the MNC of 7 diagnosis CML patients following a freeze/thaw process. Percentages of cells in the three quadrants: CD15+16+ (A), CD15+16- (B) and CD15-16- (C) were correlated with patients MNC OCT-1 activity. A significant positive correlation was found between percentage of CD15+16- cells and OCT-1 activity.

The pearson product moment was used to assess the correlation.


A
Thawed CD15+16+%
r=-0.240
p=0.605
n=7
CD15+16+ %
MNC OCT-1 Activity
B
Thawed CD15+16-%
r=0.848
p=0.016
n=7
CD15+16- %
MNC OCT-1 Activity
C
Thawed CD15-16-%
r=0.044
p=0.926
n=7
CD15-16- %
MNC OCT-1 Activity

4.3 Discussion

While immunological phenotyping has been a strong part of diagnosis and prognosis in acute leukaemia's minimal research has been conducted in the setting of CML. The few studies that have been conducted have identified an association between the expression of T and NK-lymphocyte marker CD7 and poorer outcomes to therapy. However, these studies were performed on blast crisis patients receiving chemotherapy [155, 156] or chronic phase patients receiving hydroxyurea or interferon-α [143, 157]. While imatinib treatment has produced responses which far exceed those achieved by hydroxyurea or interferon- α, there is still variable responses to imatinib and as such prognostic indicators of response are still required. MNC OCT-1 activity is one such prognostic indicator, which identifies patients who will achieve favourable or poor molecular responses to imatinib [110, 111]. As described in Chapter 3 OCT-1 activity is strongly related to cell lineage and the MNC OCT-1 activity is significantly correlated with neutrophil OCT-1 activity. The present chapter therefore examined the expression of the granulocytic surface markers CD15 and CD16 in CP-CML patients at diagnosis and its relationship to MNC OCT-1 activity as an indicator of response.

The findings from this chapter show that the expression of CD15 and CD16 is highly variable between patients. Furthermore, with 2-colour flow cytometry, the MNC population falls into three categories: CD15+16+, CD15+16- and CD15-16-. The double positive CD15+16+ cells are found to be a homogeneous population comprised of mature neutrophils which have a reduced OCT-1 activity in comparison to MNC. The CD15+16- population is a heterogeneous population of immature myelocytes, metamyelocytes and promyelocytes and the OCT-1 activity in these cells closely matches that seen in MNC. The CD15-16- population is a heterogeneous population of residual cells including lymphocytes, monocytes, blasts and red blood cells with a low OCT-1 activity.

When the OCT-1 activity and expression of CD15/16 was examined in freshly isolated MNC, no relationship was seen between the percentages of cells in the CD15+16+, CD15+16- or CD15-16- populations and the MNC OCT-1 activity. However, the predictive value of MNC OCT-1 activity was established in MNC after a freeze/thaw process [110]. This process was found to significantly reduce the OCT-1 activity in a patient's MNC and the number of "immature" CD15+16- cells in the MNC compartment. It is also possible given that the CD15+16- population is a heterogeneous group of cells, composition of this population may have changed following thawing. However, this was not assessed due to the technical difficulties in isolating pure populations post-thaw. Due to these changes in MNC following thawing the relationship between CD15/16 expression and OCT-1 activity was examined in thawed MNC. Despite only being measured in 7 patients, a significant positive correlation was seen between the percentage of CD15+16- cells and OCT-1 activity. Therefore, the predictive value of OCT-1 activity may reflect the number of immature myelocytes, metamyelocytes and promyelocytes in a patient's blood. These findings should however be interpreted cautiously and further studies are clearly required using a much larger cohort.

Only one previous study has investigated the presence of immature cells in CML patients in relation to OCT-1 [147]. The study by Racil *et al* measured the presence of immature myelocytes, metamyelocytes and promyelocytes by morphological analysis in the total WBC recovered from the PB of CML patients at diagnosis. They identified a significant negative relationship between the percentage of immature cells at diagnosis and OCT-1 mRNA expression ($r=-0.35$, $p=0.004$, $n=22$). This is intriguing given that the findings presented in this chapter suggest a significant positive correlation between percentage immature cells and OCT-1 activity. There are a number of factors which may contribute to these conflicting results. Firstly, as demonstrated in the present chapter and Chapter 3 while OCT-1 mRNA is a contributor to OCT-1 activity, there is no direct correlation between the two. Furthermore, the study by Racil *et al* did not demonstrate the prognostic significance of OCT-1 mRNA

expression. Secondly, in the present chapter no association was seen between the percentage of CD15+16- cells and OCT-1 activity in fresh samples but a significant correlation was observed in thawed samples. There is no indication in the paper of Racil *et al* if their analyses were performed on fresh or thawed cells, therefore the relevance of this factor is unknown.

In conclusion, there is a strong possibility that MNC OCT-1 activity (in thawed samples) is a feature reflective of the number of immature cells in a patient's blood. Therefore, simple immunological analysis of CD15 and CD16 may be a more efficient, safer and reliable predictor of response to imatinib therapy. The use of [^{14}C]-labelled imatinib is currently a significant problem in transferring the OCT-1 activity assay to other laboratories, due to safety and availability of reagents and equipment. In comparison, CD15 and CD16 antibodies are commercially available and most diagnostic laboratories have access to flow cytometric analysis. The correlation between the CD15+16- population and OCT-1 activity will require confirmation in a much larger patient cohort and in association with molecular responses to imatinib treatment.

5 THE EFFECT OF BCR-ABL AND IMATINIB ON OCT-1 ACTIVITY

5.1 Introduction

The expression and function of imatinib transporters may be influenced by signalling from BCR-ABL. Using a cell line model of CML, previous studies have shown that BCR-ABL up-regulates ABCG2 protein expression via the PI3-Akt pathway [163]. The ABCG2 over-expressing K562 cell line was found to have reduced surface expression and total protein expression of ABCG2 following treatment with imatinib. However, this effect was not seen in non-CML ABCG2 cell lines treated with imatinib, leading to the conclusion that this effect was due to BCR-ABL kinase inhibition. It was then demonstrated, that treatment with imatinib reduced phosphorylation of Akt in these cells, and this was found to contribute to the reduced ABCG2 protein levels. More recently, Dohse *et al* also showed that treatment with imatinib and other TKI's led to a reduction in ABCG2 surface expression in K562-ABCG2 cells [164]. Furthermore, Chen *et al* investigated the effects of imatinib in a K562 model and found that treatment with imatinib reduced both ABCB1 mRNA and total protein expression [165]. It is therefore possible that changes in BCR-ABL kinase activity and treatment with imatinib may also influence OCT-1 expression in CML cells.

To date, the transcriptional and post-transcriptional regulation of OCT-1 has not been well defined. OCT-1 transcription has been found to be activated by the hepatocyte nuclear factor-4alpha (HNF-4α) and repressed by bile acids in the liver [166]. The activity of OCT-1, with respect to the uptake of the model substrates 4-[4-(dimethylamino)styryl]-N-methylpyridinium (ASP) and tetraethylammonium (TEA), is inhibited by protein kinase A (PKA) and stimulated by the calcium/calmodulin complex and Src-like p56lck in the human embryonic kidney cell line, HEK293 [102, 103]. However, to date, no studies have reported on the regulation of OCT-1 in haematopoietic cells.

Recently, Bazeos *et al* demonstrated that OCT-1 mRNA expression in total WBC from CML patients was reduced compared with normal donors [115]. From this, they concluded that BCR-ABL kinase signalling has an inhibitory effect on OCT-1 expression. Findings presented in Chapter 3 contradict this suggestion as OCT-1 activity and OCT-1 mRNA expression in neutrophils were not found to be significantly different between CML patients and normal donors. However, this alone cannot rule-out the possibility that BCR-ABL may be mediating a direct or indirect effect on OCT-1 expression and/or function. Furthermore, findings from Chapter 3 suggest that long-term imatinib treatment may up-regulate OCT-1 expression and function, as evidenced by increased mRNA and OCT-1 activity in CML remission patients following an average 12 months of imatinib treatment. Therefore, the major aims of the present chapter were to investigate, in a controlled system, the role of 1) BCR-ABL, 2) myeloid differentiation and 3) imatinib treatment on OCT-1 expression and function.

5.1.1 Approach

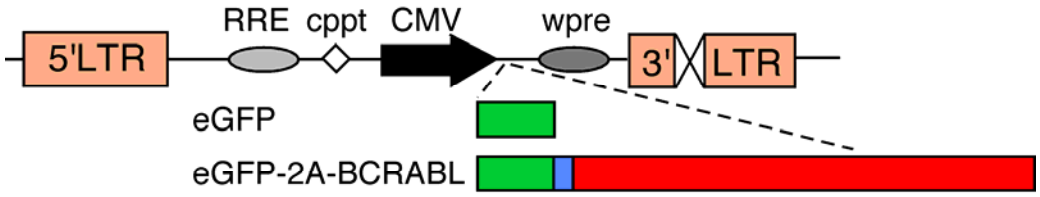
Lentivirus particles comprised of either control vector or BCR-ABL co-expressed with Green Fluorescence Protein (eGFP), were kindly provided by Professor John Rasko and Dr Charles Bailey (Centenary Institute, Sydney, Australia). Vector design and lentiviral production is described in section 2.4.4.1 and a vector map is shown in Figure 5.1A. The promyelocytic HL60 cell line was transduced with lentivirus containing control vector (HL60.CMV.CV) or ^{p210}BCR-ABL (HL60.CMV.BCRABL) and were FACS sorted based on their expression of eGFP (section 2.4.4). HL60.CMV.CV underwent a single round of sorting as shown in Figure 5.1B to obtain a pure population of cells uniformly expressing eGFP. HL60.CMV.BCRABL underwent four rounds of sorting as shown in Figure 5.1B to obtain a pure population of cells. The greater number of sorting rounds with HL60.CMV.BCRABL was due to the reduced transduction efficiency with the BCR-ABL lentiviral particles. The intracellular uptake and retention (IUR) of imatinib, OCT-1 activity (section 2.4.1) and OCT-1 mRNA expression (section 2.4.2) were assessed in these two cell lines under the following conditions: untreated, treated with DMSO

to cause granulocyte differentiation, treated with PMA to cause monocyte differentiation and treated with 2μM imatinib. The studies were designed to identify if BCR-ABL expression, granulocyte differentiation, monocyte differentiation and imatinib treatment influences OCT-1 expression and function.

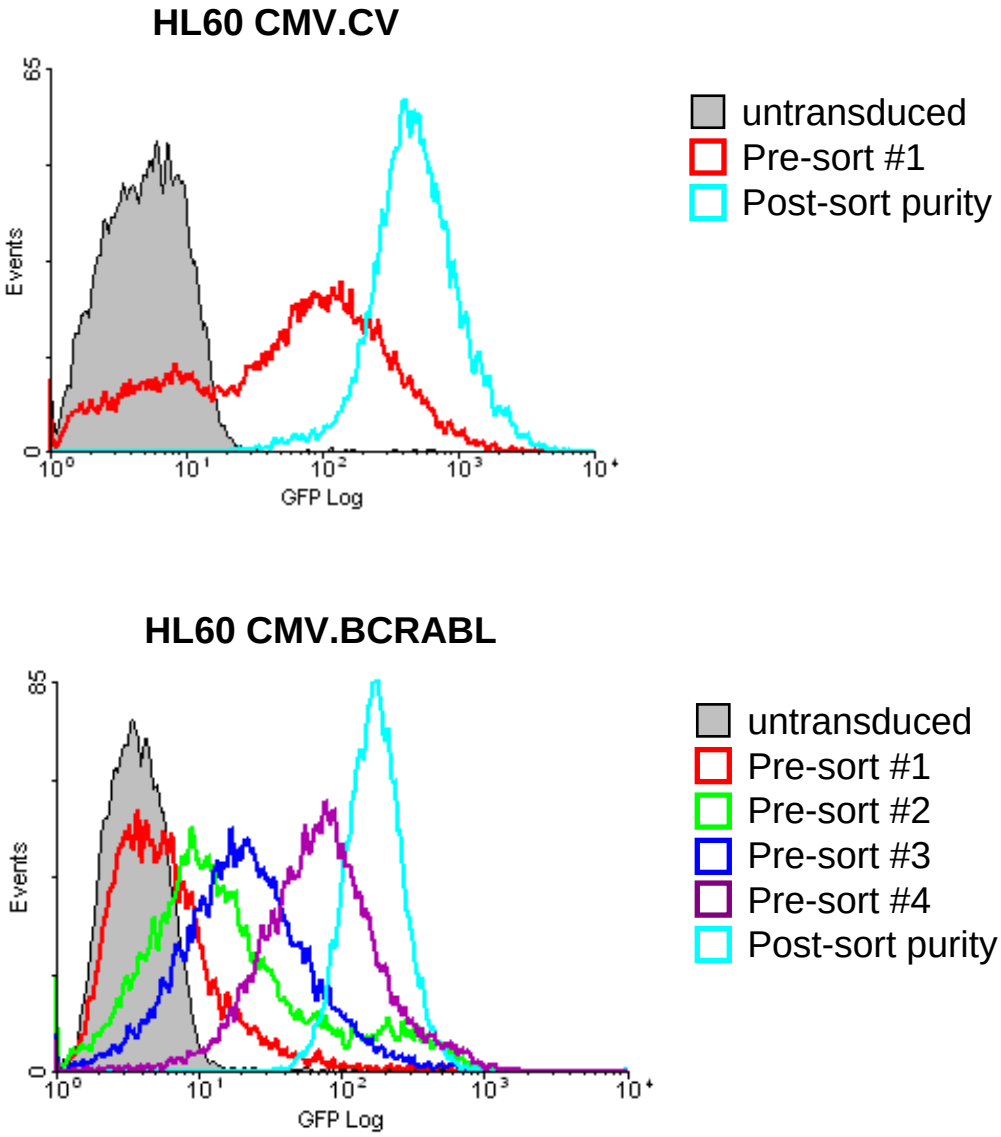
Figure 5.1 Lentiviral vector and FACS sorting of HL60 cells

(A) Schematic of lentiviral vectors. The pHIV-1SDm-derived vector {Koldej, 2005 #268} contains a 5'- and 3' HIV-1 long terminal repeat (LTR); the 3'LTR is self-inactivating. The vector backbone contains a Rev response element (RRE), a central polypurine tract (cppt), a CMV promoter and a woodchuck hepatitis virus posttranscriptional regulatory element (wpre). The eGFP and eGFP-2A-BCRABL cDNAs are cloned into the multi-cloning site. (B) EGFP expression from the lentiviral vectors. HL60 cells were transduced with concentrated lentiviral particles for 3 hours, and then cultured for 4-12 days before analysis by FACS. The control vector cell line only required a single round of sorting to isolate a pure population of eGFP expressing cells (aqua line). The BCR-ABL vector cell line required 4 rounds of sorting to isolate a pure population of eGFP expressing cells (aqua line).

A


RRE
cppt
CMV
wpre
5'LTR
3'
LTR
eGFP
eGFP-2A-BCRABL

B


HL60 CMV.CV
65
Events
0
10⁰
10¹
10²
10³
10⁴
GFP Log
untransduced
Pre-sort #1
Post-sort purity
HL60 CMV.BCRABL
85
Events
0
10⁰
10¹
10²
10³
10⁴
GFP Log
untransduced
Pre-sort #1
Pre-sort #2
Pre-sort #3
Pre-sort #4
Post-sort purity

5.2 Results

5.2.1 Effect of BCR-ABL on OCT-1

5.2.1.1 BCR-ABL and p-Crkl expression in HL60 cell lines

BCR-ABL expression and activation of downstream signalling partners were assessed in the HL60.CMV.BCRABL cell line using RQ-PCR and Western blot. BCR-ABL mRNA was expressed at levels (average 387%) similar to that observed in the CML cell line K562 (average 508%, Figure 5.2A). In addition, the levels of p-Crkl were similar to that observed in K562 cells (Figure 5.2B). No detectable BCR-ABL mRNA or p-Crkl expression was observed in the parental HL60 cell line or the control vector cell line. Treatment of the HL60.CMV.BCRABL cell line with imatinib for 2 hours inhibited p-Crkl signalling (Figure 5.2C).

5.2.1.2 Intracellular uptake and retention (IUR), OCT-1 activity and efflux in HL60 cell lines

The IUR of imatinib in HL60.CMV.BCRABL was significantly higher than that seen in HL60.CMV.CV (average 28.3 and 25.0 ng/200,000 cells respectively, p=0.034, n=14, Figure 5.3A). This difference in IUR could be due to either increased influx or reduced efflux in the BCR-ABL cell line. The addition of prazosin reduced the IUR in both cell lines (average CV: 20.5, BCRABL: 23.2 ng/200,000 cells, n=14) however the difference in IUR remained significant (p=0.017, Figure 5.3A). Interestingly, the OCT-1 activity was not found to be significantly different between the two cell lines (average CV: 4.6, BCRABL: 5.1 ng/200,000 cells, p=0.617, n=14 Figure 5.3B), suggesting that the increased IUR in HL60.CMV.BCRABL is independent of OCT-1. To investigate the role of efflux in these cell lines, the ABCB1 inhibitor PSC833 and the ABCG2 inhibitor Ko143 were utilised. Neither inhibitor was found to alter IUR in HL60.CMV.CV cells (average IUR untreated: 24.6, PSC833: 22.3, Ko143: 23.4 ng/200,000 cells, n=3, Figure 5.3C) or in HL60.CMV.BCRABL cells (average IUR untreated: 24.7, PSC833: 19.3, Ko143: 24.6 ng/200,000 cells, n=3, Figure 5.3C).

A

	BCR-ABL mRNA expression
HL60 parental	undetectable
HL60 CMV.CV	undetectable
HL60 CMV.BCRABL	387%
K562	508%

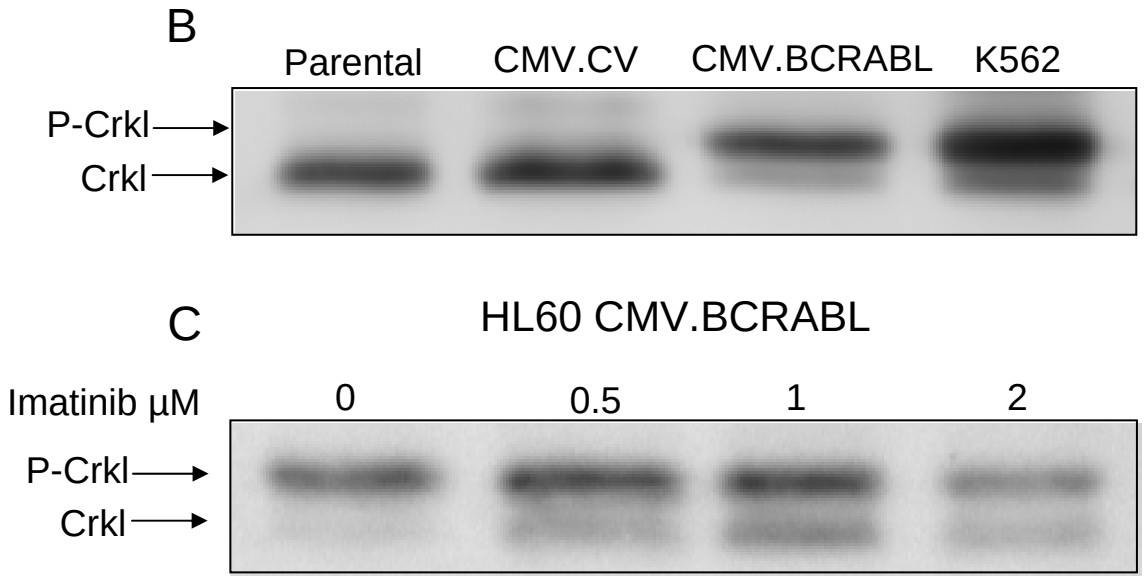


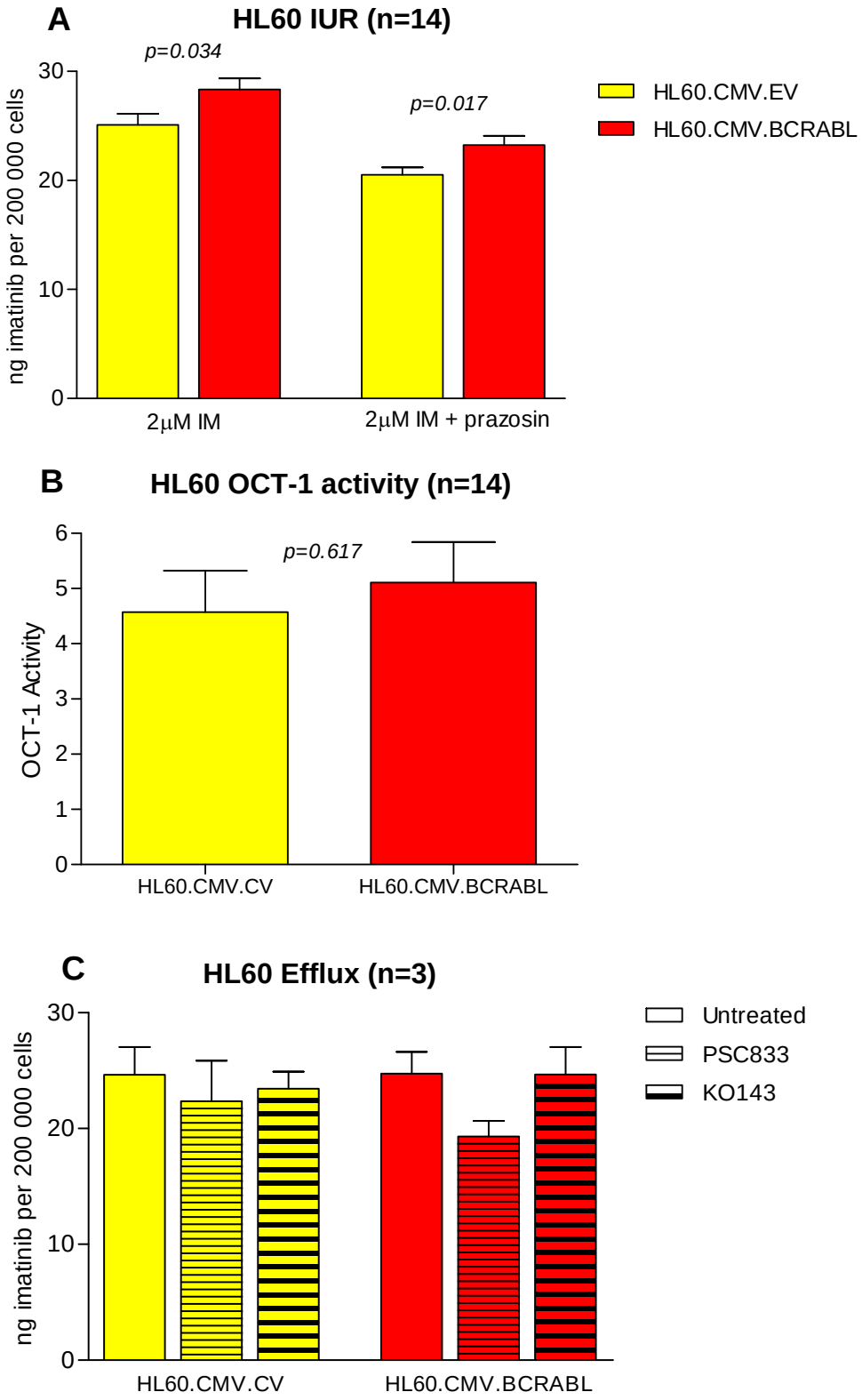
Figure 5.2 BCR-ABL expression and signalling in transduced HL60 cells

(A) BCR-ABL mRNA expression (expressed as % of BCR) confirms the presence and absence of BCR-ABL in transduced HL60 cell lines. (B) Phosphorylated Crkl (P-Crkl) expression in transduced HL60 cell lines confirms BCR-ABL signalling where appropriate. (C) Treatment with imatinib for 2 hours demonstrates a reduction in P-Crkl in the HL60.CMV.BCR-ABL cell line.

Figure 5.3 Intracellular uptake and retention of imatinib, OCT-1 activity and efflux in HL60 transduced cell lines

HL60 cells were transduced with control vector or BCR-ABL. The IUR of 2μM imatinib in the presence and absence of prazosin (A), the OCT-1 activity (B) and the effect of efflux inhibitors on IUR (C) were measured. The IUR of imatinib was slightly higher in the BCR-ABL line, but this was not due to an increase in OCT-1 activity or reduced efflux function.

Columns represent the mean plus the standard error of the mean.


A
HL60 IUR (n=14)
p=0.034
p=0.017
ng imatinib per 200 000 cells
30
20
10
0
2μM IM
2μM IM + prazosin
HL60.CMV.EV
HL60.CMV.BCRABL
B
HL60 OCT-1 activity (n=14)
p=0.617
OCT-1 Activity
6
5
4
3
2
1
0
HL60.CMV.CV
HL60.CMV.BCRABL
C
HL60 Efflux (n=3)
ng imatinib per 200 000 cells
30
20
10
0
HL60.CMV.CV
HL60.CMV.BCRABL
Untreated
PSC833
KO143

5.2.1.3 OCT-1 and ABCB1 mRNA expression in HL60 cell lines

The OCT-1 and ABCB1 mRNA expression was assessed in the HL60 cell lines. In keeping with the functional studies, no significant difference in OCT-1 mRNA expression between the two cell lines was observed (average CV: 0.64%, BCR-ABL: 0.78%, n=7, p=0.600, Figure 5.4A). In addition, no detectable mRNA expression of ABCB1 in either of the HL60 cell lines was evident. These data suggest that while BCR-ABL appears to have a modest effect on the IUR of imatinib, most likely through increased passive uptake, BCR-ABL does not appear to significantly contribute to OCT-1 or ABCB1 expression and function.

5.2.2 Effect of granulocyte differentiation on OCT-1

The HL60 cell line possesses the ability to differentiate toward a number of myeloid lineages, including granulocytes, monocytes, macrophage-like cells and eosinophils [167]. As demonstrated in Chapter 3, different cell lineages exhibited differing OCT-1 activities, and provided a rationale to examine the association between BCR-ABL, granulocyte and monocyte differentiation and OCT-1 expression and function.

5.2.2.1 Assessment of the degree of differentiation

Differentiation of the HL60 cell lines into granulocytes was induced in the presence of 1.25% (v/v) DMSO for 7 days [168, 169] and cell differentiation was monitored by cell growth, cell cycle status, cell morphology and CD11b antigen expression [170]. CD11b recognizes a human leukocyte antigen, C3bi complement receptor (CR_3) [171] and is strongly expressed on myeloid cells and weakly expressed on NK cells and some activated lymphocytes [172]. Cytospin preparations were stained with Wright's stain for morphological assessment.

DMSO treatment for 7 days resulted in a reduction in cell growth in both cell lines (Figure 5.5A). The DMSO treated HL60.CMV.CV and HL60.CMV.BCRABL cell lines had an average 9-fold (range: 8.2-9.8) and 11-fold (range: 10.2-12.6) increase in cell number, respectively. In comparison, the untreated cell lines had an average 19-fold increase in cell number (range: 15-26) over the 7 days. This reduction in proliferation was associated with an accumulation of cells

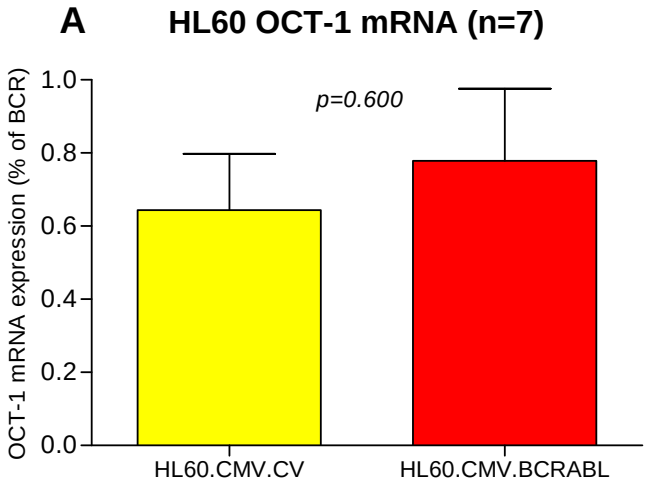
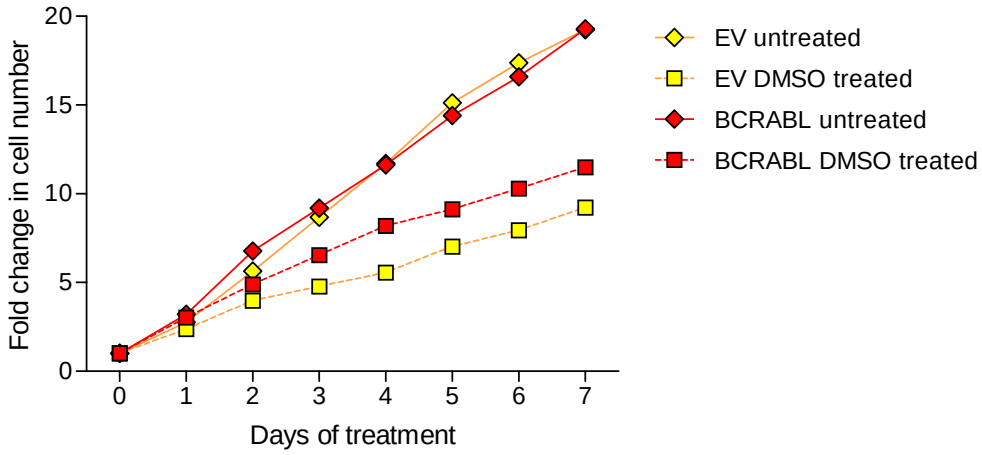


Figure 5.4 OCT-1 mRNA expression in HL60 transduced cell lines

HL60 cells were transduced with control vector or BCR-ABL. The OCT-1 mRNA expression was assessed and is expressed as a % of BCR. There is no difference in OCT-1 mRNA expression between the control and BCR-ABL cell lines.

Columns represent the mean plus the standard error of the mean.

A



B

	G0/G1	S	G2/M
CV untreated	57%	27%	16%
CV DMSO	79%	16%	5%
BCR-ABL untreated	54%	31%	15%
BCR-ABL DMSO	80%	14%	6%

Figure 5.5 Proliferation and cell cycle in DMSO treated HL60 transduced cell lines

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 1.25% DMSO for 7 days to induce granulocyte differentiation. (A) Cell proliferation over 7 days of culture was measured by trypan blue and is expressed as a fold increase in cell number. (B) Cell cycle status after 7 days of culture was measured by PI staining. Proliferation was reduced in DMSO treated cells and cells were arrested in the G0/G1 cell cycle phase. Both figures represent the average results from 3 experiments.

in the G0/G1 cell cycle phase (Figure 5.5B). The untreated cell lines had an average of 57% (CV, range: 42-66%) and 54% (BCRABL, range: 45-60%) cells in the G0/G1 phase. Following treatment with DMSO HL60.CMV.CV and HL60.CMV.BCRABL had an average of 79% (range: 63-88%) and 80% (range: 72-86%) of cells in the G0/G1 phase, respectively. This was associated with a reduction of the number of cells in both S and G2/M phases. DMSO treatment was also found to alter the cell morphology of both cell lines as evidenced by more lobular nuclei (Figure 5.6A). Furthermore, CD11b expression was up-regulated on both cell lines, from 0-6% to 35% (CV, range: 20-50%) and 57% (BCRABL, range: 38-68%) (Figure 5.6B). Notably, HL60.CMV.BCRABL was found to differentiate more than vector control cells as indicated by increased nuclear segmentation (Figure 5.6A) and increased CD11b expression (Figure 5.6B).

5.2.2.2 IUR, OCT-1 activity and OCT-1 mRNA

The IUR of imatinib remained unchanged in HL60.CMV.CV cells following granulocyte differentiation. The average IUR in untreated cells and DMSO-treated cells was 24.8 (range: 24.5-25.1) and 27.8 (range: 23.8-33.5) ng/200,000 cells, respectively (p=0.375, Figure 5.7A). The IUR of HL60.CMV.BCRABL increased slightly, although not significantly, following DMSO treatment. The average IUR in untreated cells was 27.6 ng/200,000 cells (range: 25-30) and in DMSO treated cells was 39.1 ng/200,000 cells (range: 31-49, n=3, p=0.110, Figure 5.7A). Interestingly, while the OCT-1 activity in the vector control line did not change following DMSO treatment (average untreated: 2.4, DMSO: 6.7 ng/200,000 cells, p=0.291), the OCT-1 activity in HL60 CMV.BCRABL significantly increased from an average of 5.8 (range: 5.2-6.2) to 18.6 (range: 14.5-25.7) ng/200,000 cells (p=0.024, n=3, Figure 5.7B). In HL60.CMV.CV, the mRNA expression of OCT-1 increased from an average of 0.6% (range: 0.2-0.8%) to 1.6% (range: 0.5-3.0%, p=0.239, n=3, Figure 5.7C). In HL60.CMV.BCRABL, the OCT-1 mRNA expression increased from an average of 0.6% (range: 0.1-1.5%) to 2.0% (range: 1.5-2.7%, p=0.085, n=3, Figure 5.7C). These data suggest that enforced BCR-ABL expression promotes granulocyte differentiation which in turn leads to increased OCT-1 activity.

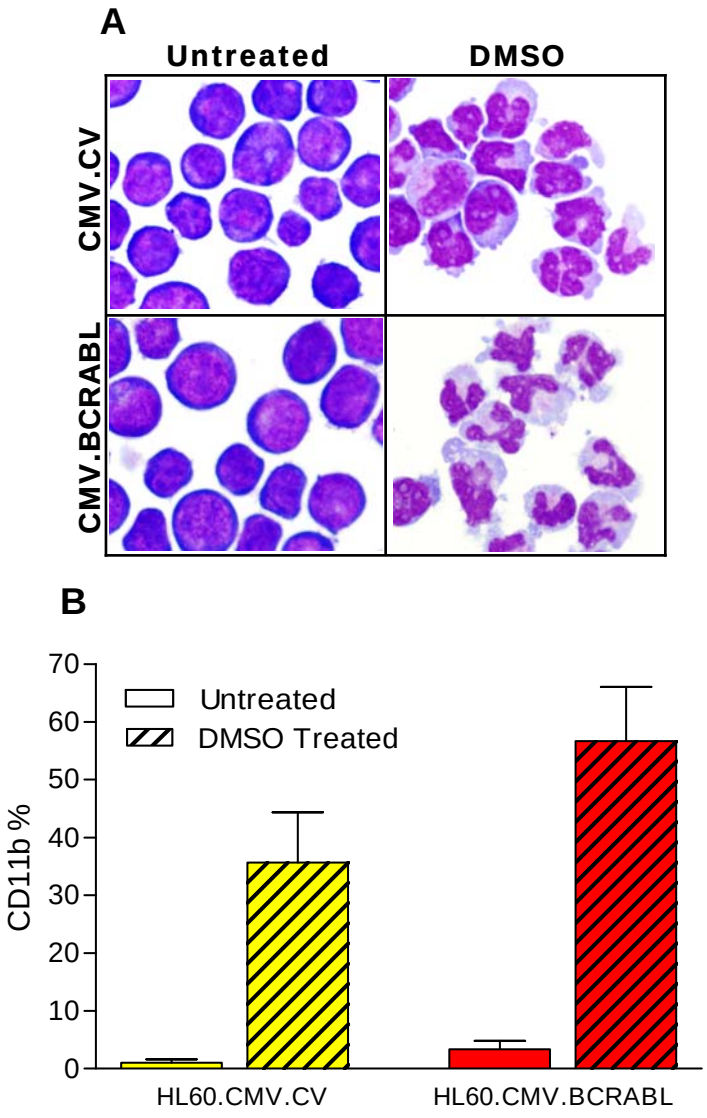


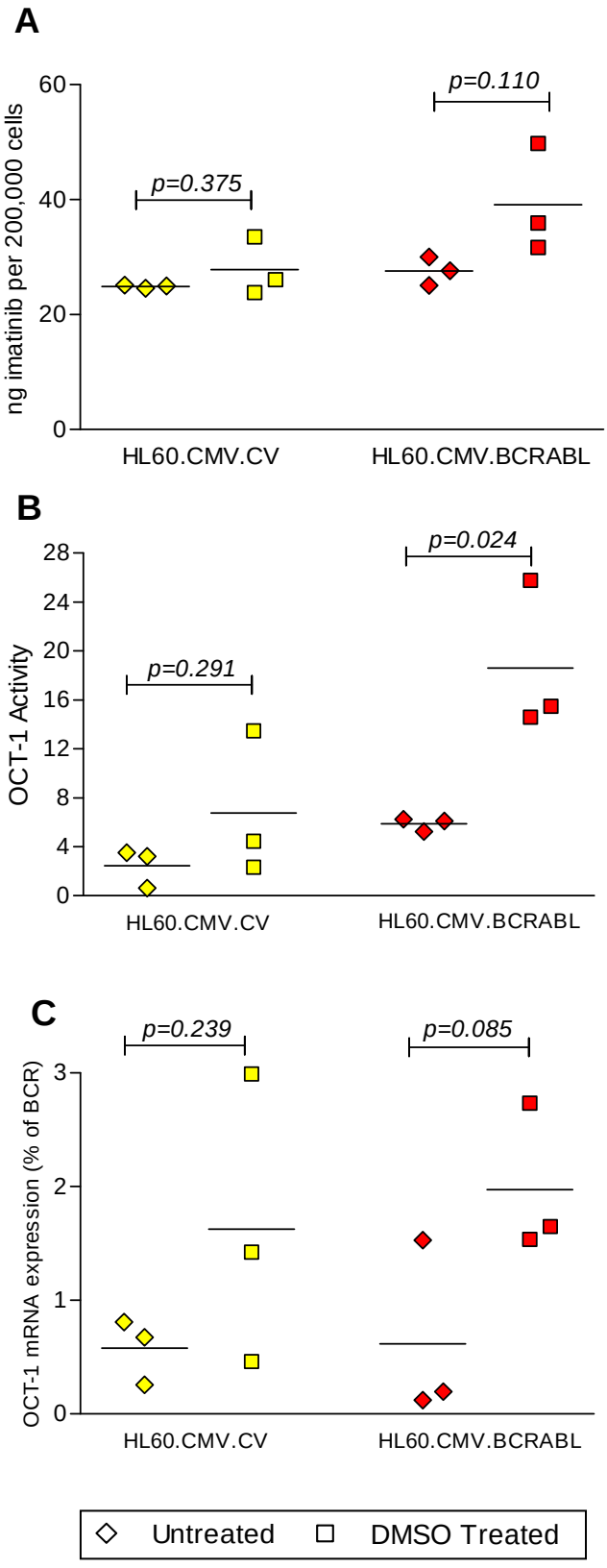
Figure 5.6 Differentiation of HL60 transduced cell lines with DMSO

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 1.25% DMSO for 7 days to induce granulocyte differentiation. (A) Examination of cell morphology on cytospin preparations using Wright's stain. (B) Cell surface expression of the myeloid differentiation marker CD11b was measured using flow cytometry. Both cell lines underwent granulocyte differentiation following treatment with DMSO, however the BCR-ABL cell line appeared to differentiate further.

Columns represent the mean plus the standard error of the mean.

Figure 5.7 Intracellular uptake and retention of imatinib, OCT-1 activity and OCT-1 mRNA expression in DMSO treated HL60 cell lines

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 1.25% DMSO for 7 days to induce granulocyte differentiation. (A) The IUR of 2μM imatinib, (B) the OCT-1 activity and (C) OCT-1 mRNA expression were measured. Treatment with DMSO significantly increased the OCT-1 activity of the HL60.CMV.BCRABL cell line but not the control vector line.


A
ng imatinib per 200,000 cells
60
40
20
0
p=0.375
p=0.110
HL60.CMV.CV
HL60.CMV.BCRABL
B
OCT-1 Activity
28
24
20
16
12
8
4
0
p=0.291
p=0.024
HL60.CMV.CV
HL60.CMV.BCRABL
C
OCT-1 mRNA expression (% of BCR)
3
2
1
0
p=0.239
p=0.085
HL60.CMV.CV
HL60.CMV.BCRABL
Untreated
DMSO Treated

5.2.3 Effect of monocyte differentiation on OCT-1

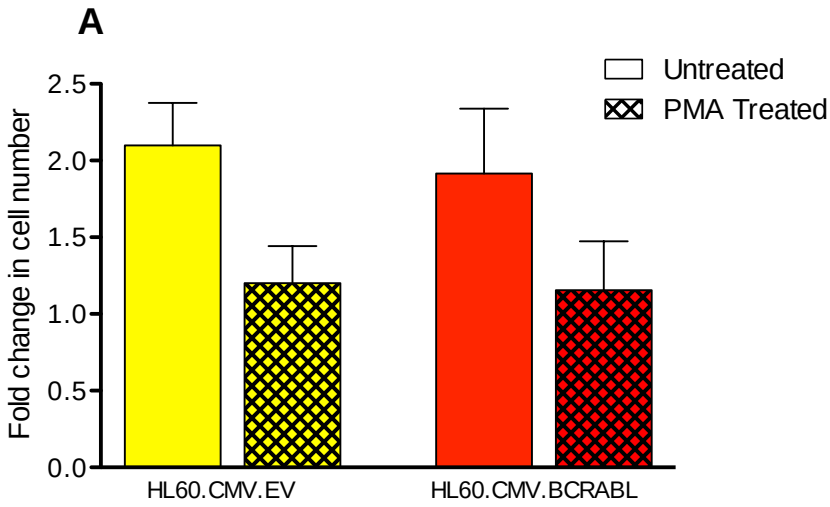
Monocyte differentiation of the HL60 cell lines was induced by treating the HL60.CMV.CV and HL60.CMV.BCRABL cell lines with 20ng/ml PMA for 24 hours [170, 173]. Both cell lines became adherent following PMA treatment, necessitating the use of trypsin to detach cells from the culture flask.

5.2.3.1 Assessment of the degree of differentiation

Cell growth, cell cycle status, cell morphology and antigen CD11b expression [170] was again monitored as described in Section 5.2.2.1. Both cell lines displayed reduced cell growth following PMA treatment (Figure 5.8A). At 24 hours the untreated HL60.CMV.CV and HL60.CMV.BCRABL cell lines had an average of 2.1-fold (range: 1.6-2.6) and 1.9-fold (range: 1.3-2.7) increase in cell number, respectively. In contrast, the PMA treated cell lines had an average of 1.2-fold (CV, range: 0.9-1.7%) and 1.1-fold (BCRABL, range: 0.7-1.7) increase in cell number. As seen with DMSO treatment, this reduction in proliferation was associated with an arrest in the G0/G1 cell cycle phase and a reduction in the S and G2/M phases (Figure 5.8B). The untreated cell lines had an average of 57% (CV, range: 42-66%) and 54% (BCRABL, range: 45-60%) cells in G0/G1. PMA-treated cell lines had averages of 89% (CV, range: 85-92%) and 87% (BCRABL, range: 84-89%) cells in G0/G1. PMA-treated cells showed signs of a differentiated phenotype in comparison the untreated cells (Figure 5.9A). CD11b surface expression was up-regulated from 0-6% to 35% in the vector control line (range: 25-45%) and 69% in the BCR-ABL line (range: 53-85%) (Figure 5.9B).

5.2.3.2 IUR, OCT-1 activity and OCT-1 mRNA

To assess the effects of trypsin detachment of cells, untreated HL60 cells were incubated with and without trypsin for 10 minutes and washed with Hanks prior to the assessment of IUR and OCT-1 activity. The IUR of imatinib and OCT-1 activity was found to increase following treatment with trypsin (Figure 5.10A&B). In view of these results, all subsequent analyses were made between trypsinised control cells and PMA treated cells.



B

	G0/G1	S	G2/M
CV untreated	57%	27%	16%
CV PMA	89%	6%	4%
BCR-ABL untreated	54%	31%	15%
BCR-ABL PMA	87%	5%	8%

Figure 5.8 Proliferation and cell cycle in PMA treated HL60 transduced cell lines

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 20nM PMA for 24 hours to induce monocyte differentiation. (A) Cell proliferation after 24 hours of culture was measured by trypan blue and is expressed as a fold increase in cell number. Columns represent the mean plus the standard error of the mean. (B) Cell cycle status after 24 hours of culture was measured by PI staining. Proliferation was reduced in PMA treated cells and cells were arrested in the G0/G1 cell cycle phase. Both figures represent the average results from 3 experiments.

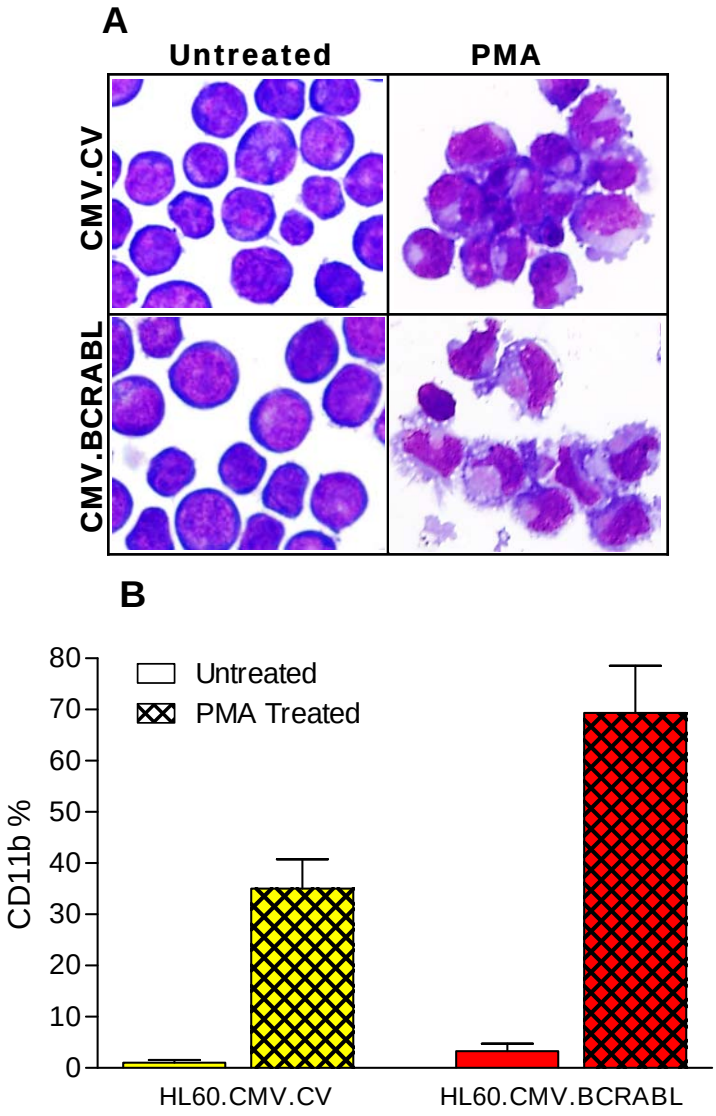


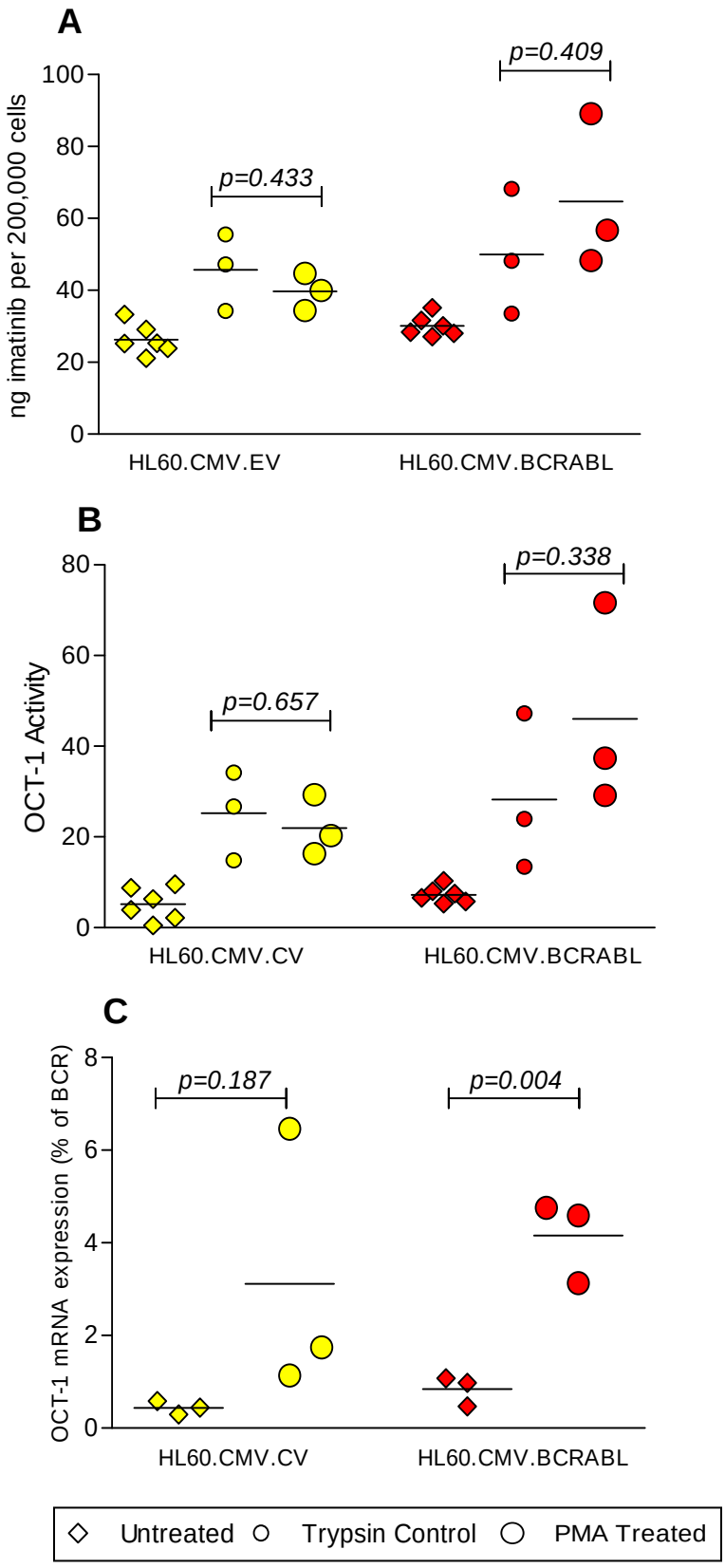
Figure 5.9 Differentiation of HL60 transduced cell lines with PMA

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 20nM PMA for 24 hours to induce monocyte differentiation. (A) Examination of cell morphology on cytospin preparations using Wright's stain. (B) Cell surface expression of the myeloid differentiation marker CD11b was measured using flow cytometry. Both cell lines underwent monocytic differentiation following treatment with PMA, however the BCR-ABL cell line appeared to differentiate further.

Columns represent the mean plus the standard error of the mean.

Figure 5.10 Intracellular uptake and retention of imatinib, OCT-1 activity and OCT-1 mRNA expression in PMA treated HL60 cell lines

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 20nM PMA for 24 hours to induce monocyte differentiation. (A) The IUR of 2μM imatinib, (B) the OCT-1 activity and (C) OCT-1 mRNA expression were measured. Treatment with PMA significantly increased the OCT-1 mRNA expression in the HL60.CMV.BCRABL cell line but not the control vector line.


A
ng imatinib per 200,000 cells
100
80
60
40
20
0
p=0.433
p=0.409
HL60.CMV.EV
HL60.CMV.BCRABL
B
OCT-1 Activity
80
60
40
20
0
p=0.657
p=0.338
HL60.CMV.CV
HL60.CMV.BCRABL
C
OCT-1 mRNA expression (% of BCR)
8
6
4
2
0
p=0.187
p=0.004
HL60.CMV.CV
HL60.CMV.BCRABL
Untreated
Trypsin Control
PMA Treated

The IUR of imatinib did not change in HL60.CMV.CV cells following treatment with PMA (control: mean: 45.7, range: 34-55; PMA: mean: 39.7, range: 34-44 ng/200,000 cells, p=0.433, n=3, Figure 5.10A). Similarly, in HL60.CMV.BCRABL cells, the IUR of imatinib did not significantly change with PMA treatment (control: mean: 50, range: 33-68; PMA: mean: 65, range: 48-89, ng/200,000 cells, p=0.409, n=3, Figure 5.10A). Similar trends were observed for OCT-1 activity, where no significant change was seen in HL60.CMV.CV cells following PMA treatment (control: mean: 25.2, range: 14-34; PMA: mean: 22, range: 16-29 ng/200,000 cells, p=0.567, n=3, Figure 5.10B) and a modest increase in HL60.CMV.BCRABL OCT-1 activity was seen (control: mean: 28.2, range: 13-47; PMA: mean: 46, range: 29-71 ng/200,000 cells, p=0.338, n=3, Figure 5.10B). Notably, a clear difference in the OCT-1 activity between PMA treated HL60.CMV.CV and HL60.CMV.BCRABL cells was observed (average 22 and 46 ng/200,000 cells, respectively, p=0.150). A non-significant increase in OCT-1 mRNA expression was seen in HL60.CMV.CV cells after PMA treatment (untreated: mean: 0.4%, range: 0.3-0.6%; PMA: mean: 3.1%, range: 1.1-6.4%, p=0.187, Figure 5.10C). However, a significant increase was observed in HL60.CMV.BCRABL OCT-1 mRNA after PMA treatment (untreated: mean: 0.8%, range: 0.4-1.0%; PMA: mean: 4.2%, range: 3.1-4.7%, p=0.004, Figure 5.10C). These data suggests that the presence of BCR-ABL assists monocyte differentiation and this increased level of differentiation results in an increase in OCT-1 mRNA expression.

5.2.4 Effect of imatinib treatment on OCT-1

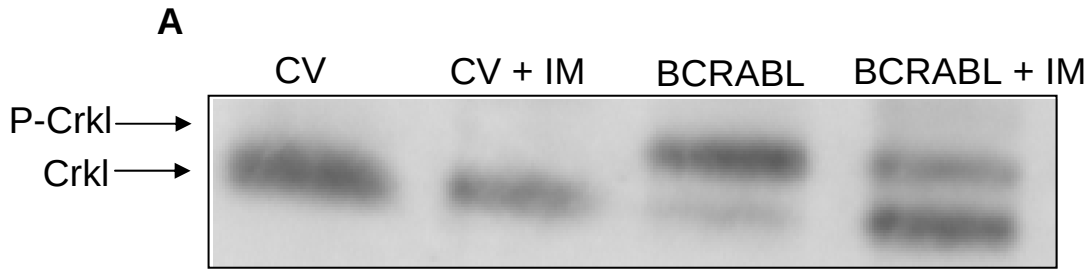
To investigate the possibility that imatinib itself had an effect on the function and expression of OCT-1, the HL60 cell lines were cultured in the presence and absence of 2µM imatinib for 24 hours and 7 days, to determine both short and long-term effects.

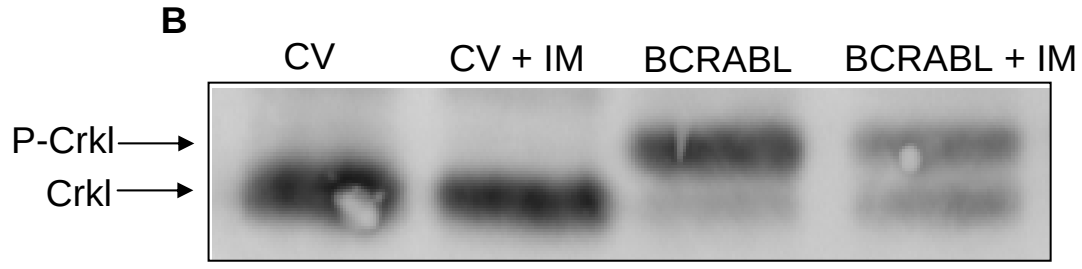
5.2.4.1 P-Crkl and BCR-ABL mRNA expression

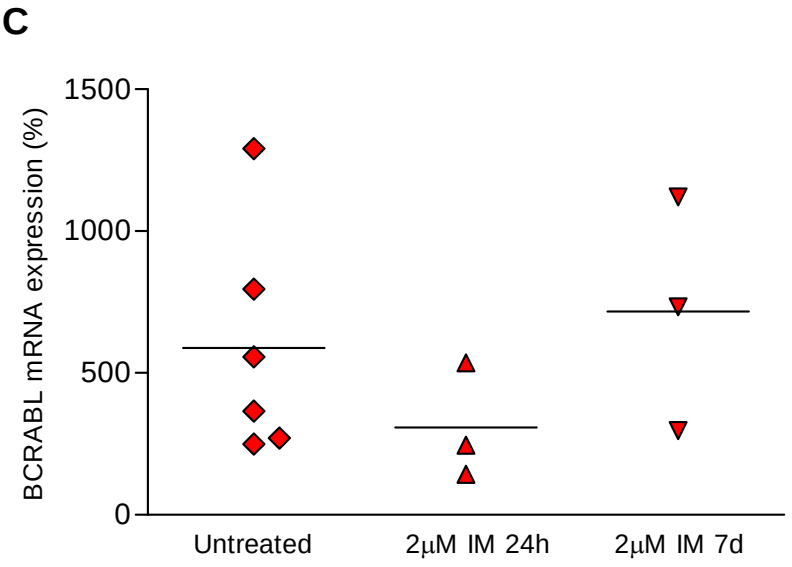
Treatment with 2µM imatinib for 24 hours and 7 days led to a reduction in detectable p-Crkl in the HL60.CMV.BCRABL cell line (Figure 5.11A&B), indicating an inhibition of BCR-ABL signalling in these cells. However, the BCR-ABL mRNA expression in HL60.CMV.BCRABL

Figure 5.11 P-Crkl expression and BCR-ABL mRNA expression in imatinib treated HL60 transduced cell lines

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 2μM imatinib for 24 hours or 7 days. Phosphorylated Crkl (P-Crkl) expression in transduced HL60 cell lines after treatment with imatinib for 24 hours (A) or 7 days (B). HL60.CMV.BCR-ABL shows a reduction in P-Crkl following treatment with imatinib. (C) BCR-ABL mRNA expression in the HL60.CMV.BCRABL line did not change following treatment with imatinib.


A
CV
CV + IM
BCRABL
BCRABL + IM
P-Crkl
Crkl


B
CV
CV + IM
BCRABL
BCRABL + IM
P-Crkl
Crkl


C
BCRABL mRNA expression (%)
1500
1000
500
0
Untreated
2μM IM 24h
2μM IM 7d

cells remained unchanged following treatment with imatinib. The respective average BCR-ABL mRNA expression in untreated, 24 hour culture and 7 day culture was 587%, 307% and 716%, respectively (Figure 5.11C).

5.2.4.2 Proliferation

Untreated HL60.CMV.CV and HL60.CMV.BCRABL cells had an average 2.6-fold (range: 1.2-4.5) and 2.5-fold (range: 1.6-3.7) increase in cell number after 24 hours culture, respectively. Similarly, imatinib treated cells had an average 2.1-fold (CV, range: 1.6-2.9, p=0.696) and 1.8-fold (BCRABL, range: 1.0-2.8, p=0.467) increase in cell number (Figure 5.12A). Surprisingly, after 7 days in 2µM imatinib, both the control vector and BCRABL cell line were viable and proliferating at the same rate as the untreated cell lines (Figure 5.12B). At day 7 the average fold change in cell number for CV untreated, CV imatinib, BCRABL untreated and BCRABL imatinib were 22.0, 22.3, 22.0 and 21.9, respectively. Therefore, while 2µM imatinib was able to inhibit BCR-ABL signalling in HL60.CMV.BCRABL cells (as evidenced by reduced p-Crkl), it was insufficient to reduce cell growth or induce apoptosis in these cells.

5.2.4.3 IUR and OCT-1 activity

The average IUR in HL60.CMV.CV cells (untreated 31.5 ng/200,000 cells, range: 23-43) did not significantly change after treatment with 2µM imatinib for 24 hours (average: 28.4, range: 23-31 ng/200,000 cells, p=0.529) or 7 days (average: 35.8, range: 28-42 ng/200,000 cells, p=0.413, Figure 5.13A). Similarly, no significant change was observed in the IUR of HL60.CMV.BCRABL after treatment with imatinib (average untreated: 33.8, range: 22-48; 24h: 33.3, range: 28-39; 7d: 41.5, range: 30-48 ng/200,000 cells, p=0.947 and p=0.309). As with IUR, no significant change in OCT-1 activity was seen in either cell line following treatment with 2µM imatinib (Figure 5.13B). In HL60.CMV.CV the average OCT-1 activity in untreated, 24 hour treated and 7 day treated cells were 9.7 (range: 1.5-22), 6.0 (range: 0.5-10.2) and 11.1 (range: 2-19) ng/200,000 cells, respectively (p=0.446 and p=0.788). In HL60.CMV.BCRABL untreated cells the average OCT-1 activity was 10.4 ng/200,000 cells (range: 2-23), which was not significantly different to that in cells treated with imatinib for 24 hours (average: 9.3, range:

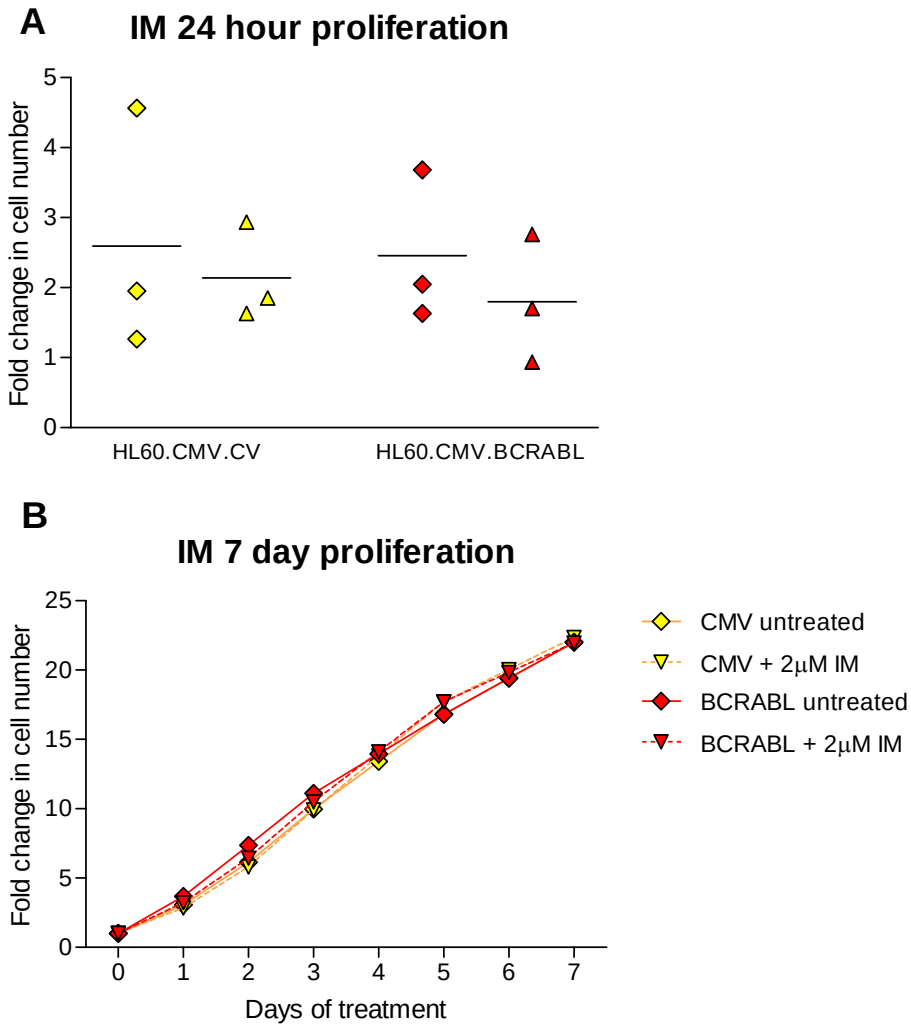
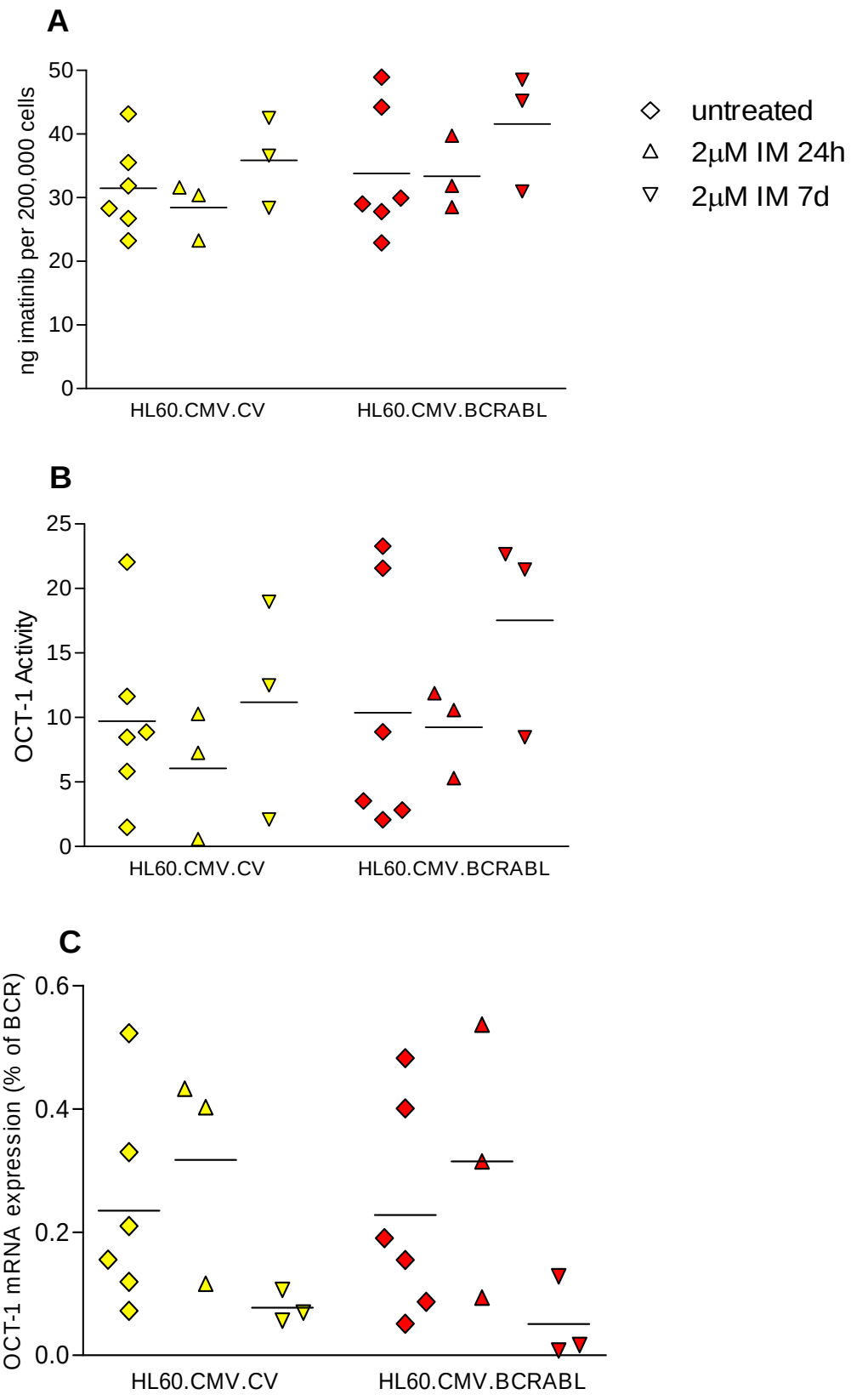


Figure 5.12 Proliferation in imatinib treated HL60 transduced cell lines

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 2µM imatinib for 24 hours or 7 days. Cell proliferation after 24 hours (A) and 7 days (B) of imatinib treatment was measured by trypan blue and is expressed as a fold increase in cell number. No change in proliferation was seen with imatinib treatment. Data represents the average results from 3 experiments.

Figure 5.13 Intracellular uptake and retention of imatinib, OCT-1 activity and OCT-1 mRNA expression in imatinib treated HL60 cell lines

HL60 cells were transduced with control vector or BCR-ABL and were treated with or without 2μM imatinib for 24 hours or 7 days. (A) The IUR of 2μM imatinib, (B) the OCT-1 activity and (C) OCT-1 mRNA expression were measured. Treatment with imatinib did not alter the IUR of imatinib, the OCT-1 activity nor the OCT-1 mRNA expression in these cell lines.


A
ng imatinib per 200,000 cells
50
40
30
20
10
0
HL60.CMV.CV
HL60.CMV.BCRABL
untreated
2μM IM 24h
2μM IM 7d
B
OCT-1 Activity
25
20
15
10
5
0
HL60.CMV.CV
HL60.CMV.BCRABL
C
OCT-1 mRNA expression (% of BCR)
0.6
0.4
0.2
0.0
HL60.CMV.CV
HL60.CMV.BCRABL

5-12 ng/200,000 cells, p=0.857) or 7 days (average: 17.5, range: 8.5-22 ng/200,000 cells, p=0.307).

5.2.4.4 OCT-1 and ABCB1 mRNA expression

The average OCT-1 mRNA expression in the untreated vector control line (0.23%, range: 0.07-0.5%) was not significantly altered following treatment with imatinib for 24 hours (mean: 0.31%, range: 0.1-0.43%, p=0.512) or for 7 days (mean: 0.07%, range: 0.05-0.1%, p=0.159, Figure 5.13C). Similarly, in the BCR-ABL cell line, the average OCT-1 mRNA expression in untreated cells (0.23%, range: 0.05-0.48%) did not significantly change following imatinib treatment (average 24h: 0.31%, range: 0.09-0.53%, p=0.535; 7d: 0.05%, range: 0.007-0.12%, p=0.144, Figure 5.13C). Furthermore, ABCB1 mRNA expression remained undetectable in both the control and BCR-ABL cell lines following treatment with imatinib for 24 hours or 7 days.

5.3 Discussion

Recent studies by Bazeos *et al* have hypothesized that BCR-ABL has an inhibitory effect on the mRNA expression of OCT-1 [115]. In addition, a number of studies have indicated that BCR-ABL up-regulates the protein expression of the efflux transporters ABCB1 and ABCG2, and that treatment with imatinib reverses this effect [163-165]. Given the importance of the functional OCT-1 activity in identifying patient response to imatinib therapy, understanding the potential role of BCR-ABL on the expression and function of OCT-1 is crucial.

This chapter describes the development of a BCR-ABL expressing HL60 cell line model. The promyelocytic HL60 cell line was transduced with lentivirus containing empty vector or ^{p210}BCR-ABL. The BCR-ABL transduced cell line, expressed BCR-ABL mRNA and p-Crkl at levels consistent with the K562 CML cell line. In addition, treatment with 2μM imatinib reduced p-Crkl levels in this cell line, consistent with BCR-ABL inhibition. However, treatment with imatinib did not induce apoptosis or a reduction in proliferation of this cell line. This is likely due to the already existing genetic abnormalities in the HL60 cell line (ie: PML-RARα), such that they do not require BCR-ABL for growth and survival.

A modest, but significant increase in the total intracellular uptake and retention of imatinib was observed in the BCR-ABL cell line compared to the control line. This increase in IUR was found not to be due to increased OCT-1 function, as the OCT-1 activity was not significantly different between the two cell lines. Moreover, this increase in IUR was not due to reduced efflux function, as inhibitors of ABCB1 and ABCG2 had no effect of the intracellular concentration of imatinib. Therefore, the increase in imatinib IUR is most likely to be due to an increase in the passive uptake of imatinib, in the BCR-ABL expressing cells. As the BCR-ABL protein provides a binding site for imatinib, this affinity may tip the passive influx/efflux balance more towards influx, resulting in increased intracellular concentrations of imatinib.

The HL60 cell line possesses the ability to differentiate toward a number of myeloid lineages [167]. In these studies, granulocyte or monocyte differentiation was induced by treating the HL60 cell lines with DMSO or PMA, respectively. In both cases, differentiation resulted in a reduction in proliferation, a G0/G1 cell cycle arrest, an increase in CD11b surface expression and morphological changes. However, with granulocyte or monocyte differentiation, the BCR-ABL expressing cell line underwent a greater degree of differentiation as evidenced by CD11b surface expression and morphological analyses. This increased level of myeloid differentiation was associated with increased OCT-1 activity and OCT-1 mRNA expression. Therefore, while BCR-ABL itself appears to have no direct effect on OCT-1, myeloid differentiation is enhanced by the presence of BCR-ABL, which in turn, increases OCT-1 expression and function.

These data are in agreement with studies in which ectopic BCR-ABL expression in cord blood-derived CD34+ cells [26] and mouse embryonic stem cells [174], led to an increase in myeloid colonies using *in vitro* colony forming assays. While other studies suggested that increased BCR-ABL expression in blast crisis mediates arrest of differentiation, this has usually been associated with additional genetic defects such as reduced p53 [175], and reduced C/EBP-α [176, 177]. Over-expression of BCR-ABL in the pluripotent primitive haematopoietic cell line, FDCP-Mix, resulted in a cell line with characteristics similar to that observed in cells recovered from “chronic phase” CML patients [178]. In this cell line, expression of BCR-ABL along with culture in myeloid differentiation media resulted in preferential granulocyte differentiation. Furthermore, BCR-ABL expression did not induce a block in differentiation as blast cell numbers decreased dramatically. Lastly, a reduction in granulocyte differentiation was observed in a CML cell line following imatinib treatment [179]. Therefore, the observation of increased myeloid differentiation with BCR-ABL expression in the present chapter is consistent with previously published findings.

The precise mechanism by which DMSO or PMA promote HL60 differentiation remains unclear. However, studies suggest that down-regulation of c-Myc [169], reduction in binding activity of AP-1 [173], NF-κB activation, PTEN up-regulation and reduced pAKT [180] all play a role in DMSO-induced granulocyte differentiation. Monocyte differentiation by PMA has been attributed to increased phosphorylation of c-Jun [170], increased c-Jun mRNA [181], increased PI3-kinase activity and activation of PKCζ [182], activation of the MEK/ERK/MAP kinase signalling pathway [183], increased Jun-B and c-fos mRNA levels and increased AP-1 binding activity [173]. BCR-ABL signalling is likely to function by enhancing one or more of these pathways through its deregulated kinase activity.

Studies described in Chapter 3 suggest that long-term imatinib treatment may influence OCT-1 expression and function, as increased OCT-1 activity and mRNA levels were seen in CML remission patients (average of 12 months imatinib treatment) compared with both diagnosis patients and normal donors. The HL60 BCR-ABL cell line model described in the current chapter represents an appropriate system in which to investigate this further. Unlike CML cells, these cells were not BCR-ABL dependent for their growth and survival, allowing for long-term culture in physiologically relevant concentrations of imatinib. In this system, culture with imatinib for 24 hours or 1 week was found to have no effect on OCT-1 activity or expression, in the BCR-ABL expressing cell line. It could be argued that the increased OCT-1 activity and expression observed in CML remission samples is due to an effect of imatinib on non-CML cells. However, the present chapter also shows that treatment of the control (non-BCR-ABL) HL60 cell line with imatinib had no effect on OCT-1.

The differences seen between the data presented in this chapter and that seen in Chapter 3 could be due to a number of additional factors. Firstly, the increased OCT-1 activity and expression in remission patients may be a result of long-term imatinib treatment and the 1 week assays conducted on HL60's may not be sufficient to recapitulate the exposure time in

patients. Secondly, micro-environmental factors within patients (ie: cytokine production), may have an influence on OCT-1 which is not accounted for in these cell line studies. Lastly, the HL60 cell line model is investigating the effect of imatinib solely on blastic cells, whereas, in patients, the effect of imatinib was measured in mature populations of neutrophils, monocytes and lymphocytes. It is therefore possible that imatinib has varied effects on different cell populations.

In conclusion, using a cell line model that, the studies presented in this chapter show that: 1) BCR-ABL expression does not directly affect OCT-1 activity and expression but enhances myeloid differentiation and maturation. 2) Myeloid differentiation increases OCT-1 expression and function and 3) short or long-term treatment with imatinib does not impact upon OCT-1 activity or expression. These data provide further evidence that OCT-1 activity in patients, and hence response to imatinib treatment, is intrinsic to the patient, is closely related to lineage and is independent of BCR-ABL.

6 THE EFFECT OF CELL MATURITY ON OCT-1 ACTIVITY

6.1 Introduction

Imatinib treatment results in cytogenetic and molecular remissions in the majority of patients with CP-CML [58]. However, residual leukaemia has been detected in the primitive stem cell compartment in patients who have achieved complete cytogenetic remissions [123, 124]. Furthermore, despite achieving complete molecular remissions, discontinuation of imatinib therapy has been associated with disease recurrence in more than 50% of cases studied [119-122]. As such, the presence of residual leukaemia in the primitive cell compartment has been implicated in long-term disease persistence.

Generally, non-committed multi-potential progenitors have been found to express the CD34 antigen but not the CD38 antigen on their cell surface (CD34+38-). Lineage-committed progenitors express both the CD34 and CD38 surface antigens (CD34+38+). In comparison, mature cells only express the CD38 antigen (CD34-38+) [184]. These surface markers have proved invaluable in isolating haematopoietic cells at varying stages of maturation in order to study their function and regulation further.

Recent *in vitro* studies suggest that primitive CML CD34+ cells have a reduced sensitivity to the anti-proliferative and pro-apoptotic effects of imatinib [125, 126, 128]. Colony formation was not completely inhibited and minimal induction of apoptosis was seen in primitive CML cells with imatinib treatment. The resistance of CML primitive cells to imatinib has been attributed to three major cellular characteristics: 1) over-expression of BCR-ABL, 2) autocrine production of cytokines and 3) altered expression of influx and efflux transporters.

It has been demonstrated that primitive CML CD34+ cells and their more primitive CD34+38- precursors express significantly greater levels of BCR-ABL mRNA than mature CML MNC or CD34- cells [131, 132]. This increase in BCR-ABL transcript levels is mirrored by an increase in

BCR-ABL protein expression and an increase in phosphorylation of one of its downstream substrates, Crkl [132]. This over-expression of BCR-ABL appears to be associated with poorer BCR-ABL inhibition by imatinib. BCR-ABL kinase activity, as measured by p-Crkl, was only partially inhibited in CML CD34+ cells and not affected in CD34+38- cells by 1-5μM imatinib [127, 132]. Furthermore, a cell line study demonstrated that increased BCR-ABL expression was correlated with greater survival in the presence of imatinib [131].

Several studies have attributed the resistance of primitive CML cells to imatinib to the autocrine production of cytokines. It has been demonstrated that primitive CML CD34+ and CD34+38- cells express significantly greater transcript levels of IL-3 and G-CSF than mature counterparts [130, 131]. Over-expression of GM-CSF has been identified in imatinib-resistant patients [185]. Furthermore, cell line studies have shown that BCR-ABL expression initiates factor-independence through the autocrine secretion of IL-3 and GM-CSF [186]. This over-production of cytokines is likely to activate BCR-ABL-independent signalling cascades through the IL-3 and GM-CSF receptors, which result in enhanced survival in these cells. Therefore, inhibition of BCR-ABL by imatinib in these primitive cells is insufficient to induce apoptosis due to the independent growth factor survival signalling.

A third mechanism by which primitive CML cells may evade imatinib-induced cell death is via alterations in the influx and efflux transporters of imatinib. It has been demonstrated that CML CD34+ and CD34+38- cells have increased mRNA expression of the efflux proteins ABCB1 and ABCG2 [93, 131] and decreased mRNA expression of OCT-1 [131] compared to mature CML cells. Therefore, these alterations in influx and efflux of imatinib may result in low intracellular concentrations of imatinib in these primitive cells, leading to poor BCR-ABL inhibition and hence resistance to imatinib.

The following chapter aimed to investigate the relationship between OCT-1 expression and function and the maturation-stage of leukaemic cells. Secondly, the studies detailed here sought to ascertain whether the OCT-1 activity of patient-derived MNC was related to the OCT-1 activity in their primitive CD34+ cells. These studies had the ultimate objective of providing insight into whether MNC OCT-1 activity provides a surrogate indicator for the efficient targeting of the primitive leukaemic population and hence the depth of response.

6.1.1 Approach

MNC were isolated from the PB, BM or apheresis samples of CP-CML patients at diagnosis and from normal BM using a lymphoprep density gradient separation (section 2.3.1). Primitive CD34+ and mature CD34- cells were isolated using magnetic cell sorting (MACS; section 2.3.3). CD34+ cell populations were assessed for purity using flow cytometry (section 2.3.5) and cell populations with purities less than 70% were discarded. The more primitive CD34+38- cells were isolated from CML CD34+ cells using fluorescence activated cell sorting (FACS; section 2.3.4) and were routinely <5% of the total CD34+ population.

The intracellular uptake and retention (IUR) of 2μM imatinib, 2μM nilotinib and OCT-1 activity were measured in all isolated populations using [^{14}C]-labelled imatinib and the OCT-1 inhibitor prazosin, and are expressed as ng of imatinib per 200,000 cells (section 2.4.1). In addition, mRNA expression of OCT-1 and ABCB1 were measured in all populations using RQ-PCR and are expressed as a % of BCR expression (section 2.4.2). Response to imatinib therapy at 12 months was assessed by RQ-PCR for BCR-ABL mRNA expression (section 2.4.2 and [133]). Major molecular response is defined as a 3-log reduction in BCR-ABL transcripts from a standardized baseline. This analysis was used to determine if OCT-1 expression and function varied between primitive and mature CML cells and to determine if CD34+ OCT-1 activity is also predictive of response to imatinib treatment. All assays performed on each individual CML patients and normal donors are shown in Tables 6.1 and 6.2, respectively.

Table 6.1: Assays performed on CML patients at diagnosis

Patient Number	CD34+						CD34+ 38-	Frozen MNC
	IM IUR	OA	Nil IUR	OCT-1 mRNA	ABCB1 mRNA	PSC833/ Ko143	IUR/OA	OA
2	■	■	■	■	■			■
3	■	■						■
4	■	■	■	■	■			■
5	■	■	■					
7	■	■	■	■	■			■
8	■	■	■	■	■			■
9	■	■	■					■
10	■	■	■	■	■			■
12	■	■	■	■	■			■
17	■	■						■
18	■	■						■
19	■	■						■
20	■	■						■
21	■	■						■
22	■	■						■
23	■	■						■
24	■	■						■
25	■	■		■	■	■		■
26	■	■						■
27	■	■	■	■	■			■
28	■	■		■	■			■
29	■	■	■	■	■	■		■
30	■	■	■	■	■		■	■
31	■	■	■					■
32	■	■	■					■
33	■	■	■	■	■			■
34	■	■	■	■	■			■
35	■	■	■					■
36	■	■	■	■	■			■
37	■	■	■					■
38	■	■	■	■	■			■
39	■	■	■	■	■			■
40	■	■						■
41	■	■						■
42	■	■						■
43	■		■					
44							■	
45	■	□	■	■	□		■	
46				■	■			
47	■	□	□	■	■	■	■	
48				■	■			
49				■	■			
50	■	■	■	■	■			
51				■	■			
52				■	■			
53	■	□	■					
54	■	□	■					

Table 6.2: Assays performed on normal individuals

Sample Number	CD34+		
	Imatinib IUR	OCT-1 activity	OCT-1 mRNA
3	■	■	
4	■	■	
5	■	■	
6	■	■	
8			■
9			■
10			■
12	■	■	
13			■
14			■
15			■
16			■
17	■	■	■ *
18	■	■	
19	■	■	■ *
20	■	■	
21	■	■	
22	■	■	
23			■
24			■
25	■	■	
26	■	■	

■ Assay performed □ Assay discarded due to poor controls

* Same individual but different sample time

6.2 Results

6.2.1 Imatinib intracellular uptake and retention (IUR) in CML and normal CD34+ and CD34- cells

In 36 CP-CML patients the IUR of 2µM imatinib was significantly lower in the primitive CD34+ population (median: 20.3, range: 7.7-23.1 ng/200,000 cells) compared to the mature CD34- population (median: 25.3, range: 16.4-51.4 ng/200,000 cells, $p<0.001$). The addition of prazosin reduced the IUR of imatinib to the same level for both CML CD34+ and CD34- cells (median CD34+: 15.6 and CD34-: 15.7 ng/200,000 cells) and as such removed the difference in imatinib IUR between the two populations ($p=0.268$, Figure 6.1A). Therefore, the predominant reason behind the lower imatinib IUR in CML CD34+ cells compared with CML CD34- cells is due to differences in OCT-1 function. In contrast to CML patients, no significant difference in the IUR of imatinib was observed between CD34+ and CD34- cells derived from normal donors with a median IUR of 12.0 ng/200,000 cells (range: 6.7-16.9) and 12.9 ng/200,000 cells (range: 9.7-20.7), respectively ($p=0.164$, Figure 6.1B). The addition of prazosin reduced the IUR of imatinib for both CD34+ (median 9.7 ng/200,000 cells) and CD34- cells (median 9.8 ng/200,000 cells) and the lack of difference remained ($p=0.505$, Figure 6.1B).

6.2.2 OCT-1 activity in CML and normal CD34+ and CD34- cells

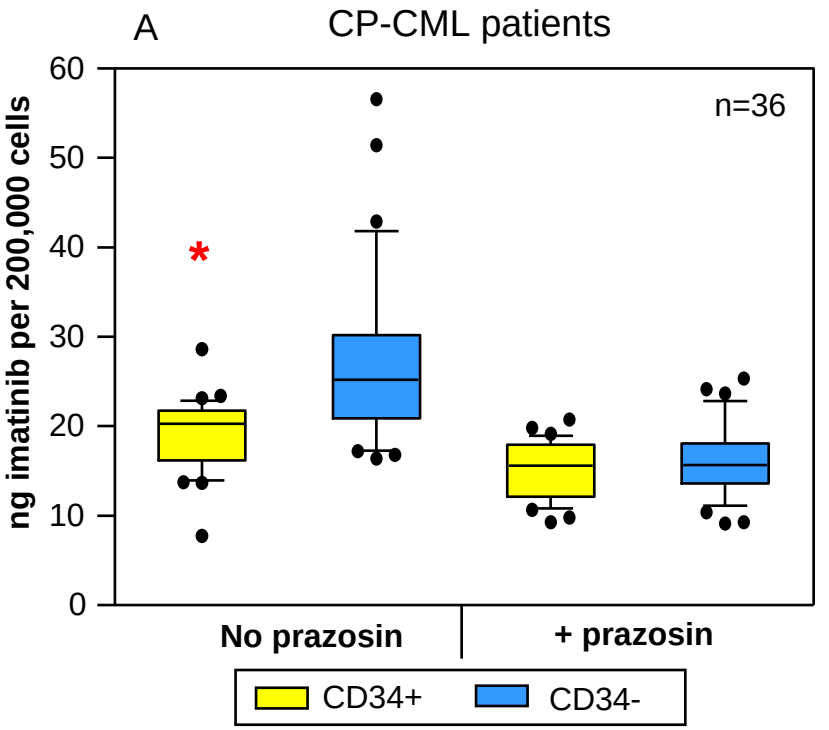
In this cohort of 36 CP-CML patients, the median OCT-1 activity in the primitive CD34+ population (mean: 4.08, range: 0-9.5, ng/200,000 cells) was significantly lower than that in the mature CD34- population with a median of 7.43 ng/200,000 cells (range: 1.6-37.1, $p<0.001$, Figure 6.2A). Furthermore, in the CD34+ population, three patients exhibited an OCT-1 activity below the level of detection (OCT-1 activity equals zero). In comparison, in the cohort of normal donors, the OCT-1 activity did not differ between CD34+ and CD34- cells with a median of 2.16 ng/200,000 cells (range: 0-6.7) and 2.45 ng/200,000 cells (range: 0-10.9), respectively ($p=0.742$, Figure 6.2B). When comparisons were made between CML and normal cells, there was no significant difference in the CD34+ OCT-1 activity ($p=0.187$). However, the OCT-1

Figure 6.1 Intracellular uptake of imatinib in CML and normal CD34+ and CD34- cells

Primitive CD34+ cells (yellow boxes) and mature CD34- cells (blue boxes) were isolated from the BM and PB of diagnosis CML patients and normal donors. The IUR of 2µM imatinib and the effect of the OCT-1 inhibitor prazosin was measured in 36 CP-CML patients (A) and in 13 normal individuals (B).

Box-plots were constructed using GraphPad Prism 5.01 software and display the median value, the upper 25th and lower 75th percentiles and whiskers encompass the 10th and 90th percentiles.

* denotes $p < 0.001$ in comparison to corresponding CD34- cells


A
CP-CML patients
n=36
ng imatinib per 200,000 cells
60
50
40
30
20
10
0
*
No prazosin
+ prazosin
CD34+
CD34-

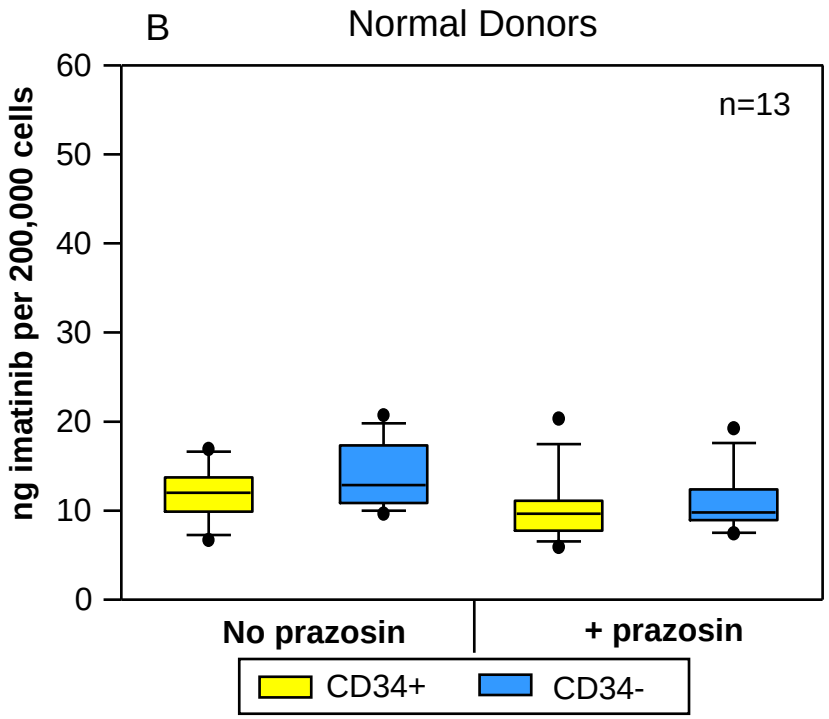
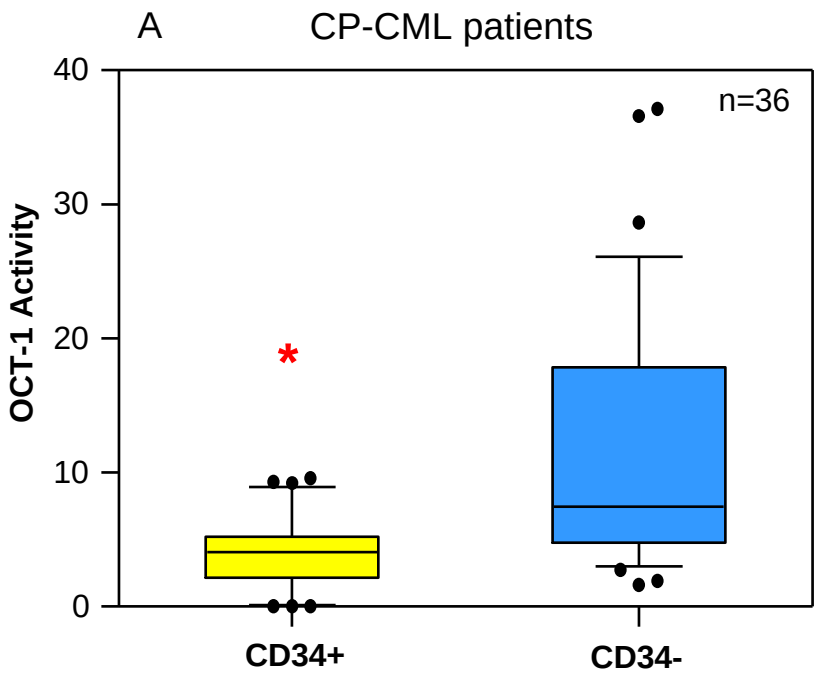

B
Normal Donors
n=13
ng imatinib per 200,000 cells
60
50
40
30
20
10
0
No prazosin
+ prazosin
CD34+
CD34-

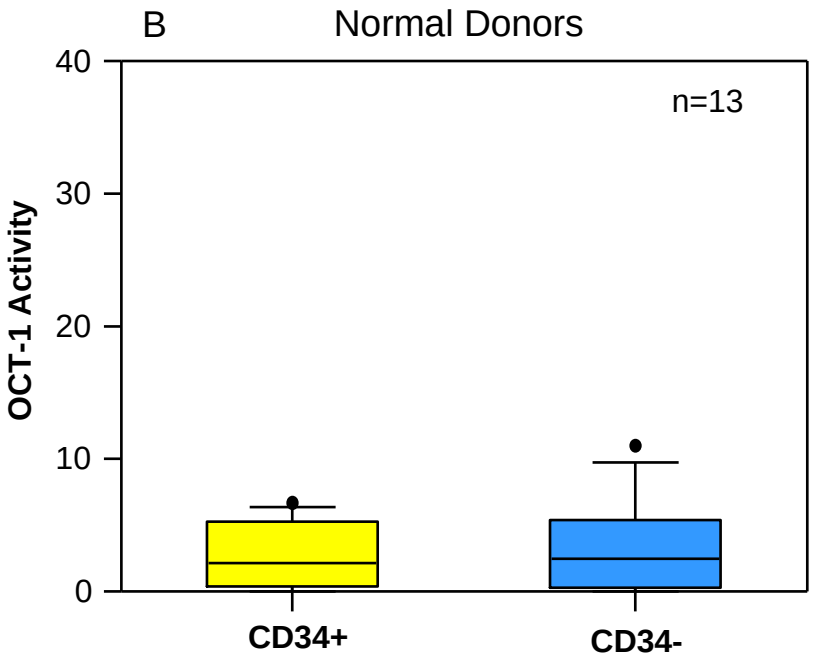
Figure 6.2 OCT-1 activity in CML and normal CD34+ and CD34- cells

Primitive CD34+ cells (yellow boxes) and mature CD34- cells (blue boxes) were isolated from the BM and PB of diagnosis CML patients and normal donors. OCT-1 activity was measured in 36 CP-CML patients (A) and in 13 normal individuals (B).

Box-plots were constructed using GraphPad Prism 5.01 software and display the median value, the upper 25th and lower 75th percentiles and whiskers encompass the 10th and 90th percentiles.

* denotes $p < 0.001$ in comparison to corresponding CD34- cells


A
CP-CML patients
n=36
OCT-1 Activity
40
30
20
10
0
*
CD34+
CD34-


B
Normal Donors
n=13
OCT-1 Activity
40
30
20
10
0
CD34+
CD34-

activity in CML CD34- cells was significantly higher than that seen in normal CD34- cells ($p<0.001$).

6.2.3 Intracellular uptake and retention (IUR) of imatinib and OCT-1 activity in CML CD34+38- cells

In four CP-CML patients, the more primitive CD34+38- population was isolated by FACS and the imatinib IUR and OCT-1 activity was measured and compared to their corresponding primitive CD34+38+ and mature CD34- populations. A stepwise increase in imatinib IUR and OCT-1 activity was observed with increasing levels of maturity. The median imatinib IUR for CD34+38- cells was 10.43 ng/200,000 cells which increased to 11.89 ng/200,000 cells and 15.40 ng/200,000 cells for CD34+38+ and CD34- cells, respectively (Figure 6.3A). The median OCT-1 activity for CD34+38- cells was 0.67 ng/200,000 cells which increased to 1.27 ng/200,000 cells and 1.90 ng/200,000 cells for CD34+38+ and CD34- cells, respectively (Figure 6.3B). Despite a lack of statistical significance due to low sample numbers (n=4), these data suggest that primitive status is associated with low OCT-1 activity.

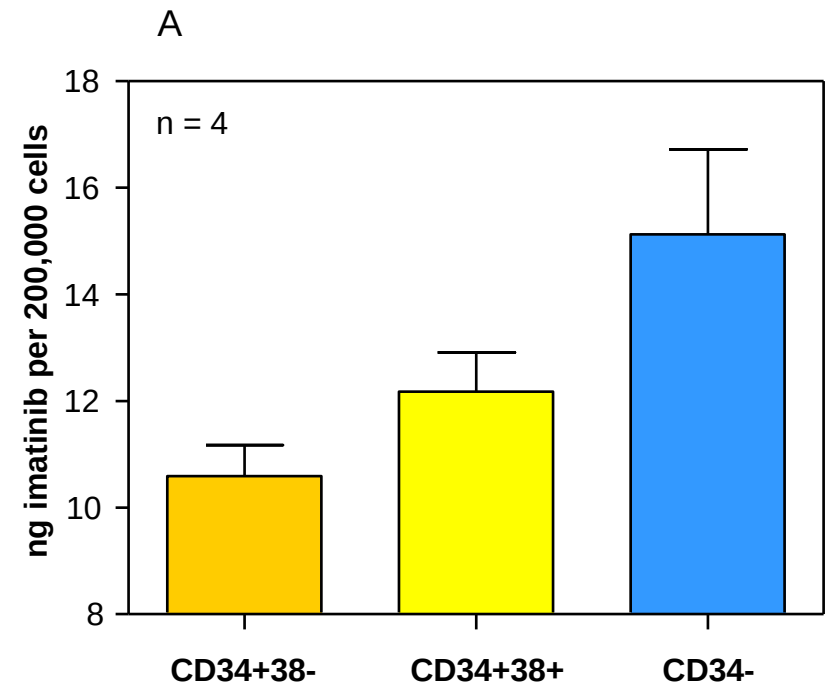
6.2.4 OCT-1 mRNA expression in CML and Normal CD34+ and CD34- cells

The differences in OCT-1 activity between CML CD34+ and CD34- cells may be due to differences in OCT-1 transcript expression in these populations. OCT-1 mRNA expression was therefore assessed in CD34+ and CD34- cells from CP-CML patients and in normal donors. From 24 CP-CML patients, the median OCT-1 mRNA expression in CD34+ cells (0.19%, range: 0.005-4.8%) was significantly lower than that of 2.87% in CD34- cells (range: 0.03-18.4%, $p<0.001$, Figure 6.4A). Similar trends were observed in normal donors, where the median OCT-1 mRNA expression in CD34+ cells (0.02%, range: 0.0005-0.1%) was significantly lower than that of 0.46% in CD34- cells (range: 0.001-3.6%, $p=0.009$, Figure 6.4B). Furthermore, no significant relationship was observed between OCT-1 mRNA expression and OCT-1 activity for either CML CD34+ ($r=0.342$, $p=0.179$, $n=17$) or CML CD34- cells ($r=0.382$, $p=0.144$, $n=16$, Figure 6.5).

Figure 6.3 Intracellular uptake of imatinib and OCT-1 activity in CML CD34+38- cells

The more primitive CD34+38- cells (gold columns), primitive CD34+38+ cells (yellow columns) and mature CD34- cells (blue columns) were isolated from apheresis samples from four diagnosis CML patients. Imatinib IUR (A) and OCT-1 activity (B) was measured in all patients.

Columns represent the mean plus the standard error of the mean.


A
n = 4
ng imatinib per 200,000 cells
18
16
14
12
10
8
CD34+38-
CD34+38+
CD34-

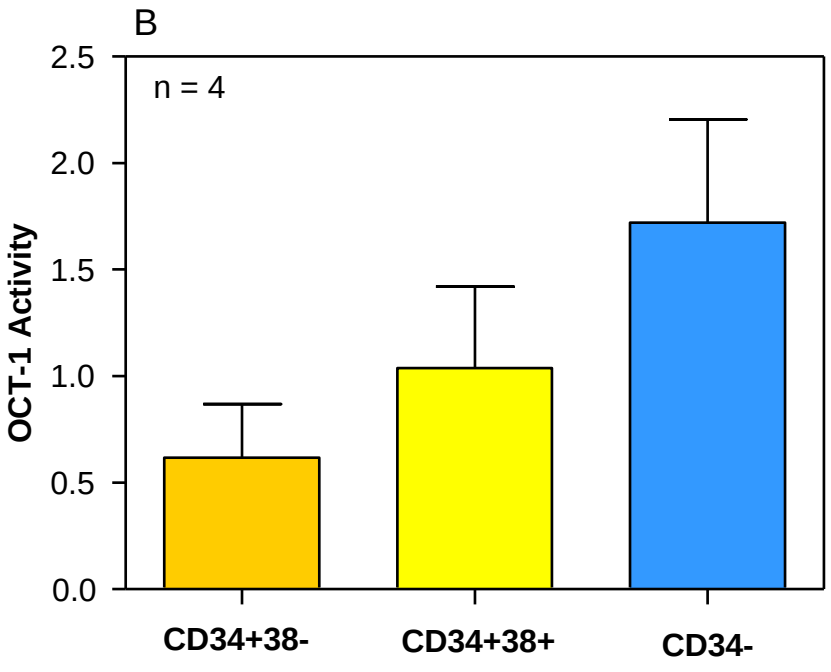
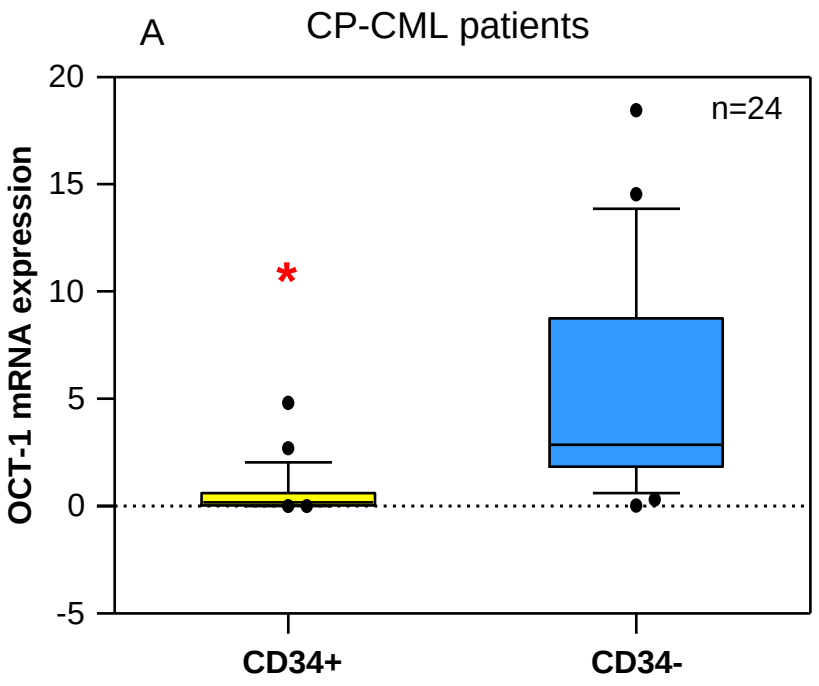

B
n = 4
OCT-1 Activity
2.5
2.0
1.5
1.0
0.5
0.0
CD34+38-
CD34+38+
CD34-

Figure 6.4 OCT-1 mRNA expression in CML and normal CD34+ and CD34- cells

Primitive CD34+ cells (yellow boxes) and mature CD34- cells (blue boxes) were isolated from the BM and PB of diagnosis CML patients and normal donors. OCT-1 mRNA expression was measured in 24 CP-CML patients (A) and in 11 normal individuals (B) and is expressed as a % of BCR expression.

Box-plots were constructed using GraphPad Prism 5.01 software and display the median value, the upper 25th and lower 75th percentiles and whiskers encompass the 10th and 90th percentiles.

* denotes $p < 0.001$ in comparison to corresponding CD34- cells


A
CP-CML patients
n=24
OCT-1 mRNA expression
20
15
10
5
0
-5
*
CD34+
CD34-

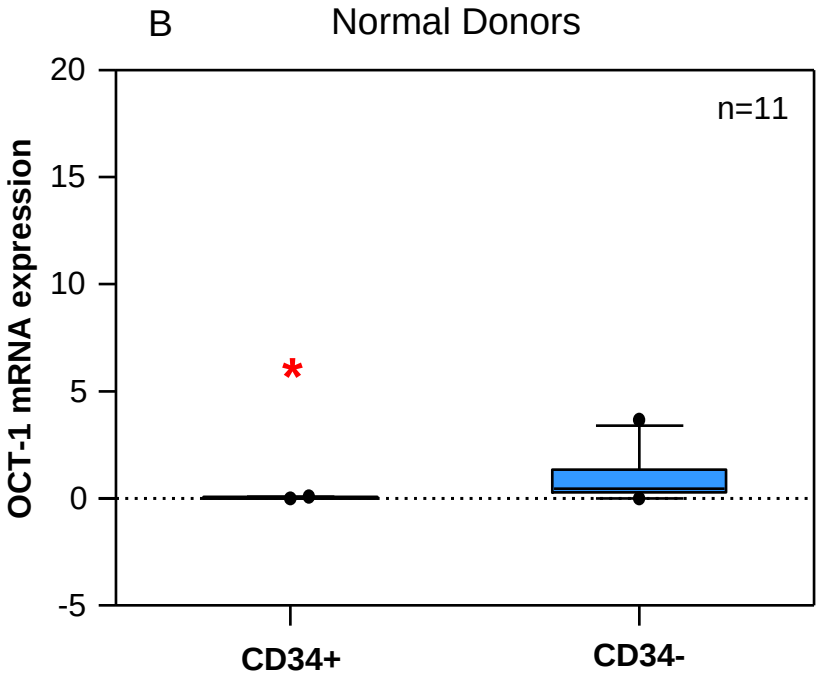
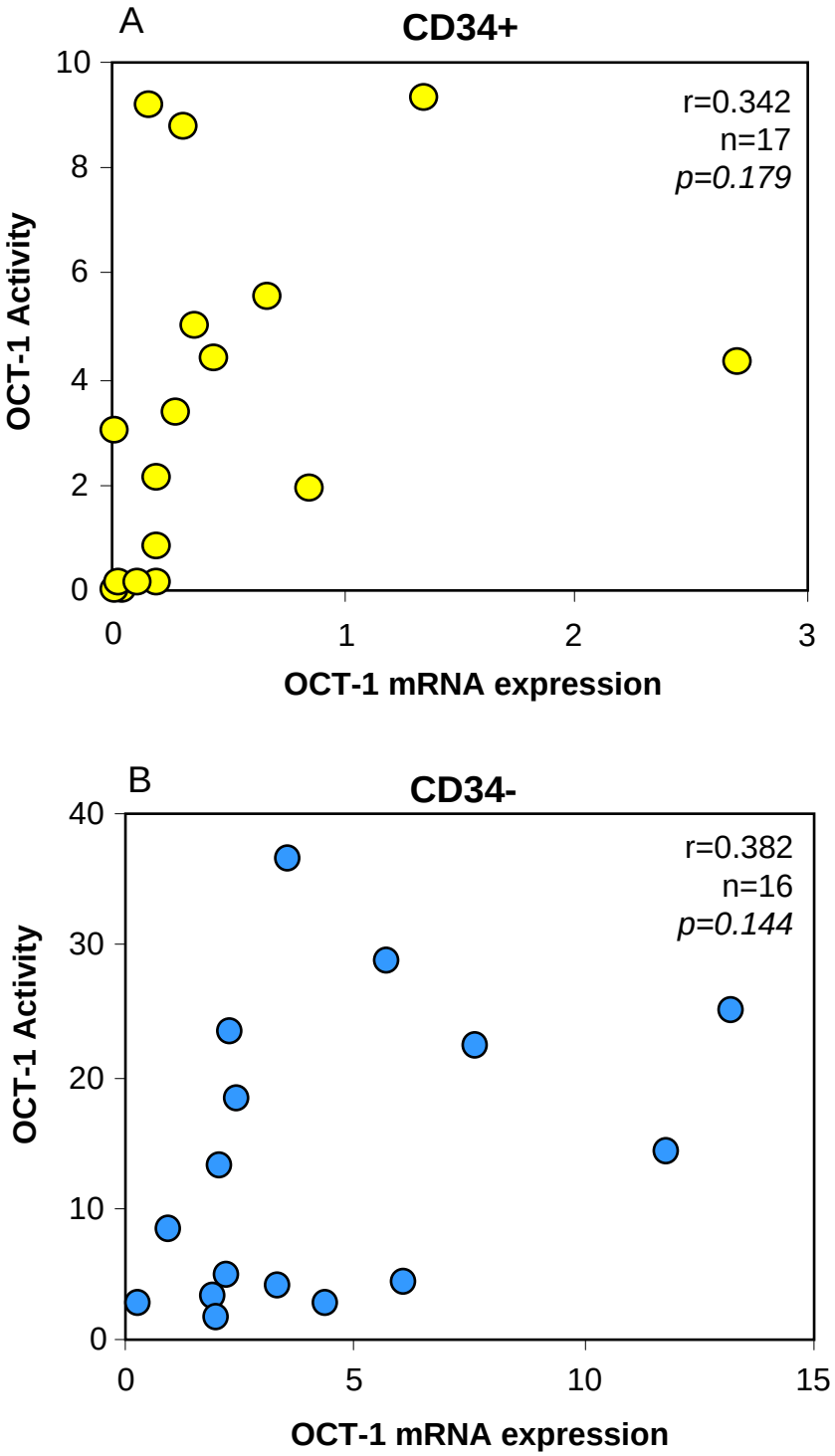

B
Normal Donors
n=11
OCT-1 mRNA expression
20
15
10
5
0
-5
*
CD34+
CD34-

Figure 6.5 Correlation between OCT-1 mRNA expression and OCT-1 activity

OCT-1 mRNA expression and OCT-1 activity were both measured in 17 CP-CML patients CD34+ (A) and CD34- (B) cells.

These two parameters were correlated using a Pearson Product Moment.


A
CD34+
r=0.342
n=17
p=0.179
OCT-1 Activity
OCT-1 mRNA expression
0
2
4
6
8
10
0
1
2
3
B
CD34-
r=0.382
n=16
p=0.144
OCT-1 Activity
OCT-1 mRNA expression
0
10
20
30
40
0
5
10
15

6.2.5 Efflux of imatinib in CML CD34+ cells

To determine the role of efflux transporters in the transport of imatinib the mRNA expression of ABCB1 was assessed in 23 CP-CML patients. ABCB1 mRNA expression was found to be significantly higher in the CD34+ cells (median: 12.7%, range: 1.5-54%) than that found in CD34- cells (median: 1.6%, range: 0.01-29%, $p<0.001$, Figure 6.6A). Therefore, high expression of ABCB1 may be a cause of low intracellular concentrations of imatinib in CML CD34+ cells. However, addition of the ABCB1 inhibitor PSC833 or the ABCG2 inhibitor Ko143 did not have any significant effect on the IUR of imatinib in CD34+ or CD34- cells from three CP-CML patients (Figure 6.6B). The average IUR in untreated, PSC833 and Ko143 treated CML CD34+ cells was 11.0, 11.3 and 10.8 ng/200,000 cells, respectively. In CML CD34- cells, the average IUR was 20.5, 14.7 and 20.7 ng/200,000 cells, respectively. Therefore, these efflux proteins appear to have a minimal role in CD34+ resistance to imatinib therapy.

6.2.6 Intracellular uptake of nilotinib into CML CD34+ and CD34- cells

The transport of nilotinib into CML cells has previously been shown to be OCT-1-independent and occurs primarily via a passive process [79, Davies, 2009 #221]. Therefore, nilotinib may be better suited to target the primitive CML compartment. To test this notion, the IUR of 2µM nilotinib into CD34+ and CD34- cells was assessed in 25 CP-CML patients where the imatinib IUR had already been measured. In these 25 patients, the IUR of imatinib was significantly lower in the primitive CD34+ population compared to the mature CD34- population ($p<0.001$, Figure 6.7). In contrast, the median IUR of nilotinib of 20.9 ng/200,000 cells (range: 9.6-36) in CD34+ cells was not found to be significantly different from the median IUR in CD34- cells (20.6 ng/200,000 cells, range: 11-38, $p=0.846$, Figure 6.7). Therefore, nilotinib targets the mature and primitive populations equally well.

6.2.7 Relationship between MNC and CD34+ OCT-1 activity in CML patients

As described in Chapter 4, the strong predictive value of MNC OCT-1 activity was originally obtained from CML patients PB-MNC following cryopreservation [110]. In order to determine if

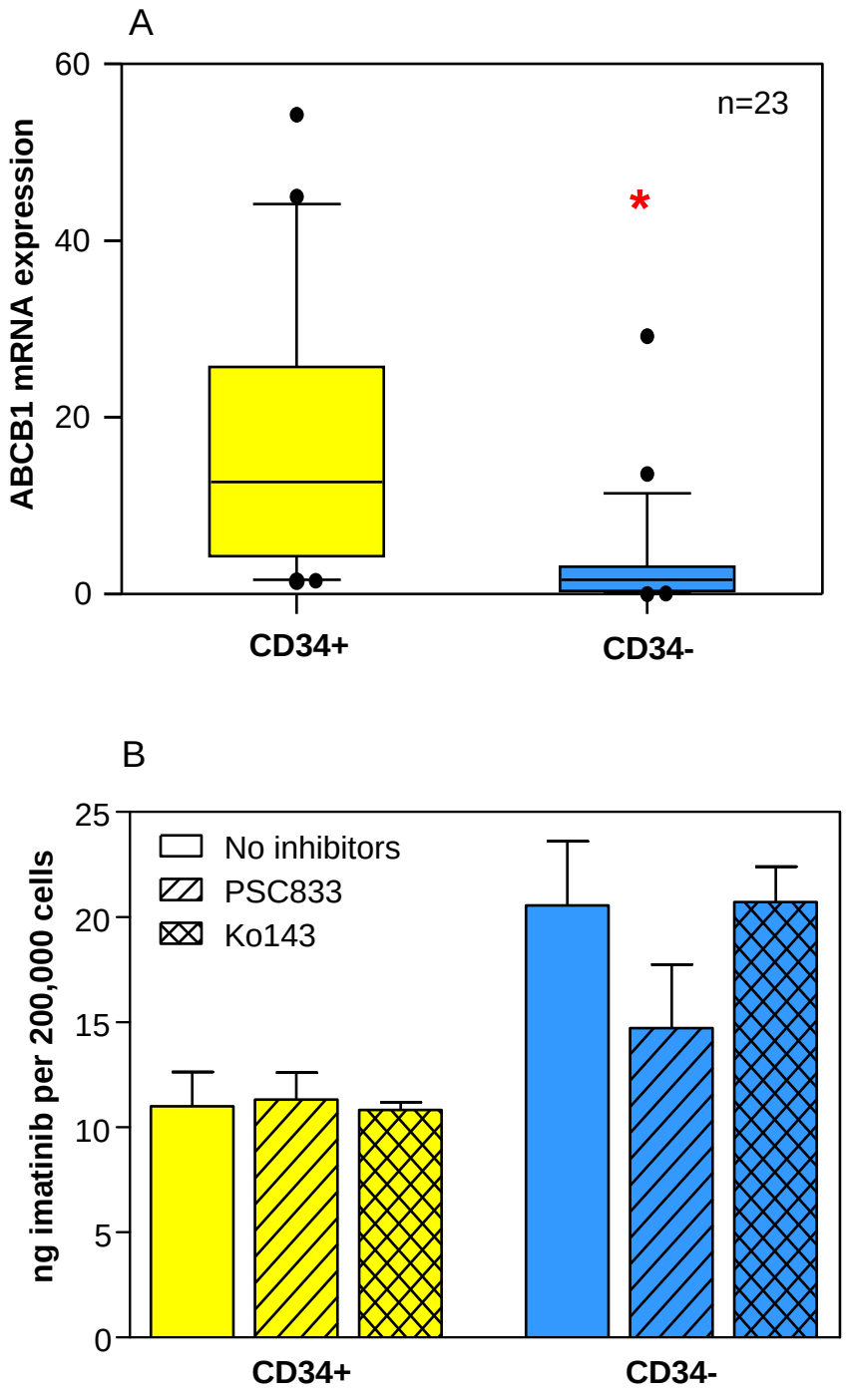
Figure 6.6 Efflux transporters in CML CD34+ and CD34- cells

Primitive CD34+ cells (yellow) and mature CD34- cells (blue) were isolated from the BM and PB of diagnosis CML patients. (A) ABCB1 mRNA expression was measured in 23 CP-CML patients and is expressed as a % of BCR expression. (B) The efflux inhibitors PSC833 (ABCB1 inhibitor) and Ko143 (ABCG2 inhibitor) were added in the imatinib IUR assay.

Box-plots were constructed using GraphPad Prism 5.01 software and display the median value, the upper 25th and lower 75th percentiles and whiskers encompass the 10th and 90th percentiles.

Columns represent the mean plus the standard error of the mean.

* denotes $p < 0.001$ in comparison to corresponding CD34+ cells


A
60
40
20
0
ABCB1 mRNA expression
n=23
*
CD34+
CD34-
B
25
20
15
10
5
0
ng imatinib per 200,000 cells
No inhibitors
PSC833
Ko143
CD34+
CD34-

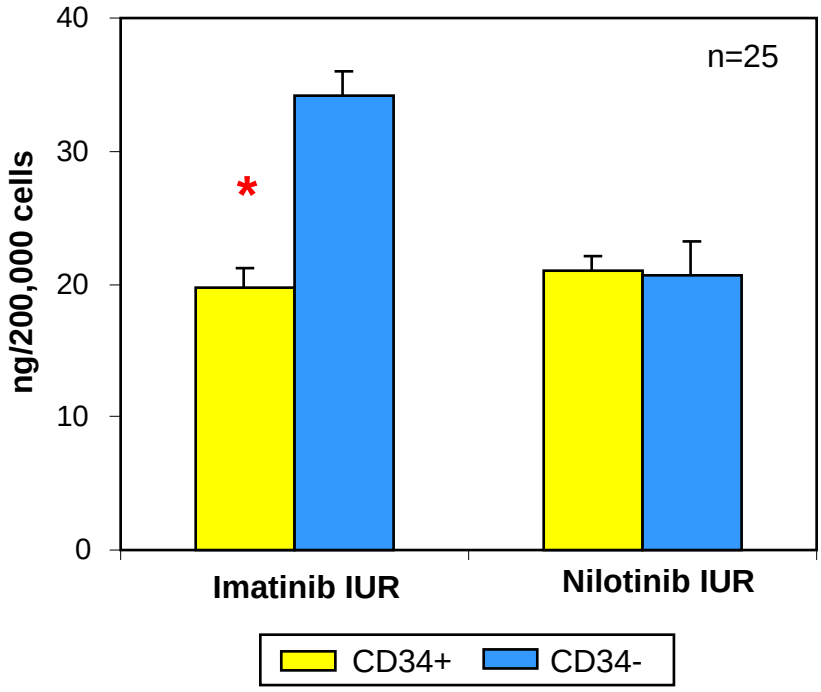


Figure 6.7 Intracellular uptake of imatinib and nilotinib in CML CD34+ and CD34- cells

Primitive CD34+ cells (yellow columns) and mature CD34- cells (blue columns) were isolated from the BM and PB of diagnosis CML patients. The IUR of imatinib and nilotinib were measured in 25 CP-CML patients.

Columns represent the mean plus the standard error of the mean.

* denotes $p < 0.001$ in comparison to corresponding CD34+ cells

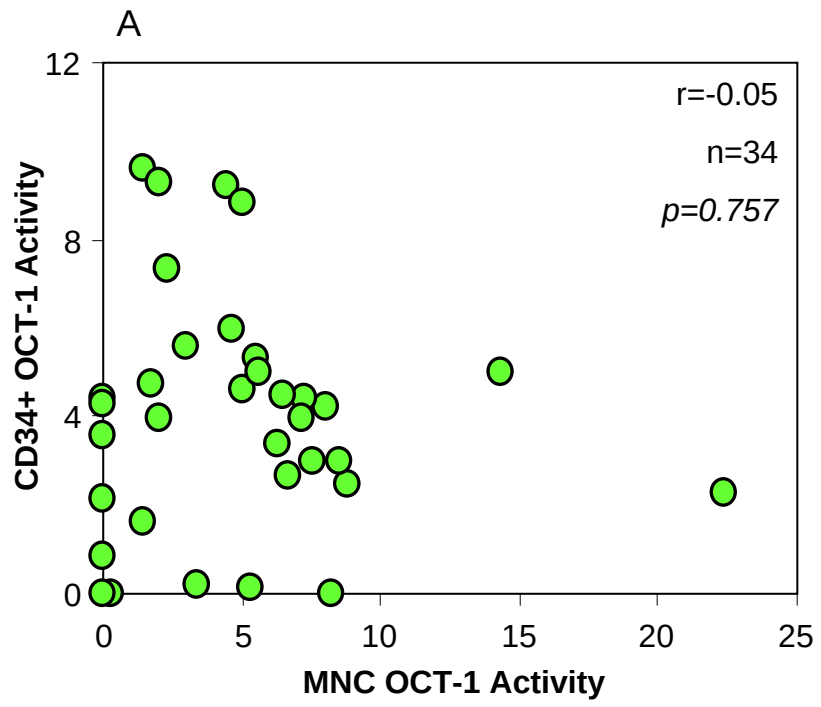
a relationship between a patient's CD34+ OCT-1 activity and their predictive MNC OCT-1 activity exists, MNC and CD34+ cells were isolated from 34 CP-CML patients and their OCT-1 activity was assessed. No significant relationship was observed between CD34+ and MNC OCT-1 activity from these matched patients (r=-0.05, p=0.757, Figure 6.8A). Patients were subsequently divided into low (n=25) and high (n=9) OCT-1 activity groups based on their MNC OCT-1 activity (about 7.2 ng/200,000 cells [110]) and the corresponding CD34+ OCT-1 activities were compared between the two groups. No significant difference in CD34+ OCT-1 activity was observed between these two groups (p=0.278, Figure 6.8B) with a median CD34+ OCT-1 activity of 4.38 ng/200,000 cells (range: 0-9.6) in the low MNC group and a median of 3.0 ng/200,000 cells (range: 0-5) in the high MNC group. Therefore, having a higher than median MNC OCT-1 activity is not associated with having a higher than median CD34+ OCT-1 activity.

6.2.8 MNC OCT-1 activity and achievement of major molecular response (MMR)

To confirm the predictive value of the MNC OCT-1 activity, MNC OCT-1 activity was assessed against individual patient's molecular response over the first 12 months of imatinib treatment in 34 CP-CML patients (Table 6.3). Patients were grouped into those who had achieved MMR (n=23) and those who had not (n=11) by 12 months of therapy. Those patients who achieved MMR had a significantly higher MNC OCT-1 activity with a median of 5.64 ng/200,000 cells (range: 0-22.4) compared with those who did not achieve MMR who had a median OCT-1 activity of 2.05 ng/200,000 cells (range: 0-6.5, p=0.028, Figure 6.9A). Furthermore, and in support of our previous findings [110], log-rank survival analysis revealed a significant association between MNC OCT-1 activity and the achievement of MMR. With 100% of patients with a high MNC OCT-1 activity (>7.2 ng/200,000 cells) achieving MMR by 12 months of imatinib treatment in comparison to 56% with a low MNC OCT-1 activity (p=0.05, Figure 6.10B). This association was also confirmed using Fisher exact analysis (p=0.017, Table 6.4).

Figure 6.8 Relationship between MNC and CD34+ OCT-1 activity in CML patients

(A) MNC OCT-1 activity and CD34+ OCT-1 activity was measured in 34 CP-CML patients. In these matched samples no correlation was observed between MNC OCT-1 activity and CD34+ OCT-1 activity. (B) In addition, patients were divided into high and low MNC OCT-1 activity groups (about the previously described median of 7.2 ng/200,000 cells {White, 2007 #218}). Their corresponding CD34+ OCT-1 activities were compared. No difference in CD34+ OCT-1 activity was seen between the two groups. Therefore, a high MNC OCT-1 activity is not indicative of whether a patient will have a high CD34+ OCT-1 activity.


A
12
8
4
0
r=-0.05
n=34
p=0.757
CD34+ OCT-1 Activity
0
5
10
15
20
25
MNC OCT-1 Activity

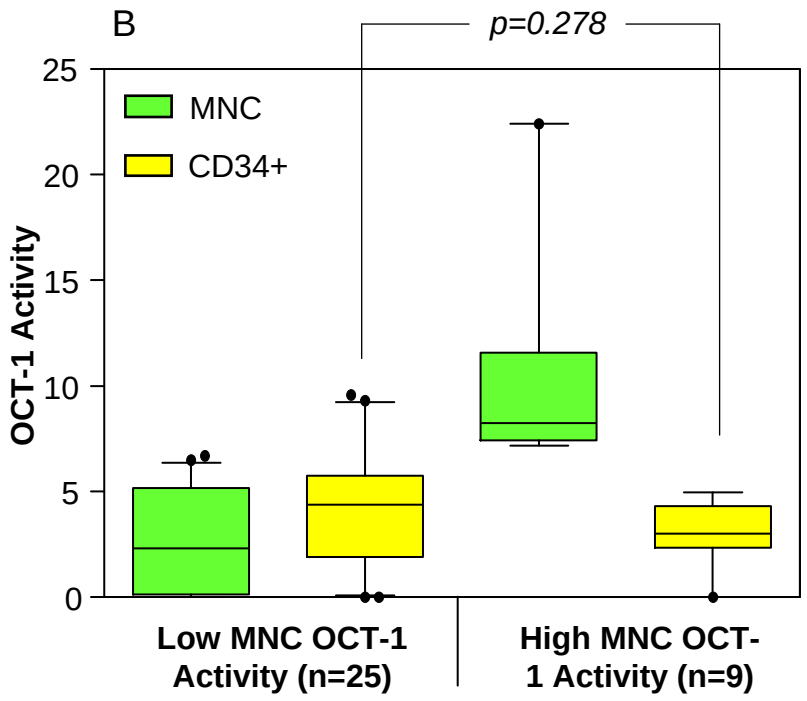

B
p=0.278
25
20
15
10
5
0
MNC
CD34+
OCT-1 Activity
Low MNC OCT-1 Activity (n=25)
High MNC OCT-1 Activity (n=9)

Figure 6.9 Relationship between MNC OCT-1 activity and achievement of MMR

(A) Patients were grouped according to the achievement of MMR by 12 months of imatinib treatment and the MNC OCT-1 activity was compared between the two groups.

Box-plots were constructed using GraphPad Prism 5.01 software and display the median value, the upper 25th and lower 75th percentiles and whiskers encompass the 10th and 90th percentiles.

* denotes $p < 0.05$ in comparison to achieved MMR

(B) Patients were grouped into high and low MNC OCT-1 activity groups (divided about the median of 7.2ng/200,000 cells). Kaplan-Meier curves demonstrate the achievement of MMR by 12 months. Significantly more patients with a high MNC OCT-1 activity achieved MMR by 12 months compared to those with low MNC OCT-1 activity.

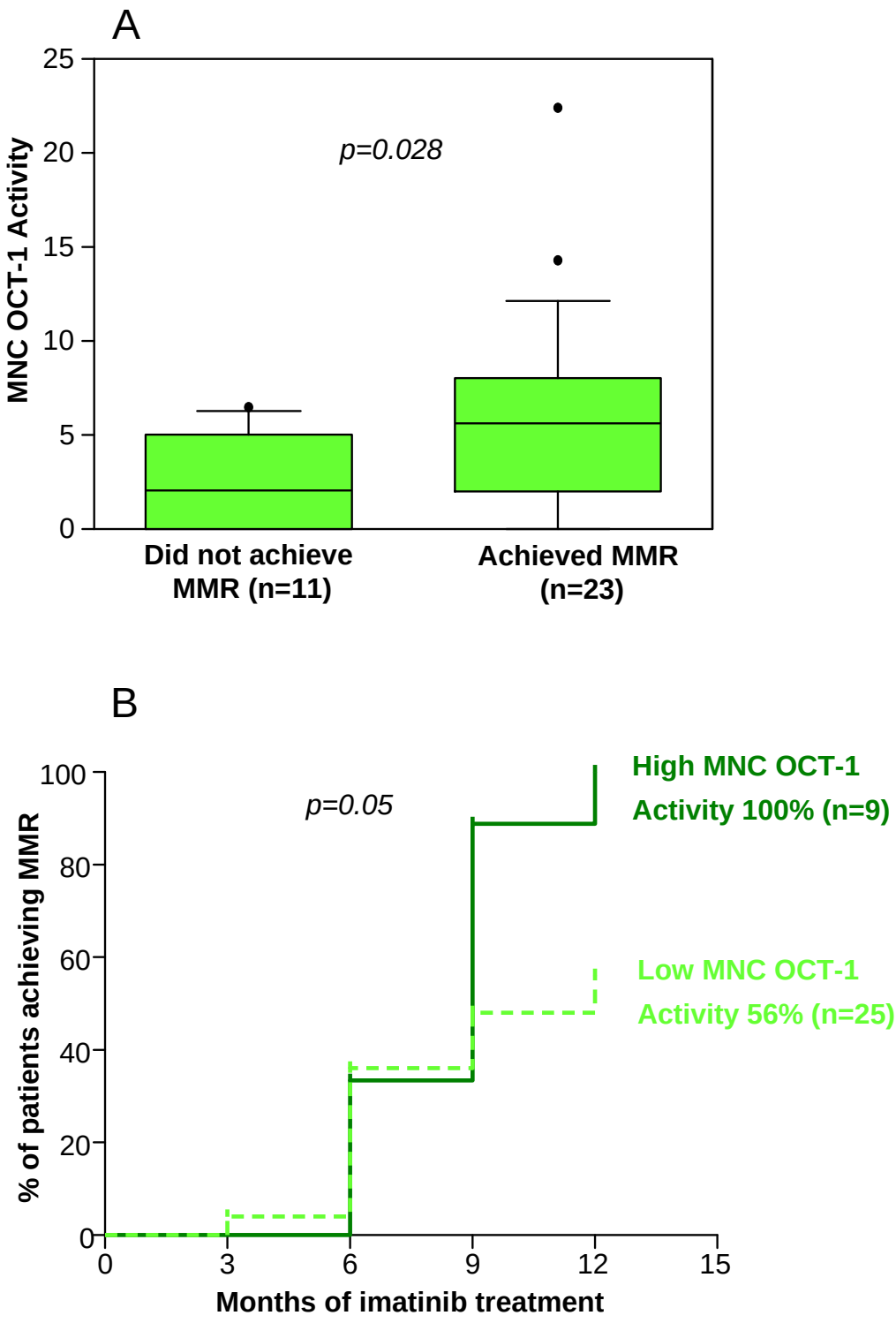
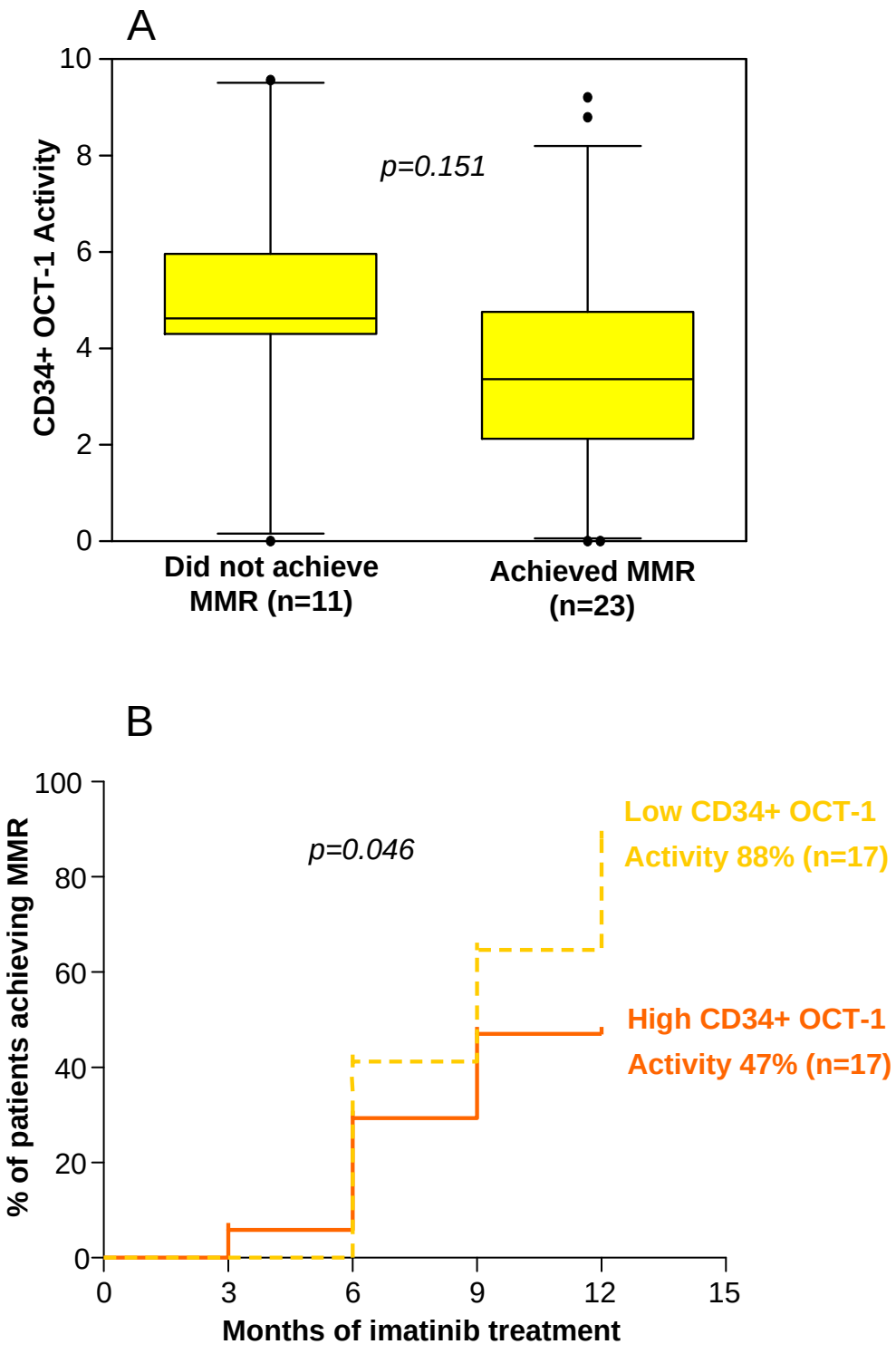

A
MNC OCT-1 Activity
25
20
15
10
5
0
p=0.028
Did not achieve MMR (n=11)
Achieved MMR (n=23)
B
% of patients achieving MMR
100
80
60
40
20
0
p=0.05
High MNC OCT-1 Activity 100% (n=9)
Low MNC OCT-1 Activity 56% (n=25)
0
3
6
9
12
15
Months of imatinib treatment

Figure 6.10 Relationship between CD34+ OCT-1 activity and achievement of MMR

(A) Patients were grouped according to the achievement of MMR by 12 months of imatinib treatment and the CD34+ OCT-1 activity was compared between the two groups. There was no significant difference in the CD34+ OCT-1 activity between patients who did and did not achieve MMR.

Box-plots were constructed using GraphPad Prism 5.01 software and display the median value, the upper 25th and lower 75th percentiles and whiskers encompass the 10th and 90th percentiles.

(B) Patients were grouped into high and low CD34+ OCT-1 activity groups (divided about the median of 4.0 ng/200,000 cells). Kaplan-Meier curves demonstrate the achievement of MMR by 12 months. In contrast to MNC, significantly more patients with a low CD34+ OCT-1 activity achieved MMR by 12 months compared to those with high. Therefore having a high CD34+ OCT-1 activity is not associated with better molecular responses.


A
CD34+ OCT-1 Activity
10
8
6
4
2
0
p=0.151
Did not achieve MMR (n=11)
Achieved MMR (n=23)
B
% of patients achieving MMR
100
80
60
40
20
0
p=0.046
Low CD34+ OCT-1 Activity 88% (n=17)
High CD34+ OCT-1 Activity 47% (n=17)
0
3
6
9
12
15
Months of imatinib treatment

6.2.9 CD34+ OCT-1 activity and achievement of major molecular response (MMR)

To identify if CML CD34+ OCT-1 activity is also predictive of molecular response to imatinib, the CD34+ OCT-1 activity from 34 CP-CML patients was assessed against their molecular response over the first 12 months of imatinib treatment (Table 6.3). Patients were again stratified into a group who had achieved MMR (n=23) or not (n=11) by 12 months of therapy. Patients who achieved MMR had a median CD34+ OCT-1 activity of 3.36 ng/200,000 cells (range: 0-9.2) which was not significantly different from those who did not achieve MMR with a median CD34+ OCT-1 activity of 4.62 ng/200,000 cells (range: 0-9.6, p=0.151, Figure 6.10A). This group of patients were subsequently divided about the median CD34+ OCT-1 activity of 4.0 ng/200,000 cells into high and low CD34+ OCT-1 activity groups and their probability of achieving MMR by 12 months was assessed. Log-rank survival analysis revealed that 47% of patients with a high CD34+ OCT-1 activity achieved MMR by 12 months in comparison to 88% with a low CD34+ OCT-1 activity (p=0.046, Figure 6.10B). Again, this was confirmed using Fisher exact analysis (p=0.026, Table 6.4). Therefore, unlike MNC, a low OCT-1 activity in patient's CD34+ cells appears to be associated with better molecular responses.

Table 6.3: OCT-1 activity and achievement of MMR

Patient Number	MNC OCT-1 activity	CD34+ OCT-1 activity	MMR at 12 months
2	4.50	9.21	✓
3	7.20	3.93	✓
4	7.58	3.00	✓
7	2.05	9.29	×
8	1.46	9.56	×
9	0.00	3.54	✓
10	5.31	0.15	✓
12	5.04	8.79	✓
17	8.03	4.20	✓
18	2.31	7.31	✓
19	7.28	4.39	✓
20	8.24	0.00	✓
21	8.85	2.45	✓
22	4.65	5.96	×
23	2.01	3.97	✓
24	5.03	4.62	×
25	0.00	4.38	×
26	6.49	4.50	×
27	5.64	4.98	×
28	6.29	3.36	✓
29	0.27	0.00	×
30	8.50	2.97	✓
31	6.70	2.66	✓
32	1.78	4.76	✓
33	0.00	0.83	×
34	0.00	2.12	✓
35	0.00	0.00	✓
36	3.40	0.16	✓
37	1.45	1.65	✓
38	0.00	4.30	×
39	2.97	5.57	×
40	22.40	2.29	✓
41	5.48	5.30	×
42	14.31	4.96	✓

Table 6.4: OCT-1 activity and molecular response		Achieved MMR (% of n)	Did not achieve MMR (% of n)
MNC (p=0.017)	High OA (n=9)	9 (100%)	0 (0%)
	Low OA (n=25)	14 (56%)	11 (44%)
CD34+ (p=0.026)	High OA (n=17)	8 (47%)	9 (53%)
	Low OA (n=17)	15 (88%)	2 (12%)

Shown is the number of patients who did and did not achieve a major molecular response (MMR) over the first 12 months of imatinib therapy. P-values were calculated using the Fisher Exact test.

6.3 Discussion

As CML is a clonal disease, arising from an oncogenic stem cell, it is not surprising that disease persistence and hence relapse has been attributed to a population of surviving leukaemic stem cells. It has been well established that primitive CML cells are resistant to imatinib-induced cell death. Firstly, *in vitro* observations showed CD34+ cells isolated from CML patients, are able to survive and produce colonies in the presence of imatinib [125, 126, 128]. Secondly, *in vivo* observations show the presence of persisting CD34+/Ph+ cells in patients who have achieved CCR [123, 124]. Studies have also suggested that increased mRNA expression of the efflux proteins ABCB1 and ABCG2 and decreased expression of OCT-1 may represent potential reasons underlying the resistance of primitive CML cells to imatinib [93, 131]. Therefore, the first aim of the present chapter was to investigate the intracellular concentration of imatinib achieved in the primitive CML CD34+ compartment and to examine the expression and function of OCT-1, ABCB1 and ABCG2 in these cells.

Our laboratory has previously demonstrated that the intracellular concentration of imatinib achieved in patients PB-MNC is significantly correlated with their level of BCR-ABL kinase inhibition *in vitro* [79]. Therefore, the balance of influx and efflux of imatinib is critical for adequate kinase inhibition in patients and hence response to treatment. This chapter demonstrates that the intracellular concentration of imatinib achieved in patient's primitive CD34+ cells is significantly lower than that found in their mature CD34- cells and the concentration is even lower in the more primitive CD34+38- cells. This is in agreement with a recent study that shows CML CD34+38- cells exhibit reduced imatinib uptake compared to CML CD34+ cells [149]. These findings raise the question, is the reduced intracellular concentration in CML primitive cells a result of reduced influx, increased efflux or a balance of both?

To investigate the role of imatinib influx in these primitive CD34+ and CD34+38- cells prazosin was utilized in the IUR assay to inhibit OCT-1 mediated uptake. The addition of prazosin was found to reduce the intracellular concentration of imatinib in both CML CD34+ and CD34- cells. Of note, the addition of prazosin removed the difference in imatinib IUR between CML CD34+ and CD34- cells, indicating that the initial difference in imatinib concentration between primitive and mature cells was likely to be due to OCT-1 mediated influx. In agreement with this notion, the OCT-1 activity was found to be lowest in the CML CD34+38- population followed by the CML CD34+ cells and highest in the mature CML CD34- cells. These data suggest that reduced intracellular accumulation of imatinib in primitive CML CD34+ and CD34+38- cells, mediated via a reduced OCT-1 activity, may contribute to incomplete imatinib-induced BCR-ABL kinase inhibition [127, 132], and may contribute to their increased survival [126], when compared to their more mature counterparts.

It is interesting to note, that when comparisons were made between primitive CD34+ and mature CD34- cells from CML and normal donors, the OCT-1 activity did not differ between CML CD34+, normal CD34+ and normal CD34- cells. These data show that primitive CD34+ cells have uniformly low OCT-1 activity, which is independent of leukaemic status. This is in agreement with the studies presented in Chapter 3, which show that OCT-1 activity is an intrinsic feature of a defined cell population, regardless of it being isolated from CML or normal donors. Furthermore, these studies are in agreement with the studies in Chapter 5, which showed that BCR-ABL itself does not have a direct effect on OCT-1 activity. Lastly, the low OCT-1 activity in normal CD34- cells and the high OCT-1 activity in CML CD34- cells is most likely due to the high proportion of lymphocytes and granulocytes, respectively, in these populations.

RT-PCR was then utilized to examine if this low OCT-1 activity in primitive CD34+ cells is a reflection of low OCT-1 mRNA expression in these cells. In the present chapter it is observed

that CML CD34+ cells have a reduced OCT-1 mRNA expression compared with mature CML CD34- cells. This is in agreement with the work of Jiang, *et al* [131] and supports the notion that this reduction in OCT-1 mRNA may contribute its reduced function in CML CD34+ cells. Low OCT-1 mRNA expression was also observed in normal CD34+ cells, again suggesting that reduced OCT-1 is a common feature of primitive CD34+ cells regardless of leukaemic status. However, no direct correlation was found between the level of OCT-1 mRNA expression and OCT-1 activity in CML patient's CD34+ and CD34- cells. This is in agreement with data presented in Chapter 3 and indicates that while low OCT-1 mRNA expression is likely to be contributing to low OCT-1 activity in primitive CML CD34+ cells, it is not the only contributing factor, and other variables may be more important in determining OCT-1 activity.

It has been reported that haematopoietic stem cells from CML patients express high levels of ABCB1 [131] and ABCG2 [93, 131], raising the possibility that efflux may play a role in stem cell resistance to imatinib. In accord with previous findings [131], the findings presented in this chapter suggest that CML CD34+ cells express significantly higher ABCB1 mRNA than mature CML CD34- cells. However, in functional assays, the findings presented here show that the intracellular concentration of imatinib was not found to be altered in CML CD34+ or CD34- cells with inhibitors of ABCB1 or ABCG2. Studies by Hatziieremia *et al* and Jordanides *et al* have demonstrated similar findings, where inhibition of ABCB1 or ABCG2 did not alter the intracellular concentrations of imatinib in CML CD34+ cells, nor enhance the anti-proliferative effects of imatinib [93, 149]. Hence, despite high mRNA expression of these efflux pumps in CML progenitors, these transporters appear to play a minimal role in the efflux of imatinib in CML CD34+ cells and are therefore not a major contributor to the reduced imatinib concentration that was observed in these cells.

Studies in our laboratory [79], and by others [187], have reported that, unlike imatinib, the OCT-1 protein is not involved in nilotinib transport and speculate that its uptake occurs primarily via

passive mechanisms. In addition, the interaction of nilotinib with ABCB1 and ABCG2 is controversial, with contradictory evidence showing that nilotinib does not interact with these proteins [187] and that nilotinib does act as a substrate, an inhibitor, or both [164, 188, 189]. In this chapter it was demonstrated that in contrast to imatinib, there was no significant difference in the intracellular concentration of nilotinib achieved between CML CD34+ and CD34- subsets. This suggests that there are no substantial differences in uptake and efflux of nilotinib between primitive and mature CML cells. It would therefore be anticipated that nilotinib may be better suited to target CML progenitors in comparison to imatinib. In agreement with this, nilotinib has been shown to induce greater p-Crkl inhibition in CML CD34+ cells in comparison to imatinib at equivalent concentrations [128]. However, despite this, nilotinib does not appear to be any more effective than imatinib in inducing apoptosis in CML CD34+ cells [127, 128]. Speculatively, this may support the hypothesis that CML progenitors survive TKI treatment through BCR-ABL independent mechanisms [128, 190].

The second aim of the present chapter was to investigate the predictive value of the CD34+ OCT-1 activity. Our laboratory has previously demonstrated that MNC OCT activity measured in a patient at diagnosis is a strong predictor of molecular response, event free and transformation free survival in response to imatinib treatment [110, 111]. It has been indicated in Chapters 3 and 4 that CML MNC OCT-1 activity may be influenced by the majority cell populations within the patient's blood at diagnosis. It is also possible that the MNC OCT-1 activity assay is providing a surrogate indicator for how efficiently the primitive population is being targeted. To address this possibility, this chapter examined the relationship between the OCT-1 activity in a patient's MNC, the OCT-1 activity in their primitive CD34+ cells and molecular response to imatinib therapy.

These studies confirmed that MNC OCT-1 activity is predictive of MMR, where significantly more patients with a high MNC OCT-1 activity achieved MMR by 12 months of therapy. It was

anticipated that CD34+ OCT-1 activity would be an equivalent, if not better predictor of response than MNC OCT-1 activity, since effective targeting of primitive cells would be considered to be a prerequisite for a deep molecular response. However, it was found that low CD34+ OCT-1 activity was associated with a greater chance of achieving MMR. Furthermore, no correlation was observed between CML patients MNC and CD34+ OCT-1 activities. This indicates that unlike MNC OCT-1 activity, high OCT-1 activity in patients CD34+ cells is not associated with superior molecular responses to imatinib. These observations raise the important question - why is the OCT-1 activity in end stage CML cells strongly predictive of response when it is presumably the fate of primitive leukaemic cells that is the determinant of the log term risk of disease progression and drug resistance?

Several studies have attributed the resistance of primitive CML cells to imatinib to the autocrine production of cytokines such as IL-3 [130, 131], G-CSF [130, 131] and GM-CSF [185]. This has been confirmed in cell line studies where BCR-ABL expression initiates factor independence through secretion of IL-3 and GM-CSF [186]. However, resistance through paracrine mechanisms is not as well understood. Studies from our laboratory have shown that mature CML MNC secrete GM-CSF and co-culturing CML CD34+ cell with mature MNC increased their proliferation and their survival in the presence of imatinib [191]. Furthermore, serum levels of GM-CSF and G-CSF have been shown to be elevated *in vivo* in CML patients compared to normal [192]. Similarly, mice transplanted with BCR-ABL transduced bone marrow display increased transcripts and serum levels of IL-3 and GM-CSF [193]. From this, we speculate that a rapid depletion of mature CML cells by imatinib (as predicted by the MNC OCT-1 activity assay) may deprive CD34+ cells of essential cytokines normally produced by the mature leukaemic population. This cytokine-depleted environment may remove the proliferative advantage of leukaemic haematopoiesis, facilitating the regrowth of the residual non-leukaemic haematopoietic cells which will be reflected in deeper molecular responses. Alternatively, the

source of drug resistance and disease progression is not from the primitive progenitor population.

The data presented in this chapter provides the basis for the following conclusions. Firstly, reduced functional activity of the OCT-1 protein in CP-CML CD34+ and CD34+38- cells appears to be a major contributor to low intracellular accumulation of imatinib in these cells. Therefore, this may significantly contribute to the persistence of these primitive leukaemic cells despite long-term imatinib treatment. Secondly, high CD34+ OCT-1 activity (unlike MNC OCT-1 activity) is not associated with favourable molecular responses to imatinib treatment in CML patients. Therefore, the depletion of mature CML cells may facilitate a lower risk of drug resistance and disease progression, possibly due to depletion of essential cytokines and chemokines. The exciting and novel implication of these findings is that direct targeting of this primitive population may not actually be essential for achievement of early and deep molecular responses, and the low risk of progression associated with these responses in CP-CML patients treated with TKI's.

7 DISCUSSION

7.1 Major Findings

7.1.1 Biological parameters underlying primary resistance to imatinib therapy

The introduction of tyrosine kinase inhibitor (TKI) therapy has revolutionized treatment for CP-CML patients. The first TKI developed, imatinib, induces molecular and cytogenetic remissions for the majority of CP-CML patients, with 86% of patients achieving MMR by 8 years of therapy [58]. However, this impressive statistic does not take into account the 45% of patients who discontinue therapy due to adverse effects, unsatisfactory response or other reasons [58]. A more recent trial, which did not censor patients who discontinued therapy, demonstrated that 27% of imatinib-treated CP patients failed to achieve CCR by 12 months of therapy [75]. Together, this indicates that approximately one third of patients' exhibit sub-optimal responses to imatinib, and it is these patients who are more likely to progress to AP or BC. As such, considerable effort has been made to define this group of poor response patients at diagnosis in order to modify their therapy and improve their outcomes.

A major determinant underlying sub-optimal responses to imatinib therapy in CP-CML patients is inadequate inhibition of the kinase activity of BCR-ABL [76] and this has been linked to low intracellular concentrations of imatinib achieved in target leukaemic cells collected at diagnosis [79]. OCT-1 has been identified as the major active influx pump for imatinib in CML cells [79, 82] and as such has been investigated as a cause of sub-optimal response in patients treated with imatinib. An *in vitro* assay which utilizes the functional status of OCT-1, has proven to be a powerful prognostic tool for the determination of short and longer-term patient responses on imatinib therapy [110, 111]. OCT-1 activity, in a patient's PB-MNC at diagnosis, while highly variable (0-31.2 ng/200,000 cells) is predictive of MMR, CMR, event-free and transformation free survival over 5 years of imatinib therapy [111]. OCT-1 activity therefore provides a strong prognostic test to identify patients who will and those who may not respond well to imatinib

therapy. However, the reasons underlying inter-patient variation in OCT-1 activity, and hence response to imatinib, are not well understood.

One hypothesis is that the observed inter-patient variation in OCT-1 activity may be related to variable OCT-1 mRNA expression. A number of studies have identified a correlation between OCT-1 mRNA levels in patients and cytogenetic responses to imatinib [96, 97]. However, as shown in Chapters 3, 4 and 6, no significant correlation between OCT-1 activity and OCT-1 mRNA expression could be identified when measured in MNC, neutrophils, monocytes, lymphocytes or primitive CD34+ cells. This observation has been described previously by White *et al*, who showed that only a weak correlation is observed between OCT-1 activity and mRNA expression [110]. As such, OCT-1 transcript levels in CP patients at diagnosis is not a reliable predictor of cytogenetic and molecular responses to imatinib treatment [109, 111].

Two recent studies have shown that polymorphonuclear cells possess a significantly higher OCT-1 mRNA expression compared with MNC, in both healthy donors and CML patients at diagnosis [115, 147]. These findings raised the possibility that the cellular composition of PB-MNC may be a strong determinant of patient variation in OCT-1 activity. As described in Chapter 3, the OCT-1 activity in MNC recovered from normal donors was significantly reduced compared to that of CML patients at diagnosis. Furthermore, the MNC compartment in CML patients at diagnosis was found to be primarily comprised of maturing (myelocytes, metamyelocytes) and mature neutrophils, while in normal donors the population was >50% lymphocytes. In view of these findings, OCT-1 activity and mRNA expression were compared between pure populations of neutrophils, monocytes and lymphocytes. In keeping with the observations of Bazeos, *et al* and Racil *et al* [115, 147], the highest OCT-1 activity and mRNA expression were consistently found in neutrophils, followed by monocytes then lymphocytes. Based on these findings, the difference in MNC OCT-1 activity between CML patients at diagnosis and normal donors is most likely a reflection of the differences in the number of

neutrophils and lymphocytes found in their peripheral blood. In addition, a significant correlation was observed between MNC OCT-1 activity and neutrophil OCT-1 activity in both CML patients and normal donors, suggesting that OCT-1 activity in the neutrophil lineage is likely to be a key contributor to overall MNC OCT-1 activity in CML patients at diagnosis.

A number of studies suggest that BCR-ABL signalling regulates the expression of the efflux transporters ABCB1 and ABCG2 [163-165]. Furthermore, it has been recently suggested by Bazeos *et al* that BCR-ABL may down-regulate OCT-1 mRNA expression [115]. As such, the relationship between BCR-ABL expression and OCT-1 activity was assessed. As presented in Chapter 3, no significant correlation was found between patient BCR-ABL mRNA expression levels and OCT-1 activity in either their MNC or neutrophil populations. OCT-1 activity was also compared between CML patients at diagnosis (BCR-ABL expressing) and CML patients in cytogenetic remission (very low BCR-ABL) and normal individuals (no BCR-ABL). When the neutrophil OCT-1 activity was compared between diagnosis CML, remission CML and normals, no significant difference was observed. Similar results were seen for both monocytes and lymphocytes. These data suggest that the presence of BCR-ABL in CML patients at diagnosis does not influence OCT-1 activity. To address these findings further, the effect of BCR-ABL on OCT-1 activity was examined using the promyelocytic HL60 cell line transduced with control or ^{p210}BCR-ABL lentivirus. As demonstrated in Chapter 5, the presence of BCR-ABL had no direct effect on OCT-1 activity or mRNA expression. Interestingly, the presence of BCR-ABL expression was found to enhance differentiation of the HL60 cell line from a blast cell to either a granulocyte or monocytes under direct stimulation with DMSO or PMA. This supports the findings from previous studies, in which increased numbers of myeloid colonies were observed from cord blood-derived CD34+ cells [26] and mouse embryonic stem cells [174] with ectopic BCR-ABL expression. The enhanced differentiation, mediated by BCR-ABL, was associated with a significant increase in OCT-1 activity. These findings suggest that while BCR-ABL does

not directly influence OCT-1 activity, it promotes myeloid differentiation, which as previously demonstrated, affects OCT-1 activity.

Taken together, these findings suggest that the greatest contributor to variability in OCT-1 activity is cell lineage. Furthermore, BCR-ABL expression has an indirect effect on OCT-1 activity by enhancing granulocyte differentiation. Two possible conclusions may be drawn from these findings. Firstly, patient OCT-1 activity may be intrinsic to the patient and independent of their leukaemia. If this is the case then their OCT-1 activity in specific cell populations (ie: neutrophils) will not change over time. This could be of importance for institutions that do not have immediate access to the OCT-1 activity assay. In these cases, therapy could be initiated at diagnosis and OCT-1 activity measured in neutrophils whenever possible. However, results from Chapter 3 suggest that long-term treatment with imatinib may influence OCT-1 activity, complicating this possibility. The second conclusion is that patient OCT-1 activity may be a consequence of their leukaemic cell of origin. While it is hypothesized that the BCR-ABL translocation arises in a HSC, it may be possible that it arises in a more committed progenitor. Furthermore, BCR-ABL may arise in different progenitor cells (ie: HSC, MPP, CMP) in different patients. Therefore, the majority cell population in a patient's blood at diagnosis may be influenced by the leukaemic cell of origin and as such causes variable responses to imatinib.

7.1.2 Prediction of primary resistance to imatinib therapy

Despite the powerful predictive value of the OCT-1 activity assay, there are major limitations in transferring the OCT-1 activity assay to diagnostic laboratories. This is predominantly due to the safety and availability of [^{14}C]-labelled imatinib and the equipment required. Therefore, identifying a technically simpler surrogate measure of OCT-1 activity is critical to establish this test as routine clinical practice.

Immunological phenotyping has long been an established method for the diagnosis and prognosis of acute leukaemia's [152, 154]. However, its use in CML has been much more limited, with studies being restricted to immunophenotypic analysis in blast crisis patients [155, 156] or chronic phase patients receiving hydroxyurea or interferon-α [143, 157]. The recent studies of Racil *et al* demonstrated that total WBC's in diagnosis CML patients contained between 0-60% immature myeloid cells (promyelocytes, myelocytes and metamyelocytes), as determined by microscopy [147]. Consistent with these findings, morphological analysis presented in Chapter 3, showed that the MNC compartment in CP-CML patients at diagnosis consists of variable numbers of mature and immature neutrophils. As demonstrated in Chapter's 3 and 4, utilizing immunophenotyping, these MNC cells were then separated into groups based on the expression of the granulocytic antigens, CD15 and CD16. CD15+16+ cells were found to be mature neutrophils, while CD15+16- cells were found to be a mix of promyelocytes, myelocytes, metamyelocytes and neutrophils (Chapter 4). The OCT-1 activity in the "immature" CD15+16- cells was greater than that seen in mature neutrophils and was most closely matched to that observed in MNC. Furthermore, after cryopreservation, a significant positive correlation between MNC OCT-1 activity and the percentage of CD15+16- cells in a patients MNC was observed. While requiring analysis in a much larger cohort, these data suggest that MNC OCT-1 activity is a feature reflective of the number of immature myeloid cells in a patient's blood.

This simple, fast and easily transportable method of measuring CD15 and CD16 expression in MNC is an ideal alternative to OCT-1 activity measurement as it is a method which can be easily adopted into any diagnostic laboratory. Having a strong and reliable predictor of response to imatinib therapy at diagnosis is critical to identify those patients who are likely to have suboptimal responses. For these patients it may be desirable to give them alternative up-front therapies to improve their responses and to prevent the development of secondary resistance. This is critical in view of studies which demonstrate that patients who do not

achieve a MCR at 6 months have an increased chance of developing BCR-ABL kinase domain mutations [61]. Current clinical practice is to start CP patients on an initial dose of 400mg imatinib daily [194]. However, increasing the initial dose of imatinib to 600-800mg has been shown to induce faster rates of molecular response [195-197]. Furthermore, the poor molecular response seen in patients with low OCT-1 activity treated with <600mg can be overcome by treatment with ≥600mg [110]. However, this increased imatinib dose is accompanied by increased levels of toxicity and as such, dose interruptions, as demonstrated in a small number of clinical trials [197]. Front-line therapy with the second-generation tyrosine kinase inhibitors nilotinib and dasatinib has shown improved outcomes compared to imatinib [69, 73]. Up-front treatment with these TKIs may be the ideal therapy choice for patients predicted to have poor response to imatinib (low OCT-1 activity/low CD15+16-%), as the influx of both nilotinib and dasatinib is not dependent on OCT-1 and is likely to be passive [79, 198].

7.1.3 The role of OCT-1 in disease persistence

Another major challenge for CML patients is that while imatinib results in excellent long-term responses for many patients, 50% of patients relapse upon imatinib cessation [119, 120, 122]. This is most likely due to the inherent resistance of primitive CD34+ and CD34+38- CML cells to imatinib-induced cell death [126, 128]. The initiating lesion in CML, most likely occurs in a HSC and as such this initiating CML cell needs to be eradicated in order to fully eliminate minimal residual disease.

Using the HL60 cell line model, it was found that the OCT-1 activity and mRNA expression increased in cells as they matured from blast cells to granulocytes or monocytes (Chapter 5). This was mirrored in patient samples where primitive CML CD34+ cells exhibited low OCT-1 mRNA expression in comparison to mature CML cells (Chapter 6), which is in agreement with the study of Jiang *et al* [131]. In addition to this, in a cohort of 36 patients, the OCT-1 activity in CML CD34+ cells was significantly reduced compared to more mature counterparts.

Furthermore, the more primitive CD34+38- CML cells had even lower OCT-1 activity compared to CD34+ cells. Therefore, a key factor relating to the resistance of primitive CML cells to imatinib treatment is likely to be low intracellular accumulation of imatinib in these cells due to low OCT-1 expression and function, resulting in inadequate BCR-ABL kinase inhibition.

It was hypothesized that patients with high MNC OCT-1 activity would also have high CD34+ OCT-1 activity. As such, good molecular responses to imatinib may be related to both the efficient targeting and de-bulking of the mature population and the eradication of progenitor CML cells. However, as Chapter 6 demonstrated, the OCT-1 activity in a patient's CD34+ cells did not correlate with the OCT-1 activity in their mature MNC cells. Therefore, patients with a high MNC OCT-1 activity and hence high uptake of imatinib into their MNC did not necessarily have high imatinib uptake in their primitive CD34+ cells. Further to this, it was confirmed that MNC OCT-1 activity is predictive of attaining MMR at 12 months of imatinib treatment. In contrast, CD34+ OCT-1 activity was not associated with the achievement of MMR. These data suggest that the MNC OCT-1 activity may actually be a measure of the efficiency of targeting the mature cell population and not a surrogate for primitive cell uptake of imatinib.

These findings raise the controversial question as to whether direct targeting of the primitive population is actually necessary to achieve good molecular responses on imatinib treatment. Longer follow up of these patients, to see if they achieve CMR, will be required in order to truly answer this question. However, it has been shown that 69% of patients who achieve MMR by 12 months of imatinib therapy go on to achieve CMR by 5 years of treatment (T.Hughes, unpublished data). In addition, MNC OCT-1 activity has been shown to be predictive of CMR at 5 years of imatinib therapy [111]. Therefore, it would be expected that there will be a lack of any relationship between high CD34+ OCT-1 activity and the achievement of CMR.

So why would efficient targeting of mature CML cells be more important for favourable long-term molecular responses rather than efficient targeting of primitive CML cells? *In vitro* studies from our laboratory have demonstrated that CML MNC secrete the growth factor GM-CSF and co-culturing CML CD34+ cells with MNC increases their proliferation and survival in the presence of imatinib [191]. While further investigations are required, we hypothesize that efficient targeting, and hence depletion of mature CML cells by imatinib, may deprive CML CD34+ cells of survival-promoting cytokines thereby making them more susceptible to eradication by imatinib. Hence, while MNC OCT-1 activity is not associated with direct targeting of progenitor CD34+ CML cells, it may be associated with indirect targeting of this primitive population.

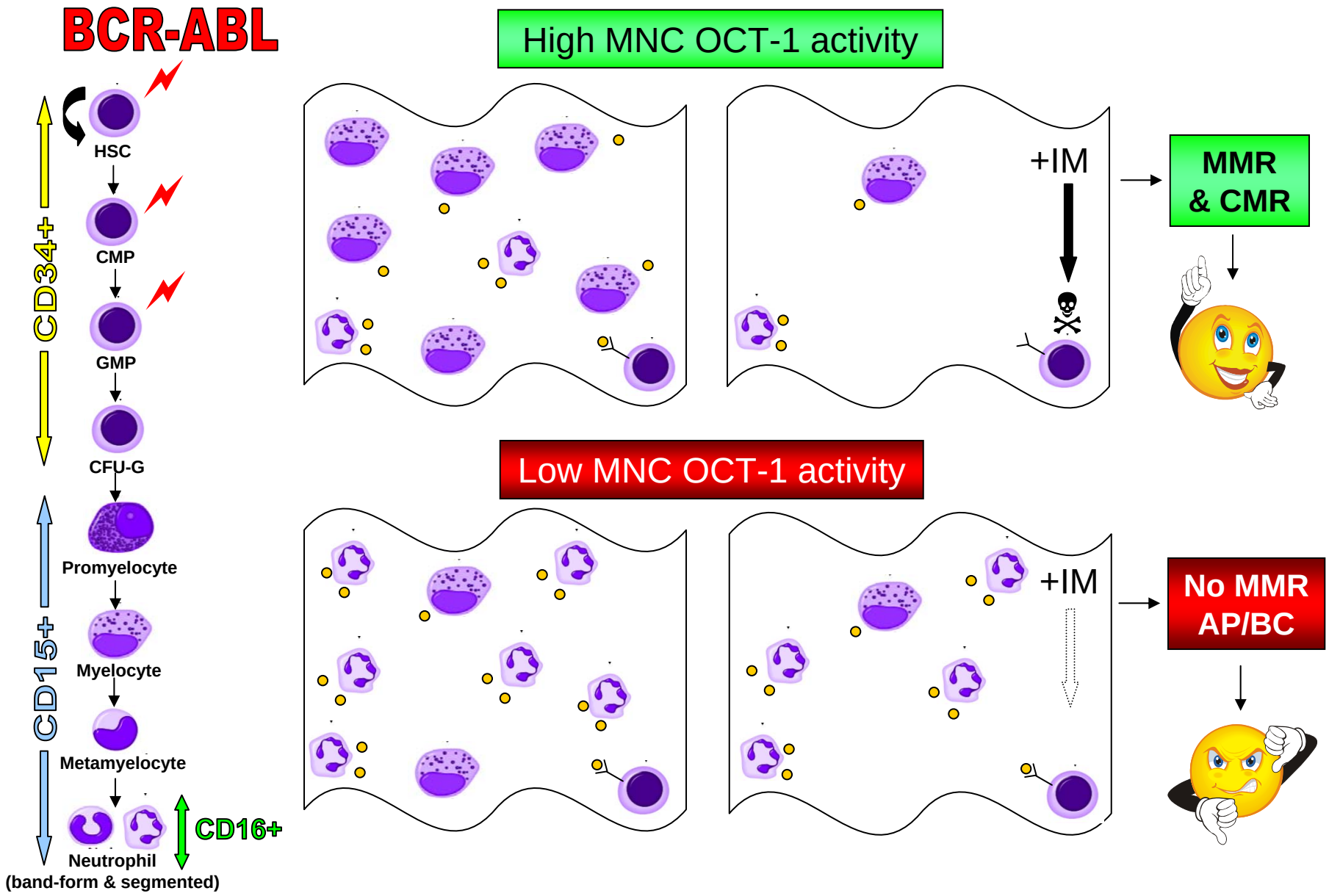
The important clinical implication of this hypothesis is that it may be unnecessary to find alternative treatment strategies to eliminate progenitor CML cells. Rather, effort should be directed towards inducing rapid and deep molecular responses to imatinib within the first 12-18 months of patient therapy. It has been shown that patients who achieve early molecular remissions go on to achieve better long-term outcomes and importantly long-term transformation free survival [199]. In keeping with this hypothesis, the data presented in this thesis suggests that efficient targeting of the mature CML cell population, resulting in early molecular responses, are likely to be associated with indirect targeting of the primitive population and as such long-term molecular remissions.

7.2 Summary

Based on the findings presented in this thesis, Figure 7.1 presents a current working hypothesis of patient responses to imatinib treatment. OCT-1 activity is a measure which is highly dependent upon cell lineage and maturation. OCT-1 activity varies between mature cells of different lineages (neutrophils, monocytes and lymphocytes), between maturation states (CD34+ and CD34-) and is independent of leukaemic status (BCR-ABL+ or BCR-ABL-).

Figure 7.1 Working model of cell type/OCT-1 activity interaction in CML patients

The formation of the Ph chromosome, and hence BCR-ABL, in a progenitor cells drives cells down the neutrophil lineage in CML patients, resulting in an excess of promyelocytes, myelocytes, metamyelocyte and neutrophils in the MNC population. Patient's who have a high OCT-1 activity in their MNC, and hence have favourable responses to imatinib treatment, tend to have a greater number of promyelocytes, myelocytes and metamyelocytes in their MNC. For these patients, treatment with imatinib results in efficient targeting of these cells, a rapid reduction in mature leukaemic cells, which depletes the local environment of cytokines and as such makes their primitive cells more susceptible to eradication and hence long-term remissions. Patients who have a low MNC OCT-1 activity, and therefore have poorer response to imatinib, tend to have fewer promyelocytes, myelocytes and metamyelocytes and more neutrophils in their MNC. Treatment with imatinib is much more inefficient in these patients, therefore not reducing leukaemic bulk sufficiently to reduce the cytokines present, as such their primitive cells survive in the presence of imatinib. Therefore, these patients are less likely to achieve molecular remissions and are more likely to progress to AP/BC.


BCR-ABL
HSC
CMP
GMP
CFU-G
Promyelocyte
Myelocyte
Metamyelocyte
Neutrophil
(band-form & segmented)
CD34+
CD15+
CD16+
High MNC OCT-1 activity
+IM
MMR
& CMR
Low MNC OCT-1 activity
+IM
No MMR
AP/BC

Transformation by BCR-ABL in a progenitor cell appears to promote differentiation and maturation toward the neutrophil lineage. This progression from primitive to mature state is associated with an increase in OCT-1 activity. Therefore, the low OCT-1 activity and intracellular concentration of imatinib achievable in primitive CML cells is likely to be a key contributor to their inherent resistance to imatinib. The cell types populating a patient's blood at diagnosis differs from patient to patient, with variable numbers of immature myeloid cells (promyelocytes, myelocytes and metamyelocytes) and mature neutrophils. Immunophenotyping of these cells identifies the percentage of immature CD15+16- cells, which strongly correlates with MNC OCT-1 activity. Therefore, those patients with a high percentage of CD15+16- cells have a high MNC OCT-1 activity, and as such have favourable responses to imatinib. In contrast, those patients with a low percentage of CD15+16- cells tend to have a low MNC OCT-1 activity, and hence poor responses to imatinib. Predicting imatinib response up-front allows for therapy changes to those patients likely to have poor responses. Treatment of these patients with nilotinib or dasatinib may be beneficial as these drugs are not transported by OCT-1.

Unlike MNC, the OCT-1 activity in primitive CD34+ cells is unrelated to response to imatinib. However, the OCT-1 activity in MNC does predict for complete molecular remission, therefore the primitive CD34+ cells must be targeted in some manner. This introduces the theory that paracrine secretion of cytokines from the mature MNC population may protect the primitive CML cells from imatinib. If these mature cells are not adequately eliminated (low MNC OCT-1 activity), the cytokine levels will be sufficient to promote the survival of primitive CML cells. As such, therapy should be directed toward intense early therapy to eradicate the bulk mature cell population, thereby making the progenitor cells susceptible to imatinib, which in turn will result in long-term molecular remissions.

7.3 Future Directions

As a result of the findings presented in this thesis the following studies have been proposed as essential for our further understanding of OCT-1 activity and will be pursued in our laboratory:

- Long-term follow-up (beyond 12 months) of imatinib treated patients who have had their CD34+ OCT-1 activity measured at diagnosis. Comparisons with MMR, CMR and survival.
- Studies more closely investigating paracrine secretion of cytokines from mature CML cells, and their protective effect on CD34+ cells. Investigate cytokine levels in the plasma of patients at diagnosis and during treatment and relate to their MNC OCT-1 activity and response to treatment.
- Increase the size of this cohort in order to assess the percentage of CD15+16- expressing cells in their MNC at diagnosis and relate to their MNC OCT-1 activity. In addition, more extensive immunophenotyping with additional granulocytic markers.

7.4 Conclusion

In conclusion, patient MNC OCT-1 activity, and hence response to imatinib treatment, is closely related to the myeloid cell populations present in their blood at diagnosis. Specifically, elevated numbers of immature myeloid cells may be associated with favourable responses to imatinib therapy. Efficient and rapid targeting of this cell population is most likely the key element in ensuring the achievement of molecular remissions and direct targeting of primitive CML cells may be unnecessary to attain this outcome.

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