Factors which impact on the response of CML patients to ABL kinase inhibitor therapy: A study of imatinib and nilotinib.

Deborah Lee Harland
(nee White)

The Melissa White Laboratory
Hanson Institute and
Institute of Medical & Veterinary Science
&
The Faculty of Health Science
Department of Medicine
The University of Adelaide
South Australia

Thesis submitted to the University of Adelaide
in candidature for the degree of Doctor of Philosophy
April 2008

Chapter 1

Introduction

1.1 Chronic Myeloid Leukaemia

Chronic Myeloid Leukaemia (CML) was first described as a specific disease entity approximately 150 years ago (Craigie 1845) (Bennett 1845). The current incidence of CML is approximately one to two in 100,000 people per year with a median age at presentation of 53 years (Sawyers 1999), with less than 10% of cases occurring in individuals less than 20 yeas of age (Cortes 2004).

CML is characterised by a biphasic course. In the majority of cases (over 80%) the disease is diagnosed in the chronic phase (Cortes 2004). The presenting characteristics of CML in chronic phase can be variable, with many patients diagnosed through routine blood examination, being asymptomatic (Cortes 2004). Of those patients with symptoms, the most commonly reported features are fatigue, night sweats, weight loss and splenomegaly, with its associated abdominal discomfort, early satiety and /or anorexia (Cortes 2004) (Savage, Szydlo & Goldman 1997).

The chronic phase blood picture consists of a benign infiltrate of excessive numbers of normal granulocytic series cells. These cells retain functionality and differentiate normally. After a variable period, but most commonly 3 to 5 years, most patients undergo an acceleration of their disease, which leads to blast crisis. This resembles an acute leukaemia in both appearance and behaviour. The blastic stage of the disease is fatal with a median survival of 3 to 9 months (Cortes 2004) (Savage *et al.* 1997).

The underlying predisposing factors, and the time required for the manifestation of the disease is unknown however, high dose irradiation is associated with a significantly increased risk of leukaemia. (Preston *et al.* 1994)

1.1.1 The Philadelphia Chromosome and BCR-ABL protein

The cytogenetic hallmark of CML is the Philadelphia (Ph) chromosome which is formed by a reciprocal translocation between chromosome 9 and chromosome 22 (Rowley 1973) (Caspersson *et al.* 1970). In this translocation the distal portion of the long arm of chromosome 22 is relocated to the long arm of chromosome 9 t(9;22)(q34;q11) Figure 1.1. Approximately 5% of patients with CML have complex or variant translocations involving additional chromosomes. These variant translocations do not appear to impact on the clinical course of CML. The Ph chromosome is also detected in 15 to 30% of patients

with acute lymphoblastic leukaemia (ALL) and in 2% of patients with acute myeloid leukaemia. (Kurzrock, Gutterman & Talpaz 1988) (Melo 1996)

The t(9;22)(q34;q11) translocation results in the c-abl oncogene being transposed from its normal position on chromosome 9 (q34) to a 5.8kb Major breakpoint cluster region (M-bcr), with in the BCR gene on chromosome 22 (22q11) (Groffen *et al.* 1984) (Heisterkamp *et al.* 1982) (de Klein *et al.* 1982).The resultant bcr-abl fusion gene translates to a protein of either 210 kD or 190kD, with a constitutively active tyrosine kinase. (Shtivelman *et al.* 1985) Figure 1.2. The resultant BCR-ABL protein specifically binds ATP resulting in phosphorylation on tyrosine residues and activation of downstream effector pathways, manifesting the disease as outlined above (Daley & Baltimore 1988) (Daley, Van Etten & Baltimore 1990).

Conclusive evidence for the involvement of BCR-ABL in the pathogenesis of CML come from various murine models. In these models where BCR-ABL has either been directly introduced into the mouse genome, or where BCR-ABL retrovirally transduced human cells have been engrafted into immunocompromised recipients, the overwhelming finding has been the presence of either a CML like syndrome, or an acute leukaemia phonotype (Daley 1993; Daley *et al.* 1990; Daley, Van Etten & Baltimore 1991; Heisterkamp *et al.* 1990; Laneuville *et al.* 1992).

NOTE: This figure is included on page 4 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.1 The formation of the Philadelphia chromosome

This diagram demonstrates the formation of the Philadelphia chromosome, which results from a reciprocal translocation of genetic materials between the long arms of chromosomes 9 and 22.
Adapted from Goldman and Melo (Goldman & Melo 2003)

NOTE: This figure is included on page 5 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2 The formation of the BCR-ABL gene leads to deregulation of normal signal transduction pathways.

The normally tightly regulated tyrosine kinase activity of the ABL proto-oncogene is deregulated by the replacement of its amino terminal sequence with that of the BCR gene. Signal transduction pathways that are vital for the regulation of normal haematopoiesis are then constitutively activated leading to deregulated proliferation, differentiation and reduced apoptosis
Adapted from Goldman and Melo (Goldman & Melo 2003)

1.1.2 Downstream effects of the BCR-ABL oncoprotein

BCR-ABL activates the Stat, (Shuai *et al.* 1996) (Ilaria & Van Etten 1996),PI-3 kinase (Gotoh *et al.* 1994), (Skorski *et al.* 1997) Myc, (Sawyers, Callahan & Witte 1992),Ras, (Mandanas *et al.* 1993) (Pendergast *et al.* 1993) and Jun (Raitano *et al.* 1995) pathways. This activation is primarily a result of phosphorylation on tyrosine residues mediated through adaptor proteins such as Grb-2, Crkl, Dok, Cbl and Shc (Ma *et al.* 1997; Muller *et al.* 1992; Puil *et al.* 1994). The constitutive activation of these pathways results in deregulated haemopoiesis, decreased apoptosis and altered bone marrow adherence (Figure 1.3).

In normal individuals haemopioetic cells are dependent on a stringent program of growth factor stimulation for differentiation and survival. In the CML setting, the presence of the constitutively active BCR-ABL protein, with its enhanced tyrosine kinase activity renders the cell growth factor independent. Additionally, it has been demonstrated that primitive CML cells are capable of producing their own growth factors. (Holyoake *et al.* 2001) (Eaves *et al.* 2003) (McGahon *et al.* 1994)

Apoptosis or programmed cell death follows terminal differentiation and may be activated by a cell upon detection of oncogene deregulation, stress induced stimuli or DNA damage (McKenna & Cotter 1997). Nuclear ABL is a pro-apoptotic protein that plays a role in the cellular response to genotoxic stress (Wang & Ki 2001). In contrast, BCR-ABL is an antiapoptotic protein and the suppression of pathways of apoptosis has been implicated in the pathogenesis of CML (Bedi *et al.* 1994). Various mechanisms for the observed decreased apoptosis have been postulated(Catlett-Falcone, Dalton & Jove 1999; Cortez, Kadlec & Pendergast 1995). BCR-ABL reportedly inhibits the interaction of STAT5 and BCL-xL (Horita *et al.* 2000). Furthermore the C-terminus of BCR-ABL has been implicated as a critical domain for facilitating increased proliferation and decreased apoptosis(Underhill-Day *et al.* 2006). The effects of BCR-ABL may also include induction of a prolonged cell cycle arrest, that allows for DNA repair after DNA damage. Such DNA damage, would most likely in normal cells be sufficient to induce apoptosis.

Normal haemopoietic progenitor cells adhere to the bone marrow stroma through interactions with fibronectin and β1 integrin receptors on their surface (Hurley, McCarthy & Verfaillie 1995). CML progenitors however are characterised by a premature release from the bone marrow microenvironment, most likely attributable to impaired stromal adherence (Gordon *et al.* 1987; Verfaillie *et al.* 1997). In contrast to normal cells, these prematurely released progenitors are then able to divide

outside of the marrow microenvironment, in extramedullary sites such as the spleen and liver. This division occurs outside of the normal integrin controlled mechanisms, and hence may be significantly enhanced under the control of BCR-ABL (Verfaillie *et al.* 1997) (Andreu *et al.* 2005; Jiang, Zhao & Verfaillie 2000) (Figure 1.3).

BCR-ABL can induce resistance to cytostatic drugs and irradiation by modulation of DNA repair mechanisms, cell cycle checkpoints and Bcl-2 protein family members. Upon DNA damage BCR/ABL not only enhances repair of DNA lesions (e.g. homologous recombination repair), but also prolongs activation of cell cycle checkpoints (e.g. G2/M) providing more time for the repair of otherwise lethal lesions (Mancini *et al.* 2007). Moreover, by modification of anti-apoptotic members of the Bcl-2 family (e.g. upregulation of Bcl-xL) BCR-ABL provides a cytoplasmic 'umbrella' protecting mitochondria from the 'rain' of apoptotic signals coming from the damaged DNA in the nucleus, thus preventing release of cytochrome C and activation of caspases (Kumar *et al.* 2003). The unrepaired and/or aberrantly repaired (but not lethal) DNA lesions resulting from spontaneous and/or drug-induced damage can accumulate in BCR/ABL-transformed cells leading to genomic instability and malignant progression of the disease (Skorski 2002). (Andreu *et al.* 2005) (Mancini *et al.* 2007) (Mazzacurati *et al.* 2004).

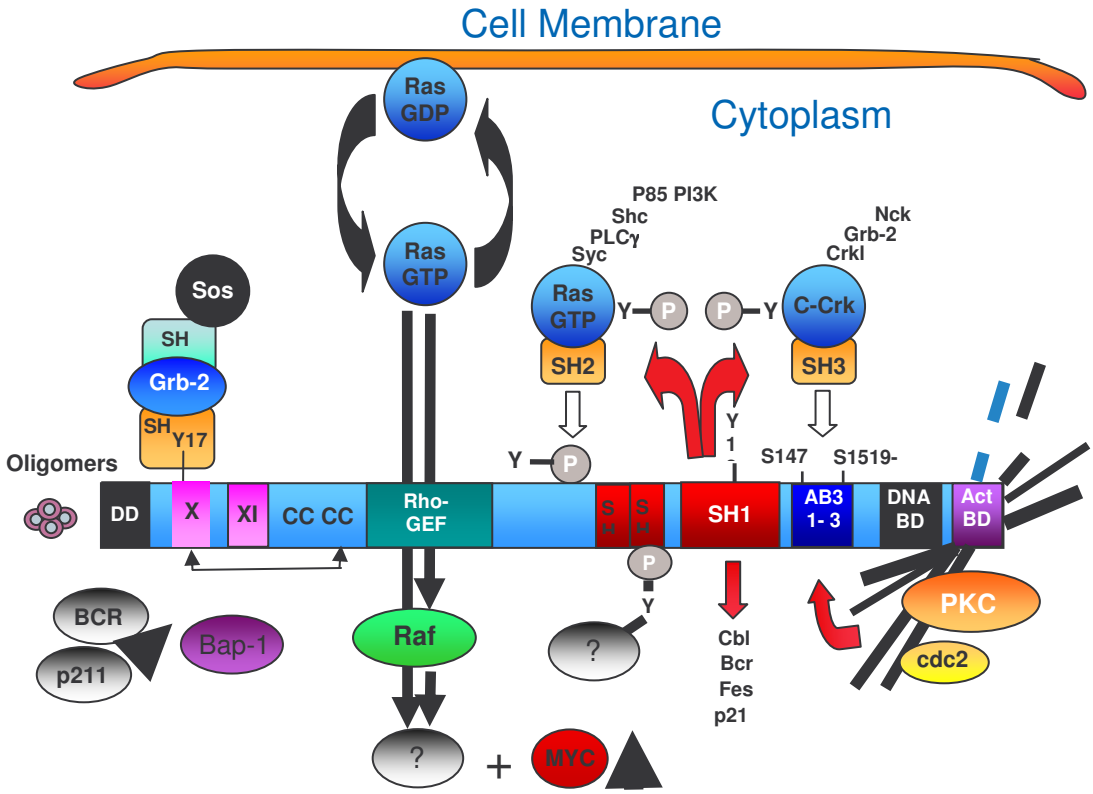


Figure 1.3 The function of the fusion protein p210 BCR-ABL

This diagram summarizes the function of BCR-ABL. BCR-ABL forms homo-oligomers and hetero-oligomers with the BCR serine threonine kinase through an N-terminal dimerisation domain (DD). The protein is localised only in the cytoplasm, which is in contrast to normal cABL which shunts between the nucleus and cytoplasm. Autophosphorylation of BCR-ABL provides docking sites for many proteins and protein substrates to bind. In turn, BCR-ABL then phosphorylates these proteins or protein substrates

Adapted from Laneuville et al (Laneuville 1995)

1.2 Treatment Options for patients with CML.

1.2.1 Allogeneic Transplantation

Presently, the only truly curative treatment for CML is allogeneic transplant from a HLA matched donor. Historically this regimen consisted of myeloablative chemotherapy and/or total body irradiation followed by bone marrow infusion. While curative, the early transplant mortality rate is high, and the treatment is available to only approximately 30% of patients because of age and donor constraints (Goldman *et al.* 1986) (van Rhee *et al.* 1997) (Clift & Storb 1996).

In more recent years, G-CSF mobilised blood transplants, again from allogeneic donors have gained in popularity in some transplant centres. This form of transplant leads to more rapid engraftment kinetics and hence, reduces to some extent, the early transplant related mortality associated with aplasia (Uzunel *et al.* 2003). However, GVHD is higher in this setting, and this approach again relies on the availability of histocompatible donors, limiting this treatment to a select group of patients.

1.2.2 Autologous Transplantation

There have been numerous attempts to treat patients without histo compatible donors, both in the chronic and blastic phases of the disease, with myeloablative chemotherapy, followed by autologous stem cell transplantation (Goldman 1996; Kantarjian *et al.* 1994; McGlave *et al.* 1994; Reiffers *et al.* 1994a; Reiffers *et al.* 1994b; Reiffers *et al.* 1993). Various methods including cytotoxic drugs, anti-sense molecules against BCR-ABL, peptide based vaccines, the use of dendritic cells to stimulate a selective anti-leukaemic cytotoxic T cell pool, and cell culture systems have been used to eliminate the leukaemic cell clone from the graft (Bocchia *et al.* 1996; de Fabritis *et al.* 1995; Gewirtz 1994; Verfaillie *et al.* 1996).

Early analysis of the outcomes from autologous transplantations demonstrated a cure rate of less than 5% (Butturini *et al.* 1990). Later reports suggested that durable remission rates may be higher, but follow-up is short and yet to be updated (Carella *et al.* 1999; Goldman 1996).

1.2.3 Conventional and intensive chemotherapy

Approximately 30 years ago it was reported that it was possible to induce cytogenetic remissions in chronic phase CML, using intensive chemotherapy and splenectomy (Clarkson *et al.* 2003). However remissions were invariably short, and only a modest survival advantage was observed (Goto *et al.* 1982). In more recent times hydroxyurea, an oral chemotherapeutic drug has been used as a palliative

treatment during the chronic phase of CML to reduce the high circulating levels of immature myeloid cells, as well as neutrophils and platelets, thereby avoiding thrombotic complications. Ninety percent of patients achieve a haematologic remission, as indicated by a return of peripheral blood cell counts and bone marrow morphology to normal. (Hehlmann *et al.* 1994) Hydroxyurea is generally well tolerated, however efficacy studies demonstrate that there is a negligible rate of reduction in the percentage of Ph chromosome positive cells, although the absolute number of circulating leukaemic cells may be reduced. The drug has limited impact on the rate of overall survival or time to progression to blast crisis

1.2.4 Interferon

Alpha- interferon (α- interferon) provided the first efficacious treatment for those patients ineligible for transplant. Treatment with α- interferon alone appears to prolong survival by about a year compared to hydroxyurea and/or busulphan (Allan, Richards & Shepherd 1995). It has also been demonstrated that some patients, particularly those with favourable prognostic indices, achieve complete cytogenetic responses (CCyR) (Hasford *et al.* 1998) (Kloke *et al.* 1996) on interferon therapy alone. A smaller percentage of patients appear also to have quite durable complete remissions, which persist even after ceasing treatment. However using sensitive techniques such as Fluorescence in-situ hybridisation (FISH) or PCR for the presence of BCR-ABL, small numbers of leukaemic cells can still be detected in the majority of patients despite the finding of CCyR by conventional cytogenetics (Hochhaus *et al.* 1995) (Lee *et al.* 1992). However, interferon results in considerable toxicity, and many patients are unable to tolerate the side effects which accompany long term therapy.

The mechanism of action of interferon is not clear, and probably complex and multifactorial. A number of possible mechanisms have been proposed including its effect on multiple gene transcription and protein phosphorylation events (Gordon *et al.* 1987; Sawyers 1999), activation of dendritic cells (Choudhury *et al.* 1997), effect on Fas-R mediated induction of apoptosis (Selleri & Maciejewski 2000; Selleri *et al.* 1998; Selleri *et al.* 1997), and down modulation of BCR-ABL mRNA.

1.3 Tyrosine Kinase Inhibitor Therapy

Since it is clear that the increased kinase activity of the BCR-ABL protein is essential for the manifestations of CML, and for the associated transformation, targeting this protein with a small rationally designed molecule, would seem an attractive approach to disease control. Some selective inhibition of the BCR-ABL tyrosine kinase was obtained with AG1112, a member of the tyrphostin

compounds,(Kaur *et al.* 1994) (Anafi *et al.* 1993) and herbimycin A, an antibiotic derived from *Streptomyces* species. (Okabe *et al.* 1992)

High throughput compound screening identified the small, 2-phenylaminopyrimidine class of kinase inhibitors. The initial compounds had low potency and specificity but optimisation produced a series of related compounds with increased potency and significantly improved specificity. The lead compound that emerged for clinical development was imatinib mesylate (STI571, glivec).

1.3.1 Imatinib mesylate (Gleevec, Glivec, STI571 and CGP571488)

Imatinib mesylate (Glivec™, formerly STI571 Novartis Pharmaceuticals) is a 2-phenylaminopyramidine compound (Figure 1.4) that is a potent inhibitor of all ABL tyrosine kinases (c-ABL, BCR-ABL and Tel/ABL) and *ABL*-Related Gene (ARG). In addition, imatinib is an inhibitor of a number of related receptor tyrosine kinases belonging to the PDGF receptor family,(Buchdunger *et al.* 1996; McGary *et al.* 2004; McGary *et al.* 2002) of ARG (Okuda *et al.* 2001) and c-Kit,(Buchdunger *et al.* 1996; Druker *et al.* 1996) and also the macrophage colony stimulating receptor, c-fms. (Dewar *et al.* 2005).

Imatinib binds at the ATP binding site of the kinase domain. This domain has a highly conserved bilobed structure consisting of an amino terminal lobe and a carboxy terminal lobe. In the interface between the two lobes are highly conserved amino acids that form the ATP binding and catalytic sites (P-loop) (Nagar *et al.* 2002).The P-loop of tyrosine kinases are critical structures and are involved in phosphate transfer and the activation of the kinase (Bossemeyer 1994).

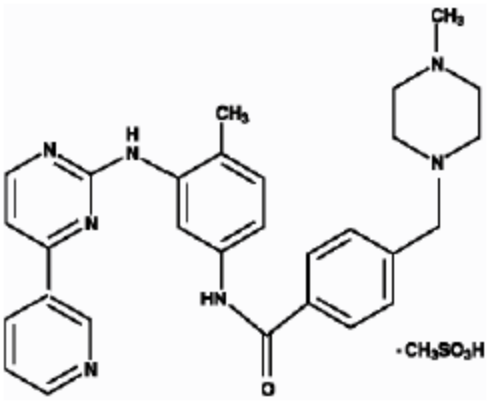


Figure 1.4 Chemical structure of imatinib mesylate

Imatinib mesylate is designated chemically as 4-[(4-Methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]- phenyl]benzamide methanesulfonate and its structural formula is shown above. Its molecular formula is $C_{29}H_{31}N_7O \cdot CH_4SO_3$ and its molecular weight is 589.7.

Figure kindly provided by Novartis Pharmaceuticals.

1.3.2 Imatinib mesylate: Mechansim of action

The activation state of kinases is dependent on the position of the activation loop, which is located in the carboxy terminal lobe. In active kinases, the activation loop is in an open conformation and faces away from the catalytic centre (Bossemeyer 1994; Nagar *et al.* 2002). The carboxy terminal segment of the activation loop acts as a platform for substrate binding and the amino terminal segment contains a strictly conserved motif of aspartate-phenylalanine-glycine. The motif is crucial for kinase activity as it coordinates the phosphate groups of ATP. The conformation of the activation loop is highly conserved in kinases when in the active state, however there are substantial differences between the inactive (closed) conformations. BCR-ABL is activated by phosphorylation of tyrosine 393 when the activation loop is in the open conformation, allowing substrates to bind. In the closed conformation tyrosine 393 points towards the centre of the kinase and is unphosphorylated. In this inactive conformation substrates can not bind to BCR-ABL (Nagar *et al.* 2002; Nagar *et al.* 2003) (Schindler *et al.* 2000) Imatinib was originally thought to act as a direct competitive inhibitor of ATP binding. However, structural resolution of imatinib with a compound closely resembling imatinib showed that it occupies only part of the ATP binding pocket (Schindler *et al.* 2000) (Nagar *et al.* 2002) Imatinib exploits the distinctive inactive conformation of the BCR-ABL activation loop and consequently achieves high specificity. It contacts 21 amino acids within the ATP binding site and the activation loop. There is an induced fit mechanism of the flexible P-loop structure within the amino terminal, which folds down upon imatinib binding to increase the surface complementarity. This arrangement is stabilised by the formation of a new hydrogen bond between tyrosine 253 and asparagine 322, forming a hydrophobic cage that surrounds imatinib (Schindler *et al.* 2000). Imatinib also forms a number of hydrogen bonds and van der Waals interactions with the kinase domain. The protein is tightly held in the inactive state which prevents ATP binding and the phosphorylation of BCR-ABL. Consequently the downstream effector molecules of BCR-ABL are not phosphorylated and the signal transduction pathways are not activated.

1.4 Imatinib mesylate:Efficacy

1.4.1 Phase I, II and III clinical Studies

Imatinib was initially studied in 83 patients who had CML in chronic phase, and in whom treatment with interferon-α containing regimens had failed (Druker *et al.* 2001). Doses of imatinib in this study ranged from 25 to 1000mg daily. There was a trend observed towards higher grades of adverse events at dose of 750mg or more. However, a clear dose response relationship was observed, with 54 patients who took 300mg or more demonstrating a 98% haematologic response rate within 4 weeks. Of these patients 31% had a major cytogenetic response (MCyR) and 13% had a complete cytogenetic

response (CCyR). No maximum tolerated dose was identified. However 400mg was recommended as the dose for further studies, largely because those patients who took 400mg had plasma drug levels known to correlate well with cell death in-vitro and with BCR-ABL tyrosine kinase inhibition in-vivo (Druker *et al.* 2001).

In June of 1999 three large, multinational phase II trials were started in CML patients with more advanced stage disease. These included treating 532 patients with late chronic phase, who had failed interferon therapy, with 400mg imatinib (Kantarjian *et al.* 2002a), treating 235 patients with accelerated phase disease with 400mg or 600mg (Talpaz *et al.* 2002) and treating 260 patients with myeloid blast crisis also with 400mg or 600mg (Sawyers *et al.* 2002). Side effects noted in these trials were oedema, mild nausea, vomiting and muscle cramps. More serious liver toxicities and fluid retention syndromes were reported but were rare. Cytopenias were also observed more commonly in advanced phase disease, implicating a compromised marrow reserve rather than direct toxicity. Overall these latter trials demonstrated that the earlier in chronic phase imatinib was started, the better the rate of cytogenetic response achieved, and importantly, the achievement of a cytogenetic response was associated with a significantly better progression free survival (Kantarjian *et al.* 2002a).

Between June 2000 and January 2001, 1106 newly diagnosed chronic phase CML patients were enrolled to the International Randomised Study of Interferon versus STI571 (IRIS) trial (O'Brien *et al.* 2003). Patients were randomised to imatinib 400mg or interferon with low dose cytarabine. Cross over between the arms was allowed if intolerance or treatment failure could be demonstrated. After an initial median follow up of 19 months, imatinib demonstrated far superior tolerability and haematological and cytogenetic response rates. Major and complete cytogenetic response rates at 18 months were 87.1% and 76.2% respectively, in the imatinib arm and 34.7% and 14.5% respectively, in the combined arm. The rate of freedom from progression was 96.7% for imatinib and 91.5% for the combination. Furthermore, there were marked differences in cross over rates between the two arms. In the imatinib cohort 12.3% had discontinued treatment and 2% crossed over to the interferon arm. In contrast, in the interferon groups 31.6% discontinued treatment and 57.5% crossed over to the imatinib arm (Hahn *et al.* 2003; O'Brien *et al.* 2003).

In a 5 year update of this trial presented at ASH in 2005 (Simonsson & On Behalf of the 2005) and later published (Druker *et al.* 2006), sustained responses have been observed with 98% of imatinib treated patients achieving a MHR, 92% achieving a MCyR and 86% achieving a CCyR by 5 years (Figure 1.5).

NOTE: This figure is included on page 15 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.5 IRIS trial 5 year update demonstrating the % of patients achieving CHR, MCyR and CCyR.

Demonstrating the percentage of patients showing a best response of 1. complete haematological response (CHR), 2. major cytogenetic response (MCyR), or 3. complete cytogenetic response (CCyR), following up to 5 years of imatinib treatment in those patients on the IRIS trial.

Adapted from Simonsson ASH 2005 (Simonsson & On Behalf of the 2005)

1.5 Imatinib resistance

While the clinical results with imatinib, particularly in the early chronic phase, have been excellent, not all patients demonstrate either initial or sustained responses. Primary and secondary imatinib resistance have been reported. Relapse occurs in almost all patients treated in blast crisis, approximately 50% of accelerated phase patients and 15 to 20% of chronic phase patients. Primary resistance occurs when the drug is ineffective from the start of therapy. Secondary resistance is defined as a loss of an initially achieved response. Heterogeneous mutations within the tyrosine kinase domain of BCR-ABL have most frequently been implicated in mechanisms of acquired resistance. The exchange of threonine for isoleucine at amino acid 315 (T315I) of the ABL protein was the first mutation described in resistant patients (Gorre *et al.* 2001). Based on crystal structure modelling, this mutation abrogates drug affinity (Schindler *et al.* 2000). There have now been over 50 different mutations described (Branford *et al.* 2002; Branford *et al.* 2003b; Hochhaus *et al.* 2002; Kreill *et al.* 2003; Shah *et al.* 2002). The biochemical and cellular impact of these mutations varies from demonstrating minor increases in inhibitory concentrations of imatinib, to virtual insensitivity (Corbin *et al.* 2003). The probability of detecting a mutation increases with each progressive stage of the disease, with mutation development being more prevalent when imatinib is initiated in the accelerated or blastic phase of the disease (Figure 1.6).

In addition to mutations which confer resistance, duplication or amplification of the BCR-ABL oncoprotein has also been reported. (Gorre *et al.* 2001; Hochhaus *et al.* 2002). Furthermore, novel chromosomal aberrations in addition to the Ph have been observed. These have historically included trisomy 8, monosomy 7 and 17p deletions (resulting in a loss of one p53 allele). In addition, other reciprocal translocations have been reported (Hochhaus *et al.* 2002).

NOTE: This figure is included on page 17 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.6 The probability of detecting a mutation by 24 months of imatinib therapy determined for each CML disease phase

The median is indicated and 95% confidence intervals (CI) are indicated in brackets. This figure clearly demonstrates that patients who commenced imatinib in accelerated phase had the highest probability of having a detectable mutation

Figure adapted from a poster presentation Branford et al.2003.

1.6 Second generation kinase inhibitors

The efficacy and tolerability of imatinib has established the paradigm that kinase inhibition is a rational treatment strategy in CML. However the vulnerability of a single agent has also been borne out by the number and frequency of kinase domain mutations which arise in patients on this treatment. There are now several second generation kinase inhibitors in clinical and pre-clinical assessment. Two of note, are Nilotinib (Tasigna. Novartis Pharmaceuticals) and dasatinib (Sprycel Bristol Myer Squibb).

Nilotinib (Figure 1.7) was designed to interact with the ATP binding site of ABL with a higher affinity than imatinib. In addition to being significantly more potent compared to imatinib (IC50<30nM), nilotinib also maintains activity against most of the BCR-ABL point mutations that confer imatinib resistance (Cowan-Jacob *et al.* 2004; Manley *et al.* 2004; Manley, Cowan-Jacob & Mestan 2005; Weisberg *et al.* 2005). The in-vitro efficacy of nilotinib translates into in-vivo activity as demonstrated by activity against a variety of imatinib-resistant BCR-ABL point mutations in animal models of myeloproliferaive disaease (Weisberg *et al.* 2005).(O'Hare *et al.* 2005). Promising results in imatinib resistant patients have been observed (Kantarjian *et al.* 2006) with this therapy. Upcoming de-novo chronic phase trials will assess efficacy in a head to head comparison with imatinib, and the mutation profile of this compound will also be assessed in newly diagnosed patients.

Dasatinib (formerly BMS-354825) (Figure 1.8) is a small-molecule, ATP-competitive inhibitor of SRC and ABL tyrosine kinases with potency in the low nanomolar range (Talpaz *et al.* 2006). In recent clinical trials of patients exhibiting imatinib resistance or intolerance, good haematological and cytogenetic responses have been observed. Although relapses have also been noted, these are generally early (within the first 6 months) in patients with blast crisis and often associated with the emergence of the T315I mutation (Cortes *et al.* 2007; Guilhot *et al.* 2007; Hochhaus *et al.* 2007; Kantarjian *et al.* 2007; O'Brien 2007; Ottmann *et al.* 2007; Quintas-Cardama *et al.* 2007; Shah *et al.* 2006). The results of on-going trials of this drug in chronic phase, previously untreated CML patients are yet to be published.

With these, and several other kinase inhibitors in phase I/II and entering phase III clinical trials it is clear that assays which predict patient's response to one or all of these inhibitors will be essential for guiding therapeutic decision making in CML patients. Chapters 2, 3 and 4 of this Thesis will discuss in detail the development of such assays and the significant contribution these developments have made to the literature that has been published in this area.

1.7 Monitoring patient response to ABL kinase inhibitor therapy

The optimal management of CML relies on accurate long term monitoring. While assessment of haematological parameters including, white cell count, differential count and haemoglobin may provide assessments of haematological responses, and gross changes may indicate a loss of response or progression, such measures are not sensitive enough to detect minor changes within the leukaemic cell clone itself. Cytogenetic analysis remains the reference method for the diagnosis of CML and for early treatment phase monitoring. The advantage of cytogenetic evaluation of metaphase spreads is that it also enables the detection of additional chromosomal abnormalities which may have prognostic implications at diagnosis, or be indicative of acceleration in advancing disease. However, with the potency of ABL kinase inhibitor therapies monitoring by cytogenetics is only efficacious until the patient achieves a complete cytogenetic response, at which time, by definition, the Ph+ chromosome becomes undetectable.

Fluorescence in-situ hybridisation (FISH) provides a further method of detecting the Ph chromosome. As this can be performed on interphase (I-FISH) cells using probes for the bcr and abl genes it has the advantage that a large number of cells can be examined, and that it can be performed on blood, rather than marrow. Due to random signal juxtaposition FISH does have a high false positive rate (5-10%), and thus is of limited value for accurate monitoring (Dewald 2002). Hypermetaphase FISH (H-FISH) does have a much improved false positive rate, and has a role for detecting Ph duplications, additional chromosomal abnormalities, and abnormal Ph translocations, but can be problematic technically (Schoch *et al.* 2002). Quantitative reverse transcriptase PCR (RQ-PCR) for BCR-ABL is unequivocally the most accurate and sensitive assay to monitor CML. RQ-PCR can detect down to one BCR-ABL positive cell in a background of 10^4 to 10^6 normal cells (Branford, Hughes & Rudzki 1999; Heid *et al.* 1996; Mensink *et al.* 1998). RQ-PCR is rapid, time and cost efficient, and can be standardised between laboratories. Furthermore BCR-ABL can be accurately monitored in blood using this technique (Branford *et al.* 1999). With the introduction of accurate monitoring by RQ-PCR a further level of response could be determined. This was termed a Major Molecular Response (MMR) and equates to a greater than 3 log reduction from standardised baseline in BCR-ABL levels (Hughes *et al.* 2003). This level of detection of BCR-ABL is approximately a log lower than that can be detected using cytogenetics (ie below a CCyR). With more recent improvements in both detection methods and patient responses a further level, CMR (complete molecular response) has also been defined, and this equates to an approximately 4.5 log reduction in BCR-ABL transcripts from standardised baseline (Ishikawa *et al.* 2006) (Jabbour *et al.* 2006) (Jabbour *et al.* 2006).

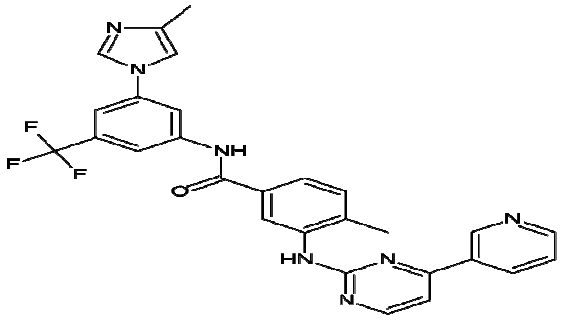


Figure1.7 The Chemical Structure of nilotinib

Nilotinib (AMN107, Tasigna) is a 4-methyl-*N*-[3-(4-methylimidazol-1-yl)-5-(trifluoromethyl)phenyl]-3-[(4-pyridin-3-ylpyrimidin-2-yl)amino] benzamide compound with a molecular weight of 529.516g/mol

Figure kindly provided by Novartis Pharmaceuticals

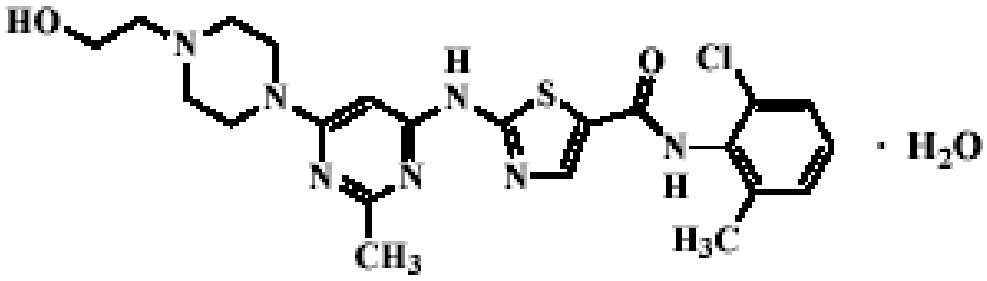


Figure 1.8 Chemical Structure of dasatinib

The chemical name for dasatinib is N-(2-chloro-6-methylphenyl)-2-[[6-[4-(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl]amino]-5-thiazolecarboxamide, monohydrate. The molecular formula is $C_{22}H_{26}ClN_7O_2S$ℜ• H_2O, which corresponds to a formula weight of 506.02 (monohydrate). The anhydrous free base has a molecular weight of 488.01. Dasatinib has the following chemical structure

Figure kindly provided by Bristol Meyer Squibb.

1.8 Research of the Author

While the efficacy of tyrosine kinase inhibitor therapy is proven in the majority of patients, predicting those patients who will respond less well to imatinib therapy in particular, remains poorly developed. Furthermore while ABL kinase domain mutations are known to be causative of poor response or frank relapse, little is known about other, intrinsic causes of resistance or sub optimal response. With this in mind the primary aims of this PhD thesis were:-

To develop assay(s) to predict imatinib (and other TKI) response pre initiation of treatment in CML patients.

To develop assay(s) which would in the early treatment period predict longer term response.

To adapt these assays to assess second generation kinase inhibitor candidates in patients with imatinib resistance (either primary or secondary)

Secondary aims which then developed out of these first aims were:-

To determine the intrinsic factors which determine imatinib response.

To develop assays to measure these intrinsic factors.

To determine what proteins control imatinib transport into target CML cells.

To assess the effect of combination TKI therapy on intracellular drug transport

To determine the effect, if any, of concomitant medications on TKI transport and efficacy.

The following chapters outline my research in the field of CML. Most chapters provide an introduction, briefly detail the research findings as published (where applicable), provide a summary of the findings, and also detail any additional relevant findings over and above that which is discussed in the relevant peer-review publication. In addition all conference abstracts relevant to the body of work are detailed. Where the work is yet to be published the chapter details all of the above points and also details all results found. It is anticipated that publications will arise from these chapters in due course and manuscripts are in many cases in preparation.

Chapter 2 details the development of an in-vitro assay to predict response to imatinib pre therapy in chronic phase, previously untreated CML patients *(Manuscript published).*

Chapter 3 details the development and application of an in-vivo assay developed to assess efficacy of kinase inhibition in the early treatment phase of imatinib therapy. *(Manuscript published)*

Chapter 4 discussed the drug transport mechanism involved in imatinib transport *(Manuscript published).*

Chapter 5 discussed the drug transport mechanism involved in nilotinib transport *(Manuscript published).*

Chapter 6 outlines the in-vitro effect of combined TKI (imatinib and nilotinib) therapy *(Manuscript published).*

Chapter 7 details the effect of concomitant medications on TKI intracellular transport *(Abstracts published)*

Chapter 8 details the importance of the functional activity of the human organic transporter 1 protein (OCT-1). *(Manuscript published)*

Chapter 9 Summary of all findings

Appendix I; Detailed Materials and Methods

Appendix II; References

Appendix III Publications arising from this Thesis

Chapter 2

The development of an in-vitro assay to predict imatinib response pre-therapy.

2.1 Introduction

While results in chronic phase CML patients treated with imatinib have been excellent in the majority of cases, a small proportion of patients fail to achieve either haematological, cytogenetic or molecular responses. The reasons for this are not clear, however if these patients can be identified pre-therapy, alternate clinical management (increased dose, or alternate TKI therapy) may improve their chances of response and longer term survival.

2.2 Historical parameters used to predict response and their applicability in the imatinib era.

2.2.1 Patient Age

Traditionally older age patients with CML have been associated with an inferior outcome. Age is a relevant variable in both the Sokal and Hasford prognostic indices (discussed later in this Chapter) (Sokal *et al.* 1984) (Hasford *et al.* 1998). For patients treated with imatinib however, age does not appear to be an independent predictor of outcome (Cortes *et al.* 2003). In previously untreated early CP-CML patients the rate of complete cytogenetic response was 87% for patients ≥60years and 79% for younger patients (*p=0.28*) (Cortes *et al.* 2003). These data suggest that age is not a predictive factor for imatinib response. However, it should be noted that this conclusion is only based on cytogenetic response. Progression free and overall survival data have not yet been analysed (or published) and the outcome of these analyses are of significance in the determination of long term predictive value of this parameter.

2.2.2 Variant Ph chromosome

In 5-10% of CML patients, one or more chromosomes in addition to chromosome 9 and 22 are involved in the translocation. Some studies have suggested that variant Ph translocations may have an adverse prognosis (Loncarevic *et al.* 2002; Potter *et al.* 1981; Reid *et al.* 2003). However, a recent report of 44 patients with variant Ph translocations treated with imatinib demonstrated similar outcomes to those of patients with classic Ph with respect to response, response duration and overall survival (El-Zimaity *et al.* 2004).

2.2.3 Deletions of derivative chromosome 9

In 10-15% of cases a portion of the derivative chromosome 9 (der[9]) immediately adjacent to the translocation breakpoint is deleted (Huntly, Bench & Green 2003a) (Sinclair *et al.* 2000) (Herens *et al.* 2000). These deletions have been reported to be associated with a poor prognosis (Huntly *et al.*

2003a) (Sinclair *et al.* 2000). An initial study in the imatinib era demonstrated poorer response in this group (Huntly *et al.* 2003b). However, a more recent series demonstrated patients with der[9] were found to have similar response rates to those patients without der[9]. Furthermore, there was no prognostic impact with respect to survival, response or response duration in multivariate analysis (Quintas-Cardama *et al.* 2005). These findings suggest that imatinib, as a potent kinase inhibitor, has overcome this adverse prognostic indicator.

2.2.4 Sokal and Hasford scores

The Sokal prognostic score (Sokal *et al.* 1984) arose from a study of 813 non-blastic Ph+ CML patients, which was designed to delineate good from poor prognostic patients. This study was conducted at a time when busulphan was the most common treatment modality, and assessed clinical parameters such as spleen size, blast cell and platelet counts as possible prognostic indicators. Using a Hazards ratio analysis the most discriminatory factors were found to be spleen size, blast cell percentage, patients ages and platelet count. The resulting hazard ratio function was derived from these variables:-

$$\lambda_i(t)/\lambda_o(t) = \mathrm{EXP}\, 0.0116(\mathrm{Age} - 43.4) + 0.0345(\mathrm{Spln} - 7.51) + 0.188\left[\left(\frac{\mathrm{Plt}}{700}\right)^2 - 0.563\right] + 0.0887(\mathrm{Blasts} - 2.10)$$

The Hazard ratio was then calculated for each patient, and patients were then grouped into low (Sokal score <0.8), intermediate (Sokal score 0.8-1.2) and high risk groups (Sokal score >1.2). In this treatment group the Sokal was found to be predictive of long term survival, and as such became a universal prognostic indicator for CML, which remains in use today.

The Hasford prognostic score was developed in the interferon era (Hasford *et al.* 1998). It was based on 908 patients and is shown here:-

Hasford prognostic score (0.6666 × age [0 when age <50 years; 1, otherwise] +0.0420 × spleen size [cm below costal margin] + 0.0584 × blasts [%] + 0.0413 ×eosinophils [%] + 0.2039 × basophils [0 when basophils <3%; 1, otherwise] +1.0956 × platelet count [0 when platelets<1500 × 109/L; 1, otherwise]) × 1000.

Both the Sokal and Hasford scores were found to be prognostic of survival in interferon treated patients (Hasford *et al.* 2003). However, there remains a degree of uncertainty about the value of these classic prognostic indicators in the imatinib era. The initial IRIS report did not show any significant difference in progression free survival among the different Sokal or Hasford categories (O'Brien *et al.* 2003). However with longer follow-up the Sokal score was demonstrated to delineate three distinct progression free survival probablities (Cervantes *et al.* 2003). Furthermore, the Sokal score was found to be predictive of molecular response. Grouping patients according to their Sokal prognostic score revealed 50% of patients within the low-risk Sokal group achieved a reduction of at least 3 log by 12 months compared with 30% in the intermediate- and 19% in the high-risk group (p=.007)(Hughes *et al.* 2003). However, this further highlights the variability of patient response and demonstrates that even in the good-risk Sokal group, molecular response is variable.

2.3 New prognostic models – imatinib treated patients

2.3.1 Factors impacting on the achievement of a Major Cytogenetic Response (MCyR)

Among 261 patients who received imatinib therapy after failing interferon, four variables were found in multivariate analysis to be associated with a lower probability of achieving a MCyR. These were haematological resistance to α-interferon, time from diagnosis to treatment exceeding 12 months, bone marrow basophils ≥ 5%, and % Ph+ metaphases >90% (Kantarjian *et al.* 2002b). Patients were then divided into 3 groups according to the number of adverse features each had. The probability of achieving a MCyR was 93% (zero to one adverse feature) 53% (two adverse features) or 34% (three or more adverse features) (Kantarjian *et al.* 2002b).

Later a model based on two adverse features, namely development of a neutropenia of <1 x 10^9/L between days 45 and 90 of therapy, and failure to achieve at least a MCyR at 3 months (Marin *et al.* 2003) was proposed. These groups were then defined with progression free and survival probabilities at 2 years of 100% (no adverse features), 66% (one adverse feature) or 15% (two adverse features). However, when this model was later applied to 260 patients the progression free survival probabilities were not significantly different between the three groups (98%, 80% and 96% respectively) (Kantarjian & Cortes 2004).

2.3.2 Imatinib drug levels and predicting response

In a recent study of 68 patients, (Picard *et al.* 2007) the association between trough plasma levels of imatinib and the achievement, or not, of CCyR and MMR was assessed. Fifty of these patients were

treated with 400mg, and 18 were treated with 600mg of imatinib. The authors concluded that superior cytogenetic and molecular responses are associated with a trough level of >1002ng/ml, and that plasma trough levels should form part of routine on-therapy disease response monitoring. This is an important body of work in the context of the "one dose for all" strategy employed for imatinib monotherapy. It is however preliminary and provides no indication for the ideal time to monitor plasma PK levels (first month, at 6 months etc?) in order to receive a good prognostic indication. Furthermore, recent publications (Blasdel *et al.* 2007) (Mahon *et al.* 2007) which highlight a small number of individual patients, demonstrate the value of imatinib PK level assessments in the case of failed response, toxicity or non-compliance.

2.3.3 Early molecular response and the prediction of long term response

BCR-ABL measurements performed early in imatinib therapy were found to predict the subsequent imatinib response (Branford *et al.* 2003a) (Hughes & Branford 2003) (Hughes & Branford 2006). In these analyses RQ-PCR for BCR-ABL was performed at 3 months post imatinib start, and patients were divided into those who failed to achieve a 1 log reduction, those achieving a 1 to 2 log reduction and those achieving a >2 log reduction. Kaplan Meier analysis of the achievement of subsequent major molecular response and probability of resistance to imatinib based on these three groups revealed a significant outcome advantage for those patients who achieved a good, early (by 3 months) molecular response. Assessing IRIS trial patients, 100% of patients who achieved a >2 log reduction by 3 months achieved a MMR by 24 months, and none demonstrated primary or secondary resistance out to 30 months of therapy. In contrast. only 13% of those patients who failed to achieve a 1 log reduction by 3 months achieved a subsequent MMR by 30 months, and 83% demonstrated either primary or secondary resistance ($p<0.001$).

2.4 Summary and research contribution

To date there is little evidence to suggest that either classical, or more recent prognostic indicators derived pre-therapy, are of significant value in the imatinib era. Furthermore, more recent and somewhat more predictive models rely on early therapeutic responses, and are as such not applicable to pre therapy screening, and hence are not readily applicable to aid in front line, therapeutic selection. With this in mind, the research described in the relevant attached paper, and conference proceedings has made a significant contribution to this area. The research described details the development of an in-vitro assay to predict the response of chronic phase, previously untreated patients to imatinib therapy. The approach taken focuses on the measurement of target inhibition and intrinsic sensitivity, and utilizes the phosphorylation status of the adaptor protein Crkl, an immediate downstream phosphorylation partner of BCR-ABL.

The following additional data are included to provide a greater understanding of the methodology developed, the background to the assay specifics, and serve to reiterate, and expand on the important findings of this body of work. Where necessary diagrams are provided to further clarify the assay specifics.

Publications

White, D., V. Saunders, et al. (2005). "In vitro sensitivity to imatinib-induced inhibition of ABL kinase activity is predictive of molecular response in patients with de novo CML." Blood 106(7): 2520-6. (White *et al.* 2005a)

Conference Presentations.

White Deborah, Verity A. Saunders, et al. (2004). The Combination of Intrinsic Sensitivity to Imatinib and Sokal Prognostic Score Is Strongly Predictive of Molecular Response in Newly Diagnosed CML Patients Treated with Imatinib. ASH, San Diego (White Deborah *et al.* 2004).

White, D. L., S. Branford, et al. (2005). "In Imatinib Treated CML Patients with Low Baseline IC50, Early Molecular Response Is Highly Predictive of Longer Term Molecular Outcome. In Patients with High IC50, Other Prognostic Indicators Are Needed." ASH Annual Meeting Abstracts 106(11): 3283-. (White *et al.* 2005b)

2.5 Assay development: Background and results.

2.5.1 Crkl as a surrogate marker for BCR-ABL kinase activity.

BCR-ABL is associated with many downstream effector proteins via its association with adaptor proteins and substrates. Several approaches can be taken to assess BCR-ABL tyrosine kinase activity. Direct measurement of the level of BCR-ABL protein is technically challenging because of the size of the protein (210kD). Total phosphotyrosine has been measured in using both flow cytometry (Schultheis *et al.* 2005) and Western blot (Tipping *et al.* 2001), and while this has demonstrated some predictive power, this approach is not specific for BCR-ABL tyrosine kinase activity alone.

A further downstream protein Stat 5 is also a potential target for measuring BCR-ABL kinase activity Stat 5(de Groot *et al.* 2000) (Signal Transducer and Activator of Transcription 5) is normally phosphorylated and activated by Janus kinases. However, in cells transformed with BCR-ABL, Stat 5 is constitutively activated by promiscuous phosphorylation. Analysis of p-Stat 5 is amenable to western blot and flow cytometry, though this has only been conclusively demonstrated in cell lines.(Jacobberger *et al.* 2003). Furthermore, pStat 5 is also present in normal cells (via Janus kinase), in some AML's (Fiedler *et al.* 2005) and in various types of non-CML myeloproliferative disorders (Aboudola *et al.* 2007; Teofili *et al.* 2007). Hence the specificity of this protein for assessment of BCR-ABL kinase activity is problematic. Furthermore it is likely, that imatinib will not be reactive against the various phosphorylation effectors for Stat 5, hence in-vitro sensitivity assays are not guaranteed to specifically read out sensitivity to imatinib induced kinase inhibition of CML cells.

Crkl (CT10 regulator of kinase like) is an adaptor protein consisting of an src homology 2 (SH2) domain and 2 tandem SH 3 domains in the absence of any catalytic domain (ten Hoeve *et al.* 1994 (Figure 2.1A)). It is related to the *crk* oncogene of the avian sarcoma virus, CT10 (Mayer, Hamaguchi & Hanafusa 1988; ten Hoeve *et al.* 1993). *Crkl* is located centromeric to the *BCR* gene on chromosome 22 and encodes a protein of 38 kDa molecular weight. ABL and BCR-ABL are capable of phosphorylating Crkl, and Crkl forms specific complexes with both proteins in COS-1 cells transfected with these proteins (Senechal *et al.* 1998). Crkl is the predominant phosphorylated protein in K562 cells, a BCR-ABL–expressing erythroblastoid cell line. (Oda *et al.* 1994). Furthermore, it has been demonstrated that tyrosine phosphorylation of Crkl (p-Crkl) occurs in cells from patients with primary CML as a direct consequence of BCR-ABL expression (Hochhaus *et al.* 2001; ten Hoeve *et al.* 1994) with levels of p-Crkl correlating well with the level of BCR-ABL protein. Importantly, p-Crkl is not detectable by western blotting in BCR-ABL–negative haemopoietic cells.

Structurally, the amino-terminal SH3 domain of Crkl has been shown to bind proteins such as C3G, SOS, PI3-K, c-ABL or BCR-ABL. The SH2 domain of Crkl can bind to tyrosine phosphorylated proteins such as CBL, HEF1, CAS or paxillin. It is not known whether Crkl is a regulator of these linked proteins. With respect to BCR-ABL, while Crkl is a prominent tyrosine phosphorylated protein in primary cells and CML cell lines, tyrosine phosphorylation of Crkl does not appear to be important for transformation, since overexpression of the Tyr^{207} to Phe Crkl mutant did not effect BCR-ABL transformation (de Jong *et al.* 1997; Sattler & Salgia 1998) (Figure 2.1B).

BCR-ABL interacts through a proline rich sequence within ABL, directly with the Crkl SH3 domain (Sattler *et al.* 1996) (Heaney *et al.* 1997). Crkl becomes phosphorylated on $Tyrosine^{207}$ binds via SH2 domain to CBL and then complexes multimerically with PI3K, cABL and BCR-ABL. Technically, the detection of p-Crkl is ideal for routine application as the phosphorylated form of the protein is only one kD larger (39kD) than the native protein, can be visualised on the same gel and can be detected using the same antibody. Furthermore the dual detection of the phosphorylated and non-phosphorylated forms provides an ideal control of protein loading for each lane. Assays were therefore set up with Crkl as an ideal surrogate for BCR-ABL kinase activity.

NOTE: This figure is included on page 32 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.1 The structure and down stream interactions of Crkl.

A Showing the structure of Crkl and the arrangement of SH2 and SH3 domains. Y207 – tyrosine 207 which is phosphorylated in association with BCR-ABL binding to the first SH3 domain. Adapted from Sattler 1998(Sattler & Salgia 1998)

B Crkl is partially localized to the cytoskeleton, and is shown here with a focal adhesion to actin filaments. The Crkl DH2 domain interacts with tyrosine phosphorylated CBL, CAS, HEF1 or paxillin. The SH3 domain binds SOS, CsG or BCR-ABL. Direct interaction of Crkl with BCR-ABL contributes to transformation. Adapted from Sattler 1998(Sattler & Salgia 1998)

2.5.2 Assay development and prediction of molecular response(White et al. 2005a).

The Ph positive erythroleukaemia cell line K562 (ATCC) was used for assay development. As the purpose of this assay was to assess patient intrinsic sensitivity, cells were exposed to increasing concentrations of imatinib in-vitro (Figure 2.2) A high concentration of imatinib is used in one tube to ensure complete kinase blockade (100 µM for cell lines and 50 µM for patient cells), and the level of p-Crkl in the absence of imatinib was used as the 100% level, to indicate the total level of Crkl phosphorylation. As described in the attached paper and Appendix I Materials and Methods, mononuclear cells (prepared by lymphoprep density gradient centrifugation) or control cell lines are incubated with varying concentration of imatinib, ranging from 0 µM to 50 µM (or to 100µM in the case of cell lines). After incubation, cells were washed once with cold phosphate-buffered saline(PBS) and lysed in Laemmli buffer (le Coutre *et al.* 2000) by boiling for 12 minutes. Cell lysates were clarified by microfugation for 5 minutes at 15 000 rcf and stored at -20°C. Protein lysate (20 µL; corresponding to 2 x 10^6 cells) were resolved on an sodium dodecyl sulfate (SDS)/10% (w/v) polyacrylamide gel. Protein was electrophoretically transferred to nitrocellulose membrane. Following blocking for 1 hour at room temperature with 2.5% membrane blocking agent (Amersham Pharmacia, Piscataway, NJ), the membrane was probed for 2 hours at room temperature with anti-Crkl antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with alkaline phosphatase–conjugated anti–rabbit immunoglobulin (Santa Cruz Biotechnology), bound antibodies were detected with enhanced chemi fluorescence (ECF) substrate (Amersham Pharmacia) and analyzed by Fluor Imager analysis (Molecular Dynamics, Sunnyvale, CA). Signals were quantified using Image Quant software (Molecular Dynamics) Figure 2.2.

Figure 2.2 Stages of the imatinib IC50 assay (K562 cells)

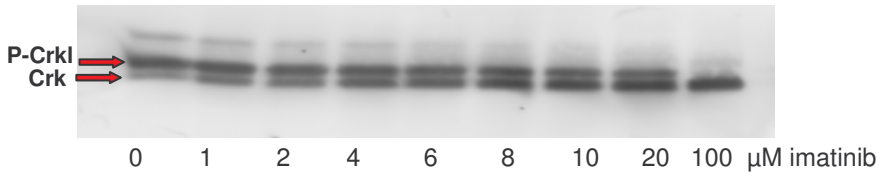
A. Western blot showing a decreasing percentage of phosphorylated Crkl (p-Crkl: upper band), and concomitant increase in non-phosphorylated Crkl (lower band: Crkl) in response to increasing concentrations of imatinib in-vitro.

B. Western blots are analysed using Image Quant software.
Densitometry readings are taken of the p-Crkl band, the Crkl band and the lane background (above the p-Crkl band) See detailed description in Appendix I Materials and Methods.
The quantified signals (average of 3 analyses per reading) are then imported into an excel spreadsheet as shown. (P-Crkl, Crkl and background).
Crkl and p-Crkl measurements are then corrected for lane background.
The ratio of p-Crkl to Crkl is then calculated and then this is converted to a %p-Crkl
The %p-Crkl at 100µM imatinib (total p-Crkl blockade- background level)) is then subtracted from all lanes and the data is normalised such that 0µM imatinib is converted to 100% p-Crkl and 100µM is 0%p-Crkl.

C The normalised data is then used to construct a graph, and the concentration of imatinib which corresponds to 50% kinase inhibition (reduction in p-Crkl) is deemed to be the $IC50^{imatinib}$

D This graph shows the actual level of p-Crkl at each imatinib concentration after lane background is substracted. This graph is not used in the calculation per se, but is used as a visual control (secondary check)for the accuracy of the Image Quant Analysis of the western blot

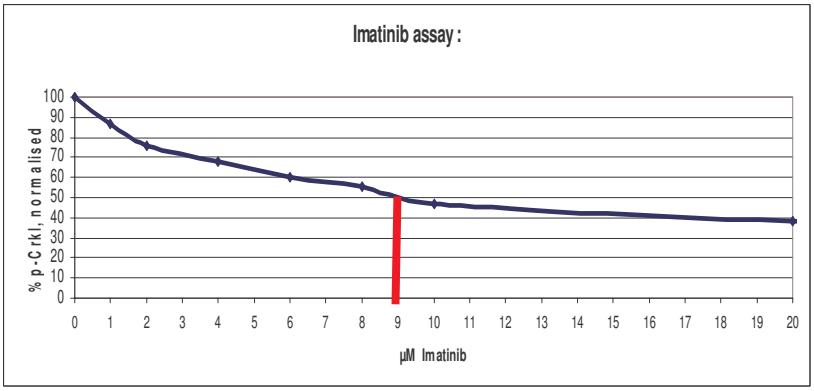
A



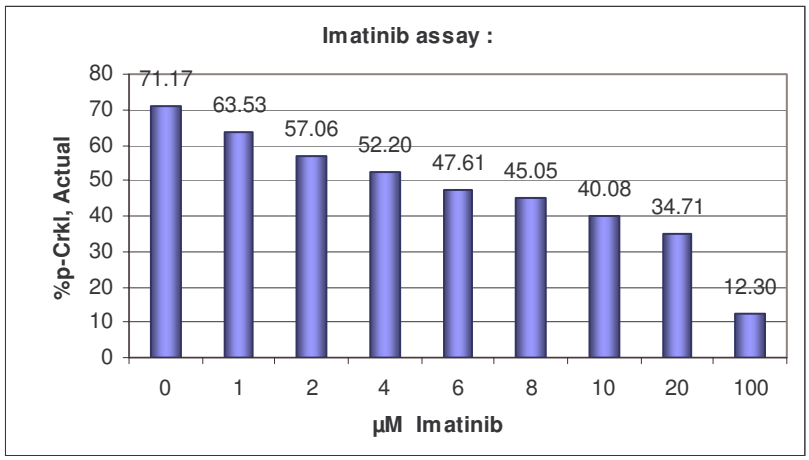
B

Lane	[imat] µM	P-CrkL	CrkL	bckgrnd	P-CrkLcor	CrkLcorr	Total CrkI	P/totalCrkI	% P-CrkL	%p - bkgn	normalise
2	0	31057925	13408120	1393747	29664178	12014373	41678550	0.711737	71.17	58.88	100.00
3	1	28905874	17167173	1341776	27564098	15825397	43389495	0.635271	63.53	51.23	87.01
4	2	24169854	18525361	1349755	22820099	17175606	39995705	0.570564	57.06	44.76	76.02
5	4	25999393	23915568	1256914	24742479	22658654	47401133	0.521981	52.20	39.90	67.77
6	6	23316006	25545707	1153822	22162183	24391885	46554068	0.476053	47.61	35.31	59.97
7	8	23701990	28661106	1152059	22549931	27509048	50058979	0.450467	45.05	32.75	55.62
8	10	20329916	29879058	1049922	19279993	28829136	48109129	0.400755	40.08	27.78	47.18
9	20	20754814	38156771	1009358	19745456	37147413	56892869	0.347064	34.71	22.41	38.06
10	100	4755970	30072019	626921.6	4129048	29445097	33574145	0.122983	12.30	0.00	0.00

C



D



2.6 Results (White et al. 2005a).

2.6.1 The IC50^{imatinib} and prediction of subsequent molecular response.

In the initial series, 62 patients enrolled to the TIDEL trial were examined. There was a surprising variation in the IC50^{imatinib} within this cohort (Figure 2.3 and 2.4) The median IC50^{imatinib} was found to be 0.6 µM in a range of 0.375 to 1.8 µM. To assess the importance of this variation, patients were dichotomised about the median into two groups, those with low, and and those with high IC50^{imatinib}. The molecular response achieved, following 12 months of imatinib therapy, was then assessed using Kaplan Meier Analysis in each group. This analysis revealed a significantly greater percentage of patients with a low IC50^{imatinib} achieved a 2-log reduction by 3 months and a 3-log reduction (MMR) by 12 months than those patients in the high group (*p=0.01 and p=0.03* respectively)(Figure 2.5). Of particular note was the difference between the groups at 3 months. In the low IC50^{imatinib} group 36% of patients achieved a 2-log reduction by 3 months. In contrast, only 8% of patients in the high IC50^{imatinib} group achieved 2-log reduction by 3 months (*p =.01*). This finding indicates that the IC50^{imatinib}, performed pre kinase inhibitor therapy, is a predictor of molecular response to 12 months in previously untreated, chronic phase CML patients.

2.6.2 Association of IC50^{imatinib} with other pre-therapy prognostic indicators(White et al. 2005a; White Deborah et al. 2004)

There was no significant correlation between the Sokal prognostic score and the IC50^{imatinib} (R=-0.08 *P>0.05*) (Figure 2.6). In this cohort, 59 patients had Sokal Scores defined at diagnosis. Dividing these patients into those with high and those with low Sokal scores about the median Sokal Score of 0.95 revealed no significant difference in molecular outcome at 2 and 3 months. However, at 12 months a significantly greater proportion of patients with low Sokal score achieved a MMR (Table 2.1). When the IC50^{imatinib} and Sokal score were both included in the analyses, it was revealed that a significantly greater proportion of patients with a low IC50^{imatinib} and low Sokal score achieved superior molecular responses at 1, 3 and 12 months (*p<0.001, p=0.0004 and p=0.023* respectively)(Table 2.2).

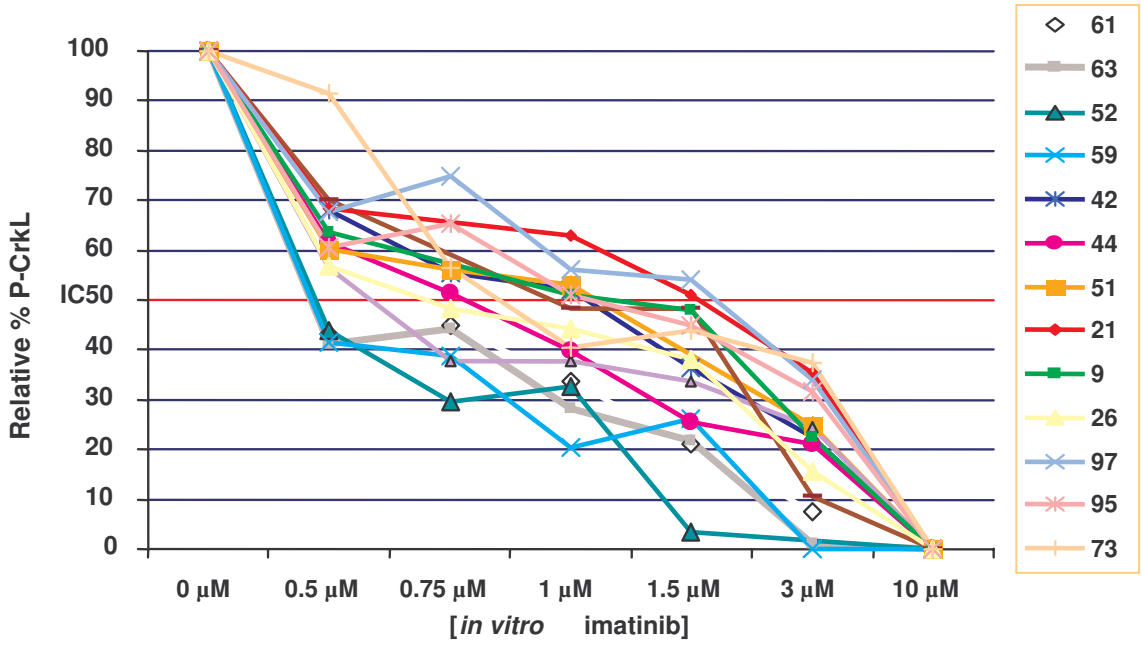


Figure 2.3 The interpatient variation in the IC50^{imatinib} was high in CP CML patients.

This graph of 15 CP, previously untreated, CML patients demonstrates the interpatient variation in IC50^{imatinib} observed. The median IC50^{imatinib} for the entire cohort was 0.6µM, with a range of 0.375 to 1.8 µM.

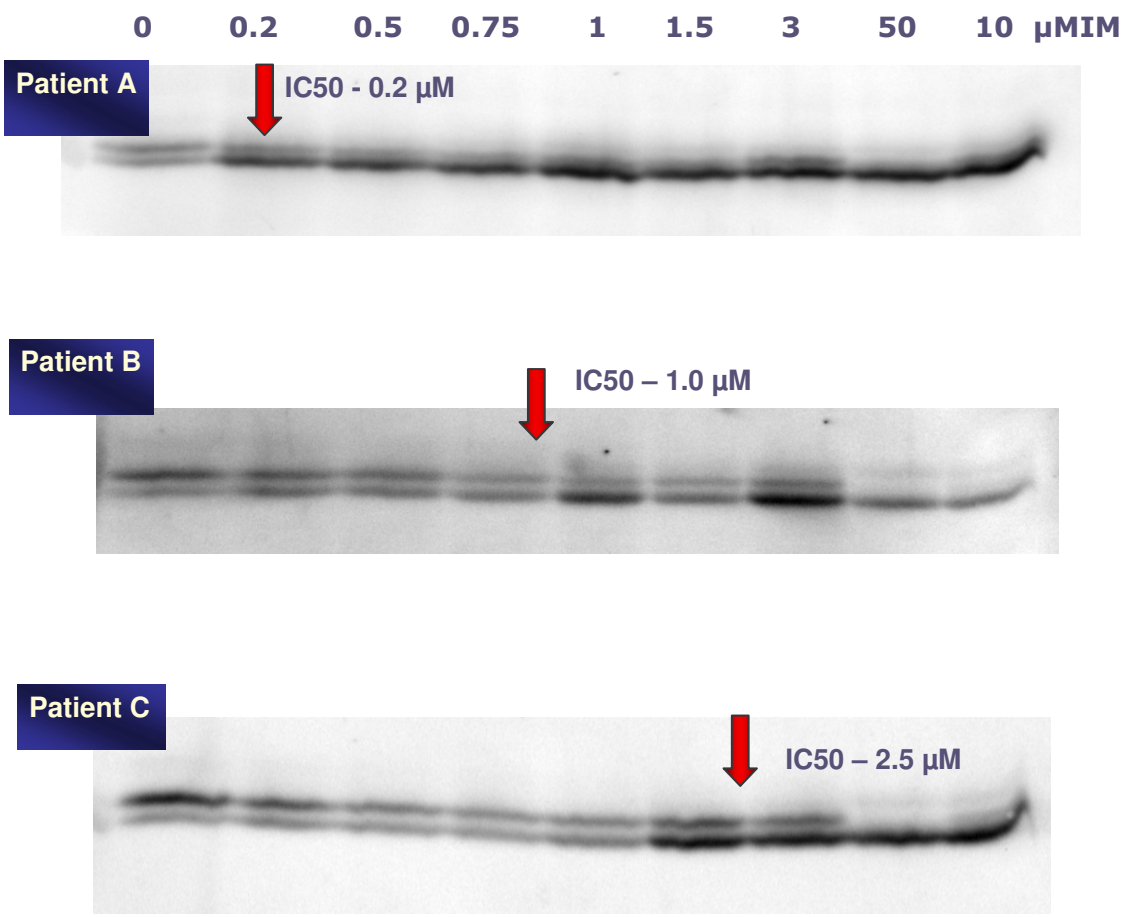


Figure 2.4 Demonstrating various IC50^{imatinib} western blots

Diagram demonstrating the variation in IC50^{imatinib} observed between patients. Patient A, B and C were all chronic phase previously untreated patients. As can be clearly seen, there is significant interpatient variation in IC50^{imatinib} between the three patients. This demonstrates the interpatient variability in intrinsic sensitivity to imatinib induced kinase inhibition.

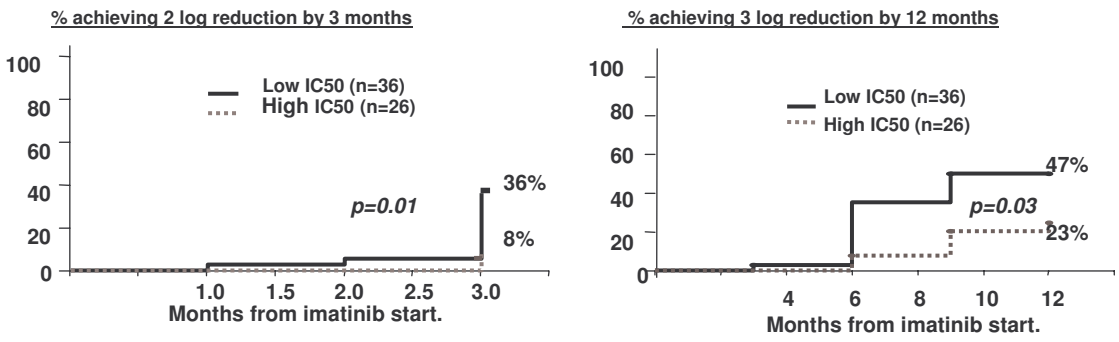


Figure 2.5 Kaplan-Meier graphs demonstrating the percentage of patients predicted to achieve 2-log reduction by 3 months and 3-log reduction (MMR) by 12months.

Significantly more patients with a low $IC50^{imatinib}$ achieve a 2 log reduction by 3 months and a 3 log reduction (MMR) by 12 months, when compared to patients with a high $IC50^{imatinib}$ (*p=0.01 and p= 0.3* respectively)

These data indicate that the $IC50^{imatinib}$ splits the good risk Sokal group (low Sokal score) into those who achieve excellent responses on imatinib therapy, and those that fail to achieve these clinical milestones. Furthermore, assessment of suboptimal response (failure to achieve a 1 log reduction in BCR-ABL by 6 months, or a 2 log reduction in BCR-ABL by 12 months) revealed that patients with a low $IC50^{imatinib}$ and low Sokal score are significantly less likely to display a suboptimal molecular response than all other groups. 15% of patients in the low Sokal/high $IC50^{imatinib}$group failed to achieve a 1 log reduction by 6 months compared to no patients with a low Sokal/low $IC50^{imatinib}$ (*p=0.013*). At 12 months, 33% of patients with low Sokal/high $IC50^{imatinib}$ group failed to achieve a 2 log reduction by 3 months in BCR-ABL compared to 7% of patients with low $IC50^{imatinib}$ (*p=0.032*). In general, patients in the high Sokal group do poorly (Table 2.1) regardless of their $IC50^{imatinib}$ The worst response group was the high Sokal score and high $IC50^{imatinib}$ group (25% achieve 2 log reduction by 3 months and 15% MMR by 12 months), though there was no statistical difference between this and the high Sokal low $IC50^{imatinib}$ group (26% achieve 2 log reduction by 3 months and 26% MMR by 12 months: *p>0.05* in both instances).

2.6.3 Chromosome 9 deletions

The incidence of deletions of the long arm of the derivative chromosome 9 (9q-), associated with poor prognosis, (Specchia *et al.* 2004) (Sinclair *et al.* 2000) (Huntly *et al.* 2003a; Huntly *et al.* 2002) (Huntly *et al.* 2001) was then assessed within the low and high $IC50^{imatinib}$ groups. Ten of the 47 evaluable patients were found to have a 9q-. There was no statistical difference in either the median $IC50^{imatinib}$or the distribution of $IC50^{imatinib}$ between the deleted and non deleted groups (*p> .05*)

NOTE: This figure is included on page 41 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.6 Dot plot comparing the IC50^{imatinib} with the Sokal Prognostic Score

In those patients for whom an IC50^{imatinib} and Sokal Prognostic score were available, there is no significant relationship between the two prognostic indicators

Figure adapted from ASH presentation Deborah White (White Deborah *et al.* 2004)

	1 log depletion BCR-ABL by 2 months (MCR)	2 log depletion BCR-ABL by 3 months (CCR)	3 log depletion BCR-ABL by 12 months (MMR)
Low Sokal (n=28)	43%	29%	54%
High Sokal (n=31)	29%	16%	19%
P value	*>0.05*	*>0.05*	***0.008***

Table 2.1 The Sokal Score and prediction of subsequent molecular response

Patients were divided into low and high Sokal scores about the median of 0.95 and the percentage of patients achieving the nominated criteria were assessed. The p values indicate that a significant difference between the groups could be found in the achievement of a 3 log reduction in BCR-ABL by 12 months (MMR) but at no other analysed time point.

NOTE: This table is included on page 43 of the print copy of the thesis held in the University of Adelaide Library.

Table 2.2 The Sokal Score and prediction of subsequent molecular response, in the cohort of patients with low IC50^{imatinib} only.

Log rank Survival analysis demonstrating the % of patients achieving the stated milestones .Only those patients who received 600mg imatinib and had a Low Sokal Score were analysed.

This demonstrates that a Low IC50^{imatinib} is necessary to achieve an early molecular response at 3 months even in those patients with Low Sokal who receive 600mg imatinib

Table adapted from ASH presentation Deborah White (White Deborah *et al.* 2004)

2.6.4 Association of IC50^{imatinib} with early molecular response (EMR)(White et al. 2005b)

It has been previously shown that the log reduction in BCR-ABL achieved at 3 and 6 months is predictive of MMR at 24 months (Branford *et al.* 2003a) (Hughes & Branford 2003) (Hughes & Branford 2006). Sixty patients treated on the TIDEL trial were examined with respect to IC50^{imatinib} and EMR. EMR (the achievement of either a 1 log reduction in BCR-ABL by 3 months or 2 log reduction by 6 months) was predictive of achievement of MMR at 12 and 24 months in this cohort ($p<0.05$ in all instances). (Figure 2.7A to D). The IC50^{imatinib} was also predictive of MMR by 12 months($p=0.026$) but not 24 months ($p>0.05$)(Figure 2.7 E and F). However, limiting assessment to only those patients with a low IC50^{imatinib}, revealed patients who also achieve a 1 log reduction in BCR-ABL by 3 months (Figure 2.7G) or a 2 log reduction by 6 months (Figure 2.7H) have a significantly greater probability of achieving MMR by 24 months. This is in contrast to those patients with a low IC50^{imatinib} who fail to achieve either of these milestones ($p<0.001$ in both instances). Analyzing those patients with a high IC50^{imatinib} and again assessing achievement of a 1 log reduction by 3 months (Figure 2.7 I) and a 2 log reduction by 6 months (Figure 2.7 J) reveals no significant difference in molecular outcome to 24 months ($p>0.05$ in both instances)..

The log reductions at both 3 and 6 months, were then assessed in the group of patients who achieve a MMR by 24 months, and comparing them to the group of patients that fail to achieve MMR. This analysis reveals a significant difference in the median log reduction between the groups (Figure 2.8A and B). Using the same grouping, but limiting analysis to only those patients with a low IC50^{imatinib} again reveals a significant difference between the two groups (Figure 2.8C and D). However there is no significant difference observed between the groups in log reduction achieved at 3 months when the analysis is limited to only those patients with a high IC50^{imatinib} (Figure 2.8E and F). There is a significant difference at 6 months however.

2.7 Summary of initial analysis

In summary, patients with a high IC50^{imatinib} were found to have a more gradual response to imatinib than those with a low IC50^{imatinib}. These data indicate that the IC50 predicts the initial slope of the leukaemic cell reduction, perhaps because it reflects the sensitivity of differentiated leukaemic cells. In patients with a low IC50^{imatinib}, the early log reduction of BCR-ABL is highly predictive of subsequent achievement of MMR. Conversely, in patients with a high IC50^{imatinib} their 3 and 6 month response is not predictive of MMR by 24 months, and suggests that in these patients other predictive tests may be

warranted. Of note, this finding is unchanged when data analysis is limited to only those patients receiving 600mg to 6 months This finding indicates that the differences in response observed, are not simply a reflection of the actual dose received (White *et al.* 2005a).

Figure 2.7 Assessment of factors which may predict for the achievement of MMR.

Patients were grouped according to achievement of MMR or not, by 24 months of imatinib therapy. These groups were then assessed on the basis of predictive indicators such as EMR and $IC50^{imatinib}$

A The % of patients achieving MMR by 12 months based on log reduction in BCR-ABL at 3 months.

B. The % of patients achieving MMR by 12 months based on log reduction in BCR-ABL at 6 months.

C The % of patients achieving MMR by 24 months based on log reduction in BCR-ABL at 3 months.

D The % of patients achieving MMR by 24 months based on log reduction in BCR-ABL at 6 months.

E The % of patients achieving MMR by 12 months based on low and high $IC50^{imatinib}$groups.

F The % of patients achieving MMR by 24 months based on low and high $IC50^{imatinib}$groups.

G The % of patients achieving MMR by 24 months based on log reduction at 3 months, Data was examined in only the low $IC50^{imatinib}$ patients.

H The % of patients achieving MMR by 24 months based on log reduction at 6 months, Data was examined in only the low $IC50^{imatinib}$ patients.

I The % of patients achieving MMR by 24 months based on log reduction at 3 months, Data was examined in only the high $IC50^{imatinib}$ patients.

J The % of patients achieving MMR by 24 months based on log reduction at 6 months, Data was examined in only the high $IC50^{imatinib}$ patients

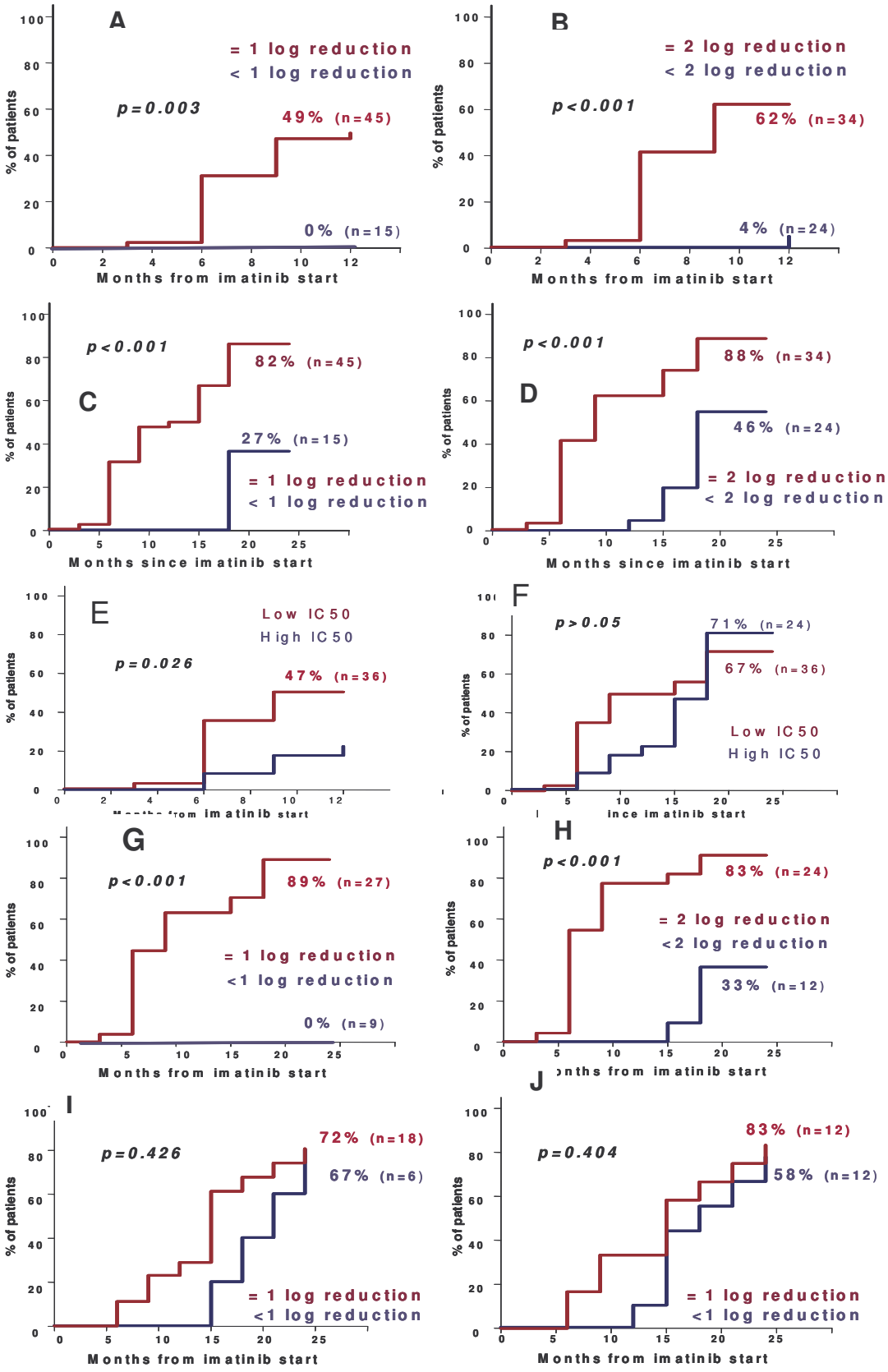

A
= 1 log reduction
< 1 log reduction
p=0.003
49% (n=45)
0% (n=15)
% of patients
Months from imatinib start
B
= 2 log reduction
< 2 log reduction
p<0.001
62% (n=34)
4% (n=24)
% of patients
Months from imatinib start
C
p<0.001
82% (n=45)
27% (n=15)
= 1 log reduction
< 1 log reduction
% of patients
Months since imatinib start
D
p<0.001
88% (n=34)
46% (n=24)
= 2 log reduction
< 2 log reduction
% of patients
Months since imatinib start
E
Low IC50
High IC50
p=0.026
47% (n=36)
% of patients
F
p>0.05
71% (n=24)
67% (n=36)
Low IC50
High IC50
% of patients
G
p<0.001
89% (n=27)
= 1 log reduction
<1 log reduction
0% (n=9)
% of patients
Months from imatinib start
H
p<0.001
83% (n=24)
= 2 log reduction
<2 log reduction
33% (n=12)
% of patients
I
p=0.426
72% (n=18)
67% (n=6)
= 1 log reduction
<1 log reduction
% of patients
Months from imatinib start
J
p=0.404
83% (n=12)
58% (n=12)
= 1 log reduction
<1 log reduction
% of patients
Months from imatinib start

Figure 2.8 The difference in median log reduction at 3 and 6 months between those patients who achieve MMR and those who fail to.

Molecular data was assessed at 3 and 6 months, based on:-

A All patients log reduction in BCR-ABL following 3 months of imatinib therapy.

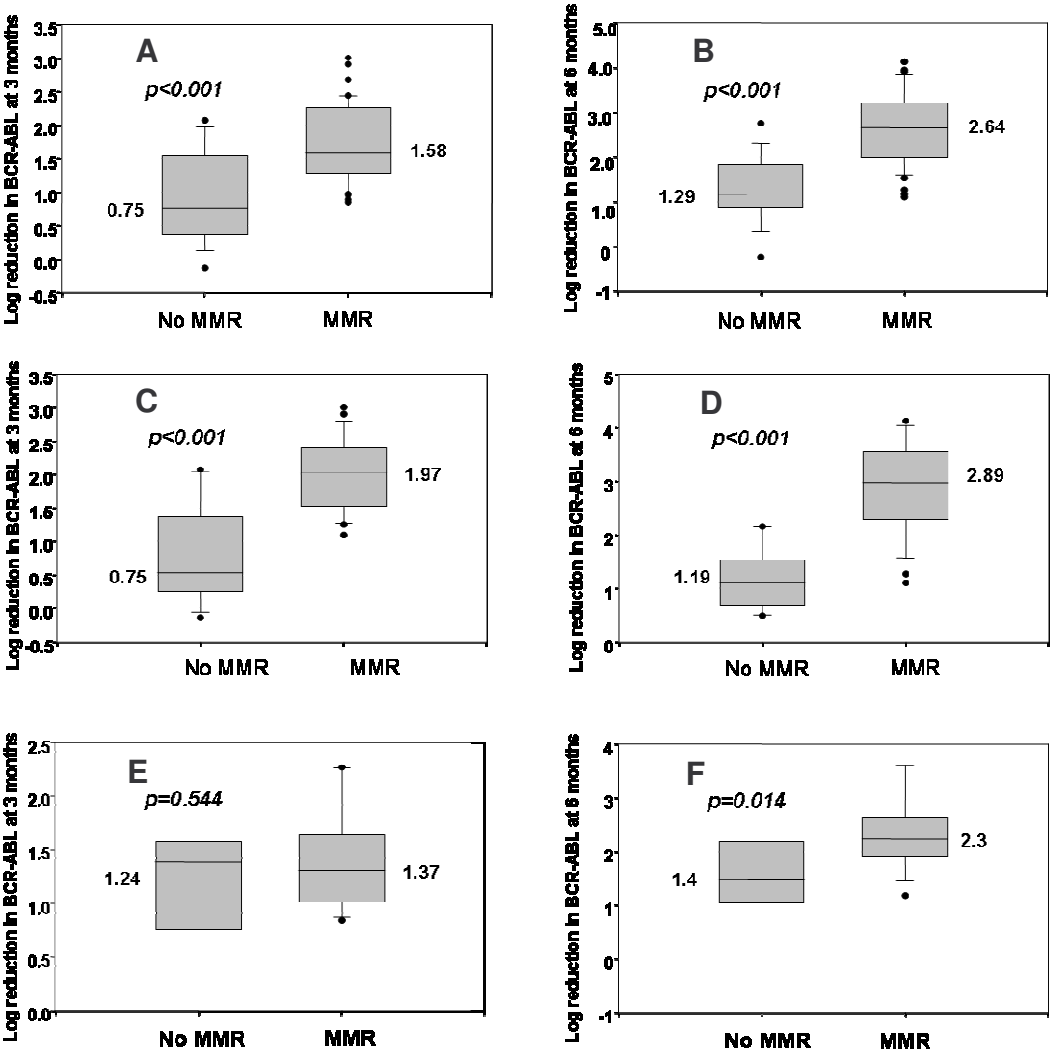
B All patients log reduction in BCR-ABL following, 6 months of imatinib therapy.

C Low $IC50^{imatinib}$ patients only, log reduction in BCR-ABL following 3 months of imatinib therapy.

D Low $IC50^{imatinib}$ patients only, log reduction in BCR-ABL following 6 months of imatinib therapy.

E High $IC50^{imatinib}$ patients only, log reduction in BCR-ABL following 3 months of imatinib therapy.

F High $IC50^{imatinib}$ patients only, log reduction in BCR-ABL following 6 months of imatinib therapy.


A
p<0.001
0.75
1.58
No MMR
MMR
Log reduction in BCR-ABL at 3 months
B
p<0.001
1.29
2.64
No MMR
MMR
Log reduction in BCR-ABL at 6 months
C
p<0.001
0.75
1.97
No MMR
MMR
Log reduction in BCR-ABL at 3 months
D
p<0.001
1.19
2.89
No MMR
MMR
Log reduction in BCR-ABL at 6 months
E
p=0.544
1.24
1.37
No MMR
MMR
Log reduction in BCR-ABL at 3 months
F
p=0.014
1.4
2.3
No MMR
MMR
Log reduction in BCR-ABL at 6 months

2.8 Subsequent analysis

As demonstrated in Figure 2.7F, the $IC50^{imatinib}$ was not predictive of subsequent molecular response at 24 months. This indicated that the $IC50^{imatinib}$ was either a time dependant predictor, or dependant on another, currently unknown, factor related to imatinib treatment. As indicated at the beginning of this thesis in the descriptor of patient cohorts and related clinical trials, patients enrolled to TIDEL were scheduled to dose escalate to 800mg at 12 months if they failed to achieve a 4 log reduction in BCR-ABL by this time point. Figure 2.7F demonstrates the molecular response for all patients irrespective of dose. In this cohort of patients only 6 achieved a 4 log reduction by 12 months, however for reasons of intolerance or toxicity, 37 patients in total failed to dose escalate to 800mg.

To explore the possible confounding issue of dose escalation, assessment of the achievement of MMR by 24 months was again performed, but in this instance, analysis was limited to only those 37 patients who remained on 600mg between 12 and 24 months (Figure 2.9). These results show that the $IC50^{imatinib}$ is predictive of the achievement of MMR at 24 months in those individuals remaining on 600mg imatinib therapy ($p=0.007$). Furthermore, it indicates that higher dosing may overcome the poor response noted in those patients with high $IC50^{imatinib}$. This finding is explored in greater detail in following Chapters which address the underlying cause for the differences observed in intrinsic sensitivities to imatinib induced kinase inhibition between patients.

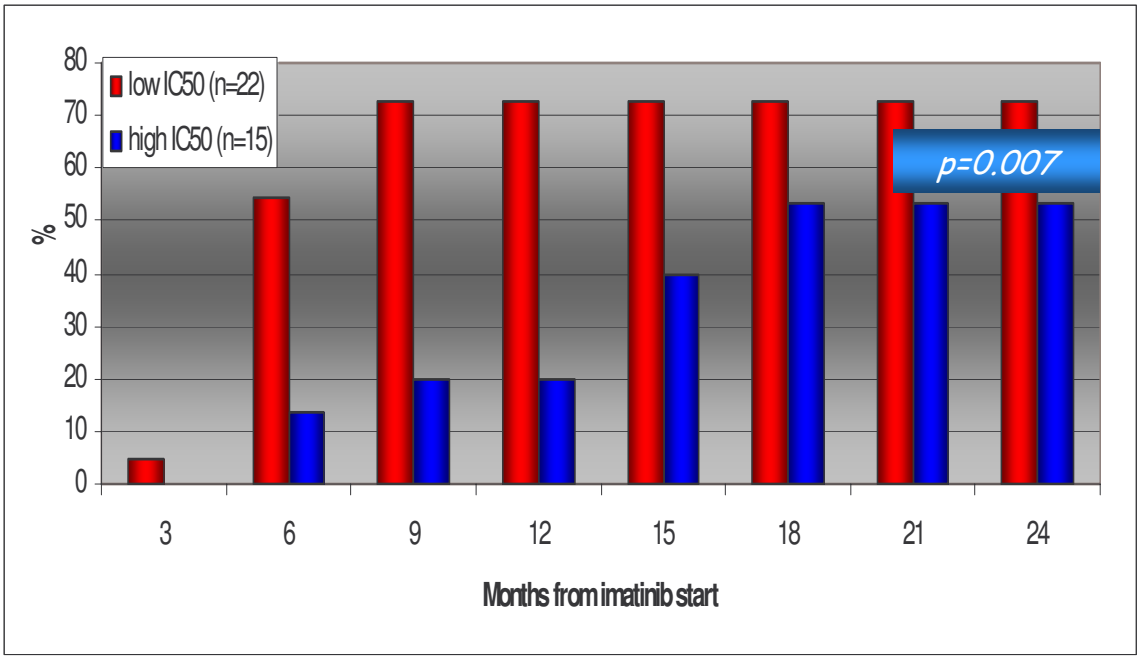


Figure 2.9 The percentage of patients achieving MMR by 24 months based on low and high $IC50^{imatinib}$ groups.

In this analysis the patient cohort examined was limited to only those patients who had remained on 600mg of imatinib per day. As is clearly demonstrated, a significantly greater proportion of patients with low $IC50^{imatinib}$ achieve MMR, compared to patients with high $IC50^{imatinib}$.

2.9 Overall Conclusion from this body of data.

The individual publication, and the supplemental data presented in conference abstracts, and in this Chapter, demonstrate that the $IC50^{imatinib}$ assay, performed pre therapy, is a significant predictor of longer term (to two years) molecular response, in CP-CML patients treated with imatinib. The predictive power of in vitro imatinib sensitivity suggests that the actual level of BCR-ABL kinase inhibition achieved is important in determining response to imatinib, particularly in patients with low Sokal score. Furthermore, it suggests that BCR-ABL kinase inhibition is incomplete in most patients. The results presented here which suggest the $IC50^{imatinib}$ may be dose related are explored in greater detail in following Chapters, which thoroughly explore the underlying causes of the observed variation in intrinsic sensitivity between patients. This is a landmark study, as the importance of the degree of kinase inhibition achieved has not been previously demonstrated. Furthermore the development of this assay has provided an invaluable starting point to explore in greater detail the observed variations in imatinib response.

Chapter 3

Development of an early treatment phase, in-vivo assay to predict long term molecular response in patients treated with imatinib.

3.1 Introduction

The striking success of imatinib therapy in CML is even more remarkable in view of the "one dose for all strategy" which is currently employed. Pharmacokinetic studies indicate that dose is the strongest predictor of overall drug exposure, and there is no necessity for imatinib dosing on a mg/kg basis (von Bubnoff *et al.* 2002) (Peng *et al.* 2004). However, the poor response observed in a minority of patients raises the possibility of inadequate drug exposure in some cases. The challenge therefore, was to develop reliable assays to predict, early in the treatment course, patients who may benefit from dose escalation.

The utility of the in-vitro kinase inhibition assay ($IC50^{imatinib}$) for predicting patient response pre therapy, has been demonstrated in previous publications (White *et al.* 2005b) (White *et al.* 2005a) and in Chapter 2. Since imatinib targets BCR-ABL, a logical approach to assess efficacy is to assay the degree of inhibition of the kinase activity of BCR-ABL, which occurs in the early treatment phase of patients treated with imatinib. In a similar approach Hochhaus et al (Hochhaus *et al.* 2002) assessed the in-vivo level of kinase inhibition achieved using p-Crkl in CML patients with imatinib resistant mutations, and in patients with non-mutation related resistance. In this current study, the biological relevance and predictive value of the in-vivo level of kinase inhibition achieved in patients was assessed over the first 28 days of imatinib therapy. This in-vivo response will result from a summation of the effects of intrinsic sensitivity ($IC50^{imatinib}$) of the patient to imatinib induced kinase inhibition, actual dose received, and pharmacokinetic factors, such as gastrointestinal absorption and hepatic metabolism. In-vivo measurement will indicate the actual level of ABL kinase inhibition achieved in peripheral blood cells, which may be the most practical and accurate way to assess the adequacy of therapy, and the potential value of dose escalation.

3.2 Summary and research contribution

The research described in the relevant attached paper, and conference proceedings have made a significant contribution to the area of early predictors of long term molecular response in CML patients treated with imatinib. This Chapter details the development of an in-vivo assay, which utilizes the phosphorylation status of the adaptor protein Crkl. This assay was a logical progression from the in-vitro assay previously established.

This data has been analyzed using two different approaches, both of which are described in the following Chapter. Additional data are included to provide a greater understanding of the methodology developed, the background to the specifics of the analyses performed, and reiterate the important findings of this body of work as published.

Publications

White, D., V. Saunders, et al. (2007). "Measurement of In vivo BCR-ABL kinase inhibition to monitor imatinib-induced target blockade and predict response in chronic myeloid leukemia." J Clin Oncol 25(28): 4445-51. (White,D *et al* 2007a)

Conference Presentations

White, D. L., V. A. Saunders, et al. (2005). "The In-Vivo Level of Imatinib Induced Kinase Inhibition Achieved in De-Novo CML Patients during the First 28 Days of Therapy Is a Powerful Predictor of Molecular Outcome." ASH Annual Meeting Abstracts 106(11): 436-. (White DL *et al* 2005)

3.3 Assay development: Background and Methodology

3.3.1 Background

There were several challenges associated with the establishment of this assay. The primary challenge was to maintain protein integrity, ensuring the actual level of kinase inhibition in the blood mononuclear cells (MNC) at the time of collection was captured, and accurately measured. As samples for this study, were collected from patients enrolled to the multi-centre TIDEL trial, conducted at 16 sites around Australia, primary attention was given to the establishment of rigorous sample processing procedures, to ensure optimum protein stability. An education process on appropriate sample preparation was established, and a schematic of the method was prepared and distributed (Figure 3.1).

3.3.2 Patient cohort

Patients in this study were enrolled to the TIDEL trial. The trial specifics have been detailed at the beginning of this Thesis (Clinical Trials Referred to in this Thesis Page XVI).

3.3.3 Method

In brief, 40 mls of blood was obtained from 49 patients prior to the commencement of imatinib therapy, and at 7 day intervals for a period of 4 weeks, once patients commenced imatinibtherapy. Mononuclear cells (MNCs) were isolated from blood using Lymphoprep (Axis Shield. Oslo Norway) density gradient centrifugation. 2 x10^6 mononuclear cells were lysed in Laemmli's buffer (le Coutre *et al.* 2000) by boiling. At this point samples were stored until all sequential samples from each patient were collected. All patient samples were run on the same gel to minimise variations due to inter-assay variability. The Western blot technique is detailed in *Appendix I: Materials and Methods*, and also outlined in Chapter 2. Examples of in-vivo assays are shown at Figure 3.3.

To calculate the degree of in-vivo kinase inhibition at any given timepoint, the lane background was firstly subtracted from phosphorylated and non-phosphorylated bands (ref Chapter 2 and Appendix I)

Following this, the % p-Crkl was determined using the following formulae:-

%p-Crkl = (pCrkl//pCrkl + Crkl)*100

The % kinase inhibition at each timepoint was calculated using the following formulae:

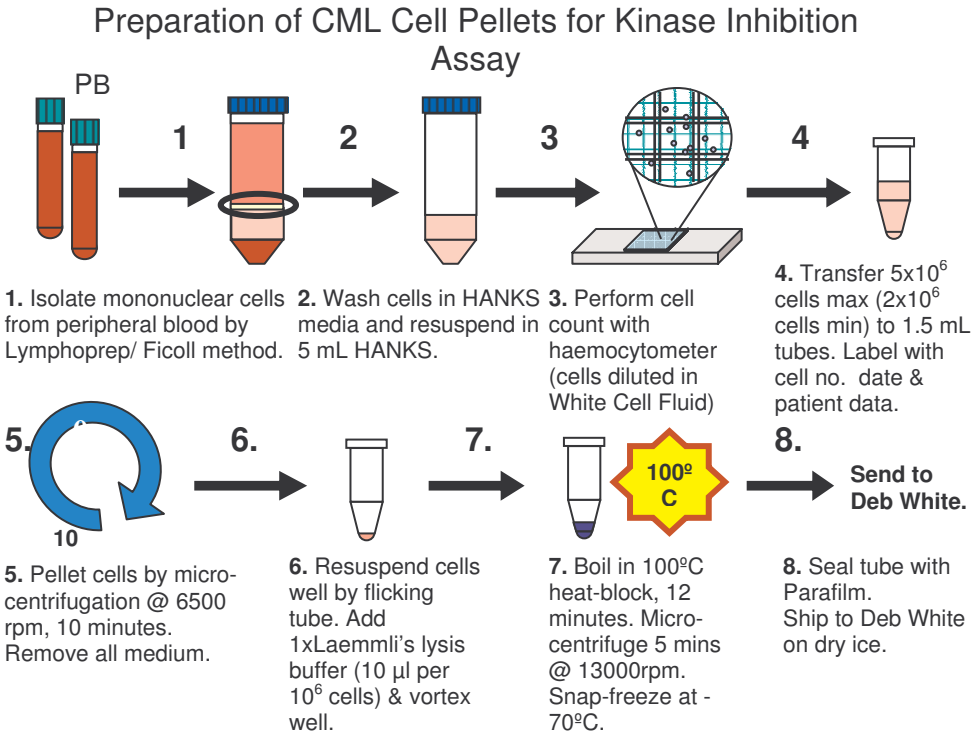
% kinase inhibition = ((% p-Crkl at baseline -% p-Crkl at designated timepoint)/ % p-Crkl at baseline) x100.

3.3.4 Assay Reproducibility.

The BCR-ABL negative cell line, VBL100 was used as a negative control for p-Crkl, and this was analysed with every in-vivo assay. Any background from this cell line was subtracted from each timepoint analysed (see Chapter 2).

3.3.4.5 Results of Reproducibility Studies

To assess the variation in protein lysate preparation, two analyses, by independent scientists, were performed on 51 samples, Independent protein preparations were run concurrently (to remove interassay variation) and compared. The X-Y plot of the data is shown at Figure 3.3. As can be seen the correlation coefficient is very strong (R=0.926), with a *p value of* $2.33x10^{-23}$.



1x Laemmli's Buffer Use

1x Laemmli's buffer for cell lysis will be sent frozen in 100 µl aliquots.
Please store aliquots at -20ºC on arrival.

**NOTE: Laemmli's buffer contains 2-mercaptoethanol.*
Wear gloves and use in fume hood.

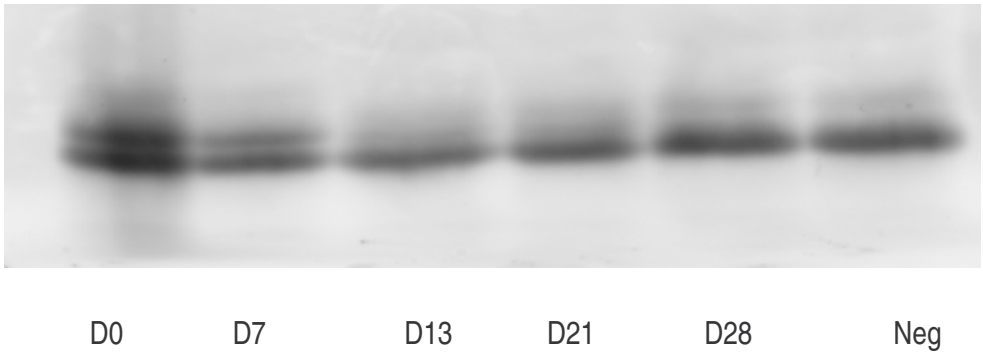
Thaw one aliquot of buffer before use.
Ensure that cell pellet is resuspended before buffer is added.
Add 10 µl buffer per 10^6 cells (i.e. 50 µl for maximum $5x10^6$ cells).
Immediately heat tube 12 minutes at 100ºC to lyse cells.
Store lysates at -20ºC.

Figure 3.1 Sample preparation guidelines for preparation of in-vivo protein lysates.

This cartoon and associated information was distributed to participating sites to aid with preparation of protein lysates for in-vivo kinase inhibition assay.

I.

A



B

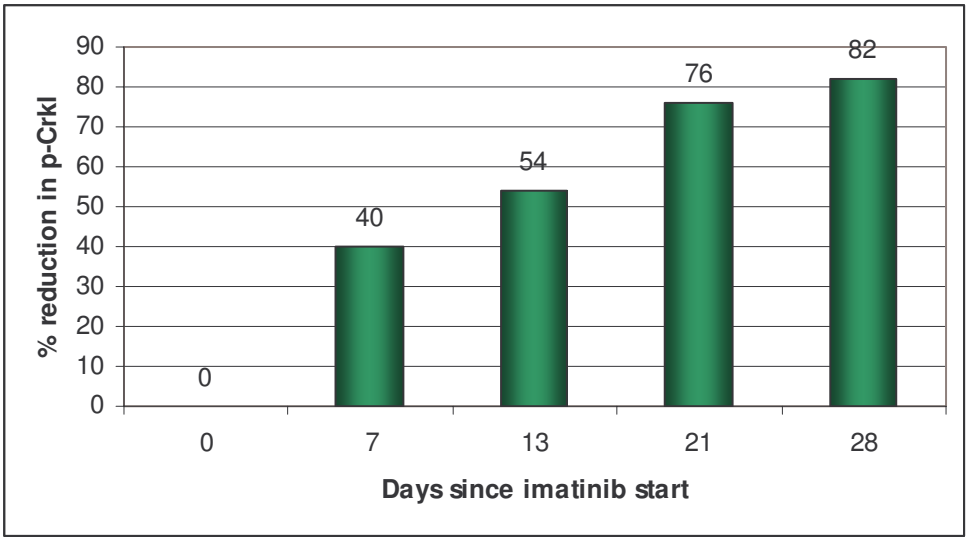


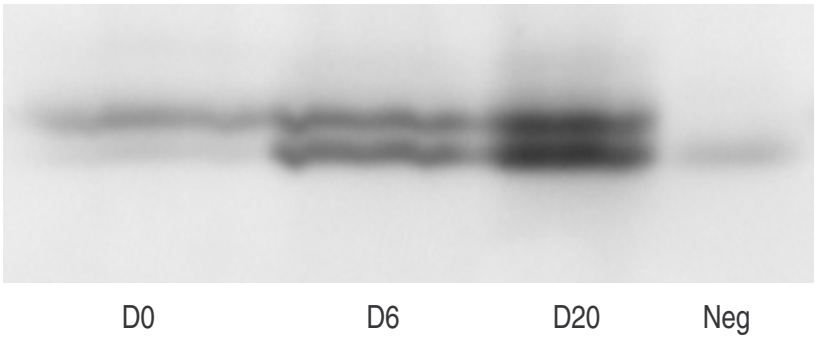
Figure 3.2 I, II and III Examples of in-vivo kinase inhibition assay.

Panel A shows the Western blot to determine the % p-Crkl (upper band) in the patient cells over for the first 28 days of imatinib therapy. The lower graph (B) demonstrates the % reduction in p-Crkl over time. Samples from this patient were collected at days 0, 7, 13, 21 and 26. There was a significant decrease in the percentage of p-Crkl over the first 26 days, resulting in an overall decrease of 82% by day 28

.

II

A



B

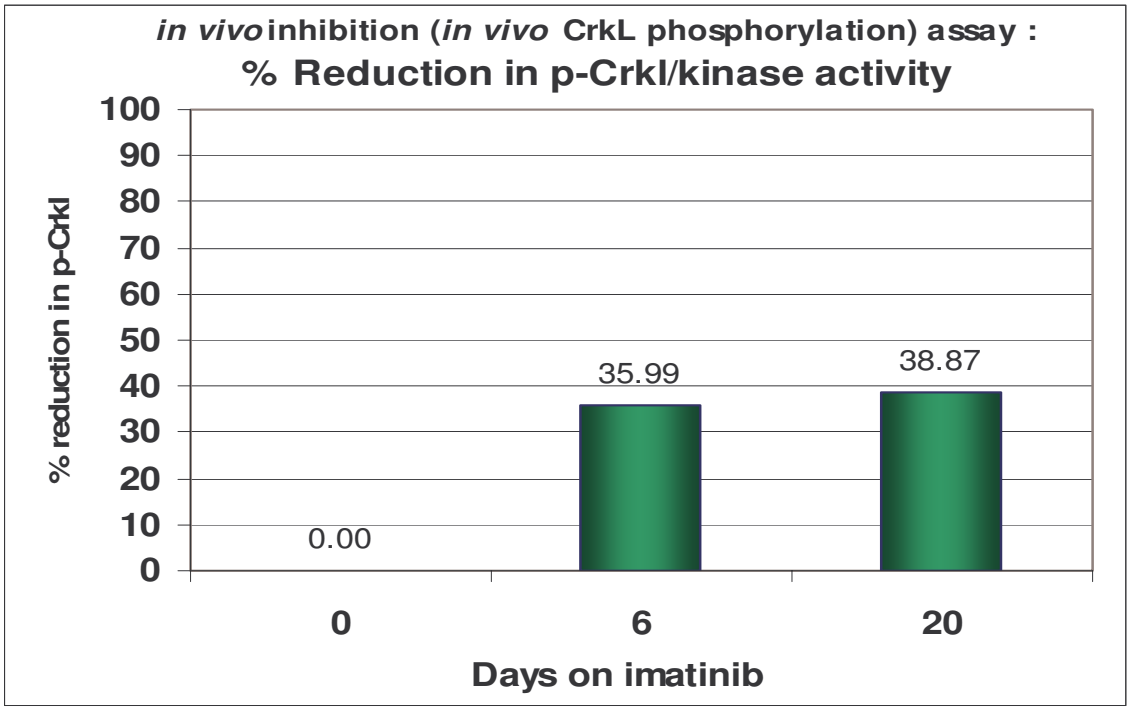
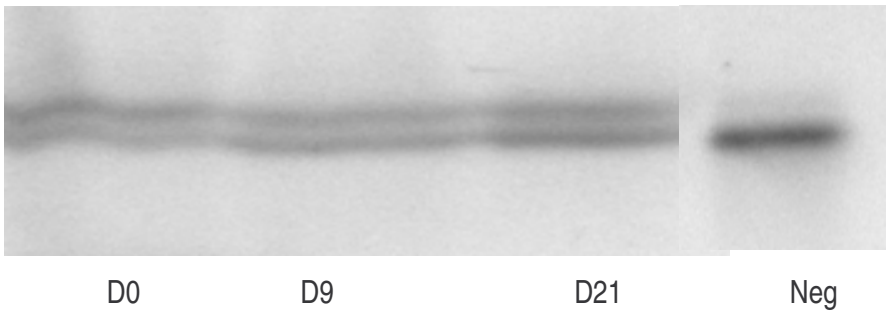


Figure 3.2 II

Panel A shows the Western blot to determine the % p-Crkl (upper band) in the patient cells over the first 28 days of imatinib therapy. The lower graph (B) demonstrates the % reduction in p-Crkl over time. Samples from this patient were collected at days 0, 6 and 20. There was a 36% decrease in the level of p-Crkl by day 6, but no significant further reduction by day 20.

III.

A



B

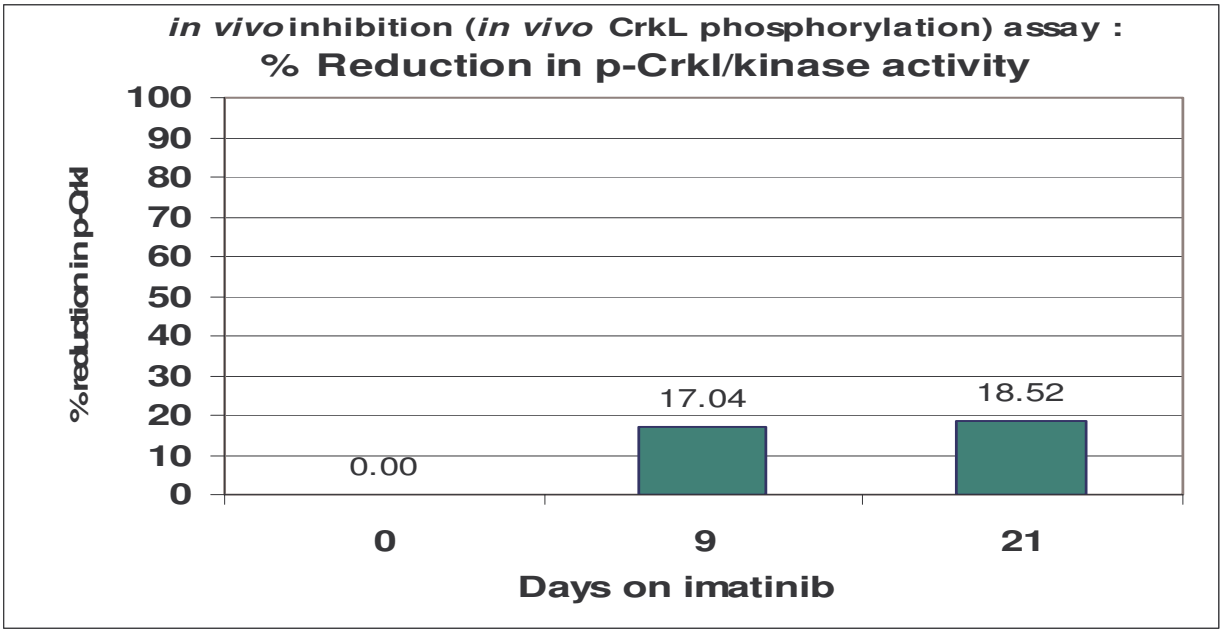


Figure 3.2 III

Panel A shows the western blot to determine the % p-Crkl (upper band) in the patient cells over the first 28 days of imatinib therapy. The lower graph (B) demonstrates the % reduction in p-Crkl over time. Samples from this patient were collected at days 0, 9 and 21. The degree of kinase inhibition in this patient was poor, with only a 18% reduction observed by day 21.

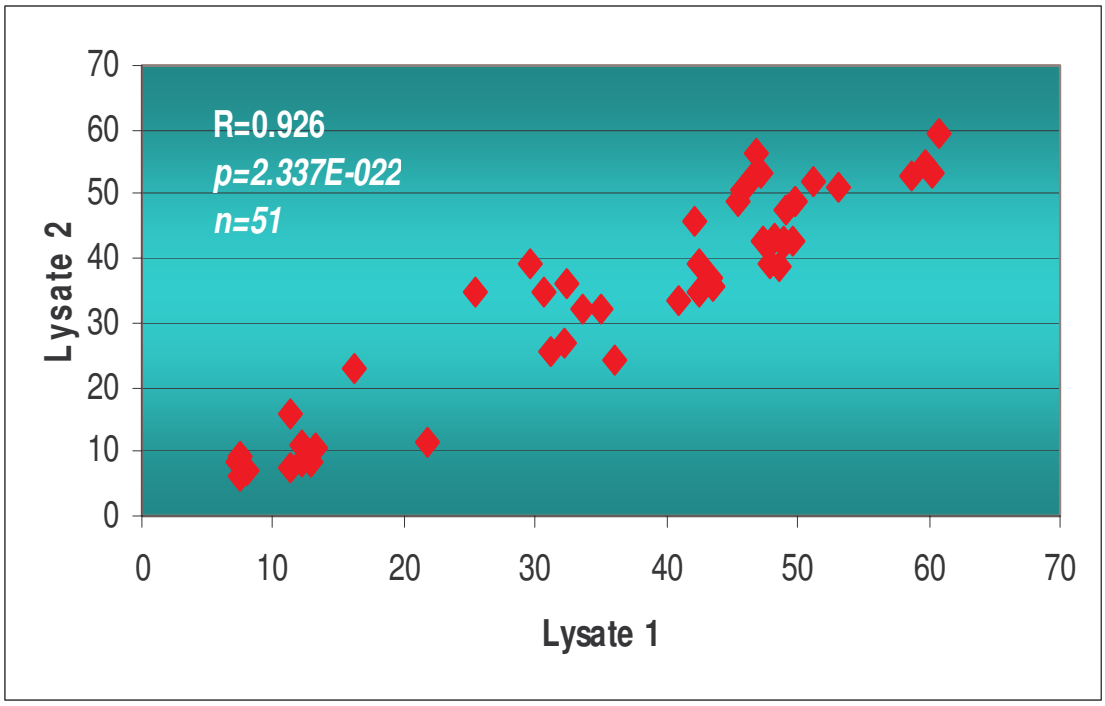


Figure 3.3 Demonstration of the correlation between the level of p-Crkl in two independently prepared lysates.

To assess for variation in p-Crkl, the same sample was prepared independently by two scientists. Fifty one clinical blood samples were divided in to two post collection, and protein lysates were independently prepared.. The samples were then analysed by Western blot. This analysis revealed a very strong correlation between the two sample preparations, and indicates that with rigorous mandating of standard procedures results generated by independent analyses correlate well.

3.4 Results

3.4.1 First Analysis (presented at ASH 2005) (White et al. 2005a)

There was wide variation in the % in-vivo kinase inhibition achieved over the first 28 days of therapy (Table 3.1, and Figure 3.4). Because not all patients had samples collected at all time points, and to facilitate statistical analyses, the data was firstly grouped, into the average % kinase inhibition achieved in the first two weeks (days 7 and 14), and that achieved in the second two weeks (days 21 and 28). The data was then dichotomised about the median % reduction for the combined timepoints (7-14 and 21-28) into low and high %kinase inhibition. Interestingly, Survival Analysis revealed there was no significant difference in the achievement of MMR between the two groups when the day 7 -14 data was examined. However, there was a significant difference, based on kinase inhibition at days 21-28, in the achievement of MMR at both 12 and 24 months (Figures 3.5 and 3.6 and Table 3.2). These data demonstrate that patients who achieve high in-vivo imatinib-induced kinase inhibition in the second two weeks of therapy, have superior molecular responses to two years when compared to those patients with a low kinase inhibition.

Despite a dose of 600mg per day being mandated for all patients in the TIDEL trial, not all patients were able to tolerate this dose, with some patients dose reducing to 400mg or less. To address the potential confounding influence of dose, the analyses were repeated, in those 40 patients who received >540mg (90% or greater of the prescribed dose) daily over the first 28 days of therapy. Again, those patients with high in-vivo kinase inhibition achieved superior molecular responses. Indeed, 100% of patients who received 600mg, and had high in-vivo kinase inhibition between days 21-28, achieved MMR by 24 months (*p=0.002*). Figure 3.7.

There was a significant correlation between the % p-Crkl at day 21-28 and the $IC50^{imatinib}$ (R^2=-0.4 *p=0.018*. n=28) indicating that a higher $IC50^{imatinib}$ is associated with a decreased level of in-vivo kinase inhibition. Stratifying patients into low and high in-vivo kinase inhibition groups and assessing the $IC50^{imatinib}$ of each cohort revealed that those patients achieving high in-vivo kinase inhibition uniformly have low $IC50^{imatinib}$ (Figure 3.8). Interestingly, there was variation in the $IC50^{imatinib}$ levels of patients with low in-vivo kinase inhibition. The finding of a high $IC50^{imatinib}$ in this cohort would be anticipated. However, the finding of a low $IC50^{imatinib}$ (high intrinsic sensitivity) in some patients with low in-vivo kinase inhibition suggests other factors, such as drug compliance, low prescribed dose, or drug interactions may present to cause low in-vivo kinase inhibition in some patients. In this cohort only 16% achieved MMR by 12 months and 38% by 24 months (Figure 3.9).

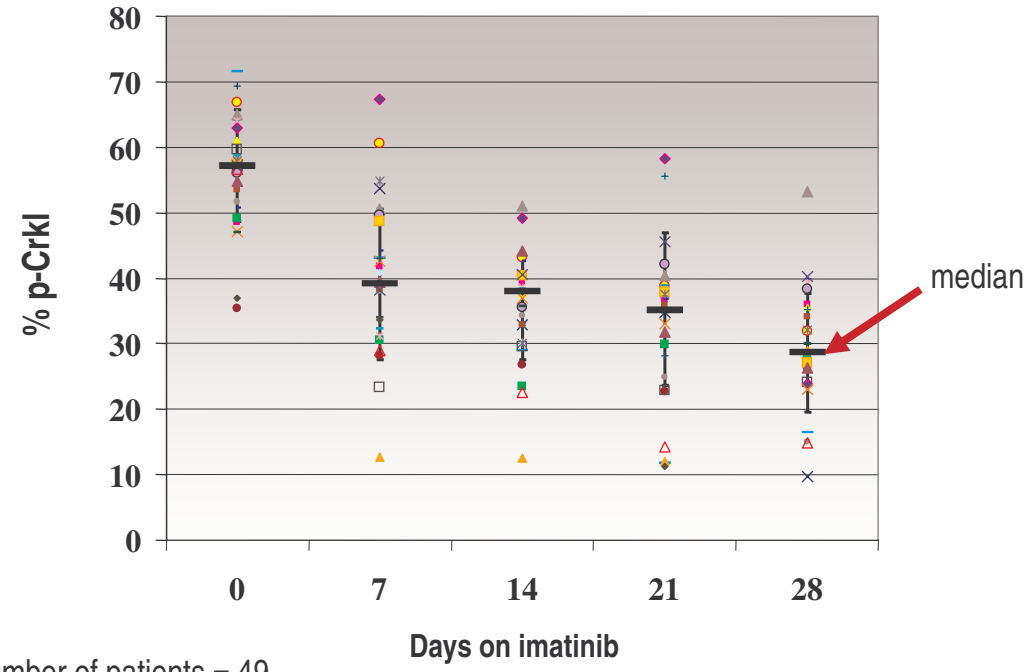
Limiting analyses to only those patients who had a low $IC50^{imatinib}$, the achievement of MMR by 24 months was assessed based on low and high in-vivo kinase inhibition groups (Figure 3.9). Despite the small patient cohort, there remained a significant difference between the low and high in-vivo groups which was significant at 6, 12, 18 and 24 months ($p<0.001$). The striking finding of this analysis was the rapid response of those patients with low $IC50^{imatinib}$, and high in-vivo kinase inhibition. Seventy two percent of these patients achieved a MMR by 6 months, and 100% by 18 months. This demonstrates that in this cohort, the extent of kinase inhibition achieved is the key determinant of the achievement of a major molecular response.

The failure to achieve a complete cytogenetic response by 18 months is one of the criteria for primary imatinib failure (Baccarani *et al.* 2006). A 2 log reduction in BCR-ABL has been previously shown to correlate well with a complete cytogenetic response (Branford *et al.* 2004). Assessment of failure to achieve a 2 log response by 18 months, as a surrogate for CCyR by 18 months, revealed that only those patients with low in-vivo kinase inhibition fail to achieve a 2 log reduction. (Table 3.2). This indicates that patients with low in-vivo kinase inhibition over the first 28 days of imatinib therapy, are more prone to therapeutic failure, than those with high in-vivo kinase inhibition ($p=0.002$).

	No of patients analyzed	Median % p-Crkl	Range % p-Crkl
Baseline	**49**	**57.2%**	**35-73%**
Day 7	**44**	**39%**	**13-67%**
Day 14	**47**	**38%**	**13-66%**
Day 21	**46**	**34%**	**11-58%**
Day 28	**39**	**27%**	**10-45%**

Table 3.1 The median and range of %p-Crkl at each analysed timepoint for the patient cohort.

The %p-Crkl was assessed at 7 day intervals over the first 28 days of kinase inhibition therapy. This analysis recealed a wide variation in the level of p-Crkl between patients at each timepoint. Interestingly, while the greatest fall in p-Crkl was seen between baseline and day 7, this reduction was not predictive of molecular response.



Number of patients = 49.

Figure 3.4 The percentage of p-Crkl observed in blood collected at 7 day intervals from 49 patients enrolled to the TIDEL trial.

As demonstrated here, there is a large variation on the %p-Crkl observed between patients at all timepoints. Interestingly, the largest decrease occurs within the first 7 days of imatinib therapy, though as the median levels indicate there is a steady decline in this level to day 28.

	Good Response Criteria 3 log reduction in BCR-ABL (MMR) by				Failure to achieve 2 log reduction by
	6 months	12 months	18 months	24months	18 months
All patients (n=49)	32%	51%	70%	74%	13%
low kinase inhibition in-vivo (n=24)	17%	28%	52%	51%	24%
high kinase inhibition in-vivo (n=22)	45%	73%	90%	96%	0%
p value	0.009	0.002	0.009	0.002	0.002

Table3.2 The percentage of patients achieving optimal and sub-optimal responses based on low and high in-vivo kinase inhibition (dichotomised about the median) to days 21-28.

Comparing the response all patients (n=49), with the low and high in-vivo kinase inhibition groups reveals that patients with high in-vivo kinase inhibition achieve significantly superior molecular responses, to those patients with low in-vivo kinase inhibition, and to the total patient pool.

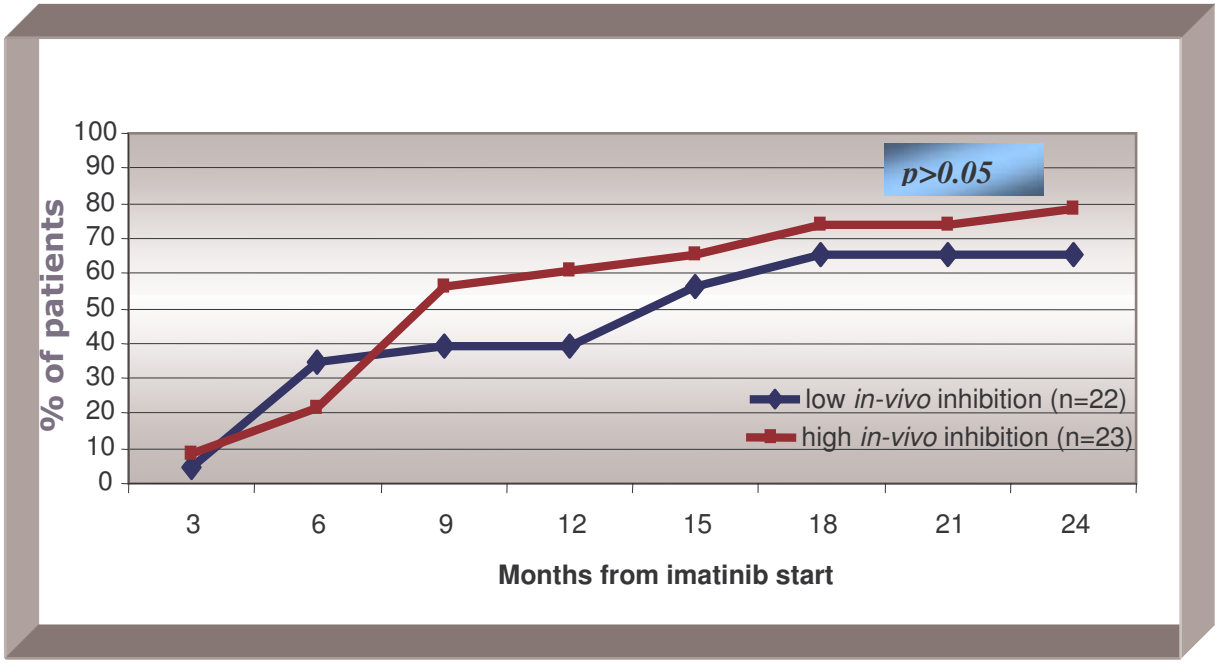


Figure 3.5. The percentage of patients achieving MMR by 24 months based on % reduction in p-Crkl over the first 14 days of imatinib therapy.

Data was dichotomised about the median % in-vivo kinase inhibition achieved over the first 14 days of imatinib therapy, into low and high groups. As demonstrated graphically there was no significant difference in the achievement of MMR between the two groups (*p>0.05*).

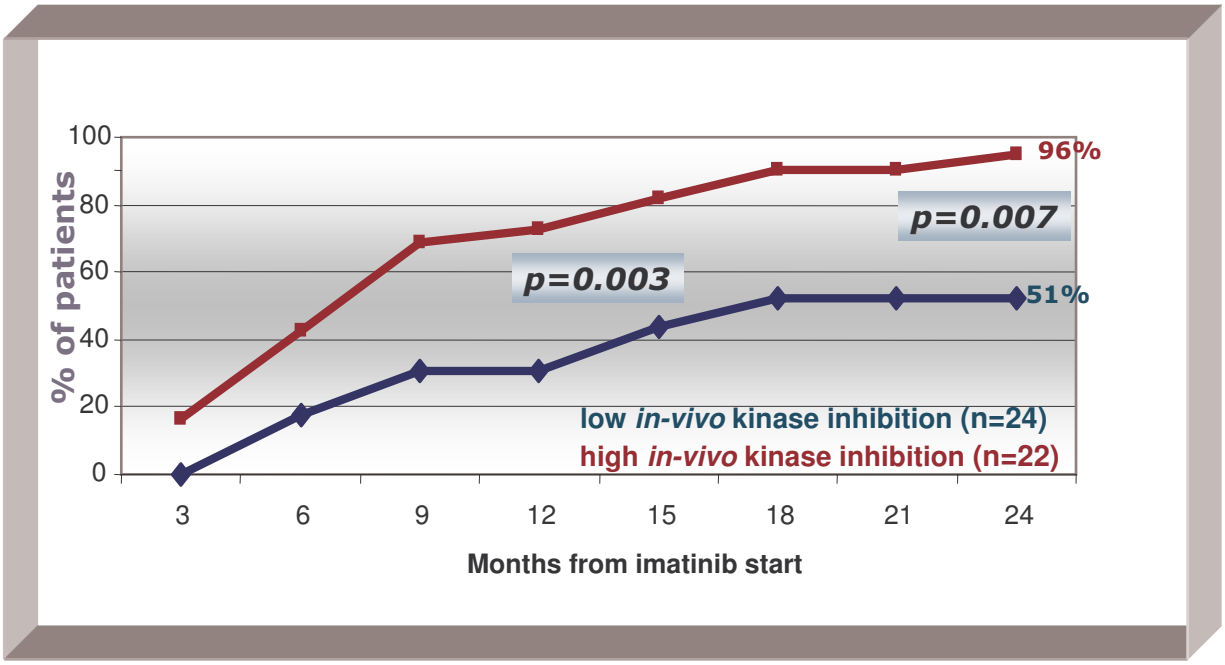


Figure 3.6 The percentage of patients achieving MMR by 24 months based on % kinase inhibition achieved from days 21 to 28.

Groups were formed by dichotomising the data about the median level of kinase inhibition achieved between days 21 and 28 of imatinib therapy. There was a significant difference in the percentage of patients achieving MMR between the low and the high in-vivo kinase inhibition groups at days 21-28 at both 12 *(p=0.003)* and 24 months *(p=0.007).*

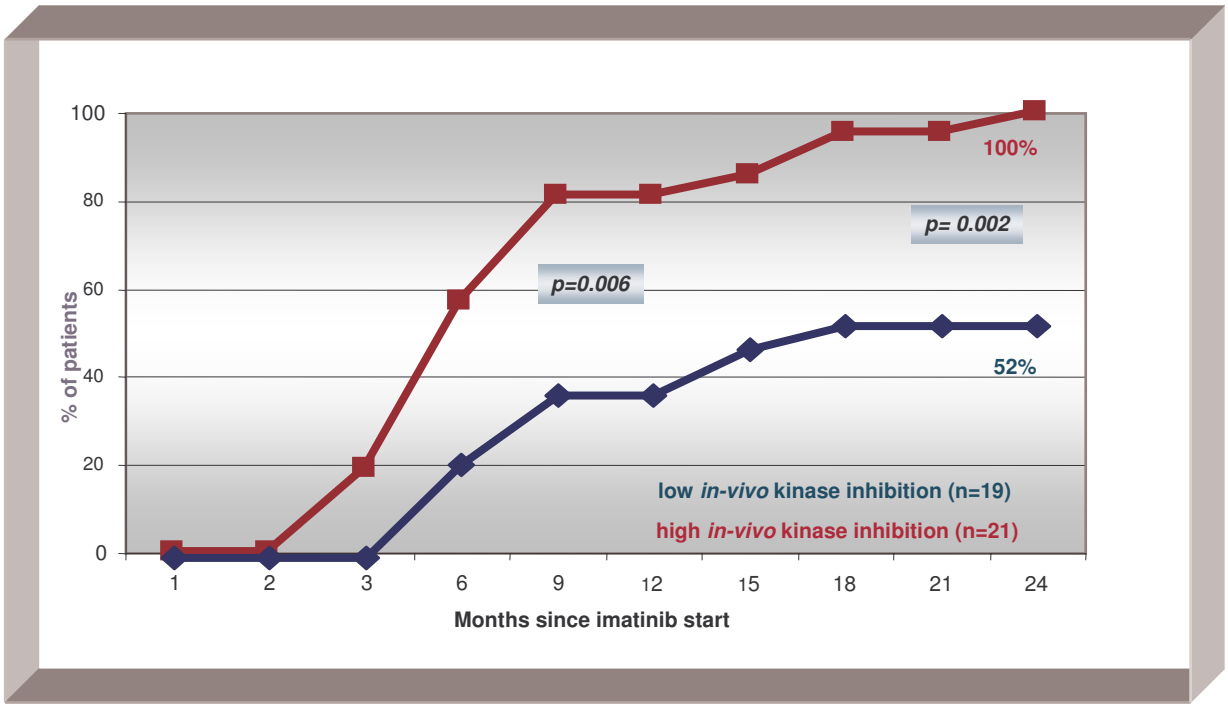


Figure 3.7 The percentage of patients achieving MMR by 24 months based on % kinase inhibition achieved from days 21 to 28 where the analysis was limited to only those patients receiving ≥540mg per day (90%of prescribed dose).

To exclude the possibility that patients with low in-vivo kinase inhibition, may have received sub-optimal dosing, the data was again examined, but this time limited to only those patients receiving >90% of the prescribed dose. As this figure demonstrates, regardless of dose received, those patients with high in-vivo kinase inhibition achieve superior responses to imatinib over the first 2 years of therapy, This demonstrates that the findings of superior molecular response, in patients with high in-vivo kinase inhibition are not simply related to actual dose received.

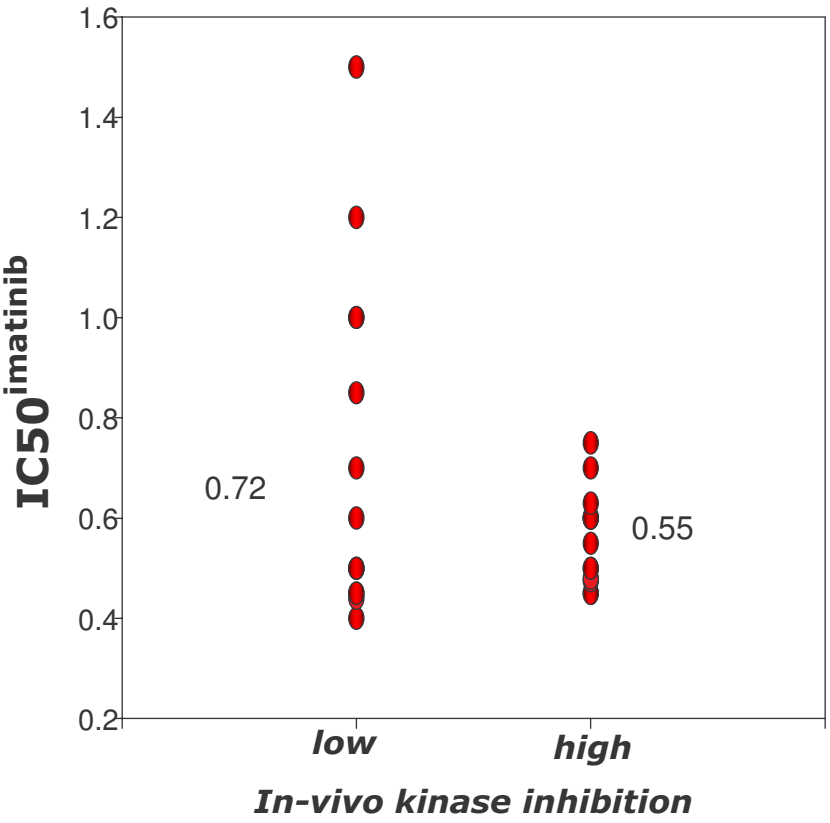


Figure 3.8 A high in-vivo kinase inhibition is associated with a low IC50^{imatinib}.

Those patients with high in-vivo kinase inhibition uniformly have a low IC50^{imatinib} (high in-vitro intrinsic sensitivity). Of interest, some patients with low in-vivo kinase inhibition, also have a low IC50^{imatinib}. This demonstrates that a low IC50^{imatinib} is essential, but not sufficient, to result in high in-vivo kinase inhibition. In patients with low IC50^{imatinib} and low in-vivo kinase inhibition, modified dose, poor compliance, or concomitant medications may also play a role

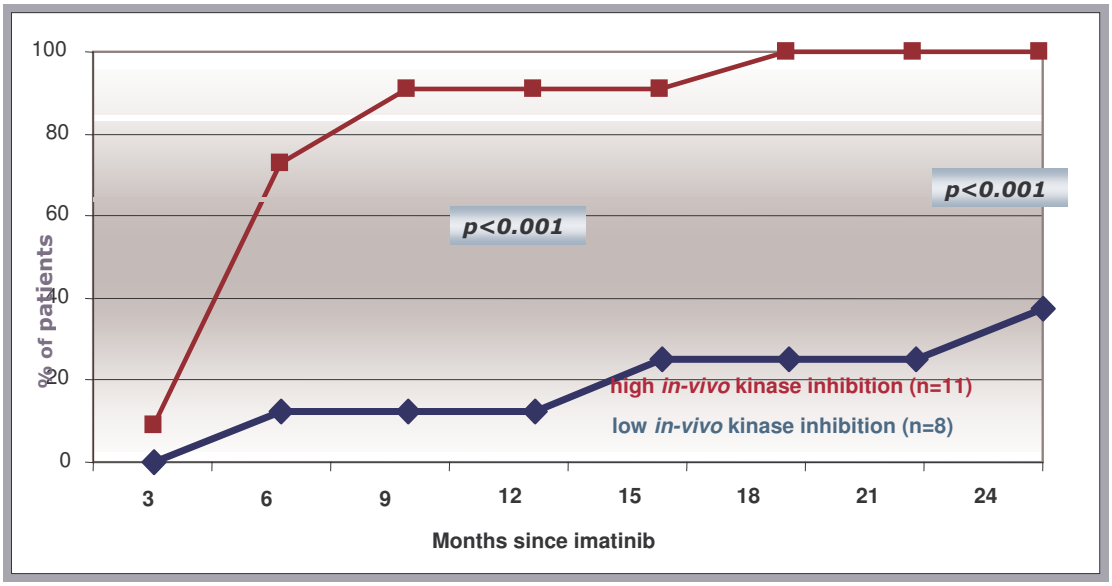


Figure 3.9 The percentage of patients achieving MMR by 24 months based on % kinase inhibition achieved from days 21 to 28, where the analysis was limited to only those patients with low IC50^{imatinib}.

This demonstrates that in the cohort of patients with low IC50^{imatinib} and high in-vivo kinase inhibition, the extent of kinase inhibition achieved is the key determinant of longer term molecular response. However in patients with low in-vivo kinase inhibition other factors are contributing to poor response.

3.5 Summary – Initial Analyses

The degree of imatinib-induced kinase inhibition achieved in-vivo, as measured by the percentage reduction in p-Crkl, provides a good early predictor of long term molecular response. This suggests that the actual level of kinase inhibition achieved is a key determinant of longer term molecular outcome and suggests that either dose escalation, or second generation kinase inhibitors, with increased potency, may result in a more uniform molecular response

3.6 Subsequent Analyses (White et al. 2007a)

3.6.1 Rationale

In initial analyses, the in-vivo kinase inhibition was shown to be a good predictor of intermediate term molecular response in imatinib treated patients. These finding were based on the dichotomisation of the data about the median for the cohort analysed. While this was a successful approach, grouping in such a manner, would be difficult to apply in the prospective setting. As a result, the data was re-examined based on the achievement of 50% in-vivo kinase inhibition by day 28. The question asked in this analysis was therefore, “do patients who achieve a 50% reduction in p-Crkl, at some timepoint in the first 4 weeks of imatinib therapy, achieve superior molecular responses”? Furthermore, the converse question, “do patients who fail to achieve 50% reduction demonstrate sub-optimal response”?, was also addressed

3.6.2 Results

3.6.2.1 Analysis of the degree of kinase inhibition achieved

The degree of kinase inhibition achieved in the first 2 weeks of imatinib treatment was compared to that achieved at the 3rd and 4th week using the Spearman Rank correlation. The kinase inhibition achieved at both day 7 and 14 correlated significantly with the degree of kinase inhibition achieved at days 21 and 28 ($p<0.001$). In patients who achieved >50% kinase inhibition on day 7, 100% maintained this level of inhibition at later timepoints. Thirty nine percent of patients who did not achieve >50% kinase inhibition by day 7 achieved this level of response by day 28. These data make it highly probable that the observed interpatient variability is due to real differences in the level of kinase inhibition achieved.

3.6.2.2 50% reduction in p-Crkl: significance and predictive value for achievement of MMR.

The achievement of 50% kinase inhibition was assessed in patient samples over the first 28 days of imatinib therapy, and the data was subsequently grouped into patients achieving, or not, this level of reduction. A greater proportion of patients who achieved 50% kinase inhibition over the first 28 days, achieved MMR by both 12 and 24 months when compared to those who failed to achieve 50% reduction (*p=0.002 and p<0.001* respectively)(Table 3.3).

No patient who achieved 50% or greater kinase inhibition over the first 28 days of imatinib therapy could be classified as having a suboptimal response to imatinib, based on the criteria of Baccarani (Baccarani *et al.* 2006), (Failure to achieve a 1 log reduction by 6 months and a 2 log reduction by 12 months). In contrast, a significantly greater proportion of patients (15% and 33% respectively) who failed to achieve 50% in-vivo kinase inhibition failed to achieve these subsequent molecular responses (*p=0.005 and p=0.002* respectively) (Table 3.3).

One of the criteria for primary imatinib failure is defined as a failure to achieve a 2 log reduction in BCR-ABL by 18 months (Baccarani *et al.* 2006). Seven of the twenty seven patients (26%) who failed to achieve 50% reduction by day 28, failed to achieve a 2 log reduction by 18 months. No patient who achieved 50% reduction failed to achieve this milestone (*p=0.002*) (Table 3.3).

NOTE: This table is included on page 75 of the print copy of the thesis held in the University of Adelaide Library.

Table 3.3 Proportion of patients failing to achieve, or achieving various imatinib response criteria

In this table sub-optimal, imatinib failure and optimal response criteria (Baccarani *et al.* 2006), based firstly on the entire patient cohort, then on achievement of 50% kinase inhibition by day 28 or not are assessed. These data demonstrate that patients who achieve 50% kinase inhibition have superior molecular responses, where as patients who fail to achieve 50% are more likely to exhibit primary imatinib failure or suboptimal response. (White *et al.* 2007a)

3.6.2.3 Comparison of the achievement of 50% in-vivo kinase inhibition and other early response predictors

The predictive value of the degree of in-vivo kinase inhibition achieved over the first 28 days of imatinib therapy was compared with that of the Sokal prognostic score in 45 patients in whom both parameters were measured. Because this sample size is small for statistical analysis, patients were grouped into low and high Sokal groups about the median for this cohort of 0.93, rather than the usual three Sokal groups. There was no correlation between the Sokal scores and the degree of kinase inhibition achieved to day 28 (r=-0.172 *p=0.261*) Figure 3.10. There was also no significant difference between the median level of kinase inhibition in low (n=23) and high (n=22) Sokal groups (*p=0.331*) (Figure 3.10). Survival analysis performed to assess the achievement of MMR by 12 and 24 months revealed a significant difference between the two Sokal groups (*p=0.024 and p=0.048*). Sokal is also predictive of achievement of 2 log reduction by 12 months (*p=0.046*) but not of any other sub-optimal criteria or imatinib failure (Table 3.4). This indicates that in this cohort of patients the degree of kinase inhibition is a superior predictor when compared to the Sokal prognostic score.

Comparison of the log reduction in BCR-ABL achieved over the first 28 days and the degree of kinase inhibition over the same time period, suggests a correlation between these two parameters (Figure 3.11A). However, dividing patients into those who achieve 50% reduction in p-Crkl and those that fail to do so reveals no significant difference in the log reduction of BCR-ABL between the two groups (Figure 3.11B). Furthermore, as demonstrated in Table 3.4, log reduction in BCR-ABL over the first month is not predictive of subsequent molecular response, in keeping with previous data (Branford *et al.* 1999). These data confirm that the degree of kinase inhibition is a superior response predictor to the log reduction in BCR-ABL over the first month.

NOTE: This table is included on page 77 of the print copy of the thesis held in the University of Adelaide Library.

Table 3.4 The proportion of patients achieving, or not various imatinib response criteria based on 1 month PCR data and Sokal score.

These data indicate that more of those patients who achieve greater log reduction in BCR-ABL over the first month of therapy achieve good subsequent molecular responses. However, there is no statistical difference between between this group and the group of patients who have low reduction in BCR-ABL over the first month of therapy. Similarly, Sokal Scores fail to be as discriminatory with respect to prediction of subsequent molecular response, as the degree of kinase inhibition over the first month.
(White *et al.* 2007a)

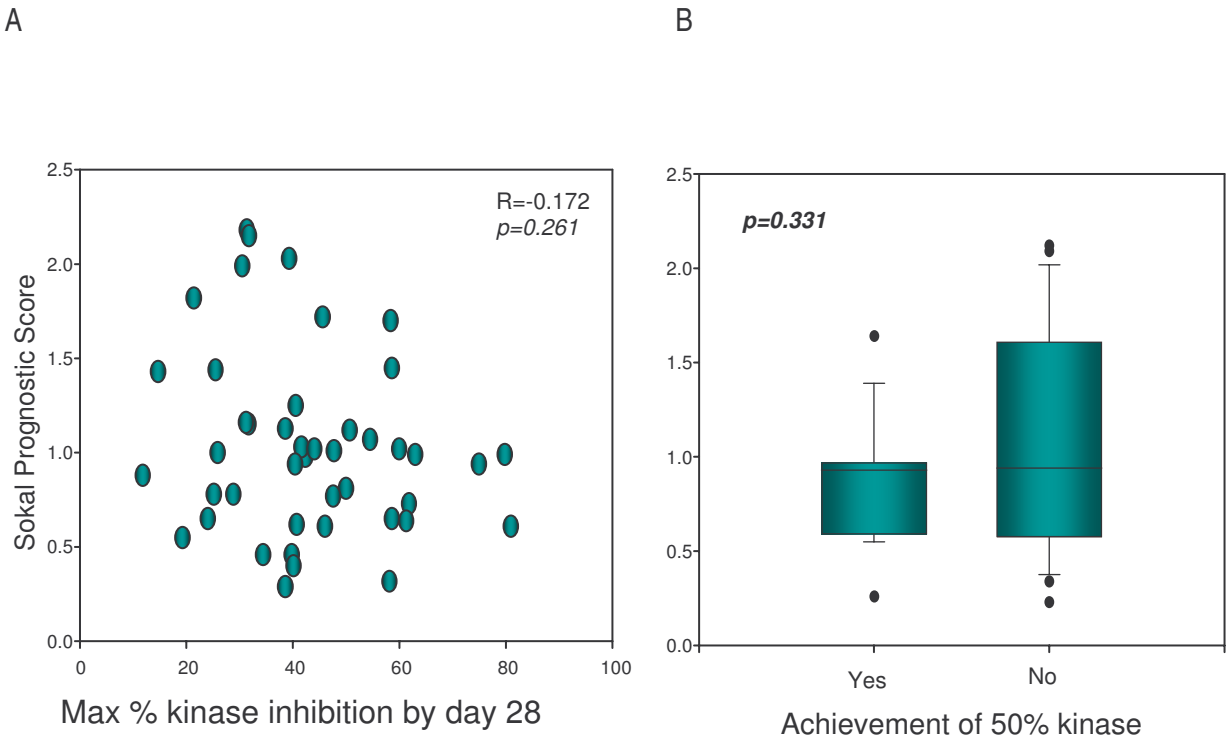


Figure 3.10 Comparison between Sokal Score and the degree of kinase inhibition achieved by day 28.

A) X-Y plot comparing the degree of kinase inhibition with the Sokal Score and demonstrating no significant correlation (*p=0.261*)

B). Box Plot demonstrating the Sokal Scores of patients grouped into those who achieved 50% kinase inhibition and those that did not. As can be seen, while there is a larger variation in the Sokal Scores of patients who fail to achieve 50% kinase inhibition the difference between the two groups is not statistically significant.

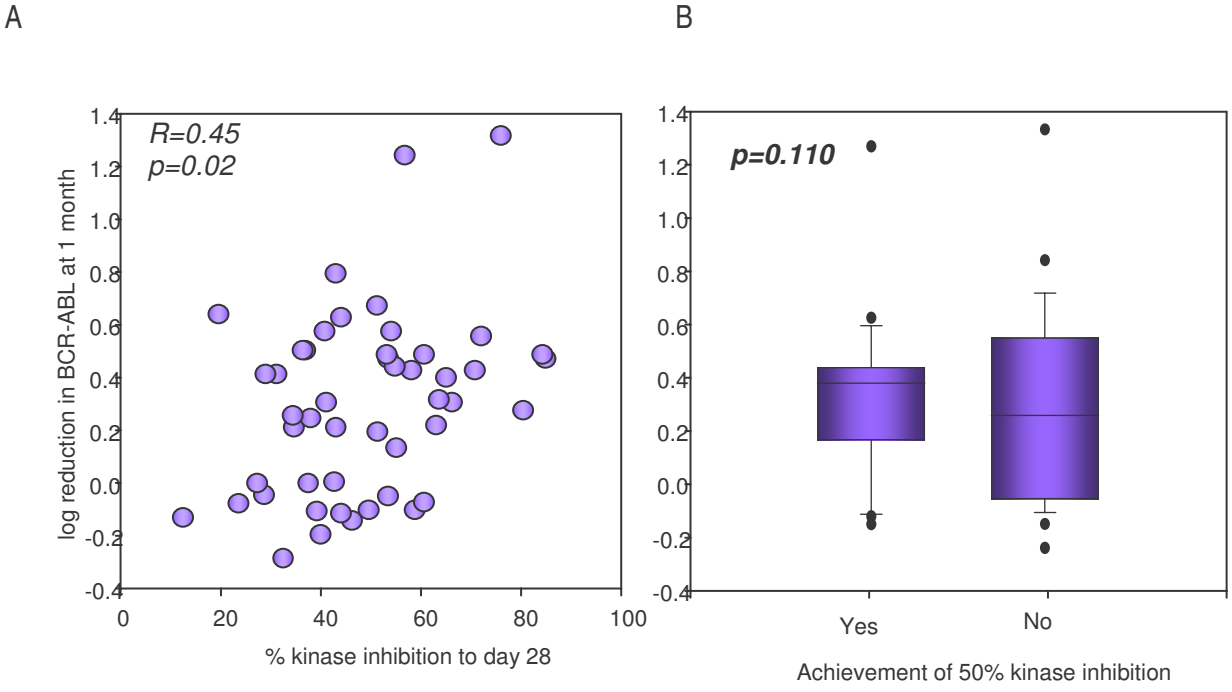


Figure 3.11 Comparison between log reduction achieved at 1 month and the degree of kinase inhibition achieved by day 28.

A) X-Y plot comparing the degree of kinase inhibition with the log reduction in BCR-ABL at one month and demonstrating a correlation (*p=0.02).*

B). Box Plot demonstrating the log reduction in BCR-ABL of patients grouped into those who achieved 50% kinase inhibition and those that did not. As can be seen, while there is some correlation, this does not translate to a significant difference in log reductions achieved by day 28 between the two groups.

As previously discussed (First Analysis), in this study $IC50^{imatinib}$ assays were performed on only 28 patients. There was good correlation between the $IC50^{imatinib}$ and the %kinase inhibition by day 28 (r= -0.403;*p= 0.019*; n=28)(Figure 3.12) indicating that a higher $IC50^{imatinib}$ predicts for poorer in-vivo kinase activity. An $IC50^{imatinib}$ of less than 0.85µM appears to be essential but is not sufficient to achieve >50% kinase inhibition (Figure 3.13). By 24 months 13/13 low $IC50^{imatinib}$ patients with >50% kinase inhibition achieved MMR, compared to only 2/9 (22%) low $IC50^{imatinib}$ patients with <50% kinase inhibition (*p=0.001*). There were insufficient patients with a high $IC50^{imatinib}$ and 50% kinase inhibition (n=6) for statistical analysis.

Patients achieving a 2 log reduction in BCR-ABL by 3 months have a significantly greater probability of achieving MMR, whereas patients who have not achieved a 1 log reduction by 3 months have a low probability of MMR (Branford *et al.* 2003a) (Hughes & Branford 2003) (Hughes & Branford 2006). To assess this parameter in the context of early kinase inhibition, patients were grouped on the basis of log reduction in BCR-ABL after 3 months of therapy, into those who failed to achieve a 1 log reduction, those achieving a 1-2 log reduction, and those achieving a >2 log reduction. As shown in Table 3.5, these data demonstrate that a >50% kinase inhibition predicts for a superior long term molecular response and that the 3 month molecular data provides no additional predictive value in this cohort. In those patients with <50% kinase inhibition the 3 month molecular data provides additional discrimination in response prediction.

Furthermore, the % p-Crkl at baseline was not predictive of MMR by 12 or 24 months (*p=.870 and p=0.584* respectively). This indicates that it is the degree of imatinib induced reduction in p-Crkl which is predictive of longer term molecular response, not the actual % of p-Crkl present at baseline.

NOTE: This table is included on page 82 of the print copy of the thesis held in the University of Adelaide Library.

Table 3.5 Comparison of the predictive value of early molecular response and kinase inhibition achieved.

Data is shown as percentage of patients achieving MMR by both 12 and 24 months, and are generated by Kaplan Meier analysis. Median time to achieve MMR shown in parentheses. *P values* generated by Log Rank Survival Analysis. These data demonstrate that a >50% kinase inhibition predicts for a superior long term molecular response, and that in this cohort, the 3 month molecular data provides no additional predictive value. In those patients who achieve <50% kinase inhibition, the 3 month molecular data provides additional discrimination in response prediction {White, 2007 #2713}

NOTE: This table is included on page 82 of the print copy of the thesis held in the University of Adelaide Library.

NOTE: This figure is included on page 83 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.12 Correlation between $IC50^{imatinib}$ and % kinase inhibition by day 28.

In the 28 patients who had both in-vivo kinase inhibition, and $IC50^{imatinib}$ measured, a significant correlation was demonstrated *(p=0.019)* (White *et al.* 2007a).

NOTE: This figure is included on page 84 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.13 Patients were grouped according to the achievement of 50% kinase inhibition or not and relevant $IC50^{imatinib}$ were plotted against these groups.

The achievement of 50% reduction in kinase inhibition over the first 28 days of imatinib therapy is associated uniformly with a low $IC50^{imatinib}$ (White *et al.* 2007a).

3.6.2.4 Is the reduction in p-Crkl merely a reflection of the normalisation of blood leukocyte parameters?

The first 28 days of imatinib therapy marks a gradual reduction in leukaemic cell burden in most patients, and the beginnings of a normalisation of white cell count and constituent parameters. Therefore, it could be suggested that the reduction in p-Crkl observed is due to the dilution of leukaemic cells with normal blood cells. To address this, patients were divided into those achieving MMR by 24 months, and those failing to achieve MMR by 24 months, and the % kinase inhibition, white cell count and neutrophil:lymphocyte ratio were assessed at 7 day intervals to day 28. The white cell count (WCC) was assessed to address the issue of white cell count normalisation, and to ask the question whether those patients achieving MMR had significantly different reductions in WCC over the first 28 days, to those who failed to achieve MMR. To address this question more specifically, with respect to reduction in the leukaemic cell numbers, the changing neutrophil:lymphocyte ratio was also assessed over the first 28 days of imatinib therapy. Presentation CML patients, pre imatinib have few lymphocytes with the majority of cells in the peripheral blood being myeloid in origin, and more particularly of the neutrophil series. As the WCC normalises, the relative neutrophil numbers decrease, with a concomitant relative rise in lymphocytes. While there is some conjecture, in general, it is thought that lymphocytes in the blood lack the Ph chromosome and are therefore not leukaemic(Hoyle *et al.* 1998; Miura 1998; Miura *et al.* 2000; Takahashi *et al.* 1998). To confirm that the reduction in p-Crkl observed was not directly related to a changing neutrophil:lymphocyte ratio, patients were again divided into those achieving and those not achieving MMR, and this ratio assessed over the first 28 days. As shown in Figure 3.14 the %kinase inhibition was significantly different between the two groups at days 7 and 21. In contrast, there was no such association observed with the other analysed parameters indicating that neither the fall in WCC, or change in neutrophil:lymphocyte ratio were predictive of long term response. This finding reinforces the importance of the degree of early in-vivo kinase inhibition in the achievement of MMR.

Further evidence for the association of in-vivo kinase inhibition and molecular response comes from the investigation of quartiles. Patients were stratified for this analysis, into four groups rather than the previous two, on the basis of the degree of in-vivo kinase inhibition achieved. These four groups were then assessed for molecular response to 24 months. There was a significant difference in the log reduction achieved at both 12 and 24 months between the first (lowest) and fourth (highest) quartiles, and between the first and third quartiles at 24 months (Figure 3.15). These data again demonstrate a

relationship between an increase in the level of kinase inhibition and increase in molecular response, and indicate that the degree of kinase inhibition achieved is a significant factor in subsequent molecular outcomes in imatinib treated patients.

Analysis of time to achieve 50% kinase inhibition was performed to determine whether early achievement of 50% kinase inhibition was advantageous with respect to achievement of MMR. Patients with good kinase inhibition were divided into those who exhibited 50% kinase inhibition in the first 2 weeks and those who achieved 50% in the second 2 weeks of therapy. All patients achieving 50% kinase inhibition in the first 28 days achieved MMR by 24 months. There was no statistical difference in the achievement of, or the timing of achievement of, MMR between those patients who achieved 50% kinase inhibition in the first or second two weeks of therapy. However, both groups were significantly different in the achievement of MMR, when compared to the group of patients with less than 50% kinase inhibition ($p=0.002$)(Figure 3.16).

NOTE: This table is included on page 88 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.14 Assessment of early treatment phase blood parameters contributing to achievement of MMR

Patients were grouped according to their achievement of MMR,or not, by 24 months. These groups were then examined relative to the following parameters measured to day 28:-

A The % kinase inhibition.

B Change in white cell count (x 109/L).

C Change in Neutrophil:Lymphocyte ratio.

Only the % kinase inhibition demonstrated a significant difference to day 28 between those patients who achieve MMR by 24 months and those that fail to achieve this (White et al. 2007a).

NOTE: This table is included on page 88 of the print copy of the thesis held in the University of Adelaide Library.

NOTE: This table is included on page 89 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.15 Assessment of quartiles of the degree of kinase inhibition achieved on subsequent molecular response

Patient's maximum level of in vivo kinase inhibition during the first 28 days was first ranked and then divided into quartiles. The average log reduction in BCR-ABL as measured by quantitative real-time polymerase chain reaction was then assessed based on these quartile groups. Statistical significance was determined using one-way repeated-measures analysis of variance (White *et al.* 2007a).

*indicates difference between 1st and 4th quartiles at 12 and 24 months.

+ indicates difference between 1st and 3rd quartiles at 24 months.

NOTE: This figure is included on page 90 of the print copy of the thesis held in the University of Adelaide Library.

Figure 3.16 The time to achieve 50% kinase inhibition, within the first month of therapy, is not a significant determinant of MMR by 24 months

Patients were divided into 3 groups: those failing to achieve 50% kinase inhibition by day 28, those achieving 50% kinase inhibition by day 14, and those achieving 50% kinase inhibition on days 21-28. Groups were compared to the log reduction in BCR-ABL over time.

There was no significant difference in the percentage of patients achieving MMR, between the groups that achieved 50% kinase inhibition by day 14 or day 28. Both groups were however statistically different to the group of patients that failed to achieve MMR. (White *et al.* 2007a)

3.7 Summary

These findings demonstrate the importance of in-vivo kinase inhibition, which reflects a summation of the effects of intrinsic sensitivity ($IC50^{imatinib}$) to imatinib-induced kinase inhibition, actual dose received, fall in CML burden and pharmacokinetic factors such as GI absorption and hepatic metabolism. These data demonstrate the degree of kinase inhibition achieved in-vivo is highly variable between patients, and the level of inhibition achieved is a significant predictor of subsequent molecular response. Furthermore, those patients who achieve a 50% reduction in the level of p-Crkl in-vivo over the first 28 days have significantly superior molecular responses when compared to those patients who fail to do so. This suggests that the degree of kinase inhibition achieved is the main determinant of the extent of molecular response achieved, and that failure to achieve MMR is due predominantly to inadequate kinase inhibition.

In the first analysis, it was demonstrated that the degree of kinase inhibition achieved in the second two weeks of imatinib therapy was more predictive than that which occurred in the first two weeks. In the subsequent analysis, while some patients achieved 50% kinase inhibition by day 14, others did not achieve this until day 28. Interestingly, patients from both groups achieved MMR, and there was no statistical difference between the two groups with respect to achieving MMR. This suggests that there is no long term advantage, with respect to molecular response, in achieving good kinase inhibition in the first 14 days. This is somewhat surprising, and the reasons for this finding remain unclear, especially since it has been demonstrated that those patients who achieve 50% inhibition do not subsequently lose this over the next two weeks. Examination of larger patient numbers may help elucidate this further.

Dividing patients into two cohorts according to maximal in-vivo kinase inhibition to analyse outcome may provide an impression that there are two distinct groups of responders – good and poor. In fact if the patients are divided into quartiles according to their maximal level of kinase inhibition achieved it appears there is a steady improvement in molecular response as the level of kinase inhibition increases. This is consistent with the concept that BCR-ABL kinase inhibition remains partial in most patients and that the optimal level of kinase inhibition is not often achieved on imatinib

3.8 Overall Conclusion

In this Chapter, a novel assay for determining the level of ABL kinase inhibition achieved in-vivo in imatinib treated CML patients is presented. The variability of kinase inhibition between patients is well demonstrated and the level of kinase inhibition achieved is highly predictive of molecular response. If the predictive value of in-vivo BCR-ABL kinase inhibition assays are confirmed prospectively this raises the possibility that imatinib dose could be titrated against in-vivo measures of kinase inhibition to achieve optimal target inhibition. Patients with suboptimal kinase inhibition may benefit from higher doses, and patients with complete kinase inhibition and significant toxicity may be able to safely dose reduce as long as kinase inhibition remained adequate.

The approach of analyzing the extent of early kinase inhibition achieved will probably represent a more effective strategy than a fixed dose for all patients and may lead to better outcomes for patients with poor ABL kinase inhibition. The value of individualized dosing with other small molecule inhibitors based on measures of target inhibition requires formal assessment. While assays of target inhibition in solid tumours will not always be as convenient as a blood test, titration of target inhibition is a promising development in the search for targeted anticancer therapy that is both safe and effective.

Chapter 4

Investigation of the intracellular transport mechanism of imatinib

4.1 Introduction

Chapters 2 and 3 detail the significant finding that the degree of kinase inhibition achieved pre (in-vitro), and in the early stages of imatinib therapy (in-vivo) , is a critical determinant of long term response in chronic phase (CP), previously untreated CML patients. The successful development of assays to measure the degree of imatinib induced kinase inhibition mediated, both in-vivo and in-vitro, has led to this finding. However the underlying intrinsic cause of this variance remains unknown. What is clear however, is that a greater proportion of those patients with low intrinsic sensitivity, will demonstrate poorer molecular responses after two years of imatinib therapy, than those patients with high intrinsic sensitivity. Indeed, a significantly greater proportion of patients with low intrinsic sensitivity demonstrate suboptimal response or therapeutic failure to imatinib monotherapy (White *et al.* 2005a 2498) (White *et al.* 2007d).

Imatinib insensitive point mutations in BCR-ABL, and BCR-ABL amplification are implicated in the development of secondary resistance. However, these factors have been excluded, through cytogenetic analysis and mutation screening, as a cause of high $IC50^{imatinib}$ (low intrinsic sensitivity) in our cohort of newly diagnosed CML patients pre imatinib initiation (White *et al.* 2005a). Drug transporters play a key role in the uptake and elimination of chemotherapeutic agents via the gastrointestinal tract. Imatinib, an oral formulation, is rapidly absorbed with peak plasma concentrations (C_{max}) achieved at 1-2 hours (Peng *et al.* 2004; Schmidli *et al.* 2005). Cytochrome P450 is the major isoenzyme responsible for imatinib metabolism, which occurs primarily in the liver by the CYP3A4 and CYP3A5 isoforms (AG; Cohen *et al.* 2002). The interpatient variation in CYP3 activity is large and this is thought to contribute to observed interpatient differences in imatinib pharmacokinetics (PK) (Peng *et al.* 2004). Furthermore, imatinib is known to be 89-96% protein bound in plasma, primarily to α1-Acid Glycoprotein (AGP). Patients with CML have significantly elevated AGP (Lyseng-Williamson & Jarvis 2001) which may reduce exposure to free imatinib in plasma, and may also be a further cause of interpatient variation (Gambacorti-Passerini *et al.* 2000; Gambacorti-Passerini *et al.* 2002; Gambacorti-Passerini *et al.* 2003). However, there remains conjecture (Jorgensen *et al.* 2002; le Coutre *et al.* 2002) about the impact of protein binding on patient response to imatinib. While these factors may, or may not impact, on the in-vivo response to imatinib they do not explain the observed differences in in-vitro sensitivity (White *et al.* 2005a).

Early investigations indicated that imatinib does not penetrate the blood-brain barrier(Petzer *et al.* 2002; Pfeifer *et al.* 2003; Takayama *et al.* 2002). As the central nervous system (CNS) is known to have high expression of the ATP-binding cassette transporters, ABCB1 (PgP or MDR1) and the breast cancer resistance protein, ABCG2 (BCRP1), and others, these finding provided the first indication that these, and possibly other efflux transporters may be involved with imatinib cellular extrusion(Dai *et al.* 2003; Gurney *et al.* 2007; Hegedus *et al.* 2002; Illmer *et al.* 2004; Mahon *et al.* 2003; Ozvegy-Laczka *et al.* 2004b). However, this is not without conjecture, with reports indicating that the impact of ABCB1 on imatinib transport is of minimal clinical impact (Ferrao *et al.* 2003; Zong, Zhou & Sorrentino 2005). Similarly, no clear consensus has been reached with respect to the impact of ABCG2 (Breedveld, Beijnen & Schellens 2006; Breedveld *et al.* 2005; Burger & Nooter 2004; Burger *et al.* 2004; Burger *et al.* 2005; Houghton *et al.* 2004; Jordanides *et al.* 2006; Ozvegy-Laczka C 2005; Ozvegy-Laczka *et al.* 2004b) on imatinib transport. A confounding issue with respect to ABCG2 transport is that imatinib may not only be transported by ABCG2, but has been reported to also inhibit ABCG2 in a concentration dependent manner. Hence, there is a potential for imatinib to modulate its own transport with respect to ABCG2 (Burger & Nooter 2004) (Burger *et al.* 2005).

While it is clear that imatinib binding efflux transporters will impact on intracellular drug concentrations, few studies have investigated the intracellular influx mechanisms for imatinib, despite their central role in drug transport. The first reported documentation of imatinib influx mechanisms was published by Thomas(Thomas *et al.* 2004), and suggested that imatinib was actively transported by the human organic transporter -1 (hOCT-1). This finding was supported by an additional study (Crossman. L, Druker B & Deininger M 2005) which suggested in a small patient cohort that levels of hOCT-1 mRNA correlated with imatinib response.

These studies therefore provided the impetus to investigate the role of intracellular drug concentration, and the impact of drug transporters on the intrinsic sensitivity to imatinib induced kinase inhibition observed in our patient cohort.

4.2 Summary and research contribution

The research detailed in this chapter, the attached manuscript and conference proceedings, have contributed significantly to understanding the impact of intracellular imatinib concentrations on intrinsic sensitivity. Furthermore, the data presented here provides further confirmation of the involvement of hOCT-1 with imatinib transport. The research in this chapter describes the development of the assay and addresses correlations with previous findings.

Publications

White DL, Saunders VA, Dang P, et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. Blood. 2006;108:697-704 (White *et al.* 2006)

White DL, Saunders VA, Dang P, et al. Most CML patients who have a suboptimal response to imatinib have low OCT-1 activity: higher doses of imatinib may overcome the negative impact of low OCT-1 activity. Blood. 2007;110:4064-4072. {White, 2007 #2750}

Conference Presentations.

White DL, Saunders VA, Engler J, et al. Low Baseline Intrinsic Sensitivity to Imatinib (High IC50) in CML Patients Is Due to Reduced Oct-1 Mediated Influx. Intrinsic Sensitivity to AMN107 Does Not Correlate with That of Imatinib and Uptake of AMN107 Is Not Oct-1 Mediated. ASH Annual Meeting Abstracts. 2005;106:1081- (White *et al.* 2005c).

White, D., V. Saunders, et al. (2006). "Molecular Response to Imatinib Is Dependent on Dose in CML Patients with Low OCT-1 Influx Activity. Patients with High Activity May Respond Equally Well to Standard or Increased Dose Imatinib." ASH Annual Meeting Abstracts 108(11): 738- (White Deborah *et al.* November 2006).

4.3 Assay development

4.3.1 Background

The major influx proteins belong to the largest superfamily of transporters, the solute carrier (SLC) superfamily (Jonker & Schinkel 2004). In humans the SLC22 sub-family consists of 12 members encompassing the polyspecific organic cation transporters (OCTs). The OCT proteins are associated with the bidirectional transport of endogenous and exogenous substrates, which exist as cations at physiological pH, across the plasma membrane. (Koepsell & Endou 2004; Koepsell, Schmitt & Gorboulev 2003). It is this family, in combination with the organic anion transporters (OATs) that are involved in the absorption, distribution and elimination of drugs in vivo (Kimura *et al.* 2002). Transporters may be oligospecific (transport a single main substrate) or polyspecific (translocate a number of different organic cations of different molecular structure).

The Organic Cation Transporter -1 (OCT-1), implicated in imatinib transport by Thomas *et al* (Thomas *et al.* 2004), is a polyspecific organic cation uniporter. Other members of this family are OCT-2, OCT-3 and OCTN1 and OCTN2 (Koepsell & Endou 2004; Koepsell *et al.* 2003). More recently OCT-6 has also been described (Gong *et al.* 2002). As shown in Figure 4.1, OCT-1 has 12 transmembrane domains, a large extracellular binding pocket, and several cytoplasmic domains.

The gene encoding OCT-1 contains 11 exons and 10 introns. OCT-1, 2 and 3 share 236 conserved amino acids. (Koepsell *et al.* 2003), and also share the ability to translocate cations with varying structures. Transport can be inhibited by other non-transported compounds, and transport occurs in an active, electrogenic fashion. While OCT-1 is largely expressed in the sinusoidal membrane of hepatocytes, mRNA has been detected in varying levels in the blood of normal individuals, in CML patients and in human cell lines (Thomas *et al.* 2004).

Given these findings, and the previous background as discussed, an assay was developed to measure the intracellular concentration of imatinib in CML cells from our newly diagnosed cohort, using radiolabelled drug. The first aim of this approach was to assess correlations between imatinib uptake and $IC50^{imatinib}$. The second aim was to further clarify the proteins involved in the transport of kinase inhibitors using known transport inhibitors. In addition the expression of key efflux proteins (ABCB1

and ABCG2) was also assessed using flow cytometry to ascertain their contribution to the intracellular drug level obtained.

4.3.2 Intracellular Uptake and Retention (IUR) Assay

[14C]-labelled imatinib was kindly provided by Novartis Pharmaceutical Basel Switzerland. The assay described here, was based on the assay of Zhang(Zhang *et al.* 1999), and is described in detail in Appendix I: Materials and Methods. Assay conditions were established to mimic those used in the IC50 assay (2hour incubation at 37oC) in IC50 media (White *et al.* 2005a). The dynamic range investigated was designed to address the predicted concentrations achievable in-vivo (ie: 0 to 2μM), and the range of $IC50^{imatinib}$ observed.

In brief, 200,000 cells were incubated for 2 hours at 37°C in the presence of varying concentrations of imatinib ranging from 0 to 2 μM. All drug uptake studies were performed in triplicate and [^{14}C]-labeled drug was admixed to the desired concentration with unlabelled drug (Thomas *et al.* 2004). Isotope was resuspended to 1 mg/ml and specific activity was 3.3MBq for [^{14}C]-imatinib. After incubation, the cellular and aqueous phases were separated, and [14C] drug incorporation was determined using a Top Count Microplate Beta Scintillation Counter (Perkin Elmer) following the addition of Microscint 20 (Perkin Elmer Boston MA) scintillation fluid. Results were displayed as ng/200,000 cells and calculated as follows:-

$$\frac{\text{\% drug incorporation}}{} = \text{cpm cells/cpm total (cells + supernatant)}$$

$$\frac{\text{ng/200,000 cells}}{} = \text{(\% drug incorporation)/100 x amount of drug added in ng.}$$

Drug concentrations used were 0, 0.5, 1 and 2μM .

All assays development was performed on the BCR-ABL expressing cell line K562.

The OCT inhibitors prazosin (Sigma Aldrich GmBH. Germany), progesterone (Sigma Aldrich GmBH.) and procainamide (Sigma Aldrich GmBH) were all used at 100μM. The ABCB1 inhibitor PSC833 was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland) the ABCG2 inhibitor fumitremorgin C (FTC; Alexis. San Diego, CA) and the fumitremorgin C analogue Ko143 (kindly supplied by Dr John Allen. Centenary Institute Sydney) were all used at 10 μM.

Human K562, KU812 and CCRF-CEM cell lines were obtained from the American Type Culture Collection (ATCC Manassus VA). The ABCB1 over-expressing variant of CCRF-CEM, CEM-VBL100 was kindly provided by Professor Leonie Ashman, University of Newcastle, Newcastle. Australia. ABCG2 overexpressing Mef cells: Mef-BCRP and the parental Mef-3.8 cell lines were kindly provided by Dr John Allen (Centenary Institute, Sydney)

4.3.3 Flow cytometric assessment of Efflux Proteins.

Expression levels of ABCB1 and ABCG2 were assessed by flow cytometric analysis using Phycoerthyrin conjugated (PE) anti- ABCB1 antibody, UIC2 (Immunotech, France) and anti-ABCG2 (R & D Systems. Minneapolis MN) were used for analysis. Analysis was performed using an Epics XL-MCL Flow Cytometer (Beckman Coulter Miami FL). Detection limits of this assay were determined using limiting dilution of the ABCB1 expressing cell CEM-VBL100 and ABCB1 negative parental line CCRF-CEM. Using this approach the assay was sensitive to the 1% level (Figure 4.2)

4.3.4 $IC50^{imatinib}$ assay

The assay was performed in accordance with the protocol described in brief in Chapter 2, and with the in-depth protocol listed in *Appendix I: Materials and Methods*. In addition assays were performed in the presence and absence of transporter inhibitors prazosin and progesterone (IC50 for OCT-1, OCT-2 and OCT-3: 3.1 µM, 27 µM and 4.3µM respectively). Both drugs were added to the $IC50^{imatinib}$ assay at 100µM, and at these concentrations, both are inhibitors of OCT-1 and OCT-3.

NOTE: This figure is included on page 100 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.1 The Human Organic Transporter – 1 (hOCT-1)

Diagrammatic representation of the proposed structure of hOCT-1 with its 12 transmembrane domains, a large extracellular binding domain, and several cytoplamsic domains.

Adapted from Shu, Yan et al. (2003) Proc. Natl. Acad. Sci. USA 100, 5902-5907 (Shu *et al.* 2003)

4.4 Results

4.4.1 Correlation between IC50^{imatinib} and IUR

The initial analysis of 25 patients revealed significant correlation between the IC50^{imatinib} and the IUR at 0.3, 0.5, 1 and 2μM imatinib (*p=0.03, 0.016, 0.017 and 0.014* respectively) (White *et al.* 2005a). More recent analysis of a greater number of patients has strengthened this analysis with the p value associated with the correlation now *p=0.00056* (Figure 4.3) (White *et al.* 2007c). Dividing patients into low and high IC50^{imatinib} cohorts revealed a significant difference in the IUR for imatinib between the low and high IC50^{imatinib} groups (*p=0.001*)(Figure 4.4). This again demonstrates the association between IC50^{imatinib} and the level of intracellular drug achieved.

4.4.2 Effect of temperature of IUR of imatinib

It has been previously suggested that the uptake of imatinib is temperature dependent (Thomas *et al.* 2004). To assess this in our patient cohort, IUR assays were performed at 4^{o}C and 37^{o}C in K562, KU812 and CCRF CEM cell lines. In keeping with this suggestion, a significant difference was found between the IUR for imatinib at the two temperatures when the drug was used at 2μM (median IUR at 37^{o}C=34.8ng; at 4^{o}C=23.2ng/200,000 cells *p=0.038*). This result suggests that the transport of imatinib is temperature dependent, and as the IUR is higher at 37^{o}C than at 4^{o}C suggests the transport process is active, with the most potent variable being uptake.

.

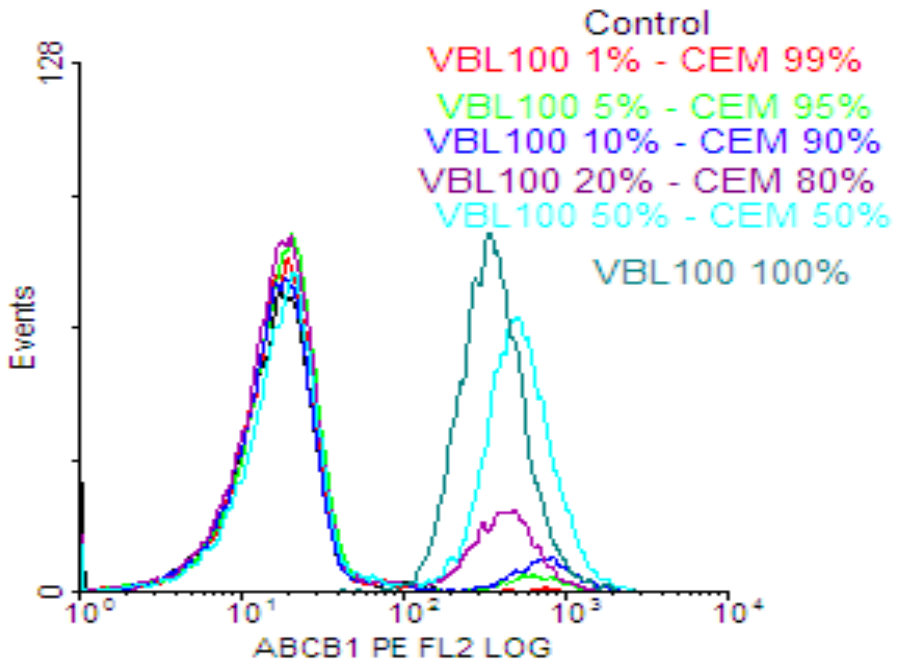


Figure 4.2 Comparison of the $IC50^{imatinib}$ and the intracellular uptake and retention (IUR) of [14C] imatinib.

A limiting dilution experiment was performed by diluting the ABCB1 expressing cell CEM-VBL100 into the ABCB1 negative parental line CCRF-CEM. As demonstrated here 1% of ABCB1 bearing cells could be identified by flow.

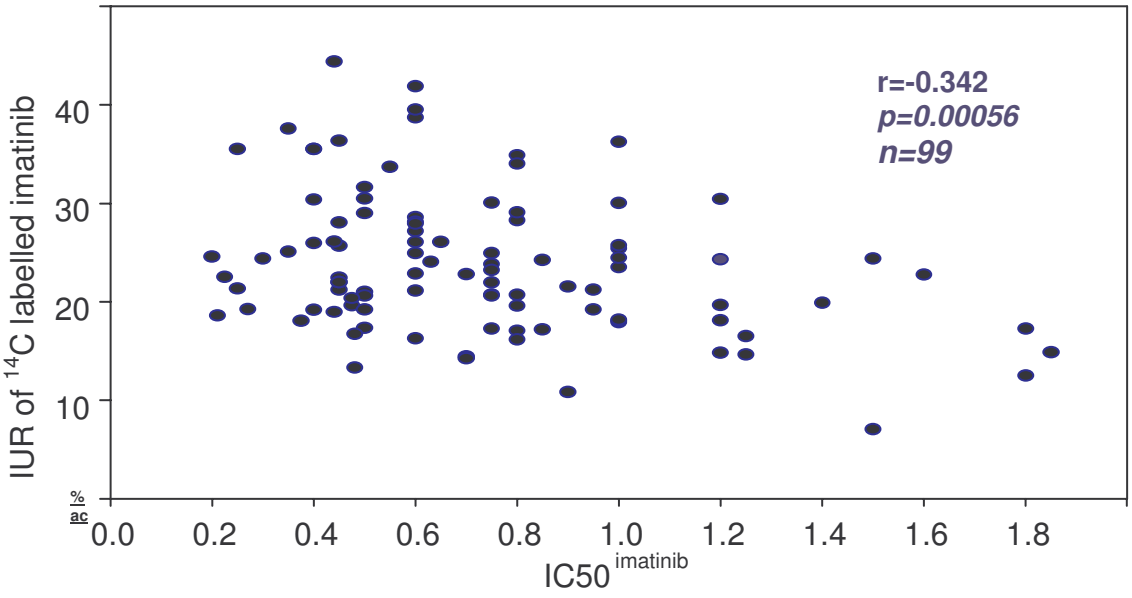


Figure 4.3 Comparison of the IC50^{imatinib} and the intracellular uptake and retention (IUR) of 2 UM[14C] imatinib.

The IC50^{imatinib} and the IUR of imatinib (at 2µM) were compared in 99 newly diagnosed chronic phase CML patients (White *et al.* 2007c). Both assays were performed at 37^{o}C with readouts occurring after a two hour incubation.

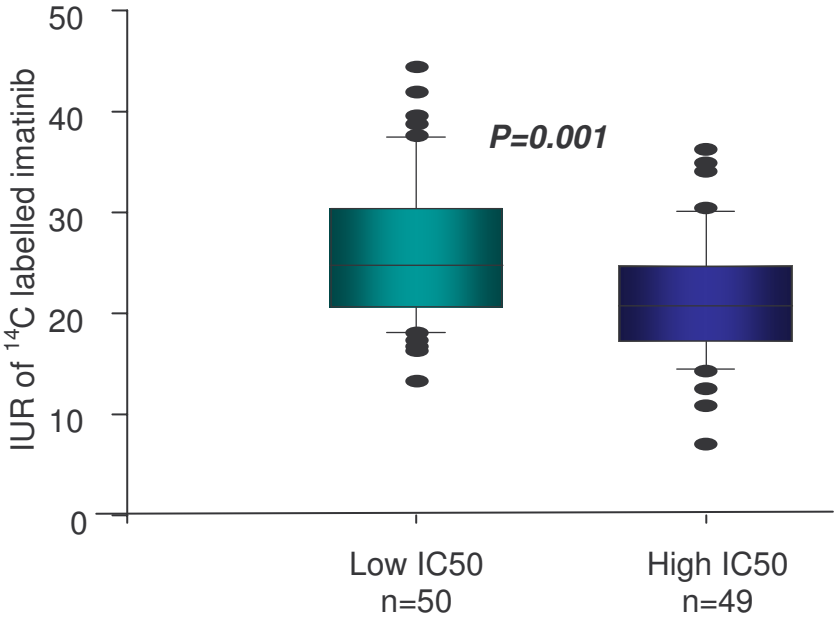


Figure 4.4 Box plots demonstrating the difference in IUR (at 2μM imatinib) between high and low IC50^{imatinib} groups.

The IUR assay was performed on 99 patients. Patients were divided into low and high IC50^{imatinib} groups about the median IC50^{imatinib}. This figure demonstrates the significant difference in IUR of imatinib between the two IC50^{imatinib} groups.

4.4.3 The effect of OCT inhibitors on the IUR of imatinib

Prazosin is a potent inhibitor of OCT-1 and to a lesser extent OCT-3 (comparative IC50 values OCT-1 / OCT-2 / OCT-3: are 1.8, >100 and 13 μM respectively) (Hayer-Zillgen, Bruss & Bonisch 2002). The addition of prazosin to the IUR assay of CP CML patients, reduced the intracellular concentration of imatinib significantly (IUR no prazosin 24.35ng; with prazosin 8.57 ng/200,000 cells $p<0.005$), suggesting imatinib uptake is being inhibited, and implicating OCT-1 or OCT-3 as the influx transporter for imatinib. Importantly, the addition of prazosin also removed the difference in IUR observed between low and high $IC50^{imatinib}$ groups (Figure 4.5) (Table 4.1) (White *et al.* 2007c), suggesting this difference in the $IC50^{imatinib}$ cohorts is attributable to the active influx of imatinib, mediated by OCT- 1 or 3.

Procainamide is an inhibitor of OCT-1 and OCT-2 (IC50's 74μM and 50μM respectively) but importantly, and in contrast to prazosin, not an inhibitor of OCT-3 (IC50 738μM) (Koepsell *et al.* 2003) (Zhang *et al.* 1999) (Gorboulev *et al.* 1997). The addition of procainamide to the IUR assay of CP CML patients resulted in a reduction in the intracellular concentration of imatinib. Dividing the patients into low and high $IC50^{imatinib}$ demonstrated a significant difference in the IUR of the low $IC50^{imatinib}$ group, but not the high $IC50^{imatinib}$ group (Table 4.1). Importantly, like the addition of prazosin, the addition of procainamide, removed the difference between the low and high $IC50^{imatinib}$ groups. Given the varied specificities of the two inhibitors, this suggests that OCT-1 is implicated in imatinib transport.

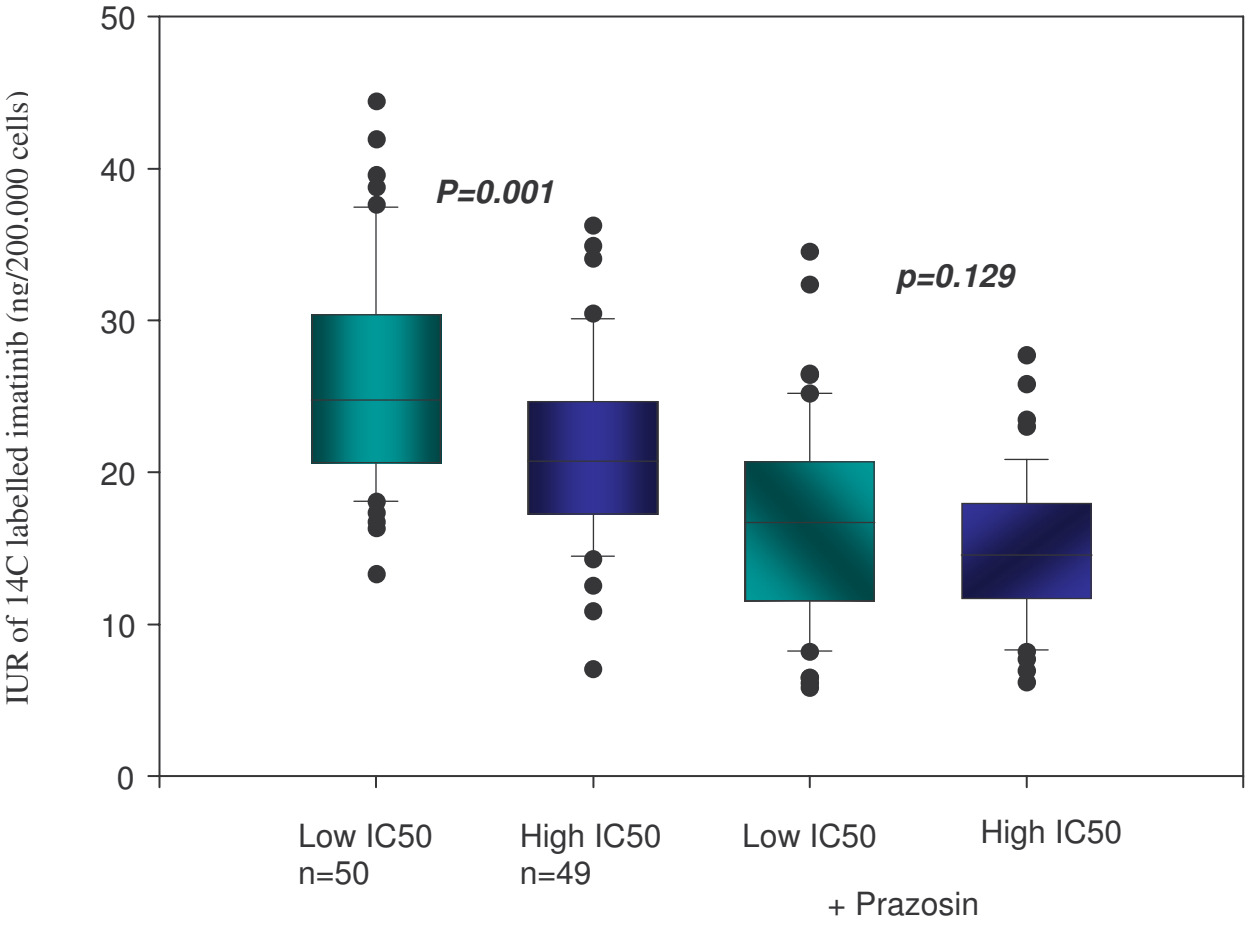


Figure 4.5 Box plots demonstrating the difference in IUR (at 2µM imatinib) between high and low IC50^{imatinib} groups, and the effect of the addition of Prazosin to these groups.

Prazosin is a potent inhibitor of OCT-1 and OCT-3. As in Figure 4.4 there is a significant difference in the IUR between the low and high IC50^{imatinib} groups. The addition of prazosin removes the significant difference in IUR between these groups, and therefore implicates OCT-1, OCT-3 or both in imatinib influx.

Table 4.1 The effect of specific influx and efflux inhibitors on IUR of imatinib on the total patient pool, and in the low and high IC50imatinib cohorts

The effect of inhibitors was assessed in 25 CP CML patients. Of these 14 had low $IC50^{imatinib}$ the remaining 11 high $IC50^{imatinib}$. As demonstrated, the addition of both Prazosin and Procainamide (OCT-1 inhibitors) result in a significant reduction in the IUR in the low $IC50^{imatinib}$ cohort.

*significant difference at the 0.05 level in comparison to no inhibitor

significant difference at the 0.05 level between low and high $IC50^{imatinib}$groups (White *et al.* 2006)

NOTE: This table is included on page 108 of the print copy of the thesis held in the University of Adelaide Library.

4.4.4 Effect of the OCT inhibitors on the IC50^{imatinib}.

To further assess the involvement of the OCT transporters, inhibitors prazosin and progesterone (IC50 for the inhibition of OCT-1 – 3.1μM) were added to the IC50^{imatinib} assay for the BCR-ABL positive cell line K562. As shown in Figure 4.5, the addition of prazosin results in an increase in the IC50^{imatinib}. The addition of the less potent OCT-1 inhibitor progesterone, also results in an increase in the IC50^{imatinib}. While the increase in the %p-Crkl was less marked in the presence of progesterone, and became limiting at 10μM imatinib, the effect on imatinib blockade was still demonstrable. These data suggest that both inhibitors reduce the amount of drug that enters the cell, and provide further evidence for the involvement of OCT-1 (though with both these drugs, at the concentrations used in the assay, OCT-3 inhibition could not be excluded).

4.4.5 Implication of the expression of ABCB1 on the transport of imatinib.

While ABCB1 has been implicated in the transport of imatinib (Dai *et al.* 2003; Gurney *et al.* 2007; Hegedus *et al.* 2002; Illmer *et al.* 2004; Mahon *et al.* 2003; Ozvegy-Laczka *et al.* 2004b),there are some conflicting reports in the literature (Ferrao *et al.* 2003; Zong *et al.* 2005). To investigate the nature of this interaction, the imatinib IUR was performed as previously, in VBL100, a cell line engineered to over express ABCB1, and in the parental CCRF-CEM line. ABCB1 is expressed in 100% of VBL100 cells as demonstrated in Figure 4.7. Triplicate experiments on these lines revealed that the uptake and retention of imatinib into VBL100 cells was greatly reduced when compared to the parental line (IUR CEM 32ng/200,000 cells; VBL100 10ng/200,000 cells). The addition of PSC833 an ABCB1 inhibitor resulted in a reversal of the reduced IUR in the VBL100 line (IUR imatinib in the presence of PSC833 25ng/200,000 cells). These preliminary results confirm the involvement of ABCB1 in imatinib transport.

However this data confirms the effect of ABCB1 only in cells which have high expression of this protein (100% of cells express ABCB1 by flow cytometry). To assess the implications of ABCB1 expression in primary cells from CP CML patients, 10 patients were screened using flow cytometry for expression of this protein. The median level of expression in these patients was 0.3% within a range of 0 to 1.9%. This indicates that high expression of ABCB1 is not a feature of the majority of CP CML patients at presentation, pre imatinib therapy. The IUR was then examined in the presence and absence of the ABCB1 inhibitor PSC833. In keeping with the flow cytometry results, there was no significant difference in the IUR in the presence, or absence of this inhibitor (standard assay) *($p>0.05$*) (Table 4.1). These data demonstrate that ABCB1 is implicated in imatinib transport, and that the expression of this protein has a significant effect of the intracellular concentration of the drug when expression levels are

high, as is the case with the VBL100 cell line. However, in this cohort of CP CML patients, measurable expression of ABCB1 is not a feature, and the addition of PSC833 has no significant effect on the intracellular concentration of the drug.

4.4.6 Implications of ABCG2 on the transport of imatinib.

Similarly ABCG2 has been implicated as a transporter of imatinib (Breedveld *et al.* 2005; Burger & Nooter 2004; Burger *et al.* 2004; Ozvegy-Laczka *et al.* 2004b) (Burger *et al.* 2004) (Jordanides *et al.* 2006; Melo 2006). Although there is some conjecture as to whether imatinib is a substrate for ABCG2, an inhibitor, or both (Houghton *et al.* 2004; Ozvegy-Laczka *et al.* 2004a). To assess this interaction, IUR assays were performed in ABCG2 expressing cells, Mef BCRP1, and parental Mef 3.8 (Figure 4.8) indicate a difference in the IUR for imatinib between the cell lines. The respective imatinib IUR's were Mef 3.8 IUR 74ng/200,000 cells: Mef BCRP1 IUR 53ng/200,000 cells Indicating a reduction in IUR in the presence of ABCG2 expression. The addition of Ko143 (ABCG2 inhibitor, an analogue of Fumitremorgin C, (Allen *et al.* 2003; Allen *et al.* 2002)) resulted in an increase in IUR in the Mef BCRP1 cell line to 72ng/200,000 cells but no such increase in the parental line (74.5ng/200,000 cells).

These findings were followed by analysis of primary CP CML cells. Flow cytometry for expression of ABCG2 was performed on 10 patients, and similar to the results for ABCB1, expression of ABCG2 was not found to be a feature of CP CML patients. The median level of expression in these patients was 0.1% within a range of 0 to 2.5%. Furthermore addition of Fumitremorgin C (FTC), an inhibitor of ABCG2, had no significant effect on the IUR for imatinib (Table 4.1). In keeping with this finding, the addition of FTC had no impact on the significant difference between low and high $IC50^{imatinib}$ groups (Table 4.1).

4.4.7 Correlation between IUR imatinib and molecular outcome.

The IUR of imatinib at 2μM was compared to the molecular response (as determined by real time quantitative reverse transcriptase PCR for BCR-ABL) at 3 months in 19 patients where molecular data was available. A significant correlation was observed between the two parameters ($R = 0.58$ *p=0.041*). Further to this, restricting analysis to the cohort of 11 patients with 12 month molecular follow-up, there was a significant correlation at both 3 and 12 months (R=0.793 *p=0.003*; R=0.643 *p=0.042* respectively). These data indicate that the IUR is predictive of molecular outcome in this small cohort of CP CML patients.

NOTE: This figure is included on page 111 of the print copy of the thesis held in the University of Adelaide Library.

Figure 4.6 The effect of OCT-1 inhibitors on the IC50^{imatinib} in K562 cells

Western blots and corresponding graphs demonstrating

A. Standard IC50^{imatinib} assay.

B. IC50^{imatinib} assay performed with the addition of 100 µM prazosin. Note the different drug concentration range used.

Concentrations where significant differences are noticed +/- Prazosin have been highlighted for reference

C. Combined graphical representation of A and B and also demonstrating the effect of the less potent OCT-1 inhibitor Progesterone.(White *et al.* 2006)

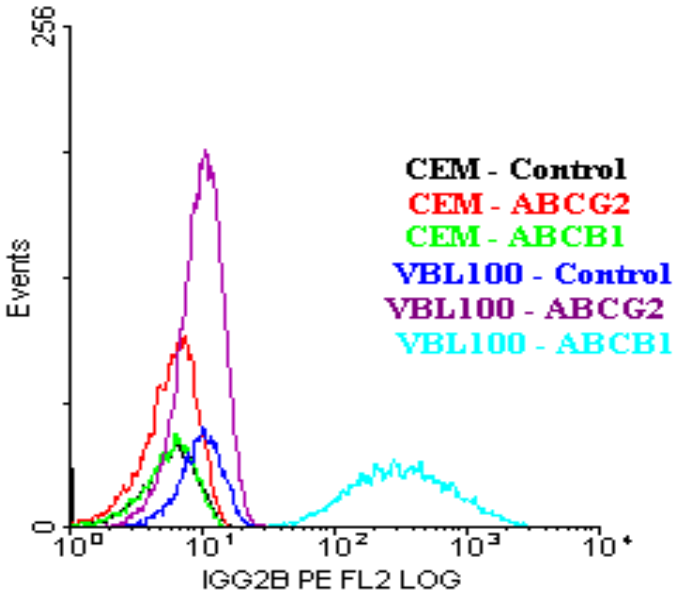


Figure 4.7 FACS Histograms showing the expression of ABCB1 and ABCG2 on VBL100 cells and CCRF-CEM cells.

VBL100, the ABCB1 over expressing variant of CCRF- CEM demonstrated expression of ABCB1 in 100% of cells. These cells do not express ABCG2. The parental line CCRF-CEM does not express ABCB1 or ABCG2.

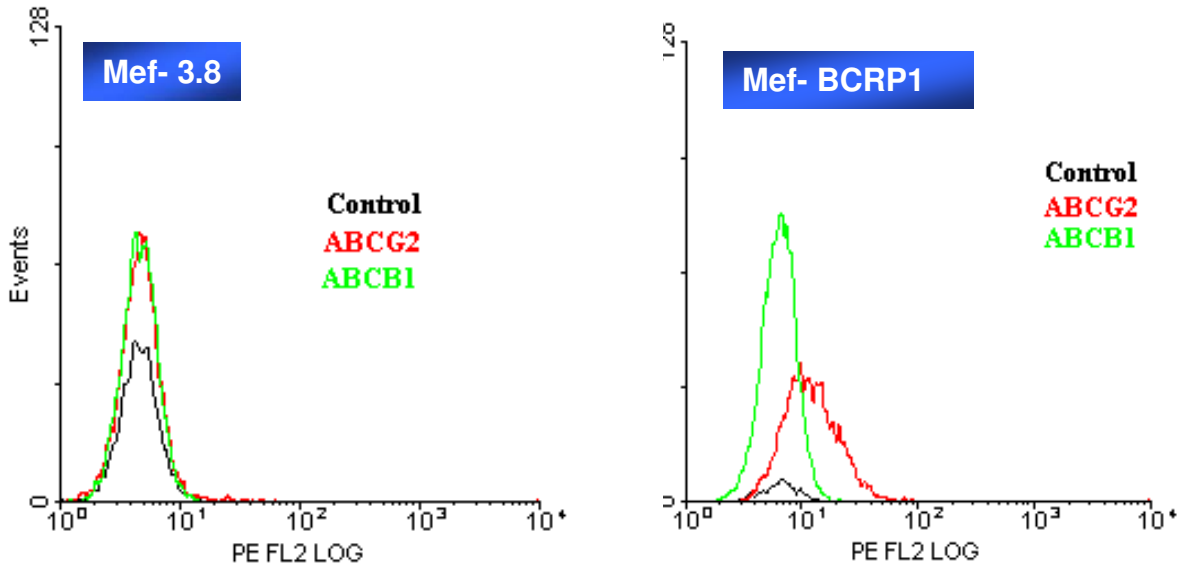


Figure 4.8 FACS Histograms demonstrating the expression of ABCG2 and ABCB1 in Mef 3.8 and Mef-BCRP1 cell lines.

Mef 3.8 (parental cells) do not express ABCG2 or ABCB1 at levels any higher than isotype matched controls. In contrast the ABCG2 over expressing variant Mef-BCRP1 expresses ABCG2, but not ABCB1.

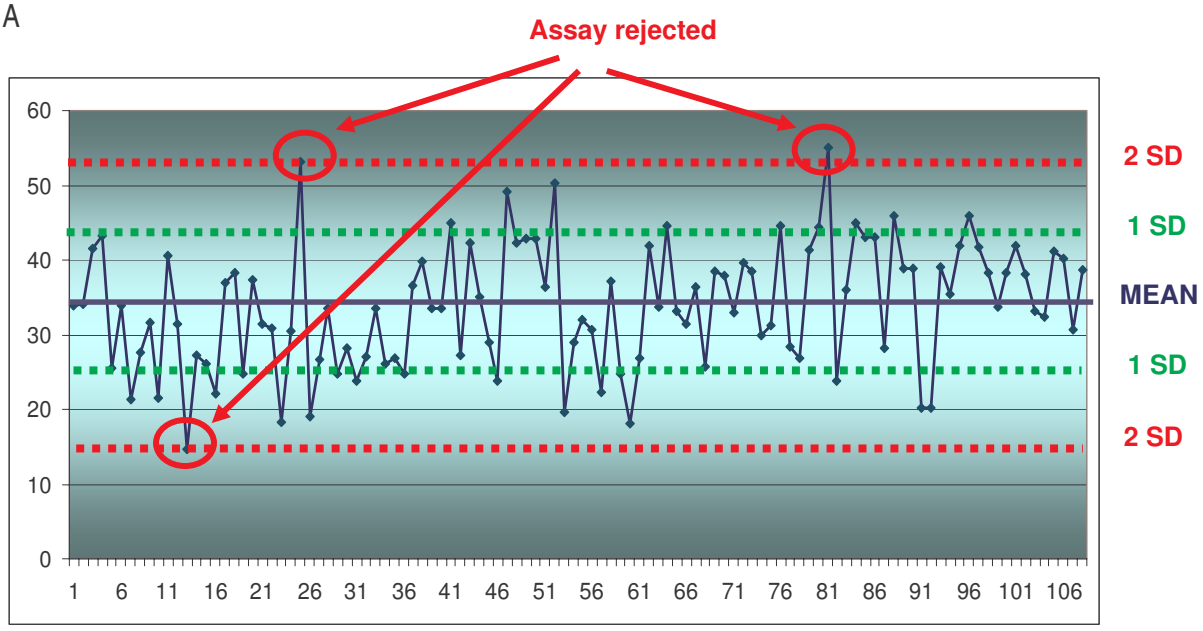
4.5 Assay Control

To control for variability in the IUR Assay K562 cells are assayed at 2uM +/- Prazosin with every assay. This is of particular importance now, since the relevance of the assay has been established, and the assay is used regularly by all members of our research group. As can be seen in Figure 4.9 the assay is very reproducible with only 3% of IUR assays rejected on the basis of falling outside of the mean +/- 2 standard deviations (SD).

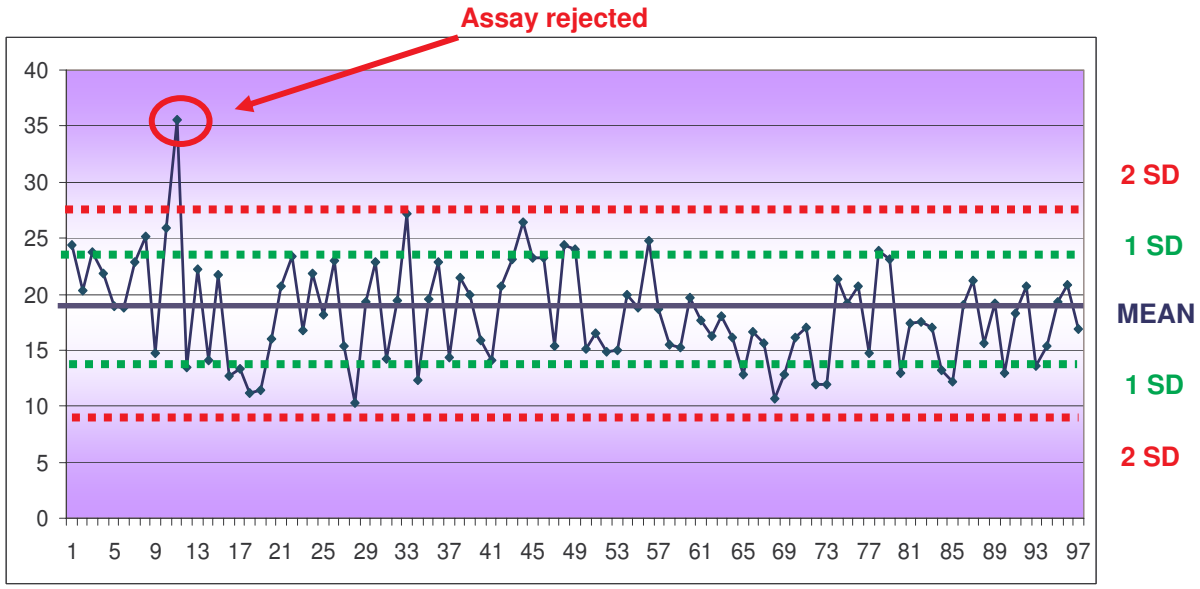
Figure 4.9 Quality Control Curves for A Intracellular uptake and retention (IUR) and B IUR + Prazosin.

Quality control rules were applied to the K562 control results to determine the acceptability of the IUR assay. If the control value was outside the 2 standard deviation range (SD), then the run was rejected (indicated by red circles. Controls were repeated by a second scientist if consecutive runs on sequential days were consistently one side of the mean or the other. If the controls were consistently repeated a new aliquot of K562 cells was thawed.

A


Assay rejected
60
50
40
30
20
10
0
1 6 11 16 21 26 31 36 41 46 51 56 61 66 71 76 81 86 91 96 101 106
2 SD
1 SD
MEAN
1 SD
2 SD

B


Assay rejected
40
35
30
25
20
15
10
5
0
1 5 9 13 17 21 25 29 33 37 41 45 49 53 57 61 65 69 73 77 81 85 89 93 97
2 SD
1 SD
MEAN
1 SD
2 SD

4.6 Summary

Using [14C]- imatinib, and identical assay conditions to that used for the $IC50^{imatinib}$ assay, a significant correlation can be demonstrated between the level of kinase inhibition achieved in vitro and the level of uptake and retention (IUR) of imatinib achieved. This finding suggests that interpatient variation in in vitro sensitivity is mainly mediated by variations in the uptake and retention of imatinib over a two hour period. This is a significant finding implicating influx and efflux processes in determining intrinsic imatinib sensitivity. Furthermore the pattern of inhibition of imatinib uptake observed in these in-vitro drug uptake studies suggests that OCT-1 is the key influx mechanism for imatinib, in keeping with previous suggestions (Crossman. L *et al.* 2005; Thomas *et al.* 2004).

Investigation into the contribution of the major efflux pumps, ABCB1 and ABCG2 on IUR, revealed that imatinib is transported by both proteins in cell lines that over express them (VBL100 and Mef-BCRP1). However, there was no evidence that primary chronic phase CML patients express appreciable amounts of these proteins. These findings indicate that unlike the influx pumps, drug efflux is unlikely to contribute to the variability in $IC50^{imatinib}$ observed in this cohort. Taken together these data further suggests that patient intrinsic sensitivity to kinase inhibition as demonstrated in the $IC50^{imatinib}$ assay is primarily a consequence of the contribution of OCT-1 mediated uptake.

This data is based on the use of known inhibitors of the functional activity of the OCT proteins. It is possible that other transporters may additionally be involved in imatinib transport, and this cannot be conclusively excluded using this approach. However, the data presented here does conclusively demonstrate the involvement of OCT-1 in imatinib transport. A more recently described OCT transporter is OCT6 (Gong *et al.* 2002). OCT6 is expressed in haemopoietic cell lines, CD34+ cells and in primary leukaemic cells, and is thus of interest in the setting of CML therapy. However, to date the substrate and inhibitor profile of this transporter has not been documented, hence imatinib affinity cannot, at this time, be examined. Similarly the inhibitor profile for OCT-N1 and OCT-N2 are not well documented, and thus imatinib transport via these proteins cannot, currently, be examined.

4.7 Overall comment on significance of this work

This Chapter describes the development of a screening assay utilizing ^{14}C-labelled imatinib, to detect the intracellular concentration of imatinib achieved in CML cells and selected cell line controls. The assay is rapid and reproducibility has been demonstrated.

In conclusion, differential expression or function of OCT-1 appears to be a significant determinant of patient response to imatinib mesylate. Whether these differences are expression or functionally related has yet to be determined, however the outcome of either of these effects is clearly demonstrable in the rapid screening IUR and $IC50^{imatinib}$ assays which have been developed and standardized.

Chapter 5

Investigation of the intracellular transport mechanism of nilotinib

5.1 Introduction

Nilotinib (AMN107, Tasigna®) is a new generation ABL kinase inhibitor. The development of nilotinib arose from rational assessment of the molecular interaction of imatinib with the ABL kinase domain. These studies were based on crystallographic examination of these complexes (Cowan-Jacob *et al.* 2004; Nagar *et al.* 2003; Schindler *et al.* 2000), and on analysis of the effects on drug:protein binding of point mutations (Cowan-Jacob *et al.* 2004). Nilotinib, like imatinib, binds to the inactive conformation of BCR-ABL. The increased potency of this compound (10 to 50 fold for proliferation, and 10-20 fold for autophosphorylation) (Manley *et al.* 2004; Weisberg *et al.* 2005) arises from engineering of a superior topographical fit for nilotinib with the target BCR-ABL protein (Figure 5.1). Furthermore, it is this structural engineering which results in nilotinib having superior activity against BCR-ABL kinase domain mutations known to affect imatinib binding (O'Hare *et al.* 2005; Weisberg *et al.* 2005).

The increased potency of nilotinib against wild type BCR-ABL, and the binding efficacy of this compound to several imatinib resistant point mutations make it an attractive therapeutic alternative to imatinib in CML. However, to date, the only clinical application of nilotinib has been in that cohort of patients who fail, or are intolerant to imatinib therapy (Kantarjian *et al.* 2006).

While binding efficiencies and potency have been extensively examined with this compound, the intracellular transport mechanism and kinetics of drug uptake, are yet to be fully addressed. There is some recent evidence that nilotinib is a substrate for ABCB1 and ABCG2 (Brendel *et al.* 2007b), but relevant influx pathways are yet to be defined. As previously shown (Chapter 4) imatinib is established as an influx substrate for the human organic cation transporter one (OCT-1) (Crossman. L *et al.* 2005; Thomas *et al.* 2004; White *et al.* 2006) (Wang *et al.* 2007), and efflux substrate for ABCB1, (Dai *et al.* 2003; Gurney *et al.* 2007; Hegedus *et al.* 2002; Illmer *et al.* 2004; Mahon *et al.* 2003; Ozvegy-Laczka *et al.* 2004b; White *et al.* 2006) (Brendel *et al.* 2007a).. Of interest therefore, is the influence of these transporters and others, on the intracellular concentration of nilotinib achievable in target cells. A further question of relevance relates to the applicability of nilotinib in the chronic phase setting, in those patients with poor uptake of imatinib mediated through decreased activity of the functional OCT-1 protein.

In this current study, the intracellular transport mechanisms for nilotinib are addressed using the same approaches developed to investigate imatinib transport (Chapter 4).

5.2 Summary and research contribution

The research detailed in this chapter, the attached manuscript and conference proceedings, have contributed significantly to understanding of the relevant transport mechanism for the second generation BCR-ABL kinase inhibitor, nilotinib. Furthermore, the data presented provides evidence for the rational selection of kinase inhibitor therapy, in chronic phase CML patients, based on transporter profile.

Publications

White DL, Saunders VA, Dang P, et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. Blood. 2006;108:697-704. (White *et al.* 2006)

Conference Presentations.

White DL, Saunders VA, Engler J, et al. Low Baseline Intrinsic Sensitivity to Imatinib (High IC50) in CML Patients Is Due to Reduced Oct-1 Mediated Influx. Intrinsic Sensitivity to AMN107 Does Not Correlate with That of Imatinib and Uptake of AMN107 Is Not Oct-1 Mediated. ASH Annual Meeting Abstracts. 2005;106:1081-. (White *et al.* 2006)

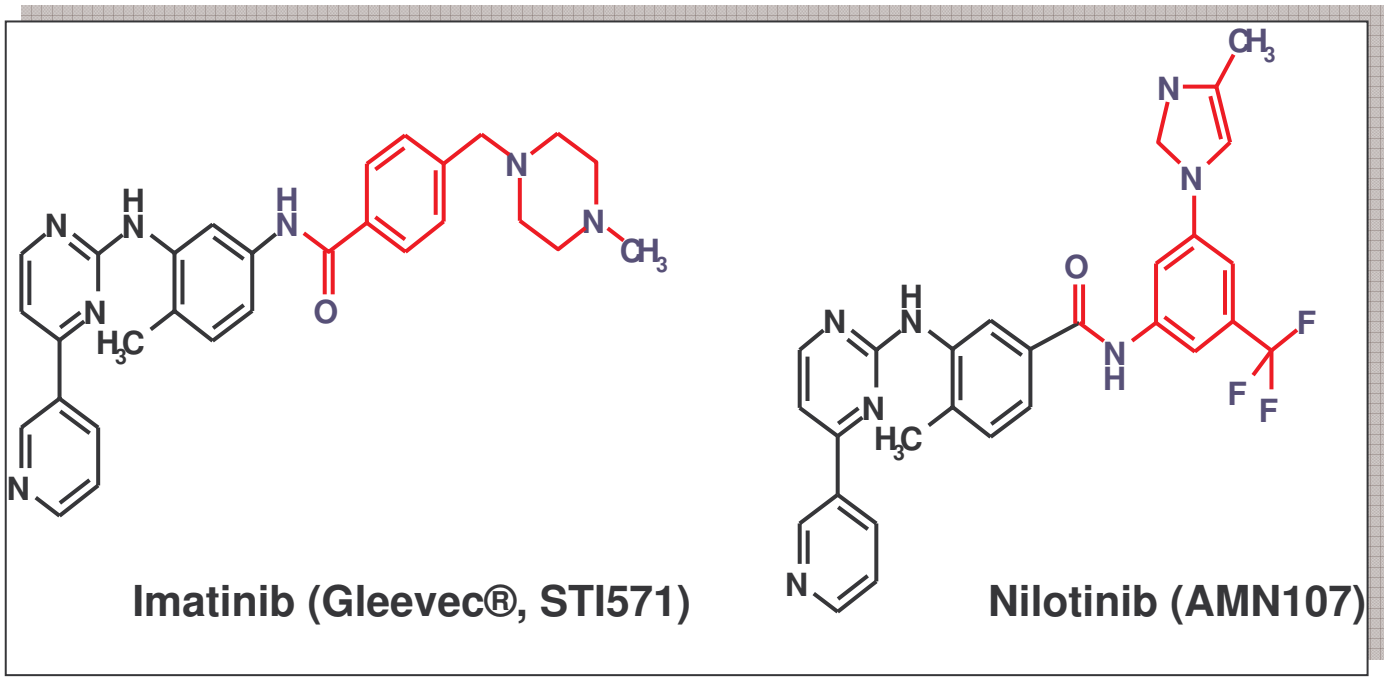


Figure 5.1 Comparative chemical structures of imatinib and nilotinib

The area indicated in black demonstrates the structures common to both drugs. The area in red indicates the structurally different regions of the structure. Of note, imatinib possesses a N-methylpiperazine group which is highly protonated at physiological pH. In contrast, nilotinib lacks this basic group and, in place possesses an N-arylimidazole which is relatively weakly basic at physiological pH. This substitution, will likely change the cationic nature of the compound, and hence the affinity of nilotinib for drug transport proteins utilized by imatinib (Verstovsek *et al.* 2005; Weisberg *et al.* 2005).

5.3 Assay development

5.3.1 Intracellular Uptake and Retention (IUR) Assay

The IUR assay was performed as previously described ((White *et al.* 2006) Chapter 4 and Appendix I Materials and Methods) and developed to assess the intracellular transport mechanisms for imatinib. [^{14}C]-AMN107 (nilotinib) was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). Stock solutions of nilotinib were prepared at 10 mM in Dimethylsulfoxide (DMSO; Sigma Aldrich GmBH. Germany) sterile filtered and stored at 4ºC. Isotope was resuspended to 1mg/ml and the specific activity was 3.31MBq for [^{14}C] AMN107.

5.3.2 The $IC50^{nilotinib}$ assay.

The $IC50^{nilotinib}$ assay was performed using the same protocol as that developed for the $IC50^{imatinib}$ assay, except that nilotinib was substituted for imatinib. Because of the increased potency of nilotinib, concentrations used in this assay were significantly less than those used for imatinib (0, 10, 25, 35, 50, 75, 100, 250 and 10 000nM). The protocol is detailed in Appendix I Materials and Methods.

5.3.3 Drugs and Inhibitors

The OCT inhibitors prazosin (Sigma Aldrich GmBH. Germany) progesterone (Sigma Aldrich GmBH.) and procainamide (Sigma Aldrich GmBH.) were each used at 100µM respectively (Koepsell *et al.* 2003). (Thomas *et al.* 2004). The ABCB1 inhibitor PSC833 was kindly provided by Novartis Pharmaceuticals (Basel, Switzerland) and the ABCG2 inhibitor Fumitremorgin C (Alexis. San Diego CA) were both used at 10uM.

5.3.4 Flow cytometry and western blot analysis for determining the $IC50^{nilotinib}$

The flow cytometry assay for expression of key efflux proteins was performed as described ((White *et al.* 2006) Chapter 4 and Appendix I Materials and Methods). $IC50^{nilotinib}$ assays were performed within the range of 0 µM to 10 µM nilotinib. All other aspects of this assay were performed as previously indicated.

5.3.5 Patient samples and cell lines

Assays were performed in newly diagnosed, CP CML patients, prior to the commencement of therapy. All patients were enrolled to the TIDEL or TOPS clinical trials (as previously described). K562

(American Type Culture Collection Rockville, MD), the ABCB1 over expressing cell lines (K562-DOX: and VBL100 kindly supplied by Professor Junia Melo Hammersmith Hospital London, and Professor Leonie Ashman) and the ABCG2 over expressing mef-BCRP1 and parental mef-3.8 were also analyzed (kindly supplied by Dr John Allen).

5.4 Results

5.4.1 Correlation between $IC50^{nilotinib}$ and $IC50^{imatinib}$.

Comparative assays for $IC50^{imatinib}$ and $IC50^{nilotinib}$ were performed on 72 chronic phase CML patients enrolled to either the TIDEL or TOPS trials. The median $IC50^{imatinib}$ was 0.75μM within a range of 0.2 to 2.7μM. This compared with a median $IC50^{nilotinib}$ for the same cohort, of 53.5nM within a range of 12.5 to 130nM, and demonstrates a 14 fold greater potency for nilotinib over imatinib, in keeping with previous reports (Manley *et al.* 2004; Weisberg *et al.* 2005) (O'Hare *et al.* 2005) (Shah *et al.* 2004). This data confirms that nilotinib is a more potent inhibitor of BCR-ABL kinase activity than imatinib in patient samples.

As demonstrated in Figure 5.2, there was no observed correlation between the two IC50's (R=0.189, *p=0.113*) (*p<0.001*; t-test) suggesting, that factors which effect the $IC50^{imatinib}$ may not be the same factors that effect the $IC50^{nilotinib}$. Furthermore all patients had $IC50^{nilotinib}$ well below the median plasma concentration of 2μM as reported in the Phase 1 trial where a maximum tolerated dose was not yet achieved (Weisberg *et al.* 2005). In contrast several patients had $IC50^{imatinib}$ which fell outside the range of the target imatinib concentration of 1μM reported in the Phase I trial(Peng *et al.* 2004).

5.4.2 Correlation between $IC50^{nilotinib}$ and the IUR for nilotinib.

In Chapter 4 the significant association between the $IC50^{imatinib}$ and the IUR for that drug was clearly demonstrated. In this study, the $IC50^{nilotinib}$ has been compared to the IUR for this drug in 57 newly diagnosed CML patients enrolled to either TIDEL or TOPS clinical trials. As can be seen in Figure 5.3, there is no correlation at any concentration (0.5,1.0 or 2.0μM) between the IUR and the $IC50^{nilotinib}$. There was also no correlation between the IUR for imatinib and nilotinib (r=0.284 *p=0.098*).

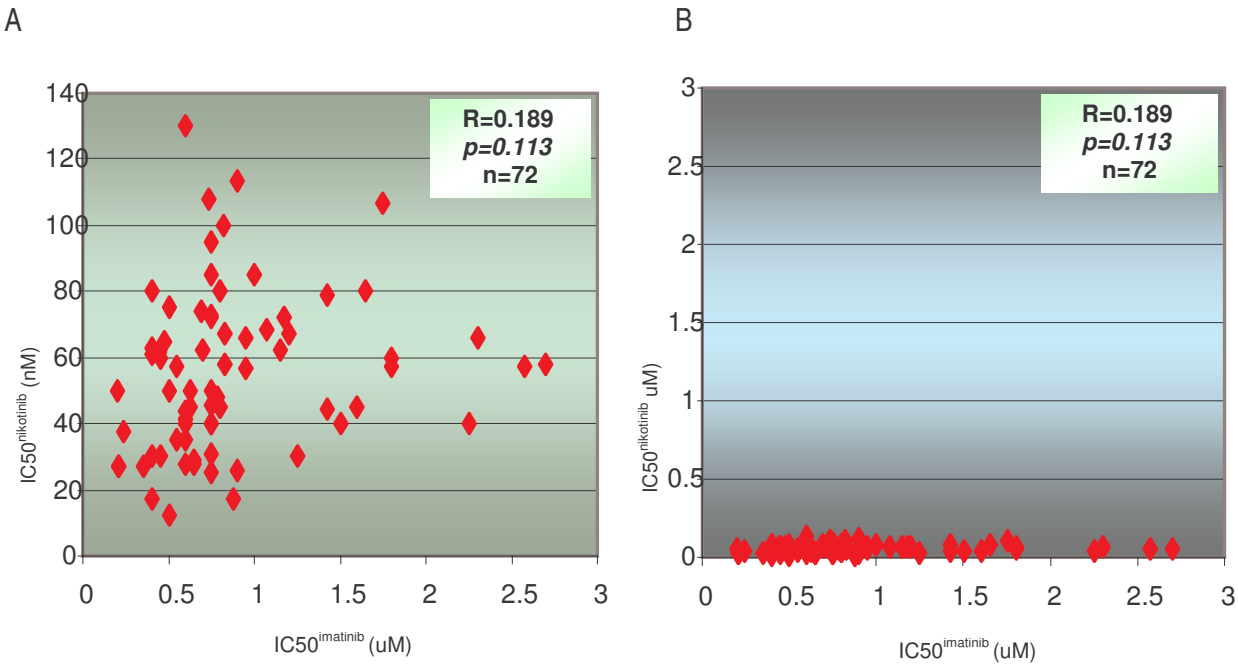


Figure 5.2 Comparison between the IC50^{imatinib} and IC50^{nilotinib} in 72 chronic phase CML patients.

Comparative IC50's were performed on 72 newly diagnosed patients enrolled to either the TIDEL or TOPS trial prior to therapy start.

Figure A . X-Y plot comparing the IC50^{imatinib} on µM vs the IC50^{nilotinib} on nM scales. As can be seen there is no correlation.

Figure B. X-Y plot comparing the IC50^{imatinib} and the IC50^{nilotinib} both plotted on µM scales, to allow comparison between the interpatient variation with the two drugs (Mean IC50^{imatinib}.0.75µM SD=0.539; Mean IC50^{nilotinib} 0.053µM SD – 0.025),

These data indicate that there is no correlation between the IC50^{imatinib} and the IC50^{nilotinib} in this cohort of patients.

Dividing patients into low and high $IC50^{nilotinib}$ groups about the median $IC50^{nilotinib}$, reveals, again in contrast to the findings with imatinib, that there is no significant difference between the IUR of nilotinib between the two groups (Table 5.1)

5.4.3 The effect of temperature on the IUR of nilotinib.

We and others have previously shown the temperature dependence of the intracellular uptake of imatinib (Thomas *et al.* 2004) (White *et al.* 2006). In this study the variation in the IUR for nilotinib, when assays were performed at 4°C and 37°C, was compared in 25 newly diagnosed patients. In contrast to the findings with imatinib, no significant difference in the IUR of nilotinib, between the two temperatures, could be demonstrated (Table 5.2). While this finding suggests that the uptake of nilotinib is largely passive, an active component cannot be conclusively excluded on the basis of this data alone.

Though not statistically significant, the intracellular concentration of nilotinib achieved was generally higher in assays performed at 4°C compared to those performed at 37°C. This is of particular note when assays were performed at 2μM. While not clearly indicative of any transport mechanism, this finding suggests a likely contribution of an active efflux component, which warrants further examination.

5.4.4 Effect of influx inhibitors on the IUR of nilotinib and $IC50^{nilotinib}$.

Prazosin and procainamide are inhibitors of OCT 1, 2 and 3 proteins to varying extents as detailed in Chapter 4. Furthermore, the addition of either prazosin, or procainamide significantly reduces the IUR of imatinib, and removes the difference observed in IUR between low and high $IC50^{imatinib}$ cohort (White *et al.* 2006) and Chapter 4. In contrast, as shown in Figure 5.4 and Table 5.3 there is no significant effect of prazosin on the IUR for nilotinib ($p>0.05$). Like prazosin, the addition of procainamide to the nilotinib IUR assay resulted in no significant difference in IUR. Further to this, there was also no significant difference when the data was grouped into low and high IC50 cohorts.

In a related series of experiments prazosin was added to the $IC50^{nilotinib}$ assay, in an experiment analogous to that performed for imatinib (Chapter 4). However, In contrast to the effect observed in the

imatinib assay however, there was no difference in the percentage of p-Crkl at any nilotinib concentration tested (Figure 5.5).

Taken together, these data, indicate that nilotinib is not a substrate for either OCT-1,-2 or -3 (Table 5.3).

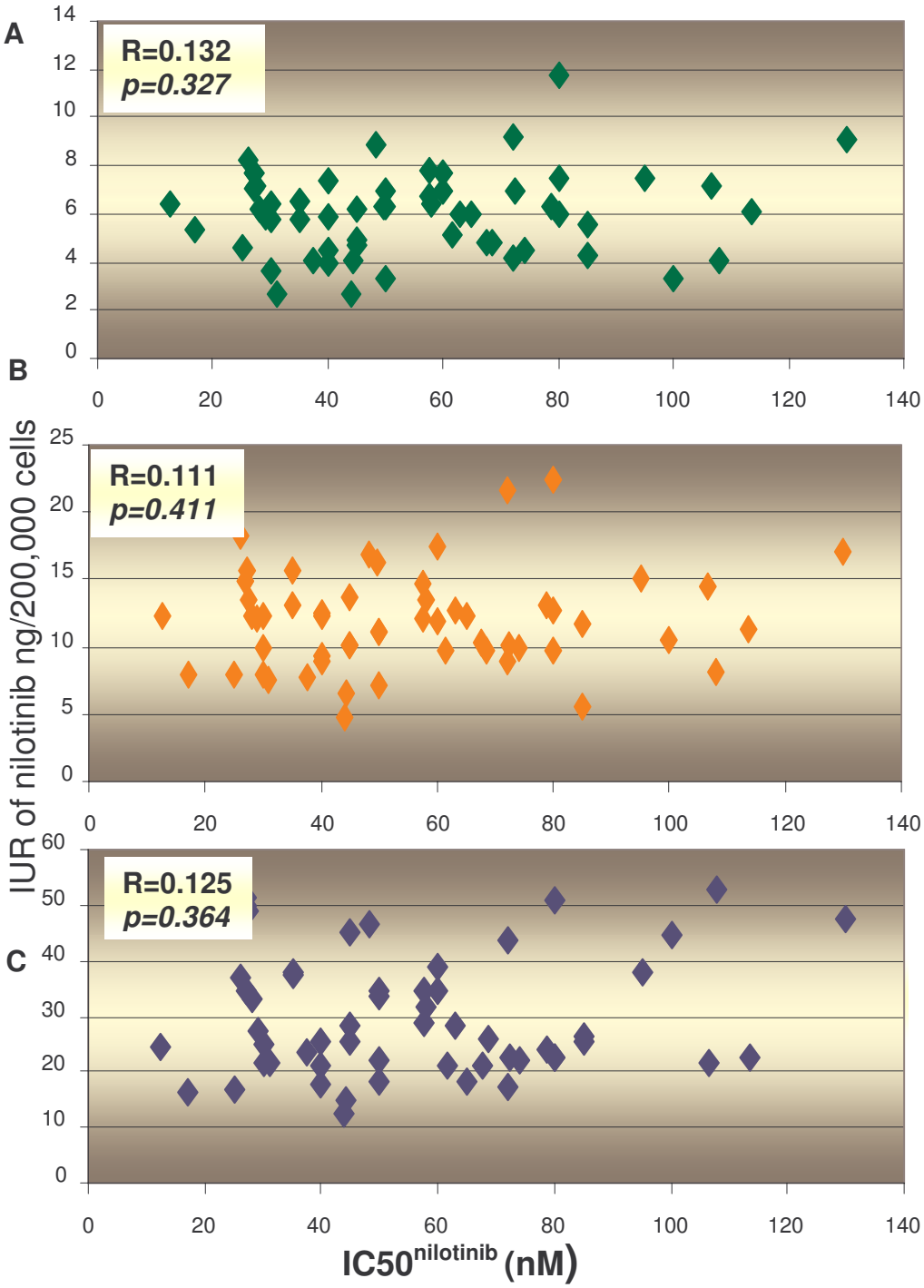


Figure 5.3. Comparison of IC50^{nilotinib} with the IUR of nilotinib.

The IUR and IC50^{nilotinib} were compared in 57 newly diagnosed CML patients prior to therapy on either the TIDEL or TOPS protocol.

A. show the IUR for nilotinib at 0.5µM, B at 1.0 µM, and C at 2.0µM. As can be seen, there is no correlation at any concentration of nilotinib between the two parameters.

	µM AMN107 added to 200,000 cells		
	0.5 µM	**1.0 µM**	**2.0 µM**
Median Uptake Total Cohort	**5.4**	**11.5**	**31.4**
Low $IC50^{nilotinib}$	**5.2**	**11.2**	**29.2**
High $IC50^{nilotinib}$	**5.7**	**12**	**33.8**

Table 5.1 The intracellular concentration of nilotinib in low and high $IC50^{nilotinib}$ patient groups.

Patients were stratified into low and high $IC50^{nilotinib}$ groups by dichotomization about the median $IC50^{nilotinib}$ for the cohort. The IUR's for the two groups were then compared, and as shown in the table above, there is no statistical significance in IUR between the two groups.

	0.5uM **[^{14}C]-AMN107**	**1.0uM** **[^{14}C]- AMN107**	**2.0uM** **[^{14}C]- AMN107**
37^{o}C	**11**	**23.5**	**59**
4^{o}C	**12.7**	**29.3**	**78**
p value	**>0.05**	**>0.05**	**>0.05**

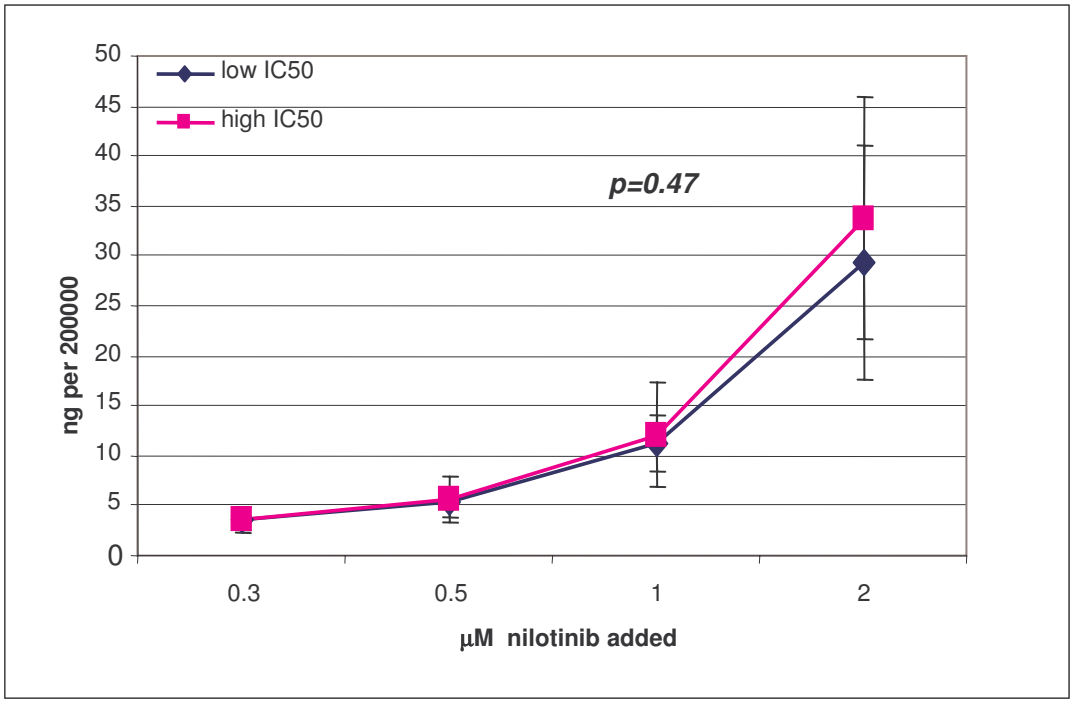
Table 5.2 Comparison of the IUR of nilotinib when assays are performed at 4^{o}C and 37^{o}C.

Comparative assays were performed on patient samples collected from 25 newly diagnosed CML patients prior to imatinib therapy. In contrast to the results observed with imatinib, there was no significant difference observed in the IUR of nilotinib between assays performed at 4^{o}C versus those performed at the normal assay temperature, 37^{o}C.

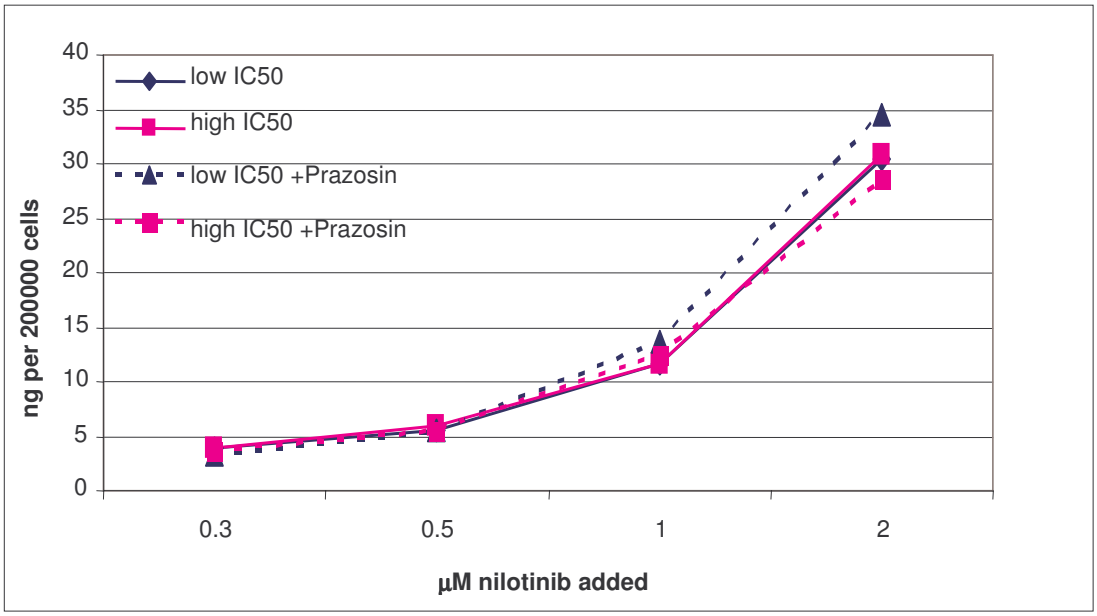
Figure 5.4 Assessment of the IUR of low and high IC50 groups with and without prazosin.

Graph A shows comparative IUR assays for nilotinib in both low and high IC50 cohorts. It is clear that there is no statistical difference with respect to nilotinib uptake, and as demonstrated in B. the addition of the OCT-1 inhibitor prazosin has no effect on the IUR for nilotinib.

A


low IC50
high IC50
p=0.47
ng per 200000
50
45
40
35
30
25
20
15
10
5
0
0.3
0.5
1
2
µM nilotinib added

B


low IC50
high IC50
low IC50 +Prazosin
high IC50 +Prazosin
ng per 200000 cells
40
35
30
25
20
15
10
5
0
0.3
0.5
1
2
µM nilotinib added

5.4.5 The role of efflux proteins in nilotinib transport.

The effect of ABCB1 on nilotinib transport was addressed by performing triplicate IUR's in an ABCB1 over expressing cell line (CEM-VBL100) and in the parental, non-ABCB1 expressing line, CCRF-CEM. In contrast, to the results observed with imatinib, there was no significant difference between the IUR of nilotinib in CEM when compared to VBL100 cell lines (CEM [mean ± SE] 34ng ± 1.08 /200,000 cells; VBL100 – 37ng ± 1.53 /200,000 cells). These data suggest that nilotinib may not be a substrate for ABCB1. The addition of the ABCB1 inhibitor, PSC833 resulted in a decreased IUR of [^{14}C]-AMN107 in both cell lines. Interestingly however, IUR assays performed in VBL 100 cells at both 4^{o}C and 37^{o}C revealed a higher intracellular concentration of nilotinib in VBL100 cells at 4^{o}C when compared to 37^{o}C (46ng ± 2.02 vs 37 ± 1.96 ng/200,000 cells respectively). This was not the case with CCRF-CEM cells (28± 1.3 vs 34 ± 1.4 ng/200,000 cells at 4^{0}C and 37^{0}C respectively). These data suggest that in VBL100 cells the IUR is higher at 4^{o}C because ABCB1 is not active at this temperature, hence less drug is actively effluxed and therefore, more is retained within the cell. This is not the case for CEM cells which have no measurable expression of efflux pumps (Figure 4.7).

To address the impact of ABCB1 on nilotinib transport further, IC50^{nilotinib} were performed on the ABCB1 over expressing cell line, K562DOX, in the presence and absence of PSC833 (Figure 5.7). The addition of PSC833 reduced the IC50^{nilotinib} for K562 DOX by 60%, from 375nM to 150nM. This finding indicates greater efficacy of nilotinib in the presence of PSC833, and hence by inference, suggests an increased intracellular nilotinib concentration. Confirmatory IUR assays indicate a rise of 22% in the intracellular concentration of nilotinib at 1μM when PSC833 is added, providing further evidence that nilotinib is transported by ABCB1.

The IUR of [^{14}C]-AMN107 was examined in mef-3.8 cells and the ABCG2 over expressing variant mef-BCRP1. Cell lines were examined in 3 separate experiments and each assay was performed in triplicate. Median uptake of nilotinib into mef-3.8 cells was 78ng ± 1.8 /200,000 cells at 2μM. This compares to 71 ± 1.6 ng /200,000 cells in the BCRP1 over expressing variant. Interestingly, the addition of the ABCG2 inhibitor fumitremorgin C (FTC) increased the intracellular concentration of nilotinib in mef-BCRP1 to 78 ± 1.5 ng, but had no effect on the IUR for mef-3.8 (77ng ± 1.8). While this data was only demonstrated in one cell line, it does suggest that there may be some minor involvement of ABCG2 in nilotinib transport. This finding warrants further investigation. Unfortunately

there are no CML cell lines available which over express ABCG2, hence $IC50^{nilotinib}$ evaluation in the presence and absence of an ABCG2 inhibitor are not currently possible.

Identical IUR assays, using [^{14}C]-AMN107, were then performed on the same cohort of patients used in Chapter 4 to assess the effect of efflux transporters in primary cells. As observed previously (Chapter 4), this cohort of patients had low to negligible expression of efflux transporters, and it is probably unlikely that any significant effect of transporter or inhibitor would be observed. In keeping with this postulation, there was no significant difference in IUR when FTC was used as an inhibitor. When PSC833 was included as an ABCB1 inhibitor, there was, as observed unexpectedly in the cell lines, a statistically significant reduction in the IUR, in all patients ($p<0.05$) (Table 5.3).

.

	[14C]-AMN107 @ 2µM				
	No inhibitor	**Prazosin**	**Procainamide**	**PSC833**	**FTC**
Total	**32.97**	**32.8**	**33.47**	**17.13***	**32.25**
low $IC50^{imatinib}$	**40.32**	**37.551**	**42.38**	**22.20**	**42.07**
high $IC50^{imatinib}$	**28.08**	**29.00**	**27.52**	**14.14***	**25.70**

Table 5.3 The effect of various transport inhibitors of the IUR of nilotinib at 2µM

These data demonstrate that influx inhibitors prazosin and procainamide have no effect on the IUR of nilotinib. Similarly there is no significant effect of the ABCG2 inhibtior FTC. In contrast however there is a significant reduction in IUR observed when PSC833 is added.

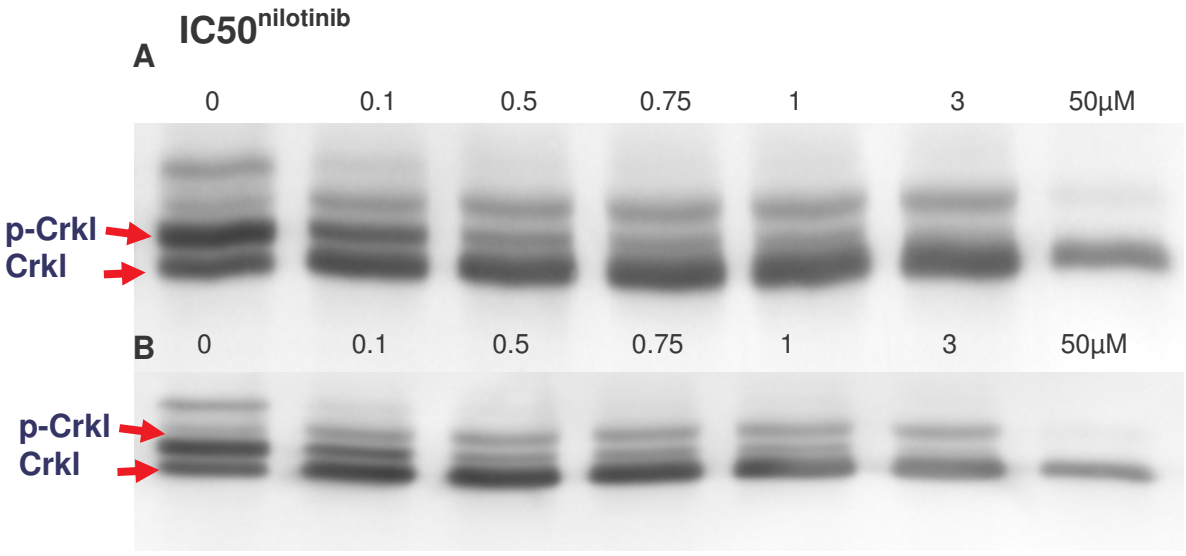
* Significant difference at the 0.05 level in comparison to no inhibitor.

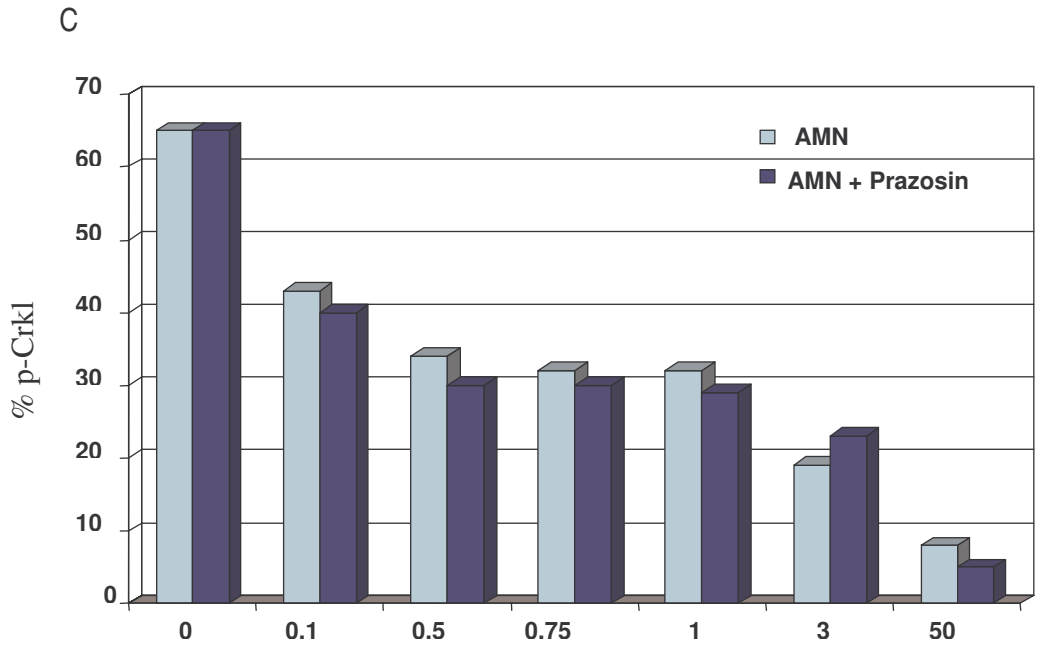
Figure 5.5 The effect of prazosin on the $IC50^{nilotinib}$.

Western blot to determine the $IC50^{nilotinib}$ in K562 cells.

Western blot performed as above but with the addition of 100µM prazosin.

Graphical representation of the western blots (A and B) showing the %p-Crkl In contrast to the effect seen with imatinib, there is no significant effect of prazosin on the level of p-Crkl observed at any nilotinib concentration.


A
IC50nilotinib
0
0.1
0.5
0.75
1
3
50µM
p-Crkl
Crkl
B
0
0.1
0.5
0.75
1
3
50µM
p-Crkl
Crkl


C
% p-Crkl
70
60
50
40
30
20
10
0
AMN
AMN + Prazosin
0
0.1
0.5
0.75
1
3
50

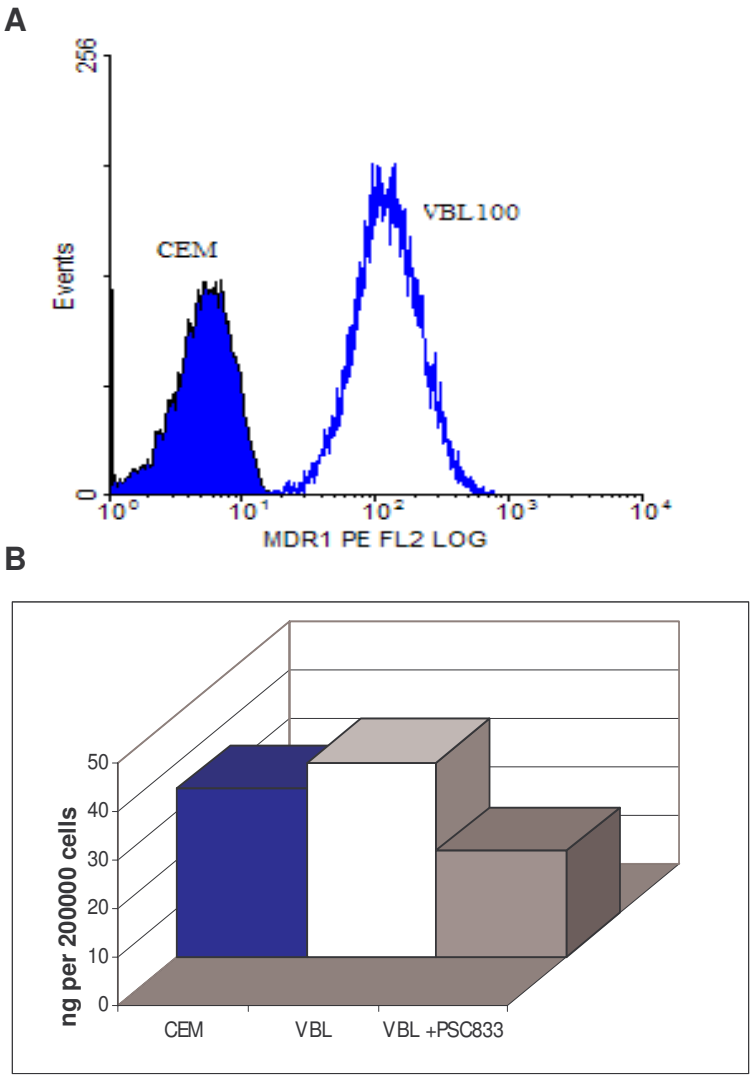


Figure 5.6 The effect of ABCB1 expression on nilotinib IUR.

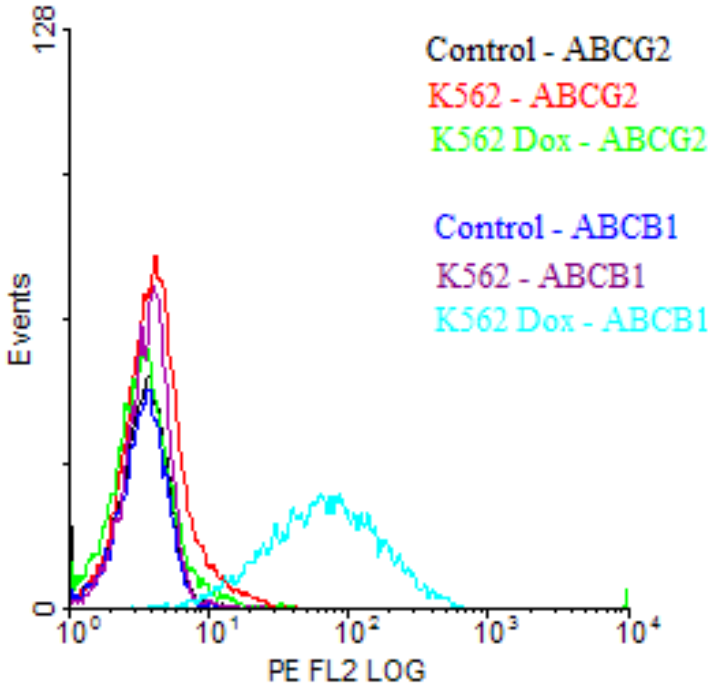
A. FACS histogram showing the level of expression of ABCB1 in the VBL100 cell line. B, Graphical representation showing the uptake of nilotinib into VBL cell is higher, at 37oC though not significantly so, than the uptake into the parental CEM cells (no expression of ABCB1). Furthermore the addition of the ABCB1 inhibitor PSC833 does not increase the nilotinib IUR of VBL cells, in fact it decreases it.

Figure 5.7 Western Blot of K562 DOX cells (ABCB1 over expressing) demonstrating the difference in the IC50^{nilotinib} in the presence and absence of PSC833.

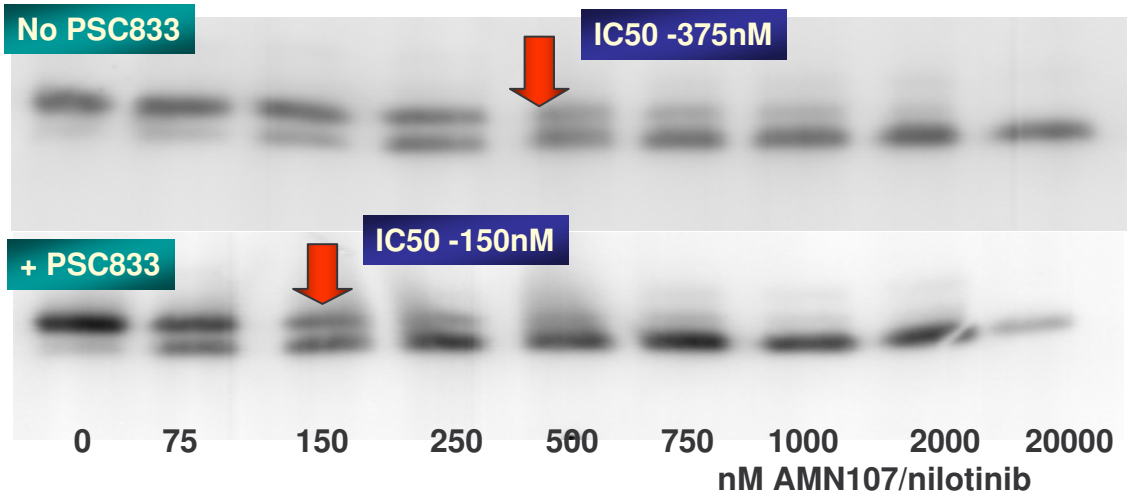
A: FACS plot demonstrating the level of expression of ABCB1 and ABCG2 in K562 DOX cells as determined by flow cytometry. As can be seen, K562 cells have no measurable expression of ABCB1 or ABCG2. In contrast K562DOX cell express high levels of ABCB1 (100% of cells), but no ABCG2.

B: Western blot for the determination of the IC50^{nilotinib} with, and without PSC833. This demonstrates a simarked reduction in the IC50^{nilotinib} for K562DOX in the presence of PSC833, and hence, implicates ABCB1 in nilotinib transport.

A


128
0
Events
Control - ABCG2
K562 - ABCG2
K562 Dox - ABCG2
Control - ABCB1
K562 - ABCB1
K562 Dox - ABCB1
10⁰
10¹
10²
10³
10⁴
PE FL2 LOG

B


No PSC833
IC50 -375nM
+ PSC833
IC50 -150nM
0
75
150
250
500
750
1000
2000
20000
nM AMN107/nilotinib

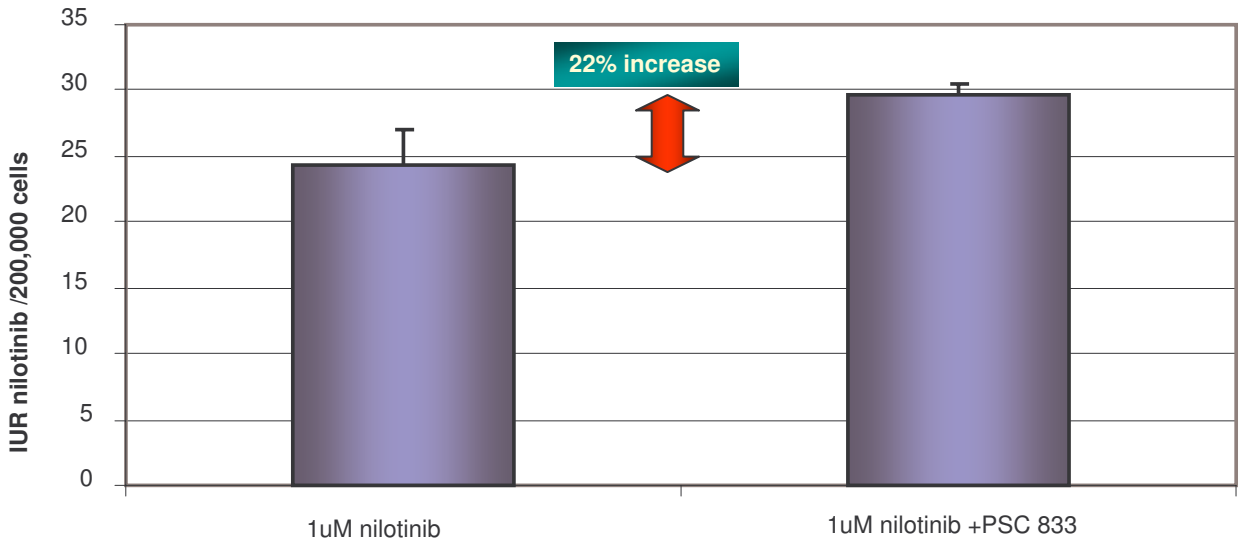


Figure 5.8 Assessing the IUR of nilotinib in K562DOX cells with and without the ABCB1 inhibitor PSC833.

In keeping with the findings in Figure 5.7, the addition of PSC833 resulted in a 22% increase in the IUR of nilotinib in K562DOX cells. This indicates a likely interaction between nilotinib and ABCB1, at least in cells which are engineered to overexpress the ABCB1 protein.

5.5 Assay Control and Quality Control

To control for variability in the IUR nilotinib assay K562 cells are used as quality control. As can be seen in Figure 5.9 the results are highly reproducible with only 4% (2 out of 50) of IUR assays rejected on the basis of falling outside of the mean +/- 2 standard deviations (SD).

However, there were some problems noted with the use of this control for the nilotinib IUR. The mean IUR for K562 cells at 2μM nilotinib was 52.4ng/200,000 cells. This compares to the median IUR for the patient samples of 31.4ng. This was not the case with imatinib, where the median uptakes of patient cells versus control cells were much closer (24.4 vs 33.6ng/200,000 cells). Hence, K562 may not be a good control for the dynamic range of the patients being analysed. Furthermore the standard error and confidence intervals of the mean (CI mean), between patient samples and K562 cells were significantly different for imatinib (Std Error 1.2 vs 0.7: CI mean 2.2 vs 1.3 respectively), but not nilotinib (Std Error 1.56 vs 1.53: CI mean 3.1 vs 3.09 respectively). This indicates that inter - assay variation cannot be excluded as a cause for the variation observed in the nilotinib IUR. However, it is possible that the level of nilotinib uptake into K562 cells may be confounding this issue, due to assay linearity problems. It is probable that direct intracellular measurement of nilotinib using Liquid Chromatography –Mass Spectroscopy – High performance Liquid Chromatography (LCMS-HPLC) may be a better measure of nilotinib intracellular concentration.

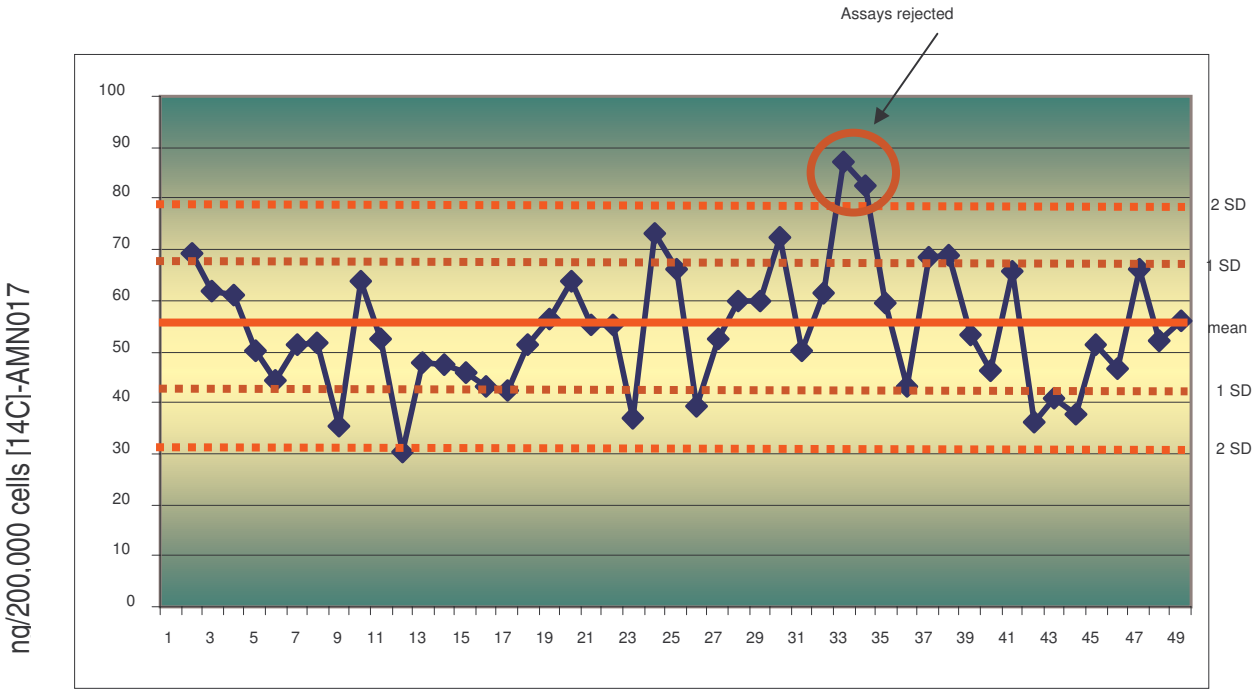


Figure 5.9 K562 Quality Control Graph for IUR Nilotinib

Quality control rules were applied to the K562 control results to determine the acceptability of the IUR assay. If the control value was outside the 2 standard deviation range (SD), then the run was rejected (as indicated).

5.6 Summary.

Nilotinib is a recently developed, potent inhibitor of ABL kinase which is structurally related to imatinib. In keeping with the documented increased potency, the $IC50^{nilotinib}$ was found to be significantly lower than the $IC50^{imatinib}$ in the 72 patient samples tested ($p<0.001$). Despite the structural similarity in these compounds there was no correlation between the IC50 for imatinib and nilotinib in these patients. In a clinical context it is significant that nilotinib blood levels achieved are generally far higher (1000 to 2000ng/ml personal communication from Novartis Pharmaceuticals Basel) than the $IC50^{nilotinib}$ indicating that unlike imatinib, achieving sufficient kinase inhibition, with nilotinib, to augment a sustained response is likely to be more uniformly achievable.

In direct contrast to imatinib, the IC50 for nilotinib does not appear, based on this dataset in patient cells, to be related to the IUR of the drug (at the tested concentrations), and there is no significant difference between the IUR in the low and high $IC50^{nilotinib}$ cohorts. There may be a number of explanations for this. Firstly, nilotinib is more potent that imatinib. This results in the $IC50^{nilotinib}$ being in the nM range in de-novo patients, in contrast to the $IC50^{imatinib}$ which is in the µM range. The IUR assay, because of the specific activity of the ^{14}C labeled compound cannot be performed in the nM range. Hence it is possible that the range assayed is inappropriate for the demonstration of a correlation, however this cannot at this stage be avoided. It may also be that there are other variables which affect the $IC50^{nilotinib}$ which obscure the impact of the IUR. Finally, the observed lack of correlation may be related to inter assay variability, as was discussed later in this Chapter.

There is also no significant difference in IUR when the inhibitors prazosin and procainamide are used, indicating that neither OCT-1,-2 or - 3 are implicated in nilotinib influx. Indeed, the uptake of nilotinib appears to be passive as indicated by assays of temperature dependent transport.

This finding may not be surprising in the light of the different structural components of both drugs. Although there is some evidence that some weak bases and uncharged compounds are transported by OCTs, the majority of substrates translocated by these transporters are organic cations(Koepsell *et al.* 2003). Consequently, it is likely that imatinib is recognised by OCT-1 in the cationic form,

esulting from the high degree of protonation of the basic N-methylpiperazine group at physiological pH. In contrast, nilotinib lacks this basic group, which is replaced by an N-arylimidazole which is relatively weakly basic. This results in nilotinib existing in the cationic state to a far lesser extent than imatinib. This greatly reduces its likelihood to be recognized by OCTs as an influx substrate (Verstovsek *et al.* 2005; Weisberg *et al.* 2005).

Investigations into the interaction of nilotinib and ABCG2 were limited, although there was some association demonstrated between this protein and nilotinib in the cell line tested. However, this finding did not translate to patient material, most likely because of low expression of ABCG2 as previously discussed. Experiments with the ABCB1 expressing cell line, CEM-VBL100, and the non-expressing CCRF-CEM parental line demonstrate no difference in [^{14}C] AMN107 uptake between the two lines suggesting nilotinib is not a substrate for this efflux pump. However, data from experiments performed to examine the effect of temperature (4^{o}C versus 37^{o}C) revealed active efflux of nilotinib in VBL100 cells, suggesting the contrary. Furthermore IC50^{nilotinib} experiments performed on another ABCB1 over expressing cell line, K562DOX do demonstrate a significant reduction (60%) in the IC50 when PSC833 is added. In keeping with this, an increased IUR is also observed with this cell line when PSC833 is added. Interestingly, and in contrast to this, the addition of PSC833 to VBL100 cells resulted in a uniform reduction in IUR, which reached statistical significance in the patient group overall, and also in the high IC50^{nilotinib} cohort. This finding is unexpected and remains unexplained. Speculatively there are two possible scenarios which would give rise to this result. Firstly PSC833 may be a competitor of nilotinib in relation to influx,, resulting in less of the drug being taken into the cell. Alternatively PSC833 may in fact complex with nilotinib. This could result in either reduced influx, or as PSC833 is a known substrate of ABCB1 (Tidefelt *et al.* 2000) result in facilitated transport of nilotinib out of the cell via this efflux pump. The results with the K562 DOX line do not however support these hypotheses. It may indeed be that this finding is unique to VBL100 cells and may be membrane related. Further experimentation is required to fully address these confounding findings.

5.7 Comment on significance of this work

In contrast to imatinib, the transport of nilotinib does not appear to involve OCT proteins. Based on our findings in this patient cohort, it is likely that response to nilotinib in CML patients at presentation will be more uniform than those responses observed with imatinib. Furthermore, selection of nilotinib as front line therapy, based on poor OCT-1 mediated uptake in the IUR assay, may provide a more rational approach for the selection of the most appropriate tyrosine kinase inhibitor in the future.

These findings may also be relevant to nilotinib activity against c-Kit and PDGF mediated diseases. In these cases potency of imatinib and nilotinib are similar(Weisberg *et al.* 2005) and more uniform uptake of nilotinib may provide a critical advantage over imatinib in this setting.

Chapter 6

The interaction of imatinib and nilotinib in drug combination studies.

6.1 Introduction

Despite the efficacy of imatinib, resistance and associated relapse do occur in approximately 15% of chronic phase patients. The relapse rate is higher in advanced stages of the disease. Relapse is frequently due to mutations within BCR-ABL which abrogate imatinib binding(Gorre *et al.* 2001) (Branford *et al.* 2002; Branford *et al.* 2003b; Cowan-Jacob *et al.* 2004; Hochhaus *et al.* 2002; Kreill *et al.* 2003; Shah *et al.* 2002; Weisberg & Griffin 2000), though BCR-ABL amplification is also thought to be causative(Campbell *et al.* 2002; le Coutre *et al.* 2000).

Nilotinib, as discussed in Chapters 1 and 5 is a second generation ABL kinase inhibitor which displays increased potency. Importantly, in in-vitro studies, nilotinib maintains activity against many point mutations which confer imatinib resistance (Manley *et al.* 2004; Weisberg *et al.* 2005). Results of phase I/II clinical trials have demonstrated the efficacy of this agent in producing cytogenetic and haematological responses in imatinib-refractory CML patients (Kantarjian *et al.* 2006).

However, it is expected that resistance to second generation kinase inhibitors may result as a consequence of unique mutation profiles. Alternatively, nilotinib resistant mutations may prove to be a small subset of the diverse mutation profile seen in imatinib resistance..In-vitro mutagenesis screens have revealed that imatinib and nilotinib display different mutagenicity profiles (Bradeen *et al.* 2006; O'Hare *et al.* 2005; Von Bubnoff *et al.* 2004). In this setting, a combination of inhibitors may be expected to result in significant clinical benefit, as each inhibitor could be effective in the suppression of BCR-ABL point mutations conferring resistance to the other inhibitor. This would appear particularly relevant in the imatinib setting where a broad spectrum of resistant mutations are described to emerge in-vivo. Recent in-vitro studies with dasatinib (BMS 354825) and imatinib have demonstrated a greatly reduced recovery of resistant clones, when the two drugs were used in combination (Burgess *et al.* 2005). Furthermore, associated studies have found that with drug combinations, maximal suppression of resistant clone outgrowth was achieved at lower concentrations compared with single agents. This suggests that drug combinations may be equipotent to higher-dose single agents, and may therefore reduce the toxicity profile associated in some cases with higher dose, single agent regimens (Bradeen *et al.* 2006).

Recently, an extensive study was performed in-vivo (murine) and in-vitro, to assess efficacy of imatinib and nilotinib in combination, against both wild type BCR-ABL and imatinib resistant mutants (Weisberg *et al.* 2007). In-vitro proliferative and clonogenic assays revealed additive to synergistic effects of the combination, with no apparent toxicity to normal precursors. Furthermore efficacy of the combination was demonstrated against a panel of 4 imatinib resistant mutants to varying extents, with enhanced induction of apoptosis of target cells being a feature. In-vivo murine experiments detail the lowest tumour burden being present in animals treated with a combination of imatinib and nilotinib.

In combination studies which involve dasatinib and imatinib, it could be postulated that the observed synergy may arise from the targeting of both the active and inactive (dasatinib binds BCR-ABL in the active conformation) conformations of the kinase. This is however not the case with nilotinib/imatinib combinations where both agent bind the kinase in its inactive conformation. Furthermore crystallographic structures reveal that BCR-ABL is only capable of binding one molecule of imatinib or nilotinib at a time (Nagar *et al.* 2002). Hence, enhanced efficacy of the combination of imatinib and nilotinib is likely to arise from other factors. Given previous studies which have demonstrated that the transport mechanisms for these two kinase inhibitors are different (White *et al.* 2006) and Chapters 4 and 5 of this thesis, it is possible that either inhibitory or synergistic interactions with these transporters may be responsible for the observed and enhanced efficacy of this drug combination.

In this current study, the effect of the combination of imatinib and nilotinib, on the intracellular transport of both drugs, is assessed using techniques developed and described in Chapters 4 and 5 of this thesis.

6.2 Summary and research contribution

The research detailed in this chapter, the attached manuscript and conference proceedings, have contributed significantly to understanding the observed synergy when imatinib and nilotinib are used in combination, based on interactions with relevant drug transport mechanisms.

Publications

White, D. L., V. A. Saunders, et al. (2007). "Imatinib increases the intracellular concentration of nilotinib, which may explain the observed synergy between these drugs." Blood 109(8): 3609-10. (White *et al.* 2007e)

Conference Presentations.

White Deborah L, Verity A Saunders, et al. (2007). The intracellular concentrations of imatinib and nilotinib, can be substantially altered by interaction with other drugs: Studies with proton pump inhibitors. European Haematology Society, Vienna. (White Deborah L *et al.* 2007)

White, D. L., Saunders AV, et al. (2007). Proton pump inhibitors interact with transport of both imatinib and nilotinib. AACR, Los Angeles. (White *et al.* 2007b)

6.3 Assay development

6.3.1 Intracellular Uptake and Retention (IUR) Assay

The IUR assay was performed essentially as previously described ((White *et al.* 2006) Chapter 4, 5 and Appendix I Materials and Methods.

For combination studies, drugs were added simultaneously using the schema in Table 1.

To assess the effect of one drug on the uptake of the other, the drug being assessed was 14C labeled, and the effector drug was unlabelled ("Cold"; not 14C).

Results for each arm of the experiment were displayed as ng/200,000 cells and calculated as follows:-

% drug incorporation = cpm cells/cpm total (cells + supernatant)

ng/200,000 cells = (% drug incorporation)/100 x amount of drug added in ng.

To assess the effect of drug combination, results were displayed as percentage of the control arm, where the control was either 2µM 14C imatinib or 14C nilotinib, with no added combination drug. Taking 1µM imatinib added to 1µM [14C]-nilotinib as an example

	[14C]-nilotinib (ng/200,000 cells)		**Calculation**	**% Change**
	Control	**+1 µM imatinib**		
CML1	**10**	**15**	**([15-10]/10)x 100**	**50%**
CML3	**10**	**22**	**([22-10]/10)x 100**	**120%**

A t-test was used to test for significance of any observed change.

6.3.2 Drugs and inhibitors

The ABCB1 inhibitor PSC833 and the ABCG2 inhibitor Ko143, were used as previously described.

6.3.3 Patient samples and cell lines

Assays were performed in triplicate, in 10 newly diagnosed CML patients in chronic phase, prior to the commencement of therapy. Cell lines HL60, K562, KU812, K562-DOX, mef-BCRP1 and parental mef-3.8 as previously described were also analyzed.

6.3.4 Cell viability

Cell viability was assessed at the beginning and at the completion of all assays to assess the effect of the drug combination on cell integrity. This was performed using Trypan Blue exclusion.

Reagents added simultaneously		[^{14}C]-imatinib		[^{14}C]-nilotinib	
		1µM	2µM	1µM	2µM
Imatinib (Unlabelled)	1µM			√	√
	2µM			√	√
Nilotinib (Unlabelled)	1µM	√	√		
	2µM	√	√		

Table 6.1 **Schema for the addition of drugs to assess the effect of combination.**

√ indicates combination ABL kinase inhibitor added

indicates no combination ABL kinase inhibitor added

This approach provides an IUR readout for the assays in singleton:

IUR of imatinib [14C]-imatinib @ 1µM
IUR of imatinib [14C]-imatinib @ 2µM
IUR of nilotinib [14C]-nilotinib @ 1µM
IUR of nilotinib [14C]- nilotinib @ 2µM
And in combination
IUR of imatinib [14C]-imatinib @ 1µM + 1µM nilotinib
IUR of imatinib [14C]-imatinib @ 1µM + 2µM nilotinib
IUR of imatinib [14C]-imatinib @ 2µM + 1µM nilotinib
IUR of imatinib [14C]-imatinib @ 2µM + 2µM nilotinib
IUR of nilotinib [14C]- nilotinib @ 1µM + 1µM imatinib
IUR of nilotinib [14C]- nilotinib @ 1µM + 2µM imatinib
IUR of nilotinib [14C]- nilotinib @ 2µM + 1µM imatinib
IUR of nilotinib [14C]- nilotinib @ 2µM + 2µM imatinib

6.4 Results

6.4.1 The effect of cold imatinib on the IUR for [14C]- nilotinib

The median IUR for the 10 patients at 1µM [14C]- nilotinib was 10.5 ng/200,000 cells. There was an increase observed in the IUR in all patients when 1µM unlabelled imatinib was added (median 12.7ng *p=0.009*). In the presence of 2µM imatinib, the median IUR rose to 13.07ng/200,000 cells (*p=0.007*) (Figure 6.1). At 2µM [14C]- nilotinib the median IUR was 26.2 ng/200,000 cells. When cold imatinib was added the intracellular concentration rose to 29.4ng/200,000 cells (1µM imatinib *p=0.342*), and to 35.5ng (2µM imatinib *p=0.054*) (Figure 6.1). This observed increase occurred in 9 of the 10 patients.

Assessing this data in terms of the % increase in the intracellular concentration of nilotinib mediated by the addition of imatinib, revealed a 34% and 42% increase (+ 1µm and 2µM imatinib). At 2µM nilotinib the median increase was only 16%, and 38% (+ 1µm and 2µM imatinib) (Figure 6.2). This indicates that the effect of imatinib on the IUR of nilotinib is more marked at lower concentration of nilotinib. Furthermore, it suggests that higher concentrations of nilotinib are able to overcome, to some extent, the effect mediated by the addition of imatinib.

6.4.2 The effect of cold nilotinib on the IUR for [14C]- imatinib

The median IUR for the 10 patients at 1µM [14C]-imatinib was 13.4 ng/200,000 cells. The addition of nilotinib decreased the imatinib IUR in 9 of the 10 patients (10ng/200,000 cells at 1µM nilotinib *p=0.09*) and in 8 of the 10 patients at 2µM imatinib (IUR at 2µM nilotinib 11.3 ng/200,000 cells *p=0.14*). (Figure 6.3). The median IUR at 2µM [14C]-imatinib was 24.6 ng/200,000 cells. The addition of nilotinib at both 1 and 2µM resulted in a decrease in the imatinib IUR in 9 of the 10 patients (22.3 and 18.1 ng/200,000 cells respectively) (p=0.53 and *p= 0.26*) (Figure 6.3).

Assessing the % decrease in the imatinib IUR at 1µM revealed 18% and 14% decreases when cold 1µM and 2µM imatinib were added. At 2µM imatinib the average decreases were 5.6%, and 13.1% respectively (Figure 6.4). These results suggest that the addition of nilotinib results in a small decrease in the intracellular concentration of imatinib, which does not reach statistical significance. Similar to the findings with nilotinib, this effect appears more pronounced at lower concentrations of imatinib.

6.4.3 Comparison of the effect of combination therapy on the intracellular concentration of both drugs.

It is evident from this small data series that the effects of combination therapy are different for both drugs. The addition of imatinib increases the intracellular concentration of nilotinib significantly. In contrast, nilotinib decreases the intracellular concentration of imatinib, though not significantly in this patient cohort. It is interesting to note, that while there is some interpatient variability, the effect on the intracellular concentration of both drugs was more marked at lower concentrations, though this effect can be overcome to some extent, by the addition of more of the competing drug (Figure 6.5).

6.4.4 Cell lines

The intracellular concentration of imatinib and nilotinib at 2µM was assessed in KU812 and HL60 cell lines. The results demonstrated the same trends as those observed in the cells derived from CML patients. The addition of 2µM imatinib increased the IUR for nilotinib by 14.4% for HL60 and 20.8% for KU812. In contrast the addition of 2µM nilotinib decreased the IUR for imatinib by 46.1% for HL60, and 19.7% for KU812 (Table 6.2).

The IUR of nilotinib at 1µM in the ABCB1 over expressing cell line, K562DOX, was 24ng/200,000. The addition of PSC833, an ABCB1 inhibitor, increased this by 21 % to 29.5ng. The addition of imatinib at 1 and 2µM resulted in 82% and >200% increases in the nilotinib intracellular concentration, respectively. Intriguingly, the addition of PSC833 and imatinib simultaneously, resulted in IUR's of 27 and 25ng at 1 and 2µM imatinib (14.5% and 3% rises respectively) (Figure 6.6).

The addition of the ABCG2 inhibitor Ko143 to the 1µM nilotinib IUR had minimal effect in the parental Mef 3.8 line (4% increase), but resulted in an 18% increase in the ABCG2 expressing Mef BCRP1. This suggests nilotinib is effluxed via ABCG2. Similarly the addition of imatinib had minimal effect on the parental line, but increased the IUR of nilotinib for the ABCG2 positive line (35% for both 1 and 2µM imatinib). The addition of both imatinib and Ko143 also increased the IUR; however the increase was 20% with 1µM imatinib + Ko143 and 26% with 2µM imatinib + Ko143 (Figure 6.7). These data indicate that the addition of Ko143 to some extent, abrogates the effect of imatinib on nilotinib transport. This finding suggests that Ko143 and imatinib may compete for ABCG2 binding sites, and furthermore, that this competition may be dose dependant.

Figure 6.1 The effect of unlabelled imatinib on the intracellular concentration of 1µM and 2µM [14C] labelled nilotinib

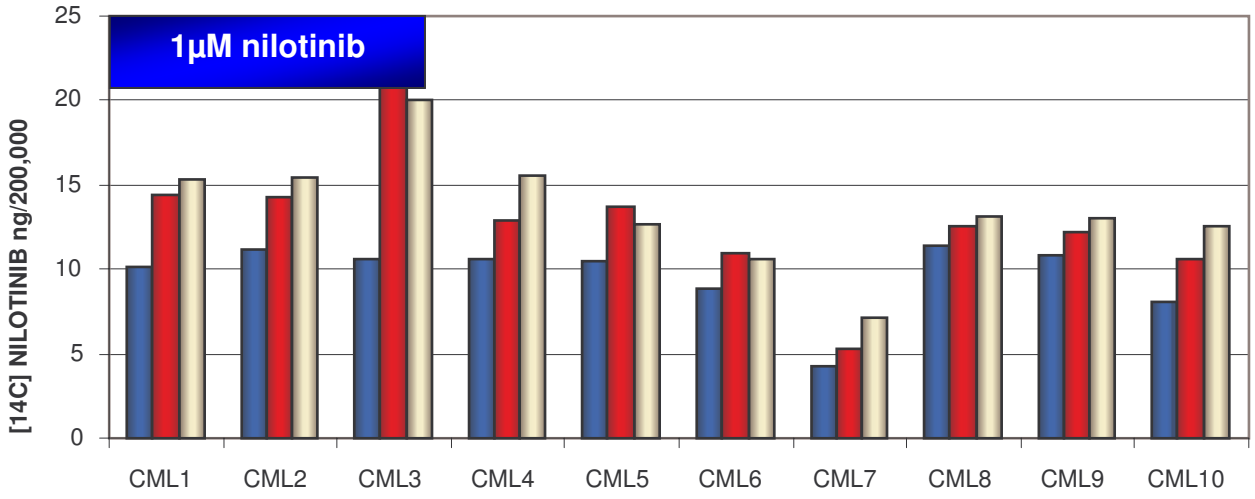
All assays were performed in triplicate. Assays were only accepted, if triplicate results were concordant within 10%, if not, assays were repeated.

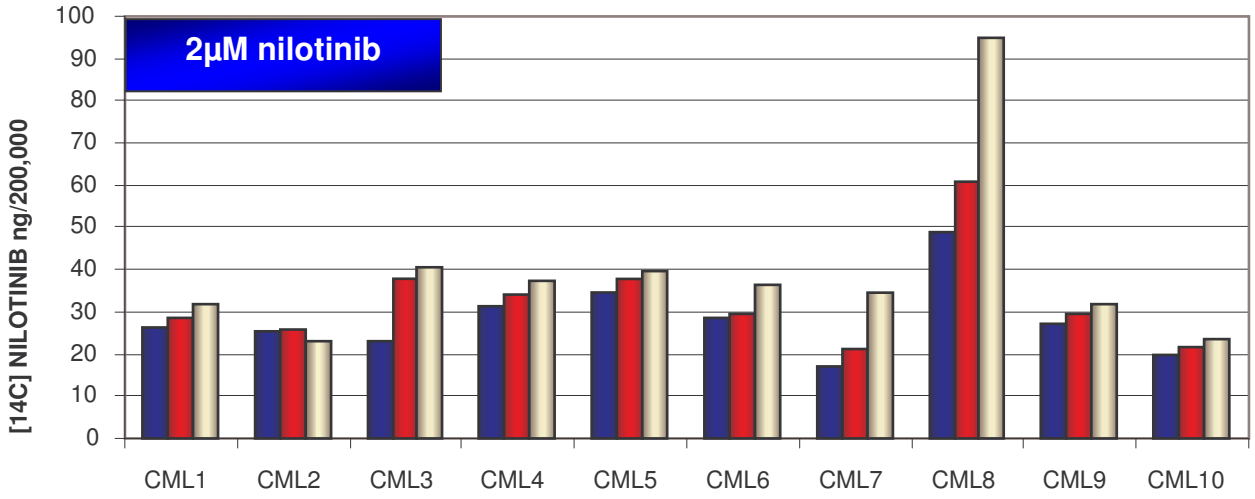
The addition of imatinib resulted in a universal increase in the intracellular concentration of nilotinib. This effect was most significant in the 1µM nilotinib IUR, suggesting that higher concentrations of nilotinib may override the effect of the addition of imatinib.

Control 1uM [14C] nilotinib

+1uM “cold” imatinib

+2uM “cold” imatinib


1µM nilotinib
[14C] NILOTINIB ng/200,000
25
20
15
10
5
0
CML1
CML2
CML3
CML4
CML5
CML6
CML7
CML8
CML9
CML10


2µM nilotinib
[14C] NILOTINIB ng/200,000
100
90
80
70
60
50
40
30
20
10
0
CML1
CML2
CML3
CML4
CML5
CML6
CML7
CML8
CML9
CML10

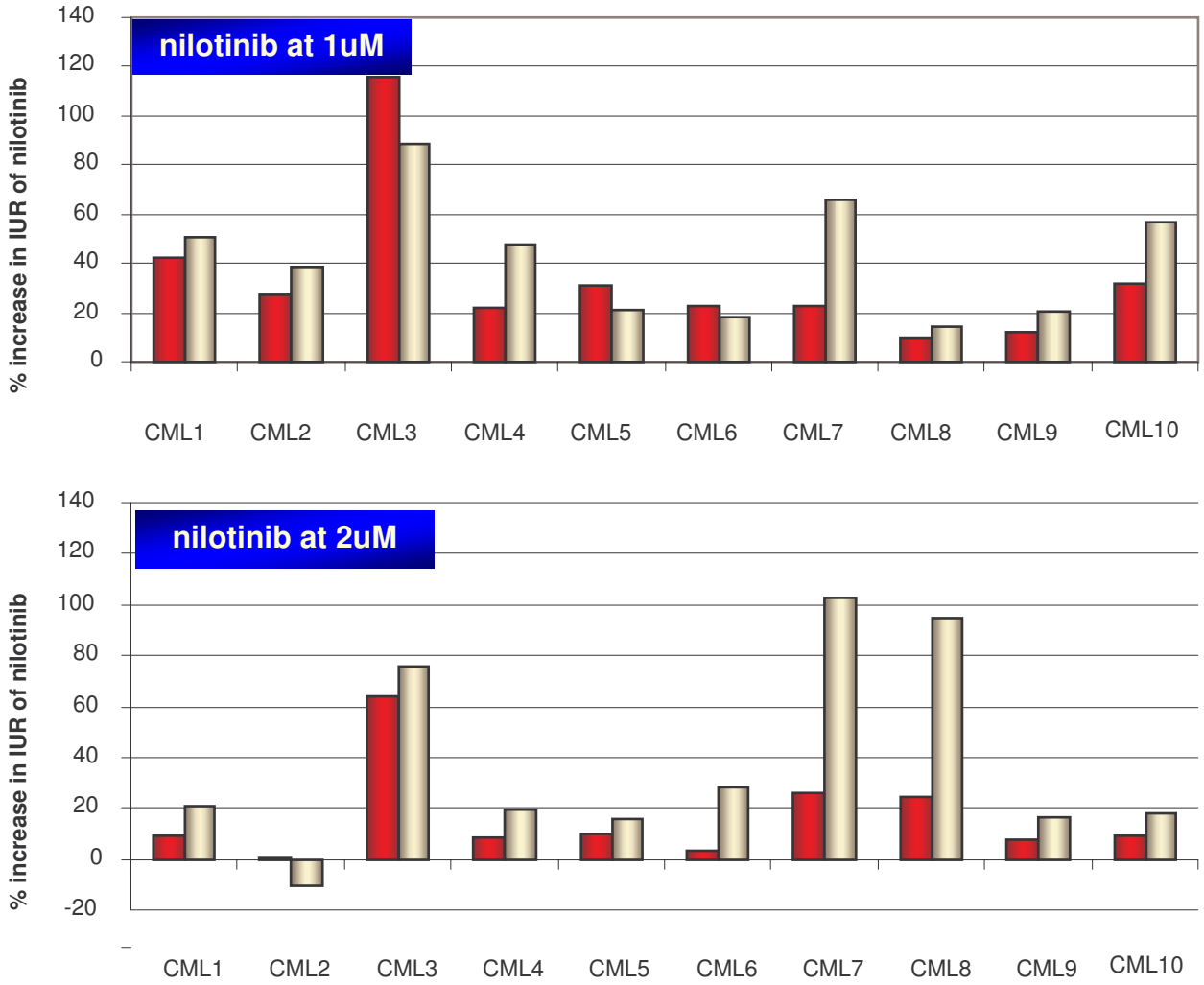


Figure 6.2 The percentage increase in the IUR for nilotinib in the presence of unlabelled imatinib.

The results of Figure 6.1 are expressed in this diagram, as the percentage change from the control (no imatinib), in the intracellular concentration of nilotinib, which occurs when 1 μM and 2μM imatinib are added to the nilotinib IUR assay.

6.4.5 Cell viability

Since in these studies the cells are being exposed to high concentrations of kinase inhibitors, the cell viability was checked at the beginning and end of the 2 hour incubation to ensure observed changes were not a result of cell death. There was no observed difference in the viability of cells from the beginning to the end of these assays, nor was there any observed decrease in viability where the drugs were used in combination even at high concentrations. Greater than 98% viability was observed in all tests.

Figure 6.3 The effect of unlabelled nilotinib on the IUR of 1µM and 2µM [14C] labelled imatinib

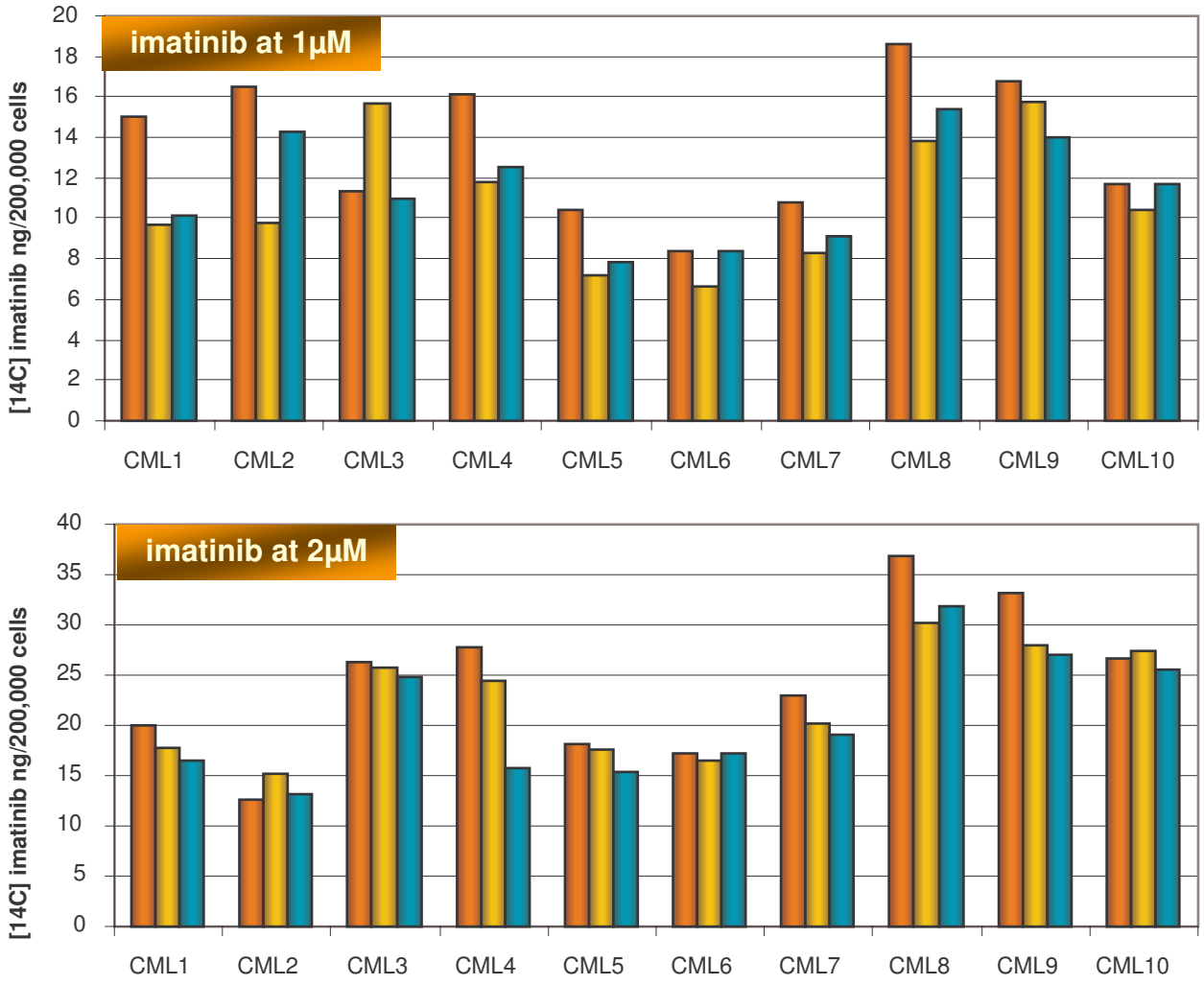
All assays were performed in triplicate. Assays were only accepted, if triplicate results were concordant within 10%, if not, assays were repeated.

In contrast to the results observed with nilotinib, the addition of cold nilotinib resulted in a decrease in the intracellular concentration of imatinib at both 1 and 2µM in the majority of patients tested.


Control 1uM [14C] imatinib


+1uM “cold” nilotinib


+2uM “cold” nilotinib


imatinib at 1µM
[14C] imatinib ng/200,000 cells
0
2
4
6
8
10
12
14
16
18
20
CML1
CML2
CML3
CML4
CML5
CML6
CML7
CML8
CML9
CML10
imatinib at 2µM
[14C] imatinib ng/200,000 cells
0
5
10
15
20
25
30
35
40
CML1
CML2
CML3
CML4
CML5
CML6
CML7
CML8
CML9
CML10

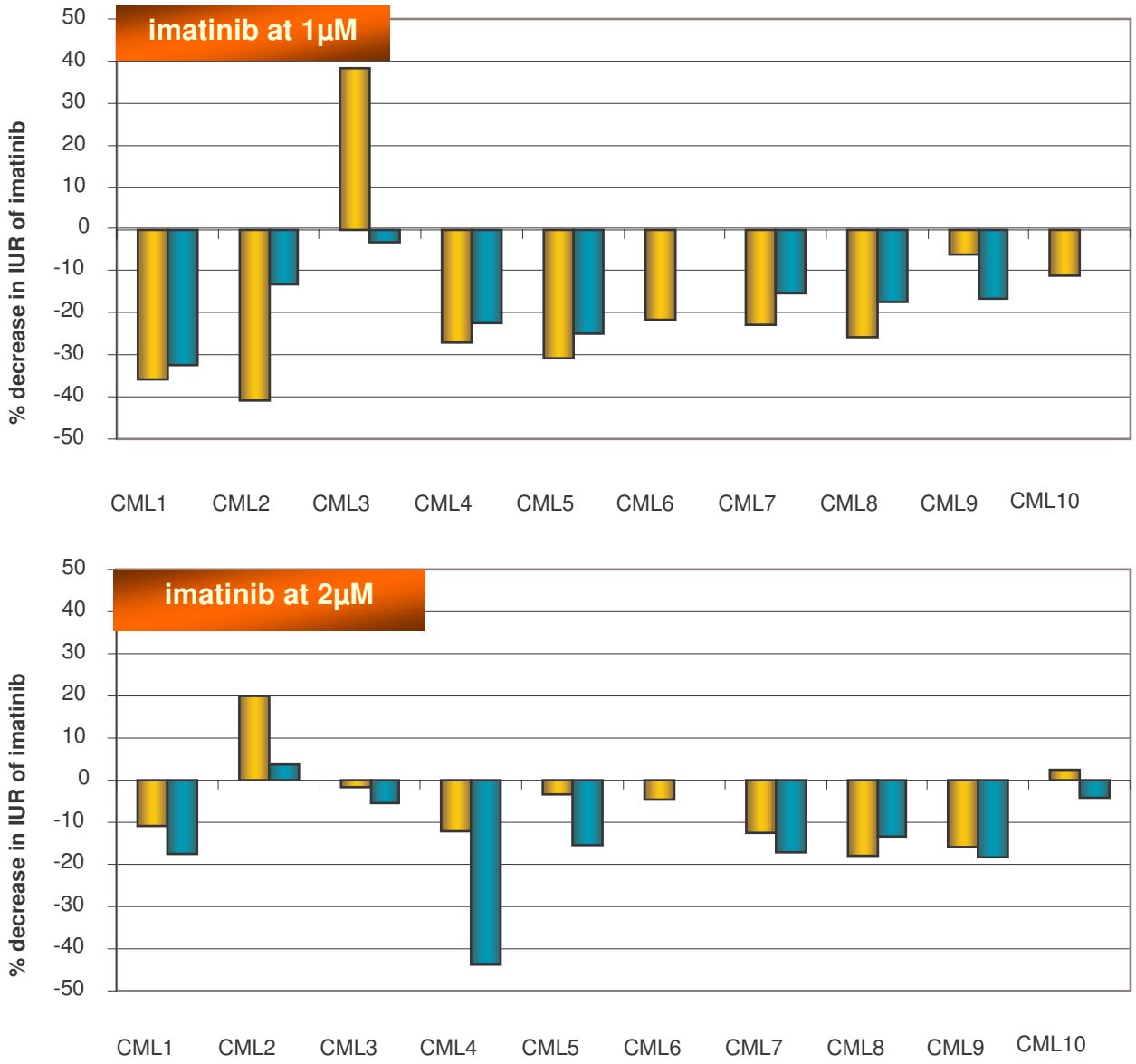


Figure 6.4 The percentage decrease in the IUR for imatinib at 1 and 2µM in the presence of unlabelled nilotinib.

There was a universal decrease, when compared to controls (no nilotinib), in the intracellular concentration of imatinib when nilotinib was added. In keeping with the findings with nilotinib IUR, this change was more significant at lower nilotinib concentrations.

+1uM "cold" nilotinib

+2uM "cold" nilotinib

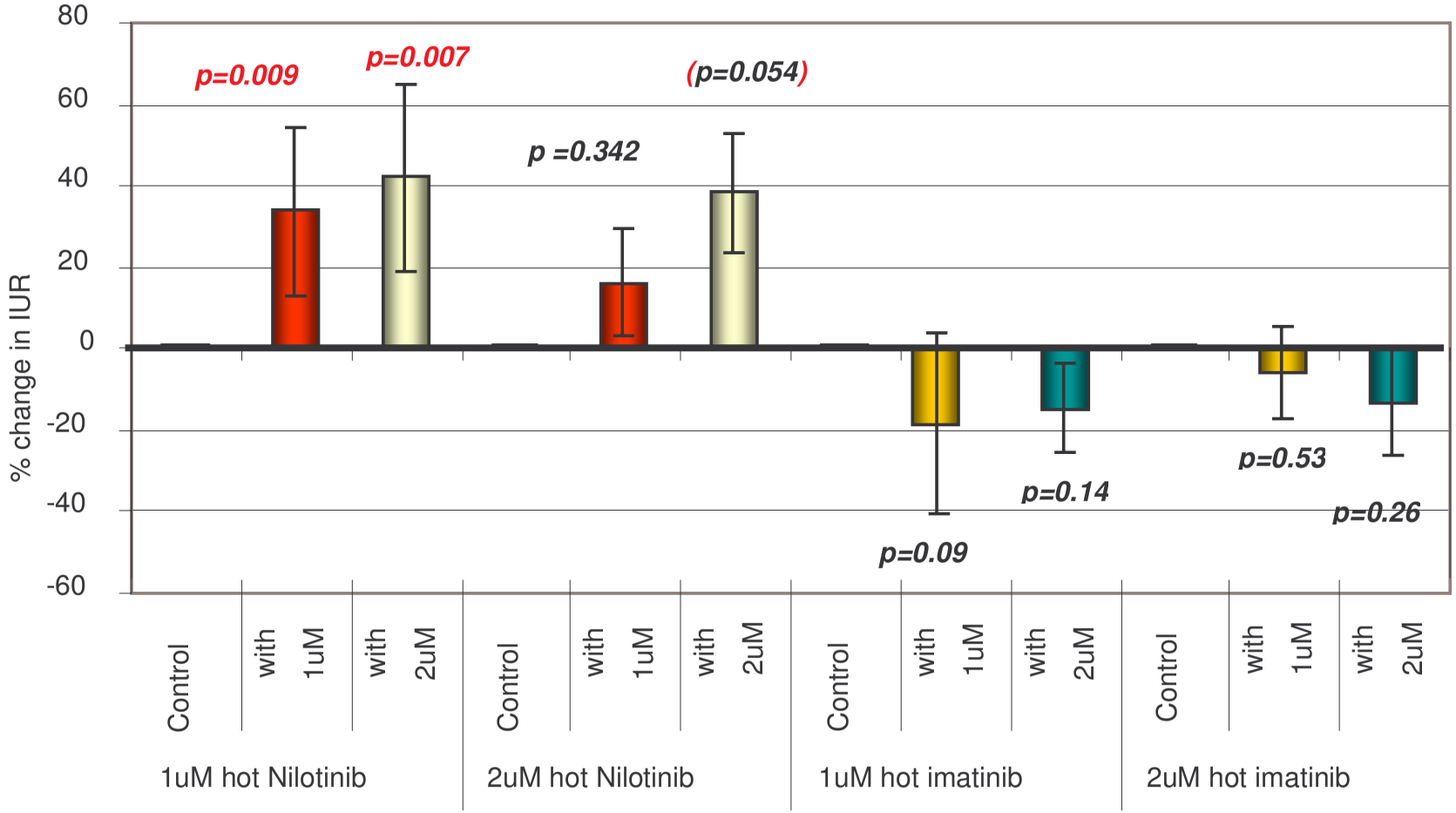
Figure 6.5 Comparing of the effect of combination therapy on the intracellular concentration of both imatinib and nilotinib

Unlababelled Imatinib at 1 and 2μM was added to the nilotinib IUR assay (1 and 2μM) and the % change in IUR, from the no imatinib control, was assessed. The addition of imatinib significantly increased the 1μM nilotinib IUR. The addition of imatinib to the 2μM nilotinib IUR also resulted in an increase, however this failed to reach statistical significance.

The addition of nilotinib to the 1 and 2μM imatinib IUR assay, resulted in a universal decrease in the imatinib IUR. This decrease failed to reach statistical significance at any concentration.

This graph clearly demonstrates the differing effects of combination therapy on the relative concentrations of each drug.

Letters in red indicate significant changes from the control. Red parentheses highlight changes close to statistical significance.


80
60
40
20
0
-20
-40
-60
% change in IUR
p=0.009
p=0.007
p =0.342
(p=0.054)
p=0.09
p=0.14
p=0.53
p=0.26
Control
with 1uM
with 2uM
1uM hot Nilotinib
2uM hot Nilotinib
1uM hot imatinib
2uM hot imatinib

% change in intracellular concentration of labeled drug	2µM [14C] – nilotinib + 2µM imatinib	2µM [14C] – imatinib + 2µM nilotinib
HL60	14.8%	-46.1%
KU812	20.8%	-19.7%

Table 6.2 The effect of combination therapy on the IUR of labelled drugs in cell lines.

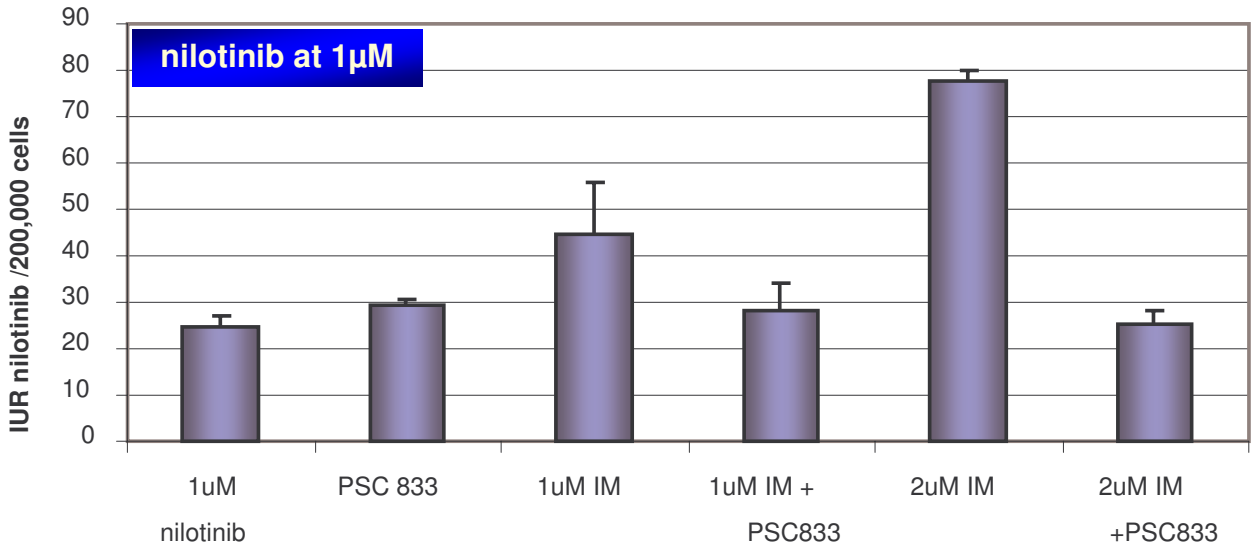
Results are in keeping with those observed in patient material, and reveal an increase in the IUR for nilotinib when imatinib is added. Conversely, a decrease in the IUR for imatinib is observed when nilotinib is added.

Figure 6.6 The effect of combination therapy in the ABCB1 over expressing cell line K562-DOX.

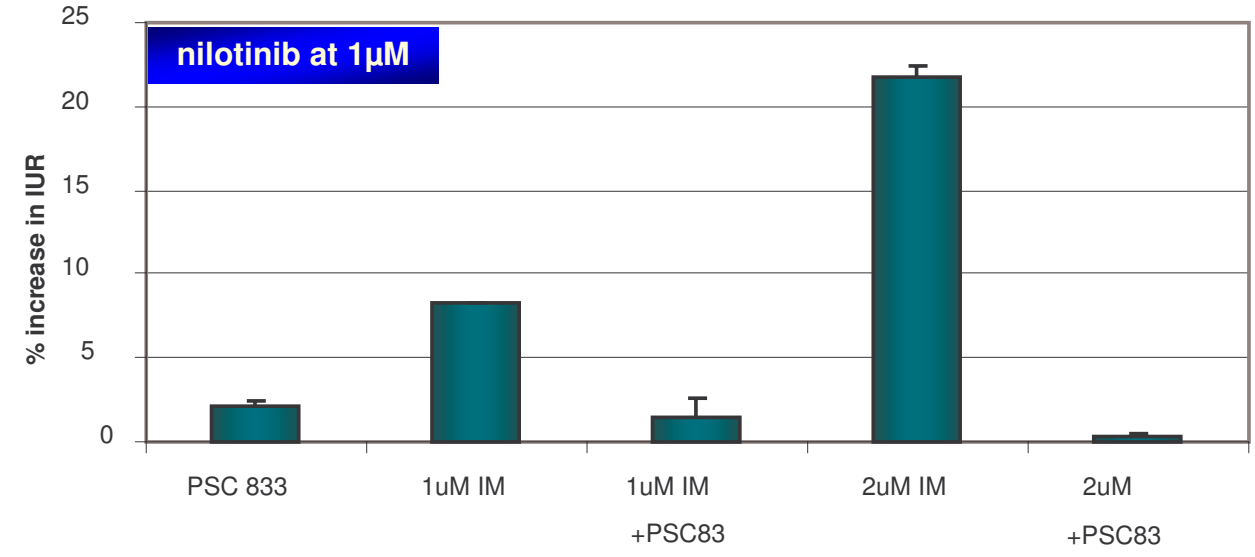
Graph A shows the IUR of nilotinib at 1μM in the presence and absence of imatinib (at 1 and 2μM), PSC833 (ABCB1 inhibitor) and imatinib + PSC833.

Graph B displays the % increase in IUR in these experiments. As is evident, PSC833 increases the IUR for nilotinib, suggesting involvement with ABCB1. The addition of both 1 and 2μM imatinib results in very significant increases in the IUR, but the effect of imatinib and PSC833 in combination, results in no significant increase in nilotinib IUR.

A


nilotinib at 1µM
IUR nilotinib /200,000 cells
90
80
70
60
50
40
30
20
10
0
1uM nilotinib
PSC 833
1uM IM
1uM IM + PSC833
2uM IM
2uM IM +PSC833

B


nilotinib at 1µM
% increase in IUR
25
20
15
10
5
0
PSC 833
1uM IM
1uM IM +PSC83
2uM IM
2uM +PSC83

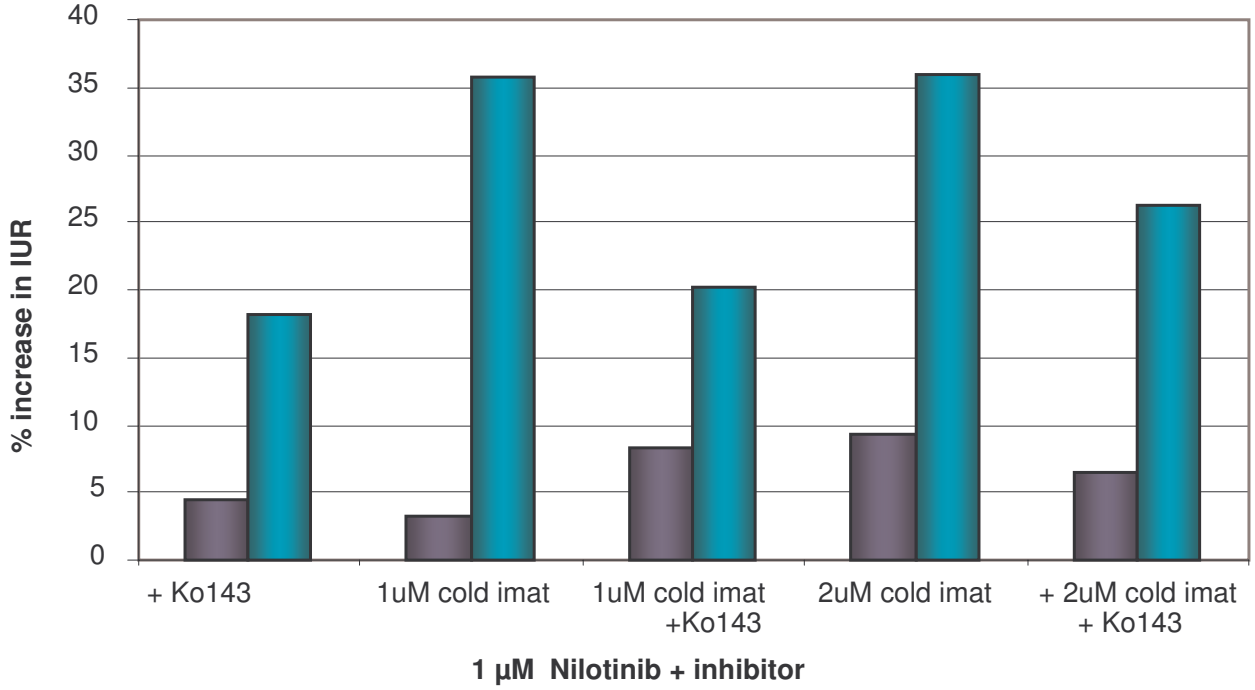


Figure 6.7 The effect of combination therapy in the ABCG2 over expressing cell line Mef BCRP1 and the parental line Mef 3.8.

The ABCG2 inhibitor Ko143 had marginal effect (<10%) on the nilotinib IUR in the parental Mef3.8 cells, as did the addition of 1 and 2µM imatinib.

In contrast the addition of imatinib and Ko143 as single agents, increased the IUR of nilotinib in the ABCG2 expressing line mef-BCRP1 The addition of imatinib and Ko143 in combination however, reduced the potency of imatinib as an inhibitor and reduced the nilotinib IUR to the level observed with Ko143 alone. This implies that nilotinib may be transported by ABCG2, that imatinib can act as a potent inhibitor of ABCG2, and that while Ko143 is apparently (based on these experiments)a less potent inhibitor than imatinib, it is likely that it binds to ABCG2 more avidly, and displaces imatinib.

Figure 6.8 Possible interactions of imatinib and nilotinib with ABC transporters.

The large red square represents an ABC transporter in this case ABCB1. The theoretical representation is of 3 interactive binding domains, each with defined specificities.

A Nilotinib binding to ABCB1, showing efflux and postulated inhibition pathways.

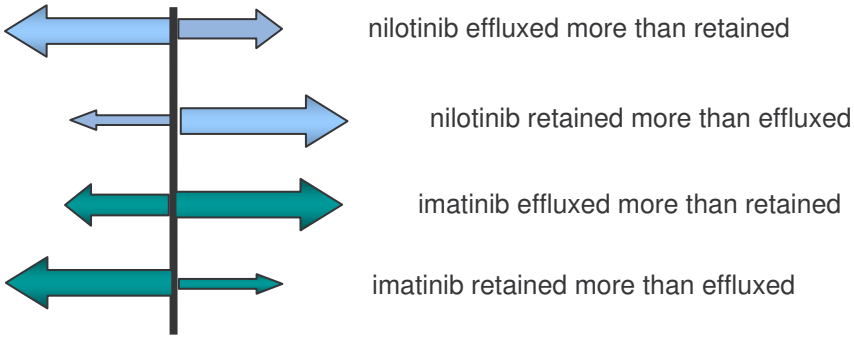
B. Imatinib binding to ABCB1, showing efflux and postulated inhibition pathways.

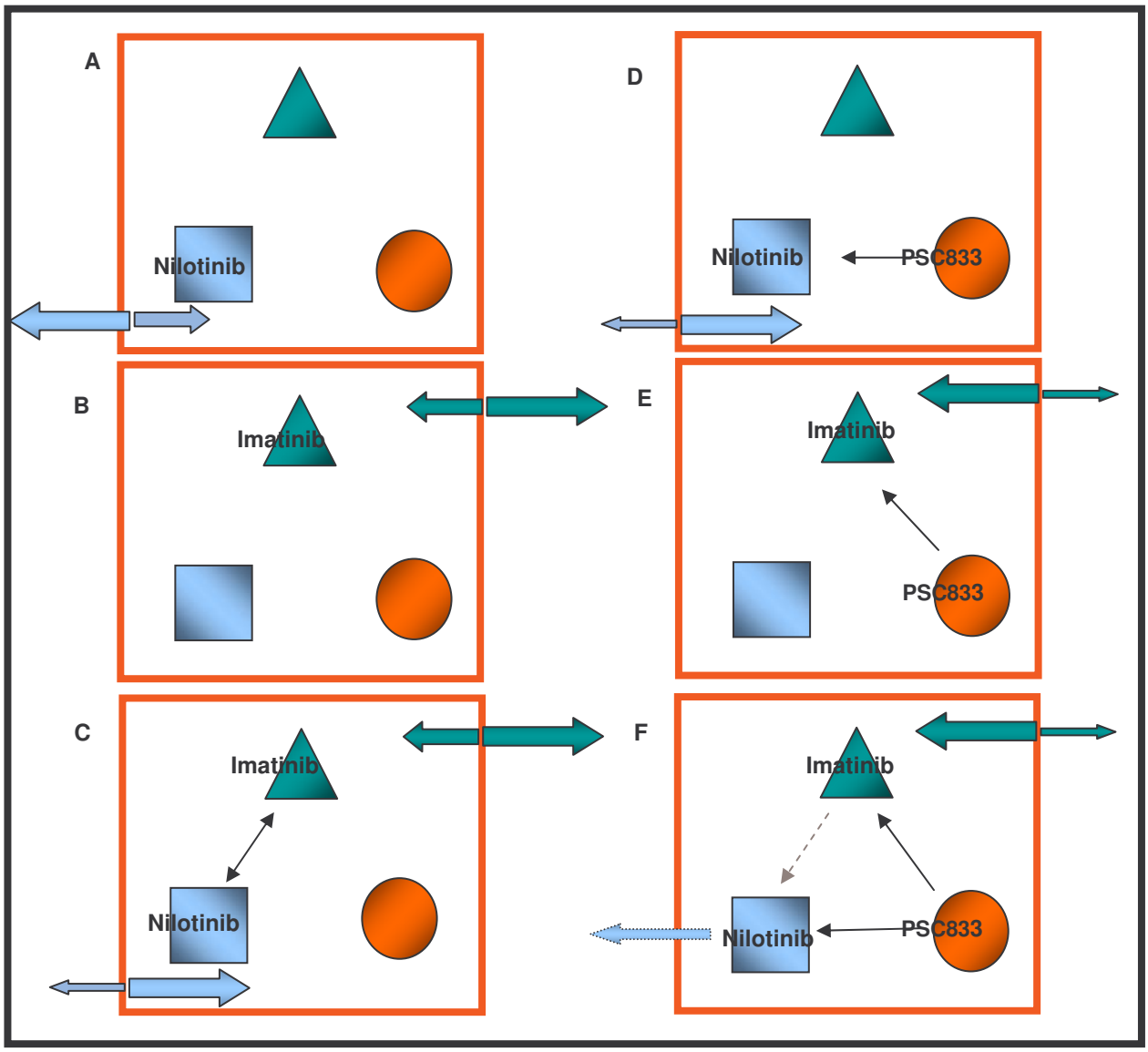
C. Imatinib and nilotinib binding each to separate domains. The binding of imatinib prevents nilotinib binding and being transported by ABCB1 to any significant effect. This situation is displayed at lower concentrations (1μM) of nilotinib. At increased nilotinib concentrations; nilotinib may by competitive binding displace some of the imatinib and thus itself be translocated.

D. PSC833 binds preferentially to ABCB1, displacing nilotinib, thus preventing nilotinib from being transported.

E. PSC833 binds preferentially to ABCB1, displacing imatinib, thus preventing imatinib from being transported.

F. When all 3 drugs are combined within the cell, PSC833 displaces nilotinib, to some degree. It also displaces imatinib and thus the effect of imatinib on nilotinib is removed. Thus in radiolabelled uptake studies of nilotinib the only effect seen is that of PSC833. In addition, the presence of all three drugs may result in some competitive binding. This may result in some displacement of PSC833 and hence reduced nilotinib intracellular concentration, through ABCB1 mediated active transport of the drug out of the cell.




A
Nilotinib
D
Nilotinib
PSC833
B
Imatinib
E
Imatinib
PSC833
C
Imatinib
Nilotinib
F
Imatinib
Nilotinib
PSC833

6.5 Summary

In previous chapters and publications, the intracellular transport mechanisms for both imatinib and nilotinib have been investigated (Chapter 4 and 5. (White Deborah L *et al.* 2007; White *et al.* 2007b; White *et al.* 2006)). From these studies it can be concluded that the influx mechanisms for imatinib are different from those for nilotinib. Imatinib is transported actively into the cell via OCT-1, while nilotinib transport is OCT-1 independent, and appears to be primarily passive. However, there appears to be some apparent overlap in efflux pathways. From the work presented here, and that of others, imatinib is an apparent substrate, and candidate inhibitor of ABCB1. This is demonstrated in cell lines engineered to over express ABCB1. Imatinib also interacts with ABCG2, though there is some conjecture as to the exact nature of this interaction, it would appear that it can act as both a substrate and inhibitor of this pump. Similarly, there is also evidence that nilotinib is transported by ABCB1 and ABCG2. It would therefore be anticipated that any synergistic or antagonistic effects observed, as a result of the use of these two drugs in combination, at least at the target cell level, would, at least partially be a result of interactions occurring between the drugs and efflux transporters.

Weisberg et al (Weisberg *et al.* 2007) demonstrated an additive to synergistic effect when nilotinib and imatinib were used in combination. This synergy was demonstrated in both in-vivo and in-vitro experiments, and was most notable at low drug concentrations. In this Chapter, it has been demonstrated that the addition of imatinib significantly increases the intracellular accumulation of nilotinib. In keeping with the findings of Weisberg et al, this occurred to a significant level at low concentrations of nilotinib. The likely mechanisms for increased cellular accumulation are either increased uptake or decreased efflux. While imatinib is actively transported via OCT-1 a passive component to intracellular uptake is also implicit (as prazosin (OCT-1 inhibitor) does not fully block intracellular accumulations). It is plausible that, imatinib facilitates the increased passive uptake of nilotinib, and that this effect is most notable at low drug concentrations, because of a dose limiting potential of the membrane and passive mechanism. However, the converse finding, that nilotinib does not increase, but indeed decreases the intracellular accumulation of imatinib, would suggest that the observed changes are unlikely to occur through increased passive uptake.

The most likely mechanism of the observed increase is through efflux pump interaction. It would appear probable that in this scenario, imatinib acts as an inhibitor of ABCB1, ABCG2 or both, in the presence of nilotinib. Therefore nilotinib is not transported out of the cell and is allowed to accumulate. The exact mechanism of substrate specificity and binding is an area of conjecture with regard to ABCB1 (Higgins 2007). There are speculated to be three or more binding sites within the ABCB1 protein (Martin *et al.* 2000) (Shapiro & Ling 1997; Shapiro & Ling 1998) (Figure 6.8), each with distinct, but overlapping substrate specificities. These binding sites are however interactive, giving rise to stimulation or inhibition effects of one domain on another. This results in enhanced or suppressed transport. In contrast to this, a single large drug binding site, with specific but overlapping specificity domains has also been speculated for ABCB1 (Higgins 2007).

The combination of imatinib and nilotinib with PSC833 suggest that, as shown in Figure 6.8, PSC833 overrides the effect of imatinib on nilotinib, substantiating the theory of linked but competing binding domains. This is further supported by the finding that with increasing concentrations of primary drug (in this case nilotinib), the effect of the competitive drug is decreased, due to a change in the equilibrium kinetics for all drugs. Alternatively, PSC833 and imatinib may competitively bind for the same ABCB1 domain, with PSC833 having superior affinity.

While data supports these findings being associated with ABCB1, a similar situation was observed with ABCG2. In this series of experiments the nilotinib IUR for the ABCG2 expressing line mef-BCRP1, was 30% lower than that observed for the parental line (mef-3.8). The addition of a known ABCG2 inhibitor or imatinib, increased the IUR for mef-BCRP1 but not Mef3.8. This increase was greater with imatinib at both 1 and 2 μM, than with Ko143. Interestingly, the addition of both imatinib and Ko143 simultaneously resulted in a smaller increase in IUR. The most likely explanation for this is that Ko143 binds to ABCG2 with a higher affinity than imatinib, but is a less potent inhibitor. This would result in imatinib being displaced and or transported out of the cell, and nilotinib allowed to accumulate, but to a lesser extent.

There is no doubt that the interaction between ABCG2 and TKI's is complex, and results in the literature to date are most likely, at least in part, influenced by the model system used. By necessity, these analyses are performed in cell lines engineered to over express ABCG2, and as such probably bear little, or no resemblance to the situation which exists in primary cells (Houghton *et al.* 2004)

(Burger *et al.* 2004) (Nakanishi *et al.* 2006) (Melo 2006). The one data set published to date which addresses this issue in primary cells uses levels of imatinib which are probably not universally achievable in vivo, and which could potentially also result in saturation, and thus loss of the ability to assess subtle interactions (Jordanides *et al.* 2006). However taking all the data together, it is likely that imatinib is both an inhibitor and substrate for ABCG2.

A further confounding issue is the finding that, by direct immunofluorescence and flow cytometry, chronic phase CML patients do not appear to express large amounts of these proteins, which would adequately account for these findings. It may also be that in an inactive conformation, pre drug exposure, that these proteins are difficult to quantify by flow cytometry (Minderman *et al.* 2002). It has however, been previously documented that exposure to both imatinib and nilotinib, as single agents, can induce the expression of both ABCB1 and ABCG2 (Burger *et al.* 2005). While this was documented over a longer time period, it does indicate that both drugs do induce expression, and speculatively, that up-regulation to a functional protein may be quite rapid when cells are exposed to high, potentially toxic, concentrations of substrate drugs in-vitro.

It is also possible that the observed reduction in the intracellular concentration of imatinib, in the presence of nilotinib, is related to the same efflux pathways. In this setting (Figure 6.8C), imatinib would be bound/and or transported preferentially by ABCB1 or ABCG2 or both. Since the observed reduction is low and below the level of significance, it is most likely that imatinib is primarily bound as an inhibitor, with some efflux transport also occurring. Any effect of nilotinib on influx of imatinib is probably unlikely given the differing transporter phenotypes for the two drugs. However on the data presented here, this cannot be conclusively excluded as a mechanism.

These data demonstrate that the interaction between nilotinib and imatinib results in opposing effects on the intracellular concentration of both kinase inhibitors. While the addition of imatinib to the nilotinib IUR assay increases the intracellular concentration of nilotinib significantly, the reverse combination results in a small, and statistically insignificant decrease in the intracellular concentration of imatinib. While other factors cannot be excluded, it seems probable from the body of data presented here, that the observed effects are mediated through the interaction of both drugs with efflux transporters, in particular ABCB1 and ABCG2 which are known to interact with both kinase inhibitors.

6.6 Overall comment on significance of this work

These analyses have to date been limited to the haemopoietic lineages. However, if these interactions hold true for other organs, the findings, as presented, do raise the possibility that in combination regimens, imatinib may exacerbate nilotinib side effects by increasing cellular concentrations of nilotinib in other tissues. While this is a consideration, significant increases in intracellular nilotinib were limited to low drug doses, suggesting additive effect/synergy may be most evident at low doses. This finding is consistent with the findings of Weisberg et al (Weisberg *et al.* 2007)

In summary, we show that the synergistic/additive effects previously reported may be mediated through an increase in the intracellular concentration of nilotinib. While other factors may also contribute to synergy, the magnitude of the increase in intracellular nilotinib induced by concomitant administration of imatinib would probably be sufficient to account for the additive and synergistic effects observed by Weisberg(Weisberg *et al.* 2007).