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In Vitro Investigation Of Intracellular Ponatinib Transport And Modeling Ponatinib Resistance In *BCR-ABL1*+ Cell Lines: Implications For Therapeutic Strategies

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Liu Lu

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Abstract

The use of tyrosine kinase inhibitors (TKIs), which target Bcr-Abl, has become the first-line treatment for chronic myeloid leukemia (CML). However, TKI resistance remains a major impediment to successful treatment of CML. The novel third generation pan-Bcr-Abl TKI ponatinib has demonstrated efficacy in overcoming single *BCR-ABL1* kinase domain (KD) mutation based resistance including *BCR-ABL1*^{T315I}, which inhibits the binding of all other available TKIs. While intracellular transport of the first and second generation TKIs have been studied, little is known about the complex interactions between ponatinib and drug transporters. Additionally, clinically significant mechanisms that may result in resistance to ponatinib remain to be elucidated. In this study, we investigated the interaction of ponatinib with drug transporters, and emerging modes of ponatinib resistance in previously TKI-naïve and dasatinib resistant *BCR-ABL1+* cell lines.

This study examined the role of ABCB1, ABCG2 and OCT transporters in ponatinib efflux and influx, as these transporters have been previously implicated in the transport of other TKIs. Results demonstrated neither ABCB1 ABCG2 nor OCT-1, play major roles in ponatinib transport. In addition, data revealed that ponatinib transport is not ATP/temperature dependent, and therefore is most likely to be passive.

To investigate potential resistance mechanism(s), ponatinib resistance was generated by exposure to increasing concentrations of ponatinib in *BCR-ABL1*+ cell-lines that either priorly treated with a TKI (dasatinib) or naïve to all TKIs. Two resistant cell lines, previously resistant to dasatinib and then treated with ponatinib, demonstrated the emergence of *BCR-ABL1* KD mutation(s). In one of these cell lines, the level of T315I increased from 44% to 66%, with *BCR-ABL1* mRNA expression also increasing. In the second cell line the compound mutation G250E/E255K developed. In contrast, the TKI (imatinib, nilotinib and dasatinib) naïve ponatinib resistant cell lines did not demonstrate *BCR-ABL1* KD mutations. Conversely, both of these resistant lines developed Bcr-Abl-independent resistance via Axl overexpression. Axl, a receptor tyrosine kinase, has previously been associated with TKI resistance. My studies are the first to report it in association with ponatinib resistance. In agreement with the observation that Axl overexpression causes ponatinib resistance, ponatinib sensitivity was restored following Axl inhibition or shRNA-mediated-knockdown of Axl.

In conclusion, the studies outlined in this thesis reveal that unlike other TKIs, ponatinib is not transported by ABCB1, ABCG2 or OCT-1 and therefore patients are unlikely to be susceptible to resistance caused by deregulation of these transporters. Moreover, the study also identified that in the setting of prior TKI-exposure, Bcr-Abl dependent mechanisms, such as *BCR-ABL1* KD mutations and *BCR-ABL1* mRNA overexpression, are likely to cause ponatinib resistance. However, in the TKI-naïve setting, Bcr-Abl-independent modes of resistance develop preferentially, and Axl

presents as a key mediator of this resistance. While further studies are required, particularly in reference to Axl expression in patients being treated with ponatinib, these data may suggest that combination therapeutic approached may be the most efficacious in the setting of up-front ponatinib use and subsequent development of resistance.

Abbreviations

µg – Microgram/s

µL – Microlitre/s

µM – Micromolar

7-AAD – 7-Aminoactinomycin D

^{14}C – Carbon-14 radioactive isotope

ABC – ATP Binding Cassette

ALL – Acute Lymphoblastic Leukaemia

AP – Accelerated Phase

ATCC – American Type Tissue Culture Collection

ATP – Adenosine Triphosphate

BC – Blast Crisis

BCR-ABL1 – Breakpoint Cluster Region-Abelson 1 (mRNA)

Bcr-Abl – Breakpoint Cluster Region-Abelson (protein)

BSA – Bovine Serum Albumin

CCyR – Complete Cytogenetic Remission

cDNA – Complementary DNA

CHR – Complete Haematological Response

CML – Chronic Myeloid Leukaemia

CP – Chronic phase

CrkL – CT10 regulator of kinase-like Ct – Cycle Threshold

DAS – Dasatinib

DEPC – Diethylpyrocarbonate

DMSO – Dimethyl Sulphoxide

DNA – Deoxyribonucleic Acid

EDTA – Ethylenediaminetetraacetic Acid

FACS – Fluorescence Activated Cell Sorting

FCS – Foetal Calf Serum

FDA – Food and Drug Administration

GUSB Beta-glucuronidase

x g – see rcf

h – Hour/s

HBSS – Hanks Balanced Salt Solution

IC50 – Inhibitory Concentration 50

IFN – Interferon

IgG –Immunoglobulin G

IUR – Intracellular Uptake and Retention

kDa – Kilo Daltons

KD – Kinase Domain

L – Litre/s

M - Molar

MDR – Multidrug Resistance Protein

MFI – Mean Fluorescence Intensity

mg – milligram/s

min – Minutes/s

mL – Millilitre/s

mM – Millimolar

MMR – Major Molecular Response

MNC/s – Mononuclear Cell/s

MQ – Milli-Q

mRNA – messenger RNA

MRP – Multidrug Resistance-Associated Protein

MW – Molecular Weight

ng – Nanogram/s

nM – Nanomolar

OCT-1 – Organic Cation Transporter 1

p – Phosphorylated Form of Protein

PAGE – Polyacrylamide Gel Electrophoresis

PB – Peripheral Blood PB

MNC/s – Peripheral Blood Mononuclear Cell/s

PBS – Phosphate Buffered Saline

PE – Phycoerythrin

P-gp – P-Glycoprotein

Ph – Philadelphia Chromosome

PON – Ponatinib

PSC – PSC-833

P value – Probability Value

PVDF – Polyvinylidene Difluoride

rcf – Relative Centrifugal Force

RNA – Ribonucleic Acid

RO – Reverse Osmosis

RQ-PCR – Real Time Quantitative PCR

SD – Standard Deviation

SDS – Sodium Dodecyl Sulphate

sec – second/s

SH1/SH2/SH3 – Src Homology Region 1/2/3

TBS – Tris Buffered Saline

TBST – Tris Buffered Saline +Tween®20

TKI/s – Tyrosine Kinase Inhibitor/s

TEA – Tetraethylammonium Bromide

U/mL – Units Per Millilitre

Chapter 1 :

Introduction

1.1 Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a myeloproliferative disease that was the first cancer type to be associated with a chromosome abnormality (1). CML arises in all age groups, though is predominantly diagnosed in the 5th or 6th decade of life, occurring in approximately 1-2 in 100,000 people (2). It is a triphasic disease when left untreated. More than 90% of patients are diagnosed in the chronic phase (CP) (3) where myeloid cells have an increased life span and proliferative index. This results in a huge expansion in granulocyte mass in the peripheral blood (4), however patients in CP are largely asymptomatic or demonstrate mild symptoms such as weight loss. If left untreated, patients usually remain in CP for 3-6 years (5)before progressing to accelerated phase (AP) and subsequently fatal blast crisis (BC) (6-9). Patients may remain in AP for a variable period, while in BC the disease behaves like an acute leukaemia, with rapid progression (3-6 months) and virtually inevitable death (5).

1.2 Philadelphia chromosome, Bcr-Abl oncoprotein and CML

In 1845, John Hughes Bennett first described CML as “hypertrophy of the spleen and liver in which death took place from suppuration of the blood” (10, 11). Around the same time, a similar case was published by Rudolf Virchow in Berlin (10, 11). In the ensuing years, CML has been distinguished from other types of leukaemia based on clinical differences and pathological characterization. A milestone was the discovery in 1960 of an abnormal chromosome in leukaemic cells of patients with CML now

called the Philadelphia chromosome (Ph) (12, 13). Several years later, the Ph chromosome (Figure 1.1) was reported to be a result of a reciprocal translocation between the break point cluster region (BCR) gene on chromosome 22 (q11) and the Ableson (c-ABL) gene on chromosome 9 (q34) [t(9;22)(q34;q11)] (14-17). This translocation induces genetic instability, clonal evolution and phenotypic alterations (18). The product of the Ph chromosome is a fusion *BCR-ABL1* gene that encodes a 210kD phosphoprotein p210Bcr-Abl, which is a constitutively active tyrosine kinase (19, 20). Bcr-Abl phosphorylates and activates a number of downstream signal transduction pathways, and results in unregulated growth of predominantly myeloid cells in the bone marrow. This massive myeloid expansion results in accumulation in the blood, which is the hallmark of CML.

The Bcr-Abl oncoprotein (Figure 1.2) contains an N-lobe and a C-lobe. Between these is an ATP pocket, which provides a binding site for adenosine tri-phosphate (ATP). ATP binds and transfers a phosphate to activate Bcr-Abl substrate proteins (Figure 1.2 and Figure 1.3). Additionally, the N-terminus coiled-coil oligomerization domain of Bcr-Abl is essential for dimerization and autophosphorylation. Phosphorylation of Tyr-393 opens the activation loop in Bcr-Abl, thus allowing the deregulated Bcr-Abl kinase to constitutively activate downstream pathways. The cytoplasmic location of Bcr-Abl enables its interaction with multiple adaptor proteins including CrkL (which binds to the C-terminus of Bcr-Abl), GRB2 (which binds to Bcr-Abl via tyrosine 177) and CBL (which binds to the SH2 domain of Bcr-Abl) (21-27) to activate signalling pathways and promote the survival of leukaemic

cells (Figure 1.4). These pathways include the Ras/Raf pathway, which is responsible for enhancing proliferation; the P13K pathway, which promotes survival of leukaemic cells; and the JAK2/STAT5 pathway, which is also critical for CML leukaemogenesis. The activation of STAT5 is through JAK2 or Src family kinases such as Lyn (28, 29). In addition, over-expression of STAT5 is associated with TKI resistance (29, 30).

Notably, the Ph chromosome is not only found in CML patients but also 20-30% of adult and 2%-10% of pediatric acute lymphoblastic leukaemia (ALL) patients (31-33). ALL is a disorder of excess lymphoblasts that can be classified into two subtypes: T cell and B cell lineage ALL. In addition, one subtype of B cell lineage ALL is Ph chromosome-like ALL (Ph-like ALL) which patients do not have Bcr-Abl but exhibit similar gene expression profile to Ph chromosome positive ALL. Both Ph chromosome positive ALL and Ph-like ALL could be targets of TKI treatment. ALL is one of the most challenging adult malignancies due to its low cure rate. Patients develop aggressive phenotypes with poor outcomes on conventional chemotherapy regimens. The overall cure rate for ALL is 80% in children and approximately 40% in adult (34, 35).

Figure 1.1

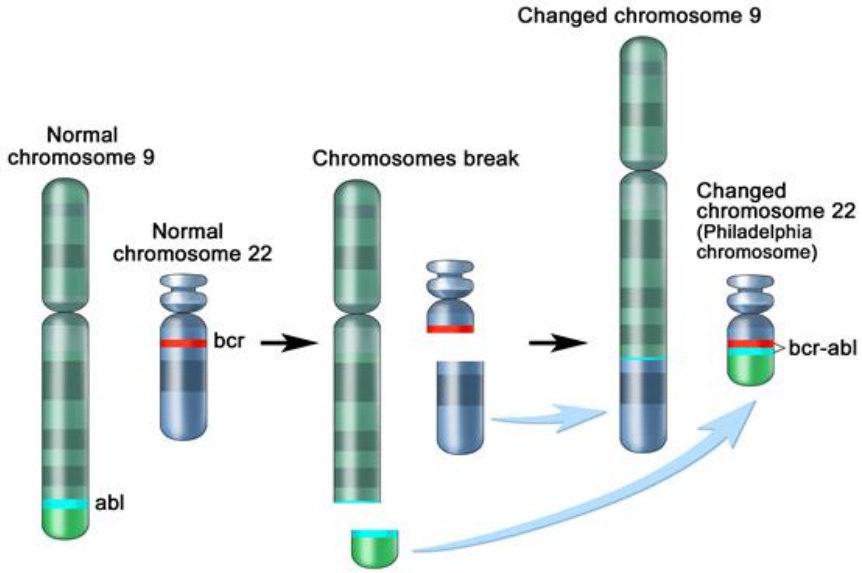


Figure 1.1. The Philadelphia chromosome. Reciprocal translocation between chromosome 9 and 22 results in the arise of Philadelphia chromosome. Adapted from National Cancer Institute (The USA), 2007, http://www.cancer.gov/publications/dictionaries/cancer-terms?cdrid=44179

Figure 1.2

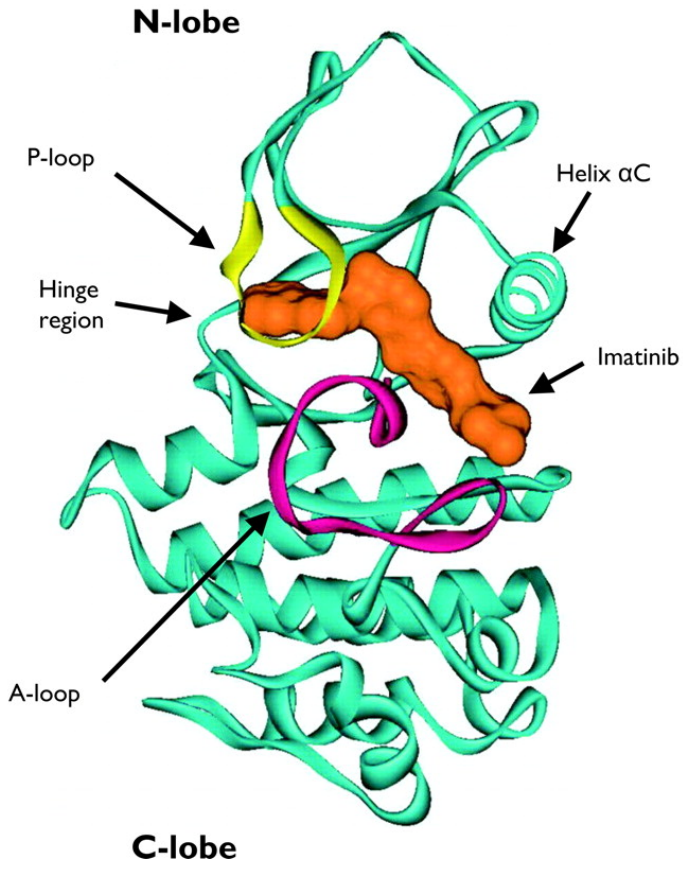


Figure 1.2. Crystal structure of Bcr-Abl. Green ribbon representation of the Bcr-Abl molecule; yellow indicates phosphate binding loop (P-loop) and purple indicates activation loop (A-loop). Orange indicates imatinib binding with Bcr-Abl kinase domain. (Adapted from Quintas-Cardama A and Cortes J, 2009, Blood)

Figure 1.3

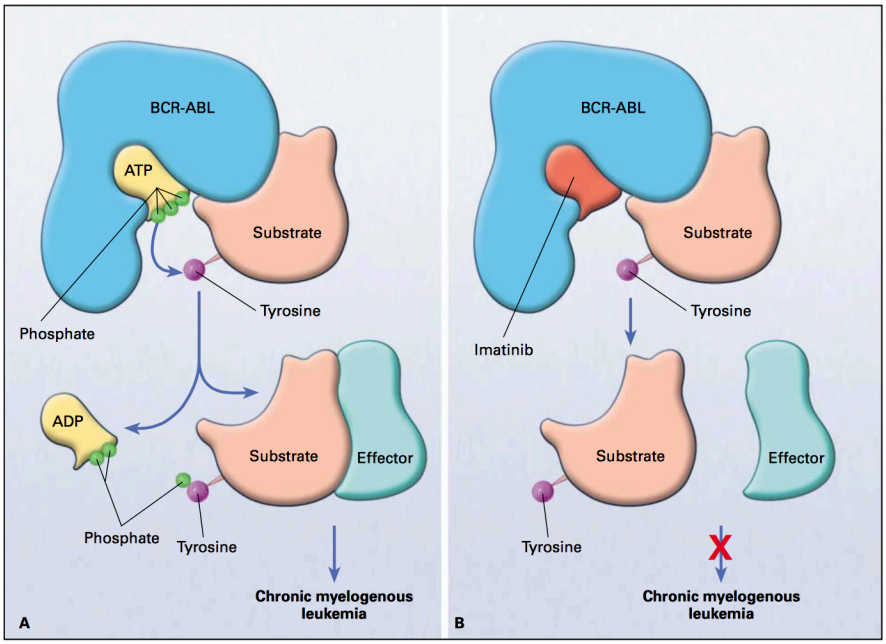


Figure 1.3. Mechanism of TKI inhibits Bcr-Abl. (A) Bcr-Abl with ATP binds to ATP-binding pocket. Bcr-Abl substrates are constitutively phosphorylated and activated by Bcr-Abl, leading to activation of downstream effector molecules and cause CML. (B) imatinib competes with ATP and binds to the ATP binding pocket in Bcr-Abl, results in the inactivation of Bcr-Abl.(Adapted from Savage D and Antman KH, 2002, New Eng J Med)

Figure 1.4

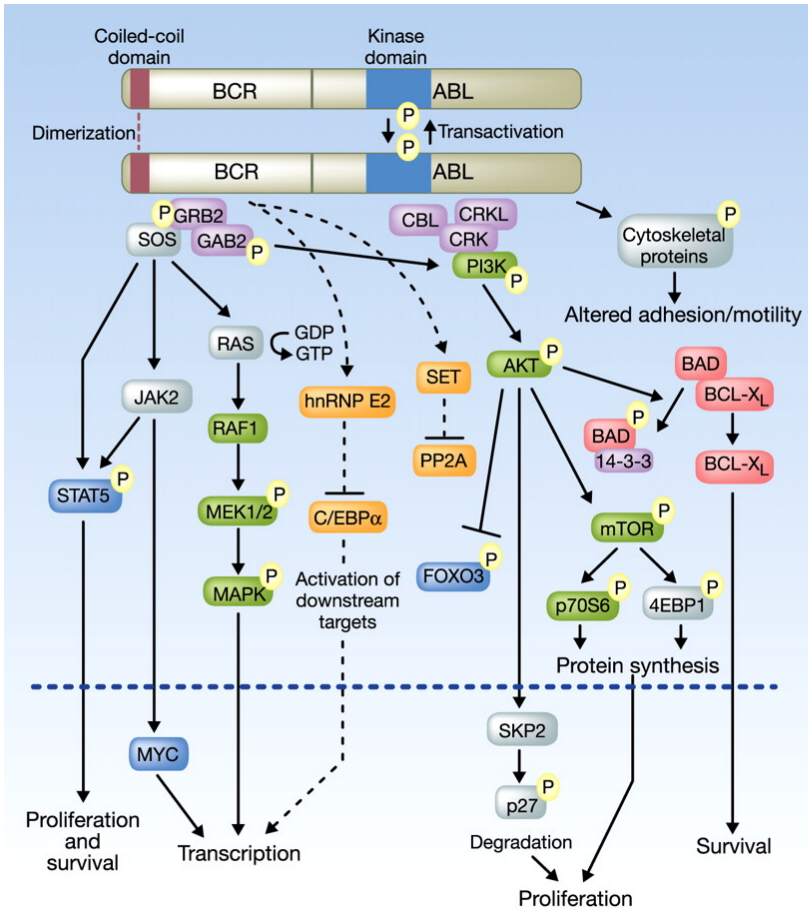


Figure 1.4. Downstream signal network of Bcr-Abl. The key pathways activated by Bcr-Abl including JAK2/STAT5, RAS/RAF and PI3K pathways. Activation of these pathways result in enhances proliferation, transcription and survival, as well as reduces in apoptosis in CML cells. Adapted from O'Hare T *et.al*., 2011,.*Clin Cancer Res* .

1.3 Treatment

In the past two centuries, advances in scientific research enabled us to gradually understand the cause of CML, and therefore developed effective and specific treatment. The history of the development of a specific treatment is a remarkable example of how medical research can lead to the development of a targeted cancer therapy

1.3.1 Stem cell therapy, interferon and hydroxyurea

There are several treatment options available to CML patients. Chemo-radiotherapy followed by allogeneic bone marrow transplantation can be curative. Unfortunately, allogeneic transplantation is not always feasible. Bone marrow donors and recipients must be human leukocyte antigen-histocompatible, and only around 30% of CML patients have a suitable matched donor (36). Transplantation is also limited by the age of patients, as older patients are intolerant to the intensive ablative therapy required for transplantation. This treatment may also be unsuitable if the patient has other diseases or medical complications in addition to CML

Before allogeneic stem cell transplantation was available, treatments in the last century included radiotherapy, busulfan (chemotherapy agents) and hydroxyurea (37). These three agents were introduced to treat CML from the early 20th centuries to the 1970s. Busulfan is a cell cycle non-specific alkylating agent. Hydroxyurea is also a chemotherapy drug that stops DNA synthesis by inhibiting ribonucleotide

reductase. Patients treated with hydroxyurea were found to survive longer than those treated with busulfan, but none of the three treatments induced any degree of Ph negativity (38). In addition, these treatments are non-specific and have low efficacy in CML. Patients treated with these therapies showed relatively low survival rates. In the 1970s, interferon alpha (IFN-α) was introduced as an efficacious CML treatment, and was found to prolong survival and induce complete cytogenetic responses (CCyR, defined as no detectable Ph+ metaphases) in a small subset of patients (39). However, the toxicity of IFN-α induced side effects in most patients, and many were intolerant of IFN-α as a long term therapy. The lack of success with standard chemotherapeutic approaches, and the growing knowledge of the Bcr-Abl protein structure, lead to the exploration of a new treatment approach for CML, the use of small molecule inhibitors.

1.3.2 Imatinib

A breakthrough was achieved in the 1990s when Imatinib mesylate (Figure 1.5) (formerly known as STI-571, Glivec; Novartis Pharmaceuticals) was synthesized to specifically target the Bcr-Abl kinase. It was the first tyrosine kinase inhibitor (TKI) approved to treat CML. Imatinib was designed to target Bcr-Abl, and was found to also have activity against PDGF receptor tyrosine kinases, c-kit and c-FMS (colony-stimulating factor receptor) (40, 41).

Imatinib competes with ATP for binding at the ATP binding pocket of Bcr-Abl, and therefore prevents ATP binding and hence prevents the phosphorylation and

activation of downstream Bcr-Abl substrates (Figure 1.3) (42, 43). In the binding complex, imatinib engages in a hydrogen bond interaction with the Thr-315 residue (the gatekeeper residue) in the kinase domain, and an amide carbonyl forms a hydrogen bond with ASP-318 to keep the activation loop closed (44). This interaction maintains Bcr-Abl in the inactive conformation, inhibiting its ability to trigger downstream signal pathways (Figure 1.4).

The International Randomized study of Interferon and STI571 (IRIS) trial (45) began in 2000, and results indicated that patients who received imatinib therapy had a remarkably higher rate of major cytogenetic response (MCyR, defined as 1-34% of Ph chromosome positive metaphases) compared with patients treated with IFN-α (87% versus 35% at 60 months). Imatinib treated patients also had higher rates of complete cytogenetic response (76% versus 15% at 60 months). The event-free survival rate at 5 years in the imatinib group was 83%, and 93% of patients who achieved CCyR with imatinib treatment did not progress to AP or BP (45). Events were defined by the first occurrence of any of the following: death from the cause of treatment, disease progression to the advance phase, or loss of a complete hematologic or major cytogenetic response (45).

Figure 1.5

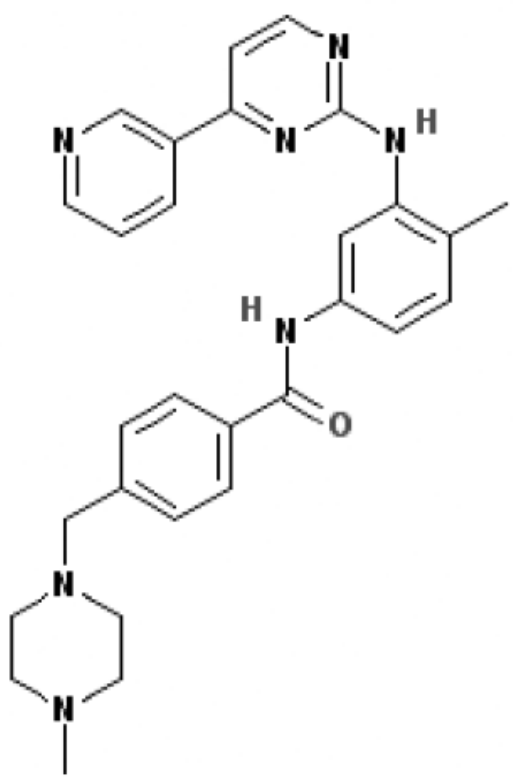


Figure 1.5. Chemical structure of imatinib mesylate. Imatinib binds to inactive form of Bcr-Abl and inhibits the kinase activity. Adapted from MM Z, GA, SM K, ML G, Leukemia 2013.

1.4 Imatinib resistance and second generation TKIs

Despite the extraordinary success of imatinib, a proportion of patients fail to achieve optimal responses due to resistance. Imatinib resistance can be divided into two groups: primary and secondary. Primary imatinib resistance is defined as lack of efficacy from the start of imatinib treatment. Primary resistance can be divided into two groups: primary hematologic resistance and primary cytogenetic resistance. Primary hematologic resistance is defined as patients who fail to achieve any level of hematologic response by 3 months (reduction in white blood cell count) or achieve complete hematologic response (platelet count <450×109/L, white blood cell count <10× 10^9/L) within 6 months of initiation imatinib therapy (46, 47). Primary cytogenetic resistance is defined as patients who fail to achieve any level of cytogenetic response (CyR, defined < 95% of Ph chromosome positive metaphases) by 6 months, a MCyR by 12 months or a CCyR by 18 months. (46-48). The majority of these patients experience poor outcomes with imatinib therapy. The major cause of primary resistance is low functional activity of the human Organic Cation Transporter 1 (OCT-1) protein; the major active transporter for imatinib in hematopoietic cells (49, 50). Patients who were initially sensitive to imatinib therapy but subsequently lose response are said to have secondary resistance. The mechanisms of secondary resistance include over-expression of the *BCR-ABL1* oncogene, deregulation of imatinib influx/efflux transporter, activation of Bcr-Abl independent signalings, and most frequently, kinase domain (KD) mutations in Bcr-Abl (51).

1.4.1 *BCR-ABL1* KD mutations

To date, more than 100 different point mutations (Figure 1.6) within the kinase domain of Bcr-Abl have been discovered in CML patients who respond sub-optimally or have treatment failure on imatinib therapy (52). Most of these mutations occur in the ATP pocket of the kinase domain (KD), and result in a conformational change that prevents imatinib binding (53, 54).

Mutation rates in patients with late CP, AP and BC phase CML have been reported to be 25%-30%, 40%-50% and 80% respectively (45, 55, 56). Interestingly, after 8 years follow-up, just 55% of patients on IRIS remained on imatinib treatment (57) with the remaining 45% of patients ceasing treatment due to resistance or intolerance, safety, unsatisfactory therapeutic outcome, death, or other reasons.

To overcome resistance attributable to these KD mutations, a number of second generation TKIs have been developed including nilotinib, dasatinib and bosutinib. Both nilotinib and dasatinib (Figure 1.7) were recently approved by the FDA (US Food and Drug Administration) and TGA (Australian Therapeutic Goods Administration) as first-line therapy for patients with CP-CML and for adults in all phases of CML with resistance to imatinib therapy. Both drugs are more potent than imatinib (58-60) and are highly effective in targeting Bcr-Abl with most known KD mutations with the exception of the T315I mutation (61-63). It has been reported that both inhibitors (dasatinib at 15 months and nilotinib at 6 months) induced MCyR in about 60% of patients with imatinib-resistant chronic phase, and induced

CCyR in approximately 45%-50% of patients ((64, 65). Despite these remarkable results, there had until recently been no currently available TKI that successfully targeted the 'gatekeeper' T315I mutation, which accounts for approximately 15%-20% of clinically observed Bcr-Abl mutations (62, 66-68). Without treatment, CML patients harbouring the T315I mutation will generally progress to fatal disease in a short period of time (56, 69). Therefore, recent research efforts have been devoted to developing compounds with activity against the T315I mutation, and a series of third generation inhibitors have been developed, including ponatinib (AP24534) (70) (will be described later in the section of 1.5).

1.4.2 *BCR-ABL1* amplification

Another resistant mechanism is increase expression of *BCR-ABL1* . This increased expression of *BCR-ABL1* can be induced by duplication of the Ph chromosome, mutations in the promoter region of Bcr-Abl, or formation of extrachromosomal double minutes (small, circular extrachromosomal DNA fragments that are transcriptionally active and frequently harbours oncogenes, and hence facilitates oncogene overexpression) carrying *BCR-ABL1* (71-73). The overexpression results in increased transcription and translation, therefore CML cells with high level of *BCR-ABL1* were shown to have decreased sensitivity to imatinib, nilotinib and dasatinib (51, 74, 75). However, *BCR-ABL1* amplification occurs in a minority of patients who are resistant to imatinib, and this mechanism is more common in advanced phases of CML (76).

Figure 1.6

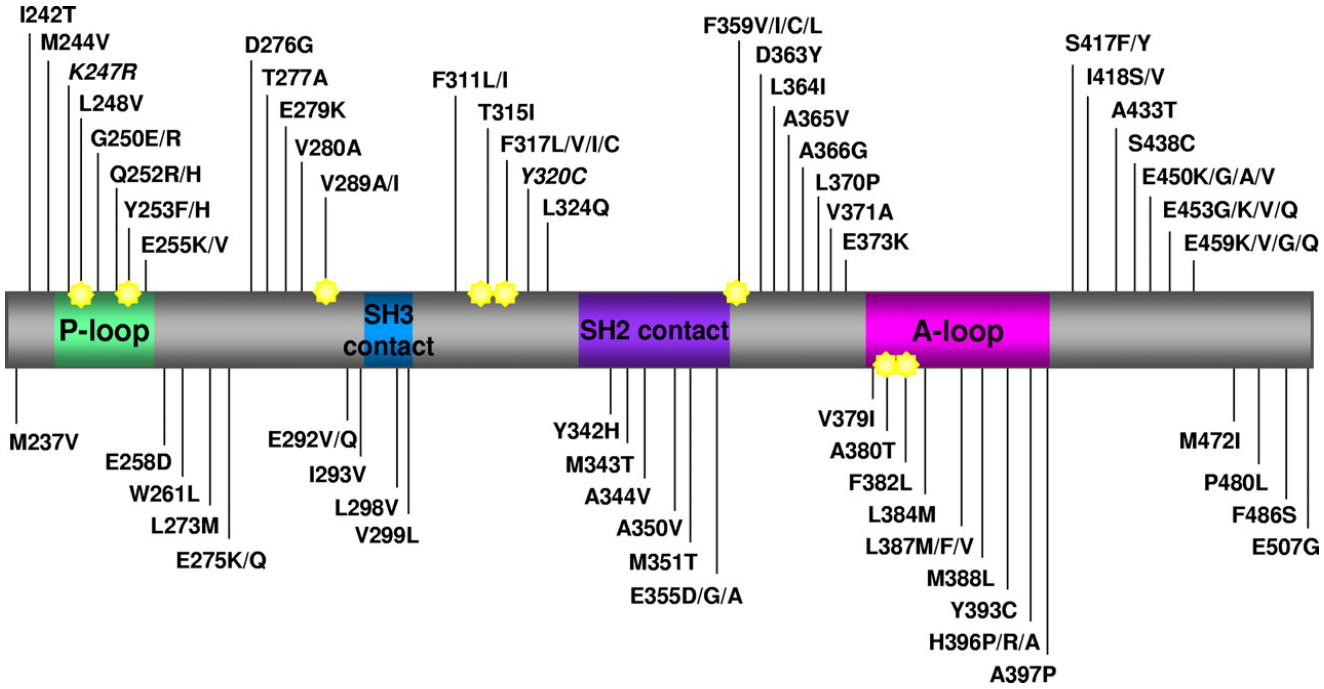


Figure 1.6. Map of *BCR-ABL1* KD mutations that are caused resistant to imatinib. Key structural motifs within *BCR-ABL*1 KD, including P-loop green), SH3 domain (blue), SH2 domain (purple), and A-loop (pink), are indicated. Yellow star indicates amino acid position involved imatinib direct binding. Adapted from Soverni S *et.al*. *Blood* 2011.

Figure 1.7

(a) nilotinib **(b) dasatinib**

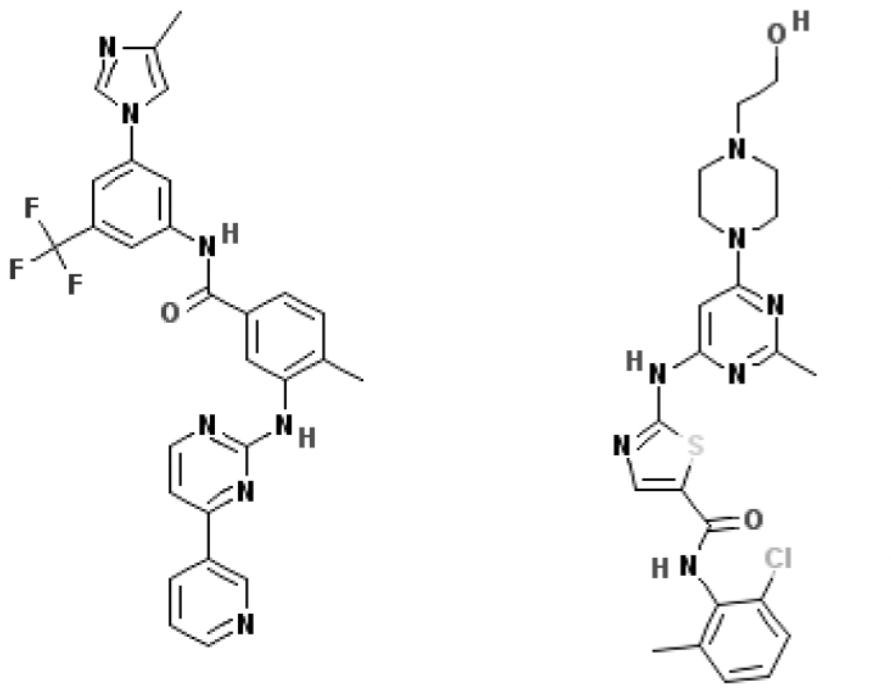


Figure 1.7. Chemical structure of nilotinib. **(a)** Like imatinib, nilotinib also binds and inhibits inactive form of Bcr-Abl. While nilotinib share a similar structure to imatinib, a substitution of the piperazine ring in imatinib with a trifluoromethyl/imidazole phenyl moiety in nilotinib results in greater potency in nilotinib compared to imatinib. **(b)** Different from imatinib and nilotinib, dasatinib binds to active form of Bcr-Abl and has greater potency in targeting Bcr-Abl compared to imatinib. Dasatinib also inhibits other kinase including Src family kinase. Adapted from Zhou T *et.al*. *Chemical biology & drug design*, 2011.

1.4.3 Drug efflux transporter mediated resistance

Since the introduction of chemotherapeutic drugs, approximately 40% of human tumours have developed resistance mechanisms to these drugs (77). Interestingly, some cells simultaneously develop acquired resistance to multiple chemically unrelated drugs. Multidrug resistance is commonly mediated by drug efflux transporters which pump multiple and different chemotherapeutic agents out of the cell, resulting in suboptimal inhibition of the target protein.

ABCB1 (multidrug resistant protein 1 or MDR1) and ABCG2 (breast cancer resistance protein 1 or BCRP1) are the two best characterized efflux pumps associated with TKI resistance. The two transporters belong to the ATP-binding cassette (ABC) superfamily. While ABC transporters are ubiquitously expressed, ABCB1 and ABCG2 are expressed at a high level in hematopoietic primitive cells (78-80). It is known that imatinib, nilotinib and dasatinib are ABCB1 and ABCG2 substrates, and that expression of these efflux pumps can significantly reduce the intracellular level of TKIs. *In vitro* studies have demonstrated that the over-expression of ABCB1 or ABCG2 significantly reduced the sensitivity of CML cell lines to these three TKIs (36, 81-83). CML cell lines resistant to TKIs were shown to overexpress ABCG2 (83, 84). Clinically, increased expression of ABCB1 is associated with sub-optimal response and disease progression in CML patients (85).

1.4.4 Drug influx transporter mediated resistance

Drug influx transporters can also be critical in facilitating resistance. Polyspecific organic cation transporters (OCTs) are the major influx proteins in the solute carrier (SLC) superfamily (86). There are twelve family members of OCTs, and the OCT family members are electrogenic transporters that transport a wide range of organic cations with different sizes and molecular structures, in both directions across the plasma membrane (87).

In humans, OCT-1 is mainly located in hepatocytes to mediate the uptake of the substrate, including small organic cations and a variety of drugs, from blood (87, 88). It was previously reported that OCT-1 is the major active influx pump involved in imatinib uptake, and this transporter is critical in regulating the intracellular levels of imatinib (49, 50, 89). Previous studies by our group indicated that functional inhibition of OCT-1 reduced the uptake of imatinib (49), and OCT-1 functional activity is an indicator of patient's response to imatinib (89-92). Furthermore, most patients with low OCT-1 activity at diagnosis exhibit suboptimal molecular response to imatinib (49, 89, 91, 92). In contrast, recent studies suggested that the influx of second generation TKIs nilotinib and dasatinib, are not mediated by OCT-1. Instead, the uptake of these two TKIs are primarily passive (49, 83).

1.4.5 Activation of alternative signalling pathways

Activation of alternative signalling pathways, or down-regulation of tumour suppressors is frequently associated with drug resistance in cancers. It was recently found that overexpression of Src family kinases, or constitutively active PI3K/AKT pathway, cause resistance to the Bcr-Abl inhibition by TKIs (75, 93-96).

Lyn is a member of the Src family kinases and is expressed mainly in myeloid cells. It plays a key role in mediating the development of resistance (97, 98). It is well known that Bcr-Abl activates Lyn, however in TKI-resistant cells, Lyn may play a more important role. Meyn and colleagues (99) demonstrated that active Lyn phosphorylates Bcr-Abl at the SH3-SH3 domain which stabilizes Bcr-Abl in the active conformation. Lyn activation and overexpression has been observed in imatinib and nilotinib resistant CML cell lines that display a reduction in *BCR-ABL1* expression. Following treatment with a Src inhibitor, this TKI resistance could be abolished (96). This raises the possibility that Lyn (acting upstream of Bcr-Abl) mediates TKI resistance by reactivation of Bcr-Abl signalling (95).

While some have suggested that Lyn interacts with Axl to mediate resistance (100), the interaction between Lyn and Axl remains unclear. Axl is a receptor tyrosine kinase (RTK) that belongs to the Tyro3/Axl/Mer (TAM) family. While the function of this kinase is yet to be determined (and more details of Axl will be described in Chapter 8), patients who are imatinib resistant were shown to have higher

expression of AXL in a study by Dufies M *et al.* (101), where it was also suggested that overexpression of Axl is associated with TKIs resistance (101).

In addition, the PI3K/Akt pathway is deregulated in many cancers including CML (94). PI3K is downstream of Bcr-Abl signalling. While activated PI3K targets Akt, Akt triggers the mTOR complex resulting in cancer cell proliferation and survival. Several studies have been performed to suggest that a mutation in the helical domain of PI3K catalytic subunit that leads to constitutive activation of the PI3K/Akt/mTOR pathway enables TKI resistant cells to survive in a Bcr-Abl independent manner (94).

1.5 Ponatinib

To overcome the suboptimal responses observed in CML patients taking 1st or 2nd generation TKIs, third generation TKIs have been designed. Ponatinib (Figure 1.8) (formerly known as AP24534, Ariad Pharmaceuticals, Cambridge, MA) is a multi-targeted third generation TKI. In addition to Bcr-Abl, ponatinib binds fms-like tyrosine kinase 3 (FLT3), and targets FLT3-ITD mutant, which is found in approximately 30% of acute myeloid leukemia (AML) patients, and thus is potentially useful for treating selected AML patients (102). Moreover, ponatinib exhibits activity against fibroblast growth factors receptors (FGFRs), vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptor α and β (PDGFRα and PDGFRβ) and angiopoietin (Tie2), and thus is

potentially useful for solid tumour therapy (102). Ponatinib was specifically designed on the basis of X-ray crystallographic analysis on the Abl kinase domain to target native and mutant isoforms of Bcr-Abl, including Bcr-Abl^{T315I}. It is unique in targeting T315I among currently available TKIs.

The T315 residue is located in the gatekeeper region of the ATP binding site. In the interaction of Bcr-Abl with first and second generation TKIs, the kinase inhibitors form hydrogen bonds with the side chain of T315 in native Bcr-Abl. Therefore, a threonine to isoleucine mutation eliminates the critical hydrogen bonding interaction between the ATP pocket and imatinib, nilotinib and dasatinib, while an extra hydrocarbon group on the side chain of isoleucine provides steric hindrance against the binding of these TKIs (54). To avoid this hydrogen bond interaction, ponatinib was designed to target the T315I side chain by making a van der Waals interaction via an ethynyl linkage (Figure 1.9). In addition, a trifluoromethylphenyl group of ponatinib makes a favorable bond with the ATP pocket, and a methylphenyl group occupies the pocket behind the gatekeeper residue (103). There are another five hydrogen bonds formed between the Glu255, Phe317, Lys247, Tyr257 and Phe359 residues of the Bcr-Abl KD and ponatinib, further stabilizing the interaction. X-ray crystallographic analysis of ponatinib binding murine Bcr-AblT315I suggests that ponatinib binds to the kinase in its inactive form, and maintains a network of protein interactions similar to imatinib (103).

Figure 1.8

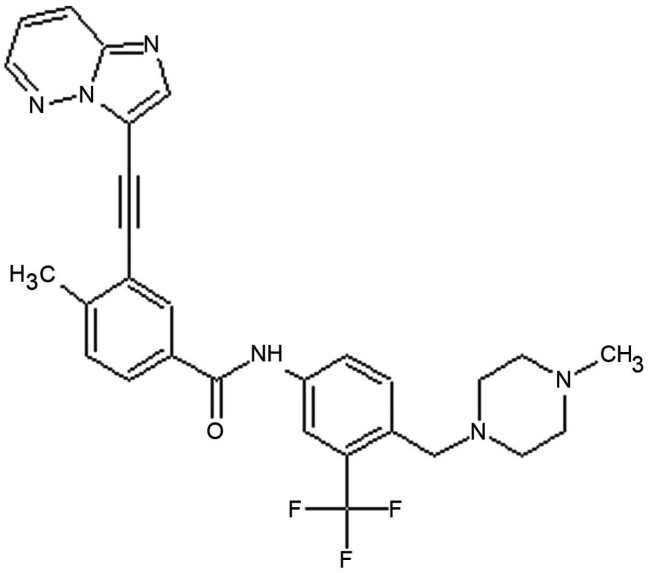


Figure 1.8. Chemical structure of ponatinib. ponatinib binds to inactive form of Bcr-Abl and has similar potency in targeting Bcr-Abl compared to dasatinib. Adapted from Sun YL *et.al*. *Oncology reports*, 2014.

Figure 1.9

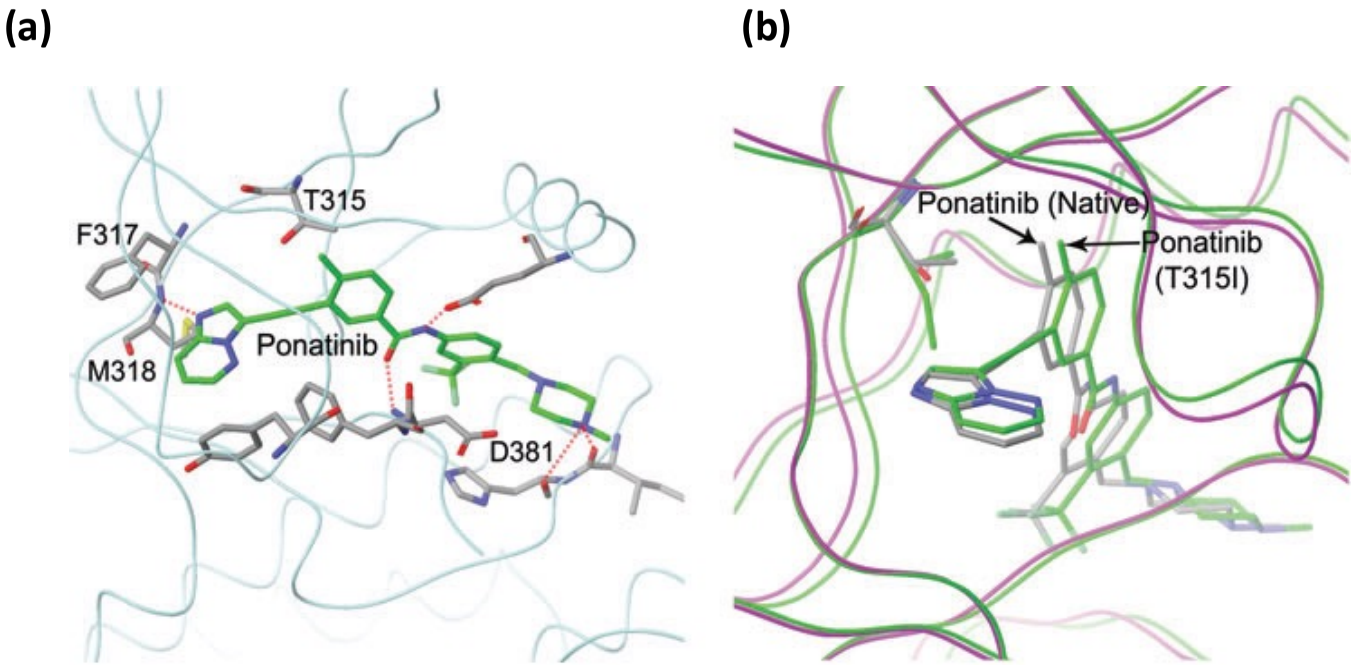


Figure 1.9. Structure of ponatinib binding to Bcr-Abl. (a) Ponatinib binds to native Bcr-Abl. Ponatinib is indicated by green while Bcr-Abl is grey (only showing the protein regions that are critical to the interaction), the red dashed lines indicate hydrogen bond interactions. (b) Ponatinib interacts with native Bcr-Abl (pink) and Bcr-Abl^{T315I} (green). Both T315I mutant and native form of Bcr-Abl form complex with ponatinib (green in ABL^{T315I} and gray in native ABL). Adapted from Savage DG *et.al*. *The New England journal of medicine*, 2002.

A phase I study of oral ponatinib in patients with refractory CML or other hematologic malignancies was commenced in 2010 and demonstrated 66% and 53% of patients with CP-CML achieved MCyR and CCyR respectively (104). Moreover, 100% of patients with Bcr-Abl^{T315I} achieved complete hematologic response, and 82% of them achieved MCyR. In the phase II trial, 56% of CP-CML patients (including 51% of these responded patients were resistant to nilotinib or dasatinib, and 70% of these patients harboured T315I mutation), and 47% Ph+ ALL patients achieved MCyR after at least 12 months treatment with ponatinib (105). No single mutation has been found to cause resistance to ponatinib in this trial. To date, ponatinib is promising for CML patients and Ph+ ALL patients who fail 1st and 2nd generation TKI therapy, especially if the patient habours the T315I mutation. In fact, ponatinib is the only FDA-approved TKI effective against the T315I mutation.

However, even given its success in the treatment of refractory CML, ponatinib was temporarily taken off market in late 2013 due to serious side effects. Therefore, following this decision, the Phase III trial, the Evaluation of Ponatinib versus Imatinib in Chronic Myeloid Leukemia (EPIC) was halted. Serious cardiovascular, cerebrovascular and peripheral vascular events were noticed in 7.1%, 3.6% and 4.9% of the ponatinib treated patients (106) respectively, and serious arterial thrombotic events were evidenced in 8.9%. This side effect of ponatinib was thought to be due to its pan activity. In addition, 45 mg/day dosing may be high for some patients as higher dose of ponatinib is more likely to target other kinases (e.g. VEGFRs), which might increase vascular risk. A recent study performed on 671

ponatinib patients indicated ponatinib dosing level associates with side effects including vascular occlusion adverse events, and therefore, a dose ranging trial of ponatinib is planned (107). Ponatinib is now available again but only for treatment of adults with CML or Ph+ ALL with T315I mutation, and adult patients for which no other TKI therapy is indicated. In addition, the side effects on ponatinib patients are being closely monitored during ponatinib treatment.

1.5.1 Ponatinib demonstrates efficacy against single KD mutations

Recently, based on the biochemical structure of ponatinib, Zhou et al (108) predicted the inhibitory potency of ponatinib against Bcr-Abl^{T315I} to be about 5-7 fold lower than against Bcr-Abl^{WT}. However, experiments by O'Hare et al (103) suggested that only ponatinib, not imatinib, dasatinib or nilotinib effectively targets Ba/F3 cells expressing Bcr-Abl^{T315I} within clinically relevant concentrations. O'Hare et al (103) also conducted a study suggesting a broad spectrum of Bcr-Abl KD mutations (e.g. Q252H, Y253F, H396P and T315I) are sensitive to ponatinib. In addition, they employed a mouse model to demonstrate that treatment with oral ponatinib resulted in prolonged survival in mice transplanted with Bcr-Abl^{WT} or Bcr-Abl^{T315I} (103). These effects were evident in a ponatinib dose dependent manner.

1.5.2 Ponatinib in targeting compound mutation

While a single KD mutation is the most common imatinib resistant mechanism, multiple mutations in *BCR-ABL1* within the same clone, known as "compound mutation", can occur and were found to confer multiple TKI resistance (109). Although only a minority of Ph+ leukaemia patients habour compound mutations, Zabriskie and colleagues (109) demonstrated that patients with 12 different compound mutations, including T315I inclusive compound mutations, are highly resistant to ponatinib and all other available TKIs. The effect of compound mutations will be more fully described/ explored in Chapter 5.

1.5.3 Influx and efflux transporters of ponatinib

While extensive modeling experiments have been performed, the interactions of ponatinib with drug transporters that impact the efficacy of other TKIs have been poorly explored. Recently, Sen and colleagues (110) tested the *in vitro* interactions between ponatinib and the ABC transporters ABCB1, ABCG2 and ABCC1, and suggested that ponatinib is a potent ABCG2 inhibitor. However, the nature of the interaction of ponatinib with ABCB1 and ABCG2 is unclear, and little is also known about ponatinib influx transport. Hence, the role of these transporters in ponatinib resistance is a main focus in this study.

1.6 Hypothesis and aims

1.6.1 Hypothesis

We hypothesize that ponatinib resistance may arise through the development of "novel" Bcr-Abl kinase domain mutations and other Bcr-Abl dependent and independent mechanisms.

1.6.2 Aims

Aim 1: To investigate the influx and efflux pumps which are responsible for transporting ponatinib.

Aim 2: To generate ponatinib resistant Bcr-Abl positive leukaemic cell lines.

Aim 3: To characterize the common resistance mechanisms for ponatinib in ponatinib resistant cell line models.

Chapter 2 :

Methods and materials

2.1 Commonly used reagents and buffer

Reagent	Supplier	Catalogue number
7-Aminoactinomycin D (7-AAD)	Life Technologies	A1310
Acetic Acid	Fisher Scientific	0400
Agarose Type I, Low	Sigma-Aldrich	A6013-250G
Ammonium Persulfate (APS)	Sigma-Aldrich	A9164
Annexin V-PE	BD Biosciences	556421
Aprotinin (from bovine lung)	Sigma-Aldrich	A1153-5MG
Axl-PE Antibody	R&D	FAB154P
β-glycerophosphate	Sigma-Aldrich	G9891
β-mercaptoethanol	Sigma-Aldrich	M6250
Benchmark Protein Ladder	Life Technologies Invitrogen	10748-010
Bis-Acrylamide solution 37.5:1 (40% Acrylamide)	Bio-Rad	161-0149
Bovine Serum Albumin (BSA)	Sigma-Aldrich	49418
Bromophenol Blue	Sigma-Aldrich	114391
Calcium Chloride (CaCl2)	Sigma-Aldrich	449709
CD243-PE (ABCB1) Antibody	Beckman Coulter	PN IM 2370U
CD44-PE Antibody	BD Biosciences	561858
Chloroform	Merck	100776B
Complete protease inhibitor cocktail, mini, EDTA-free	Roche	04693159001
Cyclosporin A	RAH Pharmacy	
Dc Protein Assay	Bio-Rad	500-0112

DEPC H_2Owater	MP Biomedicals Inc.	821739
D-Glucose	Sigma-Aldrich	G7528
Dimethyl Sulphoxide (DMSO)	Merck	K39661852
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich	D6046
dNTP Set (N=A, C, G, T)	GE Healthcare	27-2035-02
DTT 0.1 M	Invitrogen Life Technologies	18064-014
ECF Substrate	GE Healthcare	RPN 5785
Ethylenediaminetetraacetic Acid (EDTA)	APS	180-500G
Ethanol	Merck	4.10230.2511
EXO SAP-IT	Affymetrix	78201
Foetal Calf Serum (FCS)	JRH Biosciences	12003-500M
Formaldehyde, 40% w/v	BDH	10113
G-418, Geneticin	Life Technologies Invitrogen	10131-027
Glycerol	Ajax Finechem	242
Glycine	Sigma-Aldrich	G8898
Glycogen	Roche	901393
Hanks Balanced Salt Solution (HBSS)	Sigma-Aldrich	H9394
hBCRP-PE (ABCG2) Antibody	R&D Systems	FAB995P
HEPES 1 M	Sigma-Aldrich	H0887
Hydrochloric Acid (HCl, 35%)	Fisher Scientific	1100
Indomethacin (MW=357.79)	Sigma-Aldrich	I7378
Isopropanol	Ajax Finechem	425-2.5L PL
Kaleidoscope Prestained Protein Standards	Bio-Rrad	161-0375
Leupeptin	Sigma-Aldrich	L2884
L-glutamine 200 mM	SAFC Biosciences	SAFC 59202C
Lymphoprep	Axis Shield	1114547

Methanol	Chem Supply	MA004-P
Methyl Violet	BDH	34033
MICROSCINT-20 Scintillation Fluid	Perkin Elmer	6013621
Mouse IgG Antibody (AP-conjugated)	Cell Signalling	7056
Mouse IgG1-PE Antibody	R&D Systems	IC002P
Mouse IgG2a-PE Antibody	DakoCytomation	X095001
Mouse IgG2b-PE Antibody	DakoCytomation	X095101
Odyssey Blocking Buffer	Li-Cor	LCR92740010
Pantoprazole (MW=383.4)	RAH Pharmacy	
Paraformaldehyde (16%)	ProSciTech	15710
Penicillin 5000 Units/ml Streptomycin Sulfate 5000 µg/ml	Sigma-Aldrich	P4458
Phosphate Buffered Saline (PBS)	SAFC Biosciences	59331C
PMSF(Phenylmethylsulfonyl Fluroide)	Sigma-Aldrich	P7626
Prazosin Hydrochloride (MW=419.9)	Sigma-Aldrich	P7791
PSC-833 (MW=1214.7)	Novartis	
PVDF (Western Blot Membrane)	GE Healthcare	PRN 303F
PVDF membrane, Low-fluorescence, in Trans-Blot® Turbo™ RTA Midi Transfer Pack kit	Bio-Rad	170-4275
Rabbit IgG Antibody (AP-conjugated)	Santa Cruz	SC2007
Random Hexamer Primer	Geneworks	RP-6
Rhodamine-123	Life Technologies Invitrogen	R-302
RPMI-1640 Medium w/o L-glutamine	Sigma-Aldrich	R0883
SDS (Sodium Dodecyl Sulphate)	Sigma-Aldrich	L4509
Sodium Azide (NaN3)	Sigma-Aldrich	S2002
Sodium Chloride (NaCl)	Ajax Finechem	1128

Sodium Fluoride (NaF)	Sigma-Aldrich	S7920
Sodium (ortho) Vanadate (Na3VO4)	Sigma-Aldrich	S6508
Superscript II Reverse Transcriptase	Life Technologies Invitrogen	18064-014
SYBR Green Supermix	SA Biosciences	PA-012-24
Taqman Master Mix	Applied Biosystems	4318157
Tetramethyethylenediamine (TEMED)TEMED	Sigma-Aldrich	T7024
Tris (hydroxymethyl) Aminomethane	Merck	1083870500
TRIzol Reagent	Life Technologies Invitrogen	15596-018
Trypan Blue Solution (0.4%)	Sigma-Aldrich	T8154
Tween®20	Sigma-Aldrich	P9416
Western Blot Recycling Kit	Alpha Diagnostic	90100

2.2 Western blot antibodies

Reagent	Used dilutions	Supplier	Catalogue number
β-tubulin Antibody (55 kDa)	1:1000	Cell Signalling	2146
c-Abl Antibody (Bcr-Abl 210 kDa)	1:1000	Cell Signalling	2862
Crkl Antibody (C20, Crkl 36 kDa; p-Crkl 39 kDa)	1:1000	Santa Cruz	SC-319
GAPDH Antibody (37 kDa)	1:1000	Cell sSignalling	2118
Mouse IgG Antibody (AP-conjugated)	1:2000	Cell Signalling	7056
p-cAbl Antibody (pY245; 210 kDa)	1:1000	Cell Signalling	2861
Rabbit IgG Antibody (AP-conjugated)	1:2000	Santa Cruz	SC2007

2.3 Solutions, buffers and media

2.3.1 Cell culture media

90% RPMI-1640 media

2 mM L-Glutamine

50 Units/mL Penicillin

50 µg/mL Streptomycin sulphate

10% Foetal Calf Serum (FCS)

Media was stored at 4 °C and heated in a 37 °C water bath before use.

2.3.2 IMDM cell culture media

90% IMDM media

2 mM L-Glutamine

50 Units/mL Penicillin

50 µg/mL Streptomycin sulphate

2.3.3 10% Foetal Calf Serum (FCS)

Media was stored at 4 °C and heated in a 37 °C water bath before use.

Viability assay staining buffer

5 mM $CaCl_2$ in HBSS with 10 mM HEPES

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

25 mM: 40 µL of each dNTP

5 mM: 1:5 dilution of 25 mM stock in 80 µL DEPC water

2.3.5 Flow cytometry fixative (FACS fix)

1xPBS

1% Formaldehyde

111 mM D-glucose

0.02% sodium azide

2.3.6 Flow Cytometry wash (FACS wash)

94.8% 1xPBS

5% FCS

0.2% sodium azide

2.3.7 Freeze mix (cryoprotectant)

70% RPMI-1640

20% FCS

10% DMSO

Prepared fresh and pre-chilled on ice prior to use.

2.3.8 SDS-Polyacrylamide Gel

For Hoefer Ruby SE600 Electrophoresis Tank

	Resolving gel (12%)	Stacking gel (5%)
water	12.9 mL	6 mL
40% Acrylamide/Bis solution, 37.5:1 ratio	9 mL	1.26 mL
1.5 M Tris Buffer A, pH 8.8	7.5 mL	
1.5 M Tris Buffer B, pH 6.8		2.52 mL
10% SDS	300 µL	100 µL
10% APS	300 µL	100 µL
TEMED	8 µL	10 µL

For Hoefer SE260 Electrophoresis Tank

	Resolving gel (14%)	Stacking gel (5%)
water	3.8 mL	3 mL
40% Acrylamide/Bis solution, 37.5:1 ratio	3.5 mL	630 µL
1.5 M Tris Buffer A, pH 8.8	2.5 mL	
1.5 M Tris Buffer B, pH 6.8		1.26 mL
10% SDS	100 µL	50 µL
10% APS	100 µL	50 µL
TEMED	6 µL	5 µL

2.3.9 1×SDS-PAGE running buffer (pH 8.3)

25 mM Tris-HCl

192 mM Glycine

0.1% SDS

Prepared in RO water

2.3.10 1×Tris Buffered Saline (TBS)

20 mM Tris-HCL (pH 7.5)

150 mM NaCl

Prepared in RO water

2.3.11 1×Tris Buffered Saline with 0.1% Tween 20 (TBST)

20 mM Tris-HCL (pH 7.5)

150 mM NaCl

0.1% Tween.20

Prepared in RO water

2.3.12 1×SDS-PAGE Transfer buffer (pH 8.3)

25 mM Tris-HCl

192 mM Glycine

20% Methanol

Prepared in RO water

2.3.13 1× Laemmli's Buffer (modified)

50 mM Tris-HCl at pH 6.8

10% glycerol

2% SDS

5% β -mercaptoethanol

0.1% bromophenol blue

1 mM NaVanadate

10 mM NaFluoride

2.3.14 2.5% skim milk Western blocking solution

1×TBST

2.5% Non-fat milk powder

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

2.3.15 2.5% BSA Western blocking solution

1×TBST

2.5% BSA powder

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

2.3.16 Random Hexamer Primer

Stock: 500 µg/mL

Working stock: 1 in 2 dilution of the stock with DEPC water

2.3.17 Tris Buffer A (pH 8.8)

1.5 M Tris

Prepared in Milli-Q water and pH adjusted to 8.8 with HCl

2.3.18 Tris Buffer B (pH 6.8)

0.5 M Tris

Prepared in Milli-Q water and pH adjusted to 6.8 with HCl

2.4 Tyrosine kinase inhibitors

2.4.1 imatinib (MW = 589.72)

Imatinib (Symansis, Shanghai, China) was dissolved in sterile milliQ water at 10 mM, sterile filtered and stored at -70^{0}C. Serial dilutions were made water immediately prior to use.

2.4.2 nilotinib (MW = 529.5)

Nilotinib (Symansis, Shanghai, China) was dissolved in DMSO at 10 mM and stored at 4^{0}C. Serial dilutions were made in DMSO immediately prior to use.

2.4.3 dasatinib (MW = 506.2)

Dasatinib (Symansis, Shanghai, China) was dissolved in DMSO at 10 mM and stored at 4^{0}C. Serial dilutions were made in DMSO immediately prior to use.

2.4.4 Ponatinib (MW = 532.56)

Ponatinib (provided by Ariad, Cambridge, USA) was dissolved in DMSO at 10 mM and stored at 4^0C. Serial dilutions were made in DMSO immediately prior to use. 14C-ponatinib (specific activity = 19.8μCi/mL=0.72 MBq/mL) (Ariad) was dissolved in a 1:1 ratio of methanol and water at 1mg/ml (1757.4 μM) and stored at -20^0C.

2.5 Transporter inhibitors

2.5.1 Prazosin Hydrochloride

Prazosin hydrochloride (Sigma) was used at 100 μM from a 10 mM stock prepared in methanol.

2.5.2 Cyclosporin A

Cyclosporin A (Royal Adelaide Hospital Pharmacy) was used at 5 μM from a 4.15 mM stock that was diluted in 5% aqueous glucose solution (Sigma).

2.5.3 Ko143

Ko143 was kindly provided by Dr John Allen, Centenary Institute, Sydney, Australia and was used at 0.5 μM. Stocks were 10 mM in DMSO and working dilutions were 1 mM in sterile water.

2.5.4 Pantoprazole

Pantoprazole (Royal Adelaide Hospital Pharmacy) was used at 200 μM from a 10 mM stock. To make 10 mM stock, 40 mg of pantoprazole was dissolved in 10 mL 0.9% NaCl.

2.5.5 Ibuprofen

Ibuprofen (Sigma) was used at 145 μM from a 100 mM stock by dissolved in ethanol.

2.5.6 Procainamide

Procainamide (Sigma) was used at 10 mM from a 1 M stock dissolved in sterile water.

2.6 Axl inhibitors

2.6.1 R428

R428 (Selleckchem, Houston, USA) was dissolved in DMSO at 10 mM and stored at -80^{0}C. Serial dilutions were made in DMSO immediately prior to use, and was used at 1-1.25 μM working stock.

2.6.2 BMS-777607

BMS-777607 (Selleckchem, Houston, USA) was dissolved in DMSO at 10 mM and stored at -80^{0}C. Serial dilutions were made in DMSO immediately prior to use, and was used at 12.5 μM working stock.

2.7 Cell lines

The human *BCR-ABL1* negative cell line HL60 and *BCR-ABL1* positive CML blast crisis cell lines K562 and KU812, expressing native 210kD Bcr-Abl (Bcr-Abl^{p210}), were purchased from The American Type Tissue Culture Collection (ATCC, Manassas, USA). The ABCB1 overexpressing K562 variant, K562-DOX (generated by continuous exposure to doxorubicin) was kindly provided by Prof. Leonie Ashman. The ABCG2 overexpressing K562 variant, K562-ABCG2 (83) was kindly provided by Dr. Devendra Hiwase. A panel of dasatinib resistant cell lines including K562 T315I (was named as K562 200nM DAS in the original paper) and K562 DOX 55D previously generated and described by Tang *et al.* (51), were used in this study.

2.7.1 Culture of TKI-naïve and TKI-resistant cell lines

Tissue culture was performed aseptically in a Class II biohazard safety cabinet (Gelman Sciences). The cells were cultured in a 37°C /5% CO_2 incubator (Sanyo) in 25cm^2, 75cm^2 or 175cm^2 tissue culture flasks (Corning) with cell densities maintained at between 1x10^5–1x10^6 cells/mL. Cell cultures were checked every second day for contamination, counted and sub-cultured at the above concentrations. Media was pre-warmed to 37°C prior to use.

To generate ponatinib resistant cell lines, K562, K562-DOX, KU812, K562-DOX 55D and K562 T315I cell lines (as table shown below) were cultured with increasing concentrations of ponatinib by using the same method as previous studies (51, 81) (please see approach section in Chapter 4,5 and 6 for details). Parental, serially passaged TKI-naïve K562, K562-DOX, K562-DOX 55D and K562 T315I cell lines and 0.1% Di-methyl Sulfoxide (DMSO; Merck, Darmstadt, Germany) vehicle controls were maintained in parallel to the ponatinib resistant cell lines. Prior to ponatinib dose escalation, cells were harvested as follows and stored at -80°C :

3 aliquots of $2x10^6$ cells stabilized in TRIzol reagent for RNA preparations.

3 aliquots of $2x10^6$ cells were pelleted and frozen for DNA preparations.

4 aliquots of $1x10^7$ viable cells were cryopreserved and stored in liquid nitrogen.

Cell line	*BCR-ABL1* +	TKI treated	ABCB1 overexpression	Mutation	*BCR-ABL1* overexpression
K562	Yes	No	No	No	No
K562 DOX	Yes	No	Yes	No	No
KU812	Yes	No	No	No	No
K562 T315I (K562 200 nM DAS) (51)	Yes	200 nM dasatinib	No	T315I	Yes
K562 DOX 55D (K562 DOX RE55D) (51)	Yes	55 nM dasatinib	Yes	No	Yes

2.8 General and specialised techniques

2.8.1 Cell counts and viability assessment

To calculate the cell culture density (in cells per mL) a 10 µl aliquot of the culture was mixed with 10 µl Trypan Blue solution (0.4%, Sigma-Aldrich). 10 µl of this mixture was loaded onto a haemocytometer counting chamber (Neubauer Improved, Assistant, Germany) and under light microscopy live cells and dead (stained) cells were counted to calculate cell density and viability.

2.8.2 Cryopreservation of cells

Under aseptic conditions, $1x10^7$ cells were pelleted at 306 x g for 10 mins and then resuspended in 1 mL of pre-chilled Freeze Mix and transferred to cryo-ampoules (Nalgene). The cryo-ampoules were placed in an isopropanol-filled Mr. Frosty container (Nalgene) for at least 4 hours at -80 °C before transfer to liquid nitrogen (-196 °C) for long-term cryo-storage.

2.8.3 Thawing cells

Once cryo-ampoules were removed from liquid nitrogen, cells were rapidly thawed by immersing the ampoule in a 37 °C in water bath with manual agitation. Cells were immediately transferred to a 50 mL tube and cell culture media, pre-warmed to 37 °C, was added drop-wise to 10 mL with constant gentle mixing. Cells were pelleted by centrifugation at 306 x g for 10 minutes followed by removal of the media. Another 10 mL of cell culture media was than added dropwise to resuspend the cell

pellet before centrifugation again to remove all residual DMSO. Cells were then counted.

2.8.4 Viability assay

$2x10^5$ cells were spin down to remove supernatant for 3 times to remove any drugs present in the culture medium before re-culturing in 1 mL fresh culture media (with testing drug) in a 24-well plate in 37°C/5% CO_2 incubator for 72 hours. Duplicates of each condition were performed to ensure the accuracy of the viability assay. After 72 hours, cells were mixed thoroughly by pipetting and then 250 µL were transferred to a 96-well plate. Cells were washed (319 x g for 5 mins) by addition of 200 µL of binding buffer followed by staining with 0.04 µL 7-Amino-actinomycin D (7AAD) and 0.4 µL Annexin V-PE per sample for 20 minutes in the dark. After incubation, 200 µL binding buffer was added and the cells resuspended by pipetting. Flow cytometry (BD LSRFortessaTM X-20 with high-throughput platform) was performed to observe cell death. Cells stained positive for Annexin V-PE and 7AAD were considered to be apoptotic or dead respectively; the percentage of unstained cells was recorded as % viable cells. The data were analysed using FCS Express software (DeNovo Software, Los Angeles, California, USA).

2.8.5 Ponatinib Intracellular Uptake and Retention assay (IUR)

2 x 10^5 cells were incubated with 0, 0.5, 1 and 2 µM 14C-ponatinib for 2 hours at 37ºC or 4ºC in 200 µL of RPMI-1640 medium containing 10% FCS in a 96-well

Optiplate (Perkin Elmer, Boston, MA). Following incubation, organic and aqueous phases were resolved by centrifugation 417 x g for 5 minutes. 20 µl aliquot of supernatant (S/N) from the top of the media in each well was added to 100 µl Microscint-20 in a second Optiplate followed by repeated centrifugation for 5 minutes at 417 x g. Next, supernatants were by aspiration and 100 µl of Microscint-20 was added to the cells. Adhesive plastic cover-seal was applied to the plate before vortexing the plate at < 600 rpm for 15 seconds. Incorporation of 14C-ponatinib was determined using a TopCount Microplate Beta Scintillation counter (Perkin Elmer). The IUR was reported as ng ponatinib/200,000 cells.

2.8.6 Antibody staining for Flow Cytometric Analysis

Antibody	Antibody amount	Number of cells
IgG2b-PE	5 µL	5×10^5
ABCG2-PE	10 µL	5×10^5
IgG2a-PE	5 µL	5×10^5
ABCB1-PE	10 µL	5×10^5
IgG1-PE	5 µL	5×10^5
Axl-PE	15 µL	5×10^5

2.8.7 RNA extraction

200 µl chloroform was added to thawed TRIzol preparations and mixed by shaking vigorously. Samples were then incubated on ice for 3 min before centrifugation at 15871 x g for 15 minutes. Approximately 500 µl of the clear top layers were then

pipetteed into new RNase- and DNase-free tubes. RNA was precipitated by adding 500 µl isopropanol and 1 µl glycogen followed by gentle mixing and incubated on ice for 10 minutes. Next, samples were centrifuged at 15871 x g for 10 minutes and supernatants were removed. The RNA pellets were washed by addition of 1 mL of 75% ethanol. After centrifugation ethanol was removed and the RNA pellets were air dried. The RNA pellets were dissolved in 30 µl of DEPC water by incubating at 55°C for 15 minutes. The concentration of RNA was measured by NanoDrop Spectrophotometer (Thermo Scientific) and adjusted to a stock concentration of 0.25 µg/µl and stored at -80°C.

2.8.8 cDNA synthesis

1 µg of RNA was added to 250 ng of random hexamers (Geneworks) and enough DEPC water to make the total volume 11 µL in each sample. The mixture was heated to 70°C for 10 min and chilled for 5 min at 4°C. Next, 4 µL of 5x First strand buffer; 2 µL of 0.1 M DTT; 2 µL of 5 mM dNTPs and 1 µL of Superscript II were added to the previous mixture, and incubated in a Mastercycler (Eppendorf) with the conditions as follows: 25°C for 10 min, 42°C for 50 min, 70 °C for 10 min, 4°C for 5 min. cDNA was diluted 1:1 with DEPC water and stored at -20 °C.

2.8.9 RQ-PCR:

BCR-ABL1 transcript quantitation

BCR-ABL1 quantification was performed as previously described (51, 111). Firstly, cDNA was synthesized and was used as a template in a quantitative PCR (RT-QPCR) reaction. All samples expressed the B3A2 transcript. Master mixes were prepared as follows and 2.5 µL of cDNA samples were added:

Master mix of BCR	For each sample
TaqMan Universal PCR Master Mix	12.5 µL
DEPC water	9.55 µL
50 µM of BCR forward primer	0.1 µL
50 µM of BCR reverse primer	0.1 µL
BCR probe	0.25 µL

Master mix of B3A2	For each sample
TaqMan Universal PCR Master Mix	12.5 µL
DEPC water	9.55 µL
50 µM of B3A2 forward primer	0.1 µL
50 µM of B3A2 reverse primer	0.1 µL
B3A2 probe	0.25 µL

BCR Forward primer: 5’ CCT TCG ACG TCA ATA ACA AGG AT 3’

BCR Reverse primer: 5’ CCT GCG ATG GCG TTC AC 3’

TaqMan BCR probe: 5’ TCC ATC TCG CTC ATC ATC ACC GAC A 3’

b3a2 Forward primer: 5’ GGG CTC TAT GGG TTT CTG AAT G 3’

b3a2 Reverse primer: 5’ CGC TGA AGG GCT TTT GAA CT 3’

TaqMan b3a2 probe: 5' CAT CGT CCA CTC AGC CAC TGG ATT TAA GC 3'

All of the obove primers have

5' modification = 6-FAM

3' modification = TAMRA

The quantitative PCR was performed using 7500 system software (Applied Biosystems International) with conditions: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 sec and 60 °C for 1 min. *BCR-ABL1* transcript numbers were determined as *BCR-ABL1* /BCR expressed as a percentage (*BCR-ABL1* %).

2.8.10 AXL transcript quantitation

AXL transcript levels were measured by RQ-PCR. RANKL was used as positive control and GUSB was used as control gene and the expression level of AXL was calculated as a ratio of GUSB transcript levels.

The primers used for AXL amplification were as follows:

AXL Forward primer: 5' TGC ATG AAG GAA TTT GAC CA 3'

AXL Reverse primer: 5' TCG TTC AGA ACC CTG GAA AC 3'

The primers used for GUSB amplification were as follows:

GUSB Forward primer: 5' GAA AAA ATG AGG ACG GGT ACG T 3'

GUSB Reverse primer: 5' ATT TTG CCG ATT TCA TGA CTG A 3'

The primers used for RANKL amplification were as follows:

RANKL Forward primer: 5' TCA GCC TTT TGC TCA TCT CAC TAT 3'

RANKL Reverse primer: 5' CCA CCC CCG ATC ATG GT 3'

8μL master mixes were made for each primer set using the SYBR Green ROX mix (Qiagen, Venlo, Netherlands) as follows, then mixed with 2μL of cDNA. Samples were amplified in triplicate in the Rotor-Gene 3000 PCR cycler machine (Corbett Research, Cambridgeshire, UK) with the following conditions: 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 seconds and 60 °C for 1 min.

Reagent	For 1 sample
SYBR Green ROX mix	5 μL
Forward primer	0.5 μL (100 ng/ μL)
Reverse primer	0.5 μL (100 ng/ μL)
DEPC water	2 μL

2.8.11 Sequencing the *BCR-ABL1* kinase domain

2.8.11.1 Long PCR

Sequencing was performed to investigate mutations in *BCR-ABL1* kinase domains (KD). The KD of BCR-ABL (1.8 kb) was amplified using the Expand Long Template PCR System (Roche, Australia). 23 μL of master mix was prepared as follows with addition of 2 μL cDNA :

Reagent	For 1 sample
25 mM dNTP	0.75 µL
10x Buffer 3	2.5 µL
25 mM MgCl	0.75 µL
Forward primer	0.15 µL
Reverse primer	0.15 µL
Expand Enzyme Mix	0.375 µL
DEPC water	18.325 µL

Forward primer "LongF0810": 5'-ACTATGAGCGTGCAGAGTGGA-3'

Reverse primer "Long R0510": 5'-GAGGGAGCAATGGAGACACG-3'

The cycler conditions of the Long PCR were as follows:

94°C 2min

94°C 10sec, 60°C 30sec, 68°C 2min: 10 cycles

94°C 10sec, 60°C 30 sec, 68°C 2min – increase by 20 sec every cycle: 20 cycles

68°C 7min

4°C for 5 min and then stored at 4°C

2.8.11.2 2% Agarose Gel

Amplified *BCR-ABL1* transcripts (5 µL) were mixed with 2x Loading Dye (Gene Works) and electrophoretically separated on a 2% agarose-TAE gel with 0.1% GelRed (Biotium) then visualised under UV light on a Gel Doc Imaging System (Bio-Rad) to confirm products of 1.8 kb.

2.8.11.3 ExoSAP-IT purification

Long PCR products were purified using ExoSAP-IT (GE Healthcare) in the ratio of 5:2 (in 20 μL post PCR reaction product, added 8 μL ExoSAP-IT). The cycler conditions for purification were: 37°C for 15 minutes, 80°C for 15 minutes and hold at 4°C.

2.8.11.4 Sequencing reaction

The sequencing reactions were performed in both forward and reverse directions by SA Pathology Flinders Sequencing Facility by using forward primer: 5'-CGCAACAAGCCCACTGTCT-3'; and reverse primer: 5'CAAGGTACTCACAGCCCCACGGA-3'. The resulting chromatograms were analyzed using Mutation Surveyor software (SoftGenetics).

2.8.12 SDS-PAGE and Western blot

IC50 assay, as determined by CrkL phosphorylation status

2 x 10^5 cells were incubated in the presence of ponatinib (0-5000 nM) at 37oC (5% CO2) for 2 hours. After incubation, cells were washed in cold 1x PBS and lysed in 1xLaemmli's buffer (10 μL per 1 x 10^5 cells). Proteins were resolved using sodium dodecyl sulfate polyacrylamide (12% or 14%) gel electrophoresis (SDS-PAGE) and electropheretically transferred to PVDF membrane (GE Healthcare) at 65 mA overnight. Next, the PVDF membrane was incubated at room temperature for 1 h with 2.5% skim milk powder in 1xTBST, then probed for 2 hours at room

temperature with 1:1000 anti-Crkl antibody (Santa Cruz) in the same solution. After 2 hours, the membrane was washed 3 times with 1xTBST buffer for 5 minutes before incubation with 1:2000 secondary antibody (alkaline-phosphatase conjugated anti-rabbit immunoglobulin antibody) (SantaCruz) in 2.5% skim milk solution for 1 hour. Next, the membrane was washed 3 times with 1xTBST buffer for 5 minutes and 3 times with 1xTBS for 5 minutes. Bound antibodies were detected with ECF substrate (GE Healthcare) using Image Quant software and a Typhoon Fluorimager (both from Molecular Dynamics). The percentage of phosphorylated CrkL (p-CrkL) to total CrkL was determined by densitometric analysis, and the concentration of ponatinib required to reduce p-CrkL by 50% was recorded as the IC50ponatinib value.

2.8.12.1 Western blotting for proteins other than CrkL

Expression of proteins including Axl, p-Axl, c-Abl, p-c-Abl(Y245), p-Bcr(Y177), and controls β-tubulin and GAPDH, were assessed. Firstly, cell lysates prepared in NP40 Lysis Buffer (40 µg- 150 µg of total protein loaded) or 1xLaemmli's Buffer were resolved on Bio-Rad 4-15% Criterion™TGX™ Precast Gels. After electrophoretic separation, the proteins were transferred to PVDF membranes using the Bio-Rad TransBlot Turbo™ Blotting System according to the manufacturer's instructions for either 10 min (for high molecular weight proteins >100 kDa) or 7 minutes (for lower molecular weight proteins >50 kDa but <100 kDa). The PDVF membranes were incubated in blocking solution (1xTBST with 2.5% BSA) for 1 hour. Next the membranes were incubated with 1:1000 primary antibody overnight at 4°C. The

membranes were then washed 3 times 5 mins with 1xTBST buffer and probed with either alkaline-phosphatase-conjugated secondary antibody 1:2000 in BSA solution or Infrared-dye labeled secondary antibody 1:10000 in Odyssey Blocking Buffer for 1 hour at room temperature. Following incubation, the membranes were washed 3 times in 1xTBST and 3 times in 1xTBS for 5 minutes each. Proteins were detected by ChemiDoc MP imager (Bio-Rad) after incubation with ECF substrate, or by Odyssey CLx Infrared scanner (Li-Cor, Lincoln, USA). For Bcr-Abl IC50 determination (Section 6.3.6), membranes were initially probed for p-Bcr-Abl and then stripped with 1x Western Stripping Buffer (Alpha Diagnostics). Detected proteins were quantified using ImageLab software (Bio-Rad) and expression normalised to control.

2.8.12.2 Protein extraction using NP40 lysis buffer

Firstly, the following NP-40 basic stock was prepared:

	Stock concentration	Volume for 1mL
10 mM Tris-HCl pH 7.4	1 M	10 µL
137 mM NaCl	1 M	137 µL
10% Glycerol	100%	100 µL
1% NP-40 (Igepal™)	10 %	100 µL
Sterile MilliQ water		465 µL

Then the following phosphatase- and protease-inhibitors were freshly added to the NP-40 basic stock and the resultant lysis buffer kept on ice:

	Stock concentration	Volume for 1mL
NP-40 basic stock		812 µL
2 mM Sodium Fluoride	0.5 M	4 µL
β-glycerophosphate	1 M	10 µL
2 mM PMSF	0.2 M	10 µL
10 mM Sodium Pyrophosphate	0.1M	100 µL
sodium ortho vanadate	0.1 M	20 µL
1 µg/mL Leupeptin	1 mg/mL	1 µL
5 µg/mL Aprotinin	1.7 mg/mL	3 µL
1x cOmplete, mini EDTA-free protease inhibitors Cocktail tablet	10x (1 tablet in 1 mL water)	100 µL

1 x 10^7 cells were washed in cold 1xPBS and pelleted. The PBS was removed by aspiration and 270 µL of NP-40 lysis buffer was added and the cell pellet resuspended by pipetting before incubation on ice for 10 minutes. Lysates were then clarified by centrifugation at 4°C for 10 minutes at 13 000 x g and the clarified lysates were transferred to new tubes and stored at -20°C.

2.8.13 DC assay measuring protein concentration

Reagent A′ was prepared by adding 20 µl of Reagent S (Bio-Rad) to every required mL of Reagent A (Bio-Rad). In a 96-well plate, 5 µL of water was pipetted in to two blank wells and 5 µL standards were plated in duplicate (7 BSA protein standards from 0 µg/µL to 2 µg/µL as per chart below). NP40 protein lysates were diluted 1 in 5 with water and 5 µl of diluted samples were plated in duplicate. Next, 25 µl of

Reagent A′ and 200 µl of Reagent B (Bio-Rad) were added and the plate was incubated at room temperature for 15 mins for the colour to develop. The protein concentration was determined using an iMark microplate reader (Bio-Rad) at wavelength 750 nm and the result reported as µg protein per µL lysate. Typically 40-150 µg total protein was loaded per well in SDS-PAGE.

Standard Curve Chart

Std curve (µg/µl)	0	0.2	0.4	0.8	1.2	1.6	2.0
H_2O (µl)	12	10.8	9.6	7.2	4.8	2.4	0
2µg/µl BSA (µl)	0	1.2	2.4	4.8	7.2	9.6	12

2.8.14 Lentiviral transfection and transduction

HEK 293T cells were transfected by lentiviral shRNA plasmid pGFP-C-shLenti comprised of either scramble control vector or AXL knock-down with Green Fluorescense Protein (Origene, MD, USA) according to the manufacturer's instructions. Briefly, 3 x 10^5 cells were suspended in 2 mL IMDM culture media into a well of a 6-well plate. Mega Tran 1.0 Transfection Reagent (Origene), packaging plasmid (Origene), opti-MEM (Life Technologies) and viral plasmid (AXL knock-down or scramble control) were added to the cells dropwise and mixed gently followed by 72 hours incubation before harvesting the viral supernatant. Next, the

ponatinib-resistant cells were transduced with the viral supernatant and polybrene followed by centrifugation for 1 hour at 688 x g. Cells were then grown for 3-7 days until sufficient numbers were achieved for FAC sorting for GFP positivity in the The ACRF Flow and Laser Scanning Cytometry Facility (SAHMRI). The GFP level and Axl expression level were measured after sorting and before each experiment to ensure purity.

2.8.15 Statistical Analyses

Figures were developed using GraphPad Prism 6© software (GraphPad Software Inc.). Graphs with n equal or more than 3 represent the mean plus the standard deviation (SD) or plus the standard error of the mean (SEM) as indicated. All statistical analyses were performed using Student's T-test to determine statistical differences between experimental groups where the data sets passed the normality and equal variance tests. Differences were considered to be statistically significant when the probability value (p-value) was < 0.05.

Chapter 3 :

Ponatinib is not transported by ABCB1, ABCG2 or OCT-1 in CML cells

This chapter contains a published *letter to editor* in the journal of *Leukemia.* The document is located at http://www.nature.com/leu/journal/v29/n8/full/leu201535a.html

The third generation tyrosine kinase inhibitor (TKI) ponatinib (Iclusig®) was designed to target native and mutant isoforms of Bcr-Abl, including Bcr-Abl^{T315I}. It was approved for treatment of CML patients with resistance to other TKIs. However, TKI-resistance is complex. A sub-optimal response to TKI therapy can be due to the development of other Bcr-Abl dependent resistance mechanisms including reduced activity of the drug-influx transporter organic cation transporter 1 (OCT-1) (49, 50, 112), or increased expression of drug-efflux ATP-binding cassette transporters, commonly ABCB1 and ABCG2 (50, 82, 113-116). We and others (49, 50, 91, 112) have demonstrated that OCT-1 is an influx pump for imatinib, and confirmed its central role in determining the intracellular imatinib concentration (49, 50, 112). ABCB1 and ABCG2 are reported to be efflux transporters for dasatinib and other TKIs (113-115), and the expression of these proteins has been associated with TKI resistance(50, 113-115). Recent studies have implicated ponatinib as a potent ABCG2 inhibitor, and a less potent ABCB1 inhibitor (110), however, the transport mechanism for ponatinib into target leukemic cells remained elusive. In this study, we investigated whether ABCB1, ABCG2 or OCT are involved in the transportation of ponatinib.

To assess the role of ABCB1 in the cellular efflux of ponatinib, IC50^{ponatinib} values were determined for the *BCR-ABL1* -positive cell line K562 and its ABCB1-overexpressing variant K562-DOX, in the presence and absence of the ABCB1 inhibitors pantoprazole and cyclosporin (51, 83, 114, 117). IC50 values were determined using the western blotting method outlined by White *et al.* (118) to

detect CrkL (a surrogate marker of Bcr-Abl tyrosine kinase activity), whereby the percentage of phosphorylated CrkL (p-CrkL) to total CrkL was determined, and the concentration of ponatinib required to reduce this ratio by 50% was recorded as the $IC50^{ponatinib}$ value. As shown in Figure 1A, while there was an 8-fold increase in mean $IC50^{dasatinib}$ values for K562-DOX compared to K562 cells ($IC50^{dasatinib}$ 105.7 nM and 12.7 nM respectively, p=0.008, n=3), there was no significant difference between the mean $IC50^{ponatinib}$ for these cell lines (7.8 nM and 8.4 nM respectively, p=0.15, n=3). Similarly, while there was a significant reduction (p<0.001, n=3) in the mean $IC50^{dasatinib}$ in the K562-DOX cell line from 70 nM to 8 nM in the presence of the ABCB1 inhibitor cyclosporin, there was no significant reduction (16.4 nM to 16.0 nM, n=2) in K562 cells (Figure 1B), confirming transport of dasatinib by ABCB1. The addition of cyclosporin had no significant effect on the mean $IC50^{ponatinib}$ in K562-DOX (p=0.14, n=3) or K562 cells (p=0.78, n=3) (Figure 1C). Similarly, in the presence of pantoprazole, another ABCB1 inhibitor, the mean $IC50^{ponatinib}$ was not reduced significantly in either K562 or K562-DOX cells (p=0.29 and p=0.23 respectively, n=3) (Figure 1D). In summary, these data suggest that, unlike dasatinib, ponatinib is not exported from leukemic cells via ABCB1.

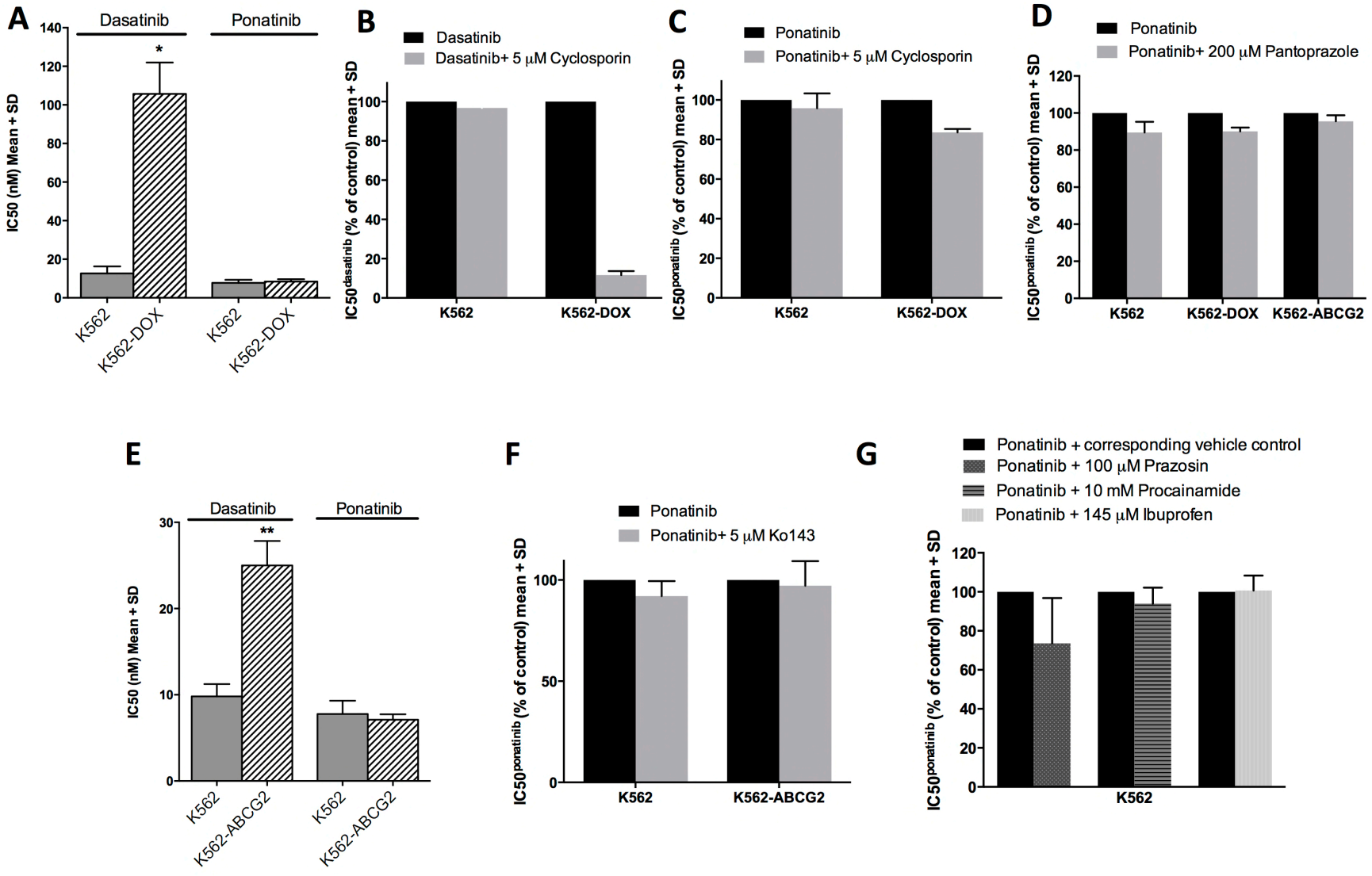
To assess the role of ABCG2 in ponatinib cellular efflux, $IC50^{ponatinib}$ was determined in K562 and its ABCG2 overexpressing variant K562-ABCG2 (11), in the presence and absence of the ABCG2 inhibitors Ko143 (83) and pantoprazole (117). While an approximate 2-fold increase in mean $IC50^{dasatinib}$ was observed in K562-ABCG2 (25 nM) compared to K562 cells (9.8 nM) (p<0.001, n=4) (Figure 1E), there was no

significant difference in the mean $IC50^{ponatinib}$ between K562 and K562-ABCG2 cells ($IC50^{ponatinib}$ 7.8 nM and 7.1 nM respectively, p=0.14, n=5) (Figure 1E). The inclusion of the ABCG2 inhibitor Ko413 did not significantly reduce the mean $IC50^{ponatinib}$ in either K562-ABCG2 (p= 0.81, n=3) or K562 cells (p= 0.61, n=3) (Figure 1F). Similarly, the ABCG2 inhibitor pantoprazole did not significantly reduce the mean $IC50^{ponatinib}$ value in either K562-ABCG2 (p=0.67, n=3) or K562 cells (p=0.29, n=3) (Figure 1D). Therefore, these results suggest that ponatinib is not exported from leukemic cells via ABCG2.

To determine whether OCT-1, 2 or 3 are involved in ponatinib influx, $IC50^{ponatinib}$ were assessed in K562 cells, in the presence of the OCT-1 and OCT-3 inhibitor prazosin (49, 83, 112), OCT-1 and OCT-2 inhibitor procainamide (49, 83, 112), and the OCT-1 inhibitor ibuprofen (119) compared to their respective vehicle controls. In contrast to imatinib, where $IC50^{imatinib}$ are increased in the presence of prazosin (49, 112), mean K562 $IC50^{ponatinib}$ reduced, but not significantly (4.6 nM to 3.0 nM, p=0.19, n=3) in the presence of prazosin (Figure 1G). In the presence of procainamide the K562 $IC50^{ponatinib}$ was reduced from 7.8 nM to 7.4 nM (p=0.33, n=3). Similarly, the addition of ibuprofen did not mediate a significant effect in K562 cells, increasing the $IC50^{ponatinib}$ from 6.8 nM to 6.9 nM (p=0.89, n=3). Overall, these data suggest that OCT-1, 2 or 3 are unlikely to be important transporters involved in ponatinib influx.

Figure 1. Unlike dasatinib, ponatinib IC50 in K562 cells was not affected by ABCB1 or ABCG2 overexpression or by the addition of ABCB1/ABCG2 inhibitors; inhibition of OCT transporters had no effect on $IC50^{ponatinib}$ in K562. (A) $IC50^{dasatinib}$ in the K562-DOX cell line was significantly higher than in the K562 control cell line (p=0.008, n=3). Comparatively, the K562-DOX cell line showed no change in $IC50^{ponatinib}$ compared to control (p=0.15, n=3). **(B)** There were 2% (n=2) and 88% (p<0.001, n=3) reductions in $IC50^{dasatinib}$ for the K562 and K562-DOX cell lines respectively with the addition of 5 µM cyclosporin (ABCB1 inhibitor). **(C)** $IC50^{ponatinib}$ values were reduced by approximately 4% and 16% following the addition of 5 µM cyclosporin in K562 and K562-DOX cells respectively and these reductions were not significantly different between the two cell lines (p=0.099, n=3). **(D)** $IC50^{ponatinib}$ values were reduced by approximately 11%, 10% and 4% (all not significant) in K562, K562-DOX and K562-ABCG2 cell lines in the presence of 200 µM pantoprazole (dual ABCB1/ABCG2 inhibitor), and these reductions were not significantly different between K562-DOX and K562 (p=0.87, n=3) nor K562-ABCG2 and K562 (p=0.21, n=3). **(E)** $IC50^{dasatinib}$ in the K562-ABCG2 cell line was approximately 2-fold higher than in the K562 control cell line (p<0.001, n=4), but $IC50^{ponatinib}$ values were similar between the two cell lines (p=0.14, n=5). **(F)** The percent reductions in the $IC50^{ponatinib}$ values of the K562 and K562-ABCG2 cell lines in the presence of 5 µM Ko143 (ABCG2 inhibitor) were not significantly different (p=0.58, n=3) to their respective vehicle treated controls. **(G)** The presence of OCT influx inhibitors did not significantly alter $IC50^{ponatinib}$ values in the K562 cell line. 100 µM prazosin decreased the $IC50^{ponatinib}$ value from 4.6 nM to 3.0 nM (p=0.19, n=3), 10 mM procainamide reduced the $IC50^{ponatinib}$ from 7.8 nM to 7.4 nM (p=0.33, n=3) and 145 µM ibuprofen caused minimal change (6.8 nM to 6.9 nM, p=0.89, n=3). Data are mean + SD. *p<0.05 and ** p<0.001. Controls were cell lines without ABCB1 or ABCG2 inhibitors and were normalized to 100%. Vehicle controls were K562 cells treated with methanol, water or ethanol for prazosin, procainamide and ibuprofen respectively and were normalized to 100%.

Figure 1


A
Dasatinib
Ponatinib
*
IC50 (nM) Mean + SD
140
120
100
80
60
40
20
0
K562
K562-DOX
K562
K562-DOX
B
Dasatinib
Dasatinib+ 5 μM Cyclosporin
IC50dasatinib (% of control) mean + SD
100
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K562-DOX
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Ponatinib
Ponatinib+ 5 μM Cyclosporin
IC50ponatinib (% of control) mean + SD
100
80
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0
K562
K562-DOX
D
Ponatinib
Ponatinib+ 200 μM Pantoprazole
IC50ponatinib (% of control) mean + SD
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IC50 (nM) Mean + SD
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K562-ABCG2
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Ponatinib+ 5 μM Ko143
IC50ponatinib (% of control) mean + SD
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K562
K562-ABCG2
G
Ponatinib + corresponding vehicle control
Ponatinib + 100 μM Prazosin
Ponatinib + 10 mM Procainamide
Ponatinib + 145 μM Ibuprofen
IC50ponatinib (% of control) mean + SD
120
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80
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40
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0
K562

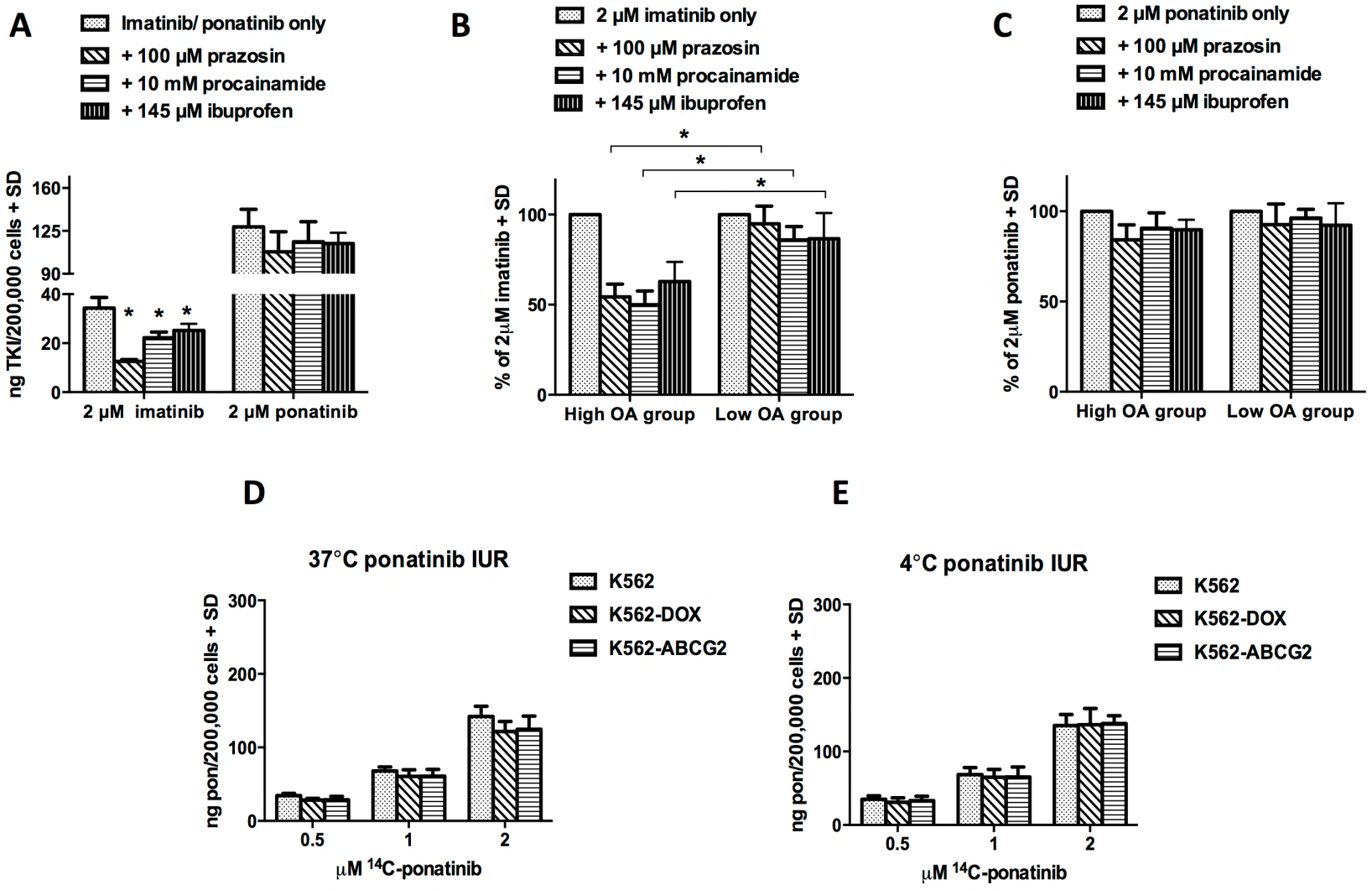
To further confirm that OCT transporters are not involved in ponatinib transport, intracellular uptake and retention (IUR) assays using 14C-labeled ponatinib were performed as described previously (49) compared with imatinib in the K562 cell line in the presence and absence of OCT inhibitors for 2 hours at 37°C. Incorporation of 14C-imatinib or 14C-ponatinib was determined and the IUR reported as ng imatinib or ponatinib/ 200,000 cells. In keeping with the $IC50^{ponatinib}$ finding, the addition of 100 μM prazosin, 10 mM procainamide or 145 μM ibuprofen did not reduce the ponatinib (2 μM) IUR significantly in the K562 cell line (p=0.1, p=0.3 and p=0.2 respectively, all n=4), but reduced imatinib (2 μM) IUR significantly (p=0.002, p=0.006 and p=0.02 respectively, all n=4) (Figure 2A). To establish clinical significance, we further assessed imatinib and ponatinib IUR in de novo chronic phase CML patient mononuclear cells (MNC) with high (n=3, 8.8 ng imatinib/200,000 cells) or low (n=3, 0.82 ng imatinib/200,000 cells) OCT-1 activity (OA). OA was defined as the difference between 2 μM imatinib IUR with and without prazosin as previously described (49). Compared to the low OA group, the addition of prazosin, procainamide or ibuprofen significantly reduced the imatinib IURs in the high OA group (p=0.006, p=0.004 and p=0.009 respectively, n=3), but this was not the case for the ponatinib IURs (p=0.3, p=0.2 and p=0.7 respectively, n=3) (Figure 2B-C). These findings confirmed that OCT-1, 2 and 3 are not ponatinib influx transporters.

The uptake of imatinib is temperature dependent, indicating primarily active, and hence ATP dependent, transport (50). To determine the role of ATP-dependent

membrane proteins in ponatinib transport, ponatinib IURs were performed at 37°C and 4°C in K562, K562-DOX and K562-ABCG2 cells. Consistent with the $IC50^{ponatinib}$ results, there were no significant differences in the ponatinib IURs between K562-DOX cells (at 0.5 μM, 1 μM and 2 μM, $p>0.05$ for all, n=3), or K562-ABCG2 cells (at 0.5 μM, 1 μM and 2 μM, $p>0.05$ for all, n=3), compared to the K562 control line at 37°C (Figure 2D). This observation further supports our finding that ponatinib is not transported by ABCB1 or ABCG2. Additionally, when the IUR assays were performed at 4°C there were also no significant differences in the IUR of K562-DOX (at 0.5 μM, 1 μM and 2 μM, $p>0.05$ for all, n=3) or K562-ABCG2 cells (at 0.5 μM, 1 μM and 2 μM, $p>0.05$ for all, n=3) compared to the K562 control line (Figure 2E). Moreover, at ponatinib concentrations of 0.5 μM, 1 μM and 2 μM, no significant changes in the IUR at 4°C compared to 37°C were observed between the 3 cells lines ($p>0.05$, all n=3) (Figure 2D-E), indicating that ponatinib is unlikely to be actively transported. However, due to the low specific activity of 14C-ponatinib (19.5 μCi/mg) and the detection limitation of the assay, concentrations below 500 nM could not be investigated, as such clinically relevant ponatinib concentrations could not be interrogated by this assay.

Figure 2. Unlike imatinib, ponatinib IUR in the K562 cell line and primary CML mononuclear cells was not influenced by inhibition of OCT transporters; Overexpression of ABCB1 or ABCG2, and temperature had no effect on ponatinib IUR in CML cell lines. (A) In K562 cells the addition of the OCT inhibitors prazosin, procainamide or ibuprofen significantly reduced imatinib IUR (all $p<0.01$, $n=4$) but not ponatinib IUR. **(B)** In the presence of prazosin, procainamide or ibuprofen**,** imatinib IUR (all $p<0.01$, $n=3$), but not **(C)** ponatinib IUR, were significantly lower in MNC from CML patients in the high OA group compared to those in the low OA group. (Note : due to heterogeneity between patients, IUR data were normalized to 100% at 2 μM and IUR data with inhibitors are presented as % of 2 μM.) **(D)** No significant difference was found in the ponatinib IUR at 37°C or **(E)** 4°C between the K562, K562-DOX and K562-ABCG2 cell lines. Neither were any significant reductions observed in the IUR at 4°C compared to the IUR at 37°C at 0.5 μM, 1 μM or 2 μM ^{14}C-labeled-ponatinib for K562, K562-DOX or K562-ABCG2 cells. All IUR assays were performed in at least triplicate, error bars represent +SD. *$p<0.01$

Figure 2


A
Imatinib/ ponatinib only
+ 100 µM prazosin
+ 10 mM procainamide
+ 145 µM ibuprofen
ng TKI/200,000 cells + SD
160
125
90
40
20
0
*
*
*
2 µM imatinib
2 µM ponatinib
B
2 µM imatinib only
+ 100 µM prazosin
+ 10 mM procainamide
+ 145 µM ibuprofen
*
*
*
% of 2µM imatinib + SD
100
50
0
High OA group
Low OA group
C
2 µM ponatinib only
+ 100 µM prazosin
+ 10 mM procainamide
+ 145 µM ibuprofen
% of 2µM ponatinib + SD
100
50
0
High OA group
Low OA group
D
37°C ponatinib IUR
K562
K562-DOX
K562-ABCG2
ng pon/200,000 cells + SD
300
200
100
0
0.5
1
2
µM 14C-ponatinib
E
4°C ponatinib IUR
K562
K562-DOX
K562-ABCG2
ng pon/200,000 cells + SD
300
200
100
0
0.5
1
2
µM 14C-ponatinib

Here, we demonstrate no significant difference in $IC50^{ponatinib}$ values between K562 and K562-DOX cell line. In addition, the ABCB1 inhibitors cyclosporin and pantoprazole had no significant effect on the $IC50^{ponatinib}$ in K562-DOX compared to the K562. Importantly, when the same concentrations of transporter inhibitors were used, in this, and previous studies (49, 51, 83, 114), unlike ponatinib, the IC50 values of imatinib, nilotinib and dasatinib, which are transported by ABCB1, were significantly reduced. Consistent with our IC50 findings, there was no significant difference in the ponatinib IUR between K562 and K562-DOX cells. These data indicate that ABCB1 is not involved in ponatinib transport in target *BCR-ABL1* + cells. Similarly our investigations into ABCG2 have demonstrated that the $IC50^{ponatinib}$ was not altered by the addition of ABCG2 inhibitors, and there was no significant difference between K562 and K562-ABCG2 in $IC50^{ponatinib}$ or ponatinib IUR. Thus, these results suggest that ponatinib is not exported by ABCG2. In contrast to imatinib, this study suggests that ponatinib is not transported by OCT-1, neither by OCT-2 or 3, as the combination of ponatinib with OCT inhibitors had no significant effect on ponatinib IC50 or IUR values in the K562 cell line or ponatinib IUR in patient MNCs. Overall, we demonstrate evidence that ponatinib is not transported by ABCB1, ABCG2 or OCT and indeed it appears to be passively transported as evidenced by similar drug transport at both 37°C and 4°C. Therefore, in contrast to other clinically available TKIs, patients are unlikely to develop ponatinib resistance mediated by altered expression of these transporters.

Statement of Authorship

Title of Paper	Ponatinib is not transported by ABCB1, ABCG2 or OCT-1 in CML cells
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted work written in manuscript style
Publication Details	Lu L, Saunders V, Leclercq T, Hughes T and White D

Principal Author

Name of Principal Author (Candidate)	Liu Lu
Contribution to the Paper	Carried out the studies and analysis, participated in the design of the study and wrote the manuscript
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 11/11/2015

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Verity Saunders
Contribution to the Paper	Participated in analysis of the IUR data, design the study and manuscript editing
Signature	Date 11/11/2015

Name of Co-Author	Tamara Leclercq
Contribution to the Paper	Participated in manuscript preparation.
Signature	Date 11/11/15

Please cut and paste additional co-author panels here as required.

Name of Co-Author	Prof. Timothy Hughes
Contribution to the Paper	Designed the study and participated in manuscript evaluation.
Signature	Date 11/11/15

Name of Co-Author	Prof. Deb White
Contribution to the Paper	Designed the study, supervised development of work, and participated in manuscript editing.
Signature	Date 11/11/15

Chapter 4 :

Ponatinib resistance in K562 T315I-R cell line is mediated by *BCR-ABL1* overexpression and increased level of T315I mutation

4.1 Introduction

4.1.1 Bcr-Abl kinase domain mutations

It is well documented how the introduction of imatinib therapy revolutionized the treatment of CML, however, more than 35% of CML patients who receive imatinib as first line treatment will require alternative therapy due to intolerance or resistance (106, 120). The development of Bcr-Abl kinase domain point mutations is the most common and well-understood mechanism of TKI secondary resistance.

To date, over 100 different mutations, which lead to over 50 different amino acid substitutions, have been described to cause Bcr-Abl resistance to imatinib. These mutations have been categorised into several groups depending on the locations: 1) mutations that are located in the ATP binding site; 2) mutations that are located in the activation loop preventing formation of the inactive conformation of Bcr-Abl, required for imatinib binding; 3) mutations that are located in catalytic domain. As Bcr-Abl mutations can develop within different locations, not all of the mutations cause the same degree of clinical resistance to imatinib: some mutations abrogate the binding of TKI/s while some mutations only reduce the binding efficacy of TKI/s. According to response to imatinib, kinase domain mutations are divided into highly resistant mutations such as T315I and moderately resistant mutations such as F359V. While the kinase with moderately resistant mutations can often be overcome by increased dosage of imatinib to 600 or 800mg per day (48), treatment

of Bcr-Abl with the highly resistant mutations requires the use of the second or third generation TKIs at a minimum.

Iulia and colleague (121) analyzed the mutation rates to different TKIs across 12 studies of in total 1698 patients. The mean mutation rate on imatinib was 9.7% (6.2%-13.3%); on dasatinib was 1.7% (0.0%-4.3%); and on nilotinib was 3.3% (0.0%-7.7%). The most common mutations included T315I, E255K and M351T, while the T315I mutation, the most common mutation and conferring Bcr-Abl resistance to all TKIs except ponatinib, constituted 58% of dasatinib related mutations and 13% of imatinib related mutations (121).

4.1.2 *BCR-ABL1* overexpression

BCR-ABL1 amplification mediated imatinib resistance was firstly reported in cell culture models and subsequently detected in CML patients (54, 122). The overexpression of *BCR-ABL1* is mostly caused by the development of mutation in the promoter region of *BCR-ABL1*, duplication of Ph chromosome or the formation of double minutes containing Bcr-Abl. To date, most CML patients are routinely screened by RQ-PCR to detect *BCR-ABL1* transcript levels to monitor the response to TKI treatment and to monitor the disease progression. Interestingly, the correlation of *BCR-ABL1* overexpression and Bcr-Abl KD mutations has been described in cell line models and in CML patients (51, 74, 123): Bcr-Abl KD mutations, including the T315I mutation, were found after a gradually increased

expression of *BCR-ABL1,* and this *BCR-ABL1* overexpression was reduced after the development of mutation.

4.1.3 Generation TKI resistance and the K562-T315I cell line

Long-term drug exposure is well utilized as a method of generating resistance, and has been employed extensively by multiple cancer research groups to develop chemotherapy resistant cell lines. We (51) and others (74, 81, 122) have previously developed TKI (imatinib, nilotinib or dasatinib) resistant human *BCR-ABL1* positive cell lines or murine *BCR-ABL1* positive cell lines via long-term culture in increasing concentrations of TKI. Although this method does not exactly mimic TKI resistance development in patients (as patients would often have therapy interruptions), the resistance mechanisms (mainly *BCR-ABL1* overexpression and Bcr-Abl KD mutations) developed during this method are representative of those that commonly arise in TKI resistant patients (74, 81, 122).

Currently, ponatinib is a second line therapy for CML or ALL patients who carry *BCR-ABL1* ^{T315I} mutation and are resistant to other TKIs. Hence, in this study we were aiming to generate ponatinib resistance in a cell line model that harbours the T315I mutation, to mimic the potential development of ponatinib resistance in T315I positive patients treated with this TKI. Therefore, we used the K562 T315I cell line as a ponatinib naïve control line to develop ponatinib resistance. The *BCR-ABL1*^{T315I} positive cell line K562 T315I (also called K562 200nM DAS) was established by Tang *et al.* by exposing K562 cells to increasing concentrations of

dasatinib up to 200 nM (51). The T315I mutation was firstly detected when this cell line was cultured with 25 nM dasatinib, and the percentage of the T315I mutation reached a peak to 44% when cultured with 200 nM dasatinib (51). The K562 T315I cell line also exhibits approximately three fold higher *BCR-ABL1* mRNA transcript expression compared to the TKI naïve K562 control line (51).

4.2 Approach

4.2.1 Generation of ponatinib resistant cell lines

In this study, the dasatinib resistant cell line K562 T315I was used to generate ponatinib resistance *in vitro*, and the intermediate stages of resistance were analyzed to monitor the development of resistance. The method used to generate ponatinib resistance was adapted from that of Mahon *et al.* and Tang *et. al*. (51, 81). K562 T315I cell line was exposed to low concentrations of ponatinib (starting concentration at 5 nM). Ponatinib concentrations were then increased by 5-10 nM increments approximately every 30-90 days, as cells tolerated each increased dose. In theory, slow ponatinib dose escalation will allow enough time for the development of resistance mechanisms and for the resistant clone to expand. Drug resistance is defined as the ability of cells to overtly resist the effects of drugs at concentrations previously toxic to them. In this study, the resistant concentration was set as 100 nM ponatinib. In addition, cells with intermediate resistance are defined by the ability to tolerate incremental increases in drug concentrations (below 100 nM ponatinib) only after a period (generally in this case 10-90 days) of

tolerance (induced tolerance). This period is likely associated with clonal selection and expansion.

In patient plasma, median peak and trough levels of ponatinib when dosed at 45 mg once daily are 145 nM and 64 nM respectively (124). Therefore, 200 nM ponatinib was firstly chosen as the final culture concentration in the resistant line for two reasons: 1. when cells survive in this concentration, it ensures the resistance to ponatinib; 2. this concentration is close to pharmacologically relevant. However, the cell culture was able to survive in no greater than 100 nM ponatinib, determining this as the final resistant concentration. Although 145 nM can be achieved in patients, the peak plasma level would not be maintained in plasma for long, hence the 100 nM ponatinib concentration still has significance in clinical settings.

The ponatinib resistant K562 T315I-R cell line was generated. The concentration of ponatinib was escalated as follows: 5 nM, 8 nM, 10 nM, 20 nM, 30 nM, 40 nM, 50 nM, 60 nM, 70 nM, 80 nM, 90 nM and 100 nM. At the 50 nM ponatinib intermediate stage, reduced proliferation and survival (by trypan blue count, 50%-60% viable) was observed. After more than three months exposure to 50 nM ponatinib, the cells became tolerant to this concentration with greater than 85% viability. A similar pattern was observed at the 80 nM intermediate stage that required a longer period (approximately 40 days) to tolerate 80 nM ponatinib.

4.2.2 Analysis of ponatinib-resistant cell line K562 T315I-R

As the aim of this study was to investigate the emergence of ponatinib resistance, samples were collected at each of the intermediate resistance stages to prepare and store DNA and RNA for later analysis. *BCR-ABL1* expression, cell surface ABCB1 and ABCG2 expression and the *BCR-ABL1* mutation status of the intermediates were monitored by RQ-PCR, flow cytometry and conventional sequencing respectively. The viability of the cells in the presence of ponatinib was measured by trypan blue exclusion analysis and flow cytometry (annexin V/7AAD). The degree of resistance was analysed by IC50 western blotting (pCrkL) as well as flow cytometry (Annexin V/7AAD). pBcr-Abl western blots were also performed to determine the kinase activity in the resistant cell line.

4.3 Results

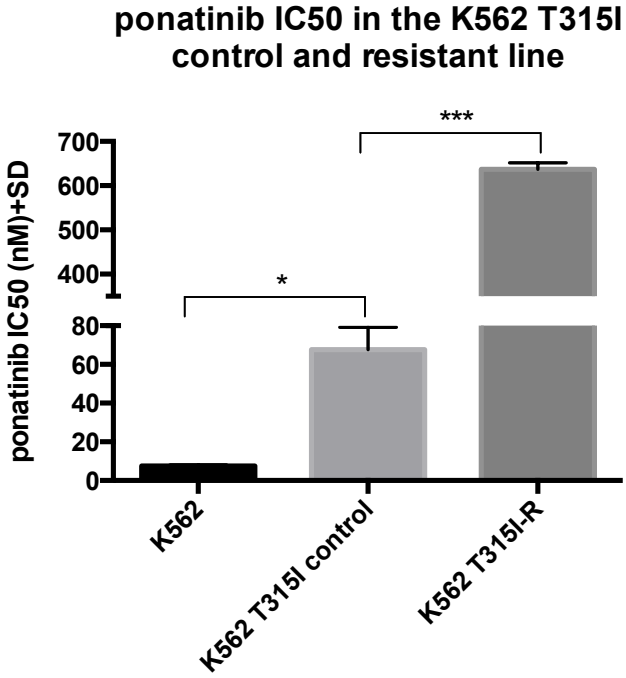
4.3.1 K562 T315I cells cultured in 100 nM ponatinib demonstrate resistance to TKIs *in vitro*

After long-term ponatinib culture, the ponatinib resistant K562 T315I-R cell line was generated. Ponatinib resistance generation in this cell line took almost three years (including twice that cells failed to survive in 100 nM ponatinib, which took approximately one and half years).

To determine the degree of ponatinib resistance in the cell line, ponatinib IC50 assays were performed in increasing concentrations of ponatinib. Increase in IC50

value indicates resistance development, as it requires higher concentration of TKI to inhibit Bcr-Abl tyrosine kinase activity. As shown in Figure 4.1(a)-(c), K562 T315I-R cells demonstrated significantly higher ponatinib IC50 (635 nM) compared to K562 T315I control cells (68 nM) ($p<0.001$, $n=3$). Notably, as K562 T315I control cell line harbours the T315I mutation and *BCR-ABL1* overexpression, it demonstrated a significantly higher ponatinib IC50 ($p<0.05$, $n=3$) compared to its original parental control line K562 (7.8 nM) (Figure 4.1(d)).

Figure 4.1 (a)



n=3

Figure 4.1 (b)

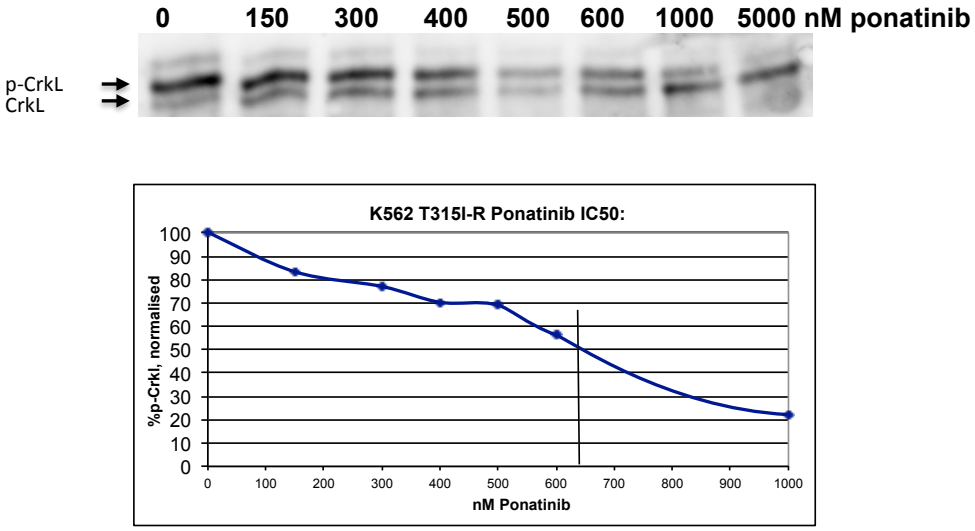


Figure 4.1 (c)

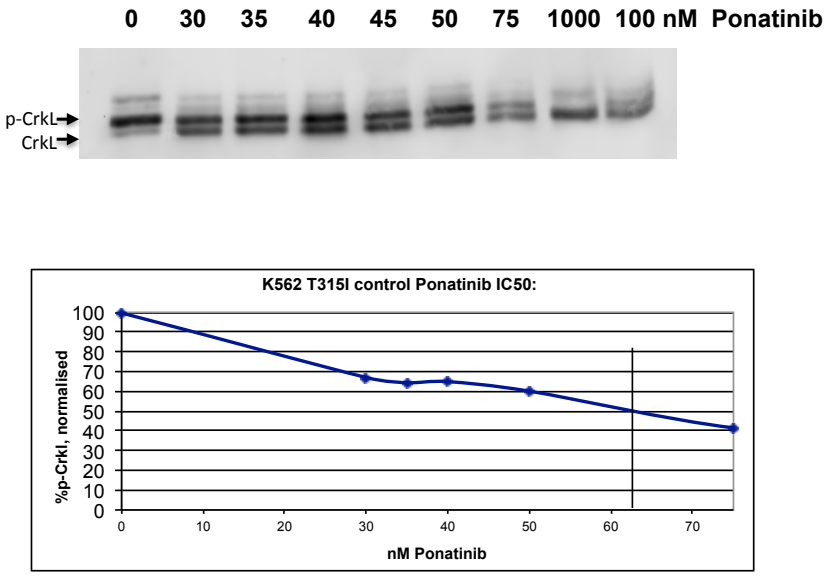


Figure 4.1 (d)

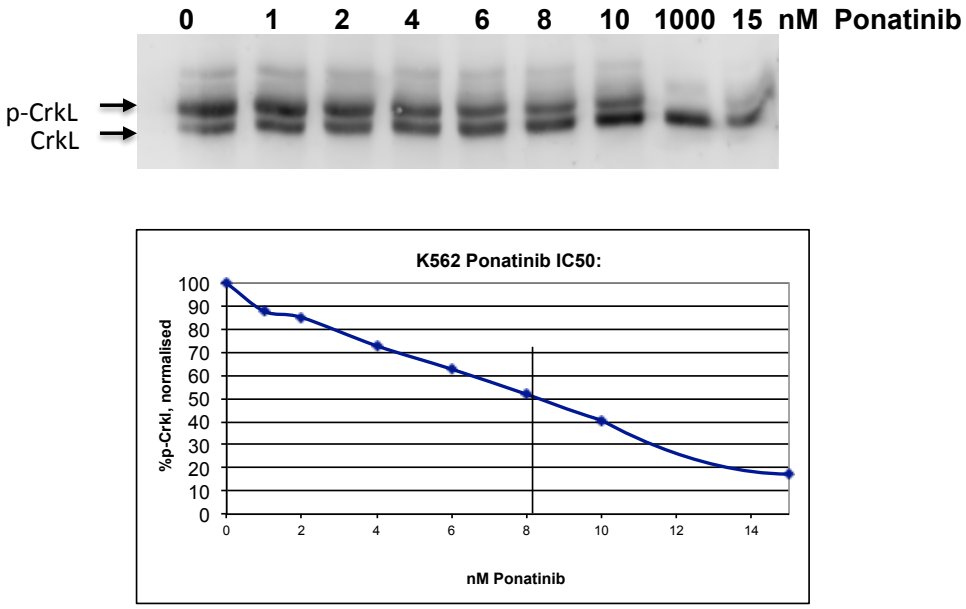


Figure 4.1: ponatinib IC50 for K562 T315I-R was significantly higher than K562 T315I control line

Cells were incubated with 2 hours with serial concentration of ponatinib and then lysed to perform p-CrkL western blot. **(a)** Increased ponatinib IC50s were observed in the K562 T315I and K562 T315I 100 nM PON cell line compared to control line. Error bars represent SD. n=3 for all data, *p<005, ***p<0.001. Representative **(b)** K562 T315I-R **(c)** K562 T315I **(d)** K562 ponatinib IC50 western blot analyzed. Top panel: representative western blotting image. Bottom panel: analysis of the representative western blot. Ponatinib concentration required to reduce the ratio of p-CrkL:CrkL by 50% (ponatinib IC50) is indicated by the black line.

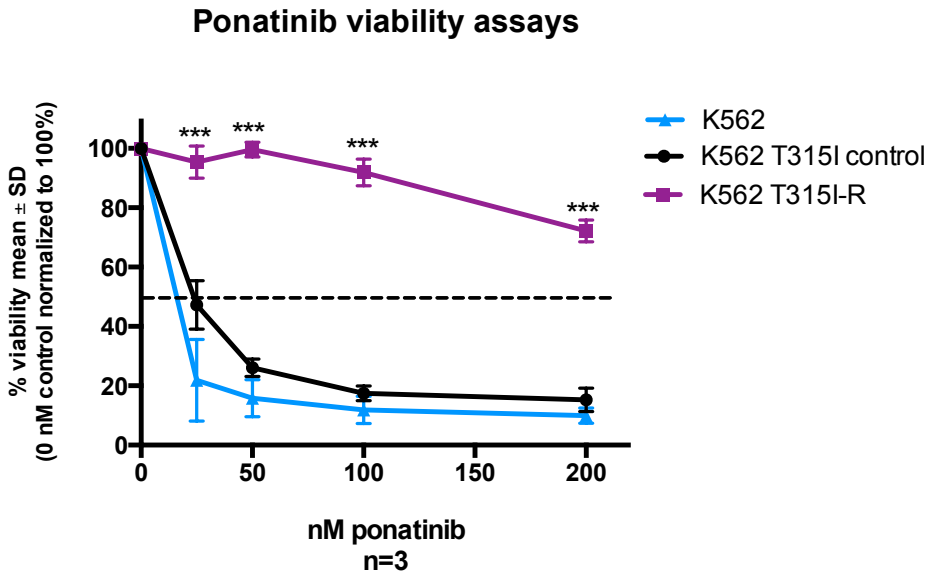
7AAD and annexin V staining for cell death in the control and resistant cell lines after 72 hours post-ponatinib exposure (Figure 4.2 (a)) revealed that K562 T315I-R cells demonstrated 1% survival reduction when exposed to 50 nM ponatinib compared to no ponatinib treatment (normalized to 100%), while a 74% reduction in survival was observed in the control line ($p<0.001$, $n=3$). When the resistant line K562 T315I-R was exposed to higher ponatinib concentration 100 nM (8% reduction) or 200 nM (46%), significantly lower reduction in viabilities compared to the control cell line (82% reduction in 100 nM and 84% in 200 nM) were observed ($n=3$, both $p<0.001$) (Figure 4.2 (a)). In addition, resistance to other TKIs including imatinib, nilotinib and dasatinib was also evaluated in the two cell lines. As the T315I mutation mediates resistance to all of the first and second generation TKIs, the control line K562 T315I exhibited resistance to imatinib, nilotinib and dasatinib (all viabilities were approximately 100% compared to the non-TKI treatment control, $n=3$, all $p<0.001$ in each tested concentration) (Figure 4.2(b)-(d)). Interestingly, the resistant line K562 T315I-R was not only resistant to imatinib, nilotinib and dasatinib, but actually survived better when cultured with these TKIs compared to TKI free media: with 2000 nM dasatinib, viability increased by 53% compared to 0 nM control; with 5000 nM imatinib, viability increased by 31% compared to 0 nM control; and with 5000 nM nilotinib viability increased by 6% compared to 0 nM control, respectively after 72 hours incubation (Figure 4.2(b)-(d)). This suggested that the presence of the first and second generation TKIs, especially imatinib and dasatinib, mediated the survival of the resistant cells (might be via maintaining the presence of the T315I mutation). However, the

mechanisms of how the TKIs facilitate the survival in resistant cells require further study.

All together, these data confirmed the cross-TKI (imatinib, nilotinib, dasatinib and ponatinib) resistance in the K562 T315I-R cell line.

Figure 4.2

(a)


Ponatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
100
80
60
40
20
0
0
50
100
150
200
***
***
***
***
nM ponatinib
n=3
K562
K562 T315I control
K562 T315I-R

(b)

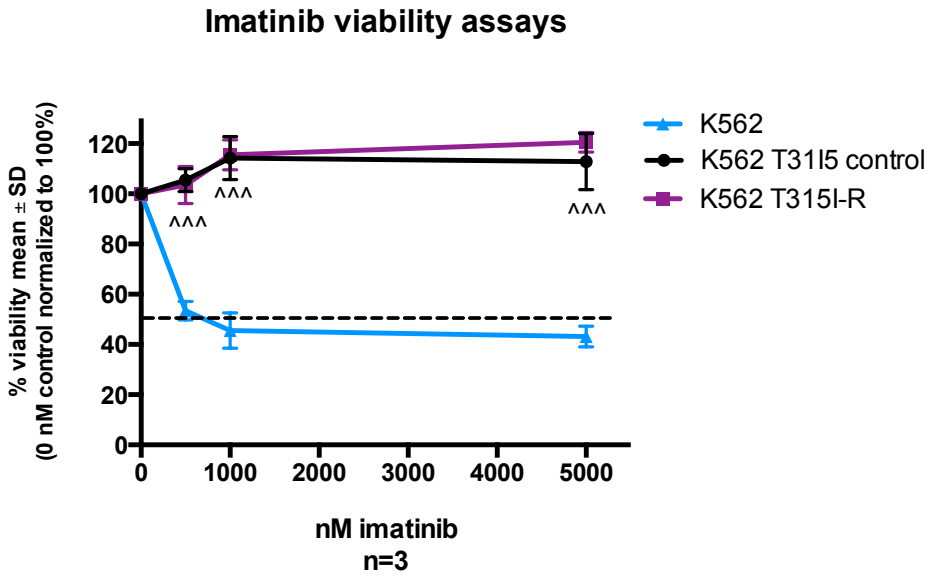
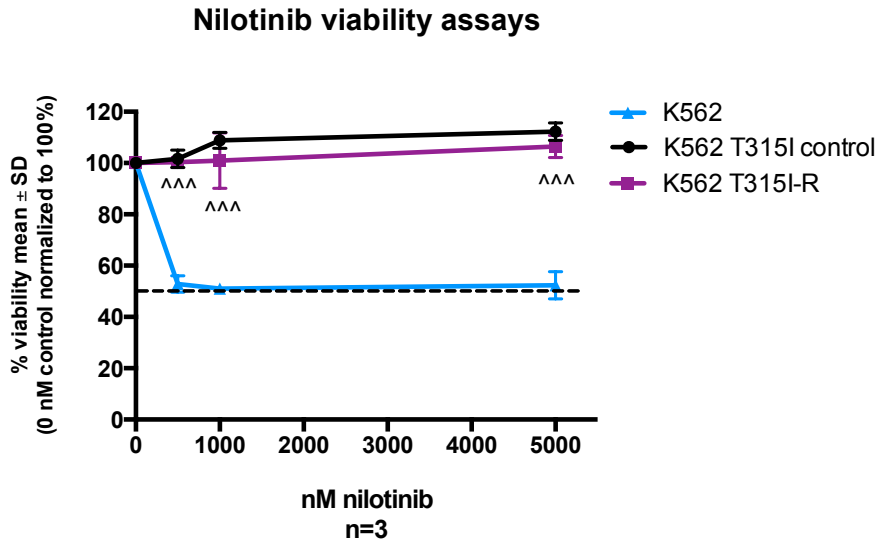

Imatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
0
1000
2000
3000
4000
5000
^^^
^^^
^^^
nM imatinib
n=3
K562
K562 T31I5 control
K562 T315I-R

Figure 4.2

(c)


Nilotinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
0
1000
2000
3000
4000
5000
^^^
^^^
^^^
nM nilotinib
n=3
K562
K562 T315I control
K562 T315I-R

(d)

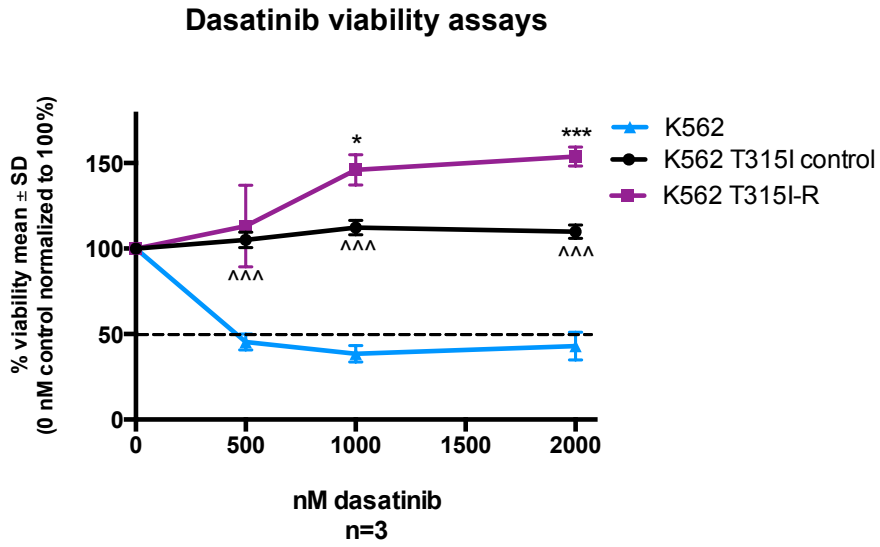

Dasatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
150
100
50
0
0
500
1000
1500
2000
*
***
^^^
^^^
^^^
nM dasatinib
n=3
K562
K562 T315I control
K562 T315I-R

Figure 4.2: ponatinib resistant K562 T315I-R cells are also resistant to imatinib, nilotinib and dasatinib mediated cell death

The K562(blue line), K562 T315I (black line) and K562 T315I-R (purple line) cells were cultured with serial concentrations of TKI for 72 hours, followed by staining with 7AAD and annexin V to determine cell death. 0 nM TKI treatment groups were normalised to 100%. The change in viabilities induced by TKI treatment were compared between the cell lines. The K562 T315I-R cells demonstrated significant improved survival when cultured with all of the tested concentrations of **(a)** ponatinib (25 nM-200 nM) compared to control line K562 T315I. The K562 T315I-R cells demonstrated similar survival when cultured with all of the tested concentrations of **(b)** imatinib (500 nM-5000 nM) and **(c)** nilotinib (500 nM-5000 nM) compared to control line K562 T315I as the control cell line demonstrate resistance to these two TKI already. The K562 T315I-R cells demonstrated significant improved survival when cultured with all of the tested concentrations of **(d)** dasatinib (500 nM-2000 nM) compared to control line K562 T315I. The control line K562 T315I show significantly improved survival (when exposed to imatinib, nilotinib and dasatinib, but not ponatinib) compared to K562 cell line. Black dash lines indicate 50% reduction in viability. Error bars represent SD. n=3 for all data, *p<0.05 and ***p<0.01 compared to K562 T315I cell line. ^^^p<0.001 compared to K562 cell line .

4.3.2 No evidence of ABCB1 or ABCG2 up-regulation in the K562 T315I-R ponatinib resistant line

Once ponatinib resistance was developed, K562 T315I-R cell line was assessed for the expression level of ABCB1 and ABCG2 via flow cytometry, as the overexpression of these two transporters results in resistance to other TKIs (36, 81-83). However, no surface expression of ABCB1 or ABCG2 was detected in the K562 T315I-R resistant or the K562 T315I control cell lines, while ABCB1 and ABCG2 expression was detected in the positive control cell lines K562 DOX and K562 ABCG2 respectively (Figure 4.3). Interestingly, while staining for ABCB1 was always negative in the intermediate stages of the ponatinib resistance development, transient ABCG2 expression was observed in the intermediate lines K562 T315I 20 nM PON and K562 T315I 30 nM PON (Figure 4.4(a)), although the staining of ABCG2 was just slightly positive in the 30 nM intermediate stage compared to the ponatinib naïve control line. Such overexpression of ABCG2 protein on the cell surface was accompanied by a significant increase in ABCG2 mRNA expression level detected by RQ-PCR in the intermediate line K562 T315I 20 nM PON (Figure 4.4(b)). However, at higher ponatinib concentrations (from K562 T315I 40 nM PON), the cell surface and mRNA expression of ABCG2 was decreased to negative (Figure 4.4(b)). In addition, no ABCG2 expression was detected in the lower ponatinib concentration cultures (lower than 20 nM). This suggests that increased ABCG2 expression is transient and possibly required for early ponatinib resistance, but that it is not the only, nor the major cause of ponatinib resistance in this cell line.

Figure 4.3 (a)

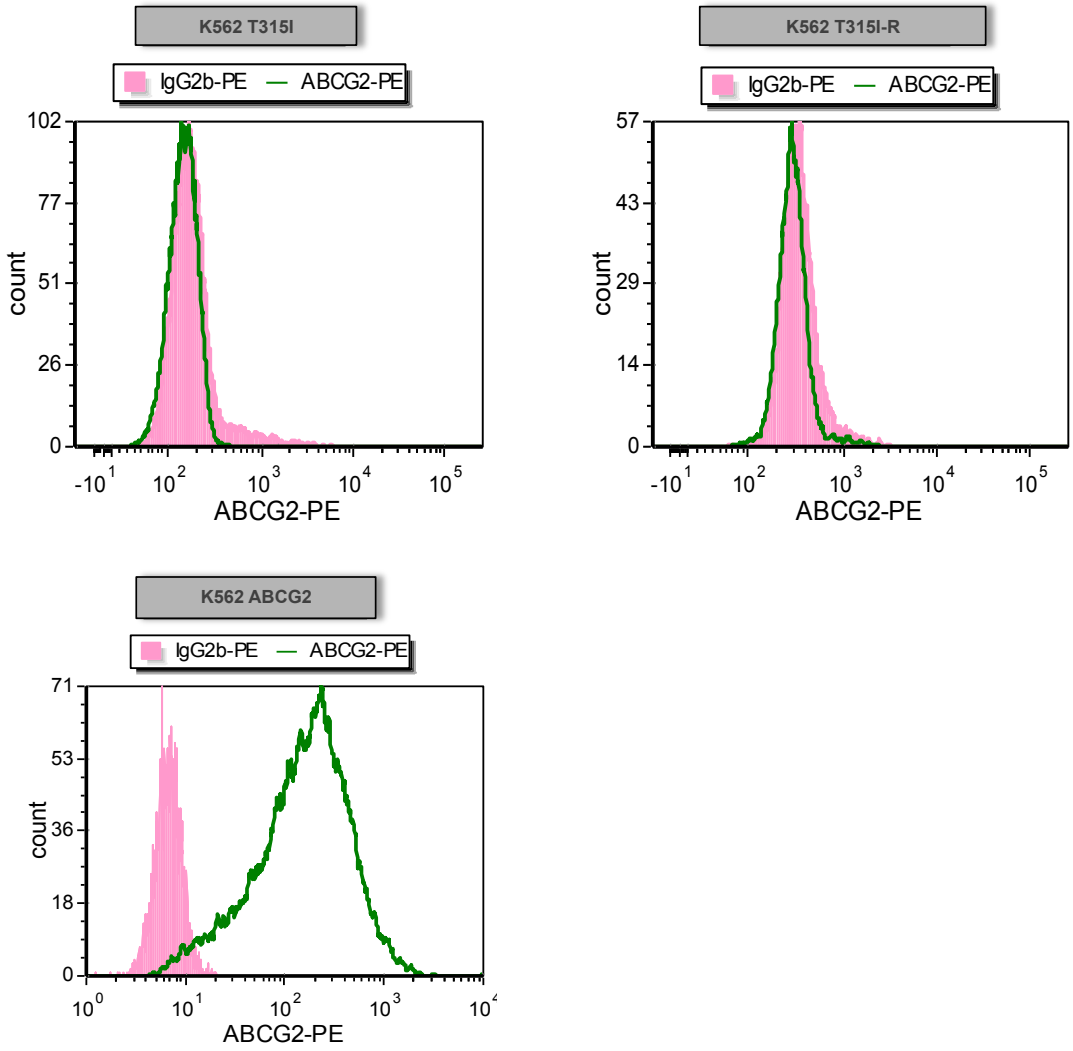

K562 T315I
IgG2b-PE
ABCG2-PE
count
ABCG2-PE
K562 T315I-R
IgG2b-PE
ABCG2-PE
count
ABCG2-PE
K562 ABCG2
IgG2b-PE
ABCG2-PE
count
ABCG2-PE

Figure 4.3 (b)

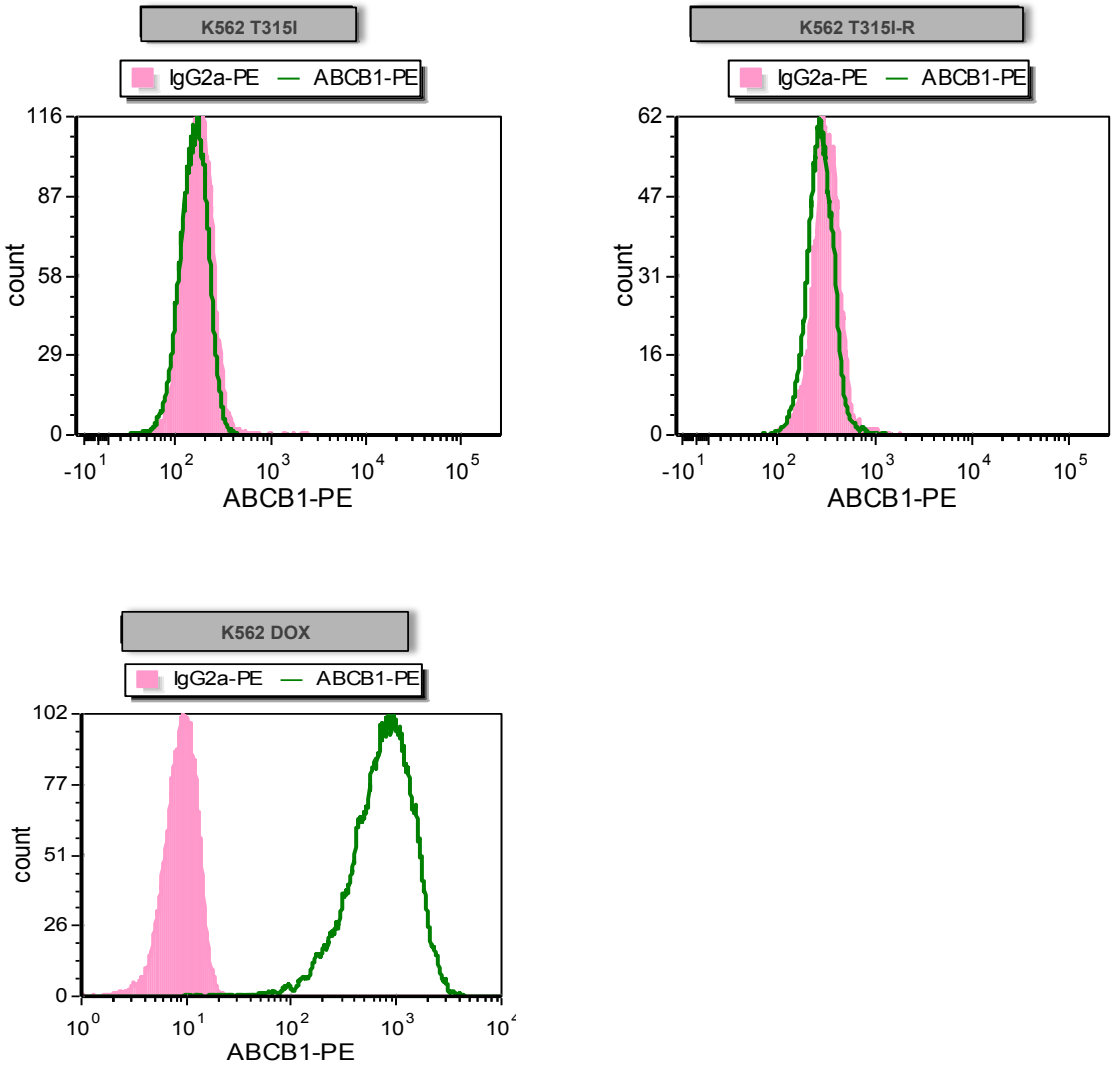


Figure 4.3: The K562 T315I 100 nM PON and K562 T315I cell lines does not express ABCG2 (a) or ABCB1 (b).
Cells from K562 T315I control line and K562 T315I-R resistant line were collected and stained with either IgG2b/ IgG2a PE isotype control antibody (pink) or the corresponding ABCG2 or ABCB1 PE antibody (green line). No ABCG2 nor ABCB1 expression was detected in the control cells K562 T315I and the resistant line K562 T315I-R. K562 ABCG2 and K562 DOX cells were used as positive control for the staining of ABCG2 or ABCB1.

Figure 4.4 (a)

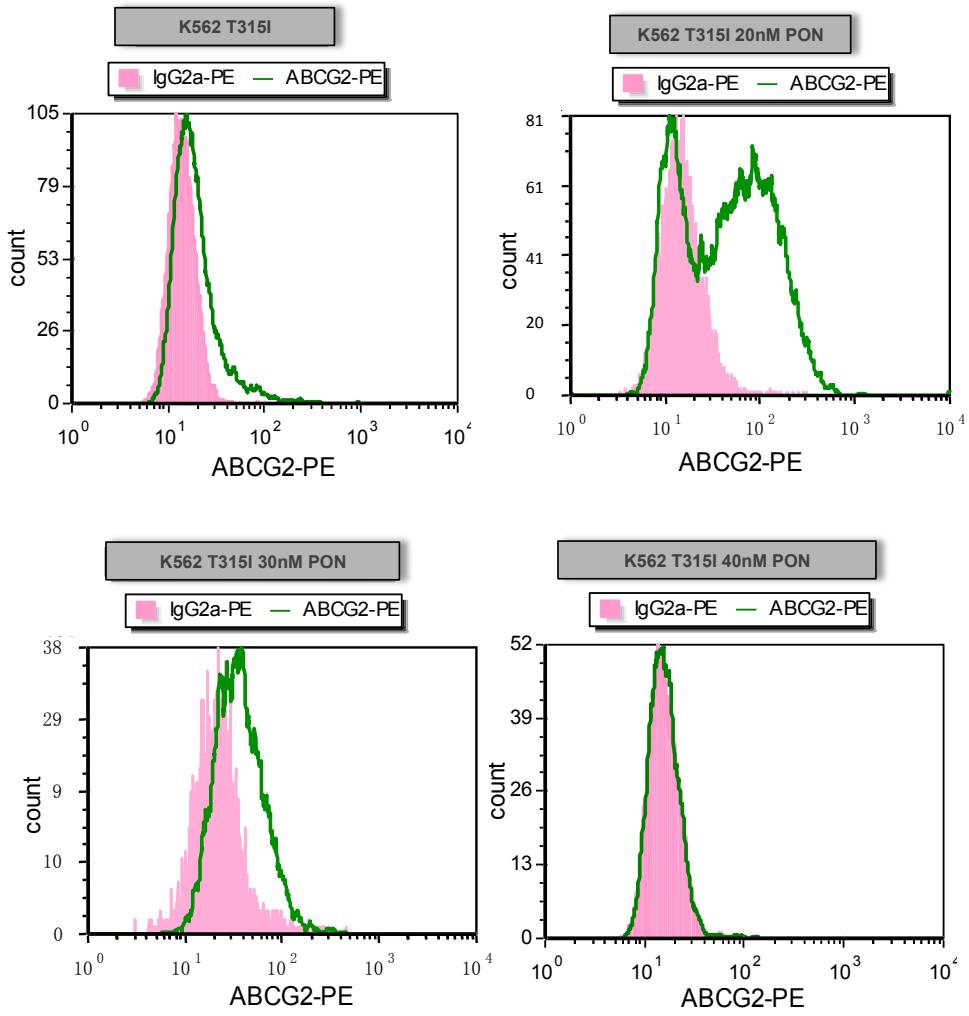

K562 T315I
IgG2a-PE
ABCG2-PE
count
ABCG2-PE
K562 T315I 20nM PON
IgG2a-PE
ABCG2-PE
count
ABCG2-PE
K562 T315I 30nM PON
IgG2a-PE
ABCG2-PE
count
ABCG2-PE
K562 T315I 40nM PON
IgG2a-PE
ABCG2-PE
count
ABCG2-PE

Figure 4.4 (b)

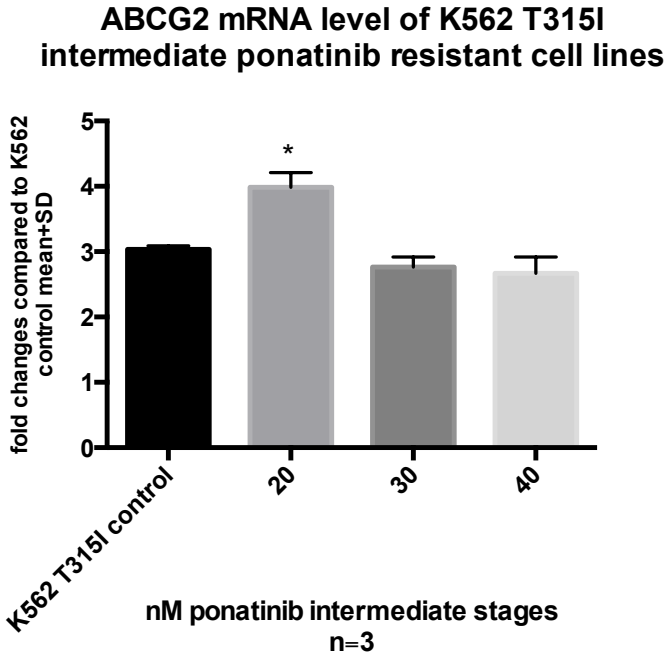


Figure 4.4: ABCG2 expression was detected in the K562 T315I 20 nM PON and K562 T315I 30 nM PON cells .
(a) Cells from K562 T315I control line and K562 T315I 20nM PON, K562 T315I 30nM PON, and K562 T315I 40nM PON intermediate lines were collected and stained with either IgG2b PE isotype control antibody (pink) or the corresponding ABCG2 PE antibody (green line). Cell surface ABCG2 expression was only detected in the K562 T315I 20 nM PON intermediate line, and slightly expressed in the K562 T315I 30 nM PON intermediate line. **(b)** ABCG2 mRNA expression level was increased in 20nM intermediate line, but was not evident in the 30nM intermediate culture, where expression had reduced back to the level evident in the K562 T315I control line. Error bars represent SD. n=3 for all data, *p<0.05.

4.3.3 K562 T315I-R resistant line demonstrates further *BCR-ABL1* mRNA overexpression, together with increased total and phospho-Bcr-Abl protein

Many studies have demonstrated that CML patients with high *BCR-ABL1* mRNA transcript levels require higher doses of TKI treatment to control their disease (51, 74, 75). As it is well known that overexpression of *BCR-ABL1* mRNA causes resistance to the first and second generation TKIs (51, 74, 75), it was necessary to determine the *BCR-ABL1* mRNA expression level in the development of ponatinib resistance. RQ-PCR was performed to quantitatively determine *BCR-ABL1* transcript number, and the result indicated that there was a significant increase in *BCR-ABL1* mRNA from 1206% in naïve cells to 8027% in the resistant line (n=3, $P<0.001$) (Figure 4.5 (a)). In addition, studies of the intermediates of ponatinib resistance development in this line revealed a step-wise increase of *BCR-ABL1* mRNA expression, peaking at 9034% in the intermediate line K562 T315I 90 nM PON (n=3, $p<0.001$) (Figure 4.5 (a)). This overexpression was not significant in the low ponatinib concentration intermediate lines K562 T315I 10nM PON and K562 T315I 20nM PON. In addition, western blotting confirmed that *BCR-ABL1* mRNA overexpression resulted in increased Bcr-Abl activity: total and phospho levels of Bcr-Abl protein were increased (four-fold) in the resistant cell line compared to control line (Appendix 1).

All together, these data suggested that *BCR-ABL1* overexpression induced increased Bcr-Abl oncoprotein expression, which assisted the development of ponatinib resistance in the K562 T315I-R cell line.

Figure 4.5

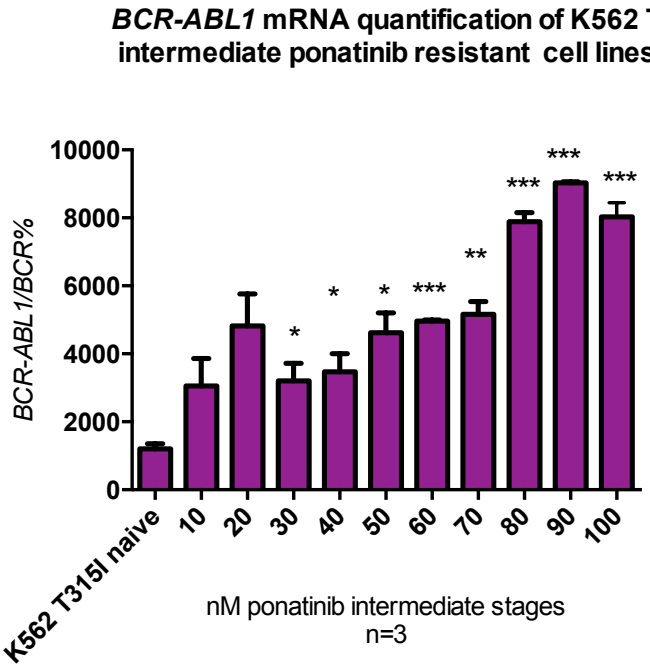


Figure 4.5: Increased *BCR-ABL1* mRNA was observed in all K562 T315I intermediate ponatinib resistant cell lines compared to naïve control line K562 T315I.
BCR-ABL1 transcript number was measured by RQ-PCR. *BCR-ABL1* overexpression was observed in the K562 T315I ponatinib resistant intermediate lines, peaking at 90 nM intermediate stage ($p<0.001$, n=3). Error bars represent SD. Data are n=3 for all data, *$p<0.05$, **$p<0.01$ ***$p<0.001$.

4.3.4 K562 T315I-R ponatinib resistant line demonstrates increased *BCR-ABL1*^{T315I} mutation level

Ponatinib is a pan-Bcr-Abl inhibitor that effectively inhibits Bcr-Abl with all of the identified single kinase domain mutations including T315I. Previous results revealed 44% T315I *BCR-ABL1* KD gatekeeper mutation in the control cell line K562 T315I as causative of resistance to dasatinib, imatinib and nilotinib, but sensitive to ponatinib. Conventional sequencing was performed on the intermediate cell lines generated during the development of ponatinib resistance and revealed a slow but steady increase in T315I% from 44% in the control line (Appendix 2) to peaking at 66% (Figure 4.6 and Appendix 3), suggestive of increasing T315I% resulting in reduced ponatinib sensitivity. Notably, during resistance development this intermediate line, T315I mutation was not detectable in 10 nM ponatinib intermediate stage, but a 49% T315I mutation was detected in the higher ponatinib concentration of 20 nM (n=2).

4.3.5 The percentage of T315I in cell lines corresponds to ponatinib sensitivity

As ponatinib was designed to specifically target *BCR-ABL1*^{T315I}, it was necessary to confirm our observation of increasing T315I% correlating to the development of ponatinib resistance. Therefore, we further assessed the effect of the T315I mutation burden on ponatinib sensitivity in an independent cell line, by conducting ponatinib IC50 analyses with a *BCR-ABL1* negative HL60 cell line transduced with either *BCR-ABL1* p210 or *BCR-ABL1*^{T315I} (Figure 4.7 (a) and (b)). *BCR-ABL1*^{T315I} cells were diluted using *BCR-ABL1* p210 cells to generate a T315I serial dilution. Mean ponatinib IC50 values gradually reduced from 56 nM in the 100% *BCR-ABL1*^{T315I}

positive HL60 cell population, to 7 nM in the 10% T315I mutation dilution (HL60-*BCR-ABL1* P210 is 6 nM (Figure 4.7 (b)). Statistically significant reductions were found in the cell populations with 75%, 50% and 25% T315I compared to the preceding dilutions ($p<0.01$, $p=0.04$ and $p=0.03$ respectively, all $n=3$). When dasatinib sensitivity was similarly assessed (Figure 4.7 (c)), incomplete kinase inhibition by the highest concentration rendered dasatinib IC50 values indeterminable for any percentage of T315I. These results suggest that even when only 10% of a cell population is T315I-positive, the mutant clone likely expands with continued dasatinib treatment resulting in overt resistance. In addition, although the sensitivity to ponatinib decreased with increasing T315I, both T315I-positive cells and non-mutant cells were still inhibited at physiologically relevant concentrations. Taken together, these dilution experiments confirm that the percentage of T315I in a cell population significantly impacts sensitivity to ponatinib treatment within the clinical relevant ranges.

Figure 4.6

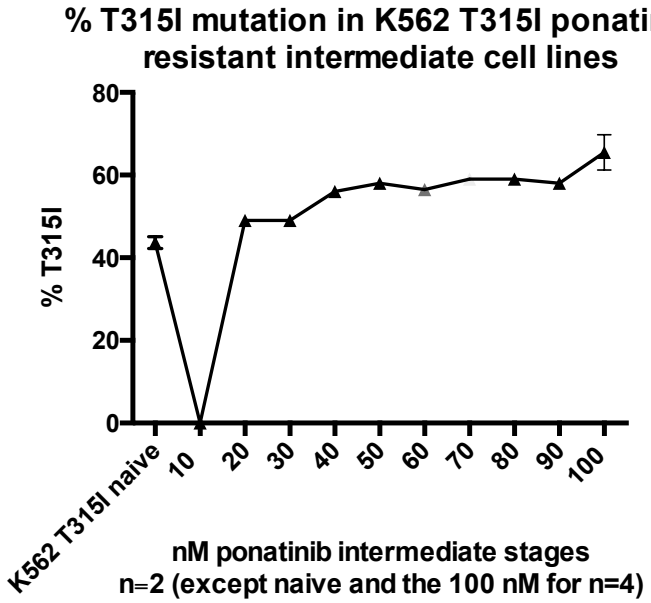


Figure 4.6: Increased T315I% was detected in K562 T315I cell line during development of ponatinib resistance (except for 10 nM intermediate line).
The percentage of T315I mutation was determined by conventional sequencing. Error bars represent SD. n=2 for all except for the naïve control K562 T315I and the K562 T315I-R resistant line.

Figure 4.7 (a)

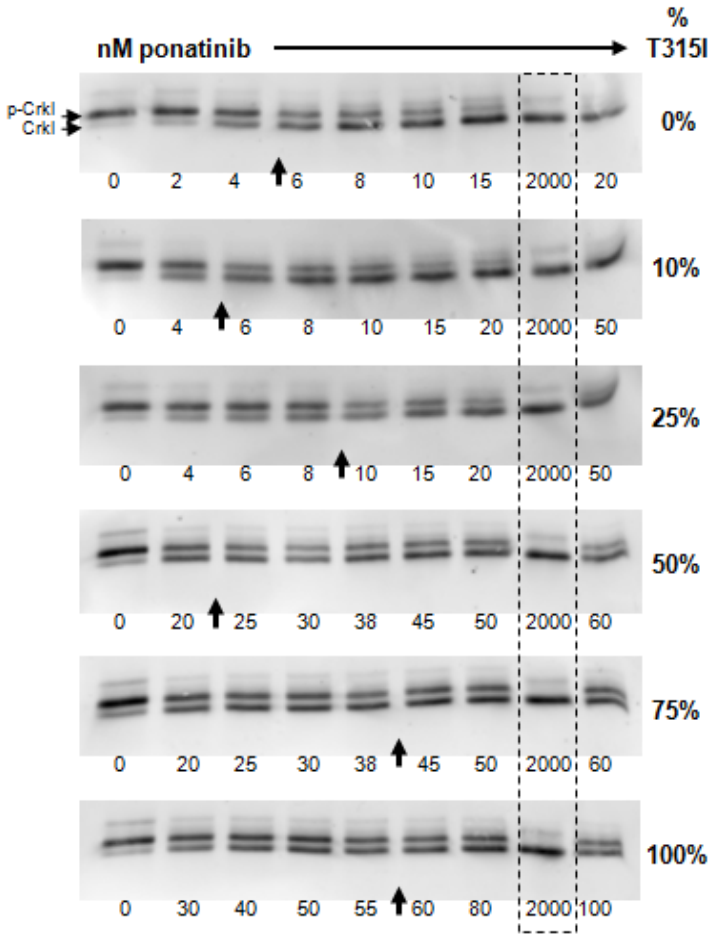

nM ponatinib
%
T315I
p-Crkl
Crkl
0%
0 2 4 6 8 10 15 2000 20
10%
0 4 6 8 10 15 20 2000 50
25%
0 4 6 8 10 15 20 2000 50
50%
0 20 25 30 38 45 50 2000 60
75%
0 20 25 30 38 45 50 2000 60
100%
0 30 40 50 55 60 80 2000 100

Figure 4.7 (b)

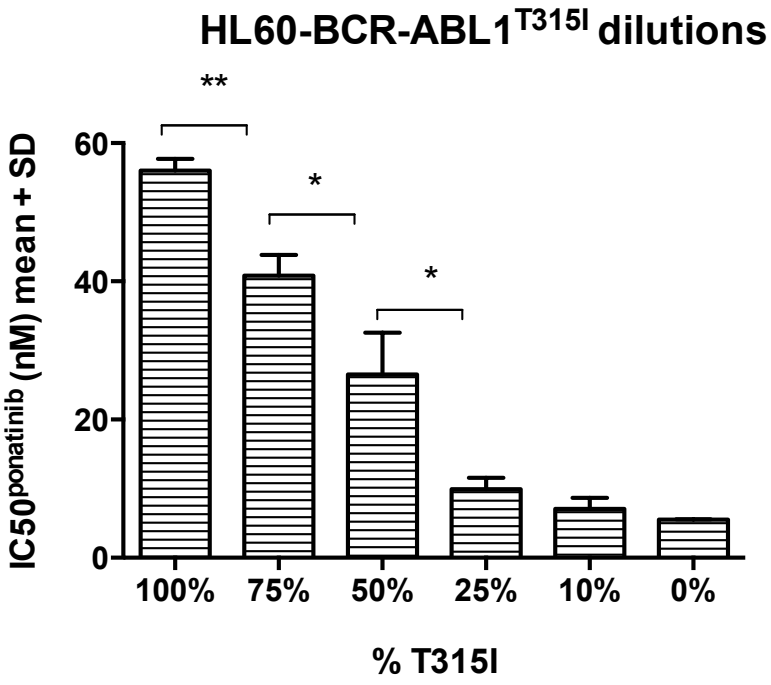

HL60-BCR-ABL1T315I dilutions
**
*
*
IC50ponatinib (nM) mean + SD
60
40
20
0
100%
75%
50%
25%
10%
0%
% T315I

Figure 4.7 (c)

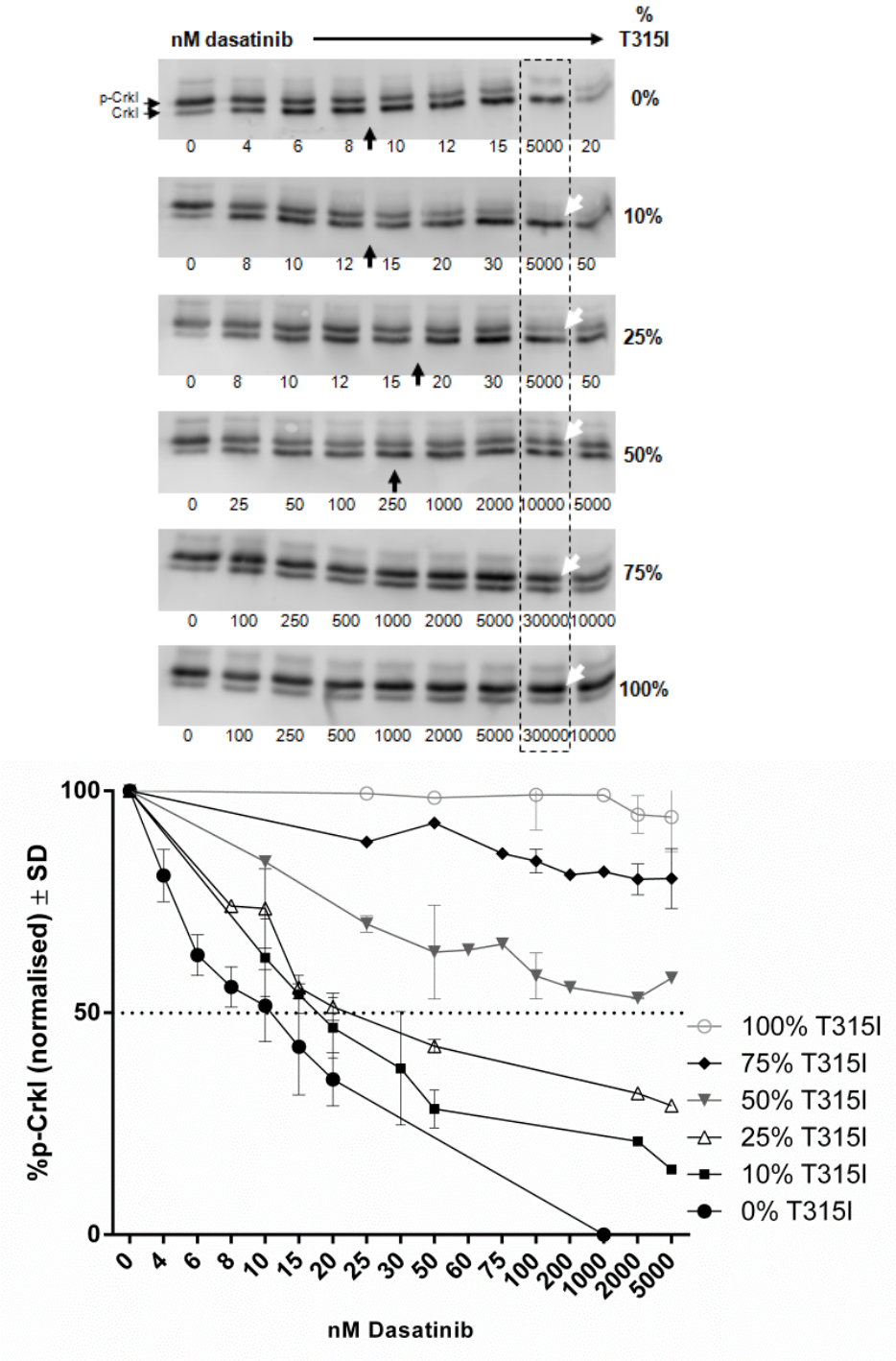

nM dasatinib
% T315I
p-Crkl
Crkl
0%
0 4 6 8 10 12 15 5000 20
10%
0 8 10 12 15 20 30 5000 50
25%
0 8 10 12 15 20 30 5000 50
50%
0 25 50 100 250 1000 2000 10000 5000
75%
0 100 250 500 1000 2000 5000 30000 10000
100%
0 100 250 500 1000 2000 5000 30000 10000
%p-Crkl (normalised) ± SD
100
50
0
0 4 6 8 10 15 20 25 30 50 60 75 100 200 1000 2000 5000
nM Dasatinib
100% T315I
75% T315I
50% T315I
25% T315I
10% T315I
0% T315I

Figure 4.7. Ponatinib IC50 decreased in response to gradual reductions in the percentage of the T315I mutant. (a) Example of the poantinib IC50 western blots of the BCR-ABL1^{T315I} transduced HL60 cell line **(b)** Mean poantinib IC50 values were gradually reduced in the BCR-ABL1^{T315I} transduced HL60 cell line following dilution of the T315I with native BCR-ABL1^{p210} transduced HL60 cells. The reductions were significant in 75% (compared to 100%, p=0.004), 50% (compared to 75%, p=0.04) and 25% (compared to 50%, p=0.03) T315I dilutions (all n=3). *p<0.05 and **p<0.005, data are mean + SD. **(c)** Representative western blotting and graphical analysis of dasatinib IC50 assays of the BCR-ABL1^{T315I} transduced HL60 cell line. Dasatinib was unable to prevent phosphorylation of CrkL at any concentration for either cell line. Data are mean ± SD, n=3. ↑ indicates IC50 value.

4.4 Discussion

The third generation TKI ponatinib is a potent pan-Bcr-Abl inhibitor that effectively targets native and mutant forms of Bcr-Abl, importantly including T315I. However, due to its serious side effects pertaining to the cardiovascular and peripheral vascular systems, this drug was temporarily off market in late 2013 and the third-phase clinical trial, which compared ponatinib with imatinib and investigated ponatinib as a front line therapy, was halted. Since its return to the market in early 2014, it has been used as a second line therapy only for CML and Ph+ ALL patients that are resistant to other TKIs and/or harbouring the T315I mutation. Therefore, a T315I positive K562 cell line model resistant to 100 nM ponatinib was generated in this study to examine potential ponatinib resistance expected to arise with its clinical use.

4.4.1 Kinetics of ponatinib resistance development in the K562 T315I-R cell line

The generation of imatinib, nilotinib or dasatinib resistant cell lines only takes approximately six months to one year using a similar method (51) to that used in this study. Conversely, the generation of ponatinib resistance in this cell line required a significantly longer time in culture with 100 nM resistance being reached after approximately three years. While the human peak and trough plasma levels of ponatinib when dosed at 45 mg once daily are 145 nM and 64 nM respectively (124), we were aiming to generate resistance to 200 nM ponatinib. However, achieving this concentration of ponatinib was difficult with less than 50% of the

cells surviving. I therefore had to choose 100 nM ponatinib to maintain a viable resistant cell line. A possible explanation is that ponatinib has a higher potency in inhibition of Bcr-Abl compared to the other TKIs (besides dasatinib): ponatinib is over 500 times more potent than imatinib (108), therefore making it more difficult for cells to acquire resistance. Additionally, the difficulties of generation of ponatinib resistance in this cell line also suggest that when this TKI is applied as a treatment for T315I positive patients, patients are unlikely to fail ponatinib therapy in a short time by secondary resistance, as it is not easy for the Bcr-Abl^{T315I} cells to develop ponatinib resistance. That means, while ponatinib is the only available TKI to target Bcr-Abl with T315I mutation, it is also the right TKI to use as the development of ponatinib resistance in the cell line habouring a T315I mutation requires a long time. Of note, as the average steady-state ponatinib plasma concentration in patients is 101 nM for dosage at 45 mg/day (109), while this study failed to generate ponatinib resistance to over 145 nM (peak plasma level in patients), the final ponatinib concentration that the K562 T315I-R resistant line was cultured with (100 nM) is still clinically relevant.

4.4.2 Increased proportion of the T315I mutation reduced ponatinib efficacy in the K562 T315I-R cell line

The TKI resistance can be divided into Bcr-Abl dependent and independent mechanisms. In theory, if the resistance is Bcr-Abl dependent, higher doses of TKI are sufficient to inhibit the kinase activity and therefore higher TKI IC50 is expected; if the resistance is Bcr-Abl independent, we would expect no change or

small change in IC50. As the ponatinib IC50 in the cell line is about ten times higher than the control line, this indicates that with the presence of ponatinib, the resistant cells still rely on the Bcr-Abl signal to survive. This confirmed by the increased level of pBcr-Abl, suggesting increased level of Bcr-Abl activity mediates the development of ponatinib resistance. Additionally, the IC50 data suggested that the resistant cell line is not only resistant to ponatinib, but also to other TKIs including imatinib, nilotinib and dasatinib, indicating that it has a TKI cross-resistance. This cross-resistance was within expectation, as when Bcr-Abl harbours the T315I mutation, it confers resistance to the first and second generation TKIs.

As described in Chapter 1, ponatinib is unique in targeting Bcr-Abl^{T315I} among other currently available TKIs (108). Importantly, *in vivo* and *in vitro* experiments (108) have previously demonstrated that ponatinib is the only approved TKI agent effective against Bcr-Abl with the T315I gatekeeper mutation. In this study, we have confirmed the *in vitro* sensitivity of *BCR-ABL1*+ cell lines with T315I mutation (in K562 T315I and in HL60^{T315I} cell lines) to ponatinib. However, in the resistant cell line with higher percentage of T315I mutation (66%), it was found to have a significantly higher IC50ponatinib value compared to the control cell line expressing lower percentage of T315I (44%). This suggests that increases in the percentage of T315I results in reduced sensitivity to ponatinib. For the first time, we show that the effect of the percentage of T315I mutation is only specific for ponatinib not dasatinib. Moreover, all of these results suggest that while the cells with the T315I mutation are only sensitive to ponatinib among currently available

TKIs, the interaction of ponatinib with Bcr-Abl with the T315I mutant remains weaker than its interaction with Bcr-Abl^{p210} (103, 108), therefore, decreasing the overall *BCR-ABL1* load increases sensitivity to ponatinib.

4.4.3 Increase *BCR-ABL1* transcript expression mediates ponatinib resistance in the K562 T315I-R cell line

While increasing levels of the T315I mutation is one of the key mechanisms for ponatinib resistance in the cell line, it is not the only reason that the cell line exhibits resistance. After a step-wise increase in *BCR-ABL1* mRNA in the intermediate lines, the expression level of *BCR-ABL1* was significantly higher in the resistant line compared to the control line. Therefore, these data raise the possibility that increased un-mutated *BCR-ABL1* mRNA expression may mediate a degree of ponatinib resistance. *BCR-ABL1* positive cells require Bcr-Abl kinase to activate oncogenic signalling pathways to proliferate and survive. As Bcr-Abl is the primary target of ponatinib, it is possible that increased *BCR-ABL1* mRNA expression results in increased Bcr-Abl protein, which then requires higher concentration of ponatinib to target all the active kinase in all the resistant cells. This finding is consistent with a previous study from our group that increased *BCR-ABL1* transcript level (approximately 1200%-1500%) in two K562 imatinib resistant cell lines resulted in 50% reduction in ponatinib sensitivities (125). Furthermore, the Bcr-Abl and the CrkL western blots confirm that the increased expression of *BCR-ABL1* mRNA in the K562 T315I-R resistant line indeed translates to increased expression of active Bcr-Abl protein. The significantly higher

IC50ponatinib values observed in the *BCR-ABL1* overexpressing resistant cell line suggests that elevated levels of *BCR-ABL1* mRNA are likely translated to increased kinase activity, and therefore required higher doses of ponatinib to inhibit kinase activity. Notably, while it is likely that increased *BCR-ABL1* mRNA contributes to the insensitivity to ponatinib demonstrated by the resistant line, it is also very likely that the increase in *BCR-ABL1* results in a proportional increase in T315I. Therefore, the increase in the percentage of the T315I in this cell line further reduces its sensitivity to ponatinib.

Interestingly, the intermediate line K562 T315I 10 nM PON was found to be T315I negative. One explanation is that the 10 nM intermediate stage contains a low level T315I mutation but this mutation could not be detected by the current method. Conventional sequencing is not sensitive enough to detect the low percentage mutation (below 10%), and the software can not reliably call any mutation lower than 20%. To verify this hypothesis, a more sensitive method, such as next generation sequencing, will be required in the further study. Another explanation is that as this intermediate line habours significant *BCR-ABL1* overexpression, this increase in *BCR-ABL1* transcripts is sufficient to reduce sensitivity to ponatinib (up to 10 nM, and from 20 nM, the T315I mutation, together with the *BCR-ABL1* overexpression, are required to maintain the survival under the stress of ponatinib). When this cell line was exposed to higher concentrations of ponatinib, *BCR-ABL1* overexpression is not enough to maintain resistance, and therefore the T315I mutation is also required to mediate survival. Hence, this raises a possible scenario

that this 10 nM intermediate cell line habours two or more populations. One population, with *BCR-ABL1* overexpression but not T315I mutation, is mildly resistant to ponatinib; while another population, containing T315I mutation as well as *BCR-ABL1* overexpression, is the highly resistant population. Under the condition of no TKI or lower doses of TKI, the highly resistant population does not have advantage over the mildly resistant clone and therefore the highly resistant population is reduced. A previous study (Tang et. al.) demonstrates a similar reduction in T315I percentage due to a possible similar clonal selection (51). When dasatinib was removed from the K562 T315I cell line, the T315I% reduced from 40% to about 10% (51). Although this was observed when drug was removed and the mutation was detectable, while in the current study the cells were still incubated with low concentration of ponatinib and the mutation was undetectable, in the both studies, the cells were under no stress or very low stress of TKI (dasatinib or ponatinib). Meanwhile, both studies demonstrate the outgrowth of the unmutated clone (with *BCR-ABL1* overexpression in the current case) over the mutated clone. Therefore, as the highly resistant population only plays a small part in the 10 nM intermediate line, the level of T315I mutation is too low to be detected by conventional sequencing. This two-clone scenario was supported by the RQ-PCR result: the *BCR-ABL1* transcript level increased from 1206% in the ponatinib naive control to 3050% in the 10 nM intermediate line, however, this overexpression is not statistically significant due to the inconsistency of the RQ-PCR result (n=3), suggesting the likely presence of two or more clones. However, this hypothesis needs to be confirmed with further clonal experiments. Moreover, the T315I

mutation positive highly resistant population was selected to grow and expand under the stress of higher concentration of ponatinib when cultured with higher dosage of the TKI. Therefore, the T315I mutation was detectable in the higher ponatinib culture intermediate stage.

4.4.4 Summary

Overall, this study suggests that increased *BCR-ABL1* overexpression together with increased T315I mutation results in ponatinib resistance in the cell line. As ponatinib is not transported by ABCB1, ABCG2 or OCT-1 (as described in Chapter 3), as expected ponatinib resistance development is not mediated by the up-regulation of these transporters.

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Chapter 5 :

BCR-ABL1 compound mutation G250E/E255K mediates resistance in K562 DOX 55D-R cell line

5.1 Introduction

5.1.1 Compound mutation in Bcr-Abl kinase domain

The best-characterized mechanism of secondary resistance to TKI therapy in CML is the development of mutations within the Bcr-Abl kinase domain (KD). Some CML patients develop two or more KD mutations, either in multiple *BCR-ABL1* clones (polyclonal mutations), or in the same *BCR-ABL1* molecule (compound mutations). Interestingly, a previous study suggested that the majority of multiple mutations are compound mutations (126). In a recent study, Khorashad and colleagues (126) analysed approximately 1700 CML patient samples (all of these samples harboured at least one Bcr-Abl KD mutation) and revealed that 11.4% of the samples contained two or more Bcr-Abl KD mutations, of these approximately 70% were compound mutations and the remaining 30% polyclonal mutations. In addition, this previous study revealed that among the observed Bcr-Abl compound mutations, T315I-inclusive compound mutations (e.g. G250E/T315I, E255V/T315I) were the most frequent (126).

While single point mutations in multiple clones can usually be targeted by one or more TKIs, Bcr-Abl with compound mutations can be highly resistant to all TKIs, especially compound mutations that involve the T315I mutation. As the T315I mutation alone is known to cause Bcr-Abl resistance to all the first and second generation TKIs, cells harbouring T315I-inclusive compound mutations are expected to be insensitive to these TKIs. While ponatinib effectively inhibits the Bcr-

Abl^{T315I}, interestingly, a study conducted by Zabriskie *et al.* (109) using BaF3 cell lines and Ph+ leukaemia patient samples (n=64) revealed that T315I inclusive Bcr-Abl compound mutations were associated with not only imatinib, nilotinib and dasatinib but also ponatinib resistance. (109). This study demonstrated that cells that harboured T315I-inclusive compound mutations including T315I/M351T, T315I/F359V, T315I/H396R and T315I/E453K were marginally sensitive to ponatinib with an intermediate IC50 85-104 nM (109). However, cells that harboured compound mutations T315I/G250E, T315I/Q252H, T315I/Y253H and T315I/F311I exhibited high-level ponatinib resistance (IC50>150 nM) (109). Furthermore, cells that harboured the compound mutation T315I/E255V were highly resistant to ponatinib (IC50: 659.5 nM), and this was the most resistant mutant combination among all of the tested compound mutations (109). Crystal structure analysis of ponatinib binding to Bcr-Abl$^{T315I/E255V}$ revealed that this compound mutation reduced the binding affinity of ponatinib to Bcr-Abl compared to Bcr-Abl^{T315I} alone, by reorientation of the P-loop and C-helix necessary to accommodate the hydrophobic V255 side chain (109). This conformational change results in narrowing of the 'channel' in which ponatinib normally binds and therefore leading to ponatinib resistance (109). Of note, cells with all of the tested non-T315I-inclusive compound mutations were sensitive to at least one TKI (109).

These two previous studies suggest that the development of compound mutations could be induced by sequential treatments with TKIs (e.g patient treated with imatinib then switched to dasatinib or nilotinib), as the composition of the

compound mutations in the Ph+ leukaemic cells in patients reflects their TKI treatment history (109, 126). The theory behind this is that leukaemia persistence requires continuous acquisition of genetic mutations, and the use of the first and second generation TKIs selects the evolution of Bcr-Abl compound mutant clones that have survival advantage under TKI pressure. However, whether the use of first/second generation TKI followed by the third generation TKI ponatinib would create an environment conducive to compound mutations development remains unknown.

5.1.2 ABCB1 overexpression

In animals, fungi and bacteria, ATP binding cassette (ABC) transporters locate in the cell membrane and are responsible for ATP-dependent efflux of various molecules. The substrates of ABC transporters include carbohydrates, amino acids, lipids, organic anions, vitamins, xenobiotics/toxins and glucoronide, glutathione conjugates (127, 128). ABC transporters are involved in defending brain and haematopoietic stem cells against foreign invading xenobiotics. Alteration in ABC transporters expression has been associated with drug resistance to chemotherapy agents, especially the upregulation of ABCB1, also known as multidrug-resistant protein 1 (129). ABCB1 is a member of ABC super family and known to interact with TKIs including imatinib, dasatinib and nilotinib (83, 130-138). As the effective treatment of CML is based on the use of TKIs, sufficient intracellular concentrations of TKIs are required to bind to and inhibit Bcr-Abl. The alteration of TKI transporter

expression, such as ABCB1, may reduce the intracellular level of TKI, and therefore affect the outcome of CML treatment.

As described in Chapter 1, it is well documented that the upregulation of ABCB1 is associated with TKI resistance as ABCB1 is the efflux transporter for all three aforementioned TKIs. However, ABCB1 is not the key efflux transporter for ponatinib as described in Chapter 3 (139), as ponatinib appears to be passively transported. A recent study (110) suggested that ponatinib is a weak ABCB1 inhibitor. In this study, the authors incubated ABCB1 overexpressing K562 cells with ponatinib and the fluorescent ABCB1 substrate 3,3'-diethyloxacarbocyanine iodide (DiOC2(3)), and observed that ponatinib marginally increased the retention of the DiOC2(3), indicating that ABCB1 was weakly inhibited by ponatinib. As this study indicated an interaction of ABCB1 with ponatinib, this chapter aims to further investigate whether ABCB1 is involved in the development of ponatinib resistance. Therefore, an ABCB1 overexpressing cell line, would be required to be used to develop ponatinib resistance.

5.1.3 The K562 DOX 55D cell line

As mentioned in section 5.1.1 and 5.1.2, this chapter aims to investigate 1. the potential for compound mutation development in a sequential TKI treatment setting, and 2. the association of ABCB1 overexpression with ponatinib resistance. Hence, an ABCB1 overexpressing *BCR-ABL1* positive cell line K562 DOX 55D, which had been exposed previously to dasatinib, was utilised to generate ponatinib

resistance in this study. This cell line was established by Tang *et al.* by exposing K562 DOX cell line to increasing concentration of dasatinib up to 55 nM with no mutation detected (51). However, when this cell line was continuously treated with incrementally increasing doses of dasatinib subsequently reaching 200 nM, a *BCR-ABL1*^{V299L} mutation emerged. Additionally, this K562 DOX 55D line was also treated with nilotinib up to 500 nM and a *BCR-ABL1*^{G250E} mutation, emerged. As two different mutations developed in this cell line after culturing with different TKIs, this cell line was considered to be 'hyper mutatable' (mutations easily develop in this cell line). Therefore, this cell line was predicted to be informative in terms of identifying key mutations when this cell line was cultured with ponatinib.

5.2 Approach

5.2.1 Generation of ponatinib resistant cell lines

During the *in vitro* generation of ponatinib resistance, the intermediate stages of resistance were analysed for the emergence of resistant clones. The same method was used to generate ponatinib resistant cell lines as described in Chapter 4, based on the methods of Mahon *et al.* and Tang *et al.* (51, 81). Firstly, dasatinib in the K562 DOX 55D cell line was withdrawn, and this line was subsequently exposed to low concentrations of ponatinib starting at 5 nM. Ponatinib concentrations were then increased approximately every 10-60 days, as cells tolerated each increased dose. In patient plasma, median peak and trough levels of ponatinib are 145nM and 64nM respectively when dosed at 45mg once daily (124). Therefore, 200 nM ponatinib

was chosen as the final culture concentration in the resistant line. As this concentration is above the upper limit of patient plasma level, it ensures the ponatinib resistance in the cells is well established. Ponatinib resistant K562 DOX 55D-R cell line was generated, and this cell line was escalated as follows (over approximately one year): 5 nM, 8 nM, 11 nM, 15 nM, 20 nM, 25 nM, 30 nM, 40 nM, 50 nM, 100 nM and 200 nM.

After the K562 DOX 55D-R resistant line was generated, compound mutations were observed in this line (will be described in the result section 5.3.4). To further investigate the compound mutations under the stress of high ponatinib concentration, the resistant line K562 DOX 55D-R was further exposed to higher concentrations of ponatinib to develop 'high-dose' resistant cell lines (cell lines that were cultured with ponatinib concentrations above clinically achievable levels): K562 DOX 55D 500nM PON, K562 DOX 55D 1μM PON, K562 DOX 55D 2μM PON (Figure 5.1). These cell lines, as their names suggest, were generated by culturing with 500 nM, 1 μM and 2 μM ponatinib respectively. To recapitulate the development of these resistance, frozen stock of the K562 DOX 55D-R (200 nM) were thawed and independently re-escalated as follows: 500 nM, 750 nM and 1 μM. The 750 nM intermediate stage was established as this cell line struggled to acclimatize to 1 μM directly from 500 nM. Therefore, another three cell lines (named re-escalate 'high-dose' resistant cell lines) were generated: K562 DOX 55D 500nM PON (2), K562 DOX 55D 750nM PON(2) and K562 DOX 55D 1μM PON(2) (Figure 5.1).

5.2.2 Analysis of ponatinib-resistant cell line

The same methods were performed to investigate and analyse ponatinib resistance as described in Chapter 4 (4.2.2)

Figure 5.1

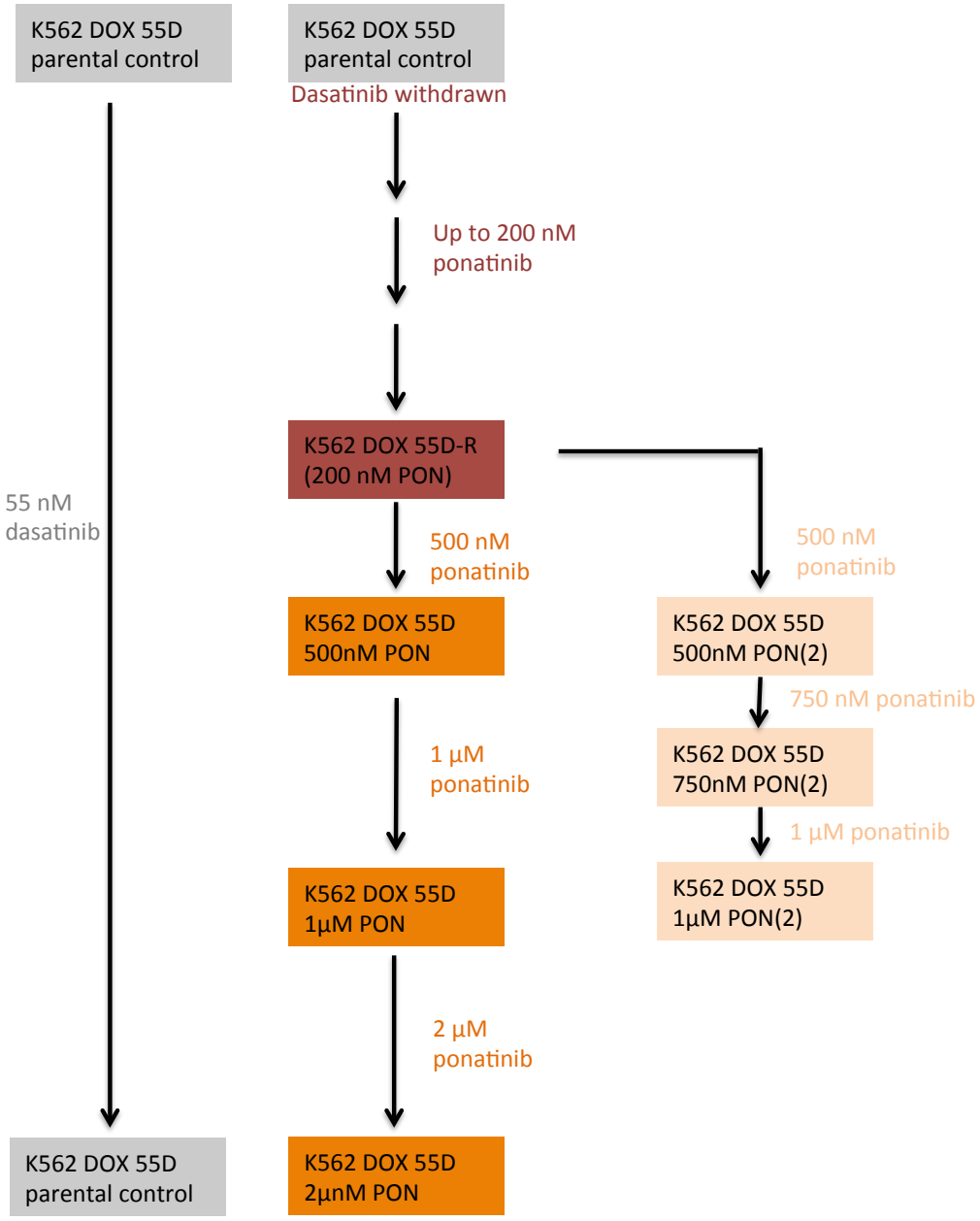


Figure 5.1: Schematic summary of ponatinib resistance generation in K562 DOX 55D cells. K562 DOX 55D cells were cultured in gradually increasing concentrations of ponatinib. Gray indicates parental control line. Red indicates 200 nM ponatinib resistant line K562 DOX 55D-R. Dark orange indicates 'high-dose' resistant cell lines. Light orange indicates re-escalated 'high-dose' resistant cell lines.

5.3 Results

5.3.1 K562 DOX 55D cells cultured in 200 nM ponatinib demonstrate resistance to TKIs *in vitro*

Ponatinib resistant K562 DOX 55D-R cells were generated by exposure to gradually increasing concentration of ponatinib from 5 nM to 200 nM. Viability of the cells in the presence of ponatinib was determined by trypan blue exclusion counts. Generation of ponatinib resistance in this cell line took approximately one year. This process took slightly longer compared to the generation of nilotinib/dasatinib resistance (approximately 8 months) in the same cell line (51). This observation is possibly due to the pan-inhibition of Bcr-Abl (effectively inhibits Bcr-Abl with all the identified single KD mutations) by ponatinib, and the higher potency of ponatinib compared to other TKIs in targeting Bcr-Abl.

CrkL based ponatinib IC50 was performed to determine the level of ponatinib resistance in the K562 DOX 55D-R cell line. As shown in Figure 5.2 (a)-(b), K562 DOX 55D-R cells demonstrated significantly higher ($p<0.001$, $n=3$) ponatinib IC50 (478 nM) compared to K562 DOX 55D control cells (51 nM) (Figure 5.2(a) and 5.2(c)). Notably, ponatinib IC50 in the ponatinib naïve control cell line K562 DOX 55D (51 nM) was significantly higher ($p=0.0016$, $n=3$) compared to the original TKI naïve parental control cell line K562 DOX (8.7 nM) (Figure 5.2 (a) and 5.2 (d)). This was due to the fact that *BCR-ABL1* mRNA and ABCB1 protein was overexpressed in this ponatinib naïve control cell line.

Figure 5.2 (a)

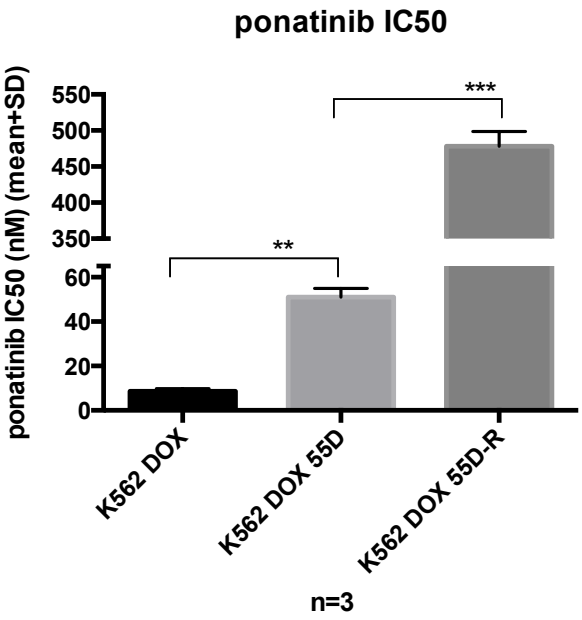

ponatinib IC50
ponatinib IC50 (nM) (mean+SD)
550
500
450
400
350
60
40
20
0
**
***
K562 DOX
K562 DOX 55D
K562 DOX 55D-R
n=3

Figure 5.2 (b)

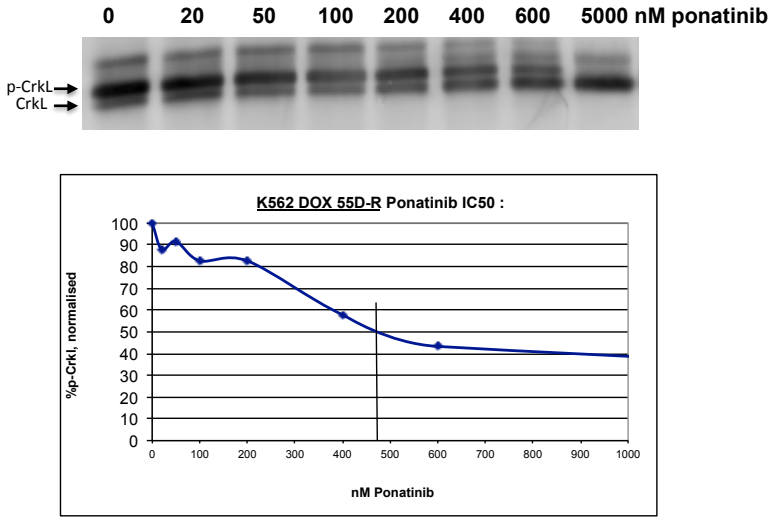


Figure 5.2 (c)

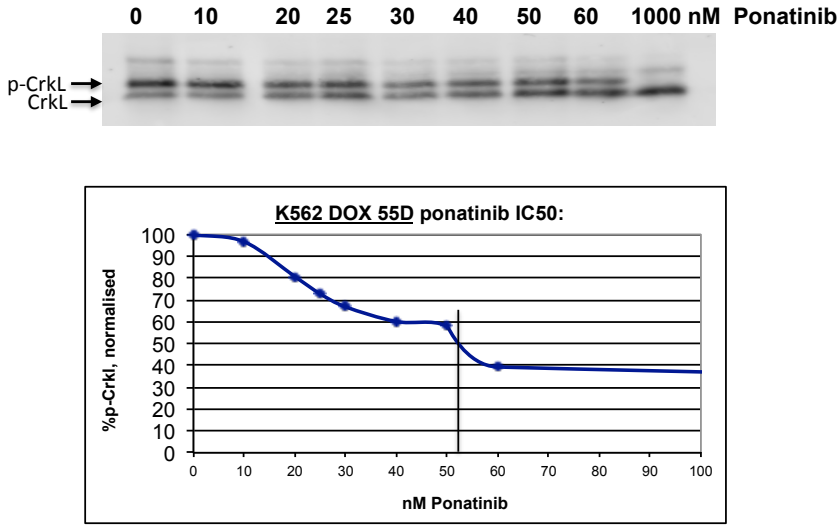


Figure 5.2 (d)

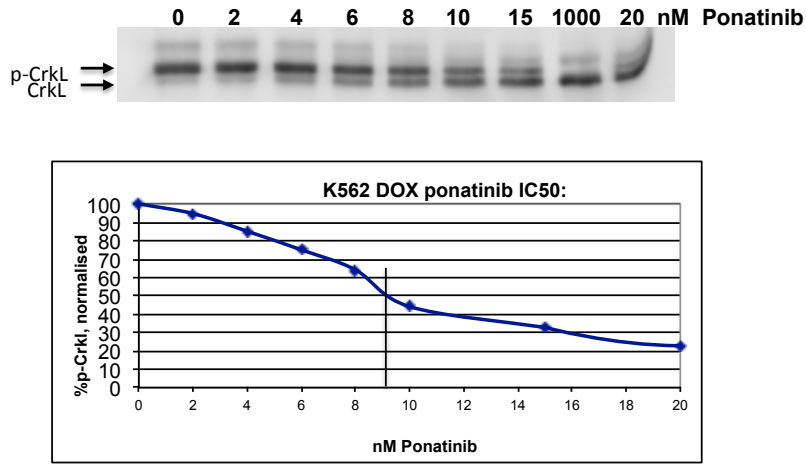


Figure 5.2: Ponatinib IC50 for K562 DOX 55D-R was significantly higher than K562 DOX 55D control line

Cells were incubated for 2 hours with serial concentration of ponatinib and then lysed to perform p-CrkL western blotting. **(a)** Increased ponatinib IC50s were observed in the K562 DOX 55D-R cell line compared to the ponatinib naive K562 DOX 55D cell line. Error bars represent SD. n=3 for all data, **p<0.01, ***p<0.001. Representative of **(b)** K562 DOX 55D-R **(c)** K562 DOX 55D and **(d)** TKI naïve K562 DOX ponatinib IC50 western blots analyzed. Top panel: representative western blotting image. Bottom panel: analysis of the representative western blot. Ponatinib concentration required to reduce the ratio of p-CrkL:CrkL by 50% (ponatinib IC50) is indicated by the black line.

Furthermore, resistance was also confirmed by 7AAD and annexin V staining after 72 hours ponatinib exposure (Figure 5.3 (a)): the K562 DOX 55D-R cells demonstrated only a 6% reduction in survival when cultured with 300 nM ponatinib compared to 0 nM ponatinib treated cells (this control was normalized to 100%), while the survival reduction was 89% in the control line K562 DOX 55D ($p<0.001$, $n=3$). In addition, compared to the ponatinib naïve control K562 DOX 55D, the K562 DOX 55D-R cell line exhibited resistance to other TKIs including dasatinib (from 50 nM to 1000 nM, 0% to 19% reduction in viabilities, all $p<0.001$, $n=3$), imatinib (from 500 nM to 5000 nM, 3% to 1% increase in viabilities compared to no imatinib control, all $p<0.01$, $n=3$) and nilotinib (from 50 nM to 5000nM, 3% reduction to 3% increase in viabilities compared to no nilotinib treatment control, all $p<0.001$, $n=3$) (Figure 5.3(b)-(d)). As the control cell line K562 DOX 55D had previously been generated with dasatinib, this cell line demonstrated reduced sensitivity to dasatinib (from 50 nM to 200 nM, 24% to 57% reduction in viabilities compared to non dasatinib treatment control, all $p<0.001$, $n=3$), imatinib (from 500 nM to 5000 nM, 0% to 34% reduction in viabilities compared to 0 nM imatinib treatment group, all $p<0.001$, $n=3$) and nilotinib (50 nM, 1% reduction in viability, $p<0.01$, $n=3$) (Figure 5.3(b)-(d)) compared to TKI naïve K562 DOX cell line.

Figure 5.3

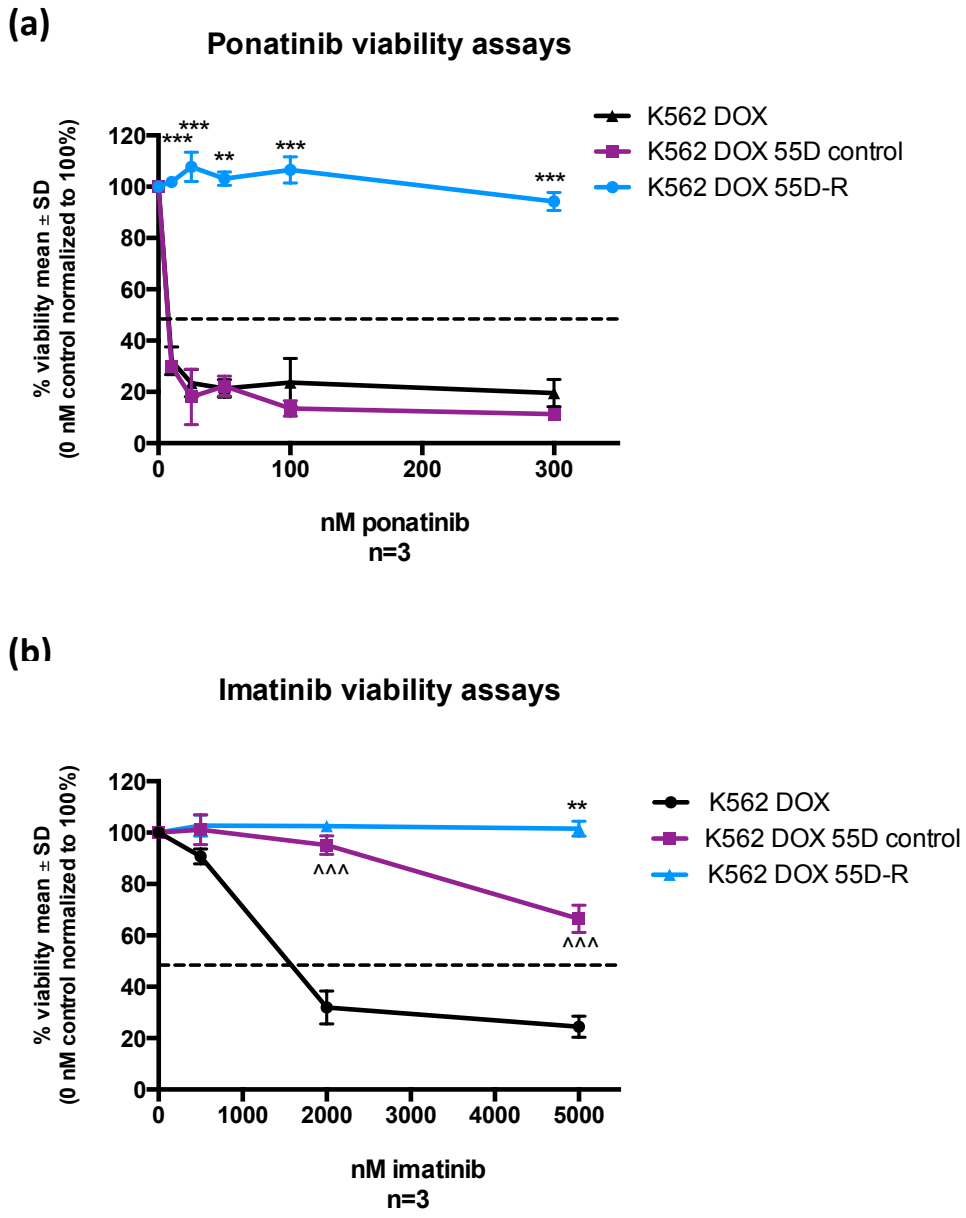
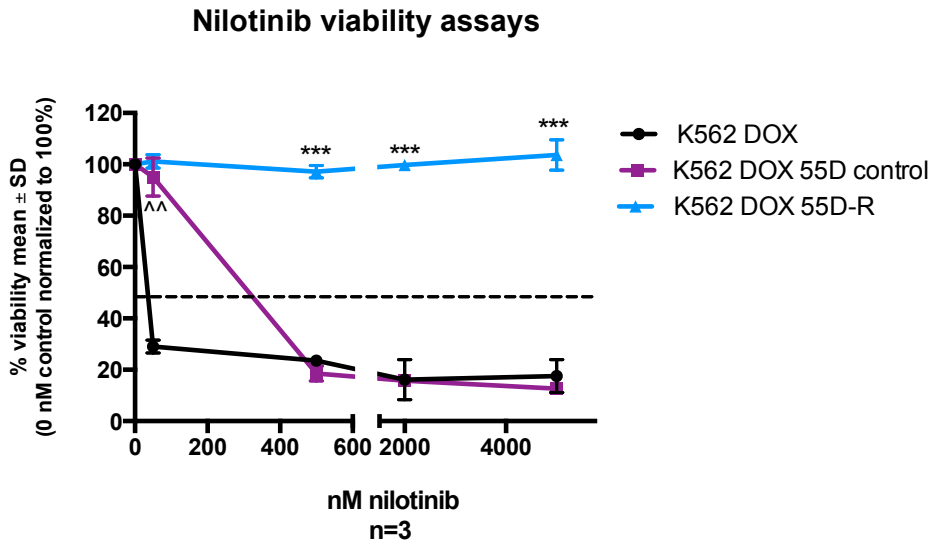

(a)
Ponatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
0
100
200
300
***
***
**
***
***
nM ponatinib
n=3
K562 DOX
K562 DOX 55D control
K562 DOX 55D-R
(b)
Imatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
0
1000
2000
3000
4000
5000
**
^^^
^^^
nM imatinib
n=3
K562 DOX
K562 DOX 55D control
K562 DOX 55D-R

Figure 5.3

(c)


Nilotinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
0
200
400
600
2000
4000
***
***
***
^^
nM nilotinib
n=3
K562 DOX
K562 DOX 55D control
K562 DOX 55D-R

(d)

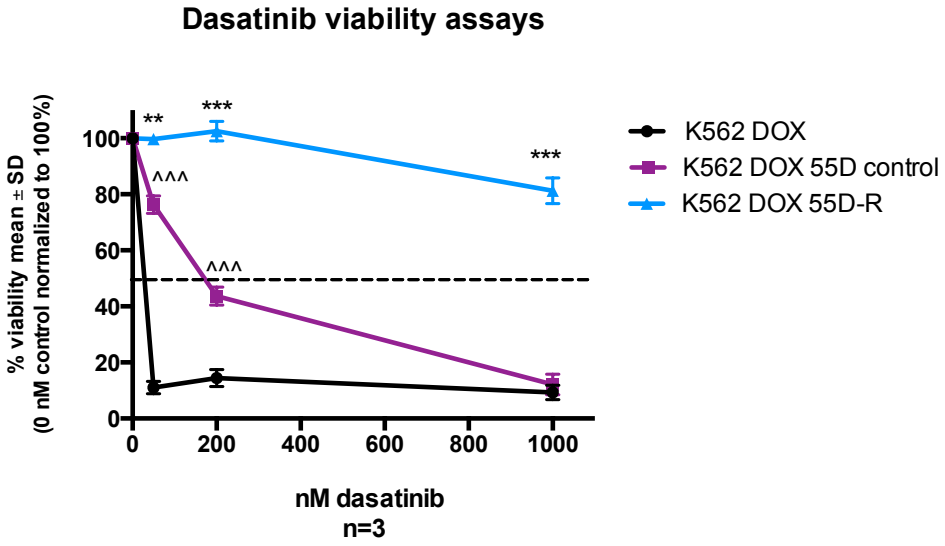

Dasatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
100
80
60
40
20
0
0
200
400
600
800
1000
**
***
***
^^^
^^^
nM dasatinib
n=3
K562 DOX
K562 DOX 55D control
K562 DOX 55D-R

Figure 5.3: ponatinib resistant K562 DOX 55D-R cells are also resistant to imatinib, nilotinib and dasatinib mediated cell death

The K562 DOX (black line), K562 DOX 55D control (purple line) and K562 DOX 55D-R (blue line) cells were cultured with serial concentrations of TKI for 72 hours, followed by staining with 7AAD and annexin V to determine cell death. 0 nM TKI treatment groups were normalised to 100%. The change in viabilities induced by TKI treatment were compared between the cell lines. The K562 DOX 55D-R cells demonstrated significantly improved survival compared to the K562 DOX 55D control line in all of the tested concentration of **(a)** ponatinib (10 nM-300 nM), **(b)** imatinib (5000 nM, not 100 nM-2000 nM), **(c)** nilotinib (500 nM-5000 nM) and **(d)** dasatinib (50 nM-1000 nM). Black dash lines indicate 50% reduction in viability. Error bars represent SD. n=3 for all data, **p<0.01 and ***p<0.005 compared to K562 DOX 55D-R group, ^^p<0.01 and ^^^p<0.005 compared to K562 DOX group.

5.3.2 ABCB1 is transiently up-regulated in the intermediate stages of the K562 DOX 55D ponatinib resistant cell line

The surface expression levels of ABCG2 and ABCB1 were measured in the resistant cell line K562 DOX 55D-R. Flow cytometry did not detect ABCG2 expression in the K562 DOX 55D-R resistant cell line or any of the intermediate lines, or in the K562 DOX 55D parental control line (Figure 5.4). K562 ABCG2 cell line was used as a positive control for the staining of ABCG2 (Figure 5.4). While the control line K562 DOX 55D exhibited a strong cell surface expression of ABCB1 compared to K562 DOX (68% increase in MFI, $p<0.001$, $n=3$), a significant reduction (28%) in ABCB1 expression was observed firstly in the 20nM PON intermediate stage ($p<0.05$, $n=3$), followed by a 60% increase in ABCB1 expression in the 30 nM intermediate stage of the resistant line (Figure 5.5 (a)). However, this significant upregulation was transient and only presented in this one intermediate line. The ABCB1 expression was significantly reduced in the later stages of ponatinib resistance (100 nM and 200 nM, 33% and 30% reductions respectively) compared to the K562 DOX 55D control (both $p<0.001$, both $n=3$) (Figure 5.5(a)-(b)). K562 was used as a negative control for the staining of ABCB1 as this cell line expresses undetectable level of cell surface ABCB1 (by flow cytometry). These results suggest that while transiently increased ABCB1 expression might possibly be an initiator of, or is required for, early ponatinib resistance, it is not the major cause of ponatinib resistance in this cell line.

Figure 5.4

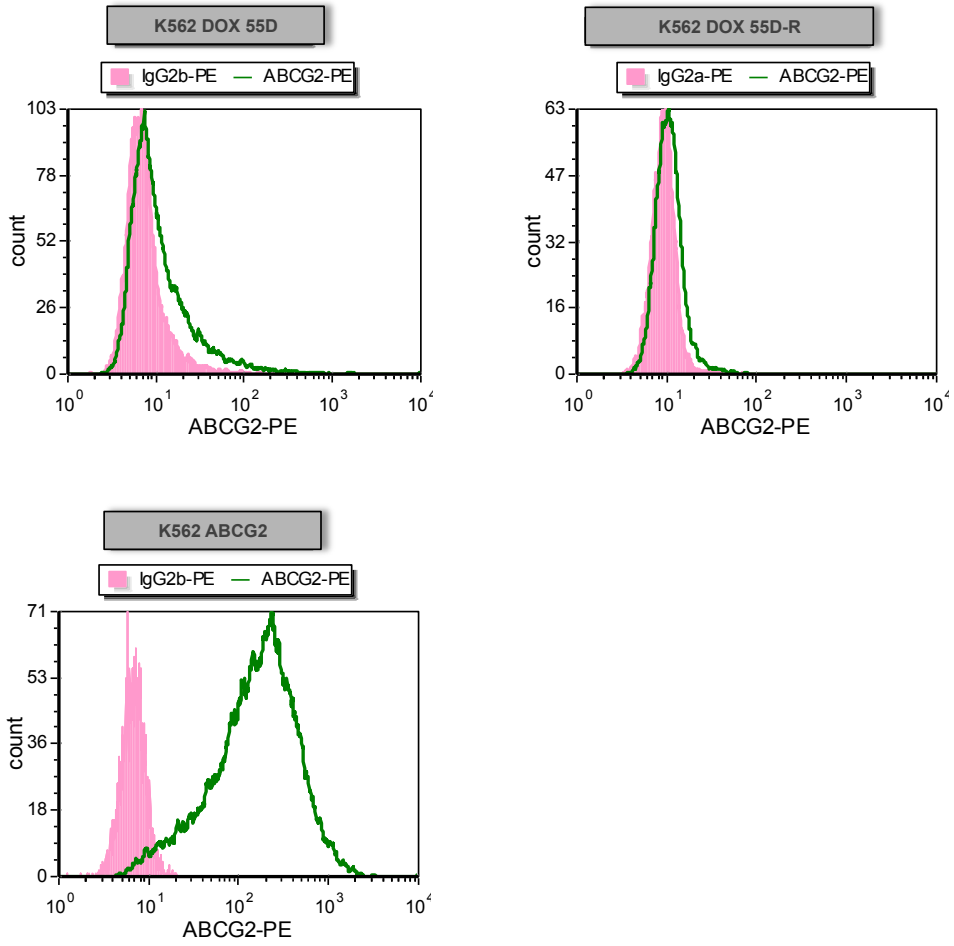


Figure 5.4: The K562 DOX 55D and The K562 DOX 55D-R cell lines do not express ABCG2. Cells from K562 DOX 55D control line and K562 DOX 55D-R resistant line were collected and stained with either IgG2b PE isotype control antibody (pink) or the corresponding ABCG2 PE antibody (green). No ABCG2 expression was detected in the K562 DOX 55D control cells or the K562 DOX 55D-R resistant cells. The staining of K562 ABCG2 cells was used as a positive control.

Figure 5.5 (a)

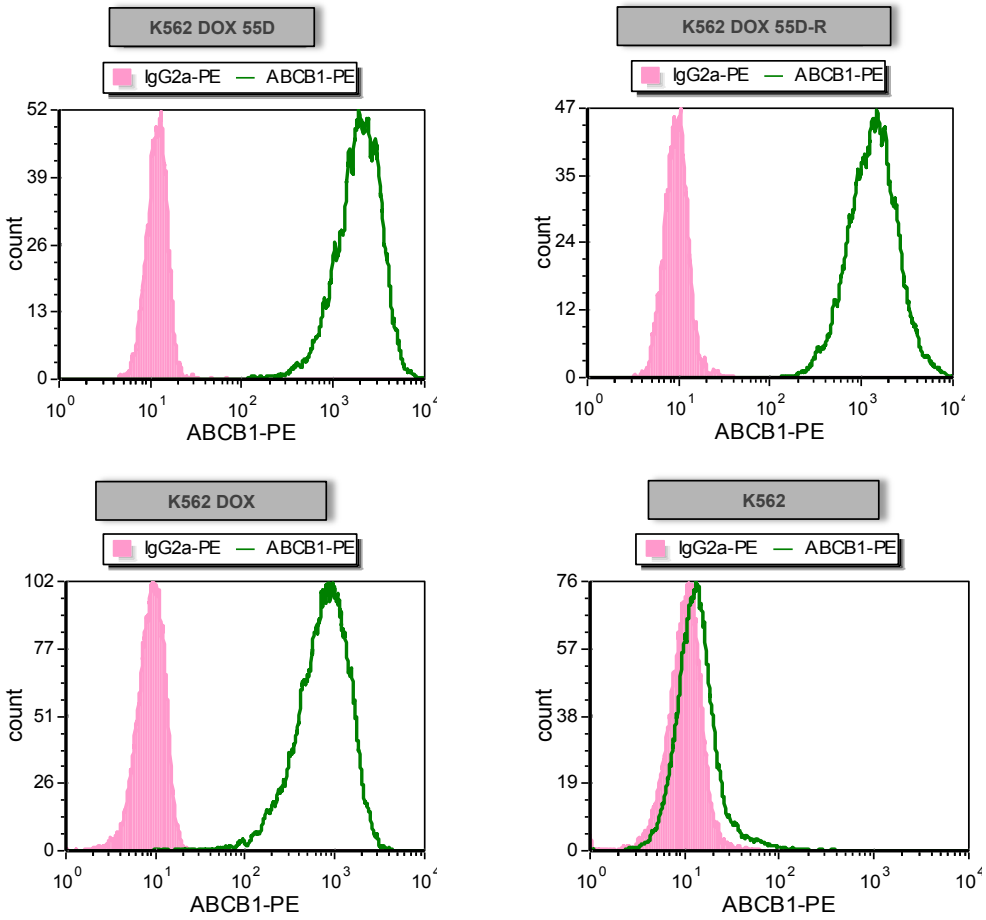

K562 DOX 55D
IgG2a-PE
ABCB1-PE
count
ABCB1-PE
K562 DOX 55D-R
IgG2a-PE
ABCB1-PE
count
ABCB1-PE
K562 DOX
IgG2a-PE
ABCB1-PE
count
ABCB1-PE
K562
IgG2a-PE
ABCB1-PE
count
ABCB1-PE

Figure 5.5 (b)

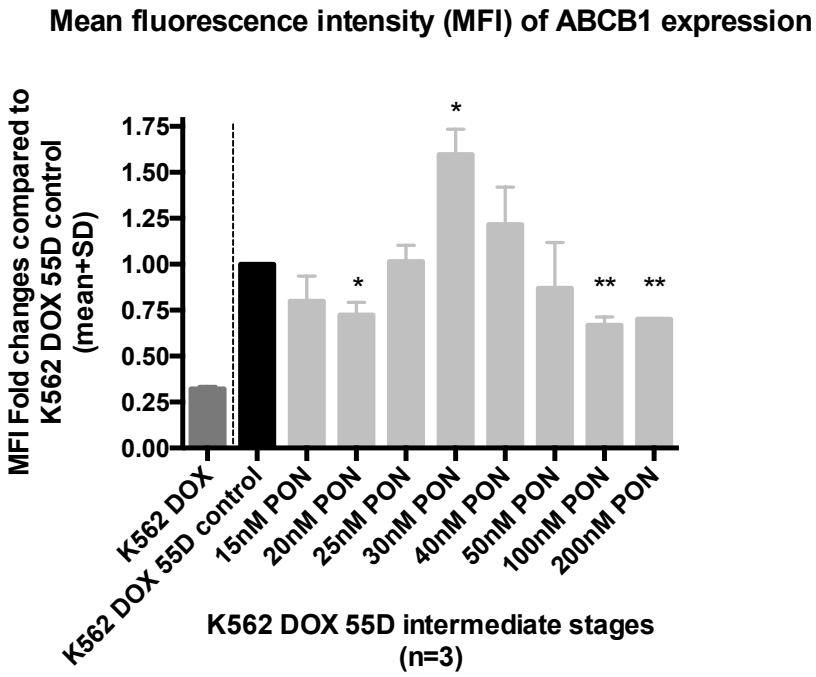


Figure 5.5: The K562 DOX 55D-R resistant cell line did not express increase level of ABCB1. K562 DOX, K562 DOX 55D and K562 DOX 55D-R cells were stained with either IgG2a PE isotype control antibody or the corresponding ABCB1 PE antibody. **(a)** Representative of flow cytometry staining of ABCB1 in the ponatinib naïve control K562 DOX 55D and the K562 DOX 55D-R resistant cells, as well as in the experimental control K562 DOX cells. K562 was used as negative control for ABCB1 staining. . **(b)** The geometric mean of mean fluorescent intensity (MFI) was compared between the ponatinib naïve control K562 DOX 55D and each intermediate stage of ponatinib resistance development as well as the final resistant cell line K562 DOX 55D-R and represented graphically as MFI fold change. n=3. *p<0.05 and ** p<0.01 compared to K562 DOX 55D cells.

5.3.3 K562 DOX 55D ponatinib resistance-developing cell lines demonstrate transient *BCR-ABL1* mRNA overexpression

Pervious studies have demonstrated that increases in *BCR-ABL1* transcript reduce sensitivity to TKIs in CML patients (54, 122), therefore, the *BCR-ABL1* mRNA level (results determined as *BCR-ABL1*/*BCR*% and expressed as a percentage) in the ponatinib resistant cell line as well as in the intermediate stages was quantitated by RT-QPCR. As shown in Figure 5.6, a step-wise significant increase in *BCR-ABL1* mRNA was observed in the intermediate stages of resistance, from 1069% in the K562 DOX 55D ponatinib naïve control cells, peaking at 3947% in the 50 nM ponatinib culture (n=3, P<0.001) (Figure 5.6). This overexpression, however, then reduced from the 100 nM intermediate stage (1818%) onwards. The K562 DOX 55D-R (200 nM) resistant cells demonstrated a further reduction in *BCR-ABL1* mRNA expression, and this expression level (1299%) was similar to the ponatinib naïve control line K562 DOX 55D (1069%) (Figure 5.6). This result suggests that the overexpression of *BCR-ABL1* mRNA only mediates early stage ponatinib resistance, similar to the observed ABCB1 overexpression (section 5.3.2).

5.3.4 K562 DOX 55D-R ponatinib resistant line harbours compound mutation G250E and E255K

To determine if the observed ponatinib resistance correlated with the development of *BCR-ABL1* KD mutations, conventional Sanger sequencing was used. This revealed a combination of two mutations G250E (64%) and E255K (55%) in the K562 DOX 55D-R (200 nM) cell line (Figure 5.7 and Appendix 6). Interestingly, a silent

mutation (means mutation do not change the amino acid sequence) V270V (40%, GTG→GTA) was also detected in the resistant line (Figure 5.7). In the ponatinib naïve control cell line K562 DOX 55D and all the intermediate stage cell lines, no mutation was detectable by conventional Sanger sequencing.

Using Sanger sequencing, mutation(s) that are present below 10% could not be detected. Therefore, samples of the resistant line and the intermediate stages were analysed by next generation sequencing (NSG) termed Single Molecule Consensus Sequencing (SMCS) (140) to investigate whether the mutation emerged at an earlier time point (sensitive to <1%). By using this method, each individual *BCR-ABL1* cDNA molecule was tagged before library amplification, hence whether the two mutations were compound mutation or polyclonal mutations could be distinguished. As demonstrated in Figure 5.6, a G250E/E255K/V270V compound mutation was detected in 14% of the 100 nM intermediate cell line. Interestingly, a single mutation E279K and a single mutation E255K were observed in 0.92% and 0.72% respectively in this 100 nM intermediate cell line. In the resistant line K562 DOX 55D-R (200 nM), compound mutation G250E/E255K/V270V (54%) was confirmed by SMCS (Figure 5.6) and there was no other mutation detected in this line.

Figure 5.6

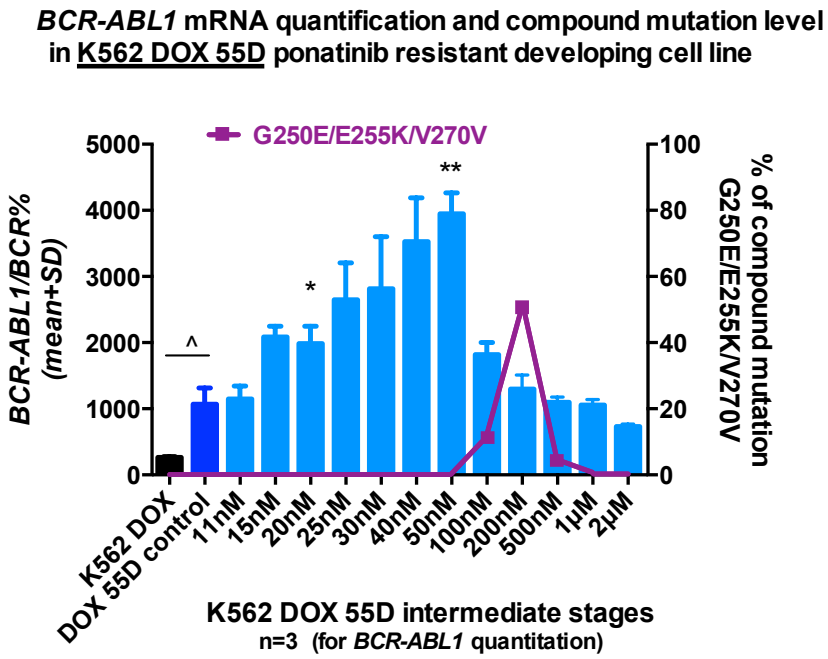


Figure 5.6: Transient *BCR-ABL1* mRNA overexpression was detected in the K562 DOX 55D ponatinib resistant cell line intermediates, followed by the development of KD mutations.

BCR-ABL1 transcript number was measured by RQ-PCR. Columns represent *BCR-ABL1* mRNA level, purple line represents compound mutation G250E/E255K/V270V level, detected by next generation sequencing. *BCR-ABL1* overexpression was observed in the ponatinib naïve line K562 DOX 55D compared to its dasatinib naïve control K562 DOX. Further *BCR-ABL1* overexpression was detected in the intermediate stages of ponatinib resistance. The reduction in the overexpression of *BCR-ABL1* in 100 nM stage followed the emergence of the compound mutation. Error bars represent SD. n=3 for *BCR-ABL1* mRNA RT-QPCR data, *p=0.02 and **p<0.01 compared to K562 DOX 55D control line, ^p=0.04 compared to K562 DOX line.

Figure 5.7

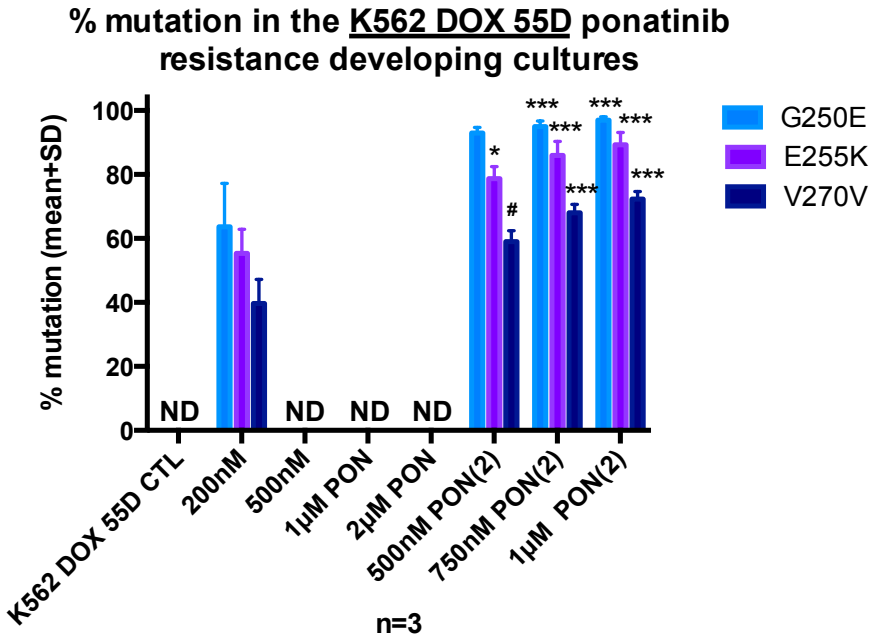


Figure 5.7: Mutations G250E and E255K, as well as single silent mutation V270V were detected in the K562 DOX 55D-R and the re-escalated 'high-dose' resistant cell lines by conventional sequencing.

Conventional sequencing was performed to determine the mutation level. Blue represents G250E. Purple represents E255K. Dark blue represents V270V. The mutations were observed firstly in the resistant line K562 DOX 55D-R by conventional sequencing. These mutations were undetectable by the same method in the following culture 'high-dose' resistant cell lines: from 500 nM to 2 μM ponatinib. However these mutations were present and expanded in the re-escalated resistant cell lines, from 500nM PON(2) to 1μM PON(2). *p=0.02 and #p=0.03, **p<0.01, ***p<0.001 compared to K562 DOX 55D-R line. ND represents not detected.

5.3.5 Undetectable or low level of compound mutation was observed in the K562 DOX 55D 1 µM PON and 2 µM PON resistant lines

To further investigate whether the compound mutation could be causative of a higher degree of resistance, a 'high-dose' resistant (beyond 200 nM ponatinib) cell line K562 DOX 55D 500nM PON was generated (Figure 5.1). The generation of this cell line, however, took approximately 180 days (from 200 nM to 500 nM). Unexpectedly, no mutation was detectable in this cell line via Sanger sequencing (n=3) (Figure 5.7). By using SMCS, however, the compound mutation G250E/E255K/V290V was detected in 2.45% in this cell line (Figure 5.6).

As the percentage of the compound mutation was unexpectedly reduced in the K562 DOX 55D 500nM PON cell line, this line was further exposed to higher concentration of ponatinib (1 µM and 2 µM) to monitor the alteration of the compound mutation. In contrast to the 500 nM ponatinib stage, the cells tolerated 1 µM ponatinib after only 14 days. After a further 14 days the cells were surviving in a concentration of 2 µM. However, as 2 µM is too high to be achieved clinically (and to be consistent with the K562 DOX 55D 1µM PON(2) cell line, which was the cell line that could not survive in 2 µM ponatinib), the K562 DOX 55D 2µM PON cell line will not be discussed further (but will be described) in this thesis. Of note, in the K562 DOX 55D 1µM PON and the K562 DOX 55D 2µM PON cell line, there was no mutation detected by SMCS.

Due to the fact that the compound mutation was reduced to undetectable level, it was necessary to investigate the mechanisms that mediated the resistance in these three cell lines. *BCR-ABL1* mRNA level was measured in the 500 nM, 1 μM and 2 μM ponatinib resistant lines. RQ-PCR results (Figure 5.6) revealed that the loss of G250E/E255K compound mutation coincided with a reduction in *BCR-ABL1* mRNA transcript number in the K562 DOX 55D 500nM PON, K562 DOX 55D 1μM PON and K562 DOX 55D 2μM PON cell lines (1097%, 1051% and 730% respectively). The *BCR-ABL1* mRNA transcript expression levels in the three cell lines were similar to the level in control cell line K562 DOX 55D (1069%) without statistical difference. In addition, the analysis of Bcr-Abl western blotting demonstrated increased total Bcr-Abl but decreased phosphor- Bcr-Abl levels in the K562 DOX 55D 1μM PON and K562 DOX 55D 2μM PON cell lines compared to control cell line K562 DOX 55D (Appendix 5). Furthermore, reductions in ponatinib IC50 in these resistant lines were observed (Figure 5.8): 46 nM and 11 nM in the K562 DOX 55D 500nM PON ($p>0.05$, $n=3$) and in the K562 DOX 55D 1μM PON cell line ($p=0.001$, $n=3$) respectively compared to 51 nM in the control K562 DOX 55D; in the K562 DOX 55D 2μM PON cell line, the pCrkL level negligible and therefore a ponatinib IC50 value (as measured by the 50% reduction in pCrkL by ponatinib) was unable to be measured (Figure 5.8(a)-(b)). These results demonstrated that Bcr-Abl kinase is sensitive to ponatinib and inactived in the K562 DOX 55D 500nM PON, K562 DOX 55D 1μM PON and K562 DOX 55D 2μM PON cell lines, indicated that the ponatinib resistance was not mediated by the activation of Bcr-Abl. Hence the resistant mechanism(s) in the three resistant cell lines are likely to be Bcr-Abl independent

(the Bcr-Abl independent resistant mechanisms will be described and discussed in Chapter 6).

Figure 5.8

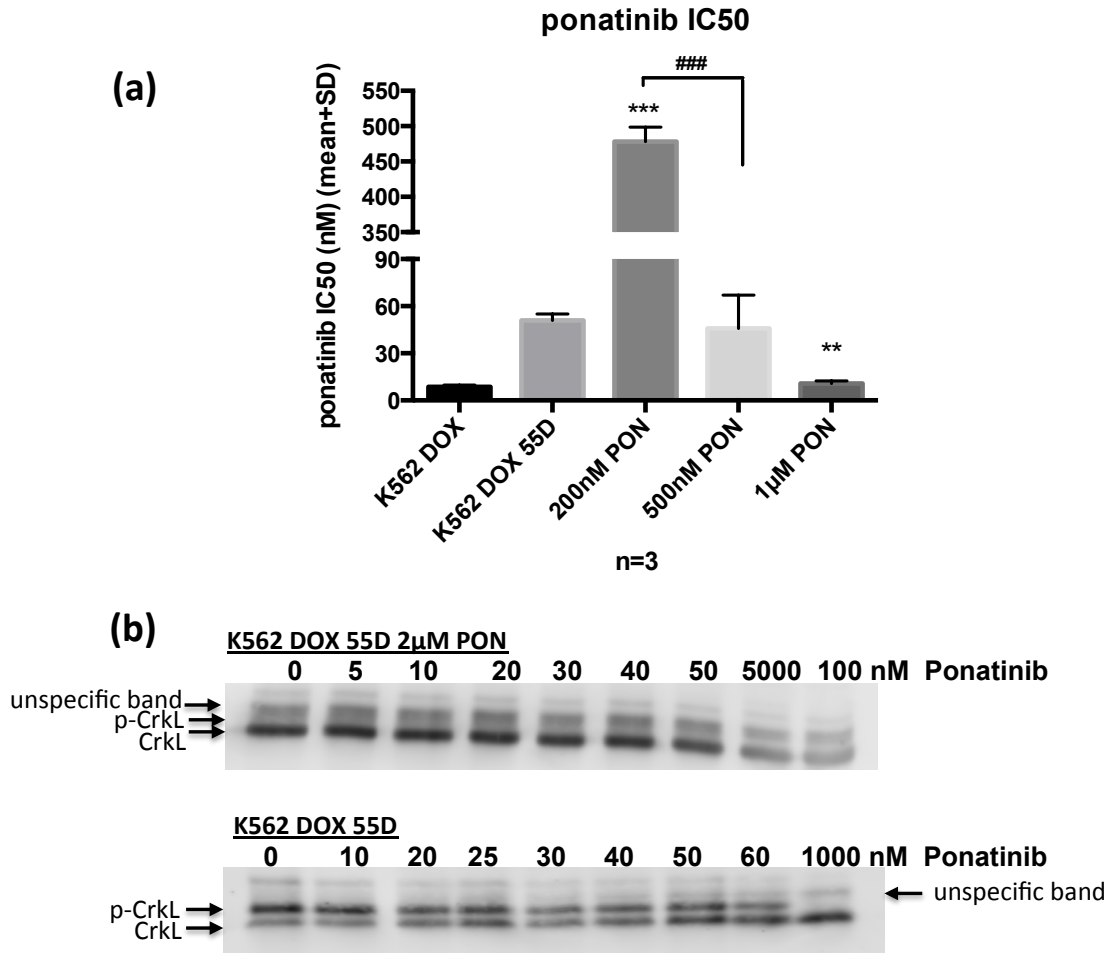


Figure 5.8: Ponatinib IC50 of K562 DOX 55D 1 μM PON resistant cell line were reduced compared to to K562 DOX 55D control line

(a) Significantly decreased ponatinib IC50 was observed in the K562 DOX 55D 1μM PON cell line compared to the K562 DOX 55D control cell line ($p<0.01$, n=3). Ponatinib IC50 was also significant decreased in the K562 DOX 55D 500nM PON resistant cell line compared to the K562 DOX 55D-R (200 nM) resistant line. Error bars represent SD. n=3 for all data, $^{**}p<0.01$, $^{***}p<0.001$ compared to K562 DOX 55D control cell line. $^{###}p<0.001$ compared to the K562 DOX 55D-R (200 nM) line. **(b)** Representative western blotting images of K562 DOX 55D 2μM PON (**top panel) and** K562 DOX 55D (**bottom panel)** cell lines. The K562 DOX 55D 2μM PON cell line exhibits very level of pCrkL , and thus the ponatinib IC50 could not be calculated.

5.3.6 K562 DOX 55D ponatinib re-escalated K562 DOX 55D 1μM PON(2) resistant line demonstrated increased percentage of mutations and *BCR-ABL1* mRNA overexpression

In order to determine whether the loss of the compound mutation could be recapitulated, re-escalated 'high-dose' resistant cell lines K562 DOX 55D 500nM PON (2), K562 DOX 55D 750nM PON(2) and K562 DOX 55D 1μM PON(2) were generated (re-escalated from 200 nM) (Figure 5.1). In contrast to the previous result, the development of K562 DOX 55D 500nM PON(2) cell line only took about three weeks. However, this cell line failed to escalate to 1 μM ponatinib directly. Hence, the K562 DOX 55D 500nM PON(2) cell line was exposed to 750 nM ponatinib, and acclimatisation took approximately 4 weeks. In addition, it took a further 200 days to escalate ponatinib concentration from 750 nM to 1 μM in these cells. Moreover, this cell line failed to survive in ponatinib concentration higher than 1 μM.

In contrast to the previous results, the compound mutation level did not fall in the K562 DOX 55D 500nM PON (2), K562 DOX 55D 750nM PON(2) and K562 DOX 55D 1μM PON(2) cell lines. Instead, these three cell lines demonstrated a step-wise increase in mutation percentages compared to the K562 DOX 55D-R line (200 nM) (determined by conventional sequencing): G250E increased from 64% to 97% in the 1μM PON(2) line ($p<0.001$, n=3); E255K increased from 55% to 89% in the 1μM PON(2) line ($p<0.001$, n=3); silent mutation V270V increased from 40% to 72% in the 1μM PON(2) line ($p<0.001$, n=3) (Figure 5.7 and Appendix 7). Due to the limit of

resources, the compound mutation level could not be measured using the SMCS method.

Coinciding with the increase in mutation percentage, the *BCR-ABL1* mRNA transcript level was also raised in the three re-escalated cell lines (Figure 5.9). Significantly increased *BCR-ABL1* mRNA was observed in the 1μM PON(2) line, from 1069% in the parental control line K562 DOX 55D and 1299% in the K562 DOX 55R (200 nM) resistant line, to 8285% ($p<0.001$, n=3). CrkL ponatinib IC50 was also increased to >1000 nM ($p<0.001$, n=3) in the same resistant cell line (5000 nM ponatinib could not fully prevent CrkL phosphorylation) (Figure 5.10). A >30-fold ($p<0.001$, n=3) increase in the level of both pBcr-Abl and total Bcr-Abl protein in the K562 DOX 55D 1μM PON(2) cell line was observed by western blotting analysis (Appendix 5), suggesting that the overexpression of *BCR-ABL1* translated into overexpressed, and active, Bcr-Abl protein, and therefore evidence that the ponatinib resistance in this cell line is Bcr-Abl dependent.

Interestingly, compared to the control line, significant reductions in ABCB1 expression (measured by flow cytometry) were observed in all of the 'high-dose' cell lines. K562 DOX 55D 500nM PON demonstrated a 89% reduction ($p<0.001$, n=3) and K562 DOX 55D 1μM PON demonstrated a 91% reduction $p<0.001$, n=3) in ABCB1 expression compared to the ponatinib naïve control line K562 DOX 55D (Figure 5.11a). Similarly, the K562 DOX 55D 500nM PON(2) demonstrated a 72% reduction ($p=0.001$, n=3) whereas the K562 DOX 55D 750nM PON(2) demonstrated

a 54% ($p>0.05$, $n=3$) reduction in ABCB1 expression compared to the control cell line (Figure 5.11a). In the K562 DOX 55D 2μM PON and the K562 DOX 55D 1μM PON(2) cell lines, the expression of ABCB1 was undetectable by flow cytometry (Figure 5.11b). The loss of ABCB1 in these two cell lines is further confirmation that ABCB1 upregulation was not required for the maintenance of ponatinib resistance especially in the 'high' degree resistance.

Figure 5.9

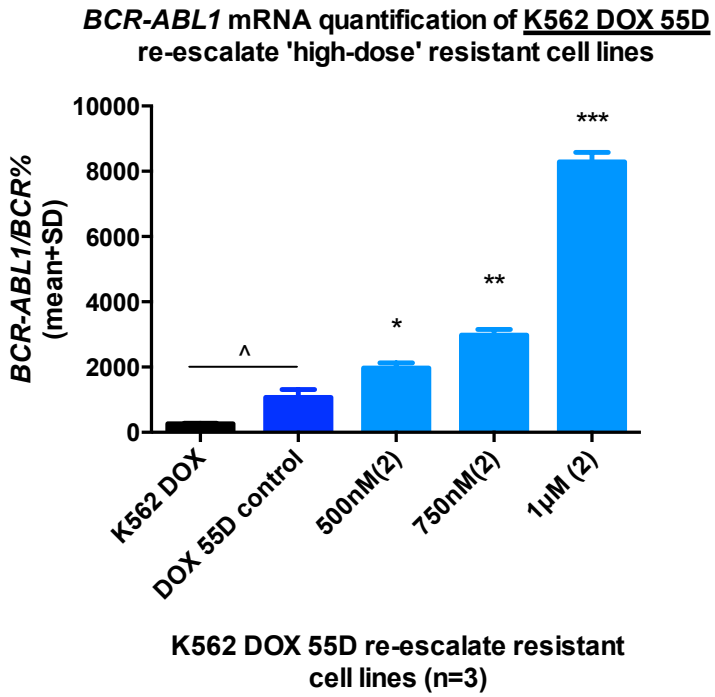


Figure 5.9: *BCR-ABL1* mRNA overexpression was detected in the K562 DOX 55D re-escalated K562 DOX 55D 1µM PON(2) resistant cell line.
BCR-ABL1 transcript number was measured by RQ-PCR. The overexpression of *BCR-ABL1* was observed in the K562 DOX 55D 500nM PON(2), K562 DOX 55D 750nM PON(2) and the K562 DOX 55D 1µM PON(2) cell lines. Error bars represent SD. n=3 for *BCR-ABL1* mRNA RT-QPCR data, *p=0.03, **p<0.01 and ***p<0.001 compared to the K562 DOX 55D control line, ^p=0.04 compared to K562 DOX line.

Figure 5.10

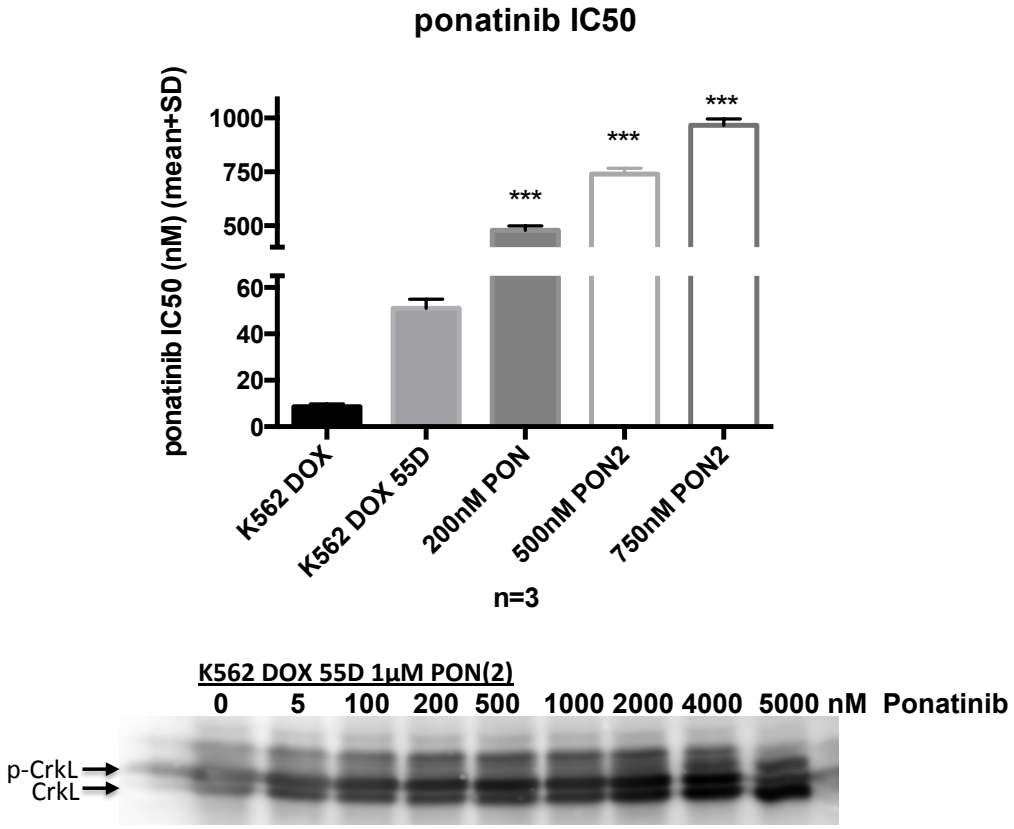


Figure 5.10: Increased ponatinib IC50 for K562 DOX 55D re-escalated 'high-dose'-resistant cell lines.
Top panel: Significantly increased ponatinib IC50 was observed in the K562 DOX 55D 500nM PON(2) and the K562 DOX 55D 750nM PON(2) cell lines compared to the K562 DOX 55D control cell line. Error bars represent SD. n=3 for all data, ***p<0.001. **Bottom panel**: representative western blotting image of K562 DOX 55D 1µM PON(2) cell line. This cell line exhibits incomplete inhibition of CrkL phosphorylation with 5000 nM ponatinib, and thus the ponatinib IC50 could not be calculated.

Figure 5.11 (a)

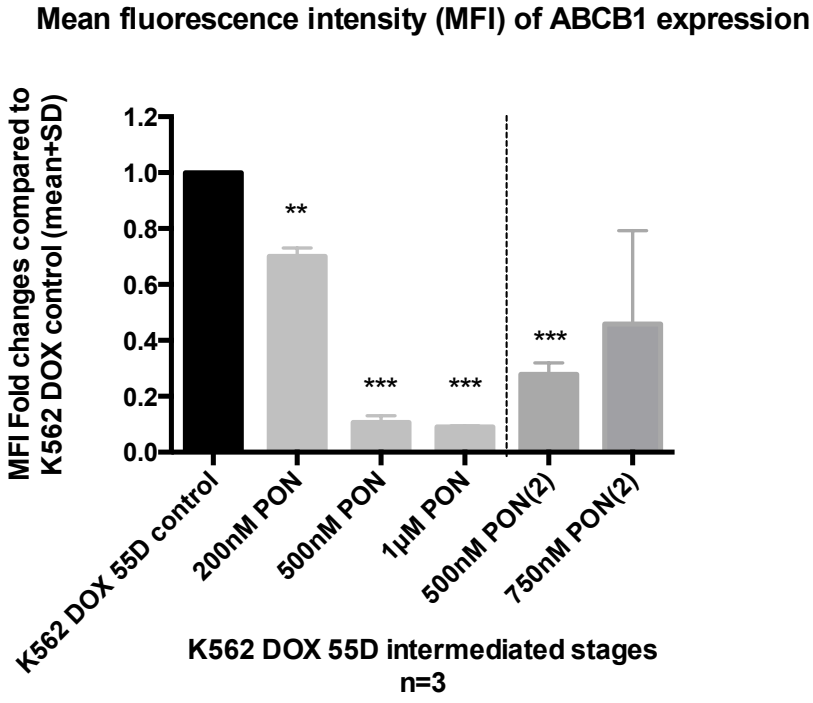

Mean fluorescence intensity (MFI) of ABCB1 expression
MFI Fold changes compared to K562 DOX control (mean+SD)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
***
***
***
K562 DOX 55D control
200nM PON
500nM PON
1μM PON
500nM PON(2)
750nM PON(2)
K562 DOX 55D intermediated stages
n=3

Figure 5.11 (b)

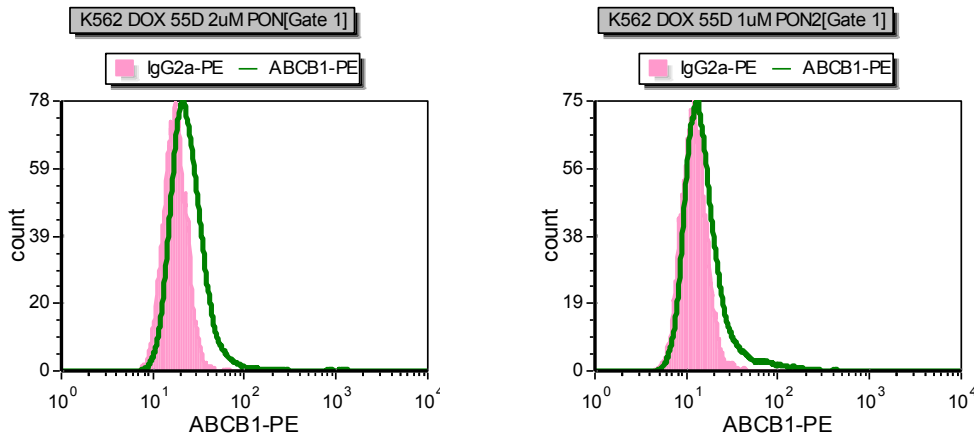


Figure 5.11: The K562 DOX 55D 'high-dose' resistant cell lines demonstrated decreased, or lost, cell surface expression of ABCB1.
Control resistant cells were stained with either IgG2a PE isotype control antibody (pink) or the corresponding ABCB1 PE antibody (green line). **(a)** The geometric mean mean fluorescent intensity (MFI) was compared between the control cell line and the resistant cell lines, and shown as fold change in ABCB1 MFI. n=3, ** $p<0.01$, *** $p<0.001$. **(b)** Representation of ABCB1 flow cytometry staining in the K562 DOX 55D 2μM PON and the K562 DOX 55D 1μM PON(2) cells, both cell lines are negative for ABCB1 staining.

5.4 Discussion

In this study, multiple mechanisms conferring resistance to ponatinib were detected in different stages of the development of ponatinib resistance in the K562 DOX 55D cell line. These mechanisms include *BCR-ABL1* overexpression, Bcr-Abl KD compound mutations and Bcr-Abl independent resistant mechanism (will be described in Chapter 6). Notably, reduced sensitivity to imatinib, nilotinib and dasatinib, but not to ponatinib was observed in the parental K562 DOX 55D cells. This reduction is most likely due to the fact that *BCR-ABL1* mRNA and ABCB1 protein was overexpressed in this cell line. This result confirms ponatinib to be the most potent TKI among the available TKIs, and also suggests that ABCB1 overexpression does not mediate resistance to ponatinib.

5.4.1 *BCR-ABL1* overexpression and the emergence of KD mutations

BCR-ABL1 overexpression, which was followed by the development of KD mutation, was the first resistance mechanism to emerge in this study. In the early stage of developing ponatinib resistance, this overexpression may reduce the sensitivity to ponatinib in the intermediate cells, and therefore enable the cells to survive under the selective pressure of ponatinib. Indeed, a previous study associated the overexpression of *BCR-ABL1* gene in CD34+ CML patient cells with enhanced proliferation, expansion, and reduced apoptosis (141). Their study also suggested that this increased expression resulted in reduced sensitivities to TKI (imatinib). The hypothesis that *BCR-ABL1* overexpression contributes to the emergence of KD

mutations has been suggested by several publications (74, 142-145). Although whether the overexpression of *BCR-ABL1* induces self-mutagenesis by increasing production of reactive oxygen species (ROS) is still uncertain (145, 146), elevation of this expression causing an increase in the risk of acquiring KD mutation was suggested by multiple studies (74, 142-146). This may explain why the *BCR-ABL1* gene overexpression emerged before the development of *BCR-ABL1* KD compound mutation in the ponatinib resistant cell line in the present study. The finding in the present study is also in line with previous research conducted by Tang *et al.* (51). By using a similar method, Tang *et al.* (51) generated 10 TKI resistant cell lines that harbour various KD mutations, and observed that increased *BCR-ABL1* expression always emerged before the development of KD mutation(s).

Additionally, according to Barnes *et al.* (74), overexpression of active Bcr-Abl may be detrimental to CML cells. Thus, when the ponatinib resistant cells develop KD mutations that enable the cells to survive in high doses of ponatinib (200 nM or higher), the initially increased expression of *BCR-ABL1* (which is likely to translate to increased Bcr-Abl protein) falls away as it is not required for cell survival and as it may be detrimental to the cells. This is also consistent with the previous study of Tang *et al.* (51) that after the development of KD mutation, the *BCR-ABL1* overexpression was reduced and superseded.

5.4.2 The development of compound mutation G250E/E255K has a significant impact in refractory CML

Bcr-Abl KD compound mutations are an emerging clinical problem in CML and Ph+ ALL, especially in the patients who receive sequential TKI treatment (109, 126). In this *in vitro* study, the compound mutation G250E/E255K was detected in the resistant cell line K562 DOX 55D-R. This compound mutation confers clinically relevant resistance to ponatinib and to other TKIs (Figure 5.2). While previous studies predicted and confirmed that some compound mutations could mediate ponatinib resistance (103, 109, 126), the present study is the first study that describes G250E/E255K compound mutation causing resistance to ponatinib. According to a previous study (126), G250 and E255 are two of a total 12 key kinase domain positions that comprise the majority of Bcr-Abl compound mutations. Hence, the compound mutation G250E/E255K that was observed in this study is most likely to have clinical impact and cause ponatinib resistance in patients.

Interestingly, a case of G250E and E255K KD mutation appearing in a Chinese patient resistant to imatinib was reported earlier this year (147). However, whether these two mutations were compound or not was unclear. In addition, the percentages of the mutations were not reported. Given that this patient developed these two mutations during the treatment of imatinib (147), it is likely that these are polyclonal single mutations. There is insufficient published evidence to support the hypothesis that the emergence of the compound mutation E255K/G250E is caused by dasatinib and ponatinib sequential treatment as demonstrated in the current

study. However, Bauer et.al demonstrated that the evolution of mutations in *BCR-ABL1*+ cell lines is significantly different depending on the order and concentration of TKI treatment (148).

Additionally, the fact that the cell line harbouring the compound mutation E255K/G250E demonstrated resistance to all of the tested TKIs, suggests a novel therapeutic approach would be required to treat this refractory disease. This is consistent with clinical observation that, although only a minority of patients harbour compound mutations, the prognosis for compound mutations is poor, and these patients currently lack targeted strategies (105, 109). Therefore, in line with previous research, the present study highlights the urgent need to develop effective therapy in compound mutation positive refractory CML, and perhaps more importantly, to adopt therapeutic strategies to avoid their development.

5.4.3 The *in vitro* formation of compound mutation G250E/E255K in the K562 DOX 55D ponatinib resistance developing cell lines

In the 100 nM intermediate of the K562 DOX 55D resistant cell line, very low levels of the single mutations E279K (0.92%) and E255K (0.72%) were detected at the same time as the compound mutation. Importantly, the emergence of the single mutation E255K may provide a clue of how the compound mutation developed in the resistant line. According to previous studies, mutations are acquired sequentially in the formation of compound mutations (103, 109, 126, 148). As there were some cells harbouring the single KD mutation E255K in the 100 nM

intermediate cell line, this mutation may have developed before the G250E mutation (Figure 5.12, left panel). After the development of the E255K mutation, the same clone expanded to (at least) two clones: one only harboured the E255K mutation; the other clone harboured the compound mutation G250E/E255K by acquiring a secondary mutation (Figure 5.12). Therefore, single mutation E255K and compound mutation G250E/E255K were both observed by using the SMCS method. When the cell line was then cultured in higher ponatinib concentration (200 nM), because the cells that harboured the single KD mutation E255K alone were not resistant enough to survive in >100 nM ponatinib concentration, this mutation was then lost in the later stage. The loss of this mutation E279K was likely due to the same reason as the loss of the E255K mutation.

However, the possibility that the G250E mutation emerged before the E255K mutation can not be completely excluded (Figure 5.12, right panel). As ponatinib binds to Bcr-Abl via the E255 position, a mutation that develops in this region leads to a higher degree of ponatinib resistance than a mutation that develops at the G250 residue. Hence the cells that harboured the E255K mutation had a greater chance to expand and hence to be detected. On the contrary, if the cells only harbour the G250E mutation, these cells are most likely to be eliminated by the increasing doses of ponatinib.

In addition, a silent mutation V270V was also detected together with the compound mutation in the resistant cell line. As V270V is a silent mutation, it is not considered

to affect the ponatinib binding by conformational changes to Bcr-Abl. While it is not clear if this silent mutation would affect the ponatinib binding in some way (most likely not), the development of V270V might not be functional in mediating the resistance and therefore just be a passenger. As the cells containing the V270V silent mutation also harboured the compound mutation, this silent mutation was maintained with the compound mutation in the K562 DOX 55D-R cell line. Hence, ponatinib resistance in the K562 DOX 55D-R cell line was caused by the compound mutation G250E/E255K.

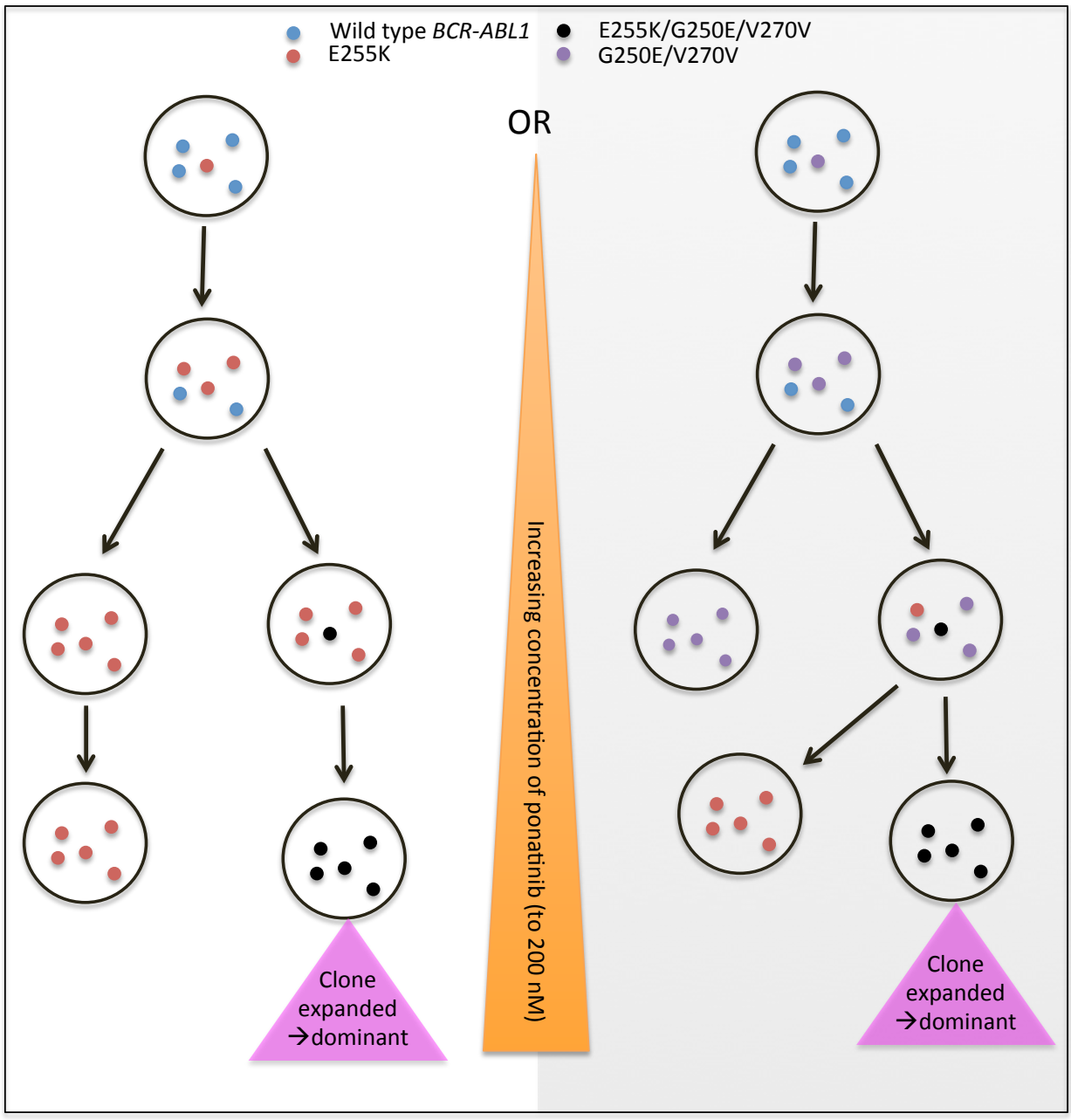


Figure 5.12 Possible clonal kinetics of compound mutation emergence. Left panel: E255K mutation raised at the first. G250E/V270V developed in the later stage from the E255K clone, and therefore the compound mutation were developed. **Right panel**: G250E/V270V developed before E255K mutation, but this clone that was killed by the increasing concentration of ponatinib (>100 nM).

5.4.4 Bcr-Abl independent resistance in the K562 DOX 55D 1μM PON cell line

The generation of the K562 DOX 55D 1μM PON and K562 DOX 55D 1μM PON(2) resistant cell lines demonstrated different modes of resistance, suggesting the emergence of resistance mechanism is stochastic. Of note, K562 DOX 55D 1μM PON(2) resistant cell line demonstrated increasing level of KD mutations as well as expression of *BCR-ABL1* transcript. This suggested that compound mutation alone is not sufficient to mediate the resistance to a higher concentration of ponatinib. Therefore, when *BCR-ABL1* transcript expression was reduced, instead of increased, in the K562 DOX 55D 500nM PON intermediate 'high-dose' resistant cell line, this cell line required another mechanism to maintain the survival under the selective pressure of high ponatinib concentration. In this case, this mechanism is the activation of alternative signalling and hence this cell line was resistant to ponatinib independently from Bcr-Abl signalling (Bcr-Abl independent resistance will be described and discussed in Chapter 6). Thus, mutations were not required in this cell line and therefore the compound mutations were eventually lost. In addition, compared to the K562 DOX 55D 1μM PON cell line, the recapitulated resistant cell line K562 DOX 55D 1μM PON(2) failed to survive in >1 μM ponatinib concentration, suggesting Bcr-Abl independent resistance mechanisms may confer a higher degree of resistance than the compound mutation G250E/E255K.

5.4.5 Kinetics of ponatinib resistance development in the K562 DOX 55D-R cell line

Ponatinib resistance development in the K562 DOX 55D cell line occurred more rapidly than the development of resistance in the K562 T315I cell line (chapter 4),

although the K562 T315I cell line confers a higher degree of TKI resistance including to ponatinib, compared to the K562 DOX 55D cell line. In addition, while both cell lines received similar TKI treatments (dasatinib and then ponatinib), *BCR-ABL1* KD compound mutation was only detected in the K562 DOX 55D-R resistant cell line (and development of compound mutation may be the reason why the generation of resistance occurred faster in this cell line), but not the K562 T315I-R resistant cell line. This observation was unexpected, but one explanation is that the development of mutations is complex and stochastic. Although multiple publications have suggested that the composition of compound mutations reflects the TKI exposure history (103, 109, 126), receiving sequential TKI treatments (even when these treatments are similar) do not necessarily mean compound mutations will develop. The fact that 86% of the CP CML patients (93% of these patients had received ≥2 TKIs treatment and 55% of them had received ≥3 TKIs treatment) that harboured the T315I mutation maintained McyR after three years ponatinib treatment (149) suggested that sequential TKI treatment does not always result in the development of compound mutation and resistance to ponatinib.

5.4.6 Transient ABCB1 overexpression

In this study, fluctuating ABCB1 expression was detected in the intermediate stages of ponatinib resistance. While ABCB1 overexpression mediating imatinib and dasatinib resistance were observed in the previous studies (51, 83, 130-138), ABCB1 is not a major efflux transporter for ponatinib, as described in Chapter 3. Therefore, ABCB1 overexpression in the resistant line would not confer advantages

for ponatinib resistance by decreasing the intracellular concentration of the TKI. Hence, as the overexpression of ABCB1 would not provide survival advantage in the presence of ponatinib, this ABCB1 overexpression was eventually lost with further exposure to ponatinib.

There are two possible explanations for ABCB1 overexpression in this study: 1. under the pressure of ponatinib, various different mechanisms are developed to maintain the survival of the cell line. However, it is not necessary that all the developed mechanisms mediate the survival. In fact, only the mechanisms that are able to mediate the resistance are maintained in the 'final' resistant cell line, and the other mechanisms, such as ABCB1 overexpression, do not have selective advantages and are eventually eliminated. 2. The second explanation is that the ABCB1 overexpression is induced by the activation of Bcr-Abl downstream signalling pathways. Previous studies (150, 151) suggested that Wnt/ β-catenin pathway, as well as MAPK signalling, could positively regulate ABCB1 expression in CML and other diseases. These two signalling pathways are downstream of Bcr-Abl and are regulated by the kinase. As the overexpression of *BCR-ABL1* is likely to translate into higher expression of Bcr-Abl protein, increased activation of the downstream signalling pathways may not only reduce sensitivity to ponatinib, but also consequently induce more ABCB1 expression as a 'side effect'. Again, as the ABCB1 overexpression did not provide survival advantage, this overexpression was only transient and was only significant in one intermediate cell line. To validate these hypotheses, an ABCB1 functional assay will need to be performed to determine

whether the inhibition of ABCB1 would restore ponatinib sensitivity in the ABCB1 overexpression intermediate line.

5.4.7 Summary

Overall, this *in vitro* study suggests that increased ABCB1 expression is unlikely to be the major cause of ponatinib resistance. Compound mutation G250E/E255K, however, could mediate the development of ponatinib resistance after sequential TKI treatment. Besides G250E/E255K, other compound mutations may also confer ponatinib resistance according to previous publications (103, 109, 126). Indeed, it is difficult to predict how many types of compound mutation will develop and mediate resistance to ponatinib. As Bcr-Abl harbouring the compound mutations characterised in this study, and in the previous researches (103, 109, 126), is resistant to all of the available TKIs, novel treatment methods are warranted to minimize the mutation escape and ponatinib resistance.

Chapter 6:

Axl overexpression mediates Bcr-Abl independent ponatinib resistance in two dasatinib naive cell lines K562-R and K562 DOX-R

6.1 Introduction

6.1.1 Bcr-Abl independent resistance

As described in Chapters 4 and 5, the most common form of secondary resistance in CML is the development of KD mutations. However, patients who lose response to therapy without harbouring *BCR-ABL1* KD mutations are also observed in clinic. Importantly, these patients may have adequate inhibition of Bcr-Abl activity (152), therefore, Bcr-Abl independent mechanisms of resistance may drive the disease in these cases. Identified Bcr-Abl independent resistance mechanisms include the deregulation of PI3K signalling, Src family kinases, JAK-STAT signalling, and TAM family receptor tyrosine kinases, particularly Axl (75, 93-96, 101).

As described in Chapter 1, Axl overexpression has been observed in a number of invasive cancers. Axl was first identified in 1991 in patients with CML (153, 154). Importantly, Axl overexpression has been identified in both imatinib resistant patients and a nilotinib resistant cell line (101). Furthermore, *AXL* mRNA overexpression represents an independent poor prognostic factor in cytogenetically normal acute myeloid leukemia (CN-AML) (155). Like other members of this receptor tyrosine kinase (RTK) family, Axl is activated by ligands, mainly via growth arrest-specific 6 (Gas6) (156, 157) and secondly by protein S (158, 159). Interestingly, a number of signalling pathways that are activated by Bcr-Abl are also targeted by Axl, including PI3K/Akt and Ras/Raf (160). By activating these downstream pathways, Axl induces proliferation, survival, cytoskeletal remodelling,

cell migration, adhesion, invasion and metastasis (160). In addition, Axl signalling also enhances the expression of epithelial-to-mesenchymal transition (EMT) genes to function in maintenance of the mesenchymal phenotype (161, 162). The use of a small molecule Axl inhibitor R428 (or BGB324), has been demonstrated to impair breast cancer cell invasion (161, 163-165) and induce apoptosis in AML cells (155). However, the role of Axl signalling and the inhibition of Axl in CML, especially in the ponatinib resistance setting, has not been described.

6.1.2 K562, K562 DOX and KU812 cell lines in the current study

The cellular events that result in Bcr-Abl independent resistance are currently poorly understood, especially for the novel TKI, ponatinib. History suggests that like the use of other TKIs, Bcr-Abl independent mechanisms mediating resistance to ponatinib are likely to occur, it is therefore critical to understand their development and function. To this end, this study aimed to investigate the development of Bcr-Abl independent ponatinib resistance by using three *BCR-ABL1*+ cell line models: K562, K562 DOX and KU812. Unlike the cell line models listed in the studies of chapters 4 and 5 (K562 T315I and K562 DOX 55D), these three cell lines were naïve to all TKIs before being treated with ponatinib. Hence, different from the previous two cell line models, Bcr-Abl dependent resistance mechanisms, especially *BCR-ABL1* KD mutations, were not detected in the K562, K562 DOX or KU812 cell lines. Therefore, Bcr-Abl independent resistance may be applicable here (although Bcr-Abl dependent resistance may still be developed).

6.2 Approach

6.2.1 Generation of ponatinib resistant cell lines

The K562, K562 DOX and KU812 cell lines were used to generate ponatinib resistance *in vitro*, and the intermediates stages of resistance were analysed to monitor the emergence of resistant clones. Both the K562 and K562 DOX cell lines were exposed to low concentrations of ponatinib (starting concentration of 0.5 nM). Ponatinib concentrations were then increased by 0.2 nM-50 nM increments approximately every 10-60 days, as cells tolerated each increased dose.

In patient plasma, median peak and trough levels of ponatinib when dosed at 45 mg once daily are 145 nM and 64 nM respectively (124). The final culture concentration in the resistant line was therefore chosen as 200 nM ponatinib, which is above the upper limit achievable *in vivo*. Ponatinib resistant K562 and K562 DOX cell line were generated, and both cell lines were escalated as follows: 0.5 nM, 0.7 nM, 0.9 nM, 1.1 nM, 1.3 nM, 1.5 nM, 2 nM, 3 nM, 4 nM, 5 nM, 6 nM, 7 nM, 8 nM, 9 nM, 10 nM, 12 nM, 14 nM, 16 nM, 18 nM, 20 nM, 25 nM, 30 nM, 35 nM, 40 nM, 45 nM, 50 nM, 60 nM, 70 nM, 80 nM, 90 nM, 100 nM, 150 nM and 200 nM. When the resistant cell lines tolerated 200 nM ponatinib, they were named K562-R and K562 DOX-R.

Notably, the KU812 cell line was unable to survive exposure to ponatinib exceeding 0.5 nM. Re-generation of a KU812 ponatinib resistant cell line was attempted but

this line failed at 0.3 nM ponatinib. Therefore, the generation of a KU812 ponatinib resistant cell line does not appear to be possible.

6.3 Results

6.3.1 K562 and K562 DOX cells cultured in 200 nM ponatinib demonstrate resistance to TKIs in vitro

Viability of the K562-R and K562 DOX-R resistant cell lines in the presence of ponatinib was determined by trypan blue exclusion. Generation of ponatinib resistance in this cell line took approximately two and half years, which is longer then the generation of dasatinib/nilotinib/imatinib resistance in these two cell lines (all less than one year) (51), or compared to the generation of ponatinib resistant cell lines K562 T315I-R and K562 DOX 55D-R. A possible explanation for this observation is that ponatinib is a pan-Bcr-Abl inhibitor (and a multi-kinases inhibitor) and it is one of the most potent TKIs to target Bcr-Abl. Thus, with ponatinib treatment, even cells harbouring some resistance mechanisms (that could cause resistance to other TKIs) such as single KD mutations, these cells would still have high sensitivity to ponatinib.

Figure 6.1 (a)

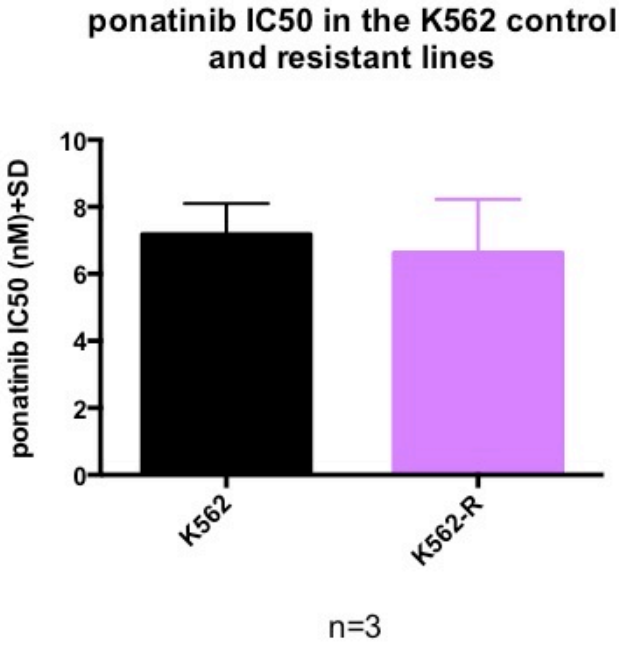

ponatinib IC50 in the K562 control and resistant lines
ponatinib IC50 (nM)+SD
10
8
6
4
2
0
K562
K562-R
n=3

Figure 6.1 (b)

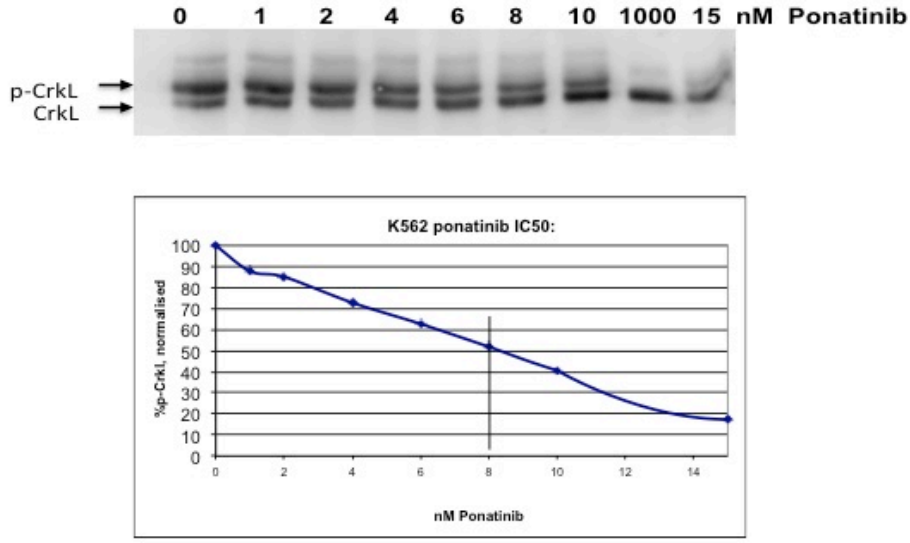


Figure 6.1 (c)

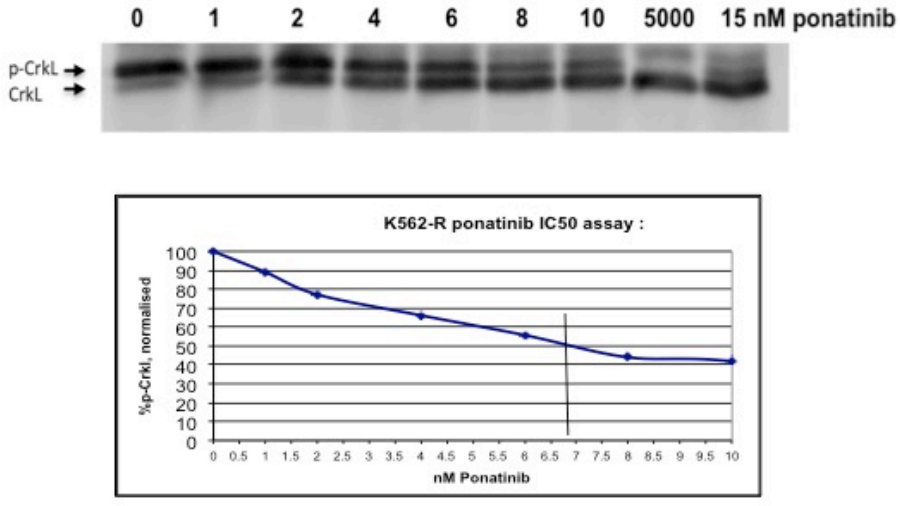


Figure 6.1: Ponatinib IC50 for K562-R was similar to the K562 control line
Cells were incubated for 2 hours with serial concentrations of ponatinib and then lysed to perform p-CrkL western blotting. **(a)** Similar ponatinib IC50s were observed in the K562-R cell line compared to the control line K562. Error bars represent SD, n=3. Representive **(b)** K562 and **(c)** K562-R ponatinib IC50 western blot analyses. Top panel: representative western blotting image. Bottom panel: analysis of the representative western blot. Ponatinib concentration required to reduce the ratio of p-CrkL:CrkL by 50% (ponatinib IC50) is indicated by the black line.

Figure 6.2 (a)

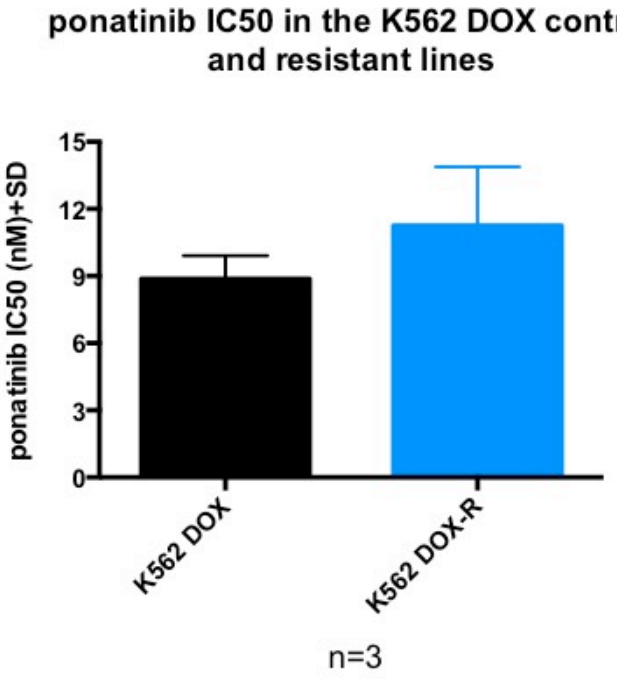

ponatinib IC50 in the K562 DOX control and resistant lines
ponatinib IC50 (nM)+SD
15
12
9
6
3
0
K562 DOX
K562 DOX-R
n=3

Figure 6.2 (b)

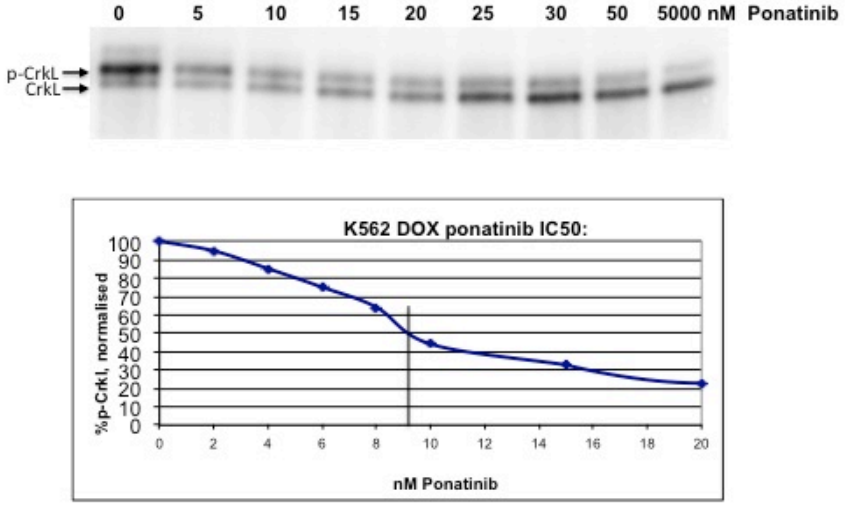


Figure 6.2 (c)

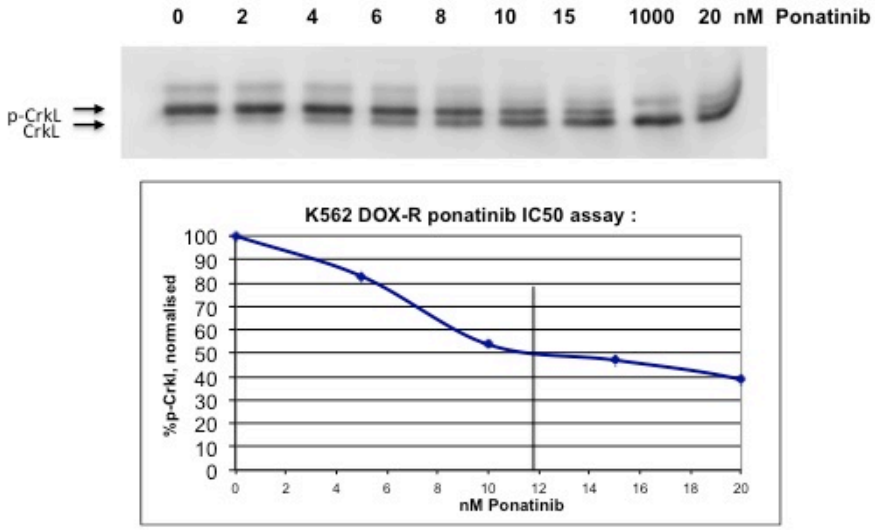


Figure 6.2: ponatinib IC50 for K562 DOX-R was similar to the K562 DOX control line

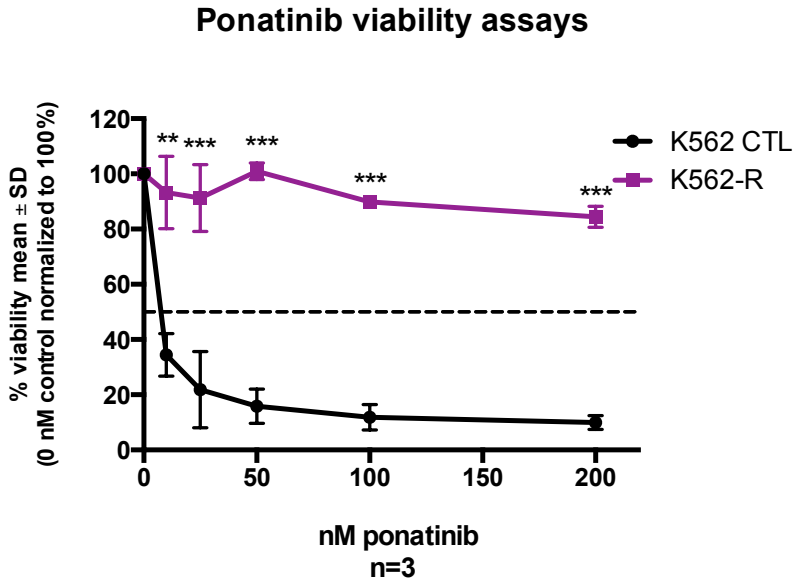
Cells were incubated for 2 hours with serial concentrations of ponatinib and then lysed to perform p-CrkL western blotting. **(a)** Similar ponatinib IC50s were observed in the K562-R cell line compared to the control line K562. Error bars represent SD, n=3. Representive **(b)** K562 DOX and **(c)** K562 DOX-R ponatinib IC50 western blot analyses. Top panel: representative western blotting image. Bottom panel: analysis of the representative western blot. Ponatinib concentration required to reduce the ratio of p-CrkL:CrkL by 50% (ponatinib IC50) is indicated by the black line.

Ponatinib IC50 was performed to determine the level of ponatinib resistance in the K562-R and K562 DOX-R cell lines. As demonstrated in Figure 6.1 (a)-(c), there was no significant difference ($p>0.05$, n=3) in ponatinib IC50 of the K562-R cell line (6.6 nM) compared to the control cell line K562 (7.2 nM) (Figure 6.1(a) -(c)). In addition, the K562 DOX-R cells (11.3 nM) also demonstrated a similar ponatinib IC50 (n=3) compared to the control line K562 DOX (8.9 nM) (Figure 6.2 (a)-(c)). These results suggested that the two cell lines were resistant to ponatinib via Bcr-Abl independent mechanism(s).

To confirm ponatinib resistance, viabilities in the K562-R and K562 DOX-R resistant cell lines were determined after 72 hours ponatinib exposure (detected by flow cytometric analysis following 7AAD and annexin V staining). The K562-R cell line demonstrated only up to a 15% reduction in survival when cultured with 10-200 nM ponatinib, compared to the control cell line K562 where the reduction in cell viability was 66% to 90% ($p<0.001$, n=3) (Figure 6.3 (a)). In addition, the K562-R cell line exhibited resistance to other TKIs including imatinib (500 nM to 2000 nM, with 21% to 33% reduction; this reduction was significantly lower compared to K562 control line, where the reduction was 43% to 56%; all $p<0.05$, n=3), nilotinib (from 100 nM to 1000nM with 14% to 23% reduction; K562 control line, this reduction was 60% to 64%; all $p<0.05$, n=3) and dasatinib (5 nM to 200 nM with 14% to 18% reduction; compared to K562 control cell line, this reduction was 63% to 73%; all $p<0.05$, n=3) (Figure 6.3 (b)-(d)).

Figure 6.3

(a)


Ponatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
0
50
100
150
200
**
***
***
***
***
K562 CTL
K562-R
nM ponatinib
n=3

(b)

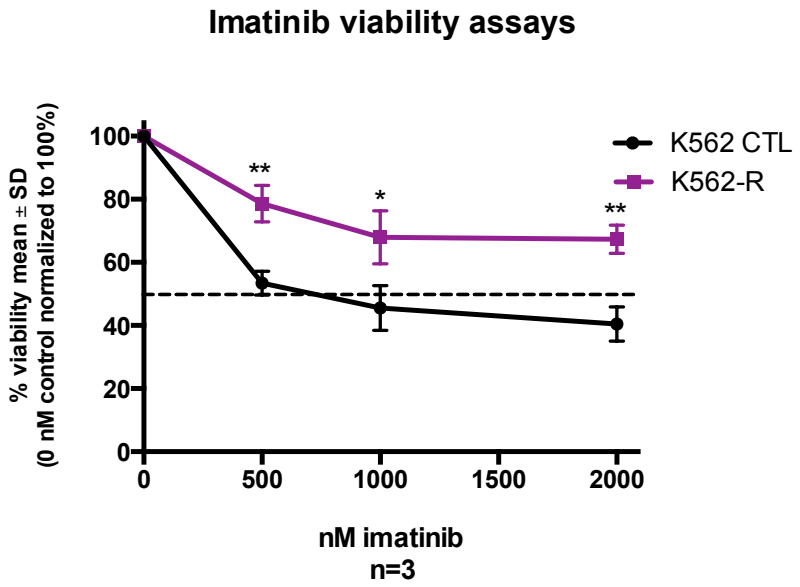
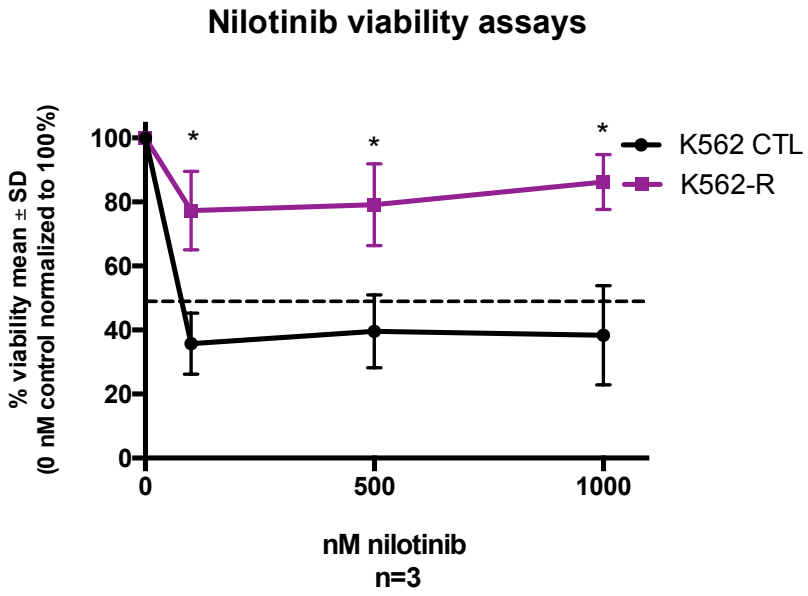

Imatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
100
80
60
40
20
0
0
500
1000
1500
2000
**
*
**
K562 CTL
K562-R
nM imatinib
n=3

Figure 6.3

(c)


Nilotinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
100
80
60
40
20
0
0
500
1000
*
*
*
K562 CTL
K562-R
nM nilotinib
n=3

(d)

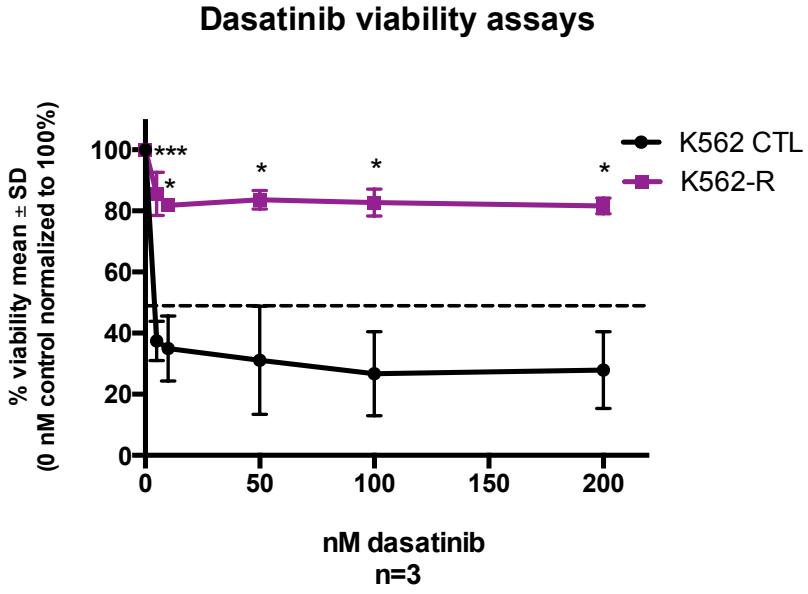
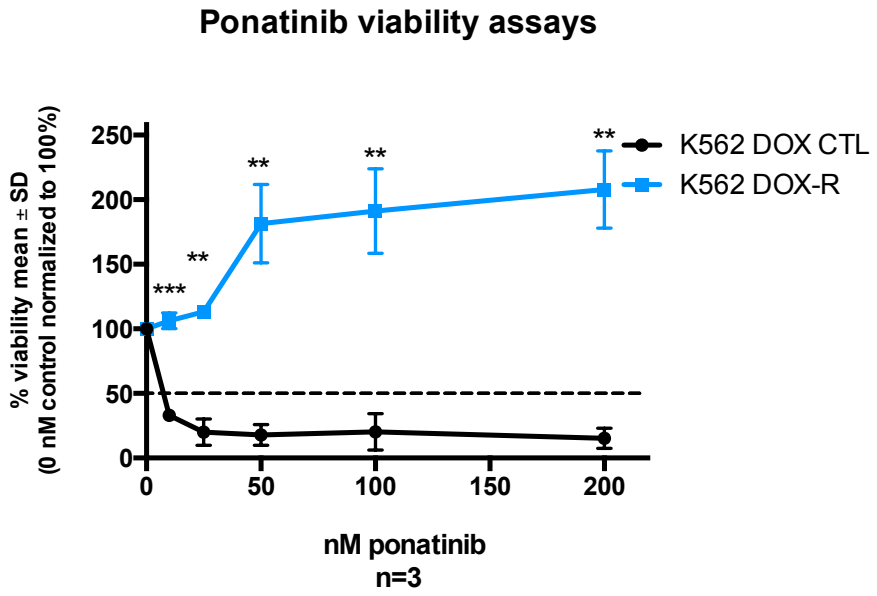

Dasatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
100
80
60
40
20
0
0
50
100
150
200
***
*
*
*
*
K562 CTL
K562-R
nM dasatinib
n=3

Figure 6.3: Ponatinib resistant K562-R cells are also resistant to imatinib, nilotinib and dasatinib mediated cell death

The K562 CTL (black line) and K562-R (purple line) cells were cultured with serial concentrations of TKI for 72 hours, followed by staining with 7AAD and annexin V to determine cell death. 0 nM TKI treatment groups were normalised to 100%. The change in viabilities induced by TKI treatment were compared between the cell lines. The K562-R cells demonstrated significantly improved survival compared to the control line in all of the tested concentration of **(a)** ponatinib (10 nM-200 nM), **(b)** imatinib (500 nM-2000 nM) , **(c)** nilotinib (50 nM-1000 nM) and **(d)** dasatinib (10 nM-200 nM). Black dash lines indicate 50% reduction in viability. Error bars represent SD. n=3 for all data, *p<0.05, **p<0.01 and ***p<0.005 compared to K562 CTL group.

Figure 6.4

(a)


Ponatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
250
200
150
100
50
0
0
50
100
150
200
***
**
**
**
**
K562 DOX CTL
K562 DOX-R
nM ponatinib
n=3

(b)

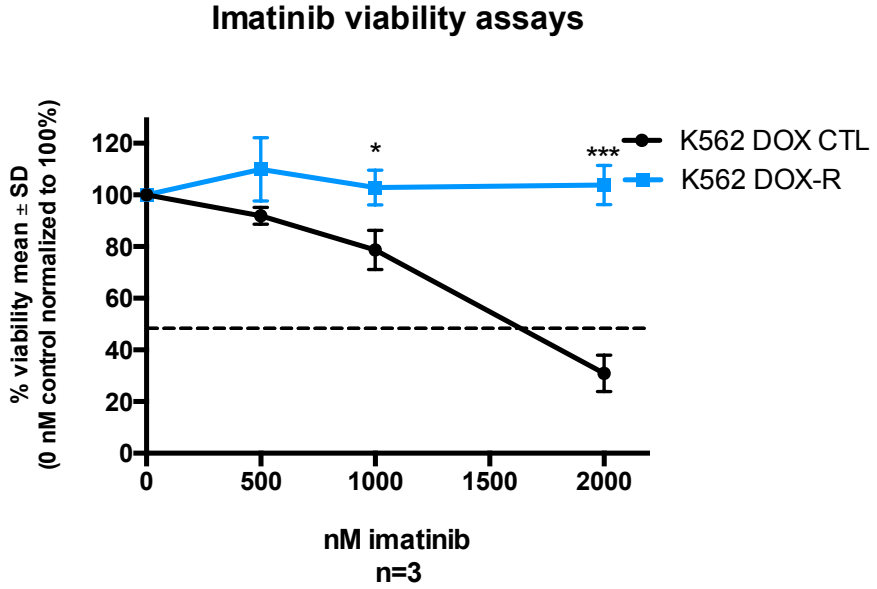
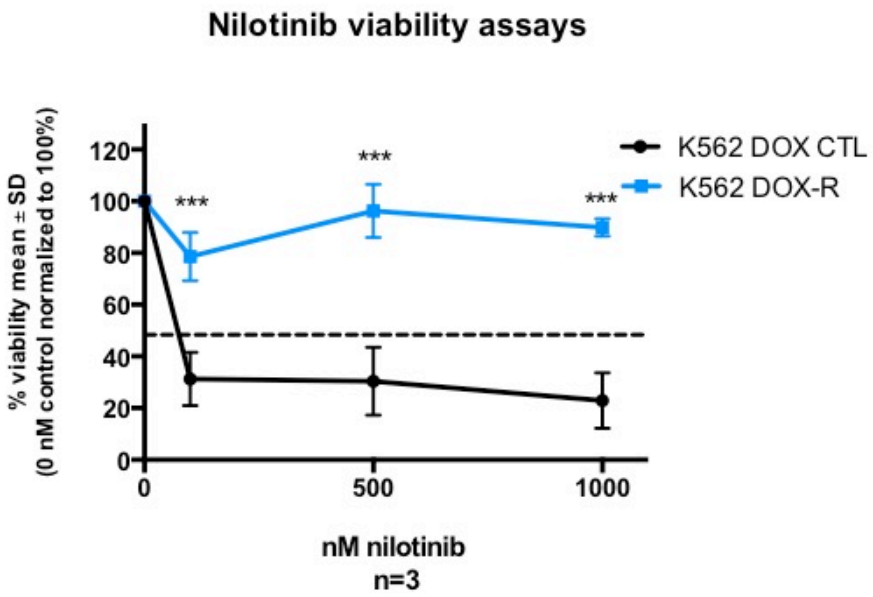

Imatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
0
500
1000
1500
2000
*
***
K562 DOX CTL
K562 DOX-R
nM imatinib
n=3

Figure 6.4

(c)


Nilotinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
***
***
***
0
500
1000
nM nilotinib
n=3
K562 DOX CTL
K562 DOX-R

(d)

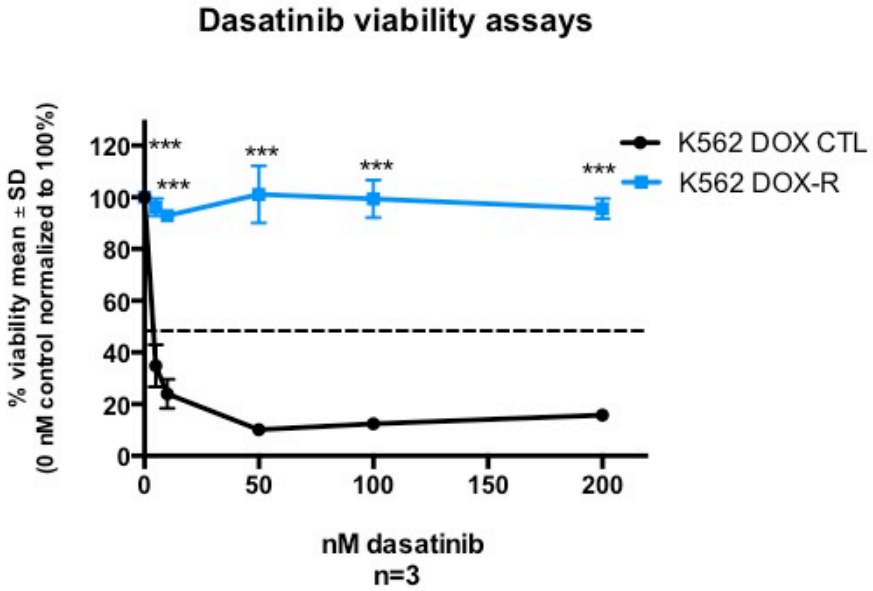

Dasatinib viability assays
% viability mean ± SD
(0 nM control normalized to 100%)
120
100
80
60
40
20
0
***
***
***
***
***
0
50
100
150
200
nM dasatinib
n=3
K562 DOX CTL
K562 DOX-R

Figure 6.4: Ponatinib resistant K562 DOX-R cells are also resistant to imatinib, nilotinib and dasatinib mediated cell death

The K562 DOX CTL (black line) and K562 DOX-R (blue line) cells were cultured with serial concentrations of TKI for 72 hours, followed by staining with 7AAD and annexin V to determine cell death. 0 nM TKI treatment groups were normalised to 100%. The change in viabilities induced by TKI treatment were compared between the cell lines. The K562 DOX-R cells demonstrated significantly improved survival compared to the control line in all of the tested concentration of **(a)** ponatinib (10 nM-200 nM), **(b)** imatinib (1000 nM-2000 nM) , **(c)** nilotinib (50 nM-1000 nM) and **(d)** dasatinib (10 nM-200 nM). Black dash lines indicate 50% reduction in viability. , *p<0.05, **p<0.01 and ***p<0.005 compared to K562 DOX CTL group.

Furthermore, the K562 DOX-R cells demonstrated less apoptosis (6% to 107% increased in survival) when cultured with 10 nM to 200 nM ponatinib compared to the 0 nM ponatinib treated group (0 nM ponatinib treated control was normalized to 100%) (Figure 6.4 (a)). That means, when 200 nM ponatinib was removed from the culture media of the resistant cell line, a 52% reduction in viability was observed, indicative of the cell line's dependence on/addiction to the presence of ponatinib. On the contrary, the control cell line K562 DOX demonstrated a 67% to 85% reduction in viability when cultured with 10-200 nM ponatinib, and this reduction was significantly higher compared to the resistant line (all $p=0.01$, $n=3$). The K562 DOX-R cell line exhibited resistance to other TKIs including imatinib (500 nM to 2000 nM with 4% to 10% increase; compared to the control cell line K562 DOX where the viability was reduced from 8% to 69%; all $p<0.005$, $n=3$), nilotinib (500 nM to 2000 nM with 10% to 21% reduction; compared to control cell line K562 DOX where the viability was reduced from 70% to 77%; all $p<0.005$, $n=3$) and dasatinib (5 nM to 200 nM with 0% to 7% reduction; compared to control cell line K562 DOX where the viability was reduced from 65% to 90%; all $p<0.005$, $n=3$) (Figure 6.4 (b)-(d)).

Taken together, the IC50 results suggest that the resistance observed in the K562-R and the K562 DOX-R cell lines is likely to be Bcr-Abl independent, and the viability data confirm the resistance of these two cell lines to ponatinib and all other currently available TKIs.

6.3.2 K562 and K562 DOX ponatinib resistant cell lines do not harbour *BCR-ABL1* KD mutations

Next, whether the observed ponatinib resistance correlated with the development of *BCR-ABL1* KD mutations was determined. Kinase domain mutations were not expected as these two cell lines demonstrate Bcr-Abl independent resistance, but for completeness Sanger sequencing was performed to detect KD mutations. As expected, there were no KD mutations detected in the K562-R or K562 DOX-R resistant cell lines. In agreement, there were also no mutations detected in the intermediate stages of the two resistant lines. This result further supports the hypothesis that the ponatinib resistance demonstrated by these two cell lines is mediated by Bcr-Abl independent mechanism(s)

6.3.3 ABCB1 is up-regulated in the K562-R ponatinib resistant cell line

The surface expression levels of ABCG2 and ABCB1 were measured in the two resistant cell lines. The K562 ABCG2 cell line was used as a positive control for the staining of ABCG2 (Figure 6.5). No ABCG2 expression was detected in the K562-R or K562 DOX-R resistant cell lines (or any of the intermediate lines), nor the corresponding control cell lines K562 or K562 DOX (Figure 6.5).

Interestingly, while the control line K562 did not express cell surface ABCB1 (Figure 6.6), cell surface ABCB1 expression was observed in the intermediate and final stages of the developing K562 ponatinib resistant culture (from 60 nM PON until the final 200 nM

PON (K562-R) resistant culture) (Figure 6.6). Notably, while ABCB1 was overexpressed in the K562-R resistant line (and the intermediate cell lines), at the same time, the ponatinib IC50 in this cell line (and the intermediate cell lines, Appendix 6A) was similar to the control line (refer Figure 6.1). These data suggest that it is unlikely that ABCB1 overexpression alters the intracellular concentration of ponatinib, and therefore this ABCB1 overexpression is not likely to mediate the observed ponatinib resistance by effluxing ponatinib.

ABCB1 expression was also detected in the K562 DOX-R resistant line. Notably, the control line K562 DOX is the ABCB1 overexpressing variant of K562. Different to the K562-R line, the K562 DOX-R line did not demonstrate ABCB1 overexpression compared to the ponatinib naïve control line K562 DOX (Figure 6.7 (a)). However, the ABCB1 expression in the K562 DOX-R ponatinib resistant developing culture fluctuated. Significant increases in ABCB1 expression were observed in the intermediate stages (5 nM, 20 nM, 30 nM and 50 nM) (determined by the further increment of mean florescence intensity) of resistance (Figure 6.7 (b)). This up-regulation was only observed in these intermediate lines, which were cultured in relatively low concentrations of ponatinib. When the ponatinib concentration reached 200 nM (K562 DOX-R), this increase in ABCB1 expression was no longer evident. These results suggest that the ABCB1 overexpression was transient and was not required for the maintenance of ponatinib resistance, although this overexpression may be required for the early stages of ponatinib resistance. Hence ABCB1 overexpression is not the major cause of ponatinib resistance in the K562 DOX-R cell line.

Figure 6.5

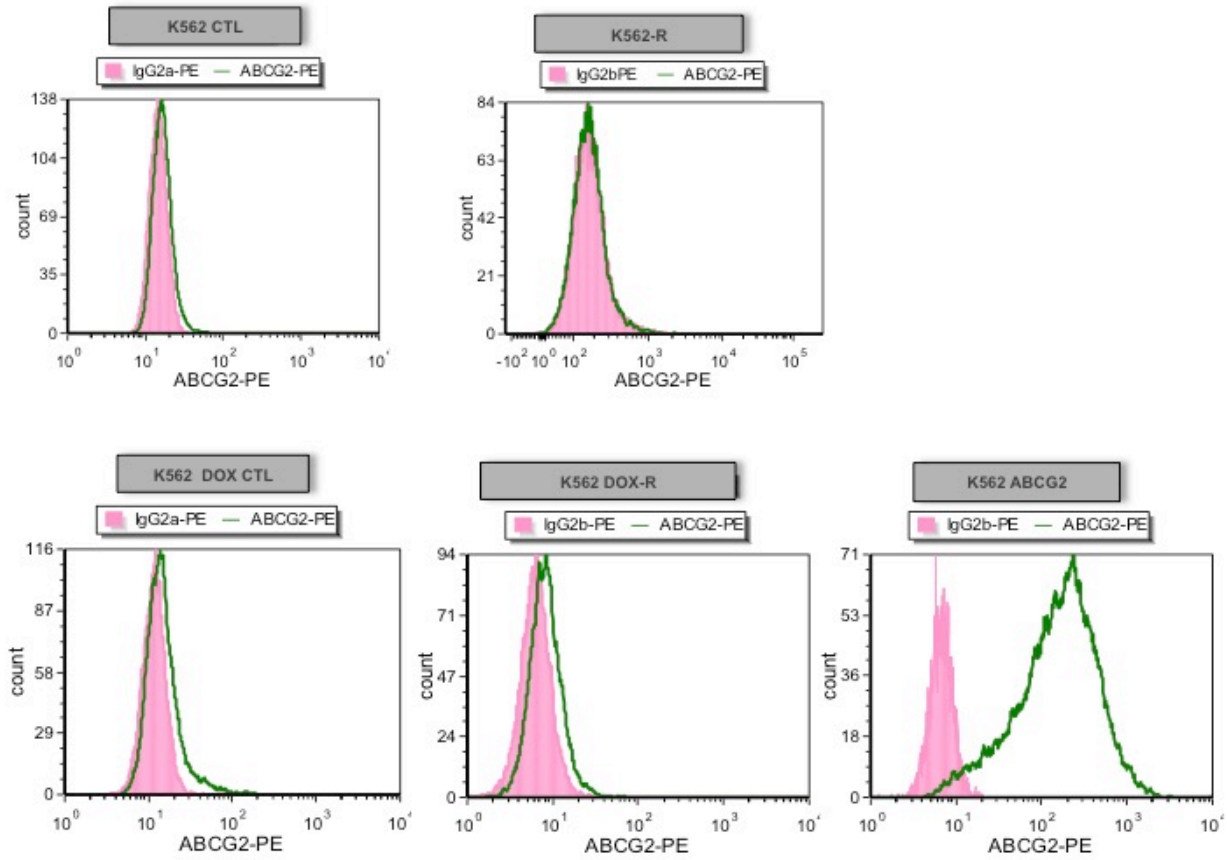


Figure 6.5: The K562-R and the K562 DOX-R cell lines do not express ABCG2.
Cells from the control cell lines K562 or K562 DOX and the resistant cell lines K562-R or K562 DOX-R were collected and stained with either IgG2b PE isotype control antibody (pink) or the corresponding ABCG2 PE antibody (green). There was not ABCG2 expression detected in the two control cell lines nor the corresponding resistant cell lines. Staining of K562 ABCG2 cells was included as a positive control.

Figure 6.6

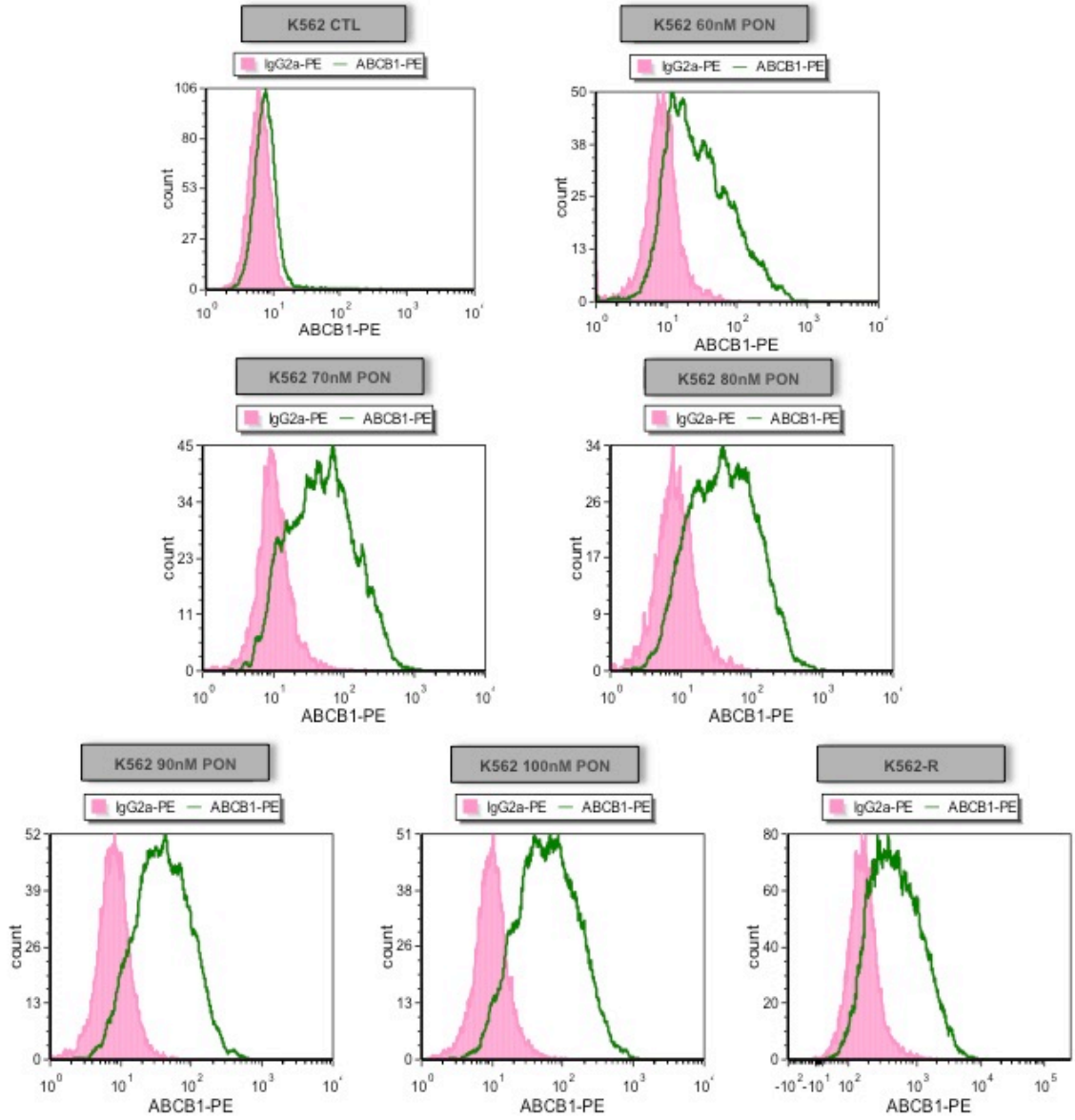


Figure 6.6: The K562-R cell line demonstrates increased surface expression of ABCB1.
Cells from the control cell lines K562 or K562-R were collected and stained with either IgG2a PE isotype control antibody (pink) or the corresponding ABCB1 PE antibody (green). There was not ABCG2 expression detected in the control cell line K562 but it was detected in the resistant cell line K562-R (K562 200nM PON) as well as all and the intermediate resistant lines from 60 nM. The expression of ABCB1 in the K562-R reduced slightly compared to the earlier intermediate lines, however, ABCB1 overexpression was still evident in this cell line.

Figure 6.7

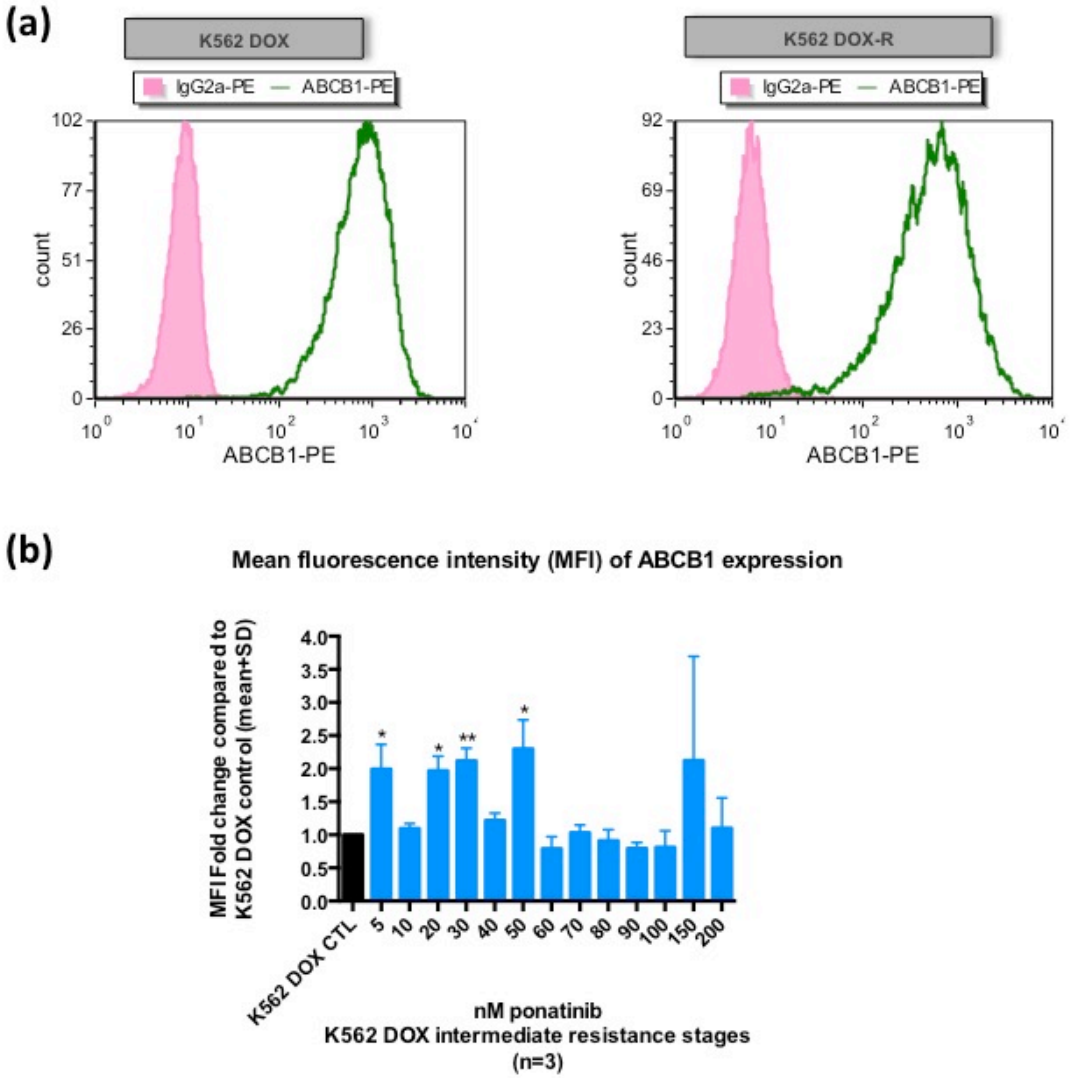


Figure 6.7: ABCB1 overexpression was detected in the intermediate stages of the K562 DOX ponatinib resistance culture but not in the K562 DOX 55D-R cell line.
Cells from K562 DOX and K562 DOX-R resistant lines were collected and stained with either IgG2a PE isotype control antibody (pink) or the corresponding ABCB1 PE antibody (green). **(a)** Example of flow cytometry staining ABCB1 in the control (left) and resistant cells (right). **(b)** The mean fluorescence intensity (MFI) from ABCB1 flow cytometric analyses was compared between the control cell line, intermediate resistant cell lines and the resistant cell line (200 nM). Error bars represent SD. n=3. *p<0.05,**p<0.01.

6.3.4 K562 DOX-R but not K562-R ponatinib resistant-developing cell lines demonstrate increased *BCR-ABL1* mRNA

The *BCR-ABL1* mRNA levels in the two ponatinib resistant cell lines as well as the intermediate stages of resistance were quantitated by RQ-PCR. As demonstrated in Figure 6.8, the intermediate stages of the K562 ponatinib resistance developing culture exhibited fluctuations in the *BCR-ABL1* mRNA transcript level, from 487% in the K562 naïve control cells, peaking at 1408% in the 40 nM ponatinib culture (n=3, $P<0.001$). The K562-R final resistant line, however, expressed slightly but not significantly increased *BCR-ABL1* mRNA level compared to the K562 control line. This result suggested that while cells with higher expression of *BCR-ABL1* are selected in the short term, they were not the dominant clones in the fully resistant cell line. Hence, the overexpression of *BCR-ABL1* mRNA may mediate early stages of ponatinib resistance, but it is not the major mechanism that caused resistance to ponatinib in this cell line. Conversely, the *BCR-ABL1* mRNA expression level in the K562 DOX-R ponatinib resistant line increased from 266% in the control line to 871% ($p=0.04$, n=3) (Figure 6.9). The intermediate cell lines of the K562-DOX-R culture also demonstrated fluctuating *BCR-ABL1* mRNA levels (Figure 6.9). Hence, the pBcr-Abl and total Bcr-Abl levels were measured in the resistant cell lines to determine whether the significantly increased *BCR-ABL1* mRNA transcript overexpression translated into increased expression of the active protein.

Figure 6.8

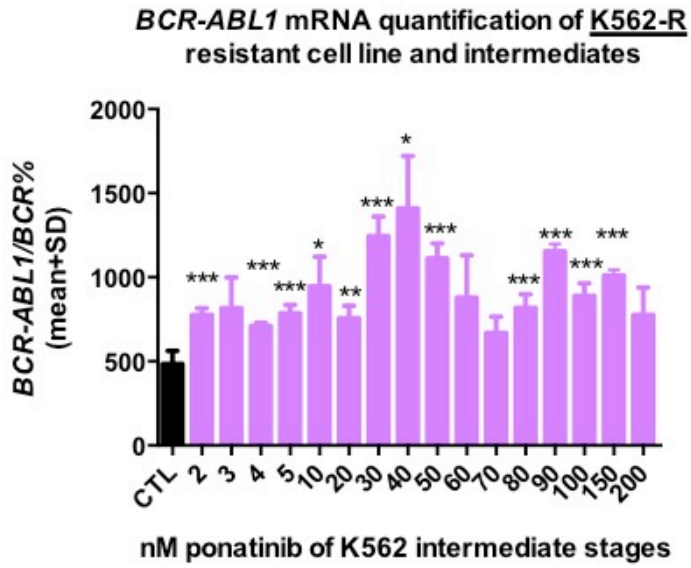


Figure 6.8: Increased *BCR-ABL1* mRNA was detected in the K562 intermediate ponatinib resistant cell lines.
BCR-ABL1 transcript number was measured by RQ-PCR. Overexpression of *BCR-ABL1* mRNA level was detected in the intermediate cell lines, from 2 nM to 50 nM. This overexpression was also observed in the later stages of intermediate resistance (80-150 nM), but not in the final 200 nM concentration (K562-R). Error bars represent SD. mRNA expression represents the mean of at least three independent experiments performed in triplicate, *p<0.05, **p<0.01 and ***p<0.005 compared to the K562 control line (CTL).

Figure 6.9

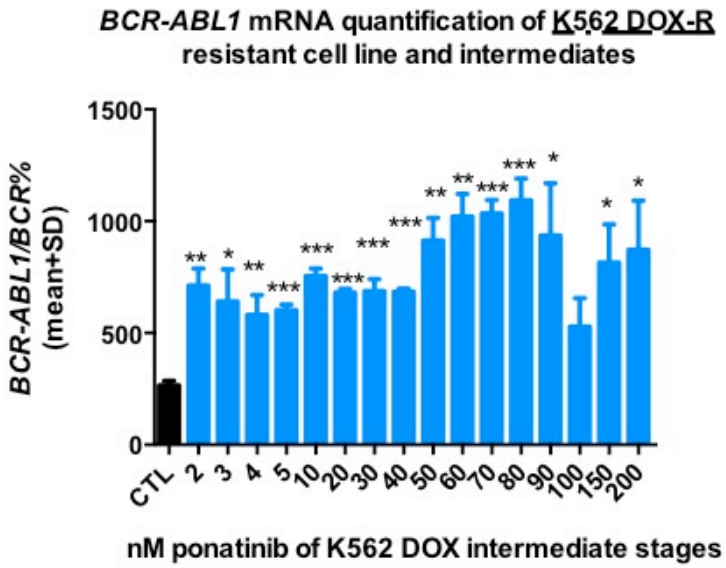


Figure 6.9: increased *BCR-ABL1* mRNA was detected in the K562 DOX-R ponatinib resistant cell line.

BCR-ABL1 transcript number was measured by RQ-PCR. Overexpression of *BCR-ABL1* mRNA level was detected in the intermediate cell lines, from 2 nM to the final 200 nM stage (but not in the 100 nM). Error bars represent SD. mRNA expression represents the mean of at least three independent experiments performed in triplicate, *p<0.05, **p<0.01 and ***p<0.005 compared to K562 DOX control line (CTL).

As represented in Appendix 7, the total Bcr-Abl protein expression level in the K562 DOX-R and the K562-R resistant cell lines were increased with ($p<0.05$)/ without statistical significance compared to the corresponding parental controls (both n=3). However, the phospho-Bcr-Abl protein level in the K562-R and K562 DOX-R resistant lines did not increase compared to their corresponding parental controls K562 or K562 DOX respectively. This is likely due to the fact that both cell lines demonstrate Bcr-Abl independent resistance to ponatinib, therefore phosphorylated Bcr-Abl was not required for survival. These results suggested that while the mRNA overexpression translated into higher level of total Bcr-Abl protein in the resistant cell lines, the overexpressed protein was not phosphorylated/activated and therefore could not function to target downstream signalling. Similarly, the K562-R cell line demonstrated decreased pBcr-Abl, confirming that the resistance mechanism(s) in these two ponatinib resistant cell lines are Bcr-Abl independent.

As detailed in Chapter 5, the Bcr-Abl independent resistant cell line K562 DOX 55D 1μM PON will be described and discussed together with the K562-R and the K562 DOX-R cell lines from this point on.

6.3.5 K562 and K562 DOX ponatinib resistant lines demonstrated increase cell adhesion

Interestingly, the three resistant cell lines K562-R, K562 DOX-R and K562 DOX 55D 1μM PON all demonstrated increased cell adherence in culture, while the control cells K562, K562 DOX and K562 DOX 55D remain completely in suspension (Figure

6.10 (a)). Due to this observation, flow cytometry was performed to assess the expression of the adherence marker CD44 (E-selection, homing cell adhesion molecule or lymphocyte homing receptor) (166) in the three resistant cell lines and their respective controls. Increased adherence in K562 imatinib resistant cells (with CD44 staining positive) were reported in a previous publication (166). As indicated in Figure 6.10 (b), the K562 control line was negative for CD44 while very small populations in the other two control lines K562 DOX and K562 DOX 55D were positive for the adherent marker staining. Conversely, the staining of CD44 was strongly positive in all three resistant lines. This supported the morphology change observed in the resistant lines, and given that the increased adhesion was only observed in the three Bcr-Abl independent resistant cell lines, suggests that the adherence is likely to be associated with the Bcr-Abl independent mechanism of ponatinib resistance.

6.3.6 K562-R and K562 DOX-R ponatinib resistant lines demonstrates Axl overexpression

As described in Chapter 1 and in the introduction of this chapter, increased adherence is a hallmark of Axl overexpression and this overexpression has previously been associated with TKI resistance. Therefore, the Axl expression level was assessed in the three Bcr-Abl independent resistant cell lines. Figure 6.11 (a) clearly demonstrates that *AXL* mRNA transcript level was significantly increased in all three resistant lines: a 6.3 fold increase in the K562-R line compared to the K562 control line ($n=3$, $p<0.005$); a 1.5 fold increase in the K562 DOX-R line compared to

the K562 DOX control line (n=3, p=0.03); and a 1.9 fold increase in the K562 DOX 55D 1μM PON line compared to the K562 DOX 55D control line (n=3, p=0.03).

Next, the Axl protein level was assessed in the three resistant cell lines by flow cytometry and western blotting. As indicated in Figure 6.11 (b), the cell surface expression of Axl in the K562-R, K562 DOX-R and K562 DOX 55D 1μM PON resistant cell lines dramatically increased compared to their corresponding control lines. All together, these data confirmed the overexpression of Axl in the three independent resistant cell lines.

Figure 6.10 (a)

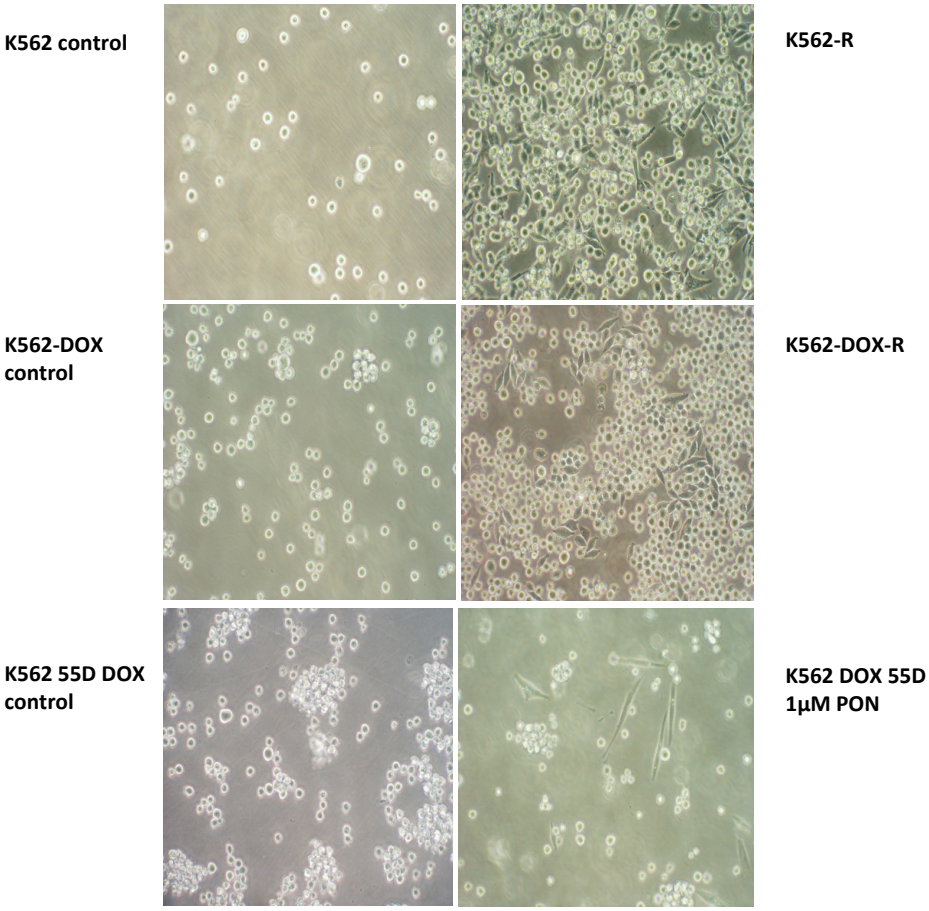

K562 control
K562-R
K562-DOX control
K562-DOX-R
K562 55D DOX control
K562 DOX 55D 1µM PON

Figure 6.10 (b)

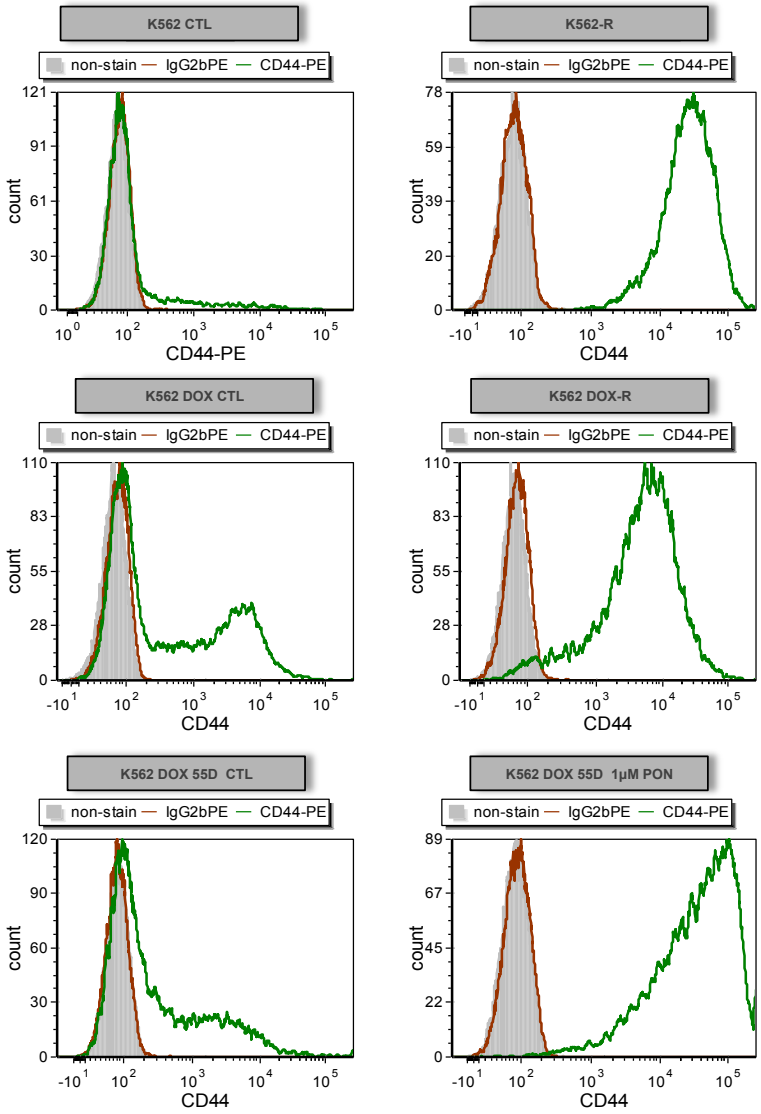


Figure 6.10: Resistant cell lines K562-R, K562 DOX-R and K562 DOX 55D 1µM PON demonstrate increased adhesion compared to their corresponding control lines.
(a) Adherent cell populations detected in all three resistant cell lines but not in their respective control lines by 20x microscopy. **(b)** Cells from the three resistant cell lines and their control lines were collected and either left unstained (grey), or stained with either IgG2b PE isotype control antibody (red) or the corresponding CD44 PE antibody (green). Flow cytometric analysis indicates that all three resistant cell lines are positive for surface E-selectin expression, when compared to their controls. The K562 DOX and K562 DOX 55D control lines demonstrated small populations of cells that were CD44 positive, but this dramatically increased in their resistant counterparts.

Figure 6.11 (a)

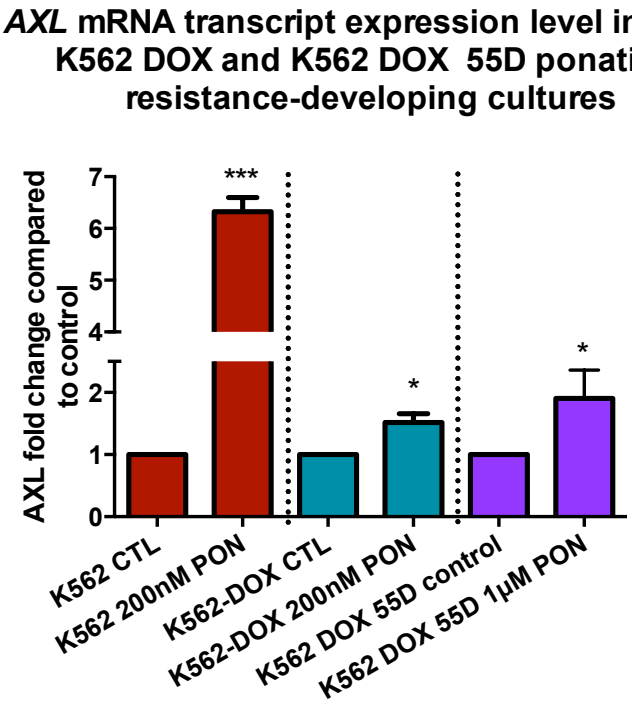

AXL mRNA transcript expression level in K562, K562 DOX and K562 DOX 55D ponatinib resistance-developing cultures
AXL fold change compared to control
0
1
2
4
5
6
7
***
*
*
K562 CTL
K562 200nM PON
K562-DOX CTL
K562-DOX 200nM PON
K562 DOX 55D control
K562 DOX 55D 1μM PON

Figure 6.11 (b)

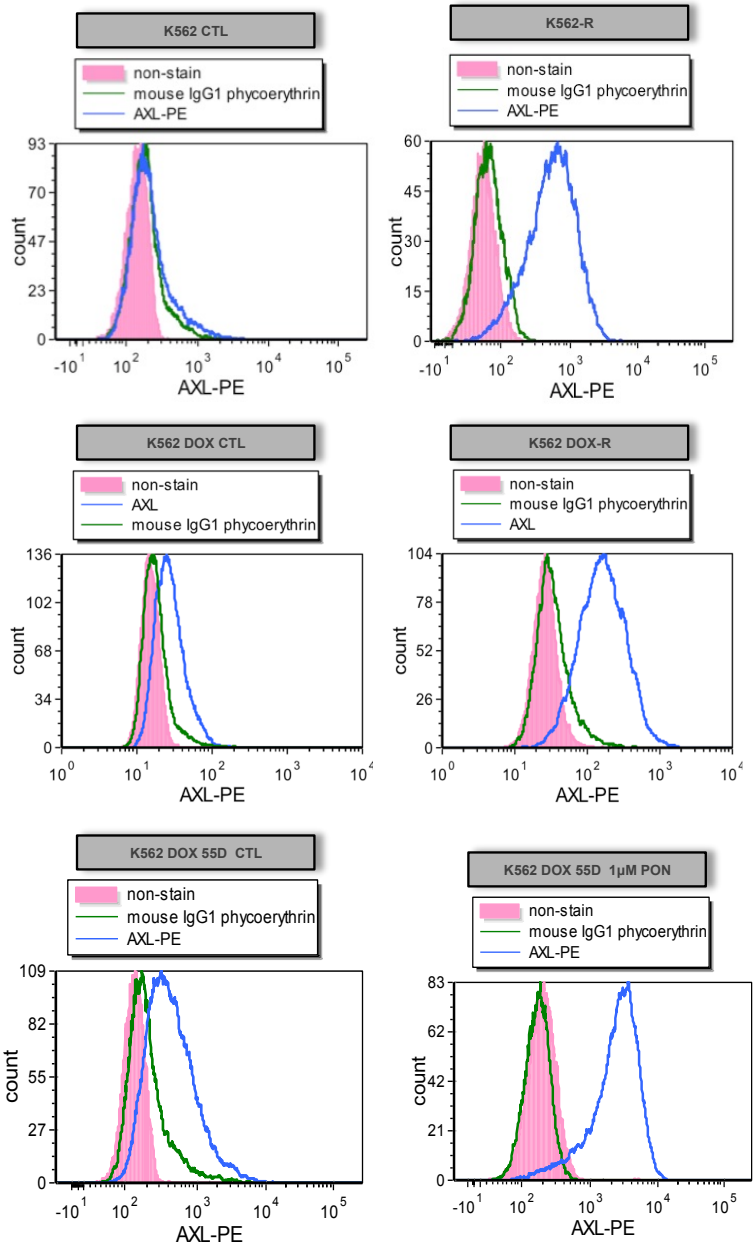

K562 CTL
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE
K562-R
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE
K562 DOX CTL
non-stain
AXL
mouse IgG1 phycoerythrin
count
AXL-PE
K562 DOX-R
non-stain
mouse IgG1 phycoerythrin
AXL
count
AXL-PE
K562 DOX 55D CTL
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE
K562 DOX 55D 1μM PON
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE

Figure 6.11: K562-R, K562 DOX-R and K562 DOX 55D 1μM PON resistant cell lines demonstrate increased expression of the pro-adhesion protein Axl
(a) *AXL* mRNA transcript was measured by RQ-PCR and the results were graphed as fold change compared to control lines. *GUSB* was used as control gene. Overexpression of *AXL* mRNA was observed in the K562-R, K562 DOX-R and K562 DOX 55D 1μM PON cell lines compared to their corresponding controls. Error bars represent SD. n=3 for all data. *p<0.05 and ***p<0.005. **(b)** Cells from the resistant cell lines K562-R, K562 DOX-R and K562 DOX 55D 1μM PON and the respective control cell lines K562, K562 DOX and K562 DOX 55D were collected and either left unstained (pink), or stained with either IgG1 PE isotype control antibody (mouse IgG1 phycoerythrin, green) or with the corresponding Axl PE antibody (blue). There was no (or low in the K562 DOX and the K562 DOX 55D) Axl expression detected in the control cell lines but all the resistant cell lines demonstrated increased Axl expression.

6.3.7 Axl inhibition in the K562-R, K562 DOX-R and K562 DOX 55D 1μM PON ponatinib resistant lines restores sensitivity to ponatinib

As Axl was overexpressed in the three resistant lines, I next sought to determine if Axl inhibition might restore ponatinib sensitivity in the resistant lines. A specific pharmacological inhibitor, R428 was employed. R428 is 50 to100-fold more specific in targeting Axl than the other TAM family members (Mer and Tyro3) (161, 163-165). Figure 6.12 (a)-(b) demonstrated that in the presence of 1 μM R428 (concentration used as per previous publications) (167-169), and 200 nM ponatinib (the culture concentration of ponatinib), significantly reduced the viability of the K562-R ((a), n=3, p=0.02) and K562 DOX-R ((b), n=4, p=0.02) resistant lines was evident. With the presence of 1.25 μM R428 (concentration also previously indicated) (167-169) with 1000 nM ponatinib (as this cell line was cultured in 1000 nM ponatinib) also significantly reduced viability in the K562 DOX 55D 1μM PON line ((c), n=3, p=0.03).

To confirm the inhibition of Axl causing reduced resistance in the three resistant cell lines, another Axl inhibitor, BMS777607, was included in the viability assay. Similar to R428, figure 6.12 (a)-(b) indicated that with the presence of 12.5 μM BMS777607 (concentration used as per previous publications) (170, 171) together with 200 nM ponatinib significantly reduced the viability of the K562-R ((a), n=3, p=0.03) and the K562 DOX-R ((b), n=4, p=0.02) resistant lines. Conversely, the presence of the BMS777607 did not reduce the ponatinib resistance in the K562 DOX 55D 1μM PON cell line (n=3). However, BMS777607 does not target Axl as specifically as R428: it is

about 3-fold more specific for Axl than c-Met, Tyro3, RON and other Met-related targets (170, 171). This may be the reason that with the presence of ponatinib, R428, but not BMS777607, reduced the viability in the K562 DOX 55D 1μM PON cell line. Another explanation is that the K562 DOX 55D 1μM PON cell line has other resistant mechanism(s) to mediate the survival other than Axl overexpression. Notably, the R428 or BMS777607 alone did not significantly reduce viability in any of the three resistant lines.

Taken together, these results indicate that Axl inhibition restored ponatinib sensitivity in the three Bcr-Abl independent resistant cell lines, suggesting that Axl is critical in mediating Bcr-Abl independent ponatinib resistance in these cell lines.

Figure 6.12 (a)

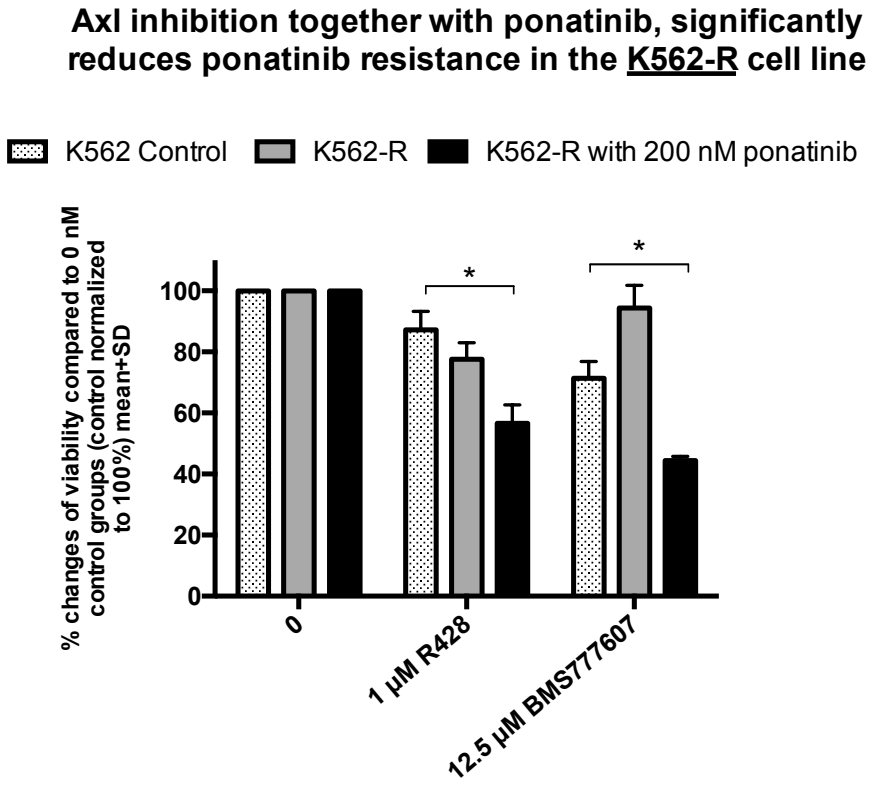

Axl inhibition together with ponatinib, significantly reduces ponatinib resistance in the K562-R cell line
K562 Control
K562-R
K562-R with 200 nM ponatinib
% changes of viability compared to 0 nM control groups (control normalized to 100%) mean+SD
100
80
60
40
20
0
*
*
0
1 μM R428
12.5 μM BMS777607

Figure 6.12 (b)

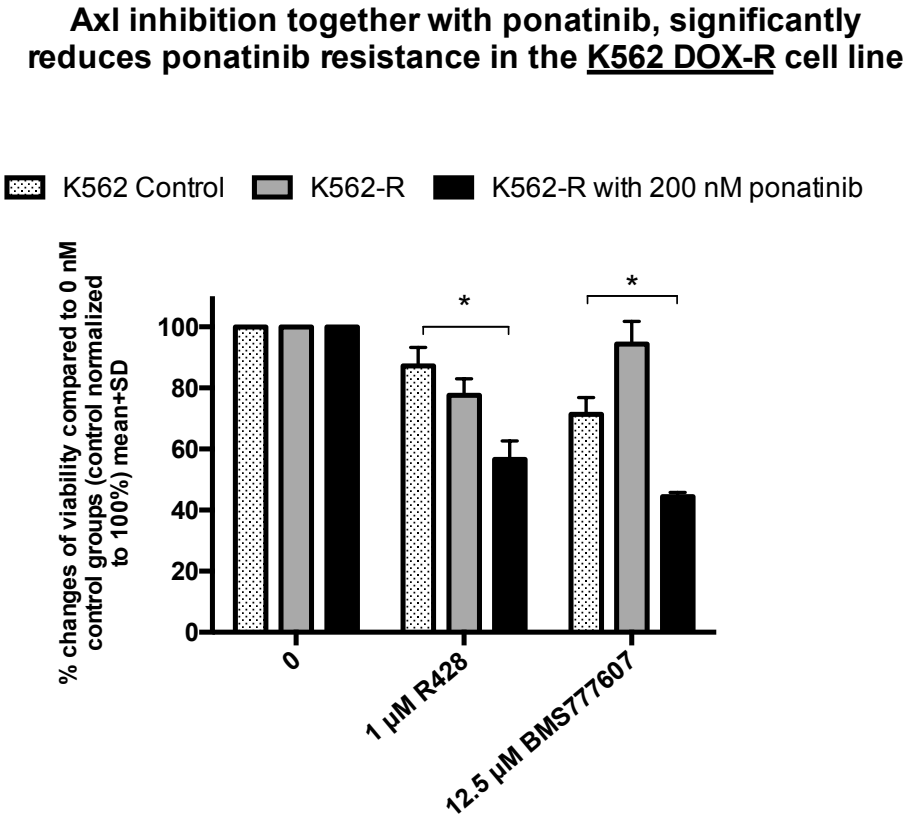

Axl inhibition together with ponatinib, significantly reduces ponatinib resistance in the K562 DOX-R cell line
K562 Control
K562-R
K562-R with 200 nM ponatinib
% changes of viability compared to 0 nM control groups (control normalized to 100%) mean+SD
100
80
60
40
20
0
*
*
0
1 µM R428
12.5 µM BMS777607

Figure 6.12 (c)

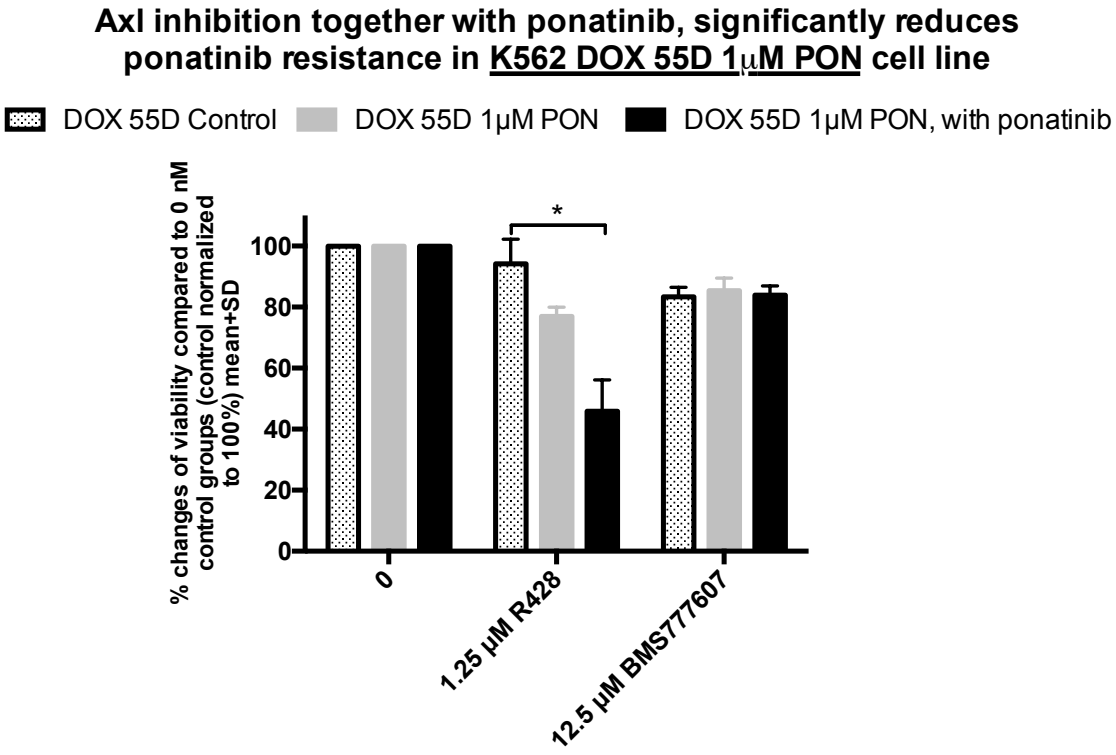


Figure 6.12: inhibition of Axl restores ponatinib sensitivity in the K562-R, K562 DOX-R and K562 DOX 55D 1μM PON cell lines

Cells were washed to remove ponatinib and incubated without ponatinib for 2 hours before performing viability assay. The presence of the Axl inhibitor, 1 μM R428, or 12.5 μM BMS777607, induced cell death significantly in the **(a)** K562-R and **(b)** K562 DOX-R cell lines when co-treated with 200 nM ponatinib (the concentration that these two cell lines are resistant to and cultured in) compared to their corresponding control lines. With the Axl inhibitors only, the cell death in the two resistant lines was increased but not significantly. **(c)** The presence of the Axl inhibitor, 1.25 μM R428, but not 12.5 μM BMS777607, induced cell death significantly in the K562 DOX 55D 1μM PON in the presence of 1000 nM ponatinib (the concentration that this cell line resistant to and cultured in) compared to its control line K562 DOX 55D**.** Error bars represent SD, and these experiments were repeated at least three times. *p<0.05 and ***p<0.005.

6.3.8 AXL knock-down in the K562-R and K562 DOX-R ponatinib resistant lines restores sensitivity to ponatinib

To further confirm that Axl plays a role in the Bcr-Abl independent ponatinib resistance, *AXL* was knocked-down in the K562-R, K562 DOX-R and K562 DOX 55D 1μM PON resistant cell lines by retroviral shRNA (with a GFP tag). *AXL* knock-down K562-R-AXL KD, K562 DOX-R-AXL KD and K562 DOX 55D 1μM PON-AXL KD cell lines were generated, as well as the scramble control lines K562-R-scramble CTL, K562 DOX-R-scramble CTL and K562 DOX 55D 1μM PON-scramble CTL. The positive expression of GFP in the *AXL* knock-down and scramble cell lines indicated the success of the transduction (Figure 6.13 (a)), which was further confirmed by the dramatic reductions in Axl expression in the knock-down cell lines (Figure 6.13 (b)).

Next, the successfully transduced cell lines were exposed to ponatinib to determine whether *AXL* knock-down reduced ponatinib resistance. As demonstrated in Figure 6.13 (c), the K562-R-AXL KD cell line exhibited a dramatic decrease (68% reduction) in viability after incubating with 10 nM ponatinib for 72 hours. This reduction was significantly higher (n=3, p=0.03) compared to the reduction in the control line that treated with scrambled shRNA (22% reduction). In addition, the level of this reduction was similar to the K562 parental ponatinib naïve control (n=3), suggesting that *AXL* knock-down restores ponatinib sensitivity in the K562-R resistant cell line to the parental control level.

The *AXL* knock-down in the K562 DOX-R resistant cell line also demonstrated a significant decrease in viability (53% reduction) compared to the corresponding control line treated with scrambled shRNA (16% reduction) (n=3, p=0.02) (Figure 6.13 (d)) after incubating with 10 nM ponatinib for 72 hours. Again, the extent of cell death in the presence of 10 nM ponatinib incubation following *AXL* knock-down was not significantly different to the K562 DOX parental control (n=3). This result suggested that *AXL* knock-down is responsible for restoring ponatinib sensitivity in the K562 DOX-R resistant cell line, to a level that was comparable to that observed in the ponatinib naïve parental control.

Figure 6.13 (a)

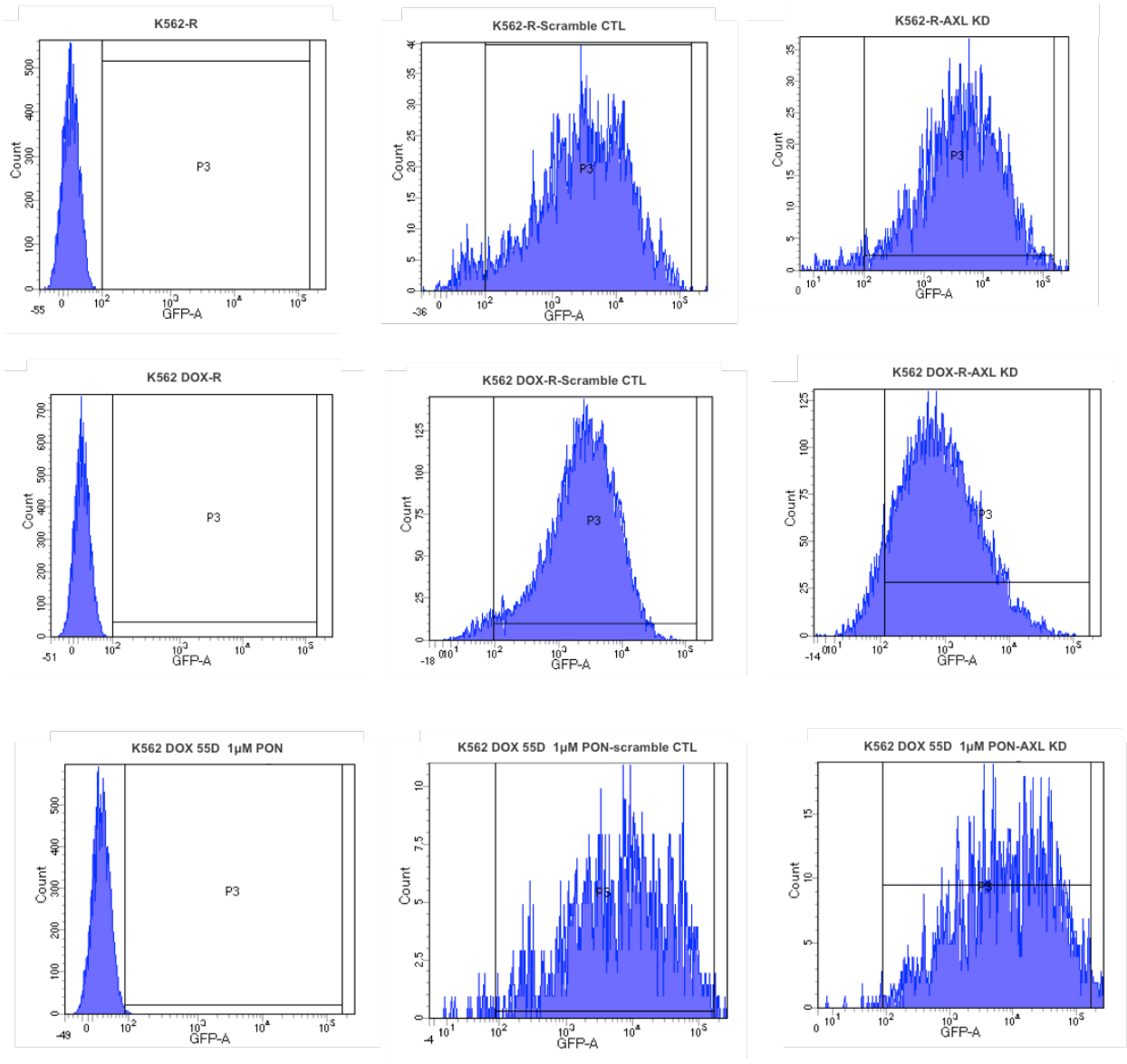

K562-R
K562-R-Scramble CTL
K562-R-AXL KD
K562 DOX-R
K562 DOX-R-Scramble CTL
K562 DOX-R-AXL KD
K562 DOX 55D 1μM PON
K562 DOX 55D 1μM PON-scramble CTL
K562 DOX 55D 1μM PON-AXL KD
Count
GFP-A
P3

Figure 6.13 (b)

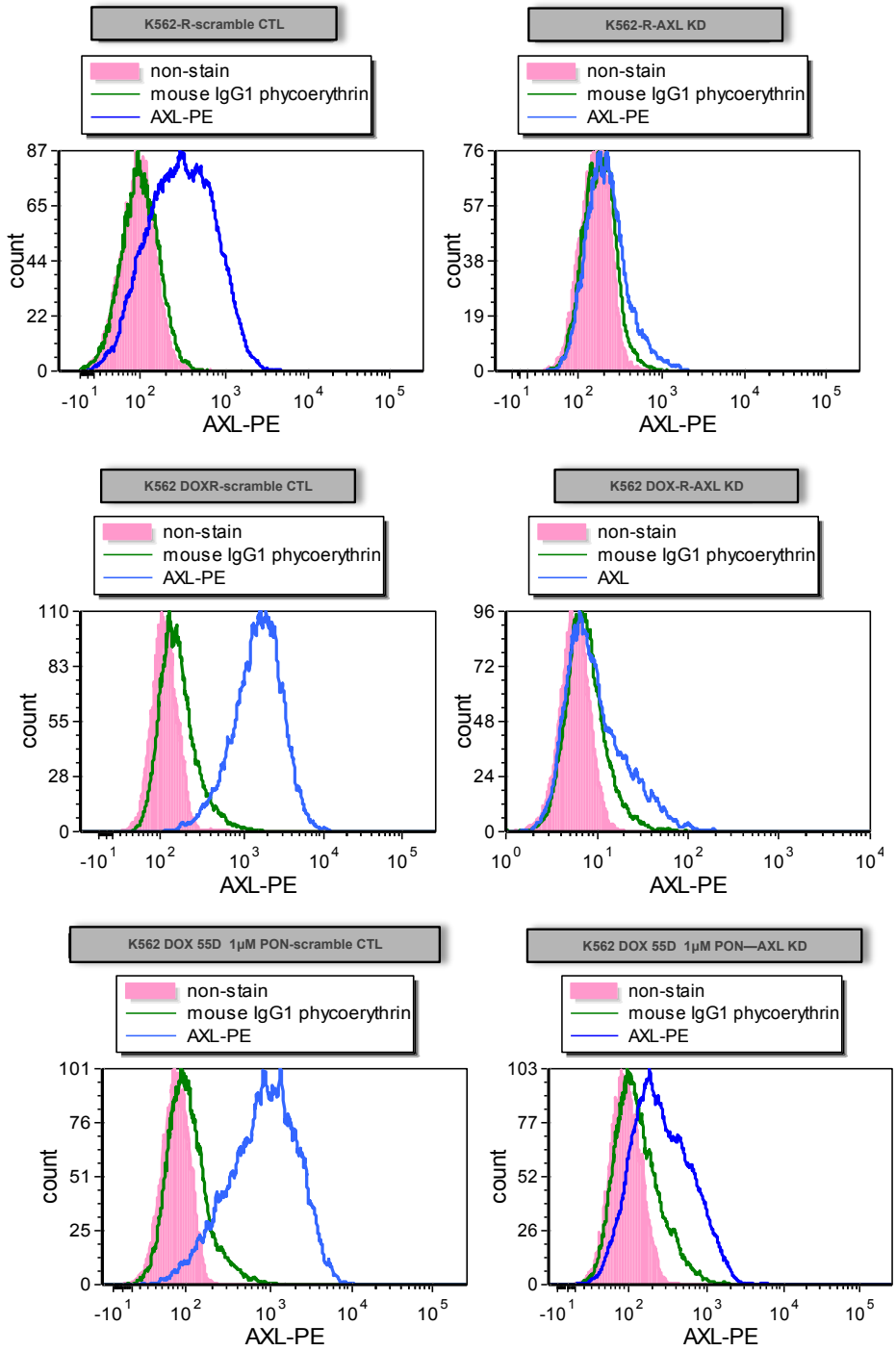

K562-R-scramble CTL
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE
K562-R-AXL KD
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE
K562 DOXR-scramble CTL
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE
K562 DOX-R-AXL KD
non-stain
mouse IgG1 phycoerythrin
AXL
count
AXL-PE
K562 DOX 55D 1μM PON-scramble CTL
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE
K562 DOX 55D 1μM PON—AXL KD
non-stain
mouse IgG1 phycoerythrin
AXL-PE
count
AXL-PE

Figure 6.13 (c)

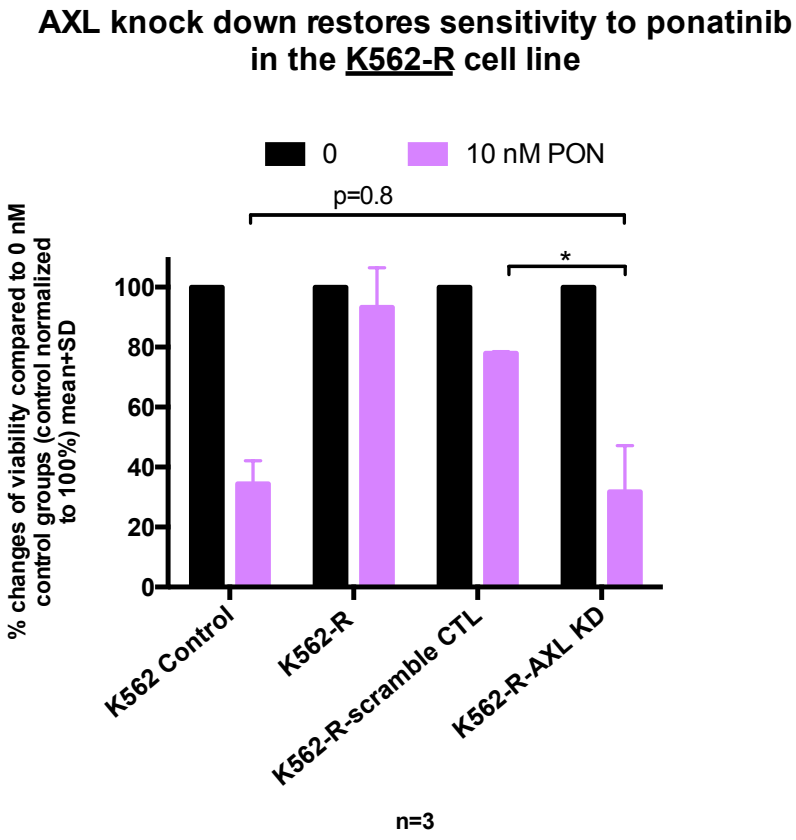

AXL knock down restores sensitivity to ponatinib in the K562-R cell line
0
10 nM PON
p=0.8
*
% changes of viability compared to 0 nM control groups (control normalized to 100%) mean+SD
100
80
60
40
20
0
K562 Control
K562-R
K562-R-scramble CTL
K562-R-AXL KD
n=3

Figure 6.13 (d)

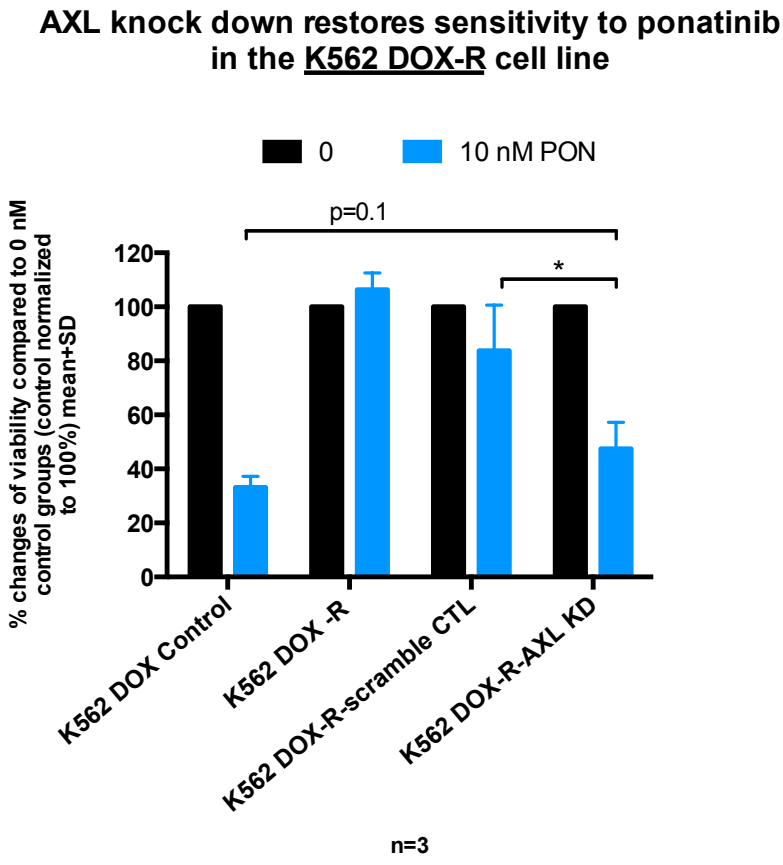

AXL knock down restores sensitivity to ponatinib
in the K562 DOX-R cell line
0
10 nM PON
p=0.1
*
% changes of viability compared to 0 nM
control groups (control normalized
to 100%) mean+SD
120
100
80
60
40
20
0
K562 DOX Control
K562 DOX -R
K562 DOX-R-scramble CTL
K562 DOX-R-AXL KD
n=3

Figure 6.13 (e)

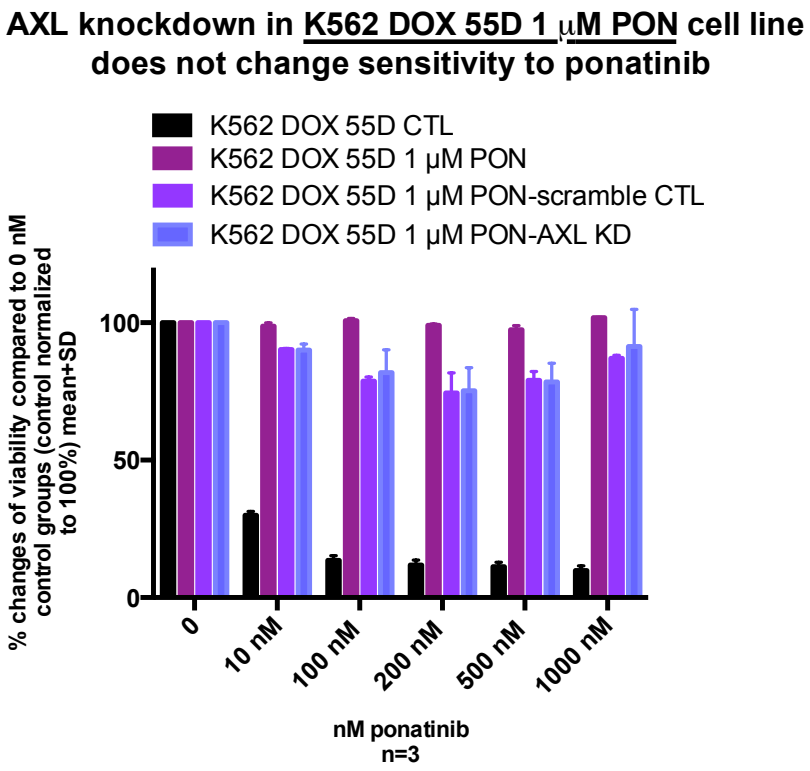

AXL knockdown in K562 DOX 55D 1 μM PON cell line does not change sensitivity to ponatinib
K562 DOX 55D CTL
K562 DOX 55D 1 μM PON
K562 DOX 55D 1 μM PON-scramble CTL
K562 DOX 55D 1 μM PON-AXL KD
% changes of viability compared to 0 nM control groups (control normalized to 100%) mean+SD
100
50
0
0
10 nM
100 nM
200 nM
500 nM
1000 nM
nM ponatinib
n=3

Figure 6.13 (f)

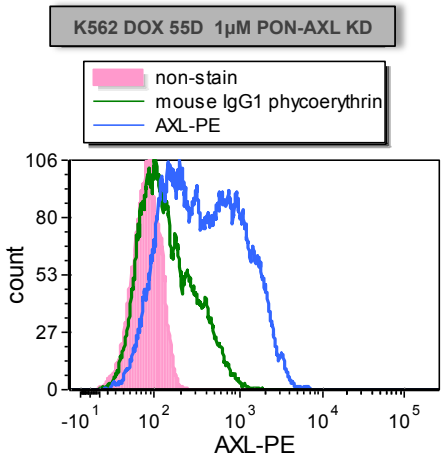


Figure 6.13 *AXL* knockdown increases ponatinib sensitivity in the K562 and K562 DOX ponatinib resistant lines.
(a) GFP expression was determined by flow cytometry following transduction of the resistant cell lines with *AXL* or scramble control shRNA. The GFP expression in all of the transduced cells was above 87% and in the experimental control (non-tranduced) cells was <1%. **(b)** Cell surface Axl expression level was measured by flow cytometry in the AXL or scramble control shRNA tranduced resistant cell lines. Compared to scramble control, *AXL* shRNA tranduced cells demonstrated a reduction in the Axl expression on cell surface. **(c)(d)** compared to scramble controls, *AXL* knockdown in the K562 and K562 DOX ponatinib resistant cell lines demonstrated re-sensitisation to 10 nM ponatinib. **(e)** *AXL* knockdown in the K562 DOX 55D ponatinib resistant line did not alter vell viability when exposed to up to 1000 nM ponatinib. **(f)** Cell surface Axl expression was measured in the K562 DOX 55D 1µM PON-AXL KD cell line and demonstrated a double peak of Axl expression assessed by flow cytometry, indicated Axl was expressed. Error bars represent SD, n=3. *p<0.05.

In contrast to the K562-R and K562 DOX-R resistant cell lines, *AXL* knock-down in the K562 DOX 55D 1μM PON cell line did not reduce resistance to ponatinib. As demonstrated in Figure 6.13 (e), a 3-day incubation with 10 nM ponatinib did not significantly affect viability of the K562 DOX 55D 1μM PON-AXL KD cells (10% reduction) compared to the experimental control K562 DOX 55D 1μM PON-scramble CTL (10% reduction) (n=3). To further verify the sensitivities to ponatinib, the *AXL* knock-down cell line was incubated with higher ponatinib concentration up to 1000 nM. Data demonstrated in Figure 6.13 (e) demonstrated that *AXL* knock-down did not reduce the resistance to ponatinib (up to 1000 nM) in the K562 DOX 55D 1μM PON-AXL KD cells (18%, 25%, 22% and 9% reduction when cultured with 100 nM, 200 nM, 500 nM and 1000 nM ponatinib respectively). Compared to the experimental control K562 DOX 55D 1μM PON-scramble CTL, this reduction was 21%, 26%, 20% and 13% respectively. Notably, increased Axl expression was observed in the K562 DOX 55D 1μM PON-AXL KD cells (Figure 6.13 (f)) (but not in the K562-R-AXL KD, K562 DOX-R-AXL KD cells) as a double peak was observed in the transduced cells. Interestingly, after sorting for the negative peak from the K562 DOX 55D 1μM PON-AXL KD cells, the viability in this line was very low and they failed to expand and survive well enough to perform viability assays. Furthermore, repeated generation of the K562 DOX 55D 1μM PON-AXL KD cell line failed due to the low viability of the *AXL* knock-down line. This raised the possibility that Axl expression is critical for the K562 DOX 55D 1μM PON cells and knock-down of the receptor tyrosine kinase may be detrimental to its survival in the resistant cell line.

Taken together, the *AXL* knock-down experiment confirmed that Axl is critical in mediating ponatinib resistance in the K562-R and K562 DOX-R resistant cell lines. Axl expression also appears to be important for the ponatinib resistance observed in the K562 DOX 55D 1μM PON cell line, although this could not be validated by routine viability assays. The fact that Axl knock-down in the K562 DOX 55D 1μM PON cell line is detrimental to its survival following transduction, strongly indicates a central role for Axl in resistance and survival of this cell line.

6.4 Discussion

In this study, two ponatinib resistant cell lines were generated using the K562 and K562 DOX cell lines. Interesting results were observed in the development of ponatinib resistance from the TKI naïve K562 and K562 DOX cell lines. While it was expected that both cell lines were resistant to ponatinib, it was not known whether the resistance mechanisms in these cell lines was Bcr-Abl dependent or independent. Determination of the pBcr-Abl levels and the ponatinib IC50s by using Bcr-Abl surrogate marker CrkL, indicated the cross-TKI resistance is mainly via Bcr-Abl independent mechanism(s). Interesting findings were observed when both the resistant lines, plus the K562 DOX 55D 1μM PON resistant cell line, were investigated for Bcr-Abl independent ponatinib resistant mechanism(s).

6.4.1 Axl overexpression is critical for Bcr-Abl-independent ponatinib resistance

AXL mRNA and protein overexpression was observed in the three Bcr-Abl-independent ponatinib resistant cell lines. With Axl inhibition via small molecule inhibitors, ponatinib effectively induced apoptosis in the K562-R (R428 and BMS777-607), K562 DOX-R (R428 and BMS777-607) and K562 DOX 55D 1μM PON (R428 only) cell lines. In addition, the knock-down of *AXL* completely restored ponatinib sensitivity in the K562-R and K562 DOX-R resistant cell lines. Consistently, *AXL* knock-down was detrimental to the survival of K562 DOX 55D 1μM PON cell line. These results indicated that Axl overexpression is critical in mediating survival and resistance in the three independent lines. In addition, while Axl overexpression has been associated with imatinib and nilotinib resistant CML (101), this is the first study linking Axl overexpression to the development of ponatinib resistance in a *BCR-ABL1*+ cell line model. While previous publications have indicated that Axl inhibition (by R428) induced apoptosis in AML (155) and chronic lymphocytic leukaemia (CLL) cells (169), the present study is the first study detailing Axl inhibition restoring ponatinib sensitivity in CML.

However, Axl overexpression in the resistant lines was not the only mechanism to maintain the survival in the three cell line models: inhibition or knock-down of Axl alone was not sufficient to induce cell death in the resistant lines, Bcr-Abl kinase inhibition mediated by ponatinib was also required. One possible explanation is that, given Axl targets similar downstream signalling pathways and induces similar cellular events as Bcr-Abl (160), when Bcr-Abl is inhibited by TKI, the

overexpression of Axl compensates for the inhibition and maintains the cellular signalling of CML. Hence, inhibition of Axl and Bcr-Abl at the same time was required to reduce resistance in the cell line models. This hypothesis however, needs to be verified by further studies focusing on the investigation of the downstream pathways, especially PI3K and MAPK pathways of Axl (as these pathways are targeted by Axl), in the resistant lines.

To this end, Axl inhibition re-sensitised the three resistant cell lines to ponatinib suggesting that combination therapy may be warranted in the Bcr-Abl independent ponatinib resistant setting. As the ATP-competitive Axl inhibitor R428 is already in Phase I clinical trial in AML and Phase II trial in lung cancer, this provides hope for clinical treatment. Moreover, measurement of Axl expression level may be used to risk stratify patients who receive ponatinib therapy to identify those likely to achieve better outcomes.

6.4.2 Axl overexpression mediates cell adhesion

As Axl also targets additional signalling pathways and induces cytoskeletal remodelling, increased adhesion was observed in the three Axl overexpressing cell lines. Interestingly, Axl and CD44 were expressed in all three resistant lines. Expression of both these proteins are associated with an epithelial-mesenchymal transition (EMT)-like phenotype (161, 162), suggesting that EMT-like phenotype may be taking place in the resistant lines. This finding is consistent with a previous publication (172) that an EMT-like phenotype can be acquired in CML. EMT-like

phenotype was observed in cells from CML patients who were treated with TKI imatinib for approximately one year. The same researchers (172) also revealed that continuous incubation of imatinib resistant CML cell lines with imatinib or nilotinib selected for the development of adherent cells with EMT-like phenotype. These cells acquired increased capacities for adhesion, migration and invasion (172). While the present study has preliminarily demonstrated that EMT-like phenotype was acquired in the resistant cell line models, further studies including mRNA or protein array that investigate the expression level of EMT gene/protein, are warranted to dissect the function and the relationship of EMT with Axl signalling.

6.4.3 ABCB1 overexpression

In this study, increased cell surface ABCB1 expression was observed in the K562-R ponatinib resistant culture. As the increased expression level of ABCB1 did not decrease the kinase inhibition (detected by ponatinib IC50), this result confirmed the observation in Chapter 3 that ponatinib is not transported by ABCB1 in *BCR-ABL1* positive cells. Hence, the function of this overexpression is yet to be determined. Several previous studies (150, 151) have suggested that MAPK signalling could positively target ABCB1 expression. Hence, the ABCB1 overexpression in the K562-R resistant cell line could be induced by the activation of downstream signalling pathways (MAPK pathway) of Bcr-Abl/Axl. However, whether the ABCB1 overexpression was just a 'side effect' of activation of the MAPK pathway, or whether this overexpression functions in mediating the resistance remained unclear, and will be investigated in future studies.

6.4.4 Kinetics of ponatinib resistance development in the TKI naïve cell line model

The generation of the two TKI naïve ponatinib resistant cell lines took approximately two and half years. This process is much longer when compared to the development of resistance to other TKIs (51, 81). This suggests that sufficient time is required for the development of ponatinib resistance from a TKI naïve setting. The preliminary evidence from the phase III ponatinib EPIC trial indicates that ponatinib has improved efficacy over imatinib in newly diagnosed CP CML patients (this trial was terminated due to the safety concerns) (173). Hence, despite these safety concerns (testing lower dose of ponatinib, to reduce the risk of side effects, is in progress), the use of ponatinib in newly diagnosis CP CML may be better than the current therapy as it offers better efficacy and slower development of resistance. Notably, this thesis has only tested the kinetics of ponatinib resistance development *in vitro*, therefore *in vivo* and clinical evidence will be required to support this hypothesis.

6.4.5 Summary

Overall, the studies in this chapter suggest that Axl overexpression is functionally critical for Bcr-Abl independent ponatinib resistance. This overexpression also induced cross-resistance to other TKIs including imatinib, nilotinib and dasatinib. Therefore, Axl inhibition combined with TKI therapy hold therapeutic promise in the clinical setting of ponatinib resistance.

Chapter 7:

Discussion

7.1 Introduction

Ponatinib is a potent oral tyrosine kinase inhibitor of native and mutated Bcr-Abl, and it is the only FDA approved TKI that effectively targets Bcr-Abl carrying the threonine-to-isoleucine mutation at position 315 (T315I). Phase I and II ponatinib clinical trials demonstrated that this TKI has significant therapeutic activity in CML and Ph+ ALL patients who are resistant or intolerant to other TKI therapies (105). Hence, ponatinib is indicated for CML or Ph+ ALL adult patients that either harbour the T315I mutation, or are resistant to other TKIs by other resistant mechanism(s). However, while ponatinib is the only FDA approved TKI that inhibits Bcr-Abl with all of the identified single kinase domain mutations, the emergence of some compound mutations has been identified as conferring resistance to ponatinib (109, 126). In addition, alternative mechanisms that could cause ponatinib resistance are not yet well explored.

A previous study indicated that ponatinib is a potent ABCG2 inhibitor and a less potent ABCB1 inhibitor at pharmacologically relevant concentrations (50-200 nM) (110). While other TKIs were reported to inhibit ABCB1/ ABCG2, they were also found to be transported by these two proteins (82, 84, 114, 131, 135, 138, 174). However, the previously mentioned study (110) only demonstrated ponatinib-induced inhibition of ABCB1 and ABCG2 but did not describe whether these two transporters efflux ponatinib, nor identify any other possible ponatinib transport mechanisms. As there are currently no other studies detailing ponatinib intra- and extracellular transport, it remains unclear whether the deregulation of transporter proteins may cause resistance to ponatinib at

clinically relevant concentrations as has been demonstrated for other TKIs (82, 84, 114, 122, 131, 135, 138, 174). Therefore, this PhD project firstly aimed to investigate ponatinib influx and efflux transporters, as the deregulation of the drug transporters may change the intracellular ponatinib concentration and therefore mediate the development of resistance.

The second aim of this project was to generate ponatinib resistant *BCR-ABL1*+ cell lines, such that a better understanding of *in vitro* resistance would be gained and hence these resistance mechanisms could most likely be relevant in a clinical setting. The findings presented in this thesis may help to develop new therapeutic strategies for high-risk disease, especially in the setting of Bcr-Abl independent TKI resistance.

7.2 Major findings

7.2.1 ABCB1, ABCG2 and OCT-1 are not major ponatinib transporters

The studies described in this thesis firstly detail the interaction of ponatinib with ABC efflux transporters ABCG2 and ABCB1, as well as the OCT influx transporter OCT-1. As demonstrated in Chapter 3, the expression of ABCG2 or ABCB1 did not significantly affect ponatinib induced kinase inhibition in *BCR-ABL1*+ cells. Moreover, unlike the other TKIs, inhibition of ABCG2, ABCB1 or OCT-1 had no impact on the inhibition of Bcr-Abl by ponatinib. These findings suggested that ponatinib is not transported by ABCB1, ABCG2 or OCT-1. Further, unlike the uptake of imatinib, the uptake of ponatinib into *BCR-ABL1*+ cell lines was temperature, and thus ATP, independent. This suggests that ATP-dependent

active drug transport does not play a role in ponatinib uptake, instead, ponatinib is likely to be passively transported. From a clinical perspective, as ponatinib is not transported by ABCG2, ABCB1 or OCT-1 (common transporters for alternative TKIs), clinically relevant ponatinib resistance is not likely to be mediated by the deregulation of these transporters and subsequently decreased intracellular concentrations of ponatinib.

However, transient upregulation of ABCG2 or ABCB1 was observed during the generation of *in vitro* ponatinib resistance in the present study. ABCG2 overexpression was identified in two intermediate stages (20 nM and 30 nM) of the K562 T315I resistance-developing cultures, but this overexpression was then lost in the K562 T315I-R final resistant cell line. This suggested that while overexpression of ABCG2 may play a role in initiating resistance, or may facilitate the development of other resistance mechanism(s), the increased expression is not required for maintenance of resistance. Similarly, overexpression of ABCB1 was transiently observed in the intermediate stages of K562 DOX and K562 DOX 55D ponatinib resistance-developing cultures. This was notably only when the cell lines were exposed to low ponatinib concentrations. When the two ponatinib-resistance developing cultures were cultured in higher ponatinib concentrations (> 50 nM), increased ABCB1 expression was not evident. The ABCB1 expression levels in the K562 DOX-R and the K562 DOX 55D-R resistant cell lines were similar (or lower) to the expression levels in the respective control cell lines. These results confirm that increase ABCB1 expression is not a sustained mechanism of ponatinib resistance.

ABCB1 expression was identified in the K562-R resistant cell line (final concentration), however, ponatinib resistance in this cell line is Bcr-Abl independent. In addition, similar CrkL ponatinib IC50 was demonstrated in the K562-R resistant cell line compared to the control cell line K562, suggesting ABCB1 overexpression is not involved in enhancing the kinase inhibition. Thus, the ABCB1 overexpression is unlikely to decrease the intracellular ponatinib concentration. Therefore, this suggests that while it is still possible that ABCB1 overexpression plays a role in ponatinib resistance, the overexpression is unlikely to efflux ponatinib. Although it is still possible that the ABCB1 overexpression increases ponatinib efflux and results in reduced inhibition of other kinase(s) (as ponatinib also targets other kinases such as fibroblast growth factor receptor, vascular endothelial growth factor and Src family kinases), but not Bcr-Abl. However, this scenario will be further investigated in the ongoing studies.

In addition, previous studies (150, 151, 175) suggest that several signalling pathways could positively regulate ABCB1 expression. These include forkhead box O3a (FOXO3a). An *in vitro* study has demonstrated that upon cellular stress condition (induced by doxorubicin), FOXO3a amplified PI3K/Akt signalling which then regulated the expression of ABCB1 (175). Hence, it raises the possibility that ABCB1 expression is targeted by FOXO3a as a cellular stress response, which is caused by ponatinib treatment in the resistance-developing cultures. However, within the scope of this study, characterisation of the exact nature of the Bcr-Abl independent mechanism(s) was not achieved. To validate this hypothesis, FOXO3a expression level will be determined in future studies.

Additionally, an ABCB1 functional assay will also be performed to determine whether the inhibition of ABCB1 would restore ponatinib sensitivity in the ABCB1 overexpression intermediate line.

Taken together, all findings presented here fill the gap in the knowledge of the transport of ponatinib in *BCR-ABL1*+ cells, and also suggest that overexpression of ABCB1, ABCG2 and OCT-1 is unlikely to be a major cause of ponatinib resistance by efflux/influx of the TKI.

7.2.2 Bcr-Abl dependent resistance, such as *BCR-ABL1* over-expression or compound mutations, is more likely to develop in the TKI pre-treated setting

In vitro cell line models have been used extensively to characterise resistance to the first and second generation TKIs in CML: our group and other groups have previously generated imatinib, nilotinib and dasatinib resistance in human and/or murine *BCR-ABL1*+ cell lines by culturing with gradually increasing concentrations of TKIs in a step-wise manner (51, 74, 81, 122). TKI resistance mechanisms generated by this method were found to be similar to that observed in the clinic (51, 74, 81, 122). For example, by using this method, a previous study demonstrated that *BCR-ABL1* KD mutations (T315I, V299L or G250E) in *BCR-ABL1*+ cell lines could induce dasatinib or nilotinib resistance (51). Clinically, these mutations have been found to mediate resistance to dasatinib or nilotinib (the T315I mutation induced resistance to imatinib, dasatinib and nilotinib) (53, 55, 176, 177). Hence the same method was employed in the

current study to investigate ponatinib resistance. Five *BCR-ABL1*+ cell lines were chosen as *in vitro* models for ponatinib resistance development within this thesis, with resistance successfully generated in 4 of the 5 cell lines (Table 7.1). Variable ponatinib responses were observed in different cell lines: the emergence of resistance mechanisms either Bcr-Abl dependent or independent.

Ponatinib resistance was generated in two dasatinib pre-treated *BCR-ABL1*+ cell lines, K562 T315I and K562 DOX 55D, by long-term incubation with increasing concentrations of ponatinib (described in Chapters 4 and 5) (Table 7.1). In both of these resistant cell lines, Bcr-Abl dependent mechanisms were identified to confer ponatinib resistance. The K562 T315I-R ponatinib resistant line demonstrated an increase in the percentage of the T315I mutation as well as increase in *BCR-ABL1* mRNA transcript level. This finding was unexpected as it has been approved that ponatinib inhibits Bcr-Abl^{T315I} kinase activity in patients and in *in vitro* models (66, 103, 105, 108). However, these previous studies did not investigate a model that contains both the T315I mutation and significant overexpression of *BCR-ABL1* mRNA transcript (66, 103, 108). Indeed this finding is consistent with previous studies demonstrating X-ray crystallographic evidence that ponatinib binds to naïve Bcr-Abl with more efficacy than to Bcr-Abl^{T315I} (103, 108), and thus an increased expression of *BCR-ABL1* mRNA translating to higher levels of Bcr-Abl protein (Chapter 4) results in an increased proportion of the T315I mutation and resultant decrease in sensitivity to ponatinib.

The K562 DOX 55D-R resistant line demonstrated Bcr-Abl dependent resistance,

with the emergence of the compound mutation G250E/E255K. This result is in line with a previous study demonstrating that, while ponatinib efficiently targets all of the identified single KD mutations, some compound mutations can induce ponatinib resistance (109). Although the G250E/E255K compound mutation has not previously been reported to cause resistance to ponatinib in CML patients, work presented here suggests this compound mutation is likely to develop. Hence, the G250E/E255K compound mutation may confer resistance to ponatinib and other TKIs (as viability assays indicated), especially in patients who have received dasatinib prior to ponatinib treatment. A previous publication studied the interaction of ponatinib with Bcr-Abl, and suggested that the P-loop mutation E255K was close to the binding site of ponatinib and could affect ponatinib efficacy. However, another P-loop mutation, G250E, did not directly affect ponatinib binding (178). While the structural interaction of ponatinib with Bcr-Abl harbouring both of these mutations remains unclear, the insensitivity of the K562 DOX 55D-R cell line to ponatinib (Chapter 5) confirms that an E255K/G250E compound mutation results in ponatinib resistance *in vitro*.

Of note, while this study only identified one compound mutation (G250E/E255K), it is still possible that other compound mutations could confer resistance to ponatinib during clinical treatment. In fact, *in vitro* and clinical studies have demonstrated that ponatinib could not target Bcr-Abl harbouring a variety of compound mutations, especially the compound mutations with T315I inclusive (109, 126). Unexpectedly, T315I-inclusive compound mutations were not observed in the current study although the T315I positive cell line model was utilised in the present research. However, as most patients receive ponatinib

therapy after developing the T315I mutation, these patients therefore have a high chance of developing a T315I-inclusive compound mutation. According to the literature, these mutations confer the highest degree of ponatinib resistance (109), resulting in failure of ponatinib therapy.

The prior TKI (dasatinib) treatment of the two cell lines may partly be the reason for Bcr-Abl dependent resistance mechanisms developing. In the K562 T315I ponatinib naïve cell line, the T315I mutation and *BCR-ABL1* overexpression conferred resistance to 200 nM dasatinib (51). Hence, these two mechanisms were easily utilised when the K562 T315I cell line was cultured with a starting dose of ponatinib and then augmented to maintain survival as the ponatinib dosage was increased. As a result, the proportion of the T315I mutation and the expression level of *BCR-ABL1* transcript further increased under the selective pressure.

In the K562 DOX 55D cell line, *BCR-ABL1* KD mutation was undetectable. However, when this cell line was cultured with increasing concentration of dasatinib to an end-point of 200 nM, a V299L mutation was detected (51). In addition, when this cell line was cultured with increasing concentration of nilotinib to an end-point of 500 nM, a G250E mutation developed (51). This suggested that this cell line could easily be mutated. The cells that have higher risks of developing mutations would have a survival advantage under the pressure of TKI treatment, so prior dasatinib exposure (55 nM) may have selected these cells to expand and become dominant in the K562 DOX 55D-R cell line. O'Hare *et.al.* hypothesised that only cells that could acquire *BCR-ABL1* KD

mutations may originally differentiate from the leukaemic stem cells that are able to sustain malignant hematopoiesis (53). Thus, when this cell line was exposed to ponatinib, mutations readily developed and mediated the resistance to ponatinib. In addition, similar to the K562 T315I cell line, Bcr-Abl dependent resistant mechanism (*BCR-ABL1* mRNA overexpression) was also detected in the K562 DOX 55D cell line. Thus, as suggested by O'Hare *et al*., once KD mutation or other Bcr-Abl dependent resistance mechanism(s) has developed in the leukaemic cells, it was difficult for these cells to activate a Bcr-Abl–independent mechanism, leaving KD mutations as the most feasible escape route (53).

All together, this study suggests that sequential ponatinib-inclusive TKI treatments could result in the development of compound mutation(s), or an increase in the percentage of a pre-existing T315I mutation. Fortunately, most newly diagnosed CP CML patients do not require multi-TKI therapy, especially ponatinib-inclusive treatment, due to high success rates with front line first or second generation TKIs. However, for those patients who fail the front line therapy and develop resistance during ponatinib treatment (especially those patients that harbour compound mutations), novel therapeutic intervention is urgently required. In addition, as the majority (93%) of these patients had been treated with ≥2 TKIs before receiving ponatinib therapy (105), the mechanisms characterised in the prior TKI treated cell line models may be more clinically relevant compared to the mechanisms that developed in a TKI naïve setting (detailed in section 7.2.3)

7.2.3 Bcr-Abl independent resistance, such as Axl over-expression, is more likely to develop in the TKI naïve setting

In this study, the ponatinib resistance developed by the K562-R and K562 DOX-R cell lines was mediated by Bcr-Abl independent mechanism (Table 7.1). These two resistant cell lines were naïve to TKI before treatment with ponatinib. This finding is expected as Bcr-Abl dependent mechanisms are less likely to develop in this setting because: 1. the development of compound mutations is mainly caused by sequential treatment of TKIs (109, 126) so compound mutation is unlikely to cause ponatinib resistance in TKI naïve cells; 2. ponatinib effectively targets Bcr-Abl with all the identified single KD mutations (103, 108) so single mutation alone is unlikely to cause resistance; 3. ponatinib is likely to be passively transported into CML cells (179) so the regulation of transporters is unlikely to decrease ponatinib intracellular levels and induce resistance; 4. in theory, *BCR-ABL1* overexpression is able to confer resistance to ponatinib, however, TKI resistance mediated by *BCR-ABL1* over-expression alone has only been demonstrated for imatinib in clinic (180). For the TKIs that are more potent than imatinib (dasatinib, nilotinib or ponatinib), *BCR-ABL1* over-expression alone has not been reported responsible for resistance in patients. Hence the most likely cause of ponatinib resistance in a TKI naïve setting is increased survival signalling through alternative kinases regardless of Bcr-Abl activity, as evidenced by the activation of Axl in this study.

The K562-R and K562 DOX-R cell lines demonstrated increased cell adherence (Chapter 6). The literature suggests that induction of adhesion is a hallmark of Axl overexpression, and Axl overexpression contributes to imatinib and nilotinib

resistance (153, 154, 160-162). Thus, Axl expression levels were measured in the resistant cell lines. Chapter 6 in this thesis demonstrated that Axl overexpression plays a critical role in mediating the survival of the resistant cell lines K562-R and K562 DOX-R (and K562 DOX 55D 1μM PON). It has been suggested that as a receptor tyrosine kinase, Axl targets MAPK and PI3 kinase pathways (160), which are downstream effectors of Bcr-Abl (26, 181-183) (Figure 7.1). Therefore, Axl overexpression may compensate for pro-survival Bcr-Abl signalling in the resistant lines when Bcr-Abl is inhibited by ponatinib (Figure 7.1). However, the exact cause of Axl overexpression in the resistant cell lines remains unclear. In addition, as Bcr-Abl and Axl are the two main kinases maintaining survival in ponatinib resistance, it is unknown if there is an interaction or cross talk between Bcr-Abl and Axl (Figure 7.1). Interestingly, as described in Chapter 1, Axl interaction with Lyn (and other Src family kinases) was demonstrated in previous studies (75, 93-96, 101). The interaction of Lyn and Bcr-Abl was also observed by a previous study (99). Therefore, it is possible that Bcr-Abl interacts with Axl directly or indirectly (through Lyn) in the Bcr-Abl independent ponatinib resistant cell lines (Figure 7.1), and this is the focus of ongoing studies.

While multiple studies have demonstrated that Axl overexpression is associated with *in vivo* and *in vitro* TKI resistance in CML (101, 153, 154), these studies did not investigate whether Axl inhibition re-sensitised cells to TKIs. In addition, these studies did not describe how the overexpression of Axl mediates TKI resistance. The limitation of patient samples may explain the lack of further investigations in these studies. This thesis however, by using extensive *in vitro*

cell line models, provides the first evidence that the inhibition of Axl and Bcr-Abl induce apoptosis in ponatinib (and TKI-cross) resistance setting. Thus, this result suggests Axl as a potential target in the ponatinib resistant scenario, and patients may benefit from the combination of an Axl inhibitor with ponatinib if refractory to ponatinib alone. Multiple Axl inhibitors are currently in clinical trials, providing hope that this combination therapy will be safe and efficacious. However, further clinical trials would be required to determine the correct dosage for each drug of the combination to prevent /diminish the occurrence of toxicity and side effects.

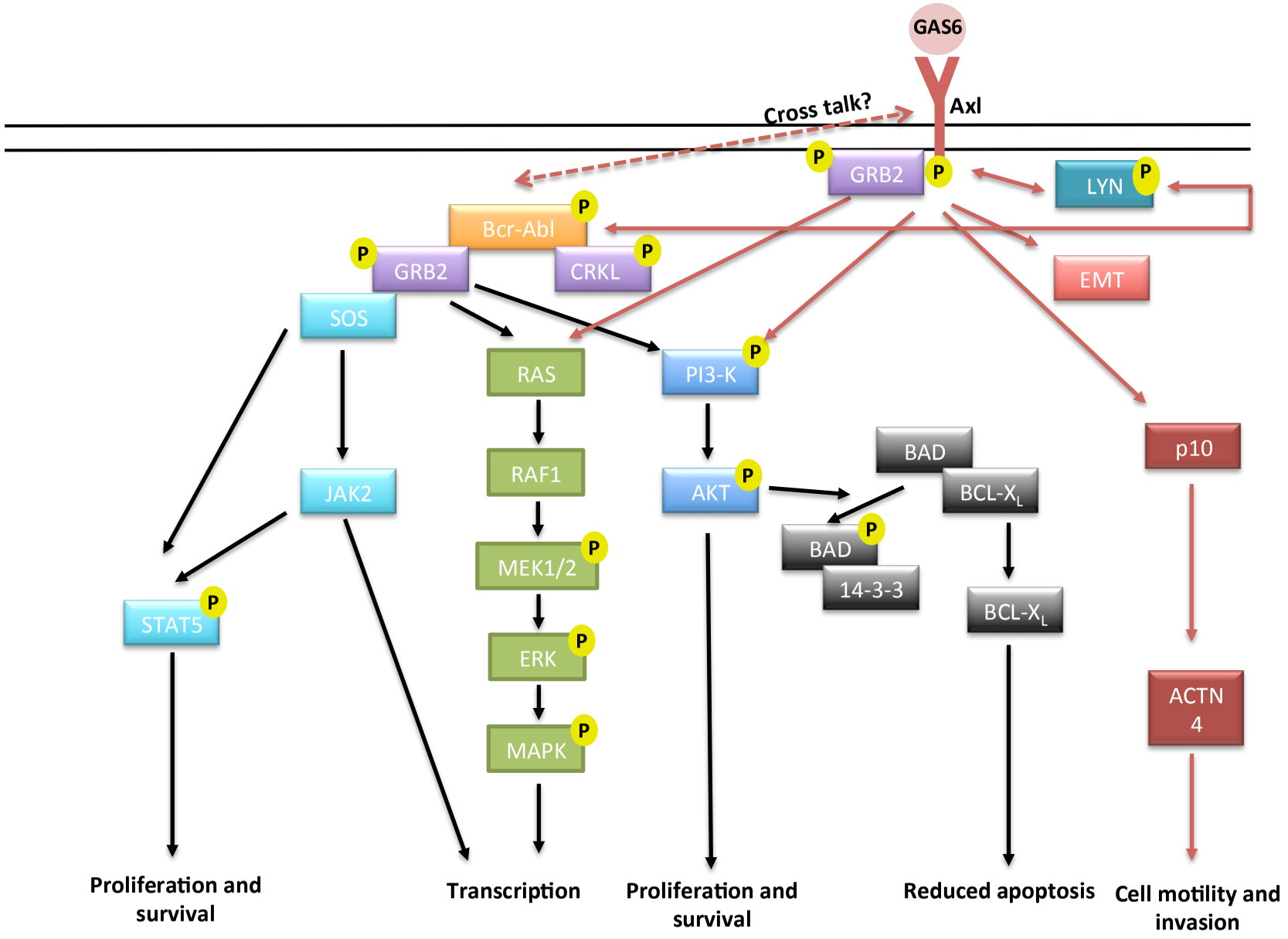
		Ponatinib cultured dosage	Pre-dasatinib treated	increased *BCR-ABL1* mRNA expression	Increased ABCB1 expression	Increased ABCG2 expression	KD mutation	Increased adherence	increased AXL mRNA expression	increased Axl protein expression
Bcr-Abl dependent resistance	K562 T315I-R	100 nM	✓	✓ ⬆⬆⬆	✗	✗	✓ T315I	✗	✗	✗
	K562 DOX 55D-R	200 nM	✓	✗	✗	✗	✓ G250E +E255K	✗	✗	✗
	K562 DOX 55D 1 µM PON (2)	1000 nM	✓	✓ ⬆⬆⬆	✗	✗	✓ G250E +E255K	✗	✗	NA
Bcr-Abl independent resistance	K562 DOX 55D 1 µM PON	1000 nM	✓	✗	✗	✗	✗	✓ ⬆⬆⬆	✓ ⬆⬆⬆	✓ ⬆⬆⬆
	K562-R	200 nM	✗	✗	✓ ⬆⬆	✗	✗	✓ ⬆⬆⬆	✓ ⬆⬆⬆	✓ ⬆⬆⬆
	K562 DOX-R	200 nM	✗	✓ ⬆	✗	✗	✗	✓ ⬆⬆⬆	✓ ⬆⬆⬆	✓ ⬆⬆⬆

Table 7.1: Summary of the characteristics resistance mechanisms detected in the six final ponatinib-resistant cell lines.
✓= yes; **x** = no. ⬆: slightly increase ⬆⬆: increase in ABCB1 expression but the level of expression is less than 80% and is lower than K562 DOX cell line. ⬆⬆⬆: dramatically and significantly increased. Increased was compared to respective control cell line. NA: not tested.

7.2.4 Ponatinib resistance is likely to confer insensitivity to all the other available TKIs

The studies in this thesis generated six ponatinib resistant cell lines including 2 that were cultured in a ponatinib concentration above a clinically relevant level (K562 DOX 55D 1μM PON and K562 DOX 55D 1μM PON(2)). (Table 7.1). Cross-resistance to ponatinib, dasatinib, imatinib and nilotinib (determined by viability assays) was shared by all of the resistant cell lines despite the different resistant mechanisms. This observation is in agreement with the fact that ponatinib is one of the most potent TKIs, especially in treating TKI resistance (as approximately 50% of the patients who were resistant to other TKIs responded to ponatinib) (105). When resistance to ponatinib developed, the mechanism was more likely to cause a higher degree of resistance, meaning resistance not only to ponatinib but also to all other TKIs, when compared to the development of resistance to other TKIs (which mostly could be overcome by treatment with ponatinib). Therefore, other available TKIs were unable to eradicate ponatinib resistant cells.

Figure 7.1: Schematic representation of the proposed Axl and Bcr-Abl signaling pathways in the K562, K562 DOX and K562 DOX 55D Bcr-Abl independent Axl overexpressing ponatinib resistant cell lines.
Axl interacts with PI3-K and Ras signaling as demonstrated in previous studies, and these two pathways are also in the downstream of Bcr-Abl. Bcr-Abl adaptor protein GRB2 is also an adaptor protein of Axl. In addition, Axl interacts with Lyn to exist in a ternary complex and this complex interacts with Bcr-Abl has been previously proposed. In the Bcr-Abl independent ponatinib resistant cell lines presented in this study, Axl overexpression is proposed to compensate Bcr-Abl signaling as common downstream pathways are share between the two kinases. Adapter proteins are depicted in PURPLE, black arrows indicate Bcr-Abl downstream pathways and red arrows indicate Axl related signalling.


GAS6
Axl
Cross talk?
GRB2
LYN
Bcr-Abl
CRKL
GRB2
EMT
SOS
RAS
PI3-K
p10
BAD
BCL-X$_L$
JAK2
RAF1
AKT
BAD
14-3-3
MEK1/2
BCL-X$_L$
STAT5
ERK
ACTN 4
MAPK
Proliferation and survival
Transcription
Proliferation and survival
Reduced apoptosis
Cell motility and invasion

From clinical observations (105), switching to ponatinib (includes >50% of patients with a T315I mutation) results in only a 40-66% rate of complete cytogenetic response, even when the T315I mutation, predicted to be sensitive to this inhibitor, was the cause of the resistance. This suggests that the remaining 34-60% of patients are likely to harbour other resistance mechanisms as demonstrated in the current studies. As exhibited in this thesis, multiple mechanisms may present at the same time and the combination effects of these multiple mechanisms (or different mechanisms may present in different clones) confer cross-resistance. Taken together, the findings presented here suggest that after the development of ponatinib resistance, other available TKIs are unlikely to be to be of clinical value. Novel therapies are therefore urgently required as ponatinib has been in clinical use for two years. Resistance to ponatinib has been observed in patients and subsequently resulted in palliative care. The novel therapies need to involve 1. combination therapy to target the activated alternative signalling pathway(s), and 2. Bcr-Abl inhibitors that interact with the kinase via binding to regions other than the mutated kinase domain. For example, the novel inhibitor ABL001 (Novartis) that binds to the myristate binding pocket of Bcr-Abl results in a conformational change to force inactivation of the kinase (184). The investigation of the efficacy of this novel inhibitor in the setting of ponatinib and other TKI resistance is ongoing in our laboratory.

7.3 Future directions

As a result of the findings presented in this thesis, a number of future investigations have been proposed as necessary for better understanding of ponatinib resistance:

- In order to investigate the downstream signalling of Axl in the ponatinib resistance setting, studies including western blotting, mRNA and protein array analysis will be performed. Quantitative measurement of active MAPK and PI3K signalling are especially important to determine if the activation of Axl and its subsequent downstream signalling, compensates for Bcr-Abl signalling.
- In order to investigate the activation of Axl, gene expression level of Gas6 and protein S (as Axl can be activated by these two ligands) will be measured by RQ-PCR. The upstream of Axl will also be determined by mRNA and protein arrays.
- Protein array will also provide better understanding of the deregulation of other signalling pathways (others than Axl) in the resistant cell lines.
- Screening ponatinib resistant CML/ Ph+ ALL patient cells for Axl expression level will provide better understanding of the importance of Axl in the clinical setting.
- Clonal studies of key intermediate cultures as well as the 'final' resistant cultures will give an insight into the make-up of cell populations and provide better understanding of the resistance development.

- Functional assays of ABCB1/ABCG2 inhibition of the key intermediate cultures will confirm whether the overexpression of these transporters provide growth advantages under the pressure of ponatinib.
- The novel Bcr-Abl inhibitor ABL001 has recently become available. This potent allosteric inhibitor was synthesised to bind to Bcr-Abl in different region than TKIs, and causes a conformational change that inactivates the kinase. We hope to determine this inhibitor's efficacy against ponatinib resistance.
- Screening of other drugs (especially those target the Bcr-Abl^{T315I}) including rebastinib, danusertib, bafetinib and omacetaxine mepesuccinate, to investigate potential treatments in the setting of ponatinib resistance *in vitro*.

7.4 Conclusion

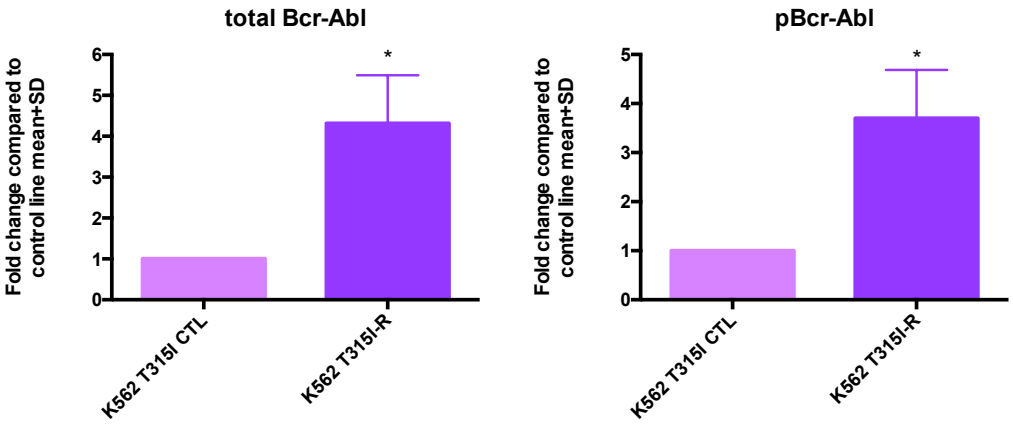
In conclusion, the work presented in this thesis firstly suggests that ponatinib enters *BCR-ABL1*+ cells passively and is not effluxed by ABCG2 or ABCB1. Secondly, this thesis demonstrates that ponatinib resistance can be generated *in vitro* and is therefore likely to be clinically relevant, as it is for all the first and second generation TKIs. This study has generated six ponatinib resistant and TKI-cross resistant *BCR-ABL1*+ leukaemia cell lines in order to investigate resistance development and mechanisms. All of the resistant cell lines demonstrate cross-resistance to imatinib, nilotinib, dasatinib and ponatinib (by viability assays), suggesting that all of these TKIs share the same broad susceptibilities to resistance.

Although there are still many questions to be answered, the identification of Bcr-Abl dependent and independent resistant mechanisms presented here has enhanced our knowledge of ponatinib resistance. This is the first study to present evidence that an increased proportion of T315I decreases sensitivity to ponatinib, and the emergence of compound mutation G250E/E255K results in ponatinib resistance. More importantly, Axl overexpression was identified to confer Bcr-Abl independent resistance. Accordingly, inhibition of Axl reverses resistance to ponatinib. From a clinical perspective, examination of Axl expression level in patient leukaemic cells may provide a predictor for their likely response to ponatinib therapy. Furthermore, determination of Axl protein expression level in patients insensitive to ponatinib therapy could be useful in designing rational combination therapeutic interventions for these patients.

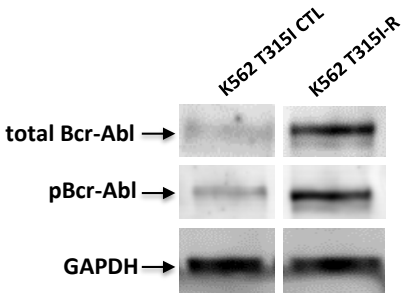
Appendix

Appendix 1

(a)



(b)

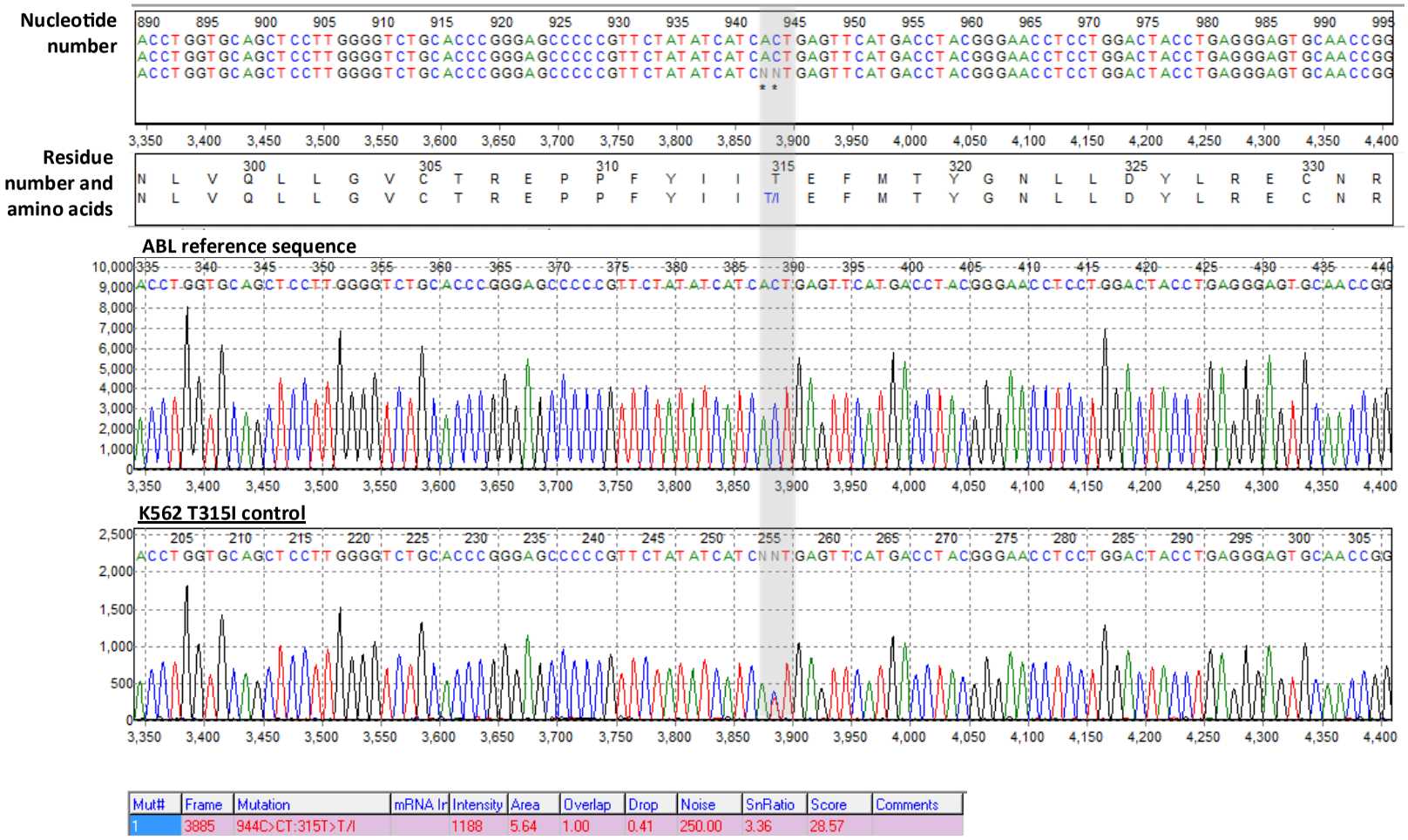


Appendix 1: K562 T315I-R resistant cell line demonstrates significantly increased total and phospho-Bcr-Abl protein.
Ponatinib was removed from the cells. Cells were then incubated with culture medium without ponatinib for 2 hours before being lysed to perform Bcr-Abl western blotting. **(a)** Significant increase in the total Bcr-Abl (approximately 4-fold, probed with c-Abl antibody) and pBcr-Abl (approximately 4-fold, probed with pAbl Y245 antibody) was observed in the K562 T315I-R resistant cell lines compared to the K562 T315I control line Data is from quantification of western blotting images (Bcr-Abl/GAPDH or pBcr-Ab/GAPDH) and represented graphically as fold change. Error bars represent SD. n=3 for all data. **(b)** Representative total and phospho-Bcr-Abl western blotting images**.** GAPDH served as the loading control.

Appendix 2: K562 T315I ponatinib naïve control cell line habours the T315I mutation

Representative DNA sequencing chromatogram of the *BCR-ABL1* kinase domain of K562 T315I ponatinib naïve control cells sequence was compared with the GenBank ABL reference sequence. The K562 T315I cell line contained 41% of the T315I mutation. Each peak represents a DNA nucleotide (A=Adenine; C=Cytosine; G=Guanine; T=Thymine; N=any of the nucleotides) in the 5'-3' direction of the ABL sequence. The nucleotide number for both the reference sequence and the sequences of interest are indicated. The amino acid and corresponding residue number are also indicated. The gray region indicates the C→T substitution at nucleotide 944 causing threonine to isoleucine change at amino acid 315 residue (T315I mutation). Mutation percentage was calculated by Mutation Surveyor software.

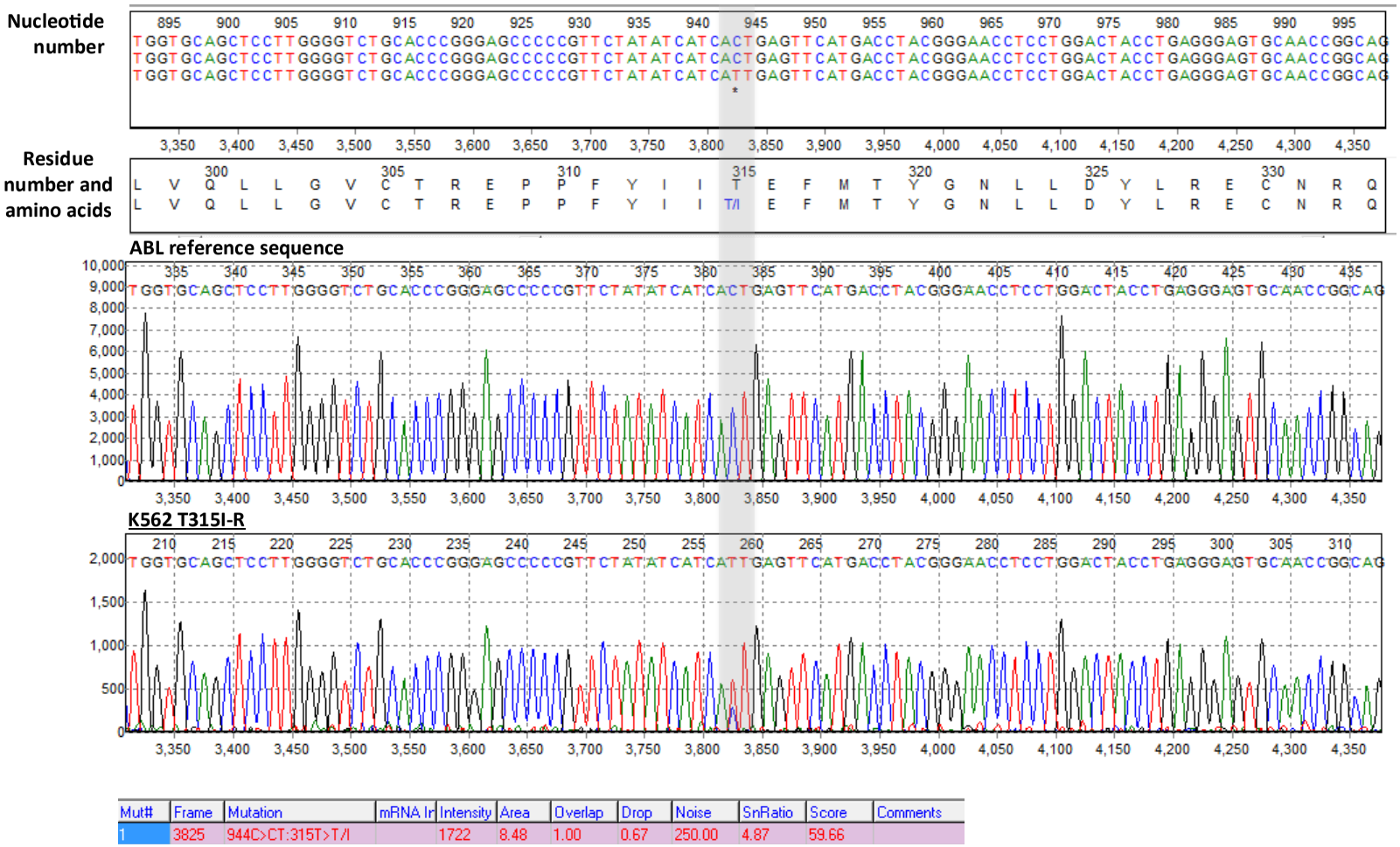
Appendix 2


Nucleotide number
Residue number and amino acids
ABL reference sequence
K562 T315I control
Mut# Frame Mutation mRNA Ir Intensity Area Overlap Drop Noise SnRatio Score Comments
1 3885 944C>CT:315T>T/I 1188 5.64 1.00 0.41 250.00 3.36 28.57

Appendix 3: K562 T315I-R resistance cell line harbours high level (67%) of the T315I mutation

Representative DNA sequencing chromatogram of the *BCR-ABL1* kinase domain of ponatinib resistant K562 T315I-R cells sequence was compared with the GenBank ABL reference sequence. The K562 T315I-R cell line contained 67% of the T315I mutation. Each peak represents a DNA nucleotide (A=Adenine; C=Cytosine; G=Guanine; T=Thymine; N=any of the nucleotides) in the 5'-3' direction of the ABL sequence. The nucleotide number for both the reference sequence and the sequences of interest are indicated. The amino acid and corresponding residue number are also indicated. The gray region indicates the C→T substitution at nucleotide 944 causing threonine to isoleucine change at amino acid 315 residue (T315I mutation). Mutation percentage was calculated by Mutation Surveyor software.

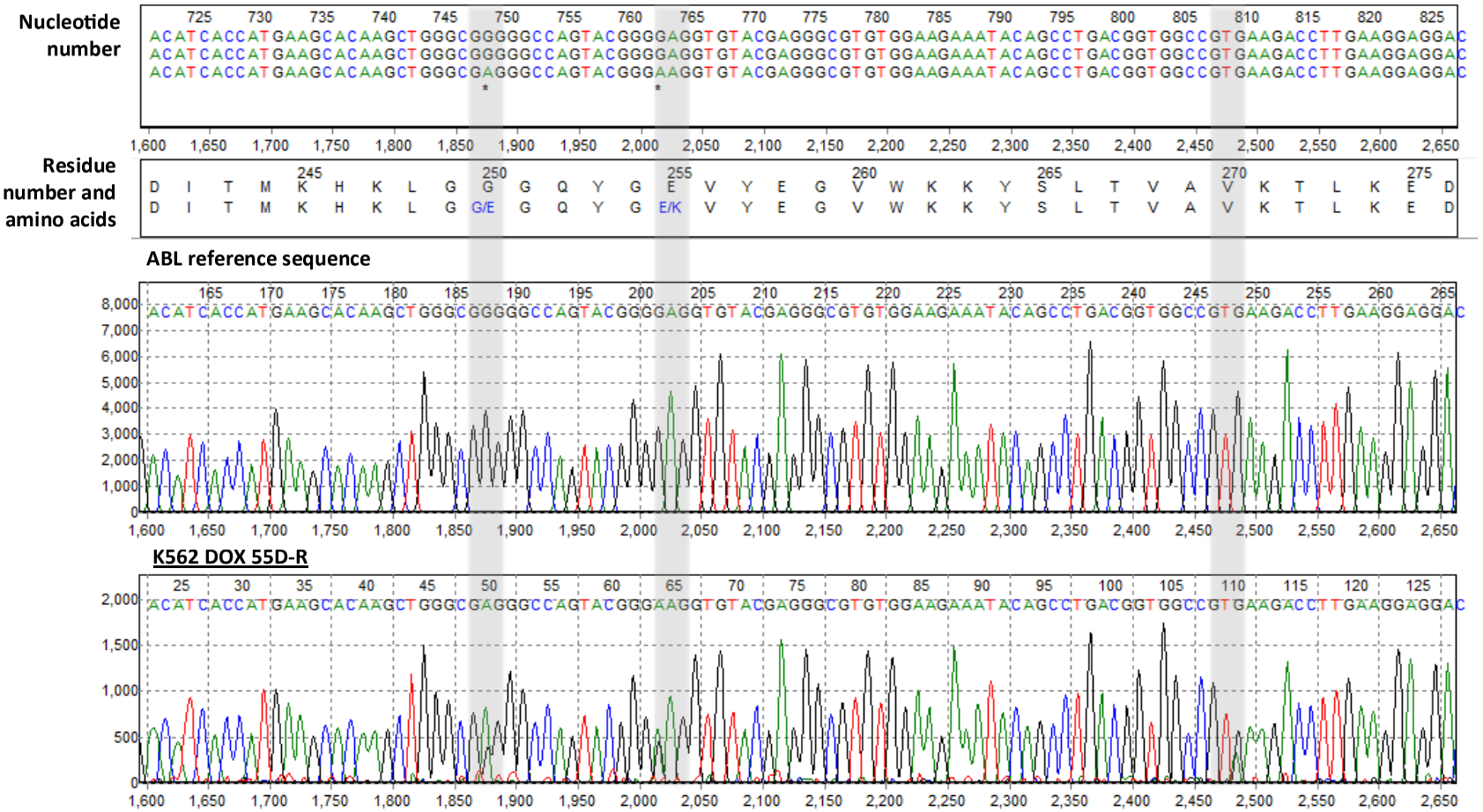
Appendix 3


Nucleotide number
Residue number and amino acids
ABL reference sequence
K562 T315I-R
Mut# Frame Mutation mRNA Ir Intensity Area Overlap Drop Noise SnRatio Score Comments
1 3825 944C>CT:315T>T/I 1722 8.48 1.00 0.67 250.00 4.87 59.66

Appendix 4: K562 DOX 55D-R ponatinib resistant cell line harbours G250E, E255K and V270V mutations

Representative DNA sequencing chromatogram of the *BCR-ABL1* kinase domain of K562 DOX 55D-R ponatinib naïve control cells sequence was compared with the GenBank ABL reference sequence. The K562 DOX 55D-R cell line contained 73% of the G250E, 53% E255K and 41% V270V (silent) mutations. Each peak represents a DNA nucleotide (A=Adenine; C=Cytosine; G=Guanine; T=Thymine) in the 5'-3' direction of the ABL sequence. The nucleotide number for both the reference sequence and the sequences of interest are indicated. The amino acid and corresponding residue number are also indicated. The gray region indicate: 1) the G→A substitution at nucleotide 749 causing glycine to glutamic acid change at amino acid 250 residue (G250E mutation); 2) the G→A substitution at nucleotide 763 causing glutamic acid to lysine change at amino acid 255 residue (E255K mutation); 3) the G→A substitution at nucleotide 810 at amino acid 270 residue, this substitution did not change the amino acid valine in this residue, and hence it is a silent mutation (V270V mutation). Mutation percentages were calculated by Mutation Surveyor software.

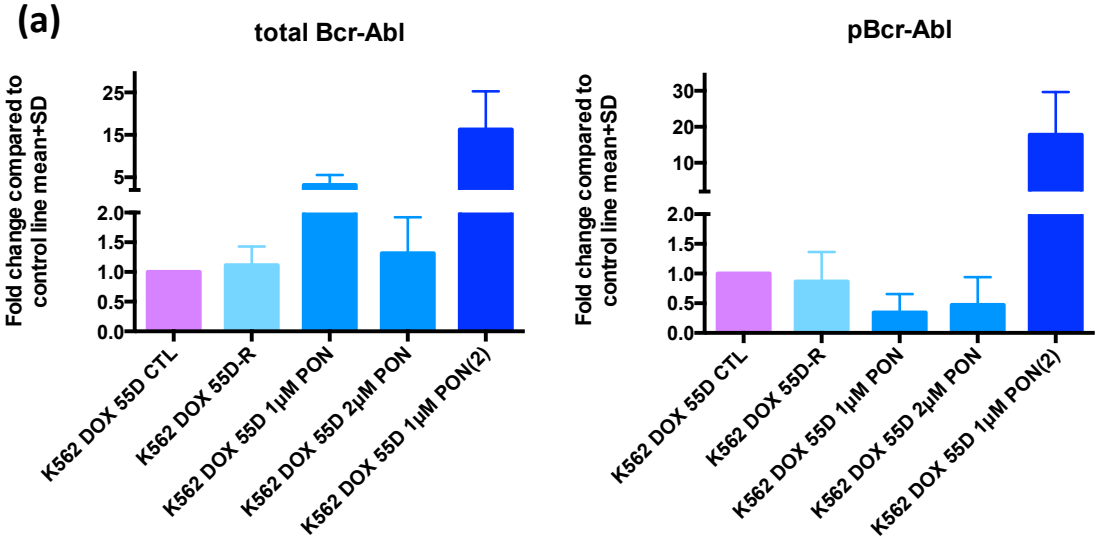
Appendix 4



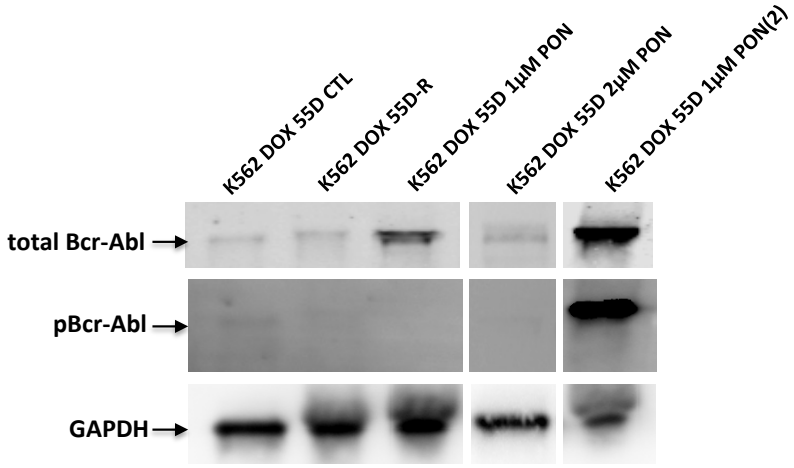
Mut#	Frame	Mutation	mRNA Ir	Intensity	Area	Overlap	Drop	Noise	SnRatio	Score	Comments
1	1875	749G>GA:250G>G/E		1884	11.27	1.00	0.73	250.00	5.33	70.42	
2	2015	763G>GA:255E>E/K		1265	7.28	1.00	0.53	250.00	3.58	34.50	
3	2484	810G>GA:270V>V/V		1116	5.76	0.64	0.41	250.00	3.16	22.43	

Appendix 5

(a)



(b)



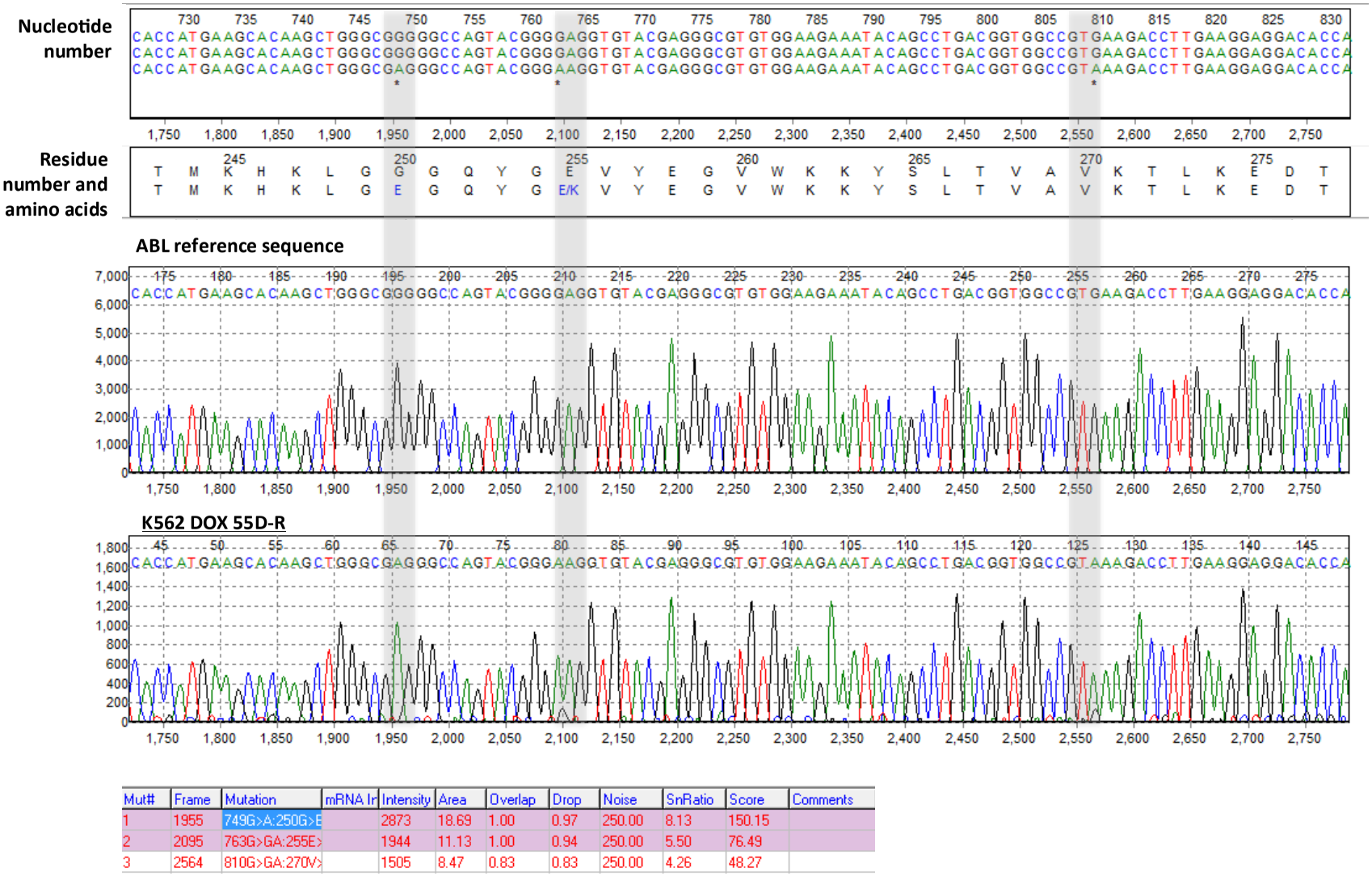
Appendix 5: K562 DOX 55D 1μM PON(2) resistant cell line demonstrate increase total and phospho-Bcr-Abl protein.

Ponatinib was removed from the cells. Cells were then incubated with culture medium without ponatinib for 2 hours before being lysed to perform Bcr-Abl western blotting. **(a)** No statistically significant increase in the total Bcr-Abl (probed with c-Abl antibody) was observed in the K562 DOX 55D-R (1-fold), K562 DOX 55D 1μM PON (3-fold) and K562 DOX 55D 2μM PON (1-fold) cell lines compared to the corresponding control line K562 DOX 55D. No statistically significant changes in pBcr-Abl (probed with pAbl Y245 antibody) were observed in the K562 DOX 55D-R (1-fold), K562 DOX 55D 1μM PON (0.3-fold) and K562 DOX 55D 55D 2μM PON (0.5-fold) cell lines compared to the control line K562 DOX 55D. Increase (both without statistical significance) in the total Bcr-Abl (16-fold) and pBcr-Abl (18-fold) were observed in the K562 DOX 55D 1μM PON(2) cell line. Data is from quantification of western blotting images (Bcr-Abl/GAPDH or pBcr-Ab/GAPDH) and represented graphically as fold change. Error bars represent SD. n=3 for all data. **(b)** Representative total and phospho-Bcr-Abl western blotting images**.** GAPDH served as the loading control.

Appendix 6: K562 DOX 55D 1μM PON(2) ponatinib resistant cell line harbours high levels of G250E, E255K and V270V mutations

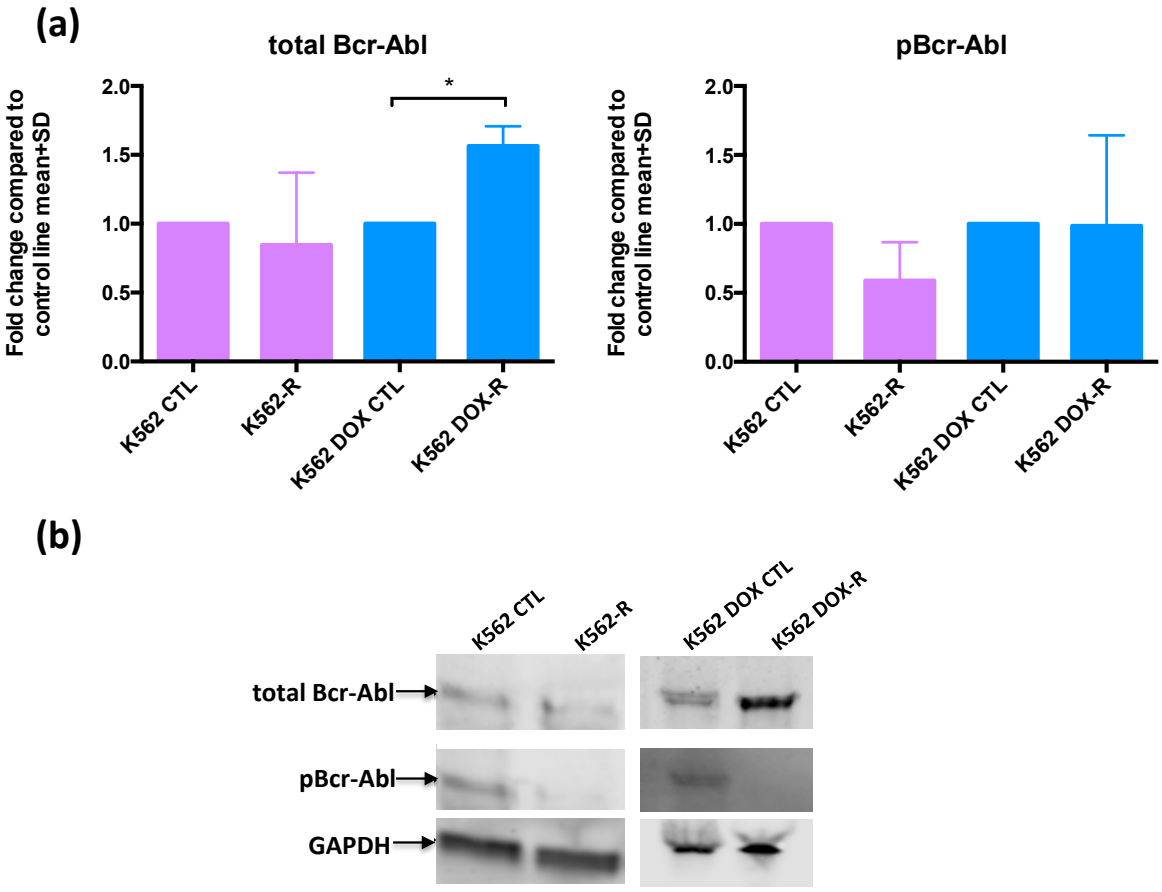
Representative DNA sequencing chromatogram of the *BCR-ABL1* kinase domain of K562 DOX 55D 1μM PON(2) ponatinib naïve control cells sequence was compared with the GenBank ABL reference sequence. The K562 DOX 55D 1μM PON(2) cell line contained contained 97% of the G250E, 94% E255K and 83% V270V (silent) mutations. Each peak represents a DNA nucleotide (A=Adenine; C=Cytosine; G=Guanine; T=Thymine) in the 5'-3' direction of the ABL sequence. The nucleotide number for both the reference sequence and the sequences of interest are indicated. The amino acid and corresponding residue number are also indicated. The gray region indicate: 1) the G→A substitution at nucleotide 749 causing glycine to glutamic acid change at amino acid 250 residue (G250E mutation); 2) the G→A substitution at nucleotide 763 causing glutamic acid to lysine change at amino acid 255 residue (E255K mutation); 3) the G→A substitution at nucleotide 810 at amino acid 270 residue, this substitution did not change the amino acid valine in this residue, and hence it is a silent mutation (V270V mutation). Mutation percentages were calculated by Mutation Surveyor software.

Appendix 6



Mut#	Frame	Mutation	mRNA Ir	Intensity	Area	Overlap	Drop	Noise	SnRatio	Score	Comments
1	1955	749G>A:250G>E		2873	18.69	1.00	0.97	250.00	8.13	150.15	
2	2095	763G>GA:255E>		1944	11.13	1.00	0.94	250.00	5.50	76.49	
3	2564	810G>GA:270V>		1505	8.47	0.83	0.83	250.00	4.26	48.27	

Appendix 7



Appendix 7: K562-R and K562 DOX-R demonstrates similar levels of pBcr-Abl compared to their corresponding control lines

Ponatinib was removed from the cells. Cells were then incubated with culture medium without ponatinib for 2 hours before being lysed to perform Bcr-Abl western blotting. **(a)** Slight reduction and a significant increase in the total Bcr-Abl (probed with c-Abl antibody) was observed in the K562-R (0.8-fold) and K562 DOX-R (2-fold) cell lines respectively, compared to their corresponding control lines. No statistically significant changes in pBcr-Abl (probed with pAbl Y245 antibody) were observed in the K562-R (0.6-fold) and K562 DOX-R (1-fold) cell lines compared to the corresponding control lines. Data is from quantification of western blotting images (Bcr-Abl/GAPDH or pBcr-Ab/GAPDH) and represented graphically as fold change. Error bars represent SD. n=3 for all data. **(b)** Representative total and phospho-Bcr-Abl western blotting images**.** GAPDH served as the loading control.

Publications and awards

Manuscript

Lu L, Saunders V, Leclercq T, Hughes T and White D. 2015. Ponatinib is not transported by ABCB1, ABCG2 or OCT-1 in CML cells. *Leukemia* 29(8):1792-4. Impact factor: 10.431

Conference Abstracts

Lu L, Saunders V, Kok CH, Wang J, Leclercq T, Hughes T and White D. Modelling Ponatinib Resistance Develops In *BCR-ABL*1+ Cell Lines. Annual Meeting of the Haematology Society of Australia and New Zealand (HAA) 2015. Adelaide. Oral presentation.

Lu L, Saunders V, Kok CH, Leclercq T, Hughes T and White D. Modelling Ponatinib Resistance Develops In *BCR-ABL*1+ CML Cell Lines. Annual Meeting of the Australian Society for Medical Research (ASMR). 2015. Adelaide. Oral presentation.

Lu L, Saunders V, Kok CH, Leclercq T, Hughes T and White D. Modelling Ponatinib Resistance Develops In CML Cell Line: Implications For Ponatinib Resistance When Used As A Treatment For TKI-Resistant Patients. Annual Meeting of the American Society of Hematology (ASH) 2014. San Francisco USA. Poster presentation.

Lu L, Saunders V, Kok CH, Leclercq T, Hughes T and White D.
Ponatinib resistance develops in a CML cell line via Bcr-Abl1 independent mechanisms. New Direction of Leukaemia Research (NDLR) 2014. Sunshine Coast QLD. Poster presentation.

Lu L, Saunders V, Kok CH, Leclercq T, Hughes T and White D.
Modeling Ponatinib Resistance in *BCR-ABL1*+ Cell Lines: Implications For Ponatinib Resistance in TKI-Resistant and TKI-naïve Patients. Florey Health Science postgraduate conference (FHS) 2014, The University of Adelaide. Poster presentation

Lu L, Saunders V, Kok CH, Leclercq T, Hughes T and White D.
Ponatinib resistance develops in CML cell line previously exposed to dasatinib, but not in TKI-naïve CML cell lines: implications for ponatinib resistance when used as a treatment for TKI-resistant patients. Florey Health Science postgraduate conference (FHS) 2013, The University of Adelaide. Poster presentation

Lu L, Saunders V, Kok CH, Leclercq T, Hughes T and White D.
Ponatinib resistance develops in CML cell line previously exposed to dasatinib, but not in TKI-naïve CML cell lines: implications for ponatinib resistance when used as a treatment for TKI-resistant patients. Centre for Personalised Cancer Medicine (CPCM) 2013 Adelaide. Poster presentation:

Lu L, Saunders V, Clackson T, Hughes T and White D.

ATP Dependent Efflux Transporters ABCB1 and ABCG2 are Unlikely to Impact the Efficacy, or Mediate Resistance to Ponatinib. New Direction of Leukaemia Research (NDLR) 2012. Sunshine Coast QLD. Poster presentation.

White D, Lu L, Saunders V, Clackson T and Hughes T.

ATP Dependent Efflux Transporters ABCB1 and ABCG2 are Unlikely to Impact the Efficacy, or Mediate Resistance to the Tyrosine Kinase Inhibitor, Ponatinib.

American Society of Haematology (ASH) 2011. Poster presentation.

Scholarships and Awards

PhD Scholarship, Adelaide Graduate Research Scholarship, 2012-2015

2015 SAHMRI PhD 3 minutes oral presentation, 2nd prize

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