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Master's Thesis

Overcoming Drug-Resistance in Chronic Myeloid Leukemia

Author
Anni Rebane

Supervisors

Gretchen Repasky, PhD
Senior Researcher

Institute for Molecular Medicine Finland (FIMM)/HiLIFE Unit
University of Helsinki

Krister Wennerberg, PhD
FIMM-EMBL Group Leader

Institute for Molecular Medicine Finland (FIMM)/HiLIFE Unit
University of Helsinki

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Key abbreviations

(*in alphabetical order*)

A-loop – activation loop

ABL1 (ABL or c-ABL) – Abelson proto-oncogene 1, non-receptor tyrosine kinase

ALL – acute lymphoid/lymphoblastic/lymphocytic leukemia

AML – acute myeloid/myelogenous/myelocytic leukemia

ATP – adenosine triphosphate

BCR (c-BCR) – Breakpoint cluster region

BCR-ABL1 – fusion oncogene composed of BCR and ABL1

CLL – chronic lymphoid/lymphoblastic/lymphocytic leukemia

CML (CGL) – chronic myeloid/myelogenous/myelocytic; chronic granulocytic leukemia (*historical*)

IC_{50} – half-maximal inhibitory score

mTOR – mammalian/mechanistic target of rapamycin

Ph+ – Philadelphia-chromosome positive

P-loop – ATP-binding domain/pocket/site

sDSS – selective Drug Sensitivity Score

SH1-3 – Src homology

SMAC mimetics – inhibitors that mimic ‘small molecule second mitochondrial activator of caspase’

TKI – tyrosine kinase inhibitor

Mutation names

T315I – “gatekeeper” mutation in which threonine is replaced by isoleucine in amino acid position 315

T315M – mutation in which threonine is replaced by methionine in amino acid position 315

E255V/T315I – compound mutation in which glutamic acid is replaced by valine in amino acid position 255, and threonine is replaced by isoleucine in amino acid position 315

Abstract

Given the success of first-line treatment in chronic myeloid leukemia (CML), the prevalence of the disease is estimated to increase and more patients are expected to develop resistance to therapy. Thus, even relatively rare point mutations are likely to become more common. In CML, the uncontrollable division of myeloid cells is caused by a reciprocal translocation of chromosomes 9 and 22, resulting in the Philadelphia chromosome. At the meeting point of the two chromosomes, breakpoint cluster region (BCR) and Abelson proto-oncogene 1 (ABL1) fuse together to form the chimeric fusion oncogene BCR-ABL1, the latter of which, the non-receptor tyrosine kinase ABL1, is the driver of the disease. Since the tyrosine kinase inhibitor (TKI) imatinib became available in 2001, the success of first-line therapy has significantly improved the prognosis of CML patients. However, up to 50% of patients with imatinib-refractory disease develop resistance due to point mutations in ABL1, and the most common mutation to emerge is BCR-ABL1 T315I. The broad-range TKI ponatinib is the only approved TKI that inhibits the kinase activity of BCR-ABL1 T315I, but adverse side effects leave patients with this mutation in need of a better, safer, and more effective treatment. The kinase inhibitor axitinib was shown to be selective for BCR-ABL1 T315I, but mutations that emerge as a consequence of axitinib-resistance have yet to be explored. Moreover, patients with the T315I mutation treated with ponatinib have been reported to develop highly drug-resistant mutations in BCR-ABL1 such as T315M and the E255V/T315I compound mutation.

The purpose of this study was to identify mutations that enable cells to develop resistance to the kinase inhibitor axitinib and to find new, potential inhibitors for cells expressing the drug-resistant mutations BCR-ABL1 T315I, BCR-ABL1 T315M, and BCR-ABL1 E255V/T315I. To this end, mouse hematopoietic cell lines were constructed prior to determining cell viability in response to inhibitors in combinations and as independent agents. As a novel finding, cells stably expressing T315M were found to exhibit sensitivity to inhibitors of topoisomerase II and mTOR. Moreover, synthetic lethality occurred in these cells in response to the combined treatment of the allosteric inhibitor asciminib and the TKI ponatinib, although not in clinically relevant doses. The highly resistant cells expressing BCR-ABL1 E255V/T315I, like cells expressing T315I and T315M, showed sensitivity to conventional chemotherapy. Notably, however, three SMAC mimetics displayed selectivity to cells expressing BCR-ABL1 E255V/T315I over cells expressing only the single T315I mutation.

Considering that CML is expected to become increasingly prevalent, more patients are estimated to develop resistance to therapy. As even relatively rare mutations in BCR-ABL1 become more common, finding an effective treatment for cells expressing these highly resistant mutations takes us one step closer to identifying a safe and effective treatment for CML patients carrying those mutations.

1. Literature Review

1.1. Cancer is diverse

1.1.1. Overview

If human life expectancy continues to rise as it has done in most countries in the past few decades, or even if it plateaus at the current level (Vaupel, 2010), an unprecedented number of people will live into old age (Christensen *et al.*, 2009). Even if the population of the world does not surpass ten billion people by the end of this century, as predicted by Lutz *et al.* (2001), the increasing number of people, including the elderly, is likely to adjust priorities in the healthcare system.

With aging, cellular processes in the body become less efficient and mutations in the genome more frequent, resulting in a range of age-related diseases (DiLoreto and Murphy, 2015). If a mutation affects genes that are responsible for the division and growth of a cell, the cell may become incapable of controlling its division and thereby contribute to cancer risk (Stratton *et al.*, 2009). In addition to increased age, risk factors for cancer include environmental factors, diet, and individual genetic background (Padma, 2015).

Although advancements in cancer treatments have added valuable years to the lives of cancer patients (Siegel *et al.*, 2016), not every patient benefits. Conventional chemotherapy, which has focused on finding treatments for large populations, has resulted in reduced rates of metastasis and relapse, and increased overall survival (Liu *et al.*, 2015). However, it also often targets all rapidly dividing cells and therefore results in frequent side effects (Bagnyukova *et al.*, 2010). In recent years, there has been increasing awareness regarding the differences in inherent characteristics leading to distinct treatment responses. Indeed, the tremendous complexity and heterogeneity in cancer revealed by a plethora of genomic and proteomic data highlights the challenge of finding a tailored approach to each individual case (Agyeman and Ofori-Asenso, 2015). Although both are considered chemotherapy, conventional chemotherapy does not target cancer cells as specifically as targeted therapy (Palumbo *et al*., 2013).

1.1.2. Targeted therapy

The purpose of targeted therapy is to design drugs that target specific genes or proteins that single out cancer cells or the microenvironment that, among other things, enables the cells to continue to divide. The benefit of the targeted approach is explained by increased specificity to the cancer cells and the reduction of side effects usually caused by off-target binding of the drug. Targeted therapy involves designing compounds that are capable of inciting autophagy or apoptosis, inhibiting the division of cancer cells, contributing to cell cycle regulation, and, most importantly, directing toxic compounds exclusively to cancer cells to kill them. In particular, the compounds used in targeted treatment include small molecule inhibitors and monoclonal antibodies, often in combination with chemotherapy or other treatments (Gerber, 2008). Consequently, targeted therapy affects fewer healthy cells than conventional chemotherapy.

1.1.3. Combination therapy

Combination therapy is another way to increase cancer treatment efficacy. The simultaneous use of multiple drugs can prove advantageous for several reasons. First, two or more compounds can strengthen a therapeutic effect by increasing efficacy. Second, the use of multiple drugs can result in decreased dosages of each drug, which, in turn, can lead to lowered toxicity and fewer side effects. Third, successful combination therapy decreases the likelihood of developing drug-resistance, a frequent challenge and a complex problem to avoid (Chou, 2006; Bukowska *et al.*, 2015).

Notably, the interaction between compounds can affect cell growth in different ways. First, compounds can act in synergy. Synergy occurs when two or more drugs, when combined, affect more cells than the sum of each would predict ($1 + 1 > 2$). When the potency of combined drugs equals to the sum of each, the effect is additive ($1 + 1 = 2$). However, antagonism occurs when the potency of two or more drugs is less than can be predicted from the discrete potency of each drug ($1 + 1 < 2$; Tallarida, 2007; Karjalainen and Repasky, 2016). Among them, synergy provides the most therapeutic benefits. In particular, selective synergy offers an efficient clinical tool by selectively targeting cancer cells instead of healthy cells (Chou, 2006). Although combinations in cancer therapy have not yet been studied and applied as much as clinical demand would suggest, some combination therapies have been shown to be clinically effective and many are in clinical trials (Karjalainen and Repasky, 2016).

Combination therapies can include conventional chemotherapy agents, targeted therapy agents, or a combination of the two. One example of therapy which combines two targeted compounds is found in AML (acute myeloid leukemia). AML is a tremendously heterogeneous leukemia with thousands of driver mutations (Papaemmanuil and Gerstung *et al.*, 2016), but the most frequent to occur are the FLT3 (FMS-like tyrosine kinase-3) activating mutations. In fact, 30% of AML cases are estimated to contain FLT3 mutations (Levis and Small, 2003), making this a desirable drug target. Notably, preclinical studies suggest that combining FLT3 inhibitors with inhibitors of HDAC (histone deacetylase) leads to synergy between the two compounds (Bali *et al.*, 2004; Pietschmann *et al.*, 2012). HDACs have been shown to be involved in activating and regulating nonhistone proteins such as HSPs (heat shock proteins). Acetylating Hsp90 renders it inactive and results in incorrect folding as well as degradation of its substrate proteins, including FLT3 (Bali *et al.*, 2004). Consequently, simultaneous inhibition of HDAC and FLT3 might provide clinical benefits to AML patients (Pietschmann *et al.*, 2012). While success does not follow many combinations expected to improve the lives of cancer patients, the combinations that lead to favorable outcomes provide an opportunity to predict successful combination therapies better.

1.2. Drug-resistance poses a problem

1.2.1. Overview

Drug-resistance in cancer can be divided into two groups: intrinsic (primary) and acquired (secondary) (Hayes and Wolf, 1990). In intrinsic drug-resistance, factors contributing to resistance are present in the tumor prior to chemotherapy. When drug-resistance is acquired, tumors that exhibited initial sensitivity can develop resistance because of emerging mutations or other adaptive

means, such as triggering an alternative signaling pathway or increasing the expression of a target site (Hayes and Wolf, 1990). Given the enormous heterogeneity in cancer (Swanton, 2012), chemotherapy that effectively terminates most of the tumor can induce drug-resistance by selecting minor but resistant subpopulations of cancer cells (clonal evolution) (Burrell and Swanton, 2014). Furthermore, undifferentiated cells known as cancer stem cells have been implicated in resistance to chemotherapy. Both primary and secondary drug-resistance can be caused by inhibition of apoptosis, DNA damage repair, epigenetic modifications, or epithelial-mesenchymal transition (which enables metastases), as well as drug inactivation, efflux, and target alteration [Figure 1] (Housman *et al.*, 2014).

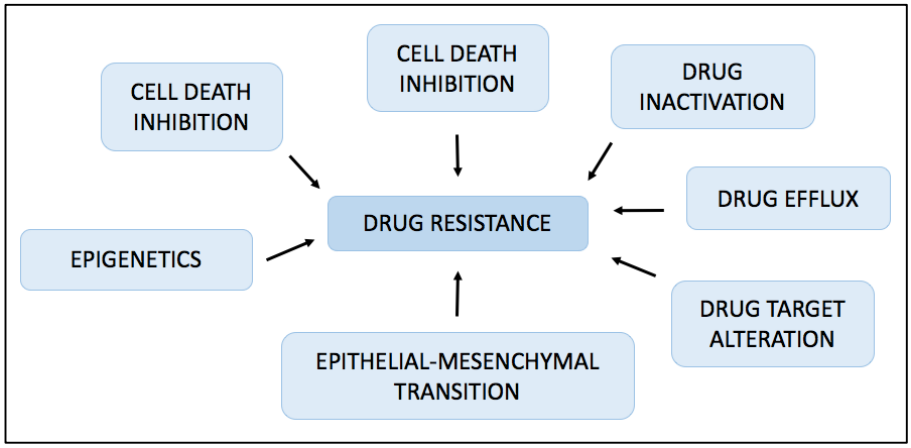

Figure 1: Mechanisms that allow or support drug-resistance in cancer are able to act independently or in various combinations (adapted from Housman *et al.*, *Cancers*, 2014)

1.2.2. Drug target alteration

In order to affect a cancer cell, antineoplastic drugs have to interact with a target. This interaction, if successful, leads to a modification or inhibition of the function of the cell. Alterations in the quality or quantity of the drug target can weaken drug efficacy (Moscow *et al.,* 2003). Altered drug targets, such as changed expression levels, mutations, or signaling pathways, are factors that affect the efficacy of a compound. These forms of drug target alterations could eventually result in resistance to a compound (Housman *et al.,* 2014). Three of the following examples provide some insight into drug target alteration.

First, while point mutations can cause uncontrolled kinase activity, the overexpression of a gene can have a similar effect. For example, 30% of breast cancer patients overexpress HER2 (human epidermal growth factor receptor 2), a receptor tyrosine kinase of the EGFR (epidermal growth factor receptor) family (Housman *et al.,* 2014). However, resistance to drugs targeting HER2 occurs in more than 65% of metastatic breast cancers (Chung *et al.,* 2013).

Topoisomerase II, an enzyme that precludes the under- or supercoiling of DNA, is targeted by some chemotherapy drugs. The complex that forms between topoisomerase II and DNA is typically short-lived, but topoisomerase II inhibitors stabilize the complex, which results in hindered DNA synthesis, DNA damage, and blocked mitotic processes. Although cells achieve resistance by

several means, mutations occurring in the topoisomerase II gene have been shown to help cell lines confer resistance to drugs targeting topoisomerase II (Housman *et al.,* 2014).

Chronic myeloid leukemia (CML) provides a good example of drug-target alteration in cancer resistance. The malignancy is driven by the uncontrolled activity of the tyrosine kinase in ABL1. Inhibitors that are available to treat CML inhibit phosphorylation in the tyrosine kinase domain, but point mutations have been shown to emerge in response to inhibitors (Soverini *et al.,* 2011). Missense mutations enable *BCR-ABL1* to encode a protein altered in size, chemistry and/or shape of the target site, often resulting in drug resistance. See Section 1.7.3. for more information.

1.3. Hematopoiesis

1.3.1. Hematopoietic stem cells

The process of hematopoiesis takes place in the bone marrow. All blood cells derive from pluripotent hemopoietic stem cells, which are capable of renewing themselves and (re-)creating all cell lineages. These pluripotent cells, therefore, give rise to myeloid progenitor cells as well as lymphoid progenitor cells. Myeloid progenitor cells give rise to red blood cells (erythrocytes), platelets, and mast cells, as well as eosinophils, basophils, neutrophils, and macrophages. Lymphoid progenitor cells give rise to natural killer cells (NK cells), B-lymphocytes (also known as B-cells) and T-lymphocytes (also known as T-cells) [Figure 2] (*Essential Haematology*, 2011).

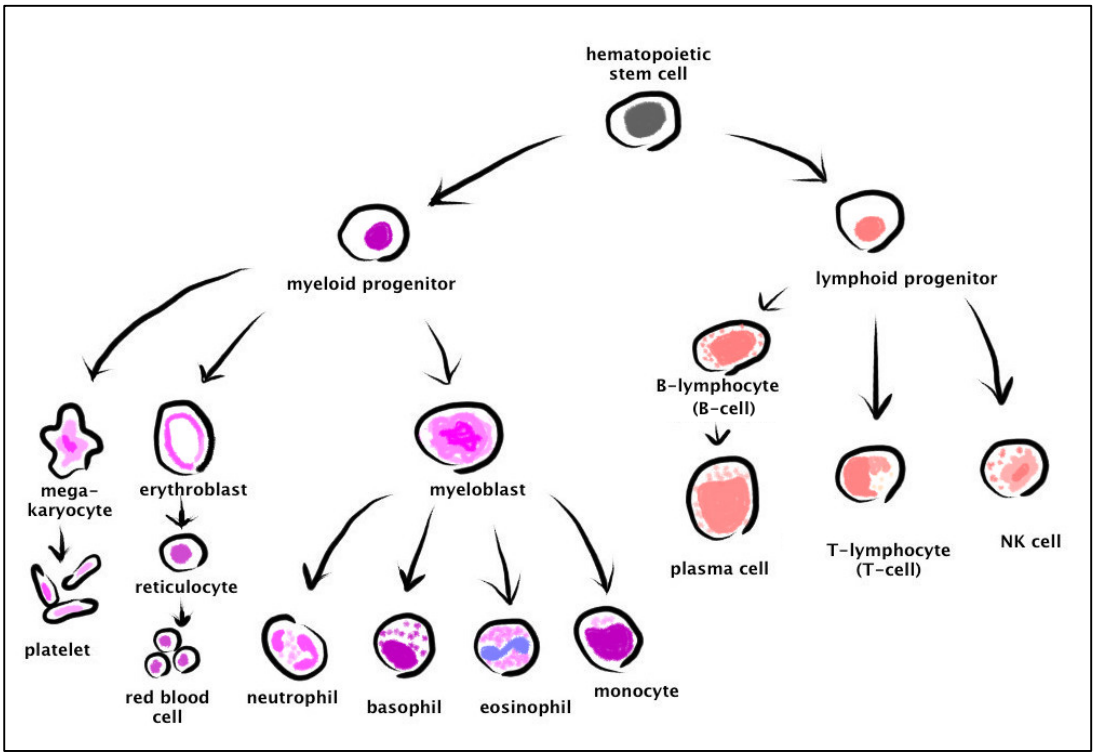

Figure 2: All blood components are created in the process of hematopoiesis. The pluripotent hematopoietic stem cell can give rise to myeloid progenitors and lymphoid progenitors, each of which, in healthy individuals, differentiates into specific cellular components of the blood.

1.3.2. Abnormal hematopoiesis can result in leukemia

If an aberration prevents the normal development of white blood cells in the bone marrow, leukemia can occur (Enciso *et al.*, 2015). Leukemia is a cancer of the blood characterized by abnormal hematopoiesis in the bone marrow and elevated numbers of white blood cells that have not fully matured. Leukemias can be divided in two groups: acute and chronic leukemias, with each further divided into myeloid and lymphoid malignancies (*Essential Haematology*, 2011). The four leukemias are estimated to make up 85% of all leukemias [Figure 3] (Cook and Pardee, 2013). Acute leukemias often have an aggressive, fast onset, while chronic leukemias progress more slowly (*Essential Haematology*, 2011). It is important to note that these four leukemias can be further subdivided into many categories and do not include all forms of hematologic malignancies (*Essential Haematology*, 2011; Taylor *et al.*, 2017).

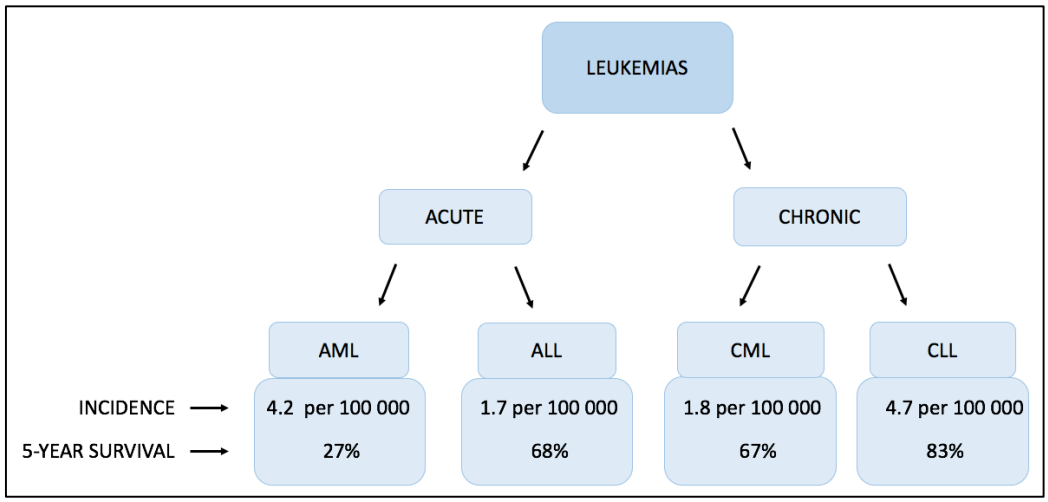

Figure 3: Hierarchy of leukemias: Four of the most common subtypes of leukemias (according to Cook and Pardee, *Cancer Metastasis Rev.*, 2013). Leukemias can be divided into acute and chronic leukemias, each of which is subdivided into myeloid and lymphoid malignancies. The numbers for incidence and 5-year survival are according to the statistics of the SEER program (Surveillance, Epidemiology, and End Results Program, National Cancer Institute; 1975-2014). Incidence is described as the number of new cases per year. AML – acute myeloid leukemia; ALL – acute lymphoid leukemia; CML – chronic myeloid leukemia; CLL – chronic lymphoid leukemia

1.4. Chronic Myeloid Leukemia (CML)

1.4.1. Overview

Chronic myeloid leukemia (CML), also known as chronic myelogenous, myelocytic, or granulocytic leukemia (CGL), is a slowly progressing cancer of white blood cells characterized by an uncontrolled production of immature myeloid cells. The worldwide incidence of leukemia is estimated to be 2,5% of all cancers (Ferlay *et al.,* 2015), and the relatively rare CML constitutes around 15% of leukemias (*Essential Haematology*, 2011) with an annual incidence rate of approximately 1-2 per 100 000 people worldwide (Dikshit *et al.,* 2011). The malignancy is slightly more common in males with a male to female ratio of 1,4:1 (*Essential Haematology*, 2011), and incidence increases with age. The median age at diagnosis is estimated to be 64 years (SEER

program, 2017). Since the introduction of the tyrosine kinase inhibitor (TKI) imatinib in 2001 (Druker *et al.,* 2001), successful first-line therapy for CML has decreased the estimated annular mortality of CML from 10-20% prior to the use of imatinib down to 1-2% since introducing the kinase inhibitor (Jabbour and Kantarjian, 2016). In spite of the unchanging incidence of CML, the prevalence of the disease is expected to increase more than six-fold by year 2050 (Huang *et al.,* 2012) because CML patients who would have died prior to the use of TKIs can now expect to live almost as long as healthy individuals (Bower *et al.,* 2016) [Figure 4].

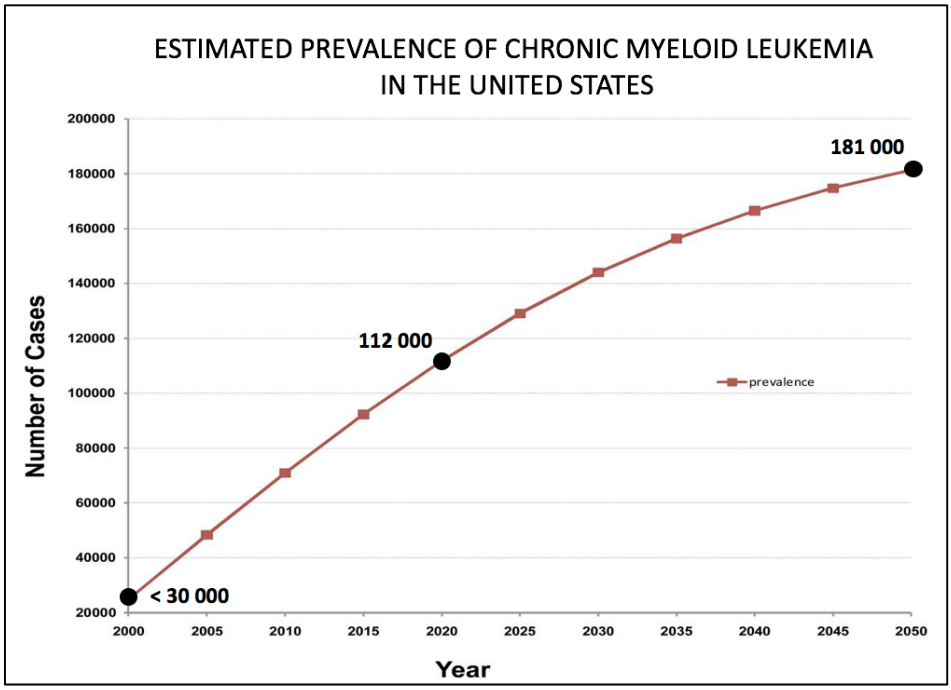

Figure 4: The number of patients living with CML has been increasing since the tyrosine kinase inhibitor (TKI) imatinib was introduced in 2001, and is predicted to reach a plateau only by the year 2050. The number of CML cases is estimated to rise more than six-fold when compared to the era prior to imatinib (used and modified with permission from Huang *et al.*, *Cancer*, 2012)

As the cancer progresses, three sequential phases can be distinguished: an initial chronic phase (which is the most common time of diagnosis), an accelerated phase, and an aggressive blast crisis phase (Jabbour and Kantarjian, 2016). Blast crisis occurs when the bone marrow and/or blood contains more than 20% of immature white blood cells (or extramedullary proliferation of blasts can be detected) (Shi *et al.*, 2015). Blast lineage is myeloid in around 70% of CML cases and lymphoid in 20-30% of cases (Derderian *et al.*, 1993; Nair *et al.*, 1995).

1.4.2. Fusion oncogene BCR-ABL1

CML is characterized by the presence of the Philadelphia (Ph) chromosome, which was the first chromosomal aberration connected to a human malignancy (Dobrovic *et al.*, 1991). The Ph-chromosome results from the reciprocal translocation of the long arms of chromosomes 9 and 22, and forms a shortened chromosome 22 (Nowell and Hungerford, 1960; Rowley, 1973) referred to as t(9;22)(q34;q11) (Prakash and Yunis, 1984) [Figure 5 and 6c]. At their meeting point, the

chimeric oncogene BCR-ABL1 is formed when breakpoint cluster region (BCR) gene from chromosome 22 fuses with Abelson proto-oncogene 1 (non-receptor tyrosine kinase; ABL) from chromosome 9 (de Klein *et al.*, 1982; Heisterkamp *et al.*, 1983a; Groffen *et al.*, 1984; Shtivelman *et al.*, 1985). The resulting uncontrolled tyrosine kinase activity in the ABL1 protein is also the driver of the malignancy (Lugo *et al.*, 1990; Deininger *et al.*, 2000a).

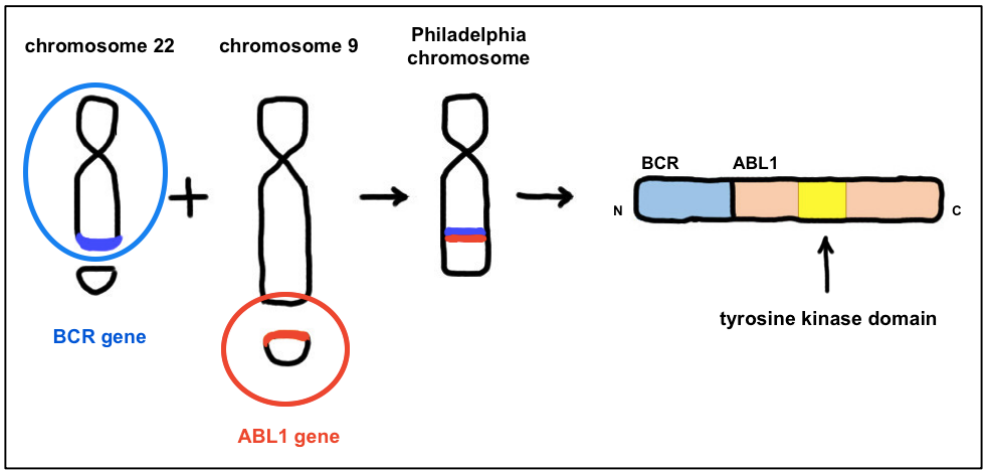

Figure 5: Reciprocal translocation of the long arms of chromosomes 9 and 22 leads to the so-called Philadelphia chromosome, where BCR (chromosome 22) fuses with ABL1 (chromosome 9), forming the fusion oncogene BCR-ABL1. The resulting constitutively active tyrosine kinase domain in ABL1 is responsible for the oncogenic transformation.

The difference in breakpoints in the BCR gene can lead to the expression of proteins of different molecular masses, namely 185/190 kDa ($p190^{BCR-ABL1}$), 210 kDa ($p210^{BCR-ABL1}$), and 230 kDa ($p230^{BCR-ABL1}$), all with constitutively activated tyrosine kinase activity. The three resulting fusion proteins comprise the same sequence of the ABL1 gene at the C-terminus but contain a different amount of BCR at the N-terminus (Li *et al.*, 1999). Since over 95% of CML patients carry the $p210^{BCR-ABL1}$ oncogenic fusion protein (Melo, 1997), descriptions of the structure of BCR-ABL1 in this thesis will focus on the $p210^{BCR-ABL1}$ form of BCR-ABL1 (referred to as BCR-ABL1 in this thesis).

1.4.3. BCR (Breakpoint Cluster Region)

The *BCR* gene is evolutionarily conserved and ubiquitously expressed (Collins *et al.,* 1987). Although the function of the *BCR* gene encoding protein in a healthy individual is largely unclear, *BCR* has been shown to code for a 160 kD protein with serine/threonine kinase (Maru and Witte, 1991) and guanosine triphosphase activities (Diekmann *et al.,* 1991). At the N-terminus of the BCR protein, a coiled-coil oligomerization motif is thought to be pivotal to the constitutive activation of the ABL1 tyrosine kinase in BCR-ABL1 [Figure 6a] (McWhirter *et al.,* 1993). The role of other BCR domains in the malignant process may be limited and include the serine/threonine SH2-binding motif, and the tyrosine residue at position 177 (Salesse and Verfaillie, 2002).

1.4.4. ABL1 (Abelson Proto-oncogene 1)

The *ABL1* gene (also c-*ABL1*), ubiquitously expressed in humans (Wang, 2014), is homologous to the v-*abl* oncogene (Heisterkamp *et al.*, 1983b) found in A-MuLV (Abelson murine leukemia virus) (Reddy *et al.*, 1983). It encodes a 145 kD protein that, among other functions, is involved in a variety of processes related to cell growth and survival, motility and adhesion, apoptosis, and reaction to DNA damage (Deininger *et al.*, 2000b; Wang, 2014).

The ABL1 protein occurs in two isoforms (1a and 1b) resulting from alternative splicing of the first exon (Deininger *et al*., 2000b). Many structural regions can be delineated [Figure 6b]. Specifically, three regions homologous to Src (SH1-SH3) are found close to the N-terminus. Most importantly, the SH1 domain contains the non-receptor tyrosine kinase domain that is constitutively activated in BCR-ABL1 (Salesse and Verfaillie, 2002). Proline-rich domains are found at the center of ABL1 (Yu *et al.*, 1994) and nuclear localization signals (NLS) are interspersed with proline-rich regions (van Etten *et al.*, 1989). DNA binding and F-actin-binding domains (Kipreos and Wang, 1992; McWhirter and Wang, 1993), as well as a nuclear export signal (NES) (Taagepera *et al.*, 1998), are located toward the C-terminal end of the ABL1 protein (Deininger *et al*., 2000b).

In ABL, the structures thought to be significant to the oncogenic transformation of BCR-ABL1 are the SH2 domain (Src Homology 2), acting-binding domain, and, most important to the work presented here, the tyrosine kinase domain. The tyrosine kinase domain in ABL1 is responsible for the uncontrolled kinase activity in BCR-ABL1 (Salesse and Verfaillie, 2002). Mutational analyses have shown that tyrosine kinase activity is crucial for oncogenic transformation of BCR-ABL, and no downstream effector is able to complement it (Lugo *et al.*, 1990; Cortez *et al.*, 1995). The tyrosine kinase domain in ABL1 is also the target of TKIs currently available to treat CML (Mughal *et al.*, 2013).

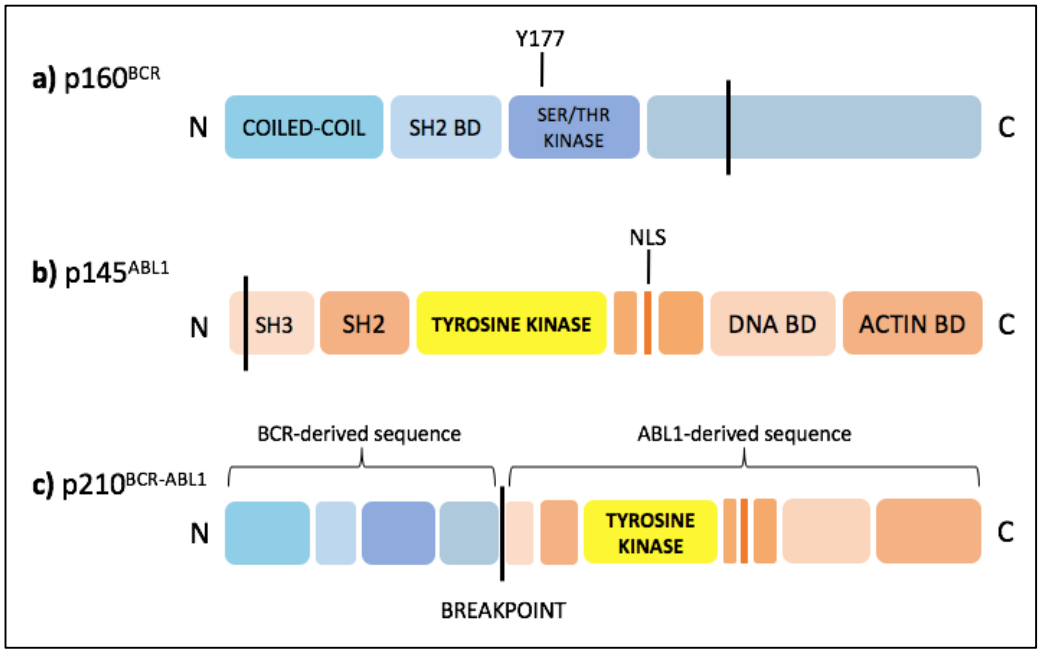

Figure 6: The BCR (p160), ABL1 (p145), and BCR-ABL1 proteins (p210). Key structural domains are indicated. **(a)** At the N-terminus, BCR contains a coiled-coil oligomerization region reported to activate the tyrosine kinase in ABL1. **(b)** ABL1 includes an SH1 non-receptor tyrosine kinase domain, which becomes constitutively activated in BCR-ABL1. **(c)** The $p210^{BCR\text{-}ABL1}$ protein isoform contains the indicated regions of BCR located toward the N-terminus, and all the ABL1 regions except for the SH3 domain toward the N-terminus. SH2 BD – Src Homology 2 binding; Y177 – tyrosine residue at position 177; SH3 – Src Homology 3; SH2 – Src Homology 2; NLS – nuclear localization signals; DNA BD – DNA binding; actin BD – actin binding (adapted from Smith *et al., Expert Rev. Mol. Med.*, 2003, and Salesse and Verfaille, *Oncogene*, 2002)

1.4.5. 3D structure of ABL1 kinase domain

The crystal structure of the ABL1 kinase domain facilitates an understanding of drug targeting [Figure 7]. In particular, the ABL1 kinase domain consists of the smaller N-lobe or N-terminal lobe, which is located above the ATP-binding pocket, and the larger C-lobe or C-terminal lobe, situated below the ATP-binding pocket (Reddy and Aggarwal, 2012). The ATP-binding pocket of ABL1 is the location that, in BCR-ABL1, enables incessant phosphorylation (Mughal *et al.*, 2013). The ATP-binding pocket (also known as P-loop) is flexible and covers the bound ATP molecule (Reddy and Aggarwal, 2012). For this reason, TKIs that bind to the P-loop are known as ATP-competitive inhibitors of ABL1. Consequently, by preventing phosphorylation, the growth of cells expressing BCR-ABL1 is restricted, or the cells die by apoptosis (Mughal *et al.*, 2013). Mutations that affect the binding of an inhibitor to the ATP site may cause drug-resistance. For example, a threonine residue (Thr^{315}) just outside of the P-loop is referred to as the "gatekeeper" (Reddy and Aggarwal, 2012), and is often replaced by isoleucine (T315I) in imatinib-refractory CML patients (Gorre *et al.*, 2001).

The myristoyl binding site is located toward the C-terminus of the ABL1 kinase domain. It is the allosteric site of ABL1, and inhibitors targeting this pocket have been shown to affect the structural dynamics of the ATP-binding pocket. This effect has been exemplified by the allosteric inhibitors GNF-2 and GNF-5 (Novartis Pharmaceuticals). Specifically, a moderate synergy has been shown

between the allosteric inhibitor GNF-5 and the ATP-competitive inhibitors nilotinib (Tasigna; Novartis Pharmaceuticals) or dasatinib (Sprycel; Bristol-Myers Squibb) when inducing loss of viability in cells expressing T315I. As single agents, none of these compounds shows efficacy toward cells expressing T315I (Zhang *et al.,* 2010; Iacob *et al.,* 2011). This finding highlights the potential of combination treatment to induce loss of cell viability in drug-resistant cells when both the ATP-binding site and the allosteric site (myristate pocket) are targeted.

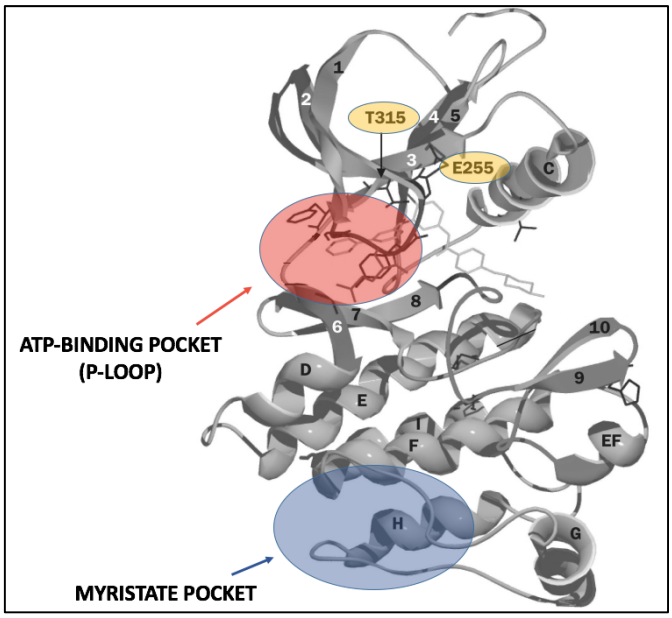

Figure 7: Crystal structure of ABL1 kinase domain. Highlighted are the ATP-binding pocket or P-loop (pink) and myristate pocket or allosteric site (blue). The amino acid residue E255 (glutamic acid at position 255) and T315 (threonine at position 315) are shown in yellow and will be elaborated on in Section 1.7.3 (picture used and modified with permission; Gambacorti-Passerini *et al.*, *The Lancet Oncology*, 2003)

1.5. History of treating CML

Before the first tyrosine kinase inhibitor (TKI) imatinib became available for treating CML in 2001 (Druker *et al.*, 2001), CML was treated with non-specific chemotherapy agents such as the alkylating agent busulfan and the antimetabolite hydroxyurea, as well as the human cytokine interferon-α (INF-α) and allogeneic stem cell transplantation (Silver *et al.*, 1999). In the 1980s, INF-α became the first-line treatment for CML patients in chronic phase who failed to clinically qualify for allogeneic bone marrow transplantation (Kujawski *et al.*, 2007). However, response to INF-α varied from successful inhibition of the leukemic clone to no 'hematologic' effect (Dowding *et al.*, 1993), and side effects and eligibility problems limited the use of the aforementioned therapies (Silver et al., 1999).

1.6. TKIs (tyrosine kinase inhibitors)

1.6.1. First-generation TKIs

The first tyrosine kinase inhibitors, referred to as tyrphostins (inhibitors of tyrosine phosphorylation), were reported in 1988 by Yaish *et al.* Through the process of random, high-throughput screening, 2-phenylaminopyrimidine was identified as a TKI that showed selectivity for the PDGFR (platelet-derived growth factor receptor) and ABL1 tyrosine kinases (Buchdunger *et al.,* 1996; Carroll *et al.,* 1997; Druker and Lydon, 2000). To optimize the compound for increased specificity and potency for PDGFR inhibition (its initial target), various compounds chemically related to 2-phenylaminopyrimidine were synthesized by analyzing the association between activity and structure (Druker and Lydon, 2000). STI-571 (CGP57148B, imatinib mesylate) surfaced as a promising compound for preclinical studies not only for inhibiting PDGFR but Kit and ABL1 tyrosine kinases as well (Buchdunger *et al.,* 1996; Druker *et al.,* 1996). STI-571 was found to bind and stabilize the ATP-binding domain (P-loop) in the catalytic region of ABL1 so that the kinase stays in an inactive state. Thus, the conformation of the P-loop is misshapen and the A-loop stays in a closed DFG (Asp-Phe-Gly)-out conformation (Schindler *et al.,* 2000). A few years after STI-571 was found to inhibit the ABL1 tyrosine kinase, preclinical research was followed by clinical trials.

In 1998, Phase I clinical trials were initiated using STI-571 for treating CML patients ineligible for INF-α treatment. When STI-571 was given in (daily) doses of 300mg or higher, 98% of CML patients achieved a complete hematologic response, and the response usually occurred within the first month (Druker *et al.*, 2001). The United States Food and Drug Administration (FDA) approved imatinib mesylate (Gleevec or Glivec; Novartis Pharmaceuticals) in 2001 (Cohen *et al.*, 2002). Five years later, the overall survival of CML patients receiving imatinib as first-line treatment was assessed to be over 85% (Druker *et al.*, 2006). For CML patients in chronic phase, the estimated eight-year survival rate has advanced from 20% historically to over 85% since imatinib became available. CML patients diagnosed in accelerated phase have an estimated 75% eight-year survival, but blast phase CML patients face a dismal median survival of seven months (Kantarjian *et al.*, 2012).

Even though long-term complete cytogenetic response is achieved in most Ph+ leukemia patients, treatment is discontinued in many due to occurrence of resistance or intolerance (Branford *et al.*, 2002). In addition to the inefficacy of imatinib in treating advanced CML (Kantarjian *et al.*, 2012), imatinib-treatment can result in the selection of point mutations in the tyrosine kinase domain of BCR-ABL1 (Soverini *et al.*, 2011), which can lead to resistance to imatinib even at increased doses (Wieczorek and Uharek, 2015).

1.6.2. Second- and third-generation TKIs

Crystallography facilitated the synthesis of second-generation TKIs, such as nilotinib (Tasigna; Novartis Pharmaceuticals), dasatinib (Sprycel; Bristol-Myers Squibb) and bosutinib (Bosulif; Pfizer). The second-generation TKIs were found to exhibit selectivity for the BCR-ABL1 mutations not affected by imatinib (Shah *et al.,* 2004; Weisberg *et al.,* 2006). However, none of these three TKIs could suppress the BCR-ABL1 T315I mutation that emerges in up to 20% of refractory patients (Nicolini *et al.*, 2009). Although the third-generation TKI ponatinib (Iclusig; ARIAD

Pharmaceuticals) inhibits this mutation, additional mutations have emerged and many of them defy inhibition even by this broad-target TKI (Lasater *et al.,* 2016).

Table 1 summarizes information on TKIs currently available for CML treatment.

Table 1: Information on FDA approved TKIs currently available for CML treatment. The first column indicates the generation of the TKI.

	Compound	FDA approval	First-line therapy	Alternative name	Brand name	Company
I	**imatinib**	2001	Yes	CGP57148B, STI-571, imatinib mesylate	Gleevec/ Glivec	Novartis Pharmaceuticals
II	**dasatinib**	2006/2010	Yes	BMS 354825	Sprycel	Bristol-Myers Squibb
	nilotinib	2007/2010	Yes	AMN107	Tasigna	Novartis Pharmaceuticals
	bosutinib	2012	No	SKI-606	Bosulif	Pfizer
III	**ponatinib**	2012 (discontinued) 2014	No, except for T315I-positive CML	AP24534, ponatinib hydrochloride	Iclusig	ARIAD Pharmaceuticals

1.7. Drug-resistance in CML

1.7.1. Overview

Drug-resistance in CML can be primary or secondary. Primary drug-resistance in CML consists of two types of resistance. One of them is primary cytogenetic resistance (15-25% of cases), which occurs when the patient has no change in the number of Philadelphia-chromosome positive (Ph+) cells in the bone marrow within 12 months. The second is primary hematologic resistance (2-4% of patients), when complete blood count, peripheral blood smear, or the size of the spleen do not return to normal within three months (Haznedaroglu, 2014; Jabbour *et al*., 2011). Point mutations in BCR-ABL1 are seldom behind primary drug-resistance (Jabbour *et al.*, 2011). The mechanisms associated with primary resistance include aberrant expression of a drug transporter or deficient protein binding. Secondary resistance occurs in patients who achieved cytogenetic and/or hematologic response initially but later developed resistance (lost response), usually due to a mutation in the ABL1 tyrosine kinase domain. Drug-resistance to TKIs can occur either through BCR-ABL1-independent or BCR-ABL1-dependent mechanisms (Bhamidipati *et al.*, 2013; Wieczorek and Uharek, 2015).

1.7.2. Mechanisms of BCR-ABL1-independent resistance

BCR-ABL1-independent mechanisms of resistance to TKIs often, but not always, occur as primary resistance, and include aberrations in drug influx, import, binding and concentration, as well as

activation of an alternative signaling pathway and epigenetic modifications (Bixby and Talpaz, 2009). For example, mechanisms independent of BCR-ABL1 include:

- elevated drug efflux due to higher expression of P-glycoprotein efflux pumps (Rumpold *et al.,* 2005);
- abnormal drug transporter expression, including hOCT1 (human organic cation transporter 1) that affects imatinib-resistance (Thomas *et al.,* 2004);
- multidrug resistance ATP-binding cassette (ABC) transporters ABCB1 or MDR1 and ABCG2 shown to influence resistance to imatinib, nilotinib, and dasatinib (Dohse *et al.,* 2010);
- serum protein α1 acid glycoprotein binding to imatinib, prevents drug-mediated inhibition of BCR-ABL1 (Gambacorti-Passerini *et al.,* 2000);
- the metabolism gene PTGS1/COX1 (prostaglandin-endoperoxide synthase 1/ cyclooxygenase 1), which codes for an imatinib-metabolizing enzyme, and thus increased transcript levels of the gene have been linked to BCR-ABL1-independent mechanisms of resistance (Zhang *et al.,* 2009).

1.7.3. Mechanisms of BCR-ABL1-dependent resistance

BCR-ABL1-dependent mechanisms of resistance include overexpression or amplification of the BCR-ABL1 gene or its encoded protein (le Coutre *et al.,* 2000), and point mutations that emerge in the ABL1 tyrosine kinase domain (Gorre *et al.,* 2001). Mechanisms dependent on BCR-ABL1 are more frequent, especially point mutations. In fact, point mutations are estimated to arise in up to 50% of CML cases that become resistant to imatinib due to impaired binding. Studies have found over 90 point mutations resulting in amino acid substitutions in the tyrosine kinase domain of BCR-ABL1, including the domain that binds ATP (P-loop), the activation loop (A-loop), the catalytic motif, and amino acids which come in contact with imatinib (Jabbour *et al.,* 2011).

The frequency of a certain point mutation, as well as its effect on the sensitivity of the cell to TKIs, varies (Jabbour *et al.,* 2011). Most of the amino acid substitutions found to be clinically relevant emerge at residues in the

- P-loop (Y253F/H, E255V/K, G250E, and M244V at the periphery of the P-loop),
- catalytic domain (F359V, M351T), and
- contact site (T315I) (Soverini *et al.,* 2006).

Substitutions at these seven residues make up 85% of all mutations linked to resistance [Figure 8] (Soverini *et al.,* 2006), with T315I alone accounting for up to 20% of resistance to first and second generation TKIs (Nicolini *et al.*, 2009). Mutations in the P-loop have been associated with shorter survival and a quicker transformation to blast crisis (Branford *et al.,* 2003), but these findings are contradictory (Jabbour *et al.,* 2006). More than one point mutation can occur in a single patient, and this happens more often when the patient has been treated with more than one TKI (Jabbour *et al.,* 2011). Highly resistant compound mutations have also been shown to emerge (Zabriskie *et al.,* 2014). The abundance of point mutations that occur in response to kinase inhibitors highlights the significance of finding compounds showing selective potency to each mutation.

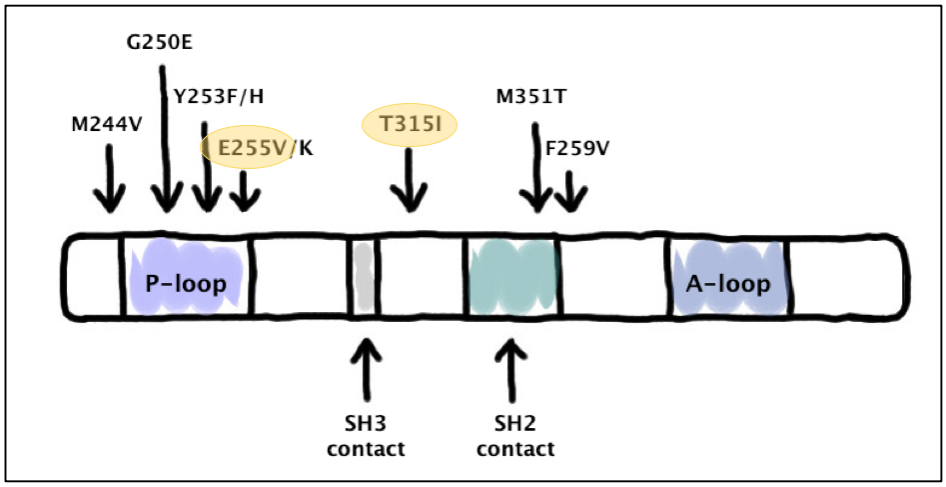

Figure 8: The BCR-ABL1 tyrosine kinase domain and the seven amino acid substitutions within it that make up 85% of mutations linked to resistance. Key structural domains in tyrosine kinase domain are shown. P-loop refers to ATP-binding loop; SH2 and SH3 contact domains indicate locations of contact with proteins that contain SH2 and SH3 domains; A-loop indicates activation loop. Amino acid substitutions relevant to this thesis are highlighted in yellow. Residue numbering follows the Ia isoform of ABL1 (adapted from Soverini *et al.*, *Blood*, 2011)

BCR-ABL1 T315I

The first identified point mutation emerging in the tyrosine kinase domain of BCR-ABL1 was a nucleotide change of C to T (ACT -> ATT) that leads to the substitution of the hydrophilic, polar threonine (T) with the nonpolar isoleucine (I) at the amino acid position 315 in the ABL1 tyrosine kinase domain (Gorre *et al.*, 2001) [Figure 9]. Threonine at position 315 (Thr^{315}) forms a crucial hydrogen bond with the TKI imatinib, but because its side chain usually provides the oxygen atom that is missing in isoleucine, the secondary amino group of imatinib can no longer bind hydrogen. However, isoleucine does not prevent ATP binding (Gorre *et al.*, 2001). Even though many point mutations have been described, T315I has remained the most significant. Importantly, this T315I mutation is referred to as the "gatekeeper" mutation because of its location in the periphery of the nucleotide-binding site (Nagar *et al.*, 2002), and tyrosine kinase activity of BCR-ABL1 with the T315I mutation cannot be inhibited by any of the first or second generation TKIs (Wehrle *et al.*, 2014).

For clarity, BCR-ABL1 T315I will be referred to as T315I in this study.

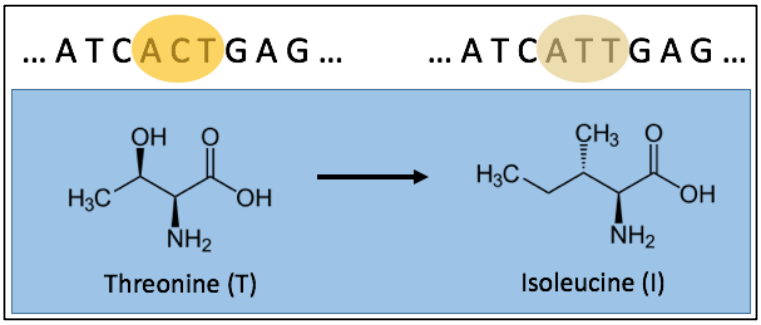

Figure 9: In T315I, threonine (T) in amino acid position 315 is replaced by isoleucine (I) because of the emergence of a missense mutation when the nucleotide C is replaced by T.

The T315I mutation in imatinib-refractory CML occurs between 2-20% of cases, with inconsistency associated with differences in patient cohort size, treatment, and mutation detection methods (Nicolini *et al.*, 2009). Ph+ ALL (acute lymphoid leukemia) patients appear to carry the T315I mutation more frequently, and those affected face a dire overall survival of only five months. The frequency of the T315I mutation in CML probably increases as TKI treatment is sustained (Soverini *et al.*, 2006 and 2007; Nicolini *et al.*, 2009).

In 2009, the presence of the gatekeeper mutation T315I was correlated with a 22-month overall survival in chronic phase CML patients (Nicolini *et al.*, 2009). The third-generation TKI ponatinib (Iclusig, ARIAD Pharmaceuticals) was designed to bind to the inactive conformation of ABL1 and, especially, $ABL1^{T315I}$. Unlike imatinib, ponatinib does not bind hydrogen with the side chain of Thr^{315} in ABL1 protein. The significant structural characteristic of ponatinib is the triple bond between two carbons, which leads to successful hydrophobic contact with the side chain of Ile^{315} and enables the thus-far cross-resistant T315I to be inhibited (O'Hare *et al.*, 2009).

In 2012, the FDA gave accelerated approval to ponatinib for treating Ph+ ALL or CML adult patients ineligible for prior TKI treatment (Bose *et al.*, 2013). However, the drug was withdrawn from the market a year later due to concerns of serious side effects, including thrombotic vascular events (Sanford *et al.*, 2015). A phase II study was carried out to determine the effects of ponatinib as first-line treatment for chronic phase CML, but it was terminated because of elevated risk of thromboembolism (Jain *et al.*, 2015). In 2014, ponatinib was again approved for CML patients carrying the T315I mutation, or when other TKIs are not indicated (Sanford *et al.*, 2015), but side effects continue to be a concern (Poch Martell *et al.*, 2016).

Notably, our group determined that the kinase inhibitor axitinib is a potential alternative to ponatinib (Pemovska *et al.*, 2015). Axitinib exhibited a high affinity for T315I in kinase assays (Davis *et al.*, 2011), and showed clinical activity in a CML patient with the T315I mutation (Pemovska *et al.*, 2015), making axitinib, previously approved by the FDA for advanced renal cell carcinoma, a desirable inhibitor for drug-repurposing (Tyler, 2012). Although concerns over adverse events have led to suggestions of decreased dosages for ponatinib, efficacy and safety may be complex to balance, and ponatinib may be incompatible in advanced cases of CML (Dorer *et al.*, 2016). Given that nearly a hundred point mutations have been shown to arise as a result of treating CML with TKIs (Soverini *et al.*, 2011), it is reasonable to conclude that point mutations would also arise in the ABL1 kinase domain of axitinib-treated patients. Yet, mutations occurring as a result of axitinib-treatment have yet to be explored.

BCR-ABL1 T315M

BCR-ABL1 T315M (hereafter referred to as T315M), although rare, presents a case of a mutation in which the amino acid encoded by a codon changes from hydrophilic, polar threonine (T) to hydrophobic, non-polar methionine (M), with the added step of two nucleotide exchanges (ACT -> ATT -> ATG) [Figure 10]. This change has been shown to occur when the gatekeeper mutation T315I is treated with ponatinib and develops resistance to the drug (Zabriskie *et al.*, 2014). In fact, cells stably expressing the mutation have displayed a high degree of resistance to ponatinib (Lasater *et al.*, 2016). No compound thus far has been shown to selectively inhibit the T315M mutation.

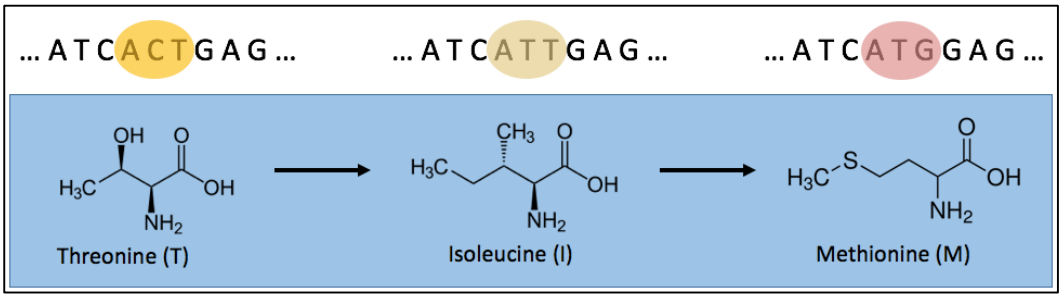

Figure 10: The T315M mutation appears to involve the amino acid substitution threonine (T) to methionine (M) at position 315, but two nucleotide exchanges are needed for this to occur.

BCR-ABL1 E255V/T315I

Unlike T315M, BCR-ABL1 E255V/T315I is a compound mutation that involves two separate nucleotide exchanges at distinct amino acid positions. T315I, as explained previously, refers to the substitution of threonine for isoleucine at position 315. In E255V, however, the polar glutamic acid is replaced by the nonpolar valine at amino acid position 255. Both positions are frequent sites for single mutations, and when they occur together as E255V/T315I [Figure 11], this compound mutation is the most frequent to emerge at 17% of compound mutations (Zabriskie *et al.*, 2014).

For simplicity, BCR-ABL1 E255V/T315I will be referred to as E255V/T315I in this thesis.

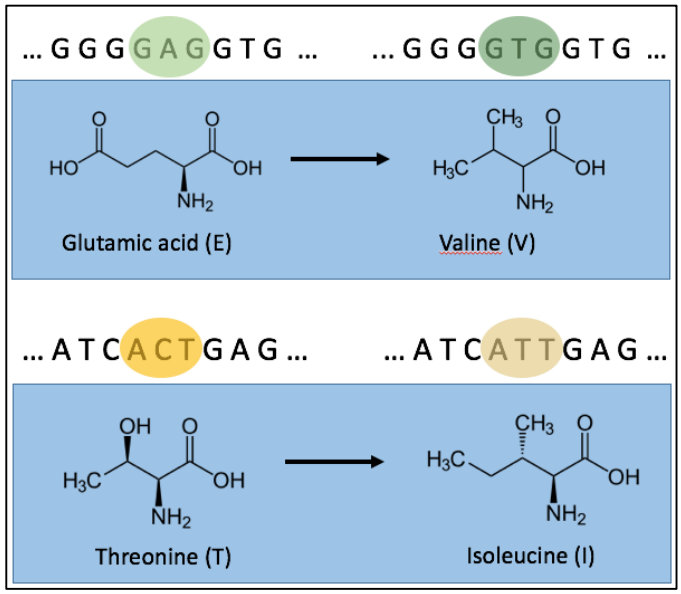

Figure 11: In E255V/T315I compound mutation, glutamic acid (E) is replaced by valine (V) at amino acid position 255, and threonine (T) replaced by isoleucine (I) at position 315.

Although ponatinib has shown the most extensive activity of all the ABL1-specific TKIs (Lasater *et al.*, 2016), the compound mutation E255V/T315I, like T315M, arises as a result of ponatinib-treatment and therefore confers resistance to the drug (Zabriskie *et al.*, 2014). No drug has so far been shown to selectively target E255V/T315I in clinically relevant quantities.

Due to the success of imatinib and the subsequent second-generation TKIs, the prevalence of CML is increasing worldwide (Huang *et al.*, 2012), making even the rare point mutations necessary drug-targets. The increasing prevalence of CML underlies the need for effective and selective inhibitors of drug-resistant mutations.

1.8. Summary

CML is a myeloproliferative malignancy defined by the fusion oncogene BCR-ABL1, which leads to uncontrolled kinase activity of the tyrosine kinase domain of ABL1. The introduction of the tyrosine kinase inhibitor (TKI) imatinib as first-line treatment for CML has rapidly improved the prognosis of the disease. However, point mutations in ABL1 account for up to 50% of acquired resistance, and T315I is the most frequent mutation to arise. The only approved TKI showing efficacy toward this mutation is ponatinib, which has severe side effects. Although axitinib has previously shown selectivity for cells expressing the T315I mutation, mutations emerging as a consequence of axitinib-therapy have yet to be examined. Furthermore, new and highly resistant mutations such as T315M and E255V/T315I have been reported in CML patients who previously carried the T315I mutation and were treated with ponatinib. Thus far, no compound has shown selective potency to cells expressing the two highly resistant mutations in clinically relevant dosages. Therefore, this study focused on finding compounds capable of inhibiting these highly resistant mutations.

1.9. Aims of this study

1. To identify new potential inhibitors for a mouse hematopoietic cell model stably expressing the BCR-ABL1 T315I, T315M and E255V/T315I mutations.

2. To identify mutations that allow cells to develop resistance to the tyrosine kinase inhibitor axitinib.

2. Experimental Research

2.1. Identifying new potential inhibitors for cells expressing the BCR-ABL1 T315I, T315M, and E255V/T315I mutations

2.1.1. Rationale

The prognosis for CML patients improved tremendously after successful introduction of the first TKI imatinib in 2001 (Druker *et al.*, 2001 and 2006). However, in spite of the prolonged lifespan of many CML patients and availability of second- and third-generation inhibitors, drug-resistance continues to be a problem (Zabriskie *et al.*, 2014). Treating CML and Ph+ ALL patients with ABL1-specific TKIs often results in the emergence of point mutations in the tyrosine kinase domain of ABL1 (Gorre *et al.*, 2001). One of the most frequent mutations to occur is T315I, which has been estimated to make up to 20% of the mutations arising from drug resistance (Nicolini *et al.*, 2009). The broad-range TKI ponatinib is available to treat patients with this mutation (Bose *et al.*, 2013). However, as a result of treatment with ponatinib, the T315M and the compound mutation E255V/T315I have been found to emerge. Although rare, these two mutations have been shown to be resistant to all approved ABL-1 specific TKIs (Lasater *et al.*, 2016; Zabriskie *et al.*, 2014). Considering the estimated increasing prevalence of CML in the world for the next three decades (Huang *et al.*, 2012), even the now-rare mutations are likely to pose a problem in the future. Thus, a safe and effective therapy is crucial to treat the increasing number of patients carrying these highly resistant mutations.

2.1.2. Materials

Reliable testing of small-molecule kinase inhibitors requires a cellular assay system dependent on kinase activity. For this purpose, the murine interleukin-3 (IL-3)-dependent pro-B cell line Ba/F3 was used. This model system is non-leukemic and dependent on the cytokine IL-3 in the absence of an oncogene but becomes independent of IL-3 after transformation by an oncogene (Gesbert *et al.*, 2000). The Ba/F3 cell model has been shown to be an efficient and effective model for testing the ability of kinase inhibitors to hinder kinase activity (Warmuth *et al.*, 2007).

Ba/F3 parental cells were maintained in growth medium consisting of RPMI 1640 (Lonza; #11675180/BE12-167F), 10% FBS (fetal bovine serum; Thermo Fisher Scientific; #16000044), penicillin-streptomycin at 100 units/ml (Thermo Fisher Scientific; #15140-122), 2 mM L-glutamine (Thermo Fisher Scientific; #25030-024), and 10 ng/ml of mouse IL-3 (interleukin-3) recombinant protein (eBioscience; #14-8031-62). Ba/F3 cells stably expressing BCR-ABL1 were kindly provided by Tea Pemovska. Ba/F3 cells stably expressing T315I, T315M or E255V/T315I, respectively, were maintained in growth medium containing RPMI 1640, 10% FBS, penicillin-streptomycin at 100 units/ml, and 2 mM L-glutamine. To avoid undesired surface adhesion, all Ba/F3 cell lines were maintained at subconfluence in non-tissue culture treated petri dishes (BD Biosciences; #351029). Cells were passed no more than 30 times for any of the following experiments.

The virus packaging cell line 90.74, referred to as CRL-11654 (derived from Human Embryonic Kidney cell line HEK293; ATCC), was maintained in growth medium consisting of DMEM (Dulbecco's Modified Eagle Medium); 10% FBS, and penicillin-streptomycin at 100 units/ml. The CRL-11654 cells were maintained at subconfluency in tissue culture treated T-75 flasks (Corning/Labnet; #430641U) and Tryple Express Enzyme (Thermo Fisher Scientific; #12605-028) was used to detach CRL-11654 cells from the flask. CRL-11654 cells used in the following experiments did not exceed 25 passages.

All cell lines were maintained in at 5% CO_2 (carbon dioxide) in a humidified incubator at 37 °C.

2.1.3. Methods

2.1.3.1. Ba/F3 cells stably expressing BCR-ABL1 T315I, T315M or E255V/T315I

Transfection of a virus-packaging cell line CRL-11654

A lipid-mediated transfection was used to produce replication-incompetent retroviruses. Three plasmids contained the respective coding sequences for pMIG-BCR-ABL1 T315I, pMIG-BCR-ABL1 E255V/T315I, and pMIG-BCR-ABL1 T315M. Next, the plasmids were packaged by the virus-packaging cell line CRL-11654. The presence of specific coding sequences within the plasmids was confirmed by capillary sequencing.

The cells were transfected using Lipofectamine 2000 Transfection Reagent according to the protocol by Invitrogen (#11668-019). On day 5 (72 h post-transfection), the growth medium containing retroviruses with the respective pMIG-BCR-ABL1 T315I, pMIG-BCR-ABL1 E255V/T315I, or pMIG-BCR-ABL1 T315M plasmids was collected with 5 ml syringes (VWR International; #613-5401) and filtered through Whatman Puradisc 0.45 µm filters (Sigma-Aldrich; #Z612510).

Ba/F3 cell infection

On day 5, Ba/F3 parental cells in non-tissue culture treated 6-well plates (Thermo Fisher Scientific; #351146) were infected with undiluted retroviruses that incorporated pMIG-BCR-ABL1 T315I, pMIG-BCR-ABL1 E255V/T315I, and pMIG-BCR-ABL1 T315M. Hexadimethrine bromide (Sigma-Aldrich; #H9268) was used to boost gene transfer effectiveness in retroviruses (Davis *et al.*, 2002) at a final concentration of 4 µg/ml. Growth medium was replaced on day 6 to Ba/F3 growth medium described above, containing 10 ng/ml of IL-3. On day 7, Ba/F3 growth medium was replaced with growth medium lacking IL-3 to select for cells stably expressing the desired plasmids. The growth medium was changed every 2-3 days. Since the presence of an oncogene, such as BCR-ABL1 T315I, BCR-ABL1 T315M, or BCR-ABL1 E255V/T315I ensures the ability of Ba/F3 cells to grow in the absence of IL-3 (Gesbert *et al.*, 2000), removing the cytokine from growth medium allowed for selection of a stably expressing population of cells. The selection was complete when the control cells (non-transformed Ba/F3 parental cells) died 10-14 days after removing IL-3.

2.1.3.2. Testing the sensitivity of cell lines to inhibitors

1. Overview

The sensitivity of cell lines to compounds described in the following sections were all tested in tissue culture-treated microplates in 384-well format (Corning; #3712). Unless stated otherwise, all plates were prepared and supplied by Laura Turunen (High Throughput Biomedicine Unit, FIMM). Wells containing dimethyl sulfoxide (DMSO) served as the negative control, and benzoyl chloride (BzCl) as the positive control. Exceptions will be stated in relevant sections.

In all three-day cell viability assays, cell viability was determined using CellTiter-Glo 2.0, which produces luminescence in response to ATP released from ruptured cells and thereby indicates metabolically active cells (Promega). To determine sensitivity of model cell lines to tested compounds, half-maximal inhibitory concentrations or IC_{50}s (concentration of a given drug that induces loss of cell viability in 50% of the cells) were calculated. Notably, the absolute concentration of compound capable of compromising cell viability in 50% of cells is not necessarily comparable between inhibitors. Ba/F3 cells are considered sensitive to a drug, e.g. ponatinib, at IC_{50} < 25 nM whereas the same cell line is considered be sensitive to imatinib at IC_{50} < 1000 nM (Zabriskie *et al.*, 2014).

2. Sensitivity of cell lines to selected inhibitors

To determine the effect of selected kinase inhibitors on Ba/F3 cells stably expressing BCR-ABL1, T315I, E255V/T315I, or T315M, cells stably expressing the respective mutations were treated with nine-point semi-logarithmic scale concentrations of 12 inhibitors [Figure 12]. The chosen concentration range for any inhibitor was either 0,1-1000 nM or 1-10 000 nM.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	empty	BzCl	2	4	6	7	9	11	12	empty	empty	empty	1	2	4	6	7	9	11	12	empty	empty	BzCl	empty
B	empty	1	2	4	6	7	9	11	DMSO	empty	empty	empty	1	2	4	BzCl	7	9	11	12	empty	empty	empty	empty
C	empty	1	2	4	6	7	9	11	12	empty	empty	DMSO	1	2	4	6	7	9	11	DMSO	empty	empty	empty	empty
D	empty	1	3	4	DMSO	8	9	11	12	empty	empty	empty	1	3	4	6	8	9	11	12	empty	empty	empty	empty
E	empty	1	3	4	6	8	9	11	empty	empty	empty	empty	1	3	DMSO	6	8	9	11	empty	empty	empty	empty	empty
F	empty	1	3	4	6	8	9	11	empty	BzCl	empty	empty	1	3	4	6	8	9	11	empty	empty	empty	empty	empty
G	empty	1	3	5	6	BzCl	10	11	empty	empty	empty	empty	1	3	4	6	8	10	11	empty	empty	empty	empty	empty
H	empty	1	3	5	6	8	10	11	empty	empty	empty	empty	1	3	5	6	8	10	DMSO	empty	empty	empty	empty	empty
I	empty	1	3	5	6	8	10	DMSO	empty	empty	empty	empty	1	3	5	6	8	10	11	empty	empty	empty	empty	empty
J	empty	1	3	5	6	8	10	11	empty	empty	empty	empty	2	BzCl	5	6	8	10	11	empty	empty	empty	empty	empty
K	empty	2	3	5	7	8	10	12	empty	empty	empty	empty	2	3	5	7	8	10	12	empty	BzCl	empty	empty	empty
L	empty	2	3	5	7	8	10	12	empty	empty	empty	empty	2	3	5	7	DMSO	10	12	empty	empty	empty	empty	empty
M	empty	2	4	BzCl	7	8	10	12	empty	empty	empty	empty	2	3	5	7	8	10	12	empty	empty	empty	empty	empty
N	empty	2	4	5	7	9	DMSO	12	empty	empty	BzCl	empty	2	4	5	7	9	10	12	empty	empty	empty	empty	empty
O	empty	2	4	5	7	9	10	12	empty	empty	empty	empty	DMSO	4	5	7	9	BzCl	12	empty	empty	empty	empty	empty
P	empty	2	DMSO	5	7	9	10	12	empty	empty	empty	empty	2	4	5	7	9	10	12	empty	empty	DMSO	empty	empty

Figure 12: 384-well plate layout containing the 12 selected compounds (plate layout by Tea Pemovska was modified by the author). Plate layout contained the kinase inhibitors (1) danusertib, (2) nilotinib, (3) saracatinib, (4) imatinib, (5) AT9283, (6) bosutinib, (7) ponatinib, (8) dasatinib, (9) axitinib, (10) rebastinib, (11) bafetinib, and (12) GNF-2. Dimethyl sulfoxide (DMSO) served as the negative control, and benzoyl chloride (BzCl) as the positive control.

5000 cells/well or 200 cells/µl were calculated using the Scepter 2.0 Cell Counter (Merck Millipore). 25 µl/well was dispensed in a 384-well format. The plates were incubated for 72 h in a

humidified incubator containing 5% CO_2 at 37 °C. Cell-Titer Glo 2.0 (Promega; #G9243) reagent was added at 25 µl/well at room temperature. The cells were incubated in the dark for ten minutes at room temperature. Cell viability was determined using CellTiter-Glo 2.0. To measure luminescence, PHERAstar *FS* microplate reader (BMG Labtech) was used, and the results were analyzed with GraphPad Prism 7 (GraphPad Software). The experiment was repeated three times.

3. High-throughput screening

High-throughput screening was performed on Ba/F3 cells stably expressing the respective oncogenes BCR-ABL1, T315I, T315M, or E255V/T315I. To observe the effect of inhibitors on non-transformed cells, Ba/F3 parental cells were included. The cells were treated with two sets of compounds. One, a collection of over 500 investigational and approved compounds in more than 50 drug classes in the FIMM oncology drug set (FO4B). Two, the cells were treated with >350 compounds in the Published Kinase Inhibitor Set (PKIS) by GlaxoSmithKline (GSK; Drewry *et al.*, 2014). Cells stably expressing BCR-ABL1 were treated with compounds in the FO4B but not PKIS. Inhibitors in both drugs sets contained a five-point logarithmic concentration of each drug.

Cell and Cell-Titer Glo 2.0 reagent dispensing was automated. Multidrop Combi nL Reagent Dispenser (Thermo Fisher Scientific) was used to measure 5 µl of growth medium in each well. Next, 20 µl of 5000 cells/well was dispensed using MultiFlo FX microplate dispenser (BioTek). 72 h later, MultiFlo FX microplate dispenser was used to distribute 25 µl of CellTiter-Glo 2.0 on the cells. Incubation, luminescence measuring, and analysis occurred as described in Section 2.1.3.2-2.

The programming necessary to analyze the results of PKIS screening was kindly done by Aleksandr Ianevski. In addition, the in-house developed software Breeze was used to analyze results and calculate drug sensitivity scores (DSS). DSS is an algorithmic approach for quantitative drug sensitivity scoring that provides the means to identify drug-sensitive (cancer) cell lines and cancer-selective drugs (Yadav *et al.*, 2014). For a better comparison, the DSS's for cells stably expressing the four respective oncogenes were subtracted from the corresponding DSS for Ba/F3 parental cells, which provided the so-called 'selective DSS' (sDSS) value. Compounds with sDSS > 5 are considered to be selective and sDSS > 10 are considered to be highly selective (Pietarinen *et al.*, 2015). Although arbitrary, this score helps to understand which drugs act selectively toward a particular cell line when compared to the other cell lines.

4. Sensitivity of cells expressing BCR-ABL1 T315M to inhibitors of mTOR, topoisomerase II, and other pathways

To refute the possibility that the effect of specific inhibitor groups on cells expressing T315M was population-specific, a new population of T315M-expressing cells was made using the protocol described in Section 2.1.3.1. 36 compounds from the FO4B drug set were chosen based on their potency and sDSS toward cells stably expressing T315M when compared to parental cells. Nine-point semi-logarithmic concentrations of the inhibitors were put on six 384-well plates according to the modified plate layout created by Tea Pemovska [Figure 12]. The selected compounds included inhibitors of

- mTOR,
- topoisomerase II,
- mitosis,
- aurora pathway,
- farnesyltransferase,
- Plk1 (polo-like kinase 1; serine/threonine protein kinase),
- VEGFR (vascular endothelial growth factor receptor),
- KSP/Eg5 (kinesin spindle protein / kinesin-5),
- poly-ADP-ribose polymerase,
- tyrosine kinase,

as well as IAP SMAC mimetics (mimics of 'small molecule second mitochondrial activator of caspase' that target inhibitors of apoptosis proteins), an anti-metabolite, a platinum-based antineoplastic agent, and an ATP-noncompetitive inhibitor that was not included in the FO4B drug set. Chosen drug concentration ranges included 0,1-1000 nM, 1-10 000 nM, 2,5-25 000 nM, or 10-100 000 nM of inhibitor.

The cell viability assay was carried out according to the protocol in Section 2.1.3.2.-3. Ba/F3 parental cells were used as the control group. Data was analyzed and IC_{50} values calculated using GraphPad Prism 7 and DSS values were kindly calculated by Swapnil Potdar.

5. Sensitivity of cells expressing BCR-ABL1 E255V/T315I to selected inhibitors

To test the sensitivity of cells stably expressing E255V/T315I to seven selected inhibitors, a viability assay was performed as described in Section 2.1.3.2.-2. Three IAP SMAC mimetics and two Hsp90 (Heat shock protein 90) inhibitors were chosen based on their potency toward cells expressing E255V/T315I. The allosteric inhibitor asciminib (ABL-001; currently developed by Novartis Pharmaceuticals) and the broad-range TKI ponatinib added up to a total of seven inhibitors. The drug plates were made manually. The selected compounds were dispensed in nine-point semi-logarithmic scale concentrations. As previously, growth medium containing DMSO equivalent to the volume of the highest concentration of compound served as the negative control, but this assay had no positive control. Cells stably expressing BCR-ABL1 were chosen as control group, and the experiment was carried out in duplicates. Concentration ranges of inhibitors contained 0,1-1000, 1-10 000 or 2,5-25 000 nM of drug.

6. Combination testing

To determine if the viability of cells stably expressing E255V/T315I or T315M, respectively, could be compromised by targeting different sites of the ABL1 kinase domain, combination testing was performed. The kinase inhibitors axitinib and ponatinib, and IAP SMAC mimetic LCL-161 were all respectively combined with asciminib [Figure 13]. Cells stably expressing BCR-ABL1 were chosen as control group to cells carrying the respective oncogenes.

A viability assay was carried out according to the protocol described in Section 2.1.3.2.-3., and the in-house developed software Breeze SynergyFinder (Ianevski *et al.*, 2017) was used to plot data and

calculate synergy according to the ZIP (Zero Interaction Potency) method. The ZIP method enables comparison between compounds, separately and in combinations, by comparing the potency differences of the dose-response curves (Yadav *et al.*, 2015). For more information on synergy, see Section 1.1.3. and 1.4.5.

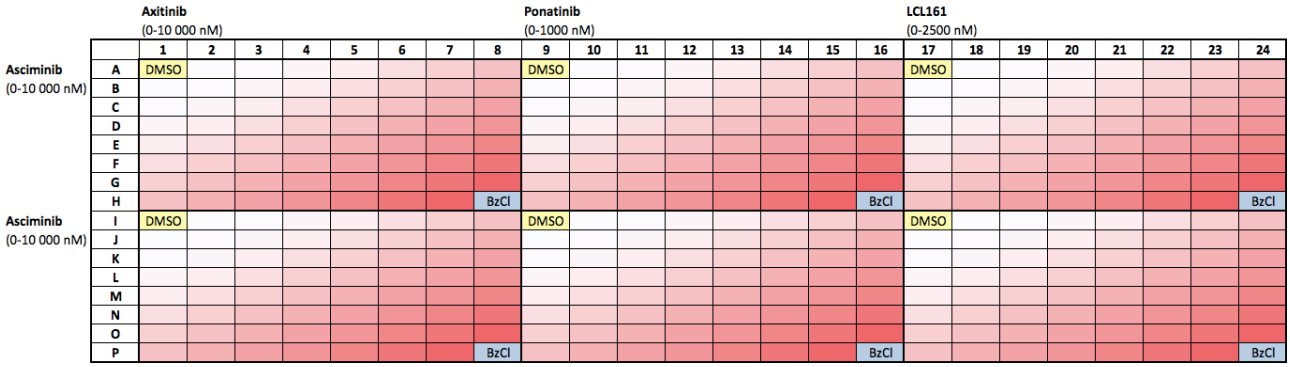

Figure 13: A 384-well plate layout for combination testing of the kinase inhibitors axitinib and ponatinib, and the IAP SMAC mimetic LCL-161, in combination with the ATP-noncompetitive inhibitor asciminib (ABL-001). The plate is divided into six sections (three replicates), with the concentration of drugs arising in a semi-logarithmic manner from left to right and from top to bottom in each section. DMSO served as the negative control, and BzCl as the positive control.

2.1.4. Results

Drug-resistance in the form of point mutations has been shown to occur when CML patients are treated with tyrosine kinase inhibitors. In particular, cells carrying T315M and E255V/T315I in the ABL1 tyrosine kinase domain display high resistance to all ABL1-specific TKIs (Zabriskie *et al.*, 2014). Although the broad-range TKI ponatinib shows potency against the "gatekeeper mutation" T315I, taking this compound can lead to worrying adverse effects (Jain *et al.*, 2015). To address this problem, mouse hematopoietic cell models were constructed. Notably, these cell lines have been shown to be useful in observing the effects of kinase inhibitors on kinase activity (Warmuth *et al.*, 2007). Furthermore, to find new potential compounds that show selective potency toward these mutations, sensitivity of cells expressing the respective mutations was tested to single compounds and combinations of them in three-day cell viability assays with Cell-Titer Glo in 384-well format.

2.1.4.1. Cells expressing BCR-ABL1 T315M showed resistance to ABL1-specific kinase inhibitors

Considering that no approved ABL1-specific tyrosine kinase inhibitor had shown selective potency toward cells stably expressing T315M (Lasater *et al.*, 2016), a mouse hematopoietic cell line expressing T315M was constructed. Next, these cells were treated with 12 selected inhibitors to determine if previously published results were reproducible as well as to find potential investigational drugs capable of compromising cell viability in T315M-expressing cells. A cell viability assay was carried out prior to data analysis with GraphPad Prism to determine half-maximal inhibitory concentrations (IC_{50}s).

Ba/F3 cells stably expressing T315M showed moderate or high resistance to the tested compounds [Figure 14]. The broad-range TKI ponatinib, although moderately resistant, showed most potency toward cells stably expressing T315M (IC_{50} = 76 nM). The cells exhibited resistance toward axitinib (IC_{50} = 390 nM). The kinase inhibitors danusertib, saracatinib, imatinib, and bosutinib compromised cell viability of T315M-expressing cells only in extremely high concentrations [IC_{50} > 1000 nM for all four inhibitors]. Moreover, T315M-expressing cells showed complete resistance to the ATP-noncompetitive inhibitor GNF-2 (IC_{50} > 10 000 nM).

The compounds included in the experiment but not mentioned here (nilotinib, AT9283, dasatinib, rebastinib, bafetinib) were excluded on the premise of not meeting the constraint criteria used in GraphPad Prism 7 because no tested amount of compound managed to induce loss of cell viability in 50% of the cells.

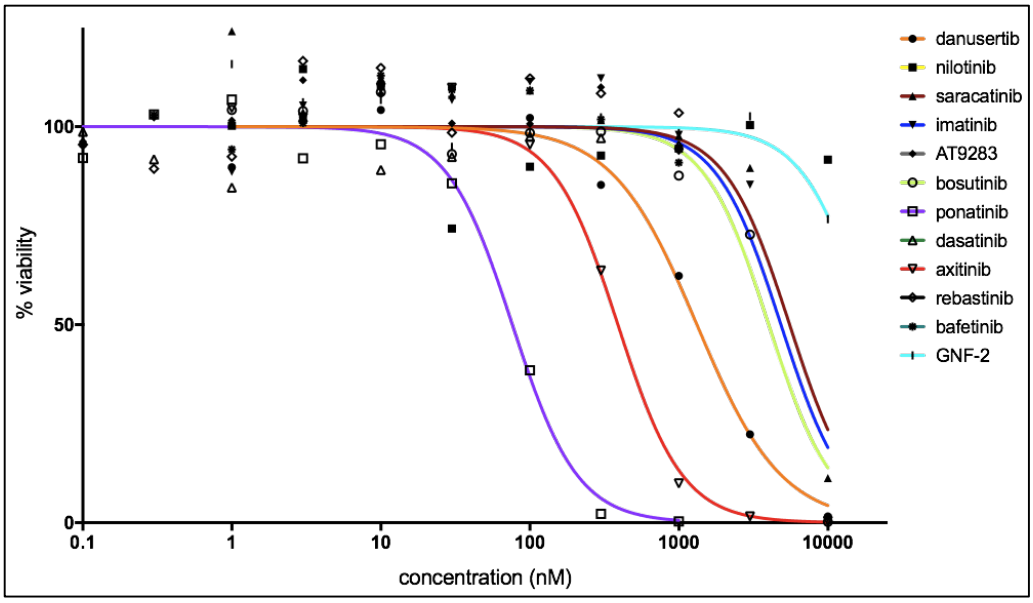

Figure 14: Cells stably expressing T315M showed moderate or high resistance to tested ABL1-specific kinase inhibitors. Data is representative of three respective experiments.

In conclusion, cells stably expressing T315M displayed moderate or high resistance to all approved and investigational TKIs as well as the allosteric inhibitor GNF-2. This result suggests that, in order to selectively compromise cell viability of T315M-expressing cells, the ABL1 kinase domain might have to be targeted at several sites. Another possibility is that compounds which target other pathways in the cell might show selective potency toward these cells.

2.1.4.2. Novel finding: Cells expressing BCR-ABL1 T315M exhibited selective sensitivity to inhibitors of mTOR and topoisomerase II

Considering that no tyrosine kinase inhibitor (TKI) displayed selective potency toward cells expressing T315M, a high-throughput screening was performed to find new potential inhibitors for these cells. In this process, cells expressing T315M were treated with over 800 compounds in the FIMM oncology collection (FO4B) and the Published Kinase Inhibitor Set (PKIS; GSK). A cell viability assay was performed prior to calculating IC_{50}s and selective Drug Sensitivity Scores (sDSS) with the software Breeze.

As a novel finding, cells stably expressing T315M exhibited selective sensitivity to various inhibitors of mTOR and topoisomerase II. After calculating the sDSS for all compounds in the two drug sets for the four cell lines expressing the respective oncogenes, a pattern of sensitivity emerged for cells expressing T315M. The selectivity of mTOR and topoisomerase II inhibitors to cells expressing T315M was only comparable to cells expressing BCR-ABL1, which was unusual [data not shown]. Among others, mTOR pathway inhibitors ridaforolimus (IC_{50} = 1 nM), everolimus (IC_{50} = 1 nM), temsirolimus (IC_{50} = 1 nM), AZD8055 (IC_{50} = 77 nM), and MLN-0128 (IC_{50} = 39 nM) showed selective sensitivity toward cells expressing T315M [Table 2]. The cells showed sensitivity toward several topoisomerase II inhibitors, including mitoxantrone (IC_{50} = 34 nM), amsacrine (IC_{50} = 21 nM), teniposide (IC_{50} = 24 nM), doxorubixin (IC_{50} = 32 nM) and aldoxorubicin (IC_{50} = 19 nM). Cells stably expressing T315M showed sensitivity to inhibitors of

various other pathways, including but not limited to inhibitors of topoisomerase I [data not shown], Plk1 (polo-like kinase 1), farnesyltransferase, KSP/Eg5 (kinesin spindle protein / kinesin-5) and the aurora pathway.

Table 2: Cells stably expressing T315M revealed a pattern of sensitivity to several mTOR and topoisomerase II inhibitors, as well as drugs targeting other pathways. Customized plates for cells stably expressing the T315M mutation ("Custom") were made after several topoisomerase II and mTOR inhibitors compromised cell viability in the cell line. Results in the columns labelled "FO4B" show the results of one high-throughput screening and columns labelled "Custom" represent an average of duplicates.

		FO4B	Custom	FO4B	Custom			FO4B	Custom	FO4B	Custom
	Compound	IC_{50} (nM)		sDSS			Compound	IC_{50} (nM)		sDSS	
mTOR inhibitors						*Aurora inhibitors*					
1.	**dactolisib**	26	14	5,4	12,9	**23.**	**alisertib**	198	53	10,5	5,8
2.	**MLN-0128**	39	35	6,4	13,1	**24.**	**tozasertib**	829*	41*	6	4,3
3.	**vistusertib**	502	321	4,1	11,1	**25.**	**GSK-1070916**	31	17	5,2	1,8
4.	**AZD8055**	77	60	9,5	11,9	*KSP/Eg5 inhibitor*					
5.	**NVP-BGT226**	18	9	6,8	9,4	**26.**	**filanesib**	4	6	12,9	5,5
6.	**gedatolisib**	54	28	3,3	10,1	*Mitotic inhibitors*					
7.	**everolimus**	1	7	16,4	7,3	**27.**	**paclitaxel**	5	2	12	8,5
8.	**temsirolimus**	1	0,7	16,4	15,2	**28.**	**docetaxel**	4	1	11,9	4,7
Farnesyltransferase inhibitors						**29.**	**vinorelbine**	6	5	16,6*	0,2*
9.	**lonafarnib**	713	373	4,3	4,6	**30.**	**ABT-751**	411	281	6,2	3,3
10.	**tipifarnib**	30	26	12,8	18,2	**31.**	**cabazitaxel**	2	1	14	17,2
Plk1 inhibitors						*Platinum-based antineoplastic agent*					
11.	**GSK-461364**	129	41	7,2	3,1	**32.**	**cisplatin**	824	1096	10,3	6
12.	**BI 2536**	51	26	7,3	6	*Poly-ADP-ribose polymerase inhibitor*					
Topoisomerase II inhibitors						**33.**	**talazoparib**	68	60	7,8	5,7
13.	**idarubicin**	6	3	5,4	3,3	*Anti-metabolite*					
14.	**etoposide**	222	53	7,7	3,3	**34.**	**gemcitabine**	33	18	7,2	5,8
15.	**teniposide**	24*	5637*	8,3*	0,1*	*ATP-noncompetitive inhibitor*					
16.	**doxorubicin**	32	15	6	4,4	**35.**	**asciminib**	–	>1000	–	0
17.	**aldoxorubicin**	19	13	8	3,7	*Broad-range TKI (control)*					
18.	**mitoxantrone**	34*	0,7*	8,5	5,1	**36.**	**ponatinib**	27	112	16,8	11,2
19.	**amsacrine**	21	10	8,5	4,1				117		10,9
IAP SMAC mimetics									116		10,9
20.	**AT-406**	456	232	5,5	15,5	*VEGFR inhibitor*					
21.	**birinapant**	93	94	5,1	9,3	**37.**	**axitinib**	258	277	16,7	3,1
22.	**LCL-161**	168	88	9,5	22,3						

IC_{50} – half-maximal inhibitory concentration; nM – nanomoles; sDSS – selective Drug Sensitivity Score; Plk1 – polo-like kinase 1 (serine/threonine-protein kinase); IAPs – inhibitor of apoptosis proteins; SMAC mimetic – inhibitors that mimic 'small molecule second mitochondrial activator of caspase' (SMAC); KSP/Eg5 – kinesin spindle protein / kinesin-5; ADP – adenosine diphosphate; ATP – adenosine triphosphate; TKI – tyrosine kinase inhibitor; VEGFR – vascular endothelial growth factor receptor

* Result pairs with an absolute fold-change that is equal to or more than 20 have been indicated with asterisks.

To ensure that the selective sensitivity of cells carrying T315M to inhibitors of mTOR and topoisomerase II was not population-specific, a new population of Ba/F3 cells stably expressing T315M was made and tested to 36 inhibitors selected from the high-throughput screening. In addition, the allosteric inhibitor asciminib was added to the customized plates.

Results of the second screening supported the first finding: the new population of cells carrying T315M also displayed sensitivity toward inhibitors of mTOR and topoisomerase II. The cells showed selective sensitivity toward mTOR inhibitors such as everolimus (IC_{50} = 7 nM), temsirolimus (IC_{50} = 1 nM), AZD8055 (IC_{50} = 60 nM), MLN-0128 (IC_{50} = 35 nM), and others. Topoisomerase II inhibitors, such as mitoxantrone (IC_{50} = 1 nM), amsacrine (IC_{50} = 10 nM), doxorubicin (IC_{50} = 15 nM) and aldoxorubicin (IC_{50} = 13 nM) showed selective potency toward cells expressing T315M. Unlike the results for the FO4B drug screening, the new population of T315M-expressing cells appeared to display resistance to the topoisomerase II inhibitor teniposide (IC_{50} > 5000 nM). However, considering the high chemical similarity between teniposide and etoposide, it is possible that this last result is an error in dispensing the drug.

In addition to inhibitors of mTOR and topoisomerase II, the new population of cells expressing T315M was sensitive to compounds that target other pathways, such as the farnesyltransferase inhibitor tipifarnib (IC_{50} = 26 nM), the Plk1 inhibitor BI 2536 (IC_{50} = 26 nM), and the SMAC mimetic LCL-161 (IC_{50} = 88 nM), among others.

Although both experiments revealed that inhibitors of mTOR and topoisomerase II showed selective potency toward T315M-expressing cells when compared to cells expressing T315I or E255V/T315I, a slight variation in results was observed. When all compounds in Table 2 are considered except for the five results with an absolute fold-change ≥ 20, the average absolute fold-change in IC_{50} values is 2,4, and the average absolute fold-change in sDSS values is 1,9. The fact that topoisomerase II and mTOR inhibitors continue to show selectivity to T315M-expressing cells even when this heterogeneity is taken into account further supports the strength of this finding. Although toxicity concerns may limit the clinical value of this selectivity, the result raises questions about using inhibitors of mTOR or topoisomerase II in combination with other compounds.

2.1.4.3. Combination testing resulted in unprecedented synthetic lethality in cells expressing BCR-ABL1 T315M

To investigate possible synergy between ATP-competitive and ATP-noncompetitive inhibitors, combination testing in cells stably expressing T315M was performed. Cells expressing BCR-ABL1 served as control. The ATP-competitive kinase inhibitors ponatinib and axitinib were tested in combination with the allosteric inhibitor asciminib. In addition, the SMAC mimetic LCL-161, which gave promising results as an independent compound in cells expressing T315M (IC_{50} = 115 nM), was combined with asciminib. Data was analyzed with the ZIP (Zero Interaction Potency) method using the software SynergyFinder.

The TKIs ponatinib and axitinib, when combined with the allosteric inhibitor asciminib, had a high synergistic growth inhibitory effect on cells stably expressing T315M. A strong synthetic lethality was observed (ZIP synergy score = 36,5) on cells stably expressing T315M cells when the allosteric inhibitor asciminib was combined with the broad-range TKI ponatinib [Figure 15 and 16].

Combining asciminib and the kinase inhibitor axitinib on cells stably expressing T315M demonstrated synergy (ZIP synergy score 11.7) centering on 100-300 nM of axitinib and 100-3000 nM of asciminib [data not shown]. However, combining the SMAC mimetic LCL-161 with the ATP-noncompetitive compound asciminib resulted in antagonism (ZIP synergy score -4.4) in cells stably expressing T315M [data not shown].

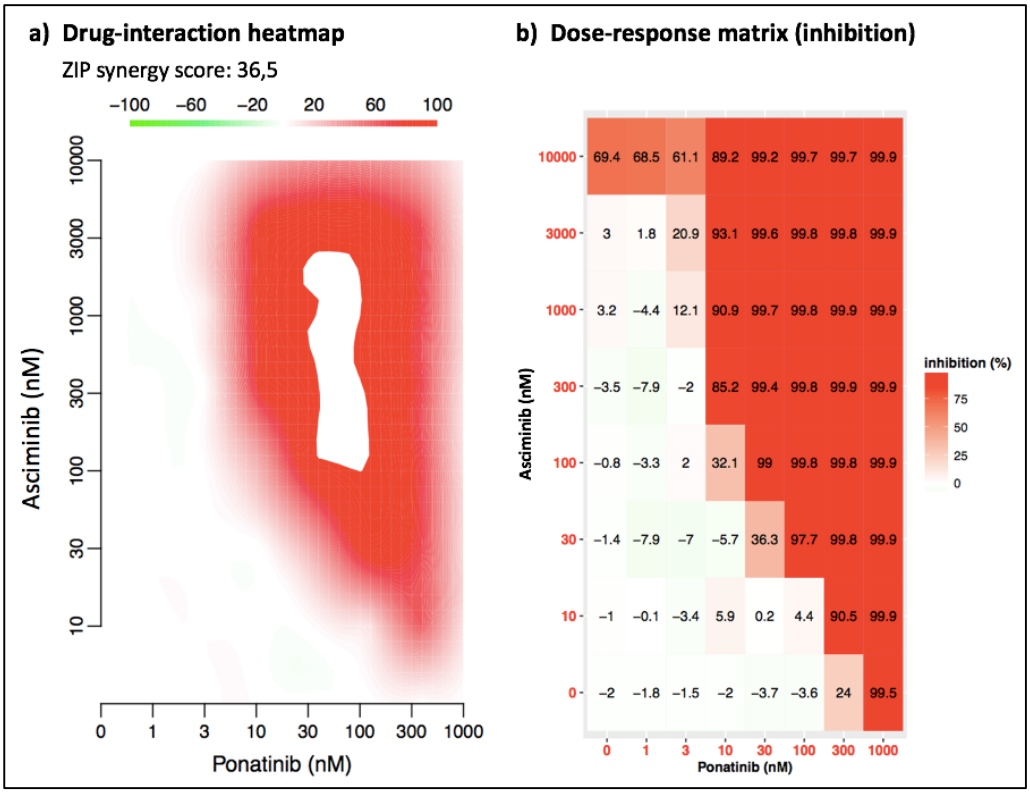

Figure 15: Combinatorial treatment of the tyrosine kinase inhibitor ponatinib and the allosteric inhibitor asciminib revealed strong synergy in high concentrations of both compounds in cells stably expressing T315M. (a) Synergy heatmap of ponatinib and asciminib in T315M-expressing cells. Red area indicates synergy, whereas green indicates antagonism. The white area surrounded by red signifies synergy >100%, which is possible when an individual drug acts antagonistically (% inhibition < 0) but the combination of two drugs in the same concentration is lethal to the cells; **(b)** Dose-response matrix of the combination treatment. Data are shown as percent inhibition when compared to negative control. The results represent duplicates.

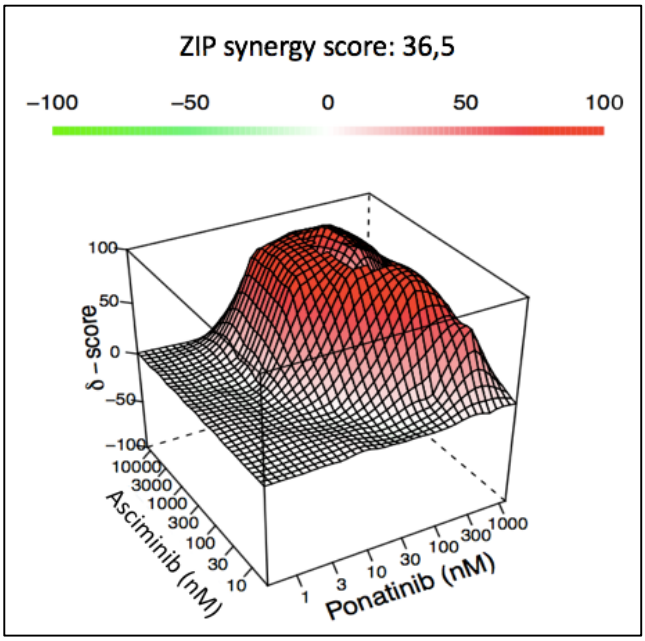

Figure 16: Dose-response landscape of the synthetic lethality between the tyrosine kinase inhibitor ponatinib and the ATP-noncompetitive inhibitor asciminib in cells stably expressing T315M. The results are representative of duplicates.

Although the combination of the TKI ponatinib and the allosteric inhibitor asciminib resulted in unusually high ZIP synergy scores, clinical relevance of the finding is doubtful because the concentrations of asciminib and ponatinib are much too high to prevent toxicity. However, the strength of this synergy suggests that combinations of other TKIs and ATP-noncompetitive drugs may exist which compromise cell viability in T315M-expressing cells at much lower concentrations. Moreover, if computer design of each compound were to be a viable option, modifications to the compounds may also lead to synergy at lower concentrations of drugs. Interestingly, this ZIP synergy score is so high as to enable consideration of the combination of asciminib and ponatinib in T315M-expressing cells as the positive control for combination testing.

2.1.4.4. Cells expressing BCR-ABL1 E255V/T315I displayed complete resistance to all approved TKIs

Given that patients with E255V/T315I mutations have been shown to display high resistance to all available tyrosine kinase inhibitors (Zabriskie *et al.*, 2014), cells stably expressing E255V/T315I were constructed. Then, a viability assay was carried out on the cells to find if E255V/T315I-expressing cells show sensitivity to any approved and investigational ABL-1 specific TKIs. Finally, to assess the sensitivity of cells to selected inhibitors, half-maximal inhibitory concentrations were calculated with GraphPad Prism.

Cells stably expressing E255V/T315I exhibited complete resistance to all 12 selected inhibitors. Although IC_{50} values could be achieved or calculated for eight compounds (danusertib, saracatinib, imatinib, bosutinib, ponatinib, axitinib, bafetinib, and GNF-2), none of the inhibitors showed potency toward cells stably expressing E255V/T315I (IC_{50} > 1000 nM for all; IC_{50} > 10 000 nM for saracatinib, imatinib, and GNF-2) [Figure 17]. Nilotinib, AT9283, dasatinib, and rebastinib failed to achieve IC_{50}s within tested concentrations.

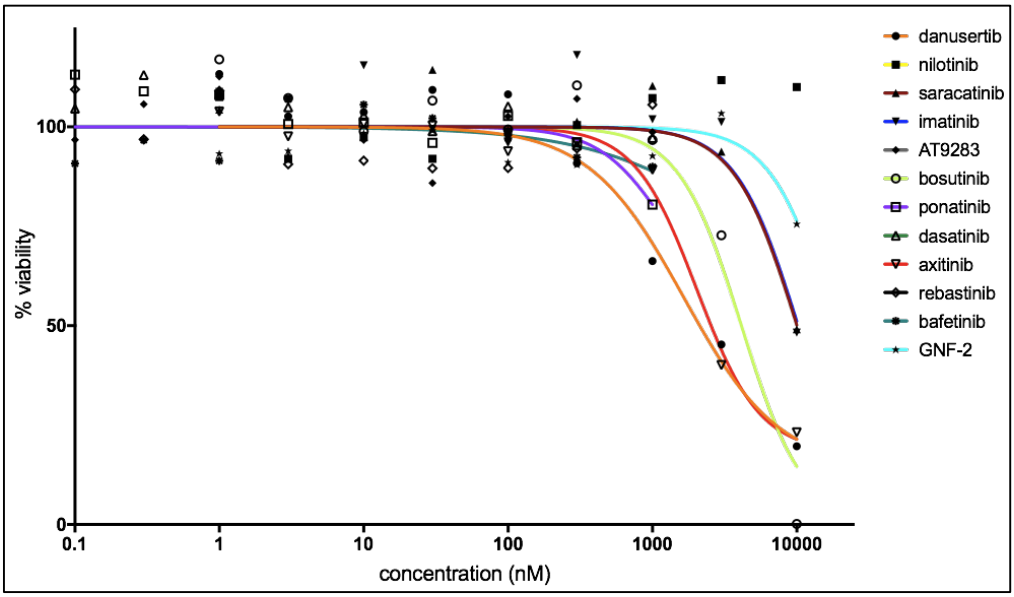

Figure 17: Cells stably expressing E255V/T315I exhibited complete resistance to all tested tyrosine kinase inhibitors, and one allosteric inhibitor. Note that when compared to cells stably expressing T315M, the dose-response curves have shifted to the right on the x-axis because a higher concentration of inhibitors is needed to compromise cell viability in E255V/T315I-expressing cells. Data are representative of three individual experiments.

To summarize, it appears that two separate amino acid substitutions in E255V/T315I-expressing cells could be responsible for the extremely high resistance of these cells to all selected inhibitors. Cells expressing E255V/T315I compare unfavorably to T315M-expressing cells, which needed lower concentrations of drugs to achieve IC_{50}s. The resistance of these cells to all selected inhibitors does, however, highlight the necessity of high-throughput screening with E255V/T315I-expressing cells.

2.1.4.5. IAP SMAC mimetics show selective potency to BCR-ABL1 E255V/T315I-expressing cells when compared to T315I-expressing cells

Considering that cells stably expressing E255V/T315I showed complete resistance to ABL1-specific inhibitors, high-throughput screening was carried out to search for compounds capable of selective potency toward E255V/T315I-expressing cells. To examine selectivity of drugs in relation to parental cells, selective Drug Sensitivity Scores (sDSS) were calculated using the software Breeze.

Ba/F3 cells stably expressing E255V/T315I showed selective sensitivity to IAP SMAC mimetics (mimics of 'small molecule second mitochondrial activator of caspase') when compared to cells expressing T315I alone [Figure 18]. With the exception of mTOR inhibitors, which showed weak selectivity to E255V/T315I-expressing cells over cells expressing T315I alone [data not shown], no drug group displayed selective potency to cells expressing E255V/T315I over T315I-expressing cells. Notably, the IAP SMAC mimetic AT-406 exhibited selectivity to E255V/T315I-expressing cells (sDSS = 6,6) when compared to cells stably expressing T315I (sDSS = 0,8). The SMAC mimetics birinapant and LCL-161, likewise, displayed selective potency to cells expressing E255V/T315I (sDSS = 3,4 and 11,1, respectively) compared to T315I-expressing cells (sDSS = 0

and -0,2, respectively). The IC_{50}s did not allow for strong conclusions to be made because the values suggested moderate resistance to E255V/T315I-expressing cells (IC_{50} = 350 nM, 124 nM, and 103 nM, for AT-406, birinapant, and LCL-161, respectively), but the values were nevertheless higher than they were for T315I-expressing cells (IC_{50} = 731 nM, 246 nM, and 526 nM, respectively). Note that SMAC mimetics did not show selective potency to E255V/T315I-expressing cells over cells expressing BCR-ABL1.

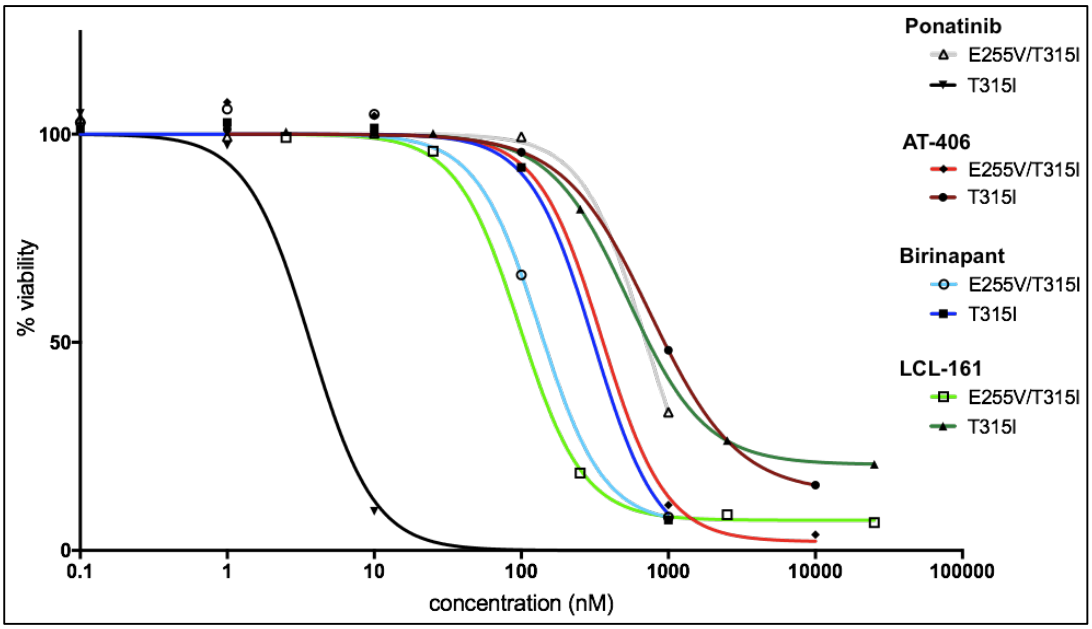

Figure 18: IAP SMAC mimetics displayed selective potency toward cells expressing E255V/T315I when compared to cells expressing T315I alone. Consider that the dose response curve for cells expressing E255V/T315I is closer to the y-axis in all cases, showing that a lower concentration of drug is needed to compromise cell viability of E255V/T315I cells than in cells expressing T315I alone. For simplicity, cell lines are referred to by the mutation they express. GraphPad Prism was used for data visualization.

Another small group of compounds that showed potency toward E255V/T315I-expressing cells included two Hsp90 (Heat shock protein 90) inhibitors: CUDC-305 (sDSS = 5,5; IC_{50} = 141 nM) and radicicol (sDSS = 5,4; IC_{50} = 229 nM). However, selectivity over cells expressing T315I was ambiguous [data not shown]. Thus, these two small groups of drugs were further investigated in E255V/T315I-expressing cells in another cell-viability assay which contained seven selected inhibitors, including the three SMAC mimetics and two Hsp90 inhibitors. The assay was carried out in duplicate and analyzed with GraphPad Prism.

The second screening with these inhibitors resulted in heterogeneous IC_{50}s. When the IC_{50}s of the three SMAC mimetics in E255V/T315I-expressing cells were considered, the average absolute fold-change between the two experiments was 2,9. However, the same comparison in cells expressing BCR-ABL1 (control cells) was inconsequential (1,5). Although results of the repeated screening with selected inhibitors remained inconclusive, SMAC mimetics continued to show consistent selectivity toward E255V/T315I-expressing cells over cells expressing T315I. Again, E255V/T315I-expressing cells displayed more sensitivity to AT-406, birinapant, and LCL-161

(IC_{50} = 170 nM, 37, and 31 nM, respectively) than to cells expressing T315I. No tested amount of the allosteric inhibitor asciminib could compromise cell viability in E255V/T315I-expressing cells.

It is important to note that cells stably expressing E255V/T315I showed sensitivity to many general chemotherapy drugs. These compounds included the RNA and DNA synthesis inhibitor dactinomycin (IC_{50} = 0,7 nM), the mitotic inhibitor vinblastine (IC_{50} = 4 nM) and the antimetabolite floxuridine (IC_{50} = 11 nM), among others. Although these inhibitors are FDA approved and potent against E255V/T315I-expressing cells, finding compounds selective toward these highly resistant cells (as opposed to drugs targeting all rapidly dividing cells) goes a long way to minimize toxicity and lead to more targeted and efficient cancer therapy.

2.1.4.6. Combining the SMAC mimetic LCL-161 and the allosteric inhibitor asciminib resulted in antagonism in cells expressing BCR-ABL1 E255V/T315I

It was not known whether the E255V/T315I-expressing cells could be simultaneously and effectively targeted at the ATP-binding pocket as well as the myristoyl pocket. Thus, to find possible synergy, combinatorial treatment of E255V/T315I-expressing cells was carried out. ATP-competitive inhibitors axitinib and ponatinib were combined with the ATP-noncompetitive inhibitor asciminib. Furthermore, considering that SMAC mimetics showed selective potency toward cells expressing E255V/T315I, asciminib was combined with the SMAC mimetic LCL-161. BCR-ABL1-expressing cells were used as control and results were analyzed with the ZIP method using SynergyFinder.

Instead of targeting the ABL1 kinase domain in a synergistic manner, SMAC mimetic LCL-161 in combination with the allosteric inhibitor asciminib had an antagonistic effect on E255V/T315I-expressing cells [Figure 19]. The combined treatment in cells expressing E255V/T315I resulted in a ZIP synergy score of -3.2. This result is similar to the antagonistic effect of LCL-161 and asciminib in T315M-expressing cells.

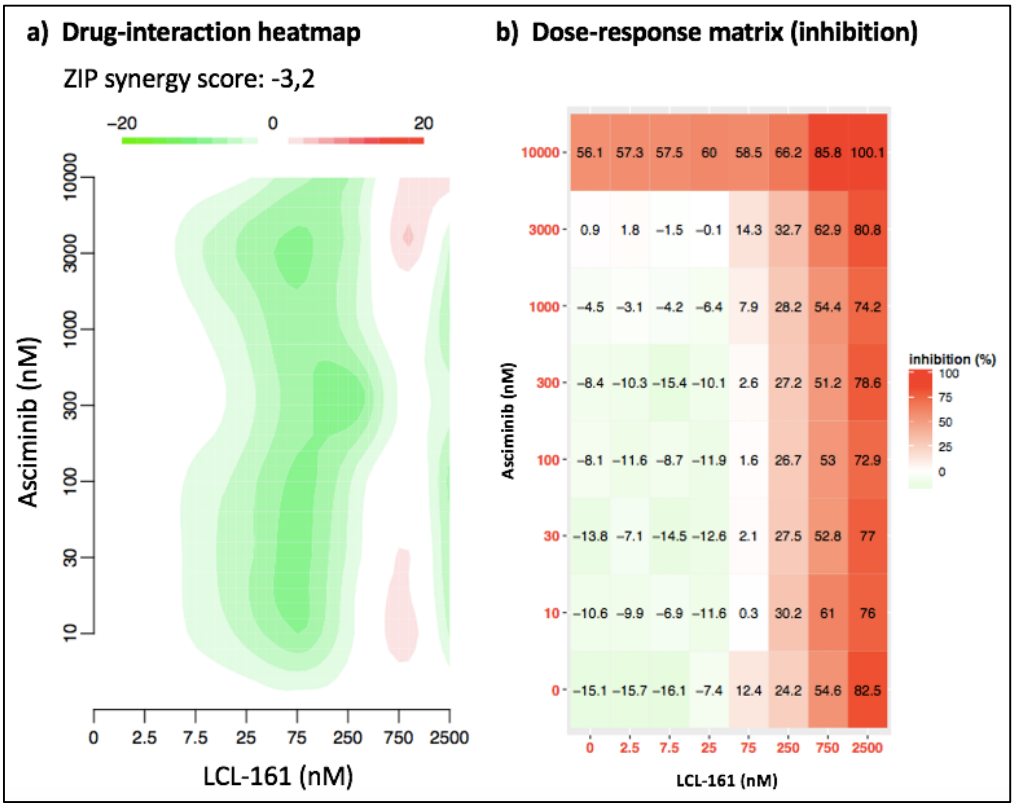

Figure 19: Combinatorial treatment of the SMAC mimetic LCL-161 and the allosteric inhibitor asciminib resulted in antagonism in cells stably expressing E255V/T315I. (a) Drug-interaction heatmap of LCL-161 and asciminib in E255V/T315I-expressing cells. Green area refers to antagonism and pink area refers to synergy. Note that, due to different legends, the intensity of color indicates a different synergy score than does the corresponding color in Figure 15 (Section 2.1.4.3.); **(b)** Dose-response matrix of the combinatorial treatment. Results are shown as percent inhibition when compared to negative control. Data represent duplicates.

Even though the ATP-noncompetitive inhibitor asciminib had no independent effect on E255V/T315I-expressing cells, combining asciminib with inhibitors that target the ATP-binding site resulted in synergy. Combining the TKI ponatinib and allosteric inhibitor asciminib in cells expressing E255V/T315I resulted in synergy (ZIP synergy score = 15,3), but in concentrations of ponatinib that show resistance (> 300 nM) [data not shown]. The tyrosine kinase inhibitor axitinib combined with asciminib had a synergistic effect (ZIP synergy score 6.4) centering on extremely high concentrations of axitinib (1000 nM) and asciminib (300-3000 nM) in E255V/T315I-expressing cells [data not shown]. Although the ZIP synergy score appears high for both combinations in this cell line, the concentration of compounds needed to achieve that synergy is much too high to consider clinical relevance. Furthermore, it can be argued that, at high enough concentrations, any toxic chemical (drug) will compromise cell viability in these cells.

All three combinations resulted in weak synergy in cells stably expressing BCR-ABL1. A modest but noticeable synergy (ZIP synergy score 2.7) could be seen between the kinase inhibitor axitinib and allosteric inhibitor asciminib in cells expressing BCR-ABL1. The strongest synergistic effect can be observed when combining 300 nM axitinib and 10 nM asciminib [data not shown]. The tyrosine kinase inhibitor ponatinib combined with asciminib revealed slight synergy (ZIP synergy

score 0.8) centered around the concentrations 10 nM for asciminib and 3 nM for ponatinib [data not shown]. Interestingly, combinatorial treatment of the IAP SMAC mimetic LCL-161 and asciminib exhibited small synergy that included low concentrations of asciminib (10 nM) but higher concentrations of LCL-161 (>250 nM) [data not shown]. It is worth noting that the concentration of asciminib needed to achieve synergy did not surpass 100 nM in any of the three duplicates, and centered around 10 nM in all cases.

Several conclusions can be drawn from these results. First, the presence of mutations in the ABL1 kinase domain, T315M and E255V/T315I, appears to alter the response of cells when compounds are combined. When ponatinib or axitinib are combined with asciminib, strong synergy can be achieved in cells expressing T315M or E255V/T315I, but only in high concentrations of either TKI. Although the combination produces synergy in BCR-ABL1-expressing cells, the synergy is faint. Interestingly, combining the SMAC mimetic LCL161 with asciminib in cells expressing BCR-ABL1 resulted in synergy, yet the same combination in cells expressing T315M or E255V/T315I lead to antagonism. This discrepancy further supports the implication that the presence of a new amino acid in the ABL1 kinase domain of these cells alters drug-drug interaction. Second, no synergy was seen in high concentrations of any drug in cells expressing BCR-ABL1, whereas, if synergy occurred in the other two cell lines, it was stronger at higher concentrations. It is important to note that, although the synergy in cells expressing BCR-ABL1 was faint, it did occur in low concentrations, which implies possible clinical relevance.

2.1.4.7. Axitinib shows selective potency against cells expressing BCR-ABL1 T315I

Given the frequent adverse side-effects of the broad-range TKI ponatinib, the VEGFR inhibitor axitinib was included among the selected inhibitors that were used to treat cells stably expressing T315I. To this end, cells expressing T315I were constructed. To confirm the selective potency of axitinib on T315I-expressing cells and reproduce published findings, a cell-viability assay was carried out, and data were analyzed using GraphPad Prism.

As has been shown previously, cells stably expressing T315I displayed selective sensitivity to the kinase inhibitor axitinib [Figure 20]. Cells stably expressing T315I exhibited more sensitivity toward axitinib (IC_{50} = 59 nM) than cells carrying BCR-ABL1 (IC_{50} = 219 nM) or T315M (IC_{50} = 199 nM). Cells expressing E255V/T315I showed complete resistance toward axitinib (IC_{50} > 1000).

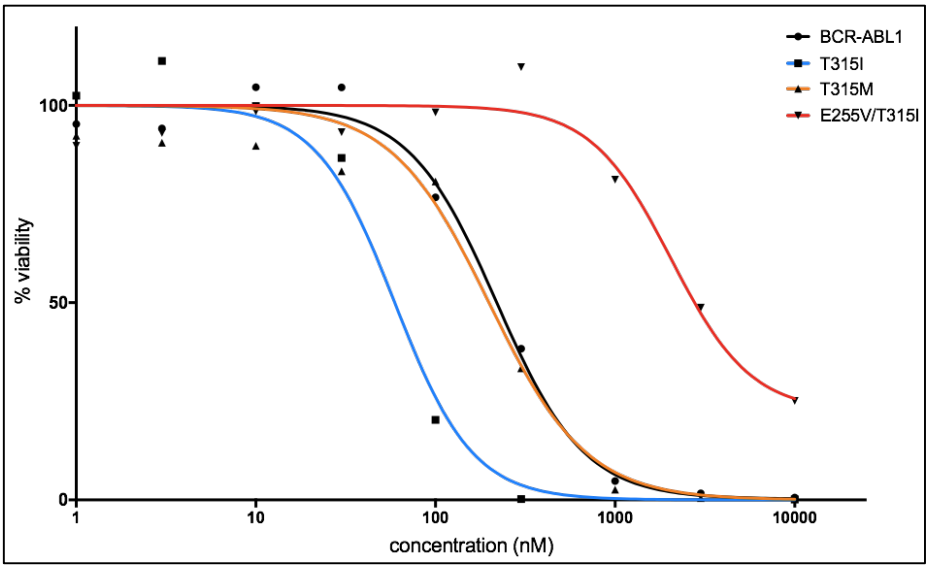

Figure 20: The effect of axitinib on cells stably expressing BCR-ABL1, T315I, T315M and E255V/T315I, respectively. For simplicity, cells stably expressing BCR-ABL1, T315I, T315M and E255V/T315I are referred to by the mutation they carry. Data represent three respective experiments.

In comparison, only cells stably expressing BCR-ABL1 showed sensitivity to the TKI imatinib (IC_{50} = 117 nM). Cells stably expressing the respective oncogenes T315I, T315M, and E255V/T315I displayed complete resistance to imatinib (IC_{50} > 4000 nM for all three) [Figure 20a]. The broad-range TKI ponatinib showed high potency to cells expressing BCR-ABL1 and T315I (IC_{50} = 1 nM for both) when compared to cells expressing T315M (IC_{50} = 17 nM) and E255V/T315I (IC_{50} = 592 nM) [Figure 20b]. Notably, cells expressing T315I showed moderate resistance to the allosteric inhibitor GNF-2 (IC_{50} = 175 nM) [data not shown].

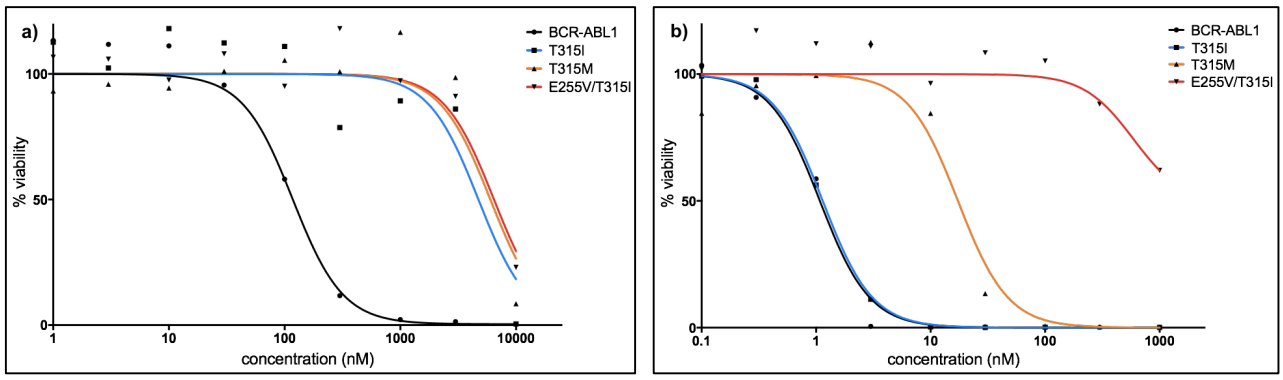

Figure 20: Comparison of the sensitivity of cells expressing BCR-ABL1, T315I, T315M and E255V/T315I to the TKIs imatinib (a) and ponatinib (b). (a) Cells stably expressing the BCR-ABL1 plasmid are considerably more sensitive to imatinib than cells expressing T315I, T315M, or E255V/T315I; **(b)** Ponatinib exhibited potency toward cells expressing the BCR-ABL1 and T315I when compared to cells expressing T315M or E255V/T315I. Data represent three independent assays.

When compared to the other cell lines, cells stably expressing BCR-ABL1 (control) showed most sensitivity to the 12 selected inhibitors. Cells expressing BCR-ABL1 exhibited most sensitivity to the TKIs dasatinib (IC_{50} = 0,4 nM), ponatinib (IC_{50} = 1 nM), bafetinib (IC_{50} = 4 nM), nilotinib (IC_{50} = 8 nM), and imatinib (IC_{50} = 117 nM). Of the tested compounds, cells expressing BCR-ABL1

were least sensitive to the kinase inhibitors axitinib and saracatinib (IC_{50} = 275 nM), and the ATP-noncompetitive inhibitor GNF-2 (IC_{50} = 577 nM) [data not shown]. Notably, cells stably expressing BCR-ABL1 showed selective sensitivity to the BRAF (serine/threonine protein kinase B-Raf) inhibitor CEP-32496 at IC_{50} = 24 nM (sDSS = 31,8).

To find other potential compounds capable of selective potency toward cells expressing T315I, the cells were treated with inhibitors from > 50 drug classes in the FO4B and PKIS drug sets. As in previous assays, cell viability measurement was followed by analysis using Breeze and Graphpad Prism. Supporting the results of the first experiment, ponatinib and axitinib showed remarkable selectivity toward T315I-expressing cells (sDSS = 27 and 22, respectively). The cells showed resistance to imatinib, nilotinib, dasatinib, and bosutinib (IC_{50} > 1000 for all four). However, six compounds in the PKIS showed potency toward cells expressing T315I [data not shown]. The cells showed most sensitivity to the PKIS inhibitor GW683134A (IC_{50} = 86 nM), and all six compounds that displayed selectivity to T315I-expressing cells are known inhibitors of ABL1. Note that Ba/F3 parental cells and not cells expressing BCR-ABL1 were used as control in the PKIS screening.

T315I-expressing cells showed more sensitivity to the selected inhibitors than cells expressing T315M or E255V/T315I, but it is worth noting that, with the exception of ponatinib, no TKI approved to treat CML compromised cell viability in T315I-expressing cells in clinically relevant concentrations. However, the sensitivity of the cells to the TKI axitinib shows promise in treating patients carrying the gatekeeper mutation.

2.1.5. Discussion

2.1.5.1. Summary of Key Results

Cells expressing the BCR-ABL1 T315M mutation displayed resistance to all first- and second-generation TKIs, and moderate resistance to ponatinib. However, as a novel finding, several mTOR and topoisomerase II inhibitors selectively compromised cell viability in T315M-expressing cells. Combination testing revealed strong synthetic lethality when high concentrations of the ATP-noncompetitive inhibitor asciminib and ponatinib were combined in these cells. Next, cells stably expressing E255V/T315I showed complete resistance to all approved TKIs. The cells were affected by general chemotherapy but few compounds showed selectivity. Among them, three SMAC mimetics displayed selective potency to E255V/T315I-expressing cells over cells expressing T315I. As reported previously, cells stably expressing the gatekeeper mutation T315I displayed sensitivity to the TKIs ponatinib and axitinib, but the first- and second-generation TKIs failed to induce compromised cell viability in T315I-expressing cells. All five TKIs induced loss of cell viability in cells stably expressing BCR-ABL1 in clinically relevant concentrations. Interestingly, the BRAF inhibitor CEP-32496 displayed selective potency toward cells expressing BCR-ABL1.

2.1.5.2. Novel finding: Inhibitors of mTOR and topoisomerase II showed selective potency toward T315M-expressing cells

As a novel finding, inhibitors of the mTOR pathway and topoisomerase II induced cell viability loss in T315M-expressing cells. This result was supported by two screenings with two separate T315M-expressing cell populations.

The reason behind the selectivity of topoisomerase II inhibitors toward cells expressing T315M over cells carrying T315I or E255V/T315I can be speculated. Although the polar threonine is replaced by the non-polar methionine in amino acid position 315 in the ABL1 kinase domain of cells expressing T315M (Gorre *et al.*, 2001), both BCR-ABL1-expressing cells and T315M-expressing cells showed selective sensitivity toward inhibitors of topoisomerase II. Thus, the threonine to methionine substitution is unlikely to be the cause of sensitivity of cells expressing T315M cells to topoisomerase II inhibitors. It is reasonable to conclude that the presence of methionine instead of isoleucine might imply changes in pathways within cells carrying T315M. Moreover, a potential change in pathways with similar implications—sensitivity to topoisomerase II—does not appear to occur when the T315I mutation is accompanied by another point mutation, E255V, because inhibitors of topoisomerase II did not show selective potency toward cells stably expressing E255V/T315I.

Uncontrolled activation of the PI3K (phosphatidylinositol 3-kinase)/Akt (protein kinase B)/mTOR pathway has been detected in CML. Aberrant mTOR activity could enable drug resistance, but it could also be an effective target for inhibitors (Dinner and Platanias, 2016). Suppressing the Akt/mTOR pathway with the anesthetic propofol has been shown to improve the efficacy of TKIs in CML (Tan *et al.*, 2017). In addition, two respective clinical trials have combined imatinib with the mTOR inhibitors everolimus and temsirolimus in treating CML (ClinicalTrials.gov ID: #NCT00093639 and #NCT00101088). Although no results have been published, this appears to be an active area of research. Considering the selective sensitivity of cells expressing BCR-ABL1 or

T315M to mTOR inhibitors, it is possible that the mTOR signaling that may be constitutively activated in cells expressing BCR-ABL1 is also activated in cells expressing T315M. The occurrence of T315M may affect the PI3K/Akt/mTOR pathway in a way that is dissimilar to cells stably expressing T315I. Another possibility is that the presence of T315M returns the PI3K/Akt/mTOR pathway back to its "original" constitutively active state seen in cells expressing the BCR-ABL1. Given that mTOR inhibitors showed potency for T315M-expressing cells only comparable to BCR-ABL1-expressing cells, combining an mTOR inhibitor with a TKI might induce loss of cell viability in a synergistic way in either cell line.

2.1.5.3. Combination testing resulted in synthetic lethality for cells expressing T315M

Considering that compound mutations containing T315I have been linked to ponatinib-failure (Zabriskie *et al.*, 2014), it would be reasonable to conclude that combining TKIs targeting the ATP-binding pocket with the ATP-noncompetitive asciminib would result in synergy in cells expressing T315M or E255V/T315I. Asciminib has a different pattern of resistance mutations than second-generation TKIs do. The allosteric inhibitor has been shown to be active against all mutations in the ATP-binding site while inhibitors targeting the ATP-binding site showed activity against all asciminib-resistant mutations. In spite of the (expected) acquired resistance in mice treated with single compounds, combining the TKI nilotinib with the allosteric inhibitor asciminib resulted in complete control of leukemia and elimination of CML xenograft tumors. Furthermore, leukemia did not recur after combination therapy had ended (Wylie *et al.*, 2017).

Combining two inhibitors with non-overlapping resistance profiles that target different sites of ABL1 has tremendous potential. Indeed, combining asciminib and ponatinib in treating cells expressing T315M resulted in such high ZIP synergy score (36,5) as to suggest using this combination as the positive control for synergy. In this case, combining 30 nM of ponatinib with 100 nM of asciminib has little clinical relevance because both concentrations are too high for safety. 30 nM of ponatinib alone is considered to be "moderately resistant" by Zabriskie *et al.*, 2014. Nevertheless, combining all available TKIs with all available allosteric inhibitors might lead to combinations that compromise cell viability in cells stably expressing T315M or E255V/T315I in concentrations low enough to imply clinical relevance.

2.1.5.4. Cells expressing T315M showed moderate resistance to the broad-range TKI ponatinib

Cells stably expressing T315M showed resistance to the four first- and second-generation TKIs, but the cells showed more sensitivity toward ponatinib (IC_{50} = 44,5 nM, an average of four experiments) when compared to results published by Lasater *et al.*, 2016 (IC_{50} = 204 nM). According to Lasater *et al.* (2016), the T315M mutation is highly resistant to ponatinib because the hydrophobic side chains of T315M are too big to allow ponatinib to bind to the ATP-binding pocket. Although the average of three replicates on the new T315M custom plates reveals a higher result (IC_{50} = 115 nM) and remains consistent within the experiment, cells expressing T315M were found to be more sensitive to ponatinib than previous publications suggest (Zabriskie *et al.*, 2014; Lasater *et al.*, 2016). Regardless of the difference between the published sensitivity to ponatinib and

one described here, even the IC_{50} achieved in this thesis calculated as an average of all experiments (IC_{50} = 75 nM) would be too high to consider in a clinical setting (for cells expressing T315I, IC_{50} = 8 nM), especially when the side effects of ponatinib are considered (Cassuto and Dufies *et al.*, 2012; Jain *et al.*, 2015).

2.1.5.5. IAP SMAC mimetics show selective potency toward cells expressing E255V/T315I over cells carrying T315I alone

Although few drugs were selective toward E255V/T315I-expressing cells, three IAP SMAC mimetics showed selectivity when compared to cells stably expressing T315I. In the initial screening, cells expressing E255V/T315I displayed complete resistance to all TKIs, including ponatinib. The reason behind the lower affinity of ponatinib to the compound mutation compared to the gatekeeper mutation T315I alone has been suggested to be the re-orientation of the P-loop in order to fit the hydrophobic valine side chain in position 255 (Zabriskie *et al.,* 2014). Although the high-throughput screening found general chemotherapy drugs capable of inducing loss of cell viability in cells carrying this highly resistant mutation, few compounds showed selectivity to these cells. Among them, three IAP SMAC mimetics stood out: AT-406, LCL161, and birinapant. A repeated three-day viability assay gave heterogeneous results regarding the IC_{50}s of these drugs, but both screenings showed consistent selectivity of IAP SMAC mimetics to cells expressing E255V/T315I over cells expressing T315I alone.

The selective potency of several SMAC mimetics to E255V/T315I-expressing cells over cells that express T315I alone is an exciting prospect. No other drug class showed such a marked and consistent selectivity to cells expressing E255V/T315I over T315I. Moreover, to the best of our knowledge, this selectivity has not been shown before. Importantly, SMAC mimetics displayed selective potency to E255V/T315I-expressing cells over T315I-expressing cells even when the heterogeneous IC_{50} values of the second experiment were considered.

However, a few caveats have to be considered. First and foremost, the three SMAC mimetics were tested only once in cells expressing T315I in the FO4B screening. The second screening included cells expressing BCR-ABL1 as control. Given the difference in results of the first and second assay, it is not unreasonable to question whether the drugs would also display less potency to T315I-expressing cells in the second screening. Second, only three SMAC mimetics were included in FO4B screening and in this thesis. It is possible that this selective potency, if supported by further evidence, is limited to only a few SMAC mimetics. And third, even if the finding is supported by further evidence, the concentrations of drugs needed to achieve cell viability loss in mouse cells might not translate well to drug concentrations needed by humans. All in all, however, this supposed selectivity is an exciting discovery and should be considered for further investigation.

IAPs are expressed in many cancers, and SMAC mimetics induce cell death by imitating the IAP antagonist Smac (Fulda, 2015). This selectivity, if true, could be a pathway change affected by the presence of valine at position 255. These pathways might be associated with an abnormal IAP activity which could be changing the sensitivity of these cells to SMAC mimetics. However, further research is necessary to determine if this result can be repeated, and if so, how much clinical relevance this finding would hold.

2.1.5.6. Combining the TKI ponatinib with the allosteric inhibitor asciminib resulted in synergy at low concentrations of drugs in cells expressing BCR-ABL1

Regardless of the low ZIP synergy score in BCR-ABL1-expressing cells when the TKI ponatinib and the allosteric inhibitor asciminib were combined, synergy occurred at low concentrations of either drug. Considering that ATP-competitive and ATP-noncompetitive inhibitors have been shown to have non-overlapping mutation profiles (Wylie *et al.*, 2017), low concentration of ponatinib combined with asciminib might have value in clinical setting as means to prevent drug-resistance that is driven by point mutations in the ABL1 kinase domain.

2.1.5.7. T315I-expressing cells showed sensitivity to the TKI axitinib

As published by Pemovska *et al.* (2015) and shown here, cells stably expressing T315I exhibited sensitivity to the VEGFR inhibitor axitinib (IC_{50} = 59 nM). In addition, the result for the broad-range TKI ponatinib in these cells falls in line with that of Cassuto and Dufies *et al.* (2012), who found ponatinib to be capable of inducing loss of cell viability at a similar concentration (IC_{50} = 8 nM). Moreover, as shown previously by Redaelli *et al.* (2009), cells stably expressing T315I showed resistance to first- and second-generation TKIs (imatinib, nilotinib, dasatinib, bosutinib). In comparison to cells stably expressing T315M or E255V/T315I, the problem of resistance in T315I-expressing cells might not appear as complex now that ponatinib is in the market, but the adverse side effects of the drug and lack of FDA-approved alternatives must be considered when comparing the mutations. Furthermore, given that the gatekeeper mutation T315I is considered to be the most frequent mutation to occur in CML patients (COSMIC database) and that the prevalence of CML is expected to rise six-fold by 2050 (Huang *et al.*, 2012), the importance of finding an alternative to ponatinib cannot be overstated.

2.1.5.8. Conclusions

Finding drugs with high affinity toward cells expressing these three mutations, especially CML patients carrying these mutations, will be a challenge. Treating drug-resistance as well as anticipating its occurrence may often have to be considered together. First, successful and long-term inhibition of cells carrying highly resistant mutations may have to include combination testing to target several sites of the molecule and find drugs that act in synergy. Second, crystallography of the ABL1 kinase domains of T315M and E255V/T315I may be needed to fully understand the molecular dynamics of drug-binding and the implications of each mutation. The structure, chemistry and size of each mutation may be incompatible with drug-binding. Therefore, understanding the characteristics of each mutation may allow computer modeling to design for an inhibitor that binds and inhibits the constitutively active ABL1 kinase domain. Third, CML patient samples would be invaluable in testing drugs and combinations of drugs to establish the efficacy of the treatment and bring findings closer to clinical trials.

2.2. Identifying mutations that enable cells resistance to the kinase inhibitor axitinib

2.2.1. Rationale

The kinase inhibitor axitinib has not only been shown to have high affinity for T315I in kinase assays (Davis *et al.*, 2011), the inhibitor has also shown clinical activity in a CML patient carrying T315I (Pemovska *et al.*, 2015). Considering the frequent adverse side effects that accompany ponatinib-treatment (Cortes *et al.*, 2013), axitinib shows promise for CML patients carrying the T315I mutation. However, mutations that enable cells to develop resistance to axitinib have so far remained unexplored. Knowing which point mutations enable axitinib-resistance is key to understanding how to address resistance in CML patients. To this end, Kesarwani *et al.* (2014) have successfully introduced a library of random mutations in JAK2 (Janus kinase 2) kinase domain prior to transduction of Ba/F3 cells and inducing drug-resistance. All of the mutations they identified using this method were later observed in patients (Kesarwani *et al.*, 2014). Therefore, in my study Ba/F3 cells were infected with retroviruses containing a library of random mutations introduced to BCR-ABL1 T315I and treated with axitinib to observe which mutations are likely to emerge as a result of axitinib-resistance.

2.2.2. Materials

All cell lines were maintained in a humidified incubator containing 5% CO_2 at 37 °C (see Section 2.1.2). The XL1-Red bacterial strain (from *Escherichia coli*) and β-mercaptoethanol were purchased from Agilent Technologies (#200129) and stored at -80 °C. XL1-Red competent cells are characterized by three impaired primary DNA repair pathways, which create an approximate 5000-fold increase in mutation rate when compared to wild-type (Agilent Technologies, #200129). SOC medium (Super Optimal broth with Catabolite repression) was kindly provided by Daria Bulanova.

2.2.3. Methods

2.2.3.1. XL-1 Red Mutagenesis

To introduce random mutations in a BCR-ABL1 gene containing the T315I mutation, an XL1-Red bacterial strain was used according to a modified protocol by Agilent Technologies (#200129). Note that 100 ng of DNA (pMIG-BCR-ABL1 T315I) was used in the experiment. The plates were incubated for 30 h at 37 °C. No colonies grew on the control plate. Colonies containing the random mutations introduced to pMIG-BCR-ABL1 T315I were collected prior to DNA isolation using the protocol provided by QIAGEN Plasmid Plus Midi Kit (Qiagen; #12943).

2.2.3.2. Construction of cell lines and selection of drug-resistant cells

Ba/F3 parental cells were infected with a retrovirus from the virus packaging cell line CRL-11654 containing the random plasmid collection introduced to pMIG-BCR-ABL1 T315I using the

protocol in Section 2.1.3.1. Selection was complete 10 days after the removal of IL-3 from the growth medium.

Ba/F3 cells stably expressing the randomized plasmid collection introduced to BCR-ABL1 T315I were treated with axitinib in order to select for an axitinib-resistant population. 8,3 x 10^5 cells/well on a 6-well plate were divided into three sections based on the concentration of axitinib: 0 nM (DMSO), 200 nM, and 500 nM. DMSO volume corresponding to the amount of axitinib needed to achieve the highest concentration (500 nM) of drug served as the control. Rapid growth of Ba/F3 cells stably expressing mutagenized BCR-ABL1 T315I in wells containing 500 nM of axitinib was detected 20-25 days after the beginning of axitinib-treatment.

2.2.3.3. Drug-screening of axitinib-resistant cells

A three-day CellTiter-Glo Luminescent Cell Viability Assay was performed on Ba/F3 cells stably expressing (1) BCR-ABL1 T315I, (2) random mutations introduced to BCR-ABL1 T315I, and (3) axitinib-treated cells stably expressing random mutations introduced to BCR-ABL1 T315I according to the protocol described in Section 2.1.3.2.-2. The 384-well plate layout, originally designed by Tea Pemovska, was modified to contain the ATP-noncompetitive inhibitor asciminib (0,1-1000 nM) in addition to 10 compounds listed in Figure 12 (excluding AT9283 and rebastinib).

2.2.3.4. Sequencing the kinase mutations that allow resistance to axitinib

QIAprep Spin Miniprep Kit was used to isolate plasmid DNA from the three cell lines according to the protocol provided by Qiagen (#27106). Amplicon sequencing of the ABL1 kinase domain helped detect the mutations that emerged under treatment with axitinib. Amplicon sequencing was performed by the FIMM Sequencing Unit with the following locus-specific primers:

ABL1_cDNA_1-F	CCATTATCCAGCCCCAAAG
ABL1_cDNA_1-R	CCTTCACCAAGTGGTTCTCC
ABL1_cDNA_2-F	AGATCTTGCTGCCCGAAACT
ABL1_cDNA_2-R	CCTGCAGCAAGGTACTCACA

The following adapter primers were attached to the locus specific primers (5'-3'):

F-primers	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
R-primers	AGACGTGTGCTCTTCCGATCT

The standard PCR (polymerase chain reaction) protocol by Finnzymes Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific; #F531L) was carried out. Note that the PCR process included a total of 30 cycles and that annealing occurred at 60 °C. Basic Local Alignment Search Tool (BLAST) was used to identify individual nucleotide mutations.

2.2.4. Results

Although the broad-range TKI ponatinib shows potency toward cells carrying T315I, adverse effects have been shown to occur in patients (Jain *et al.*, 2015). As a potential alternative, the VEGFR inhibitor axitinib has displayed clinical activity in a T315I-positive CML patient (Pemovska *et al.*, 2015). Given that Kesarwani *et al.* (2014) have successfully predicted clinically relevant mutations by expressing a library of random mutations in a Ba/F3 cell model and sequencing the kinase domain after treating the cells with their drug of interest, this approach was used to predict mutations that might arise after axitinib-treatment.

2.2.4.1. Cells stably expressing BCR-ABL1 T315I displayed sensitivity to asciminib and axitinib

Three different cell lines were constructed and treated with the selected inhibitors: cells stably expressing (1) BCR-ABL1 T315I, (2) the library of random mutations introduced to BCR-ABL1 T315I, or (3) axitinib-resistant cells expressing random mutations introduced to BCR-ABL1 T315I. A three-day cell viability assay using Cell-Titer Glo in 384-well format was carried out. Next, to determine sensitivity of a cell line to a particular drug, half-maximal inhibitory concentrations were calculated using GraphPad Prism.

Of the three cell lines, cells expressing T315I displayed the most sensitivity to the selected compounds [Figure 21]. Notably, cells stably expressing T315I showed sensitivity toward the kinase inhibitor axitinib (IC_{50} = 48 nM) and the allosteric inhibitor asciminib (IC_{50} = 1 nM). In addition, the TKIs ponatinib (IC_{50} = 1 nM) and dasatinib (IC_{50} = 5 nM) exhibited potency toward T315I-expressing cells. The cells displayed moderate resistance to the allosteric inhibitor GNF-2 (IC_{50} = 81 nM), and the TKIs danusertib (IC_{50} = 179 nM) and bosutinib (IC_{50} = 514 nM). T315I-expressing cells showed complete resistance to nilotinib, saracatinib, imatinib, and bafetinib (IC_{50} > 1000 for all four compounds).

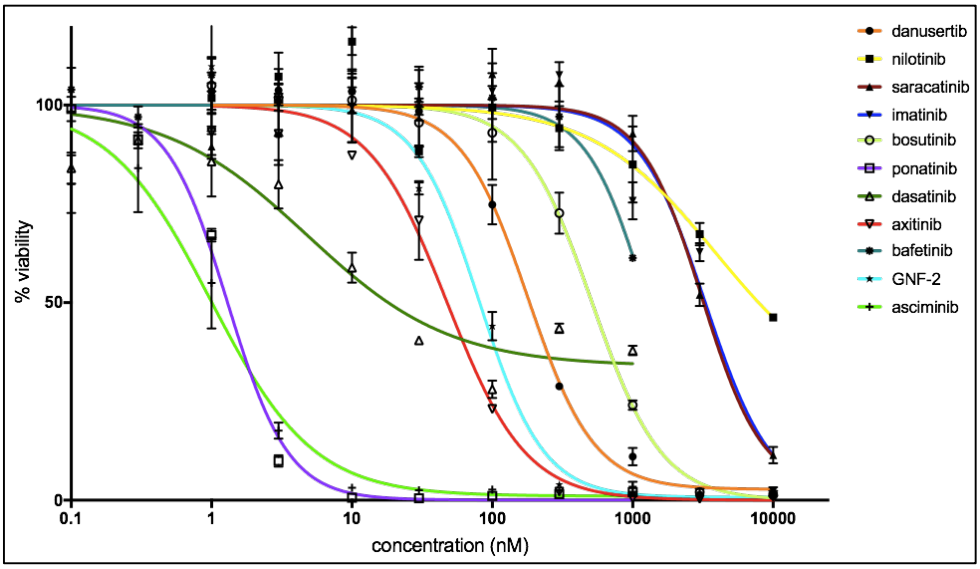

Figure 21: Cells stably expressing T315I showed sensitivity to the allosteric inhibitor asciminib, the kinase inhibitor axitinib, and the TKIs ponatinib and dasatinib. The cells exhibited the most sensitivity toward the broad-range TKI ponatinib and the ATP-noncompetitive inhibitor asciminib, moderate resistance to the allosteric inhibitor GNF-2, the TKIs danusertib and bosutinib, and complete resistance to nilotinib, saracatinib, imatinib, and bafetinib. Error bars indicate standard deviation between two replicates.

Of note, this finding further supports the sensitivity of T315I-expressing cells to axitinib, but it also highlights the potential of the ATP-noncompetitive inhibitor asciminib to compromise the viability of cells expressing the gatekeeper mutation. However, the apparent potency of dasatinib to T315I-expressing cells is a clear outlier when compared to the previous four experiments, in which no IC_{50} could be reached in T315I-expressing cells. With this exception, the results of this experiment compare favorably to the previous experiments and can be considered a reliable control for cells expressing the random mutations introduced to BCR-ABL1 T315I, and axitinib-treated cells expressing random mutations introduced to BCR-ABL1 T315I.

2.2.4.2. Cells stably expressing random mutations introduced to BCR-ABL1 T315I displayed more resistance to selected inhibitors than T315I-expressing cells

Cells stably expressing random mutations introduced to BCR-ABL1 T315I showed more resistance to all tested compounds when compared to T315I-expressing cells [Figure 22]. The cells exhibited moderate resistance to ponatinib (IC_{50} = 86 nM), the allosteric inhibitor asciminib (IC_{50} = 226 nM), and the kinase inhibitors danusertib (IC_{50} = 242 nM) and axitinib (IC_{50} = 303 nM). The cells showed resistance to saracatinib, imatinib, and bosutinib (IC_{50} > 1000 nM), and the allosteric inhibitor GNF-2 (IC_{50} > 10000 nM). No IC_{50} could be reached in these cells with nilotinib, dasatinib, and bafetinib.

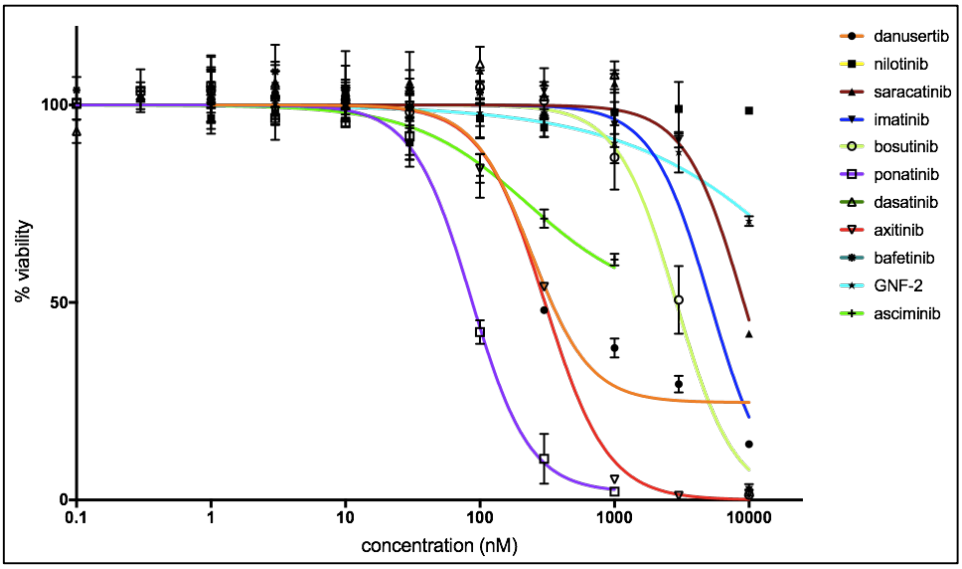

Figure 22: Cells stably expressing the random mutations introduced to BCR-ABL1 T315I showed more resistance to the tested compounds than cells expressing the T315I alone. Ponatinib, although resistant, showed most sensitivity to cells expressing the collection of random mutations introduced to BCR-ABL1 T315I. The allosteric inhibitor asciminib, and the kinase inhibitors danusertib and axitinib showed moderate resistance to cells carrying the mutagenized BCR-ABL1 T315I. The cells displayed complete resistance to bosutinib, imatinib, saracatinib, and GNF-2. Lines indicate standard deviation within duplicates.

2.2.4.3. Axitinib-resistant cells expressing random mutations introduced to BCR-ABL1 T315I conferred complete resistance to selected inhibitors

Axitinib-resistant cells expressing random mutations introduced to BCR-ABL1 T315I showed complete resistance to all tested inhibitors [Figure 23]. Once more, the broad-range TKI ponatinib showed potency to the cells at the lowest concentration of drug (IC_{50} = 646 nM). The cells were completely resistant to the TKIs danusertib, saracatinib, imatinib, and bosutinib (IC_{50} > 1000 nM for all four). Interestingly, although the cells displayed resistance to axitinib, axitinib still induced loss of cell viability (IC_{50} > 1000 nM). No IC_{50} could be reached for nilotinib, dasatinib, bafetinib, GNF-2, and asciminib.

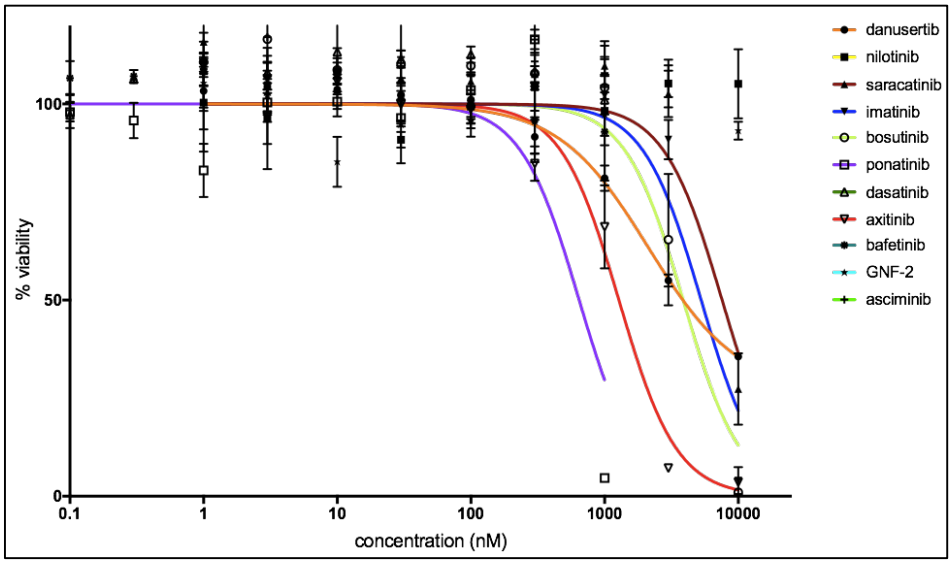

Figure 23: Axitinib-treated cells expressing mutagenized BCR-ABL1 T315I conferred resistance to all tested inhibitors. The TKI ponatinib showed most potency to axitinib-resistant, mutagenized BCR-ABL1 T315I-expressing cells. The cells showed complete resistance to danusertib, saracatinib, imatinib, bosutinib, and axitinib. Nilotinib, dasatinib, bafetinib, GNF-2, and asciminib failed to show potency to axitinib-resistant, mutagenized BCR-ABL1 T315I-expressing cells in any measured concentration. Note that, when compared to cells expressing BCR-ABL1 T315I or mutagenized BCR-ABL1 T315I, more drug is needed to reach the IC_{50} and, therefore, the dose response curves have shifted to the right. Standard deviation within two replicates is indicated by lines.

2.2.4.4. Cells expressing random mutations introduced to BCR-ABL1 T315I showed remarkable similarities to T315M-expressing cells

Cells carrying the mutagenized BCR-ABL1 T315I showed remarkable similarities to cells expressing T315M [Table 3]. Two exceptions were observed. First, the aurora kinase inhibitor danusertib compromised cell viability in cells expressing the mutagenized BCR-ABL1 T315I at 242 nM when compared to >1000 nM for cells expressing T315M. Second, the allosteric inhibitor asciminib showed more potency toward cells expressing random mutations introduced to BCR-ABL1 T315I (IC_{50} = 226 nM) than toward T315M-expressing cells (IC_{50} > 1000 nM). However, both cell lines showed sensitivity in similar concentrations to all the other selected inhibitors. Table 3 illustrates the increasing resistance of cells expressing random mutations introduced to BCR-ABL1 T315I, and cells expressing those random mutations are treated with axitinib.

Table 3: Sensitivity of cells stably expressing BCR-ABL1 T315I (T315I), cells stably expressing mutagenized BCR-ABL1 T315I (mut. T315I), and axitinib-treated cells stably expressing mutagenized BCR-ABL1 T315I (axi-res. mut. T315I), to selected inhibitors. T315M shows results of the effect of selected inhibitors on cells expressing T315M described in Section 2.1.4.1., and asciminib in Section 2.1.4.2. Cells expressing random mutations introduced to BCR-ABL1 T315I (mut. T315I) appear highly similar to cells expressing T315M.

IC50s (nM)					
	Compound	T315I	mut. T315I	axi-res. mut. T315I	T315M
1.	danusertib	179	242	>1000	>1000
2.	nilotinib	>1000	N/A	N/A	N/A
3.	saracatinib	>1000	>1000	>1000	>1000
4.	imatinib	>1000	>1000	>1000	>1000
5.	bosutinib	514	>1000	>1000	>1000
6.	ponatinib	1	86	646	76
7.	dasatinib	5	N/A	N/A	N/A
8.	axitinib	48	303	>1000	390
9.	bafetinib	>1000	N/A	N/A	N/A
10.	GNF-2	81	>10 000	N/A	>10 000
11.	asciminib	1	226	N/A	>1000

IC_{50} – half-maximal inhibitory concentration; nM – nanomoles; N/A – IC_{50} could not be reached with the measured concentrations

Amplicon sequencing confirmed the nucleotide substitution C -> T (99,9% of sequences) that defines the change from threonine to isoleucine in the ABL1 kinase domain of T315I-expressing cells. However, cells previously thought to be stably expressing T315I plasmid collection with random mutations were revealed to be expressing primarily two point mutations that are present in T315M: C -> T substitution (threonine to isoleucine), and the following T -> G substitution (isoleucine to methionine) in amino acid position 315, both in 99,8% of sequences. Two less frequent mutations occurred in these sequences in two distinct locations. First, C -> G substitution was found at amino acid position 420 (TCC -> TCG), but both DNA codons code for serine. Second, another C -> G substitution occurred at amino acid position 422 (GTC -> GTG), and, as with the previous substitution, both codons encode valine. The C -> G substitutions in amino acid positions 420 and 422 occurred in 2,2% and 2,1% of sequences, respectively. Therefore, although the nucleotides changed, the encoded amino acids remained the same.

Sequencing of the ABL1 kinase domain from axitinib-resistant cells expressing the random plasmid collection introduced to T315I also revealed nucleotide substitutions characteristic of T315M: C -> T substitution in 99,8% of sequences and T -> G substitution in 99,6% of sequences. However, the two mutations that were observed less frequently in cells expressing the random plasmid collection introduced to T315I were not seen in the ABL1 kinase domain of axitinib-treated cells.

2.2.5. Discussion

2.2.5.1. Summary of Key Results

Cells stably expressing T315I showed sensitivity to the VEGFR inhibitor axitinib (IC_{50} = 48 nM) and the allosteric inhibitor asciminib (IC_{50} = 1 nM). Cells expressing random mutations introduced to BCR-ABL1 T315I displayed more resistance to all tested compounds when compared to T315I-expressing cells. Also, the sensitivity of cells carrying mutagenized BCR-ABL1 T315I showed remarkable similarity to cells expressing T315M. Indeed, amplicon sequencing of the ABL1 kinase domain of cells expressing the random mutations introduced to BCR-ABL1 T315I revealed that 99,8% of sequences contained the missense mutations C -> T and T -> G (in amino acid position 315) characteristic of T315M. Axitinib-treated cells expressing the random mutations introduced to BCR-ABL1 T315I conferred resistance to all tested compounds. In addition, sequencing of the ABL1 kinase domain of these cells revealed the same two point mutations present in cells expressing T315M. Yet, the two mutations that were observed less frequently in cells expressing the mutagenized T315I(M) were not found in the ABL1 kinase domain of axitinib-treated cells.

2.2.5.2. Mutations characteristic of T315M appear to have occurred as a result of random mutagenesis of the T315I plasmid

Given the success of Kesarwani *et al.* (2014) to anticipate clinically relevant drug-resistant mutations by sequencing drug-resistant Ba/F3 cells expressing random mutations, creating an axitinib-resistant population of cells expressing mutagenized T315I was expected to reveal point mutations that arise as a result of axitinib-resistance.

However, amplicon sequencing of the ABL1 kinase domain revealed that instead of a library of random mutations, nucleotide substitutions were limited to four. Two of these are characteristic of T315M (ACT -> ATT -> ATG) present in 99,8% of sequences, and the other two occur infrequently and have no effect on the amino acid that is being produced. The reasons for the emergence of mutations characteristic of T315M could be as follows: (1) either the one nucleotide substitution that is needed between isoleucine and methionine (ATT -> ATG) is so likely to happen in random mutagenesis that this occurred in most bacterial cells in this study; (2) the bacterial cells that received the necessary nucleotide substitution for T315M outnumbered colonies containing other mutations; (3) an error occurred in the dispensing of the pMIG-BCR-ABL1 T315I and T315M plasmid was chosen instead. Although it is possible, the last option does not seem likely since the same pMIG-BCR-ABL1 T315I plasmid was used to create Ba/F3 cells stably expressing pMIG-BCR-ABL1 T315I, which sequencing showed to have only the C -> T (ACT -> ATT) nucleotide substitution characteristic of T315I at 99,9% frequency.

The lack of random mutations as a result of random mutagenesis of the pMIG-BCR-ABL1 T315I plasmid allows limited conclusions to be drawn. To better understand what caused the T315M mutation to arise as a result of mutagenizing pMIG-BCR-ABL1 T315I, this experiment should be repeated. If the bacterial cells containing the T315M mutation outnumber the other random mutations over time, limiting the incubation time to 18 or 24 hours might help to understand the ratio of mutations prior to T315M outnumbering the others. Most importantly, repeating the full experiment might give insight into which mutations are (1) likely to arise as a result of axitinib-

treatment, and (2) sensitive to available inhibitors. If, however, the single nucleotide substitution allowing T315I to become T315M is so likely to happen in random mutagenesis of the pMIG-BCR-ABL1 T315I plasmid that T315M would always be the most frequent mutation to arise, another method for creating mutations in the kinase domain of BCR-ABL1 T315I should be considered.

2.2.5.3. The TKI axitinib and the allosteric inhibitor asciminib show potency to T315I-expressing cells

As expected, cells stably expressing T315I showed selective sensitivity toward the VEGFR inhibitor axitinib (IC_{50} = 48 nM). Notably, however, the cells also showed sensitivity to the allosteric inhibitor asciminib (IC_{50} = 1 nM), which targets the myristoyl pocket of ABL1 instead of the ATP-binding pocket, as has been shown previously by Wylie *et al.* (2017). As seen in Aim 1, asciminib alone failed to induce cell viability loss in either T315M-expressing or E255V/T315I-expressing cells. However, asciminib showed powerful synergy in combination with ponatinib in cells expressing T315M, further highlighting the importance of combination treatment in targeting the ABL1 kinase domain.

2.2.5.4. Conclusions

Although Kesarwani *et al.* (2014) successfully predicted clinically relevant drug-resistant mutations by sequencing the kinase domains of drug-resistant Ba/F3 cells expressing random mutations, this approach failed to provide potential mutations emerging as a result of axitinib-resistance in this study. However, this experiment supported several findings. First, the consistency with which the TKI axitinib continued to display selective potency toward T315I-expressing cells encourages consideration of this inhibitor as an alternative to ponatinib. And second, the ATP-noncompetitive inhibitor asciminib, which did not achieve IC_{50}s in cells expressing T315M or E255V/T315I, showed remarkable potency toward T315I-expressing cells. In conclusion, the potency of axitinib and asciminib to T315I-expressing cells can be considered the silver lining of this experiment, especially considering the adverse side effects of ponatinib and the highly resistant mutations found to occur as a result of ponatinib-treatment.

3. Conclusions

Successful first-line therapy for CML with tyrosine kinase inhibitors has resulted in improved prognosis for the malignancy for nearly two decades (Bower *et al.*, 2016). Although the disease is relatively rare and the incidence of CML has not been shown to increase, the prevalence of CML is expected to rise by more than six-fold by the year 2050 (Huang *et al.*, 2012). Patients who had few options prior to tyrosine kinase inhibitor therapy are now able to live nearly the lifespan of a healthy individual (Bower *et al.*, 2016). However, drug-resistance due to point mutations has been estimated to occur in up to 50% of imatinib-refractory CML patients (Jabbour *et al*., 2011), which poses a severe problem considering the increasing prevalence of the disease. Nearly a hundred mutations have been observed in the tyrosine kinase domain of ABL1 (Jabbour *et al.*, 2011), and among them are the T315I, T315M, and E255V/T315I.

As a novel discovery, cells stably expressing BCR-ABL1 T315M showed selective sensitivity toward inhibitors of topoisomerase II and mTOR. Eleven inhibitors of the mTOR pathway and nine inhibitors of topoisomerase II showed higher selective Drug Sensitivity Scores (sDSS) for cells stably expressing BCR-ABL1 T315M than for cells expressing BCR-ABL1 T315I or E255V/T315I. The sDSS values were comparable to cells expressing BCR-ABL1, which implies a possible difference in pathway signaling when isoleucine has been substituted with methionine. The finding opens the possibility of combining mTOR inhibitors with TKIs to induce loss of cell viability in cells expressing BCR-ABL1 T315M. Combination treatment of the allosteric inhibitor asciminib and broad-range TKI ponatinib in cells expressing BCR-ABL1 T315M resulted in unprecedented synthetic lethality. The ZIP synergy score could be suggestive of using this cell line and combination of drugs as the positive control for synergy. Moreover, combining other TKIs with ATP-noncompetitive inhibitors may lead to finding a combination that targets cells expressing BCR-ABL1 T315M in clinically relevant concentrations.

Cells stably expressing BCR-ABL1 E255V/T315I exhibited incredibly high resistance to all TKIs. Although general chemotherapy drugs compromised cell viability in cells carrying the double mutation, few drugs showed selective potency toward these cells. Among these were three IAP SMAC mimetics: AT-406, LCL161, and birinapant showed selectivity over cells expressing T315I. However, it is important to note that the experiment was performed once in T315I-expressing cells and would have to be repeated to draw stronger conclusions from the finding. Furthermore, treating E255V/T315I-expressing cells with both asciminib and ponatinib resulted in synergy, but in incredibly high concentrations. Because compound mutations that contain T315I have been linked to ponatinib-failure (Zabriskie *et al.*, 2014), combining the non-ATP inhibitors with inhibitors that target the ATP-binding site could lead to synergy capable of inducing cell viability loss in these cells in concentrations that are clinically useful.

The gatekeeper mutation T315I, considered to be the most frequent point mutation in CML (COSMIC database), occurs in up to 20% of TKI-resistant patients (Soverini *et al.*, 2009). Given that the VEGFR inhibitor axitinib has shown clinical activity in a CML patient carrying T315I (Pemovska *et al.*, 2015), a model system was set up that would allow to predict mutations that enable axitinib-resistance in patients. Although the method allowed Kesarwani *et al.* (2014) to accurately predict clinically relevant mutations, this approach failed to provide mutations emerging

as a result of axitinib-resistance in this study. For further investigation, another method for creating random mutations in the BCR-ABL1 T315I kinase domain should be considered.

Importantly, the allosteric inhibitor asciminib showed high potency toward cells stably expressing BCR-ABL1 T315I (IC_{50} = 1 nM), as has been shown previously by Wylie *et al.* (2017). This ATP-noncompetitive inhibitor presents an exciting opportunity for combination testing to target both the myristoyl site with asciminib and the ATP-binding pocket with an ATP competitive inhibitor. This prospect is particularly exciting because mutations that emerge as a result of asciminib-treatment and as a result of TKI-treatment do not overlap (Wylie *et al.*, 2017). Moreover, combining asciminib and ponatinib in cells expressing BCR-ABL1 resulted in synergy in low concentrations of either drug, implying clinical relevance.

The three mutations occur seldom in a rare disease, but it is important to remember that even rare mutations will become more frequent as the prevalence of CML increases. Finding solutions may be possible by drug-repurposing, finding synergy in combination therapy, or solving the structures of the kinase domains containing the drug-resistant mutations to design new drugs. Solutions are likely to be found one step at a time.

This study focused on ways to overcome drug-resistance that is caused by point mutations in CML. However, drug target alteration, although important, is only a fraction of the elaborate machinery of drug-resistance. Drug-resistance in cancer is a difficult problem, and tackling it requires understanding of the broad mechanisms underlying drug-resistance and using that knowledge to find solutions. Although efficient treatments exist for several cancers, including CML, many lives are lost due to resistance. Thus, it is crucial to persist in efforts to find treatments for patients who are resistant to available therapies.

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