# TARGETING TYROSINE KINASE INHIBITOR-INSENSITIVE CML STEM/PROGENITOR CELLS BY EFFECTIVE INHIBITION OF A NOVEL PP2A-AHI-1-BCR-ABL-JAK2 COMPLEX

**by**

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**B.Sc., University of Ottawa, 2012**

**A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF**

**MASTER OF SCIENCE**

**in**

**The Faculty of Graduate and Postdoctoral Studies**

**(Experimental Medicine)**

**THE UNIVERSITY OF BRITISH COLUMBIA**

**(VANCOUVER)**

**August 2015**

## Abstract

Imatinib Mesylate (IM) and other tyrosine kinase inhibitors (TKIs) have had a major impact on treatment of early phase Chronic Myeloid Leukemia (CML) patients. However, TKI monotherapies are not curative and initial and acquired resistance remain challenges. Particularly, CML stem cells are less responsive to TKIs and are a critical target population for TKI resistance. Thus, improved treatments targeting key elements active in CML stem cells are needed. One candidate is Abelson helper integration site-1 (AHI-1), an oncogene that is highly upregulated in CML stem cells and interacts with multiple kinases, including BCR-ABL and JAK2. AHI-1-mediated complexes regulate TKI response/resistance of CML stem/progenitor cells, indicating that AHI-1 is a new therapeutic target in CML.

By screening the Prestwick Chemical Library, a specific growth inhibitory compound that potentially targets AHI-1 was identified: Cantharidin (CAN), an inhibitor of protein phosphatase 2A (PP2A). CAN is toxic however, so two new PP2A inhibitors, LB100 and LB102, were identified for this study. These new inhibitors specifically inhibit PP2A activity and suppress growth of CML cell lines. Importantly, these new PP2A inhibitors selectively target CML stem/progenitor cells while sparing healthy stem/progenitor cells. When combined with TKIs there is significant further suppression of growth in cell lines and in $CD34^+$ treatment-naïve IM-nonresponder cells. Furthermore, this combination effect was determined to be synergistic. Cell cycle analysis showed that treatment with PP2A inhibitors alone induced a shift from G1 to G2/M phase. Confocal microscopy confirmed that the G2/M arrest led to mitotic catastrophe. However a similar shift in cell population was observed after combination with IM, suggesting that the G2/M phase arrest is solely due to PP2A inhibition. Mechanistically, the PP2A-PR55α subunit was identified as a new AHI-1 interacting protein. Western blot analysis showed that, compared to single agents, the combination treatment greatly suppresses protein expression of AHI-1, BCR-ABL, JAK2, STAT5, AKT, β-catenin, P-38 and JNK. The combination treatment

also affected PP2A and BCR-ABL-mediated β-catenin dephosphorylation/phosphorylation. These results indicate that simultaneously targeting both BCR-ABL and PP2A activities in CML stem/progenitor cells may provide a novel treatment option for CML patients, through destabilization of the protein-protein interactions mediated by AHI-1.

## Preface

All experiments were conducted by Damian Lai, except for the parts stated below. I designed the experiments presented in this thesis, analyzed and interpreted all the data, composed and edited the thesis, under the supervision of Dr. Xiaoyan Jiang at the Terry Fox Laboratory, BC Cancer Research Centre.

Preliminary studies in chapter one including the AHI-1 drug screen and the initial testing of cantharidin were performed by my colleagues Min Chen and Will Liu respectively. All work involving the new PP2A inhibitors LB100 and LB102 was performed by me.

The studies described in this thesis that were performed with primary samples from healthy donors or patients with CML were approved by the University of British Columbia-Clinical Research Ethics Board, certificate number H12-02372.

Dr. Xiaoyan Jiang contributed to the experimental design, data interpretation, and editing of the thesis.

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## List of Abbreviations

ABCB1: ATP Binding Cassette B1

ABCG2: ATP Binding Cassette G2

AHI-1: Abelson Helper Integration Site 1

AKT: Protein Kinase B

APC: Allo-phyocyanin

ATP: Adenosine Tri Phosphate

BAD: BCL2 Associated Agonist of Cell Death

BC: Blast Crisis

BCL2: B-cell Lymphoma 2

BCR-ABL: Breakpoint Cluster Region - Abelson Murine Leukemia Viral Oncogene Homolog 1

BIM: BCL2 Interacting Mediator of Cell Death

BSA: Bovine Serum Albumin

CAN: Cantharidin

CFC: Colony Forming Cell Assay

CI: Combination Index

CKIα: Casein Kinase 1 Alpha

CLP: Common Lymphoid Progenitors

CML: Chronic Myeloid Leukemia

CMP: Common Myeloid Progenitors

CO2: Carbon Dioxide

DA: Dasatinib

DMEM: Dulbecco's Modified Eagle's Media

DMSO: Dimethyl Sulfoxide

DOX: Doxycyclin

EB: Erythroblast

ED: Effective Dosage

ENSA: α-endosulfine

ERK: Extracellular Signal Regulated Kinase

FBS: Fetal Bovine Serum

FITC: Fluorescein-isothiocyanate

GAB2: GRB2 Associated Binding Protein 2

GAPDH: Glyceraldehyde-3-Phosphate-Dehydrogenase

G-CSF: Granulocyte Colony-Stimulating Factor

GM-CSF: Granulocyte Macrophage Colony-Stimulating Factor

GMP: Granulocyte-Macrophage Progenitors

GRB2: Growth Factor Receptor Bound Protein 2

GSK3β: Glycogen Synthase Kinase 3 Beta

IC50: 50% Inhibitory Concentration

IgG: Immunoglobulin G

IL: Interleukin

IM: Imatinib

IP: Immunoprecipitation

JAK2: Janus Kinase 2

JNK: C-Jun N-Terminal Kinases

kD: Kilodalton

LEF/TCF: Lymphoid Enhancer Factor / T-Cell Factor

LiCl: Lithium Chloride

LSC: Leukemic Stem Cell

MAPK: Mitogen Activated Protein Kinase

MB: Monoblast / Myeloblast,

MEK: Mitogen Activated Protein Kinase Kinase 1

MEPs: Megakaryocyte-Erythrocyte Progenitors

MKP: Megakaryocyte Progenitor

mm: Milli Metre

mM: Milli Molar

MPPs: Multipotent Progenitor Cells

NBM: Normal Bone Marrow

NK: Natural Killer

NL: Nilotinib

nM: Nano Molar

NR: Non-Responders,

OCT1: Organic Cation Transporter-1

PADs: Phosphastase Activating Drugs

PBS: Phosphate Buffered Saline

PEI: Polyethylenimine

PEST Sequences: Proline, Glutamic Acid, Serine, Threonine Rich Sequences

Ph+: Philadelphia Chromosome Positive

Phospho: Phosphorylated

PI: Propidium Iodide

PI3K: Phosphoinositide 3 Kinase

PIC: Protease Inhibitor Cocktail

PMSF: Phenylmethylsulfonyl Fluoride

PP1: Protein Phosphatase 1

PP2A: Protein Phosphatase 2 A

PP4: Protein Phosphatase 4

PSB: Phosphorylation Solubilisation Buffer

PXXP: Proline Rich Motifs

qPCR: Quantitative Polymerase Chain Reaction

R: Responders

RAF: Rapidly Accelerated Fibrosarcoma

RAS: Rat Sarcoma

RPMI: Rosewell Park Memorial Institute

RT: Room Temperature

SEM: Standard Error of the Mean

Ser: Serine

SET: Protein Phosphatase 2 A Inhibitor (I2PP2A)

SH2: Src Homology 2

SH3: Src Homology 3

SOS: Son of Sevenless

STAT5: Signal Transducer and Activator of Transcription 5

ST-HSCs: Short Term Hematopoietic Stem Cells

TBST: Tris-Buffered Saline with Tween 20

Thr: Threonine

TKI: Tyrosine Kinase Inhibitor

Tyr: Tyrosine

WNT: Wingless Type MMTV Integration Site Family

YFP: Yellow Fluorescent Protein

β-catenin: Beta Cadherin Associated Protein

μCi: Microcurie

μL: Microlitre

μm: Micrometre

μM: Micromolar

## Acknowledgements

First and foremost I would like to thank my supervisor Dr. Xiaoyan Jiang for giving me the opportunity to join her laboratory. I have grown immensely under her supervision and learned to ask the right questions and how to pursue their answers. I really appreciated that you gave me the freedom to explore my interests but made sure I kept an eye on the big story.

I would also like to express my gratitude to my committee member Dr. Catherine Pallen for all her support and helpful criticisms of my work. Your suggestions have been invaluable and I am grateful for your expertise.

I thank the members of the Jiang lab past and present for all their support. I would particularly like to extend special thanks to Min and Will who originally took me on as an innocent undergraduate student and really mentored me and provided a foundation for me to build upon. They were both essential to any successes I have and hopefully will achieve. Katharina, your advice and expertise really made a difference in helping me to develop my eye for the small details which can make or break an experiment. Josephine, Sharmin, Rachel, Kyi Min, Clark, Kevin, Helena, Akie, Jonathan, and Elianne, you have really contributed an absolutely positive and energetic atmosphere to the lab. The everyday banter and support is something I looked forward to everyday. Leon, your friendship has really helped to get me through the hurdles of graduate student life. I'll see you at happy hour!

I would also like to acknowledge the Canadian Institute of Health Research for their financial support during my MSc.

My greatest supporters are my parents and I thank them for their unconditional love and support. You have allowed me to put my passions first and chase my interests. You taught me how to dream big and aim high. My journey is only just beginning and your belief in me fuels my motivation to never give up and succeed. Finally I would like to thank Orchid for her support and patience. Never have I ever made someone wait so many times because I was 'finishing up' an experiment. I will always appreciate the moment you laughed when I was 3 hours late for our anniversary dinner because I was engaged in battle with an uncooperative flow cytometer!

Thank you all.

*I dedicate this thesis to my loving grandparents.*

*I will make you proud.*

# Chapter 1: Background

## 1.1 Introduction to Chronic Myeloid Leukemia (CML).

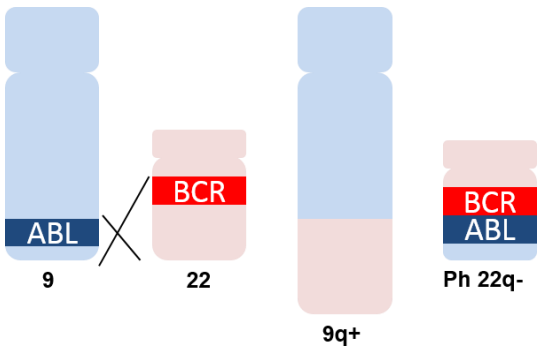
CML is a clonal, hematopoietic stem cell disorder that is characterized by a reciprocal translocation between the long arms of chromosomes 9 and 22 resulting in the Philadelphia chromosome ($Ph^+$). Chromosome translocation (9; 22) results in the juxtaposition of the human analogue of the ABL gene from chromosome 9 with the BCR gene on chromosome 22 to produce the fusion BCR-ABL gene (Figure 1A). This fusion gene is ultimately transcribed and translated into the $p210^{BCR/ABL}$ oncoprotein in almost all patients with CML (4).

CML occurs with an incidence rate of about 1 individual per 100,000 per year. The disease is triphasic, with most patients presenting in the initial chronic phase. This chronic phase can last several years and is characterized by an accumulation of myeloid precursors and mature cells in bone marrow, peripheral blood, and extramedullary sites (5). At this stage symptoms can be controlled but without treatment patients will progress into an accelerated phase lasting 4 to 6 months. As the patients progress through the accelerated phase there is an increase in the frequency of progenitor/precursor cells which are unable to differentiate resulting in a deficiency of healthy mature differentiated cells (6). The patient then reaches a terminal stage called blast crisis, characterized by the rapid expansion of a population of differentiation-arrested blast cells which can be myeloid or lymphoid in nature (7). Patients are classified into the blast phase if they have at least 20% blast cells in the peripheral blood or bone marrow (8).

The characteristic translocation mutation is thought to initially occur in the more primitive hematopoietic stem cell compartment. The resultant production of the oncoprotein BCR-ABL is the initial malignant transformation event that triggers the onset of CML (Figure 1B). Expression of BCR-ABL has been shown to be both necessary and sufficient for this malignant

transformation (9-11). This discovery made BCR-ABL an attractive target for therapeutic intervention. Importantly, the constitutively high levels of tyrosine kinase activity derived from the kinase domain of ABL are crucial for BCR-ABL to drive the pathogenesis of the disease (12, 13). This led to the development of small molecule drugs specifically designed to inhibit BCR-ABL tyrosine kinase activity. Imatinib Mesylate (IM) is a BCR-ABL tyrosine kinase inhibitor (TKI) approved in 2001 by the US Food and Drug Administration (FDA) as a first line treatment for Philadelphia chromosome positive CML. The drug was touted as a major breakthrough in CML therapy and it has led to the development of newer, more potent TKIs. BCR-ABL can phosphorylate its substrates by transferring a phosphate from ATP to tyrosine residues. IM binds to the ATP-binding site of BCR-ABL thus preventing BCR-ABL-mediated tyrosine kinase activity (14). IM binds to the ABL kinase domain in its inactive conformation, effectively locking ABL in the inactive conformation (15, 16). Nilotinib (NL) is a second generation TKI that is structurally derived from IM but with over 30 times the potency (17). It binds to the ABL inactive conformation in a similar manner to IM but with higher binding affinity (18). Dasatinib (DA) is another second generation TKI that is structurally distinct from IM. It inhibits both BCR-ABL and the SRC family kinases with up to 250-fold higher potency (19, 20). Unlike IM, DA binds to the ABL kinase domain in its active conformation (19). All three compounds are susceptible to the T315I mutation, leading to the development of ponatinib, which was specifically designed to accommodate the T315I mutation (21, 22).

**A**



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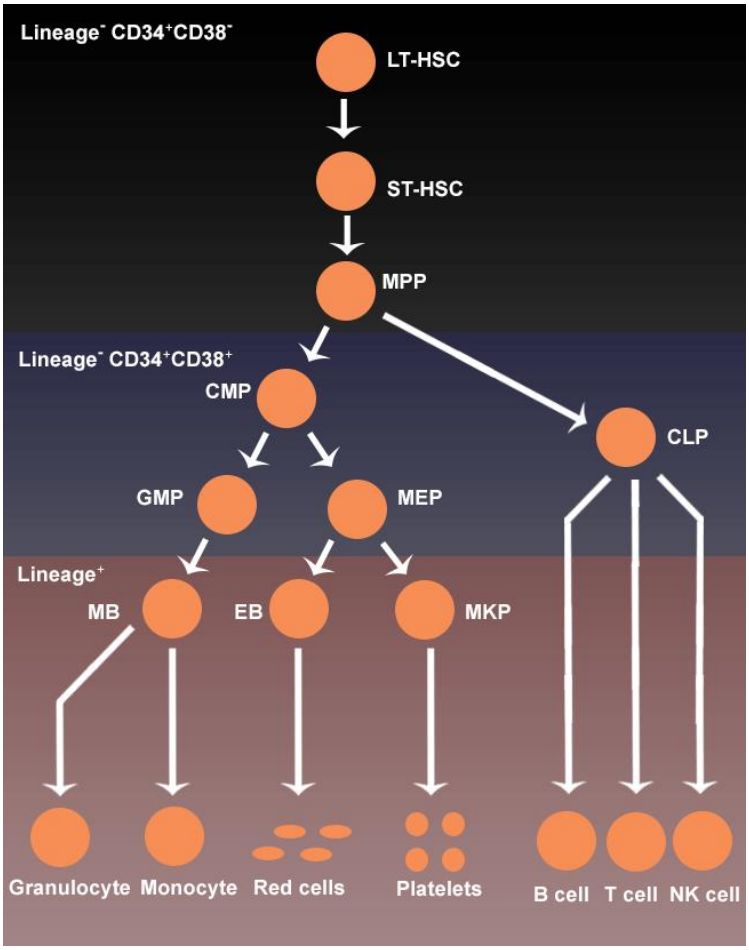



**Figure 1: Acquisition of the Philadelphia Chromosome and the CML Hierarchy. (A)** Reciprocal translocation between the long arms of chromosome 9 and 22 results in the Philadelphia chromosome (Ph 22q-), encoding the fusion gene BCR-ABL. **(B)** Primitive multipotent cells maintain hematopoiesis with the capacity for both self-renewal and differentiation along all hematopoietic lineages. The initial transformation event that results in the production of the oncoprotein BCR-ABL is thought to occur in the most primitive long-term hematopoietic stem cells (LT-HSCs). These leukemic stem cells are able to take over the hierarchy of hematopoiesis and drive leukemogenesis. (ST-HSCs: short term hematopoietic stem cells, MPPs: multipotent progenitor cells, CMP: common myeloid progenitors, CLP: common lymphoid progenitors, GMP: granulocyte-macrophage progenitors, MEPs: megakaryocyte-erythrocyte progenitors, MB: monoblast/myeloblast, EB: erythroblast, MKP: megakaryocyte progenitor, NK: natural killer. Adapted from O'Hare et al, 2012 (3)**.**

**1.2 Clinical Challenges in the Treatment of CML.**

Until the development of the BCR-ABL TKI Imatinib Mesylate, the only way to prevent disease transition from chronic phase to blast crisis was through allogeneic bone marrow transplants (23). However, this is not a feasible treatment option due to the older age of patients and a lack of suitable stem cell donors. IM soon became the treatment of choice for chronic phase CML patients. In newly diagnosed patients with chronic phase CML, IM induces complete hematologic response in more than 90% of patients. However while patients with more advanced phases of CML may also respond to IM, this occurs much less frequently (14, 24-30). Even in patients with complete cytogenetic response, BCR-ABL transcripts continue to persist. CML patients in remission continue to show minimal residual disease, detected by quantitative polymerase chain reaction (qPCR), in their peripheral blood or bone marrow. This residual disease indicates that the treatment with IM alone may only be able to control disease development but not cure CML patients (4, 31-35). Indeed the major problem with IM treatment is relapse of the disease due to resistance.

The most frequent mechanism of resistance is the appearance of point mutations in the kinase domain of the BCR-ABL protein that impairs drug binding (Figure 2A, left) (36-39). While the second generation TKIs like dasatinib (DA) and nilotinib (NL) have shown much promise by targeting alternative regions of BCR-ABL, many mutations like the T315I mutation have been very difficult to overcome. More recently, the third generation TKI ponatinib has shown promise in treating the T315I mutation (5, 38, 40). The persistence of BCR-ABL positive cells in patients on TKI therapy indicates that alternative pathways may need to be targeted to fully eradicate leukemic cells (28, 41-44). The failure of targeted therapy to cure CML patients despite inducing clinical remission suggests that there could be a small population of leukemic stem cells (LSC) capable of sustaining the disease (3). Further investigation confirmed the presence of a small population of primitive LSCs that are quiescent in nature and insensitive to TKI

treatment. This population of cells provides a reservoir of leukemic cells that are capable of expanding after cessation of TKI treatment (Figure 2B) (44-50).

BCR-ABL duplication or over-amplification represents a BCR-ABL dependent mechanism of resistance to TKIs (Figure 2A). Increased BCR-ABL activity can lead to errors in the splicing of GSK3β (51). Lack of GSK3β kinase activity leads to reduced degradation of β-catenin and increased self-renewal of the leukemic stem cells that TKIs are unable to eradicate. (52). It has also been reported that CML stem cells may not be fully dependent on BCR-ABL for their survival (43, 53, 54). Put together, these findings could explain the insensitivity to TKI treatments, as well as emphasize the need for alternative molecular targets other than BCR-ABL that are essential for CML stem cell survival. Other BCR-ABL independent mechanisms of resistance include deregulation of transporters like OCT1, ABCB1 and ABCG2 that are responsible for drug influx and efflux, effectively hampering the achievement of effective drug levels in target cells (Figure 2A, right). Impaired signalling pathways, deficient DNA repair, as well as micro-environment induced resistance all represent other potential mechanisms for TKI resistance (39, 45, 52, 55-59). Thus, clinical evidence indicates that TKI monotherapies may not cure most patients as molecular remissions are rare (32, 41-43, 60). These observations emphasize the need to develop new agents and new combination strategies to prevent continuous development of resistant subclones in leukemic stem cells.

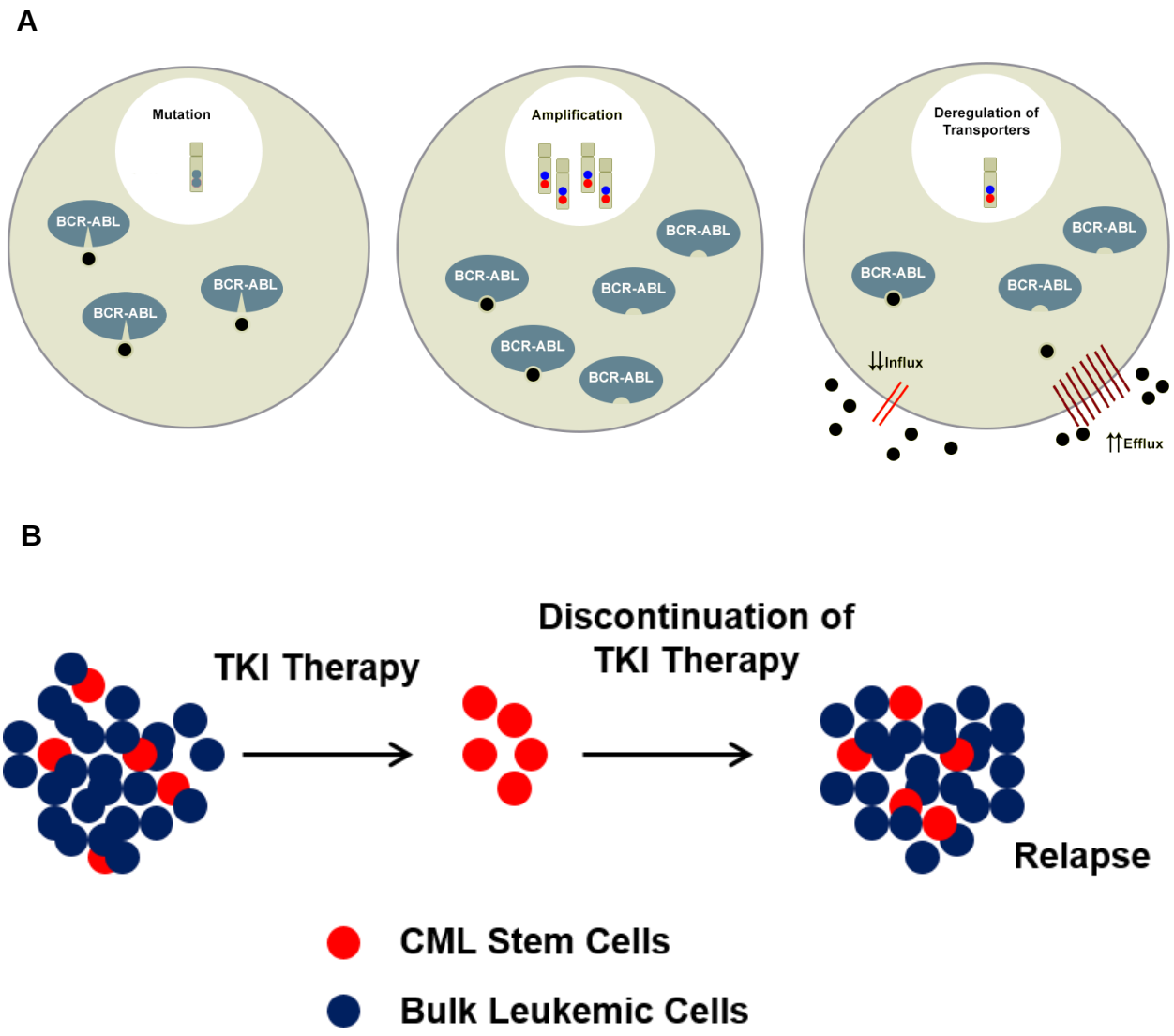



**Figure 2: Mechanisms of Resistance to TKI therapy. (A)** Schematic of BCR-ABL-dependent and independent mechanisms of resistance. Left: Mutations in BCR-ABL kinase domain lead to ineffective binding of TKIs. Middle: Amplification leads to overexpression of BCR-ABL kinase. Right: Downregulation of influx transporters inhibits effective TKI shuttling into the cell, and overexpression of efflux transporters leads to low levels of TKIs in the cell. **(B)** Schematic of CML stem cells response to TKI therapy. Without CML stem cell targeted therapy, TKI-resistant CML stem cells are able to regenerate the bulk leukemic population leading to relapse in patients.

**1.3 BCR-ABL Signalling.**

The best characterized example of a malignancy initiated and driven by the unrestrained activity of a protein kinase is CML. BCR-ABL exerts its oncogenic activity through a network of pathways that promote proliferation and survival of leukemic cells (Figure 3). BCR-ABL is known to drive the JAK-STAT pathway, the PI3K-AKT Pathway, as well as the RAS-RAF-MEK1/2-ERK pathway (4). The Wnt/β-catenin signalling pathway is also known to have an important role and BCR-ABL has been shown to physically interact with β-catenin and regulate its stability in CML cells (44, 61, 62).

Activation of the transcription factor STAT5 is a well-known signalling hallmark of CML. It is critical for leukemia initiation and maintenance (63), as well as contributing to CML progression and resistance to TKIs (64). Its activation occurs through the phosphorylation of its Tyr694 residue leading to SH2 domain-dependent dimerization and consequent transcriptional activation (65). Upstream of STAT5 are the JAK kinases, with JAK2 in particular understood to be the predominant activator of STAT5 (66). However it has recently been suggested that in CML, STAT5 may be under direct control of BCR-ABL and the canonical JAK2-STAT5 pathway may be rewired (64, 67). Activation of JAK2 was verified in several cell lines expressing BCR-ABL as well as primary leukemic cells derived from CML patients. The link between BCR-ABL and JAK2 activity was further confirmed by the observation that IM treatment of CML cell lines was able to reduce tyrosine phosphorylation of JAK2 (68). There is a reciprocal phosphorylation between BCR-ABL and JAK2. BCR-ABL phosphorylation of JAK2 is required for its activation, and JAK2 seems able to phosphorylate BCR-ABL on its Tyr177 residue (69). The phosphorylation of the Tyr177 residue of BCR-ABL by JAK2 is critical for BCR-ABL's ability to activate the PI3K/AKT and RAS pathways (70-72).

The GRB2-GAB2 complex acts as an amplifier in the BCR-ABL signalling pathway. Tyr177 lies within a GRB2 binding motif in BCR-ABL and the phosphorylation of this residue

facilitates recruitment of GRB2 (70). This GRB2-GAB2 complex is required for activation of the PI3K/AKT and RAS/ERK pathways. Through its association with GRB2, BCR-ABL recruits the scaffold adapter GAB2 and regulates its tyrosine phosphorylation. Phospho-GAB2 presents binding sites for the SH2 domains of the p85 subunit of PI3K and SHP2, which is one of the steps required for activation of the RAS-ERK pathway (70). GRB2 also binds SOS, a guanine-nucleotide exchanger of RAS, indicating RAS can also be directly activated by BCR-ABL through the GRB2-SOS complex (4, 73).

The RAS/RAF/MEK/ERK pathway is a complex signalling cascade that can regulate apoptotic regulatory molecules as well as modify the activity of transcription factors to regulate gene expression. In CML, activation of this pathway by BCR-ABL can prevent apoptosis and promote proliferation by altering the phosphorylation status of apoptotic proteins (74).

The PI3K/AKT pathway is another major survival signalling pathway that plays roles in normal hematopoiesis and is mutated in many solid tumors and hematological malignancies. (75). It is established that upregulation and activation of this pathway confers a growth advantage to CML cells. BCR-ABL has been shown to deregulate this pathway and IM treatment can promote further PI3K/AKT activity. This contributes to the development of resistance through the role of AKT in activation of the antiapoptotic mTOR kinase (76) as well as inactivation of GSK3β (72) which, among its roles in cell cycle progression and self-renewal, is an essential component of the β-catenin degradation complex (77, 78).

The Wnt/β-catenin signalling pathway is known to play essential roles in a variety of different processes in hematopoiesis, including regulation of both proliferation and self-renewal of hematopoietic stem cells. Interestingly, it has been reported that BCR-ABL can physically interact with β-catenin and control its protein stabilization in CML cells (61). β-catenin has also been shown to play a crucial role in the survival of CML leukemic stem cells (LSCs), which are resistant to IM (62), (79). In a mouse model, β-catenin deletion was shown to synergize with IM

to delay recurrence of the disease after IM discontinuation. Serial transplantation assays showed that β-catenin deficiency reduces the transplantability of CML into untreated tertiary recipients, further emphasizing the potential of β-catenin as a LSC target (80). While TKI monotherapy targets leukemia bulk cells, it is not able to deplete LSCs. In contrast inhibition of Wnt signalling inhibits LSCs but not the bulk differentiated leukemia cells (72). Drug treatments which combine to target BCR-ABL as well as β-catenin activity could potentially eradicate both the LSCs and the bulk population. Indeed a β-catenin inhibitor, AV65, was shown to synergize with IM to inhibit proliferation of CML cell lines (81).

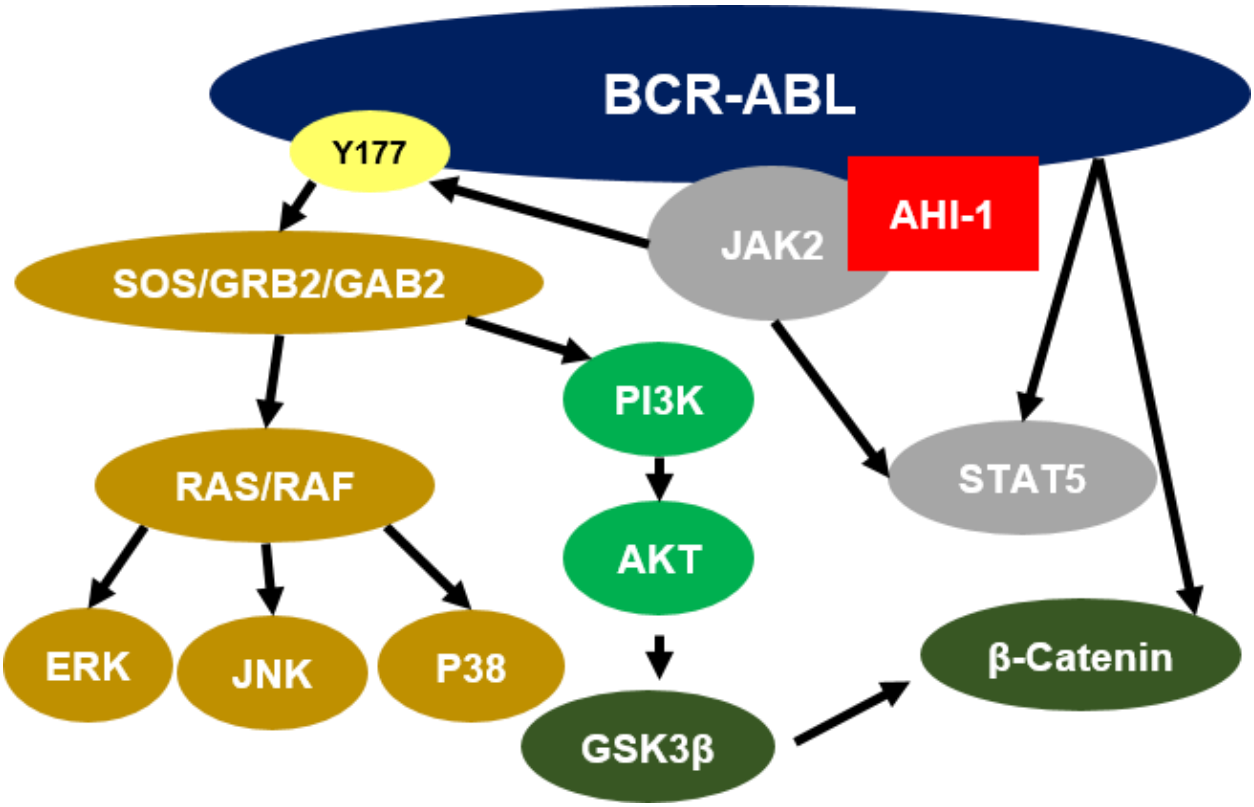



**Figure 3: Key Proteins Involved in the BCR-ABL Signalling Pathway.** BCR-ABL exerts its oncogenic activity through a network of pathways that promote proliferation and survival of leukemic cells.

**1. 4 Identification of Abelson Helper Integration Site 1 (AHI-1).**

Murine Ahi-1 was identified as a cooperating oncogene in a v-abl-induced murine lymphoma model. It is commonly activated by provirus insertional mutagenesis in various murine leukemias and lymphomas (82). AHI-1 encodes a unique protein with multiple SH3 binding sites, an SH3 domain, two PEST motifs and seven WD40-repeat domains. These are all known to be important mediators of protein-protein interactions indicating that AHI-1 may have an important function as a signalling protein. The human form of AHI-1 contains an additional coiled coil domain at its N-terminal region. Human AHI-1 encodes three major isoforms, suggesting that this gene is also subject to alternative splicing. Isoform 1 and 3 are similar in that they contain all the domains, while isoform 2 lacks the C-terminus suggesting it may have a different function than the other two (Figure 4). The high frequency of Ahi-1 mutations observed in certain virus-induced murine leukemias and lymphomas suggests that Ahi-1/AHI-1 may be involved in leukemogenesis (82).

**1.5 AHI-1 Expression in Normal and Leukemic Hematopoietic Cells.**

It has been demonstrated that Ahi-1/AHI-1 expression is regulated at multiple stages of hematopoiesis in a manner that is highly conserved between mice and humans. Ahi-1 transcript levels are highly expressed in the most primitive hematopoietic cells, while its expression is downregulated as cells begin to differentiate (59, 83). Interestingly, the transcript levels of Ahi-1 are found to be highly deregulated in the K562 CML cell line, as well as in $Ph^+$ primary leukemic cells, particularly in the leukemic stem cell-enriched population ($lin^-CD34^+CD38^-$ cells) (83). These observations indicate that downregulation of AHI-1 expression could be an important step in the regulation of differentiation of normal primitive hematopoietic cells and deregulation of AHI-1 expression may contribute to the development of human leukemias.

A recent study investigating the biological effect of AHI-1 in K562 cells showed that suppression of AHI-1 resulted in increased sensitivity to IM treatment, while overexpression of AHI-1 resulted in greater resistance to treatment (59). In experiments where primary $lin^{-}CD34^{+}$ CML cells were assessed for their sensitivity to IM, DA, and NL, samples from patients who were clinically diagnosed as IM-nonresponders or in blast crisis showed increased sensitivity to TKI treatment when AHI-1 was suppressed. Analysis of the AHI-1 transcript levels of these cells showed that the IM-nonresponders and blast crisis patients displayed significantly higher levels compared to patients in the chronic phase (59). Collectively these data highlight a correlation between resistance of CML stem/progenitor cells to TKIs and AHI-1 expression levels. This gives evidence that AHI-1 may play a significant role in this BCR-ABL driven disease.

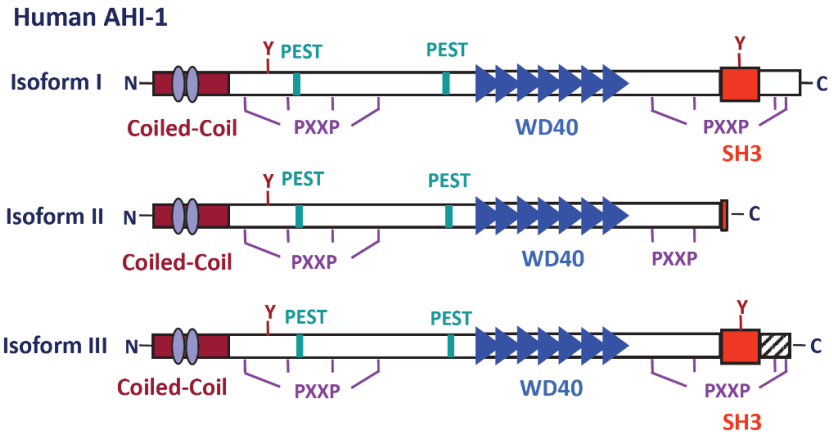



**Figure 4: Structure of Abelson Helper Integration Site 1 (AHI-1)**. There are 3 existing isoforms of human AHI-1. AHI-1 is thought to function as an adaptor protein to mediate protein-protein interactions. Domains involved in protein interactions include: one SH3 domain (red box), seven WD40 repeats (blue triangles), proline rich motifs (PXXP), PEST sequences (green boxes), a coiled-coil domain (purple ovals), and tyrosine phosphorylation sites. PEST sequences: proline (P), glutamic acid (E), serine (S), threonine (T) rich sequences. Reproduced with permission from Esmailzadeh et al., 2011 (2).

### 1.6 Significance of an AHI-1-BCR-ABL-JAK2 Interaction Complex in CML.

It has been reported that the more primitive $lin^{-}CD34^{+}CD38^{-}$ CML cells express higher levels of both BCR-ABL and AHI-1, suggesting a possible cooperative role between the two in regulation of LSC functions (83). Importantly, overexpression of AHI1 alone can transform hematopoietic cells and this effect is significantly enhanced by BCR-ABL (59).

Previous studies investigated the cooperative relationship between these two oncoproteins (59). In a model where full-length BCR-ABL and Ahi-1 were overexpressed in the murine BaF3 cell line, a murine interleukin-3 dependent pro-B cell line, overexpression of BCR-ABL and Ahi-1 alone led to an increase in cellular proliferation and growth factor independence. Overexpression of both oncogenes significantly enhanced these phenotypes thus suggesting a dual oncogenic cooperative role in leukemogenesis. This was further demonstrated in an *in vivo* study where BaF3 cells, and BaF3 cells transduced with either BCR-ABL or Ahi-1 or co-transduced with both, were injected into mice. While BaF3 cells did not display any disease following injection for the duration of the experiment, the singly transduced AHI-1 and BCR-ABL expressing BaF3 cells developed a lethal leukemia. The injection of co-transduced cells led to mice developing a lethal leukemia in an even shorter time, thus further indicating a cooperative role between AHI-1 and BCR-ABL in leukemogenesis (59).

To investigate the importance of the various domains of Ahi-1, mutants of Ahi-1 were stably transduced into BaF3 cells together with BCR-ABL (84). The Ahi-1 mutants included an N-terminal deletion mutant (containing both SH3 and WD40 domains), an SH3 deletion mutant (containing N-terminal and WD40 domains), as well as an SH3 and WD40 deletion mutant (containing the N-terminal domain). Compared with BaF3 cells transduced with BCR-ABL alone, the addition of full-length Ahi-1 drastically reduced the sensitivity of cells to IM treatment, reflected in less apoptosis and reduced ability of these cells to form colonies in colony forming cell (CFC) assays. BCR-ABL transduced BaF3 cells also expressing the SH3/WD40 double

deletion mutant displayed dramatically increased sensitivity to IM compared to BaF3 cells transduced with BCR-ABL and full length AHI-1, while the N-terminal deletion and SH3 deletion mutants also showed increased sensitivity to a lesser extent. This indicated that the WD40 domain of Ahi-1 in particular may be important in the response of BCR-ABL$^+$ cells to IM treatment. AHI-1 may have a significant role in how BCR-ABL drives this disease.

Interestingly, it was observed that manipulation of AHI-1 expression in K562 cells affected the amounts of total and phosphorylated BCR-ABL (59). Suppression of AHI-1 led to a significant reduction while overexpression of AHI-1 increased the amounts. Several other proteins known to be involved in BCR-ABL-mediated signalling were also affected, with the greatest differences occurring in the JAK2-STAT5 pathway. Suppression of AHI-1 expression led to decreased expression of JAK2 and STAT5 while overexpression of AHI-1 showed the opposite effect, with increased levels of these proteins. Interestingly, co-immunoprecipitation experiments in K562 cells revealed a direct interaction between BCR-ABL and AHI-1 as well as between AHI-1 and JAK2 (59). More recently, it was shown that the WD40 repeat domain of AHI-1 interacts with BCR-ABL, while the N-terminal region interacts with JAK2 (84). Tyrosine phosphorylated BCR-ABL and JAK2 also interact with AHI-1. However, IM treatment of K562 cells resulted in an inability to detect the interaction of either tyrosine-phosphorylated BCR-ABL or JAK2 with AHI-1. These results provide strong evidence that AHI-1 forms a complex with BCR-ABL and JAK2 that modulates sensitivity to treatment with IM in CML cells.

Having demonstrated that suppression of AHI-1 expression results in a reduction of BCR-ABL and JAK2 protein expression and phosphorylation, it was hypothesized that dual inhibition of BCR-ABL and JAK2 may disrupt an AHI-1-BCR-ABL-JAK2 interaction complex and could be more effective against primitive CML cells. IM treatment in combination with the JAK2 inhibitor TG101209 (TG) significantly inhibited growth of AHI-1 overexpressing K562 cells, K562 IM-resistant cells as well as TKI-insensitive CML stem/progenitor cells compared to single

agents (84). Another study with a JAK2 inhibitor currently in clinical trials (BMS-911543) showed that dual inhibition of BCR-ABL and JAK2 resulted in a statistically significant synergistic reduction in growth of these cells (85). These results further support the importance of a stable AHI-1-BCR-ABL-JAK2 complex in driving leukemic activities. Specifically targeting AHI-1 could potentially destabilize this complex and disrupt the multiple protein interactions mediated by AHI-1. However there are no known specific inhibitors of AHI-1 available.

### 1.7 Identification of PP2A as a Potential AHI-1 Interacting Protein.

Having established that AHI-1 has an important role in BCR-ABL-mediated leukemogenesis, a drug screen was conducted by Min Chen and Kaiji Hu from the Jiang and Dunn laboratories in order to find an AHI-1 specific growth inhibitory compound (Figure 5A). The drug screen used the Prestwick compound library, a library of 1200 small molecules which have known bioavailability and safety in humans. The drug library was tested on AHI-1-transduced K562 cells containing a yellow fluorescent protein (YFP) marker, alone and in combination with 0.1μM IM. The concentration of 0.1μM IM was used because this does not have a significant effect on cell viability but does reduce BCR-ABL phosphorylation (Figure 5B). The rationale for the combination with IM was so that a compound that may potentially synergize with IM could be identified. After treatment, the cells were fixed and analyzed using a High Content Screening system. This allowed for comparison of the rate of growth, as well as assessment of AHI-1 expression by measuring YFP intensity levels.

Out of the library of 1200 compounds, 28 were identified that inhibited cell growth by more than 80%, while 3 compounds inhibited AHI-1 expression by more than 80% according to fluorescence levels of YFP. From this list of compounds cantharidin (CAN), an inhibitor of protein phosphatase 2A (PP2A) was identified. CAN is extracted from dry blister beetles and

has been used in folk medicine for many decades, primarily as a topical agent for treating warts (86). Interestingly, about 30% inhibition of growth was observed with CAN alone but this was remarkably enhanced to 93% by its combination with IM (Figure 5C), suggesting that PP2A may have a relationship with AHI-1 and that dual inhibition of PP2A and BCR-ABL could have potential as a therapeutic combination treatment.

CAN was first identified to target PP2A in 1992 (87). CAN demonstrates 10-fold selectivity for PP2A over Protein Phosphatase 1 (PP1), the other major serine (ser) / threonine (thr) phosphatase, but also potently inhibits Protein Phosphatase 4 (PP4) (88). Many studies have used treatment with CAN as a model to study the effects of PP2A inhibition (86, 89-93). CAN is quite potent across a diverse panel of cell lines with $IC_{50}$ values mostly in the micromolar range (90). However, the clinical use of CAN is limited due to severe side effects such as dysphagia, dysuria, hematemesis, liver congestion, and renal toxicity (94). Efforts have been made to generate analogues of CAN to target PP2A but avoid toxicity. This led to the development of inhibitors like LB100 and LB102 that will be used for this study.

In our preliminary study, CAN was found to potently inhibit K562 and K562 IM-resistant cells. Combination treatment with IM significantly prevented growth and induced apoptosis compared to single treatments (Figure 6A). However CFC assays conducted using $CD34^+$ normal bone marrow (NBM) cells showed that treatment with 2.5μM CAN is highly toxic (Figure 6B), confirming the limits of CAN's potential in the clinic. This preliminary study confirmed that PP2A represents a potential target for inhibition in combination with TKIs but an alternative, less toxic, specific inhibitor of PP2A needs to be identified.

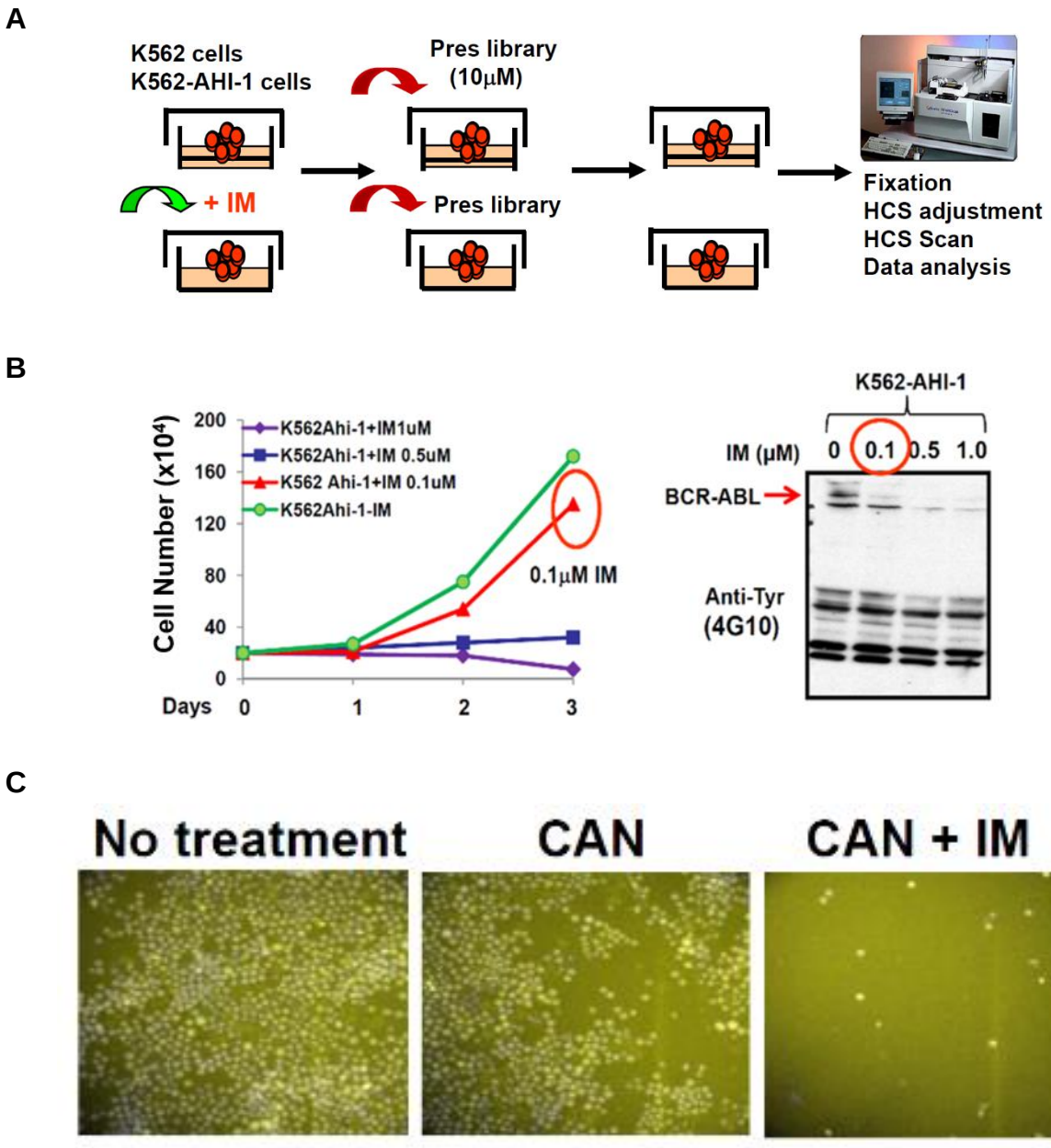



**Figure 5: Identification of New Growth Inhibitory Compounds in AHI-1-transduced CML Cells. (A)** Schematic of experimental design to identify growth inhibitory compounds by screening the Prestwick chemical library against AHI-1- transduced K562 (K562-AHI-1) cells for 48 hours in the presence or absence of IM. **(B)** Optimization of inhibitory effects of IM in K562-AHI-1 cells. Treatment with 0.1μM IM did not inhibit cell growth (left) but suppressed BCR-ABL phosphorylation (right). **(C)** Representative imaging of cells after CAN treatment alone and in combination with IM. These experiments were performed by my colleagues Min Chen and Kaiji Hu.

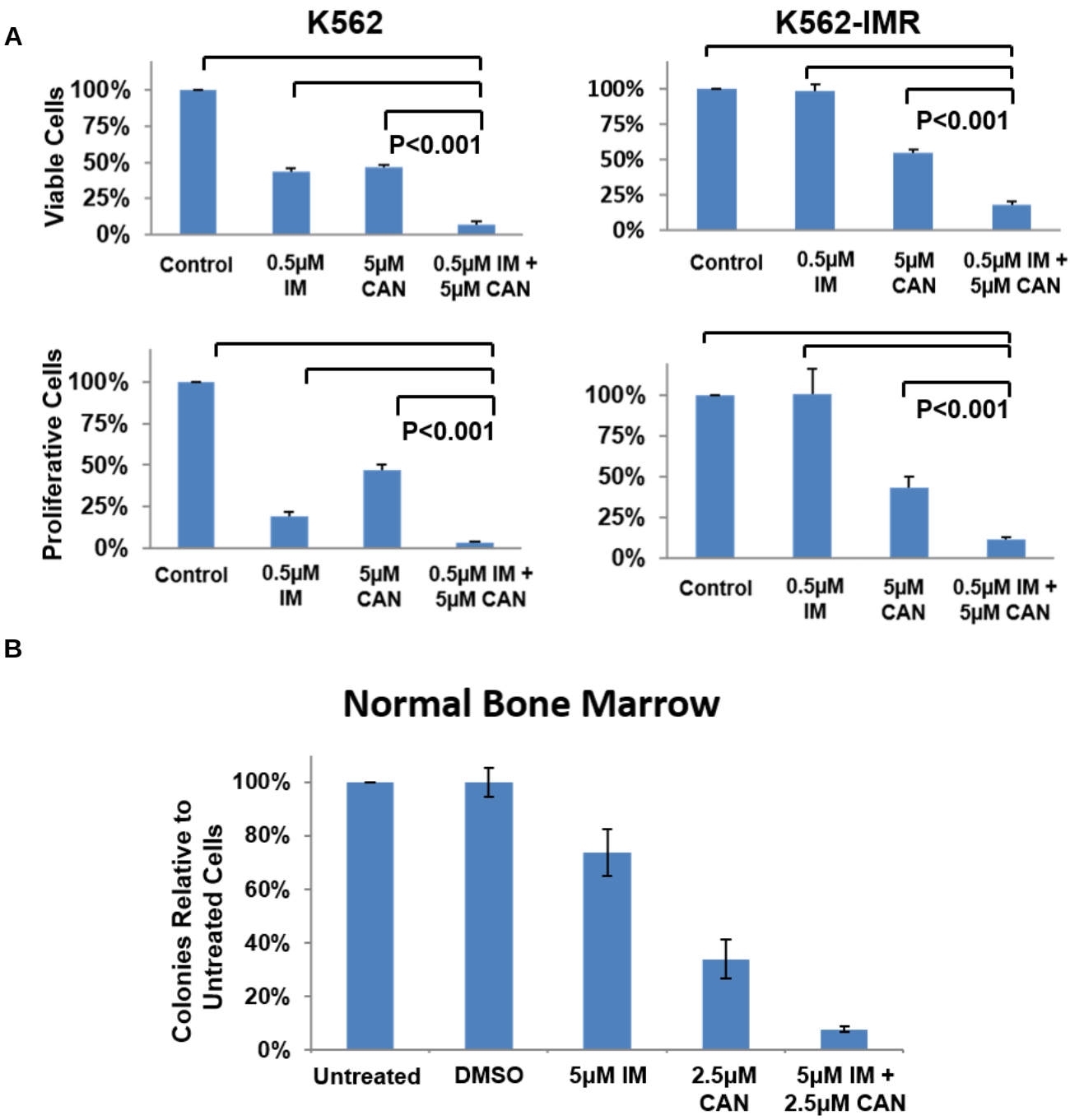



**Figure 6: Potency of CAN in the K562 and K562 IM-resistant Cell Lines, as well as Primary CML and Normal Bone Marrow (NBM) Cells. (A)** Inhibition of proliferation in K562 and K562 IM-resistant cells (K562-IMR) by 48 hour combination treatment with CAN and IM. These experiments were performed by my colleague Will Liu. **(B)** CFCs of primary $CD34^+$ NBM cells with single and combination treatments of CAN and IM. I conducted this experiment.

**1.8 Introduction to PP2A.**

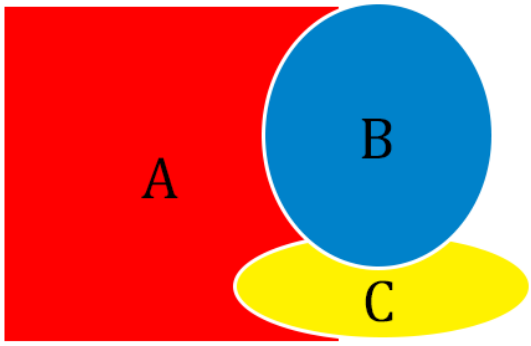
With over 500 kinases encoded by the human genome, nearly every cellular process is regulated by the reversible phosphorylation of specific residues on proteins. Up to 70% of all eukaryotic proteins can be phosphorylated on a Ser, Thr, or Tyr residue (95). Decades of studies have identified crucial roles for protein kinases in cell proliferation, survival and other important processes. Genetic alterations that lead to unrestrained constitutive activation of kinases are known to be drivers of cancer development, like the role of BCR-ABL in CML. Protein phosphatases antagonize the action of protein kinases and are just as important in maintaining correct phosphorylation balance (96).

PP2A is a highly conserved family of Ser/Thr phosphatases that along with PP1 constitutes the bulk of Ser/Thr phosphatase activity (97). PP2A has a role in a wide range of signaling pathways and is involved in the regulation of cell proliferation and death (98, 99), cell division (100), adhesion and migration (101), and metabolism (102). The PP2A holoenzyme is a heterotrimeric complex consisting of a catalytic C subunit, a scaffolding A subunit, and a regulatory B subunit (Figure 7). In humans there are 23 different isoforms of the B subunit (1, 103), which can be divided into four distinct families. These B regulatory subunits dictate the substrate specificity of the catalytic C subunit as well as PP2A subcellular localization based on where they are expressed in the cell (104). Recent reports have suggested that PP2A has a tumor suppressive role in leukemogenesis and that modulation of PP2A activity could be beneficial for treatment of hematologic malignancies (105-107).

PP2A has a major role in the maintenance of normal cell division (108, 109). PP2A controls the G1/S transition, where a specific PP2A complex involving the B56y3 regulatory subunit accumulates in the nucleus and its methylation levels change from G0 throughout the G1 and S phases of the cell cycle (110). PP2A activity is also thought to regulate mitotic exit. A PP2A complex involving the PR55α regulatory subunit prevents transition from G2 to M and is

also thought to control mitotic spindle breakdown, chromatin decondensation, and post-mitotic reassembly of the nuclear envelope and Golgi apparatus (111). Many studies have indicated that PP2A predominantly displays pro-apoptotic functions, particularly in the extrinsic pathway (88, 112, 113). PP2A promotes pro-apoptotic activity by negatively regulating the PI3K/AKT pathway. It directly dephosphorylates AKT leading to inactivation of antiapoptotic BCL-2, and activation of proapoptotic factors BAD and BIM (99).

Interestingly, PP2A can act as both a positive regulator and a negative regulator of the Wnt signalling pathway. Its dominant role likely depends on the status of the cell as well as the expression of the various regulatory B subunits in the specific type of cell (98). PP2A has also been shown to stabilize β-catenin directly through complexing with E-cadherin and β-catenin to stabilize both proteins. Ablation of PP2A expression results in β-catenin being redistributed to the cytoplasm where it is rapidly degraded by the proteasome (98, 106, 114). Studies have also shown that PP2A directly dephosphorylates β-catenin, and treating cells with okadaic acid, a PP2A inhibitor, leads to an increase in the phosphorylation of β-catenin (115). This is explained by the role of PP2A in antagonizing the kinase activity of GSK3β and CKIα in the β-catenin degradation complex (78). PP2A can dephosphorylate the same residues (thr41/ser45) that these kinases phosphorylate.




| Subunit | Name | Gene |
|---|---|---|
| Scaffold (A) | PR65a | PPP2R1A |
| | PR65B | PPP2R1B |
| Catalytic (C) | PPP2cα | PPP2CA |
| | PPP2AcB | PPP2CB |
| Regulatory (B) | PR55α/B55α | PPP2R2A |
| | PR55β/B55β | PPP2R2B |
| | PR55γ/B55γ | PPP2R2C |
| | PR55δ/B55δ | PPP2R2D |
| Regulatory (B') | PR56α/B56α | PPP2R5A |
| | PR56β/B56β | PPP2R5B |
| | PR56γ/B56γ | PPP2R5C |
| | PR56δ/B56δ | PPP2R5D |
| | PR56e/B56ε | PPP2R5E |
| Regulatory (B") | PR72 | PPP2R3A |
| | PR70 | PPP2R3B |
| | G5PR | PPP2R3C |
| | PR59 | PPP2R3D |
| Regulatory (B"') | PR110/Striatin | STRN |
| | SG2NA | STRN3 |
| | PR53/PTPA | PPP2R4 |

**Figure 7: PP2A Structure and Regulatory Subunits.** PP2A is composed of a heterotrimeric complex involving a structural subunit (A), regulatory subunit (B), and a catalytic (C) subunit. The B subunits confer substrate specificity as well as subcellular localization. Adapted from Kiely et al., 2015 (1).

### 1.9 Therapeutic Potential of Manipulation of PP2A in CML.

Given the ability of PP2A to act as both a positive and negative regulator of the Wnt and MAPK signalling pathways (98), it is not surprising that strategies of increasing as well as decreasing PP2A activity have been proposed to treat cancers. The SET protein is an inhibitory regulator of PP2A, and its overexpression has been observed in various solid tumours and leukemias (99). Inhibition of PP2A is thought to be cancer promoting, acting by inducing the phosphorylation and activation of kinases like JNK, ERK, p38, Akt, PKC which drive signalling pathways that promote cell proliferation (116). Recent studies have shown that pharmacological activation of PP2A by inhibition of SET can target leukemic cells (117, 118). In blast crisis CML, induction of SET expression is thought to be controlled by BCR-ABL. It was demonstrated that restoration of PP2A activity by overexpression of the PP2A catalytic domain (PP2A-C) or stable knockdown of SET led to SHP-1-mediated BCR-ABL inactivation and degradation (119). Pharmacological activation of PP2A suppressed cell growth, impaired clonogenicity and enhanced apoptosis without considerable toxic effects on normal hematopoiesis (120). Inhibition of SET, especially in combination with TKIs, has shown some biological effects in CML cells (106, 107, 121).

It was recently reported that PP2A-activating drugs (PADs) can reduce survival of primitive CML cells through targeting of the JAK2-PP2A-β-catenin network (106). These PADs increased PP2A activity by inhibiting SET, and JAK2 activity seemed necessary for BCR-ABL kinase-independent induction, and the nuclear localization and transcriptional activity of β-catenin in primary CML $CD34^{+}CD38^{-}$ cells. It was also demonstrated that increasing PP2A activity, through treatment with PADs or transduction with PP2A, suppressed β-catenin expression. A LEF/TCF luciferase assay measuring β-catenin downstream transcriptional activity showed significant suppression after treatment with a JAK2 inhibitor or PADs. In these experiments IM treatment alone was not able to suppress β-catenin expression or downstream

transcriptional activity. GSK3β, an integral component of the β-catenin degradation complex, can be activated by PP2A with specificity thought to be conferred through the PP2A-B56α regulatory subunit (122, 123). GSK3β activity was shown to be essential for the PADs to target quiescent CML stem cells as shown by combining the PADs with GSK3β inhibitors LiCl and SB216763. Altogether, this study suggests that treatment with PADs could be a potential treatment strategy for patients who have mutations in their BCR-ABL kinase domain and do not respond to mono-TKI therapy.

Interestingly, it has also been convincingly shown that pharmacological inhibition of PP2A can lead to cancer cell death (124). There are kinase-dependent anticancer pathways that are induced by treatment with PP2A inhibitors. Kinase-dependent anticancer mechanisms triggered by PP2A inhibition could include JNK-dependent growth inhibition and NF-kB apoptosis induction (125). It was reported that PP2A inhibition with CAN suppressed pancreatic cancer cell growth and migration through enhancing the phosphorylation and further degradation of β-catenin. This could be attributed to the down-regulation of genes downstream of the Wnt/β-catenin pathway like c-Myc and cyclin D1 (61, 92). In addition, it has been reported that PP2A activity is 20-fold lower in the G2/M phase of cell cycle than in S-phase and that PP2A affects phosphoproteins associated with microtubules (126). This may explain how PP2A inhibitors like CAN induce G2/M phase arrest in cancer cells (91, 93). PP2A inhibitor-induced cell death is likely due to improper cell cycle progression caused by premature entry from G1/S phase into G2/M, as well as deficient exit from mitosis; ultimately leading to mitotic catastrophe. This rationale lays the foundation for the following strategy based on the role of PP2A in DNA damage and DNA repair signalling (127, 128). PP2A inhibition in combination with general DNA-damaging chemotherapy treatment enhances the effectiveness of chemotherapy by blocking DNA damage induced defence mechanisms. The susceptible cancer cells are forced into cell cycle progression and continue to accumulate DNA damage, resulting

in mitotic catastrophe or apoptotic cell death (129, 130). PP2A inhibition has thus been proposed as a potential strategy to eradicate cancer cells of slow growing tumours, or even the quiescent cancer stem cells (99).

### 1.10 Use of new PP2A inhibitors: LB100 and LB102.

Pharmacological inhibition of PP2A has primarily been studied using naturally produced, but relatively toxic compounds (88). Despite the potency of CAN, especially in combination with IM, toxicity issues indicate that alternative drugs with a similar mechanism of specific PP2A inhibition but with less toxicity need to be identified. Two competitive small molecule inhibitors of PP2A, LB100 and LB102, were recently developed and have been studied in various cancer models (Figure 8). LB100 is a water soluble compound while LB102 is a lipid soluble homolog of LB100. Both compounds have been shown to be inhibitors of PP2A (130-132). These two compounds have been studied in gliobastoma (130, 133), sarcomas (134), pheochromocytoma (135), nasopharyngeal and hepatocellular carcinomas (136, 137), as well as in breast, ovarian and pancreatic cancers (132, 137-139). As demonstrated with CAN, treatment with LB100 and LB102 promoted mitotic catastrophe and apoptosis. While these studies demonstrated the potency of the compounds as both chemo- and radio- sensitizers without significant systemic toxicity, there have not been any published studies to date on the use of these drugs in hematological malignancies. Interestingly these studies have shown that inhibition of PP2A with these compounds can overcome cancer cell senescence and induce cellular differentiation in progenitor cells (140). Given the important role of β-catenin in leukemic stem cell renewal and the potential for PP2A inhibition to destabilize β-catenin, these findings support the use of PP2A inhibitors to potentially target leukemic stem cells, including quiescent stem cells. With strong evidence that TKI monotherapies are only able to eliminate the bulk differentiated leukemic cell population but not the leukemic stem cell population, a combination of these new PP2A

inhibitors with current TKIs represents a new treatment strategy to specifically target both BCR-ABL-dependent and BCR-ABL-independent mechanisms that are operative in CML stem/progenitor cells. LB100 has shown particular promise and is now being evaluated in human clinical trials. These two inhibitors have thus been selected for this study to investigate the therapeutic potential of inhibiting PP2A in combination with TKIs as a new and more effective therapeutic approach for treatment of CML, particularly in patients at high risk of TKI resistance and disease progression.

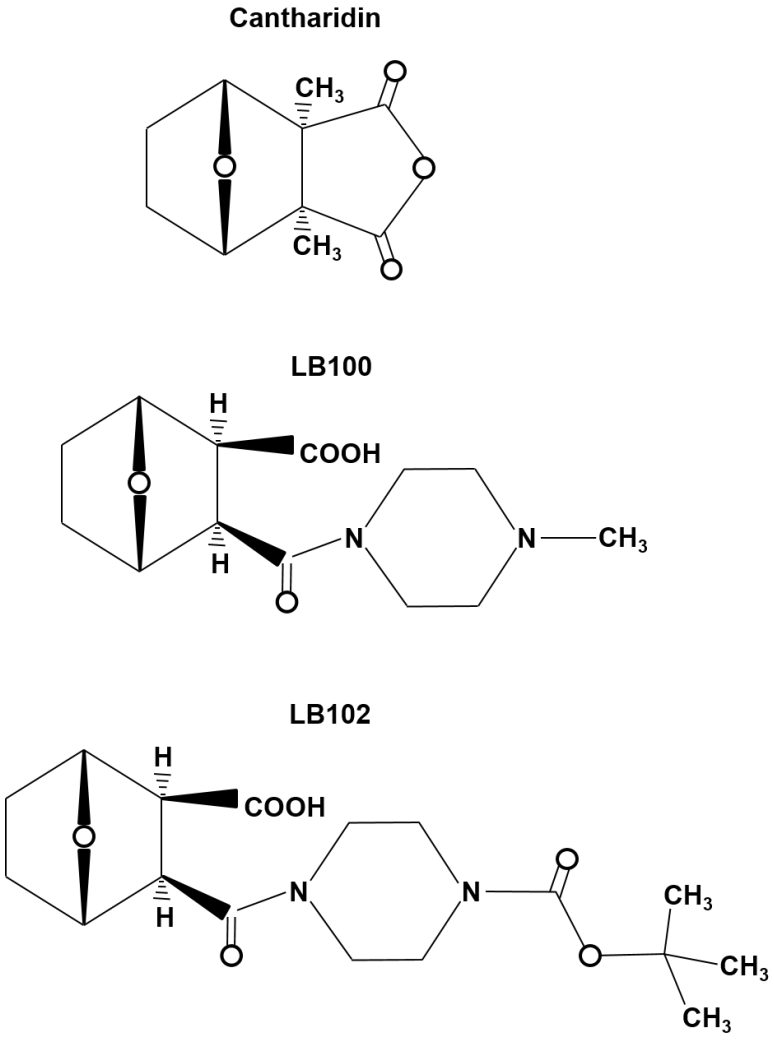



**Figure 8: Structural Comparison of PP2A inhibitors.** Structures of Cantharidin, LB100, and LB102 are shown.

## 1. 11 Experimental Outline

The major goal of this thesis project is to investigate the therapeutic potential of inhibiting both BCR-ABL and PP2A as a new combination treatment strategy for targeting TKI-insensitive leukemic stem cells in CML. As described above, there has been great success with the use TKIs to treat CML, but relapse and resistance to treatment are significant problems. This is likely due to a remaining reservoir of leukemic stem cells that are capable of expanding and regenerating the leukemic cell population. While there are many potential mechanisms for these cells to resist current treatments, there is an overall need to develop new agents and combination strategies to prevent the continuous development of these resistant subclones. Alternative targets to BCR-ABL that are essential for leukemogenesis must be identified and AHI-1 represents such a target. While studies into the exact role of AHI-1 in the mediation of BCR-ABL signalling activities are ongoing, the correlation of its expression with BCR-ABL in leukemic stem cells highlights its potential as a molecular target. The identification of a PP2A inhibitor (CAN) through an AHI-1 specific drug screen suggests that PP2A has a role in how AHI-1 supports BCR-ABL driven activities. It is therefore important to study the biological effects of inhibiting PP2A, its function and mechanism of action in CML, and how it may contribute to AHI-1-mediated protein interactions.

**Hypothesis:**

- New PP2A inhibitors in combination with TKIs are more effective than current TKI monotherapies in eliminating leukemic stem cells by selectively targeting and destabilizing AHI-1-mediated complexes.

**Objectives:**

- To investigate the role of PP2A in CML and evaluate the therapeutic potential of two PP2A inhibitors, LB100 and LB102, alone and in combination with TKIs, to inhibit growth of CML stem and progenitor cells.

**Specific Aims:**

- To investigate the potential of combining new PP2A inhibitors with TKIs to inhibit the growth of IM-resistant cells and primary CML stem/progenitor cells *in vitro*.
- To investigate the molecular mechanisms of AHI-1's interaction with specific proteins, including PP2A (PR55α) and β-catenin, and the biological significance of these interactions in the regulation of BCR-ABL transforming activity, cell cycle control and drug resistance in CML stem/progenitor cells.

# Chapter 2: Materials and Methods

## 2.1 Cell Lines

The human CML cell lines K562, K562 IM-resistant cells (K562-IMR), AHI-1-transduced K562 cells (K562lenti-AHI-1), AHI-1-depleted K562 cells (AHI-1 sh4) and BV173 cells were cultured in Rosewell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and $10^{-4}$ M β-mercaptoethanol (STEMCELL Technologies), at 37°C, 5% CO2, in a humidified cell culture incubator. This cocktail of RPMI 1640 will be referred to as complete RPMI 1640 media. Parental and Ahi-1 transduced BaF3 cells were supplemented with 5ng/mL murine IL-3 (STEMCELL Technologies). 293T cells were grown in Dulbecco's modified Eagle's media (DMEM) with 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and L-glutamine (STEMCELL Technologies). These cells were maintained as suspension cultures in 10cm Falcon® tissue culture dishes (Corning Inc.). Cell counts were assessed using trypan blue dye exclusion.

## 2.2 Human Cells

Primary CML cells were obtained from newly diagnosed patients with CP CML before initiation of any TKI therapy. Subsequent IM-responders were classified as achieving complete hematologic remission within three months, major cytogenetic remission within 12 months, and complete cytogenetic remission within 18 months, based on the European Leukemia Net treatment guidelines (141). Conversely, IM-nonresponders did not achieve these response criteria or had evidence of loss of response later. Fresh bone marrow (BM) cells were obtained from healthy donors (ALLCELL). Informed consent was obtained, and the procedures used were approved by the Research Ethics Board of the University of British Columbia. $CD34^+$ cells were isolated immunomagnetically by positive selection of cells utilizing EasySep CD34 selection kits (STEMCELL Technologies). Purity was verified by restraining the isolated cells

with a fluorescein-isothiocyanate (FITC)-labelled anti-human CD34 mouse monoclonal antibody or with mouse monoclonal anti-human CD34 allo-phyocyanin (APC) (BD Biosciences) antibody and analyzing the cells by flow cytometry.

### 2.3 Reagents

Imatinib (IM) was obtained from Novartis (Novartis, Basel, Switzerland. Dasatinib (DA) was provided by Bristol-Myers Squibb (Princeton, USA). The two PP2A inhibitors LB100 and LB102 were provided by Lixte Biotechnology (EastSetauket, NY, USA). Stock solutions of 10mM were prepared with water (IM and LB100) or with dimethyl sulfoxide (DMSO) (DA and LB102) and stored at -20°C. Antibodies used for immunoprecipitation, immunofluorescence, and western blotting included: anti-N-terminal-AHI-1, murine anti-C-terminal-Ahi-1 (Applied Biological Materials), anti-phospho-tyrosine (4G10; Millipore), anti-ABL (8E9: BD Biosciences), anti-phosphoY177-BCR (Cell Signalling), anti-phospho-JAK2 (Cell Signalling), anti-JAK2 (Cell Signalling), anti-phospho-STAT5 (Cell Signalling), anti-STAT5 (Cell Signalling), anti-phospho-T41,S45-β-catenin (Cell Signalling), anti-phospho-Y86-β-catenin (Cell Signalling), anti-β-catenin (BD Biosciences), anti-phospho-AKT (Cell Signalling), anti-AKT (Cell Signalling), anti-β-Actin (Applied Biological Materials), anti-GAPDH (Sigma Aldrich), anti-Caspase-8 (Cell Signalling), anti-Caspase-3 (Cell Signalling), anti-PP2CA (Cell Signalling), anti-PPP2R2A (Cell Signalling), anti-SET (Santa Cruz), anti-phospho-ERK (Santa Cruz), anti-ERK (Santa Cruz), anti-phospho-P38 (New England Biosciences), anti-P38 (New England Biosciences), anti-JNK (New England Biosciences), anti-phospho-GSK3β (Cell Signalling), anti-GSK3β (Cell Signalling), anti-α-Tubulin (Sigma Aldrich).

### 2.4 Cellular Viability Assay

Total cell counts and viability of cells was assessed using trypan blue dye exclusion. For cell lines, $5x10^5$ cells were seeded in 6-well Falcon plates in 4mL of complete RPMI1640 media per

well with or without drugs. For primary $CD34^+$ cells, $2x10^4$ cells were seeded in 24 well falcon plates and cultured in 500uL Serum Free Medium containing 80% ISCOVE MEM (STEMCELL Technologies), 20% BIT (STEMCELL Technologies), 1% L-Glutamine, and 1% β-mercaptoethanol (STEMCELL Technologies) supplemented with 100 ng/mL Flt3, 20ng/mL IL-6, 20ng/mL IL-3, 20ng/mL Granulocyte Colony-Stimulating Factor (G-CSF). The cells were seeded both with and without the various drug treatments.

**2.5 Apoptosis Assay**

Cells were seeded in complete RPMI 1640 medium and cultured at 37°C, 5% CO2 in a humidified cell culture incubator 48 hours. Cells were then pelleted and washed in Hanks Buffer (STEMCELL Technologies) and 2% FBS then resuspended in 50µL of 1× Annexin buffer (eBioscience). 2.5 µL of Annexin V-APC (eBioscience), and 2.5 µL of propidium iodide (PI) (eBioscience) were added to each sample and incubated on ice for 20 minutes at room temperature (RT) in the dark. After the 20 minute incubation, 200 µL of 1× Annexin buffer was added to the mixture followed by pelleting of the cells and resuspension in another 200uL of 1x Annexin buffer. Cells were analyzed using a FACSCalibur™ flow cytometer (BD Bioscience). The % apoptotic cell numbers were calculated as the % of cells that were Annexin $V^+$. Data was analyzed with FlowJo software.

**2.6 Thymidine Incorporation Proliferation Assay**

$2 \times 10^4$ cells were cultured in triplicate in round-bottom 96-well Falcon plates in 100µl of complete RPMI 1640 media per well. The cells were cultured for 48 hours at 37°C, 5% CO2 in a humidified cell culture incubator. The amount of tritiated [3H]-thymidine incorporation during a 4-hour pulse of the culture was measured. Briefly, 1 microcurie (µCi) of tritiated [3H]-thymidine was added to each well. After 4 hours, the cells were harvested onto a membrane using a

Skatron instruments combi Harvester (LKB Wallace-PerkinElmer). The amount of tritiated [3H]-thymidine was measured with a LKB Betaplate scintillation counter.

### 2.7 Protein Extraction and Quantification

Cell lysate was prepared by pelleting cells after washing with Dulbecco's Phosphate Buffered Saline (PBS) (STEMCELL Technologies) followed by protein extraction in a lysis buffer. Lysis buffer consisted of 900µL phosphorylation solubilisation buffer (PSB), 100µL NP-40 Alternative Protein Grade Detergent (Calbiochem), 5µL phenylmethylsulfonyl fluoride (PMSF,Sigma-Aldrich), and 5µL protease inhibitor cocktail (PIC, Sigma-Aldrich). The lysate was then centrifuged at 13000 rpm for 15 minutes at 4°C and the supernatant was collected and stored at -80°C. The Bradford assay was used to quantify the protein concentration. Bio Rad Protein Assay Dye Reagent was diluted in deionized super Q water at a ratio of 1:5. Protein lysates were diluted at a ratio of 1:10. Purified bovine serum albumin (BSA) was used to generate a standard curve and formula. 20µL of diluted protein lysate and 200µL of diluted Bio-Rad dye were added in duplicates to a 96-well Falcon plate. The absorbance of standards and samples were measured at 630nm using the Elx808TM Absorbance Microplate Reader (BioTekk Instruments).

### 2.8 Western Blotting

Samples were prepared using 30µg protein lysate, 6X loading buffer, and deionized super Q water followed by heating at 90°C for 10 minutes. Samples were then separated on 8%-15% SDS-page gels with 1.0 mm wells alongside PageRuler Prestained Protein Ladder (Fermentas). The gel was run under reducing conditions at 100 V for 1.5 hours. Proteins were then transferred from the gel onto Nitrocellulose 0.2-µm membrane (Bio-rad) using NuPAGE Transfer Buffer (Invitrogen) at 33 V for 1.5 hours. The membrane was then blocked in Tris-buffered saline Tween 20 (TBST) with 5% BSA for 1 hour at RT, washed with TBST for 2x5 minutes,

then incubated with a primary antibody overnight at 4°C. After incubation with the primary antibody, the membrane was then washed with TBST for 3x10 minutes and incubated with corresponding horseradish peroxidase–conjugated secondary antibody for 1 hour at RT. After incubation with the secondary antibody the membrane was washed with TBST for 4x10 minutes. Target proteins were visualized by incubation with enhanced chemiluminescence reagent for 2 minutes and then exposed on KODAK BioMax XAR autoradiography film.

**2.9 Immunoprecipitation**

For Immunoprecipitation (IP), between 500-1000µg of total cell lysate was incubated with 1ug of antibody on a rotator at 4°C overnight. The same amount of normal immunoglobulin G (IgG) (Santa Cruz) was also used as a negative control. The next day, the immune complexes were captured by adding 30µL of protein A/G bead flurry (Santa Cruz) to the mixture and incubating for another 2 hours on a rotator at 4°C. The beads were then pelleted by centrifugation at 4000G for 4 minutes followed by 3 washes with cold PBS. After the final wash 4uL of 6X loading buffer was mixed with the beads followed by heating at 90°C for 10 minutes. Supernatants were then used for western blotting.

**2.10 Phosphatase Assay**

PP2A phosphatase assays were carried out using the PP2A IP phosphatase assay kit from Millipore. Briefly, cells were lysed through sonication in a phosphate free buffer described by the manufacturer's protocol. Protein lysates (50µg) were incubated with 4µg of PP2Ac antibody (Millipore), and 25uL of Protein A-agarose beads. The IP was carried out on a shaking rack at 4°C for 2 hours. IPs were washed and used in the phosphatase reaction according to the manufacturer's protocol. Malachite green was used to assess the amount of phosphates released during the reaction. Normal mouse IgG was used instead of PP2Ac antibody as a negative control blank to determine the background level of phosphates present in the reaction. The absorbance of standards and samples were measured at 630nm using the Elx808TM

Absorbance Microplate Reader (BioTekk Instruments). The amount of immuno-precipitated PP2A was monitored by Western blots with anti-PP2Ac primary antibodies.

### 2.11 Colony Forming Cell Assay

Colony-forming cell (CFC) assays were performed using primary $CD34^+$ cells from CML patients and healthy individuals. Briefly, 3000 cells were mixed in 3mL methylcellulose medium (STEMCELL Technologies) supplemented with 20 ng/mL IL-3, 20ng/mL IL-6, 20ng/mL G-CSF, and 20ng/mL Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) (STEMCELL Technologies) as well as with or without inhibitors. The mixture of cells in medium was evenly distributed onto 2 CFC culture dishes (STEMCELL Technologies). Colonies produced were counted after 14 days of incubation.

### 2.12 Transfection of HA-tagged AHI-1 into 293T Cells

HA-tagged AHI-1 plasmid (84) was transfected into 293T cells with polyethylenimine (PEI). 24 hours before transfection, $6 \times 10^6$ cells were plated in 10-cm Falcon® tissue culture dishes in 10mL of DMEM with 10% FBS and were cultured at 37°C, 5% CO2, in a humidified cell culture incubator. The culture medium was changed 4 hours prior to transfection. A total of 18µg of HA-tagged AHI-1 plasmid was used for the transfection of each dish. The cells were then incubated for 48 hours. After 48 hours, transfected cells were washed with PBS, pelleted and used for western blotting or stored at -80°C.

### 2.13 Double Thymidine Block for Cell Cycle Synchronization

Thymidine was used to synchronize cells at the beginning of the S phase of the cell cycle (142). High thymidine concentrations in the cell have a negative feedback effect on nucleotide biosynthesis. Without proper supply of nucleotides, DNA replication is halted leaving cells stranded in the S phase. K562 cells were cultured in fresh, complete RPMI 1640 media with 2mM Thymidine (Sigma-Aldrich) for 24 hours at 37°C, 5% CO2, in a humidified cell culture

incubator. The cells were then released from the first thymidine block by washing once with PBS and culturing in fresh, complete RPMI 1640 media 12 hours. The second thymidine block was then applied by culturing the cells in complete RPMI 1640 media with 2mM Thymidine for another 24 hours. Following the second thymidine block the cells were washed once with PBS and allowed to recover in fresh, complete RPMI 1640 media for 3 hours. The status of the cells could then be assessed by flow cytometry or confocal microscopy.

### 2.14 Cell Cycle Analysis

Propidium iodide (PI) was used to assess cell cycle distribution. PI is an intercalating agent that binds to nucleic acids. The amount of fluorescing DNA in an individual cell can then be quantified using flow cytometry in order to identify the stage of the cell cycle the particular cell is in. The fluorescence of cells in G2 will be twice as high as in G1 phase and these 2 peaks can be used to quantify the % of cells in each stage of the cell cycle. Briefly, cells were collected, fixed, and permeablized with ice-cold 100% ethanol. After treatment with 10µg/mL of DNAse-free RNAse (Thermo Scientific), the cells were stained with 50 µg/mL of PI. A FACSCalibur™ flow cytometer (BD Bioscience) was used to measure the PI fluorescence of each cell in each sample. FlowJo software was used for analysis to determine the % of cells in each stage of the cell cycle.

### 2.15 Immunohistochemistry Staining and Confocal Microscopy

Cells ($1 \times 10^5$/per slide) were placed and adhered on poly-L-lysine coated slides (Electron Microscopy Sciences) for approximately 20 minutes at RT. Slides were then dipped into PBS to wash away the non-adherent cells. Cells were then fixed and permeabilized with cold methanol for 10 minutes. The cells were incubated for 20 minutes at RT with PBS containing 5% BSA. Cells were then incubated with anti-α-tubulin rabbit polyclonal antibody (GeneTex) diluted at 1:500 ratio in PBS containing 2% BSA. The cells were washed with PBS 3X followed by incubation with secondary antibodies conjugated to Alexa Fluor® 488 (Invitrogen). DAPI

(Vector Laboratories) was used as a nuclear stain and slides were viewed on an Olympus Fluoview 1200 confocal microscope. The percentage of catastrophic cells after treatment was determined by counting the number of catastrophic cells in a field of view. The main feature used to identify catastrophic cells was the observation of disrupted spindles with multipolar cell division.

### 2.16 RNA Extraction and Quantitative Real Time PCR (qPCR)

Total RNA was extracted with TRIzol (Life Technologies) (143). Glycogen (10ug/mL, Life Technologies) was added as a carrier to facilitate visibility of the RNA pellet. RNA (100ng) was reverse transcribed into cDNA with SuperScript® VILO ™ Master Mix (Life Technologies). Quantitative real-time PCR was performed with specific primers to detect AHI-1 transcripts as previously described (59).

### 2.17 Statistical Analysis

All data are shown as mean ± standard error of the mean (SEM) of measurements from at least three independent experiments. Differences between groups were compared using the two-tailed Student's t-test for paired samples. Statistical analyses were performed using Microsoft Excel. P-values $<0.05$ were considered statistically significant.

### 2.18 Analysis of Drug Interactions

Analysis of drug interactions in combination treatment groups was assessed after 48 hours of suspension culture with drug exposure using total viable cell counts. Cells were exposed to a wide range of IM, LB100 and LB102 using constant-ratio drug combinations and analyzed using the median-dose effect method (144). The combination index (CI) was calculated using CalcuSyn software (Biosoft, Cambridge, United Kingdom). $CI < 1$, $CI = 1$, $CI > 1$ represent synergistic, additive, or antagonistic effects respectively.

## Chapter 3: Results

### 3.1 New Inhibitors LB100 and LB102 Inhibit Growth of BCR-ABL$^+$ Leukemic Cells and Specifically Reduce PP2A Activity.

Two recently developed inhibitors of PP2A, LB100 and LB102, were obtained from Lixte Biotechnology Holdings Inc. Viability assays using trypan blue were conducted on K562 (a myeloid blast crisis cell line), K562 IM-resistant and BV173 cells (a lymphoid blast crisis cell line) to assess the ability of these two new PP2A inhibitors to affect the growth of these cell lines (Figure 9A). Treatment with these inhibitors reduced growth after 48 hour with a 50% Inhibiting Concentration (IC50) of approximately 3μM for both LB100 and LB102 in K562 cells, 5μM in K562 IM-resistant cells, and 1μM in BV173 cells.

To determine the ability of these two new inhibitors to specifically target PP2A, immunoprecipitation phosphatase assays were first conducted in K562 cells to assess PP2A phosphatase activity after treatment with the new inhibitors. Cells were treated with 5μM of either LB100 or LB102 for 1, 3, 5, 7 and 12 hours. After treatment the cells were harvested and sonicated to extract the protein. Immunoprecipitation with an anti-PP2AC antibody specific to the catalytic domain of PP2A and subsequent exposure to phospho-peptides allowed the enzymatic activity of PP2A to remove phosphate groups from the phospho-peptide. Malachite green was used to assess the amount of free phosphate released by PP2A activity. Interestingly, PP2A activity gradually decreased over time and reached a plateau at around 25% of original activity after 5 hours of treatment in K562 cells (Figure 9B).

To further confirm the ability of the inhibitors to inhibit PP2A activity, phosphatase assays were conducted in parallel on K562, K562 IM-resistant and BV173 cells. K562 and K562 IM-resistant cells were treated with 5μM of LB100 or LB102 while BV173 cells were treated with 2.5μM of either drug. In all three cell lines PP2A activity was reduced to lower than

25% of activity. Interestingly the higher sensitivity of BV173 cells to drug treatment, reflected by the lower IC50 value, was consistent with a lower concentration of drug reaching the same level of PP2A inhibition compared to K562 and K562 IM-resistant cells. These results indicate that, similar to CAN, the inhibitors LB100 and LB102 do indeed inhibit PP2A phosphatase activity and inhibition of PP2A with these drugs alone can reduce viability of BCR-ABL$^{+}$ myeloid and lymphoid blast crisis cells.

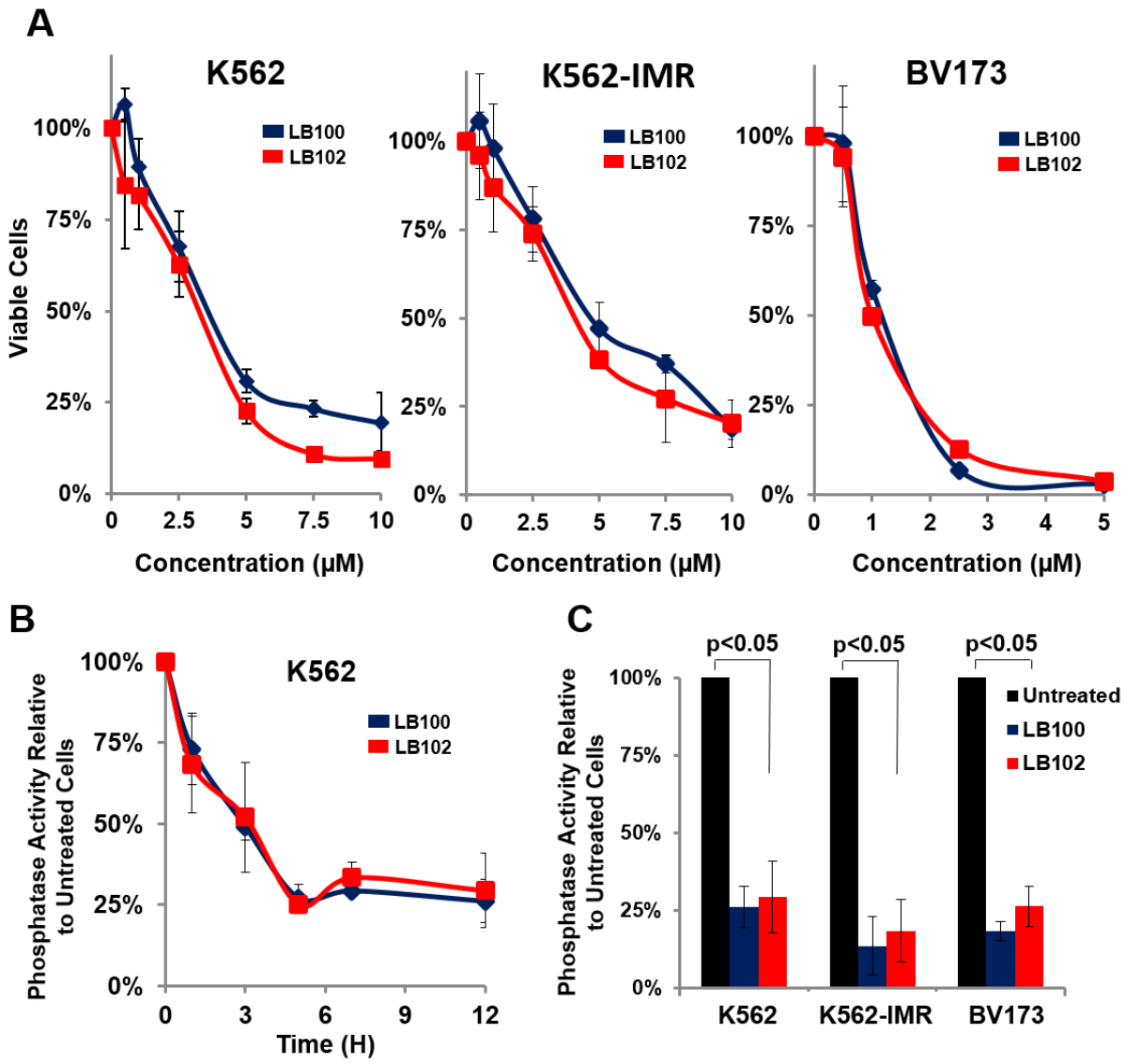



**Figure 9: New PP2A Inhibitors LB100 and LB102 Inhibit Growth of CML Cells and Specifically Reduce PP2A Activity. (A)** Viability of K562, K562 IM-resistant (K562-IMR) and BV173 cells was assessed after being cultured for 48 hours in the presence of various concentrations of LB100 and LB102. Viable cell numbers were determined by counting trypan blue dye-excluding cells. These were then expressed as a percent of the frequency of viable cells in parallel cultures where no drugs were added. A 50% Inhibiting Concentration (IC50) of LB100 and LB102 respectively was determined to be 3.5μM and 3μM in K562 cells, 5μM and 4.5μM in K562-IMR cells and 1μM for both in BV173 cells. **(B)** Treatment with 5μM LB100 and 5μM LB102 inhibits phosphatase activity of K562 cells in a time-dependent manner. **(C)** Treatment with LB100 and LB102 for 12 hours inhibits phosphatase activity of K562, K562-IMR and BV173 cells. K562 and K562-IMR cells were treated with 5μM of each inhibitor while BV173 cells were treated with 2.5μM of each inhibitor. Data shown are mean ± SEM from three independent experiments.

### 3.2 Knockdown of AHI-1 Mediates Response to PP2A Inhibitors in BCR-ABL$^+$ Leukemic Cells.

PP2A was identified as a potential target through a drug screen designed to identify an AHI-1 specific inhibitor. The sensitivity of K562 cells to IM treatment has been shown to correlate with AHI-1 expression (59). It was shown that when AHI-1 is overexpressed in K562 cells, they become less sensitive to IM treatment. On the other hand when AHI-1 expression is depleted the cells become more sensitive to IM. This can be explained by the role of AHI-1 in mediating phosphorylation of BCR-ABL and JAK2-STAT5 in CML cells (59).

In order to determine whether manipulation of AHI-1 can affect the sensitivity of K562 cells to the PP2A inhibitors, AHI-1 expression was depleted through lentiviral-mediated RNA interference (59). In these AHI-1 depleted K562 cells, AHI-1 expression was reduced by 40% as measured by qPCR (Figure 10A). The sensitivity of these cells to PP2A inhibition was then assessed with cell viability assays. Increased sensitivity to IM was confirmed in AHI-1-depleted cells after 48 hours treatment. Interestingly, the AHI-1 depleted cells were significantly more sensitive to LB100 and LB102 treatment alone compared to parental K562 cells (~2 fold, $P<0.05$, Figure 10A), suggesting the sensitivity of K562 cells to PP2A inhibitors could be modulated by the expression of AHI-1.

### 3.3 AHI-1 Physically Interacts with PR55α, a Regulatory Subunit of PP2A.

An IP-Mass Spectrometry experiment was previously conducted (unpublished data) to identify key proteins that interact with AHI-1. This experiment identified one of the PP2A regulatory subunits PR55α (also B55α) as a potential AHI-1 interacting protein. To confirm the interaction between AHI-1 and this PR55α regulatory subunit of PP2A, co-IP experiments were conducted in Ahi-1/AHI-1 transduced BaF3 and 293T cells. Since these cells have very low endogenous expression of Ahi-1/AHI-1, BaF3 cells were thus stably transduced with full length

murine Ahi-1 while 293T cells were transiently transfected with an HA tagged human full length AHI-1 construct. Co-IP experiments were performed in both cell lines, where PR55α was pulled down followed by Western blot to detect Ahi-1/AHI-1 using the Ahi-1/AHI-1 specific antibodies. The results showed that Ahi-1/AHI-1 does indeed interact with this specific PP2A regulatory subunit in both Ahi-1/AHI-1 transduced cells (Figure 10B). An 80kD band likely indicating an alternate isoform of AHI-1 was also observed in the BaF3AHI-1 cells when detecting AHI-1. These results suggest that PP2A could be involved in AHI-1-mediated protein interactions, including the BCR-ABL-AHI-1-JAK2 complex. Previous studies have shown the therapeutic potential of dual inhibition of BCR-ABL and JAK2 to destabilize this complex and prevent BCR-ABL driven leukemic activity. Dual inhibition of BCR-ABL and PP2A may also result in a potent synergistic effect.

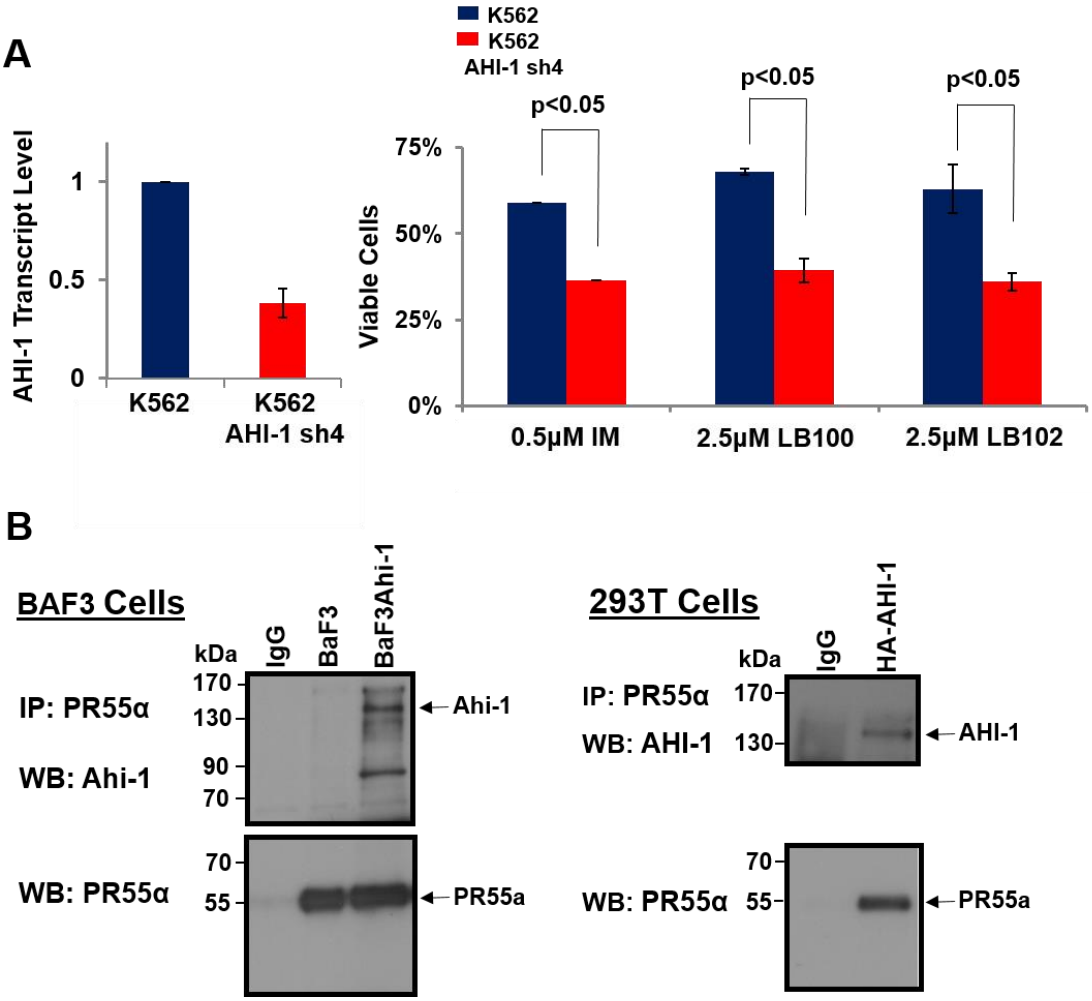



**Figure 10: Knockdown of AHI-1 Expression Mediates Response of PP2A Inhibitors in CML Cells and AHI-1 Physically Interacts with PR55α. (A)** AHI-1 transcript levels measured by Q-RT-PCR in control K562 and AHI-1-depleted K562 cells (AHI-1sh4). AHI-1 transcript levels detected in AHI-1-depleted cells were then compared to control K562 cells and normalized to GAPDH (left). Sensitivity of AHI-1-depleted cells to treatment with 0.5μM IM, 2.5μM LB100 or 2.5μM LB102 compared to control K562 cells (right). Data shown are mean ± SEM from three independent experiments. P-values were calculated using a two-tailed Student's t-test. **(B)** PR55α was immunoprecipitated from cell lysates of BaF3 cells stably transduced with full length murine Ahi-1 and 293T cells transfected with an HA tagged human full length AHI-1 construct. The immunoprecipitates were then probed with anti-Ahi-1 and anti-AHI-1 antibodies as well as an anti-PR55α antibody. Western blots shown are representative of three independent experiments.

### 3.4 Combination Treatment with PP2A Inhibitors and IM Demonstrates Synergistic Effects on BCR-ABL$^+$ Leukemic Cells and IM-resistant Cells.

To determine if IM in combination with the new PP2A inhibitors is more effective at eradicating CML cells, the biological responses of the K562 and K562 IM-resistant cell lines to treatment with IM and PP2A inhibitors, alone or in combination, were assessed with viability, proliferation, and apoptosis assays.

In K562 cells combination treatments of 0.5μM IM and 2.5μM or 5μM of the PP2A inhibitors (LB100 and LB102) significantly reduced the percentage of viable cells remaining after 48 hour treatment compared to single treatments ($P<0.01$, Figure 11A). Proliferation assays supported the greater potency of the combination treatment after both 24 hour and 48 hour treatments (> 80% inhibition, $P<0.01$, Figure 11B). Apoptosis assays assessing the percentage of Annexin V positive cells after 48 hour treatment further confirmed that the combination treatment with IM and PP2A inhibitors lead to more cell death than any drug inhibitors alone ($P<0.01$, Figure 11C). Western blots analyzing the expression of the caspase 8 and caspase 3 showed increased cleavage of the caspases with the combination treatment (Figure 11C, right). This suggests that dual inhibition of PP2A and BCR-ABL induces greater activation of the apoptotic cascade of caspase proteases.

Since IM on its own is already quite potent in K562 cells, these biological assays were performed in IM-resistant K562 cells. Similar to the parental K562 cells, viability, proliferation, and apoptosis assays showed that the combination treatment of IM and PP2A inhibitors significantly suppresses proliferation and induces apoptosis in IM-resistant cells compared to the single treatments ($P<0.01$, Figures 11A, 11B, 11C). As shown in figure 9, the two PP2A inhibitors also potently reduce PP2A activity and reduce cell viability in BV173 cells with an IC50 value of 1μM. BV173 cells are derived from a lymphoid blast crisis patient and shown to generate a lethal leukemia in mice (84). They express BCR-ABL and represent an alternative

BCR-ABL positive cell line to assess the potency of the combination treatment of the TKIs IM and DA, and PP2A inhibitors. Viability assays showed that while low concentrations of 0.5μM IM or 0.05μM DA and 0.5μM of either PP2A inhibitor alone did not affect the viability of BV173 cells, combination treatments were able to significantly reduce their viability ($P<0.01$, Figure 12A).

While statistically significant, an important question was whether dual inhibition of BCR-ABL and PP2A led to a synergistic inhibition of growth or if this combination of drugs simply led to an additive effect. A wide range of concentrations of single and combination treatments were assessed in both K562 IM-resistant and BV173 cells at a constant ratio. The Compusyn software, based on Chou-Talalay's combination index theorem (144), was used to determine whether the combination treatment was synergistic. A combination index (CI) value less than 1 indicates a synergistic interaction. The relationship between both LB100 and LB102 with IM, across a range of effective dosages (ED), was determined to be synergistic in both cell lines (Figure 12B). ED50 represents the effective dose where 50% of cells are killed by the combination treatment. At ED50, K562 IM-resistant cells had a CI value of 0.59 for the combination of IM and LB100 and 0.64 for IM and LB102. In comparison, BV173 cells had a CI value of 0.21 for the combination of IM and LB100 and 0.43 for IM and LB102 (Figure 12B).

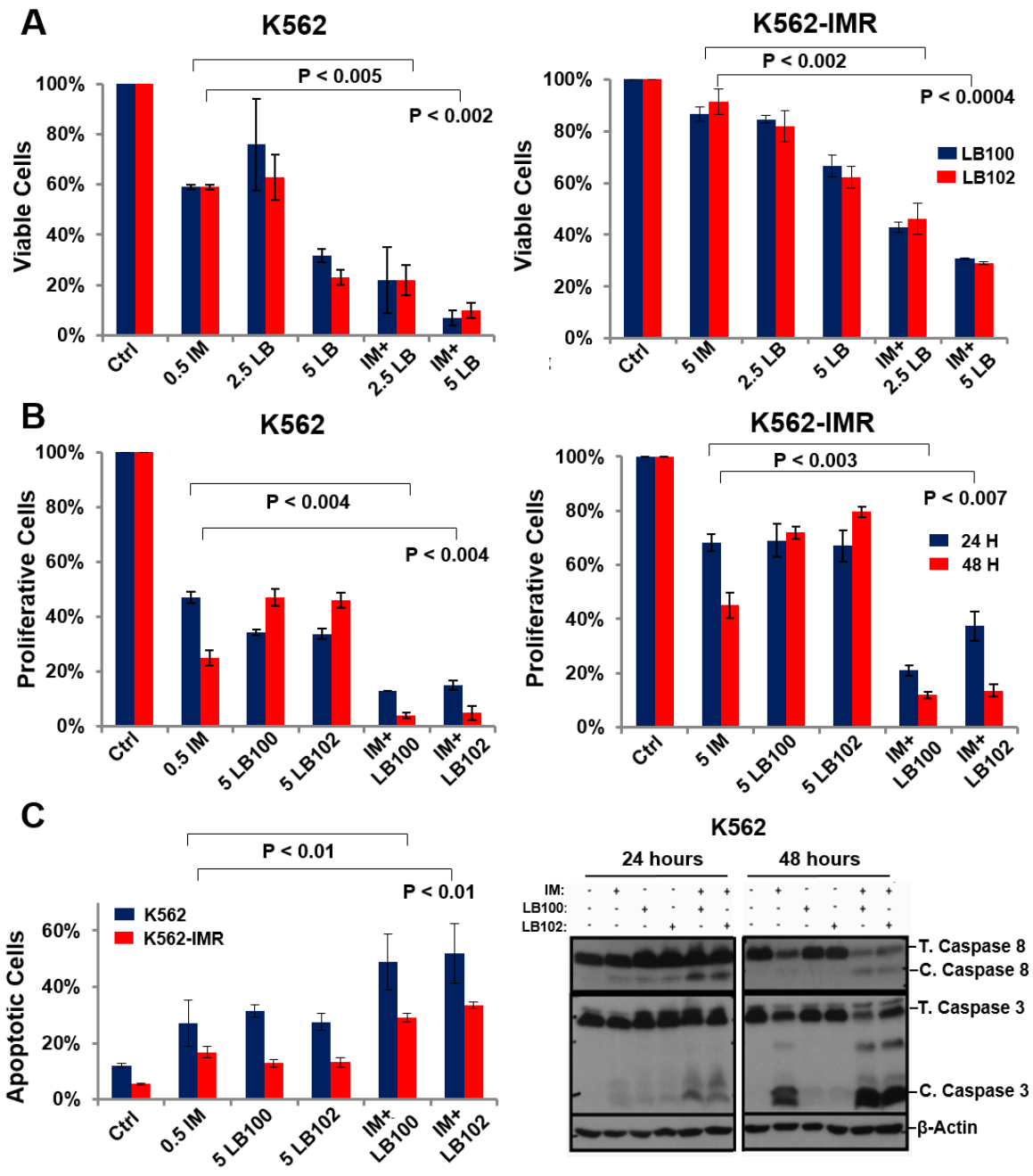



**Figure 11: Combination Treatment of PP2A Inhibitors with IM more Effectively Eradiate K562 and K562 IM-resistant Cells than Single Agents. (A)** Combination treatments of 2.5μM and 5μM of LB100 or LB102 with 0.5μM or 5μM IM for 48 hours reduced viability of K562 and K562 IM-resistant cells (K562-IMR), as compared to single agent treatments. **(B)** Combination treatments of LB100 or LB102 with IM for 24 hours and 48 hours reduced proliferation of these cells. **(C)** The same combination treatments induced apoptosis in K562 and K562-IMR cells compared to single agent treatments after 48 hours (left). Western blot analysis of total (T. Caspase 8) and cleaved caspase 8 (C. Caspase 8) and caspase 3 in the same treated K562 cells. Data shown are mean ± SEM from three independent experiments. P-values were calculated using a two-tailed Student's t-test. Ctrl=untreated cells.

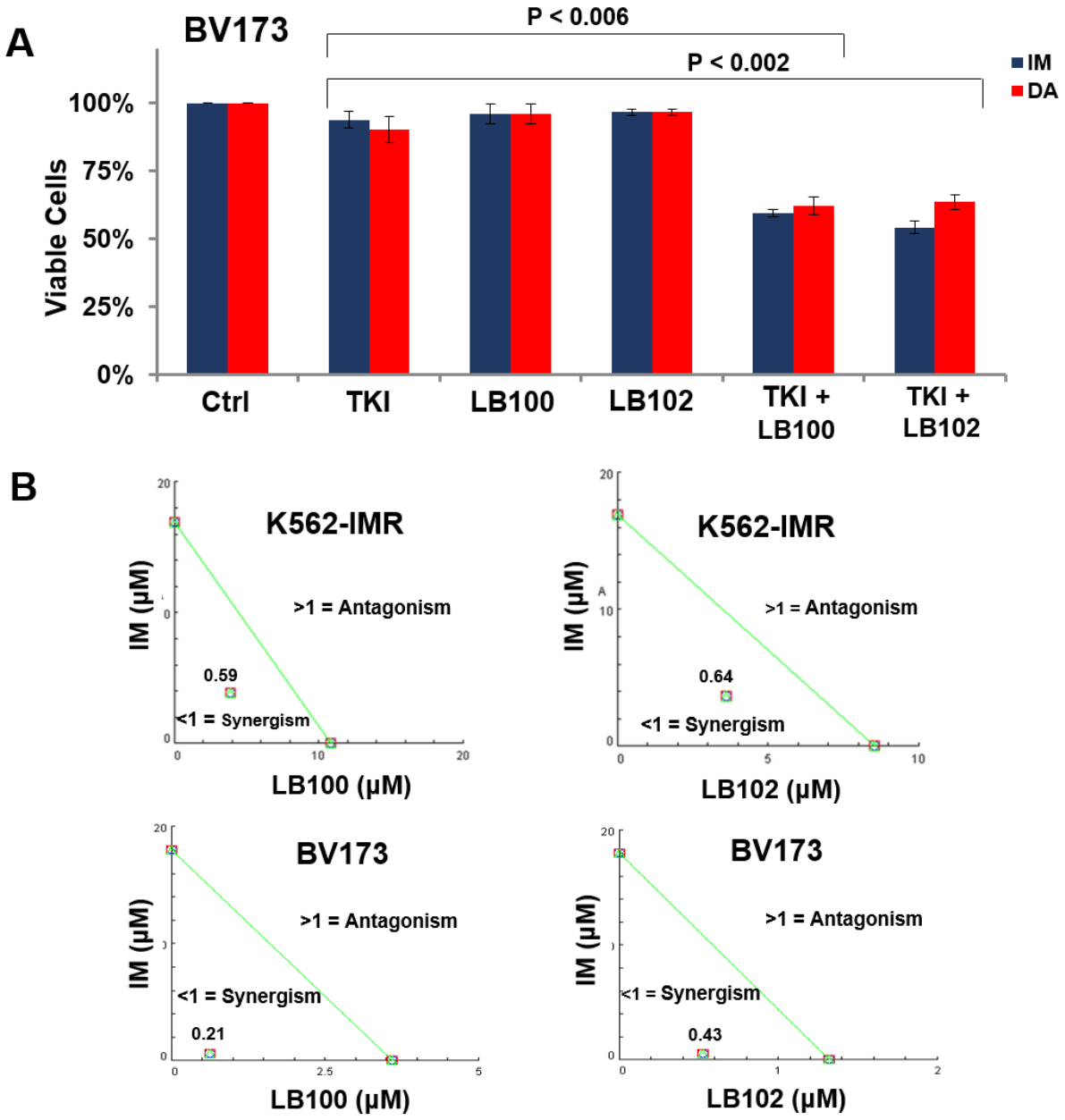



**Figure 12: Combination Treatment of PP2A Inhibitors with IM Exhibits Synergistic Cytotoxicity in K562 IM-resistant and BV173 Cells. (A)** Combination treatments of 0.5μM IM or 50nM dasatinib (DA) with 0.5μM of LB100 or LB102 in BV173 cells cultured for 48 hours more effectively suppressed growth of BV173 cells than a single agent. Data shown are mean ± SEM from three independent experiments. P-values were calculated using a two-tailed Student's t-test. **(B)** Isobologram analysis of combining IM with LB100 or LB102 in K562 IM-resistant (K562-IMR) and BV173 cells. Each line designates a combination index (CI) value where CI=1 (additive effect). CI<1 indicates synergism while CI>1 indicates antagonism. The combination data points indicate the synergism at an effective dose (ED50). Ctrl=untreated cells.

### 3.5 Combination Treatment with PP2A Inhibitors and TKIs more Effectively Targets CML Stem/progenitor Cells.

To further investigate the potential of combining TKIs with PP2A inhibitors, the ability of the drugs to prevent growth of primary CML $CD34^+$ stem/progenitor cells was assessed. Primary blood or bone marrow samples were obtained from newly diagnosed patients with chronic phase CML who had not been treated with any TKI therapy. Samples were obtained specifically from patients who would then go on to be classified as IM-nonresponders as this is the subset of patients who would benefit most from alternative therapy. $CD34^+$ cells were isolated and purified so that the ability of the drugs to target the more primitive leukemic stem and progenitor cells could be assessed.

Viability assays showed that after 72 hour treatment with IM, DA, LB100, or LB102 alone, approximately 40-50% of cells were still viable. Combination treatments of TKIs (IM or DA) with PP2A inhibitors (LB100 or LB102) led to a significantly greater reduction in the percentage of viable cells remaining after treatment (< 20%, $P<0.02$, Figure 13A). Normal bone marrow (NBM) samples from healthy donors were also obtained to assess the toxicity of the drug treatments. Individual treatments with 2.5μM of the PP2A inhibitors resulted in only slightly lower remaining percentage of viable cells than the IM and DA treatments (>80%). Similar observation were obtained by the combinations of TKIs with LB100 or LB102.

CFC assays were also performed with these samples to assess their ability to differentiate and form colonies. The primary samples were cultured in semi solid medium with or without the inhibitors for 2 weeks. Colonies were then counted and the treatments were compared to the untreated control. Similar to the viability assays performed in liquid culture, the CFCs showed that after combination treatments of IM or DA with LB100 or LB102 significantly reduced the ability of the CML primary cells to form colonies ($P<0.05$, Figure 13B). Importantly,

single treatments with LB100 or LB102 versus CAN as well as combinations of IM with LB100 or LB102 versus IM with CAN showed much less toxicity in the NBM CFCs ($P<0.01$, Figure 13C).

These results show the potential of combining TKIs with PP2A inhibitors to target the CML stem/progenitor cells, a population that has so far resisted treatment with TKI monotherapies. While CAN was limited in its potential due to concerns regarding its toxicity, the two new PP2A inhibitors LB100 and LB102 are much less toxic. A synergistic approach resulting in great inhibition of growth in TKI-resistant cells has been identified but the mechanism which allows for the success of this combined approach is unclear. The next question to investigate was how inhibition of PP2A induced cell death and mechanistically how it is able to synergize with TKIs to more potently target the CML cells.

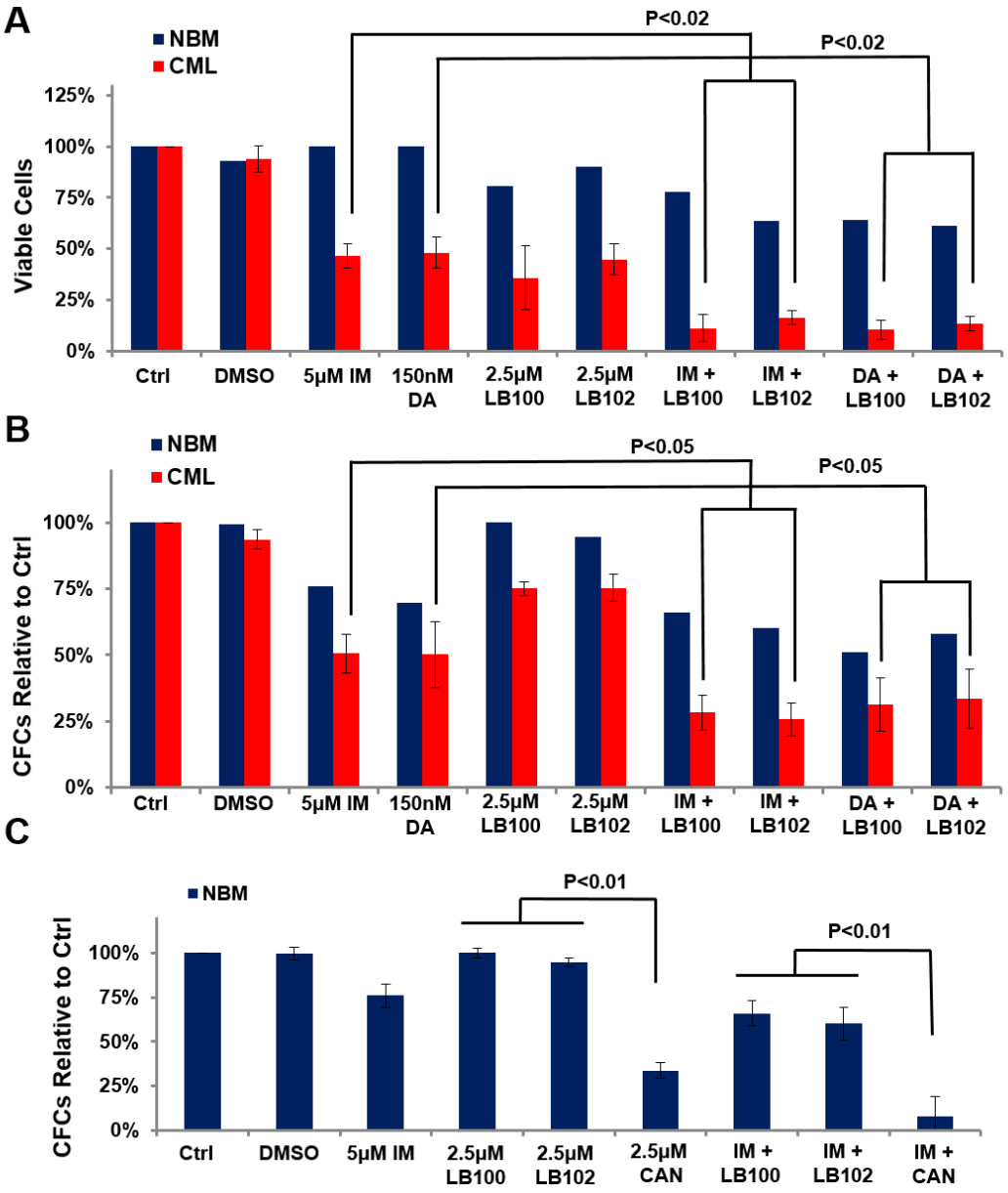



**Figure 13: Combination Treatment with PP2A Inhibitors and TKIs more Effectively Targets CML Stem/progenitor Cells but not Normal BM Cells. (A)** Viability of $CD34^+$ normal bone marrow (NBM, n=2) and CML cells from IM-nonresponders (n=3) cultured for 72 hours with single and combination treatments of IM, dasatinib (DA) and PP2A inhibitors (LB100 and LB102). Data shown are mean ± SEM for assays with CML samples and the average for assays with NBM samples**. (B)** $CD34^+$ NBM and CML cells were plated in colony forming cell (CFC) assays with single and combination treatments of TKIs and PP2A inhibitors. CFCs were expressed as a % of values obtained in control plates where no drugs were added. Data shown for assays with CML cells are mean ± SEM of independent experiments with 4 CML patient samples. Data shown for assays with NBM cells are averages of experiments with 2 NBM patient samples. **(C)** Comparison of toxicity of new PP2A inhibitors (LB100 and LB102) versus cantharidin (CAN) in $CD34^+$ NBM cells plated in CFC assays. Data shown are mean ± SEM of independent experiments with 3 NBM samples. P-values were calculated using a two-tailed Student's t-test

### 3.6 Inhibition of PP2A Disrupts Cell Cycle Control and Induces Mitotic Arrest.

It has been well documented that PP2A has a major role in the maintenance of normal cell division (108, 109). In particular, the PR55α-PP2A heterotrimeric complex is thought to prevent transition through mitosis (103). Given the importance of PP2A in regulating the cell cycle and the identification of PR55α as an AHI-1 interacting partner (Figure 10B), the suppression of growth observed through inhibition of PP2A activity could potentially result from interference to the control of cell cycle.

Propidium iodide (PI) was used to assess cell cycle distribution. PI binds to nucleic acids and the amount of fluorescent DNA in the cell can be quantified using flow cytometry. Following DNA replication, the fluorescence of cells in G2 will be twice as high as in G1 phase and these 2 peaks were used to quantify the % of cells in each stage of the cell cycle. K562 and K562 IM-resistant cells were subjected to treatments of 0.5μM or 5μM IM, and 1μM, 2.5μM and 5μM of LB100 or LB102. The cells were treated for 18 hours, fixed with 70% methanol, stained with PI and then analyzed by flow cytometry. In untreated control cells, the majority of the population was in G1/S phase (Figure 14). Interestingly for both cell lines, while single treatment with IM did not affect the cell cycle distribution of the cells, treatment with either of the PP2A inhibitors led to a significant, dosage dependent shift in the population towards G2/M phase ($P<0.05$, Figure 14A & B). Combination treatments of PP2A inhibitors with IM led to a similar shift suggesting that the IM treatment does not contribute to the observed shift in the cell cycle distribution (Figure 14A). This suggests PP2A inhibition alone is sufficient to induce the arrest in G2/M phase which is reflected in the increased percentage of cells in this phase of the cell cycle.

It has been suggested that improper cell cycle progression caused by premature entry into G2/M as well as deficient exit from mitosis can induce cell death through mitotic catastrophe (111). When looking at the cells under a regular light microscope, some of the cells seemed

distorted while dividing after treatment with PP2A inhibitors. The mitotic spindle is an essential component of the cell machinery that allows cells to divide properly. Disruption of the spindle is a key feature of mitotic catastrophe. To determine if treatment with PP2A inhibitors alone can lead to mitotic catastrophe in BCR-ABL$^+$ cells, K562 cells were synchronized in S phase through a double thymidine block then treated with 5μM of LB100 or LB102. The cells were fixed and permeablized on slides with methanol and then stained with an anti-α-tubulin antibody followed by mounting with DAPI to distinguish the nucleus. Thecells were then assessed by confocal microscopy. One of the distinguishing characteristics of mitotic catastrophe is a disrupted spindle with multipolar cell division. This was observed after treatment with the PP2A inhibitors, while no such disruption was observed with the untreated cells (Figure 15A). The percentage of catastrophic cells in multiple fields of view for each treatment was determined. Treatment with 5μM of LB100 or LB102 induced significantly more catastrophic cells with about 40% of the synchronized cells going into mitotic catastrophe after treatment with either LB100 or LB102 ($P<0.001$, Figure 15B). This data confirms the findings from other studies that PP2A has a role in regulating mitosis in cancer cells and demonstrated a similar effect in BCR-ABL$^+$ leukemic cells. It suggests that induction of mitotic catastrophe may be a mechanism by which PP2A inhibition is able to prevent proliferation and induce cell death. However since combination treatments of these PP2A inhibitors with IM did not significantly affect the percentage of cells arrested in G2/M and their consequent catastrophe, it is unlikely that the synergistic combination effect observed with dual inhibition of BCR-ABL and PP2A is due to disruption of the cell cycle.

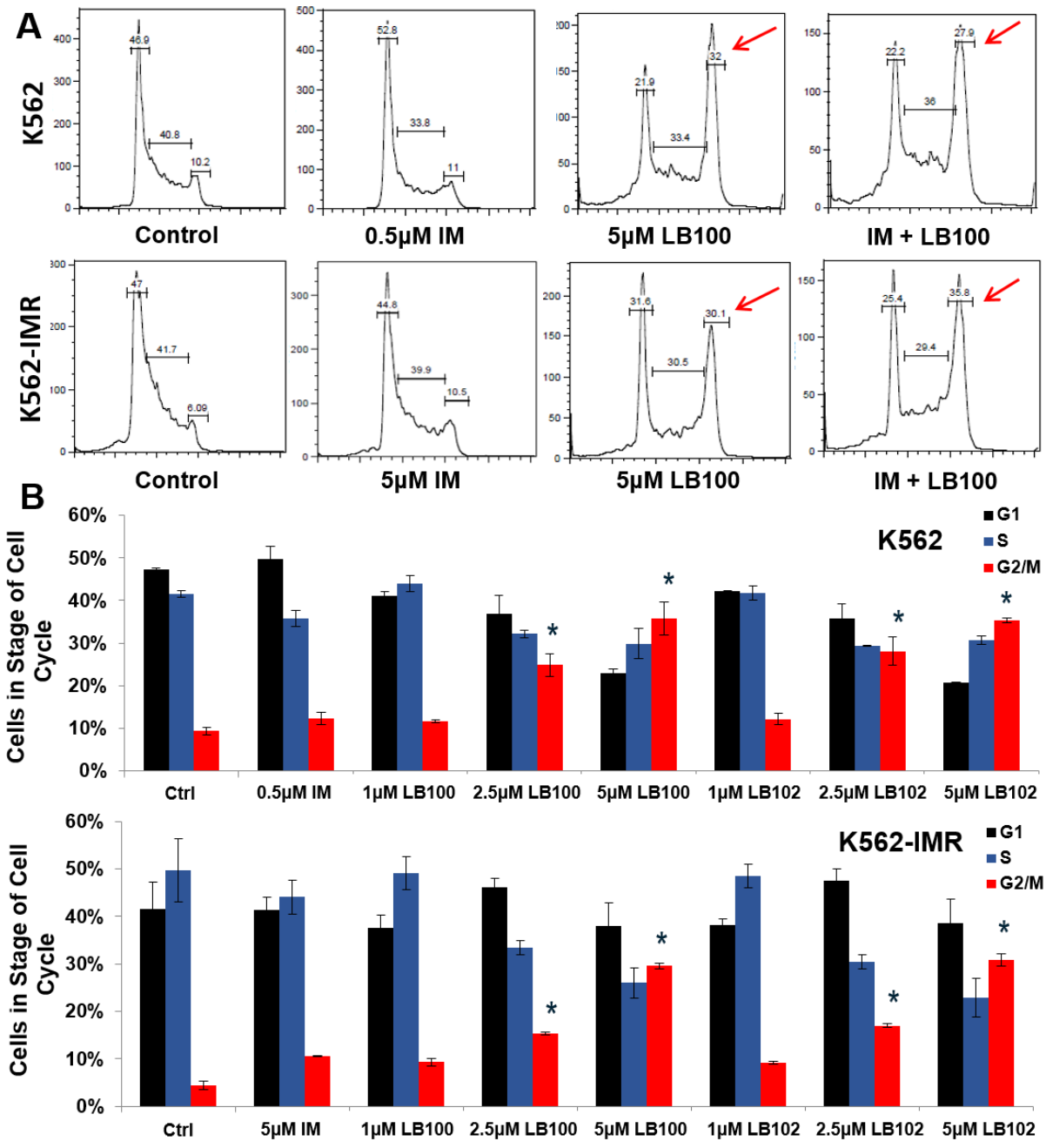



**Figure 14: Inhibition of PP2A Disrupts Cell Cycle Control. (A)** Representative FACS plots showing the shift in cell cycle distribution in K562 and K562 IM-Resistant cells (K562-IMR) after treatment for 18 hours with 5μM LB100 alone or in combination with IM. **(B)** Percentage of K562 and K562-IMR cells in each phase of the cell cycle after treatment for 18 hours with various concentrations of LB100 and LB102 or IM. Data shown are mean ± SEM from three independent experiments. P-values were calculated using a two-tailed Student's t-test. Asterisk indicates significant difference ($P<0.05$) between untreated cells (Ctrl) and drug-treated cells.

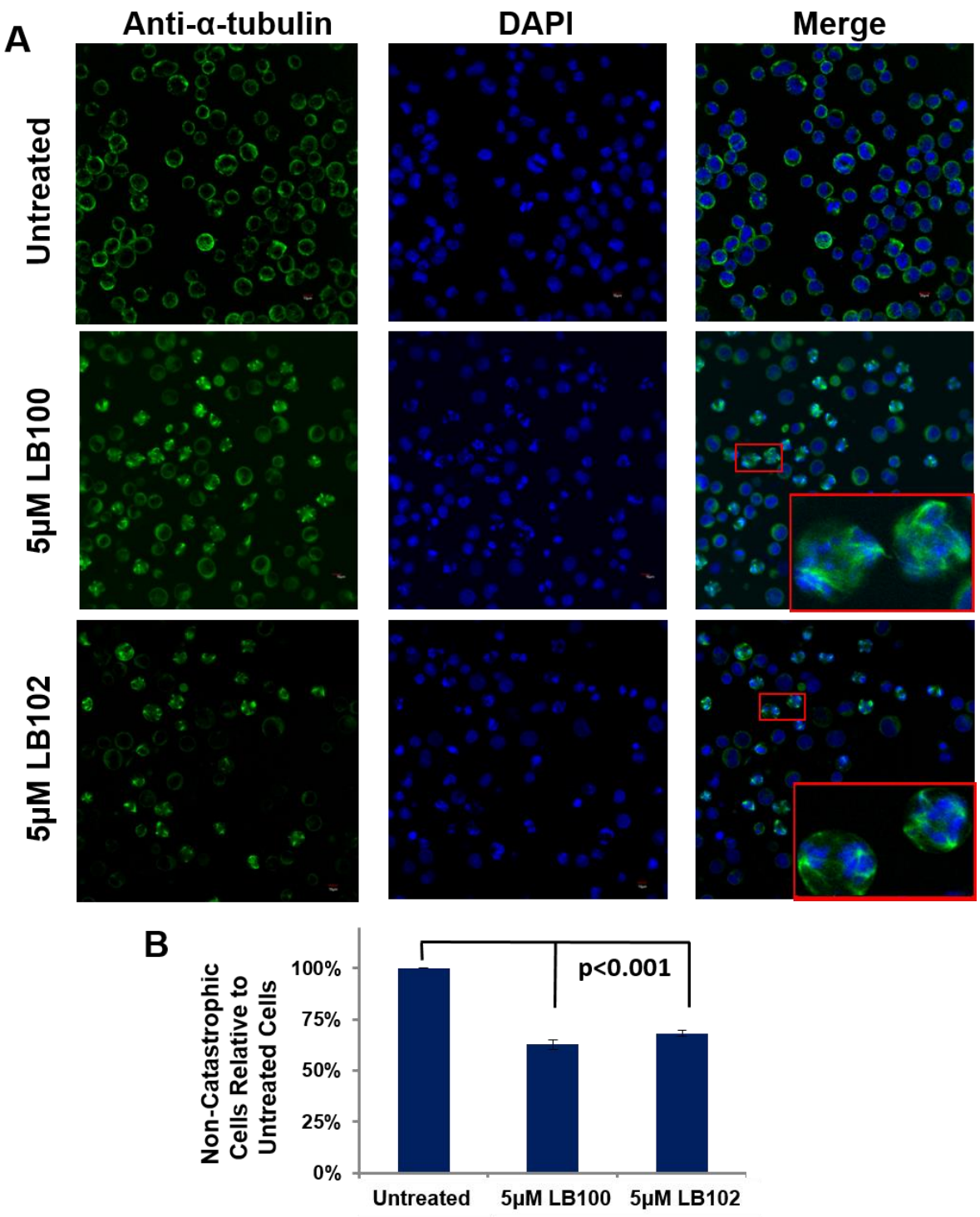



**Figure 15: Inhibition of PP2A Induces Mitotic Arrest in CML Cells. (A)** Representative images of confocal microscopy analysis of K562 cells stained with an anti-α-tubulin antibody (green) and DNA binding dye DAPI (blue) in the presence of 5μM LB100 or 5μM LB102. Images are from a 20X lens and scale bars are as indicated. **(B)** Percentage of non-catastrophic cells remaining after treatment with 5μM LB100 or 5μM LB102. Values were determined by counting the number of catastrophic cells in three fields of view. Data shown are mean ± SEM from five independent experiments.

### 3.7 PP2A Inhibition in Combination with IM Affects Phosphorylation and Protein Expression of Key Signalling Proteins in BCR-ABL-mediated Pathway.

To further investigate how the combination of BCR-ABL and PP2A inhibition could effectively synergize and eradicate leukemic cells, Western blots were run to examine the expression level of proteins known to play a major role in the BCR-ABL signalling pathway. It has been shown that treatment of K562 cells with IM, or even combination treatments of IM with JAK2 inhibitors results in a reduction in phosphorylation of BCR-ABL, JAK2, and STAT5 but not much change is observed at the total protein expression level of these proteins (84, 85).

In K562 IM-resistant cells, 48 hour treatment with a high dose of 5μM IM reduced the amount of general tyrosine phosphorylation of BCR-ABL (4G10), phospho-JAK2 and phospho-STAT5 but as expected there was no change in the total protein levels of these proteins (Figure 16). IM treatment alone slightly reduced the protein expression of AHI-1 and β-catenin. Treatments with 5μM LB100 or LB102 alone did not affect the phosphorylated or total protein levels of any of these proteins (Figure 16). In contrast, the combination treatments of 5μM IM with 5μM LB100 or LB102 resulted in a remarkable reduction in the protein expression of BCR-ABL, JAK2, STAT5, AHI-1, and β-catenin. This was a fascinating observation because this dramatic change was only observed after the combination treatments and correlates with the synergistic reduction in growth of these cells after treatment with both BCR-ABL and PP2A inhibitors. Similarly, while IM alone did not have any effect on the phosphorylation of the Tyr177 residue of BCR-ABL, the combination treatment resulted in dramatically reduced phosphorylation of this BCR-ABL residue. This residue is critical for BCR-ABL's ability to activate the PI3K/AKT and RAS/MAPK pathways (70-72). Correspondingly, the combination treatment also greatly reduced the levels of phosphorylated and total AKT, phosphorylated and total P38, as well as JNK. However, there was no reduction in total ERK. Furthermore, despite the inhibition of PP2A activity, there was no change in the expression of either the catalytic

subunit PP2A-C or the potential AHI-1 interacting PP2A regulatory subunit PR55α. This indicates that inhibition of PP2A activity does not degrade the protein, or induce increased expression through negative feedback. There was also no change in the expression of SET, the endogenous inhibitor of PP2A.

Another interesting observation was that while IM alone partially reduced the total protein expression of AHI-1, there a residual amount was still observed. In contrast, after the combination treatment there was almost a complete absence of AHI-1. This is particularly interesting because the strategy of inhibiting PP2A initially came through the drug screen specifically looking for AHI-1 related inhibitory compounds. It has been suggested that the presence of a BCR-ABL-AHI-1-JAK2 complex enables BCR-ABL to drive leukemic activities. AHI-1 likely has a role in the stabilization of this complex since it does not have any kinase or phosphatase activity but has many motifs known to mediate protein-protein interactions. With such a reduction in levels of the AHI-1 protein, this could compromise the stability of this protein interaction complex resulting in the observed reduction in the amount of the other important proteins in the BCR-ABL signalling pathway. There is also unpublished data suggesting that AHI-1 and β-catenin can also directly interact. While β-catenin's potential role in this complex of proteins has yet to be established, it has been shown that β-catenin interacts with both BCR-ABL and PP2A (61, 78).

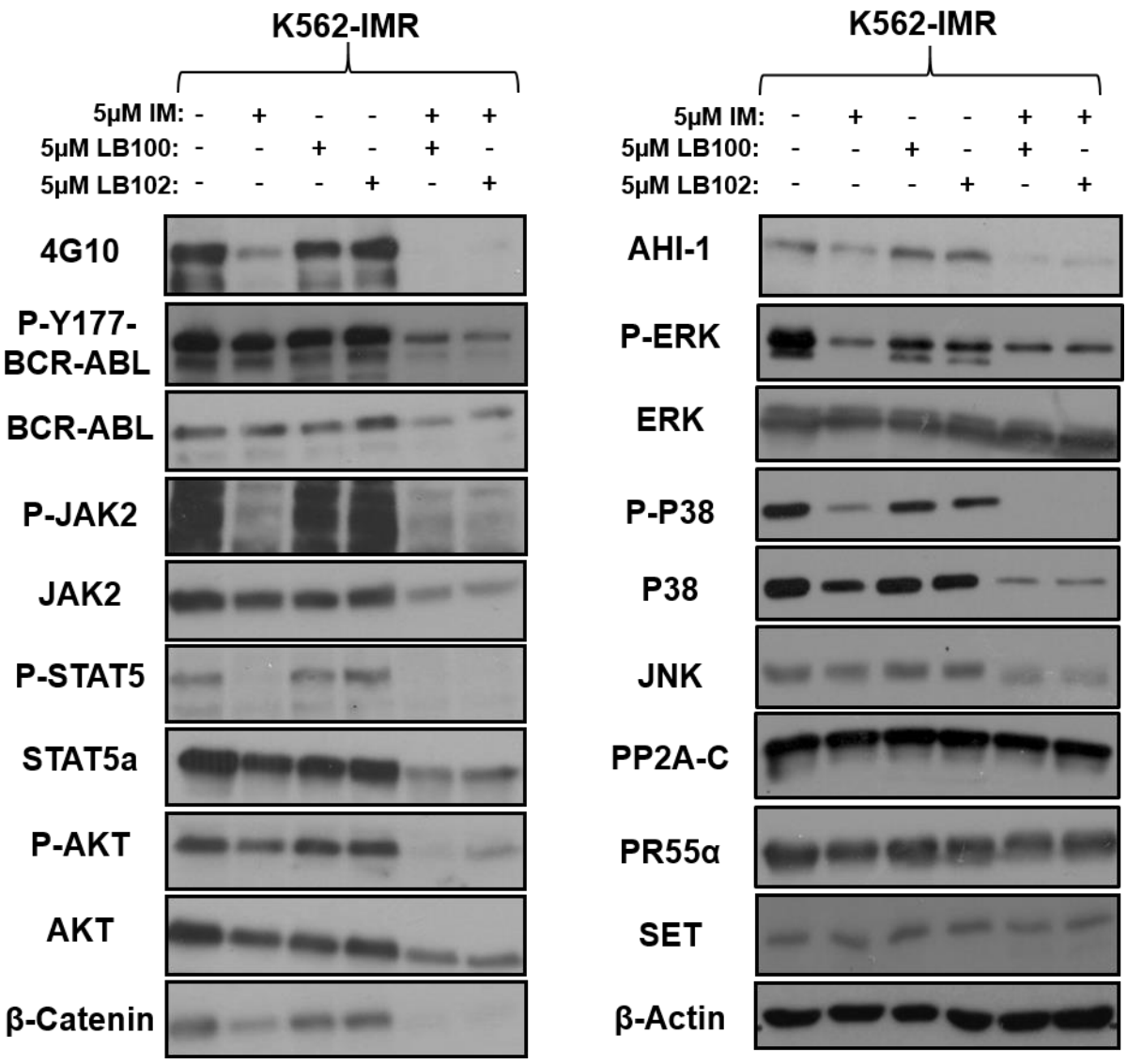



**Figure 16: PP2A Inhibition in Combination with IM Affects Phosphorylation and Protein Expression of Key Signalling Proteins in BCR-ABL-mediated Pathway.** K562 IM-resistant cells (K652-IMR) were treated with the indicated inhibitors, either alone or in combination for 48 hours. The expression and phosphorylation of several key signalling proteins were analyzed by western blotting. Data shown are representative blots from three independent experiments.

### 3.8 Dual Inhibition of PP2A and BCR-ABL Modulates Phosphorylation and Protein Expression of β-catenin.

PP2A has been shown to play a role in the stabilization of β-catenin (78, 92, 106, 114, 115, 122, 145-147). Specifically it is thought to antagonize the kinase activities of GSK3β and casein kinase I α (CKIα). Normally these kinases would phosphorylate the thr41 and ser45 residues of β-catenin to signal it for degradation. In agreement with this role, inhibition of PP2A through treatment with LB100 or LB102 for 24 hours led to increased phosphorylation of the thr41, ser45 residues of β-catenin (Figure 17A). These residues were similarly more highly phosphorylated with the combination treatment while there was no increase in phosphorylation observed after treatment with IM only (Figure 17A). This suggests that increased phosphorylation of these residues is due to PP2A inhibition alone rather than IM. Interestingly, PP2A inhibition also led to increased phosphorylation of the ser9 residue of GSK3β. The GSK3β kinase is inactivated when it is phosphorylated on its ser9 residue (77, 78). At this 24 hour treatment time point, β-catenin expression after the combination treatment was still comparable to untreated cells despite a slight reduction with IM alone. The GSK3β total protein expression levels also remained the same after the various treatments. The ability of the combination treatment to drastically reduce total β-catenin expression was not observed until after 48 hours treatment.

To determine if the phosphorylation of the thr41, ser45 residues is still dependent on GSK3β kinase activity, K562 cells were treated with lithium chloride (LiCl), an inhibitor of GSK3β. Western blots showed that inhibition of GSK3β with LiCl treatment alone for 24 hours led to a dosage dependent accumulation of ser9 phosphorylated GSK3β (Figure 17B). The amount of total GSK3β protein remained unchanged, indicating that LiCl treatment led to a greater proportion of inactivated GSK3β. At the same time there was a drastic dosage dependent increase in the levels of total β-catenin expression which correlated with a dosage

dependent decrease in the amount of phosphorylated β-catenin. This confirms that treatment with LiCl alone prevents GSK3β kinase activity resulting in greater stabilization and accumulation of β-catenin.

The increasing dosages of LiCl were also combined with 5μM of LB100 to determine if inhibiting GSK3β kinase activity could reverse the increased phosphorylation induced by PP2A inhibition. Interestingly, compared to treatment with 5μM of LB100 alone, there were no changes in phosphorylated GSK3β and levels of phosphorylated β-catenin were only marginally decreased (Figure 17B). While β-catenin accumulated with increasing concentration of LiCl, this accumulation was not observed when LiCl treatment was combined with LB100. Ultimately treatment with both LiCl and LB100 still led to increased levels of phosphorylated β-catenin. Despite inhibition of GSK3β activity, this increased phosphorylation induced by PP2A inhibition was not reversed (Figure 17B). These results suggest that the phosphorylation of the tyr41 and ser45 residues of β-catenin after PP2A inhibition is not dependent on GSK3β activity (Figure 17C).

BCR-ABL has been shown to phosphorylate β-catenin on its tyr86 residue (61). After 24 hour treatment with IM alone, there was an observed reduction in the phosphorylation of the tyr86 residue of β-catenin (Figure 17A). However at this same time point, the phosphorylation status was unchanged after combination treatment with both IM and LB100 or LB102. As described earlier, at 24 hours after the combination treatment there was no change in the levels of total β-catenin either. In contrast, 48 hours the combination treatment led to an almost complete reduction in the levels of tyr86 phosphorylated BCR-ABL. Similarly it was only after 48 hours that the combination treatment led to the drastic reduction in total β-catenin expression (Figure 17A).

Put together, the drastic decrease in total β-catenin expression after the combination treatment with PP2A inhibitors and IM for 48 hours was preceded by changes in its

phosphorylation status. Deregulated BCR-ABL and PP2A activities have been separately implicated to have a role in the stabilization of β-catenin, thus encouraging its ability to promote downstream transcription activation. Importantly, dual inhibition of BCR-ABL and PP2A could lead to degradation of β-catenin and the consequent repression of downstream transcriptional activity. Thus, β-catenin represents a potential target that this combination treatment may converge on (Figure 17C).

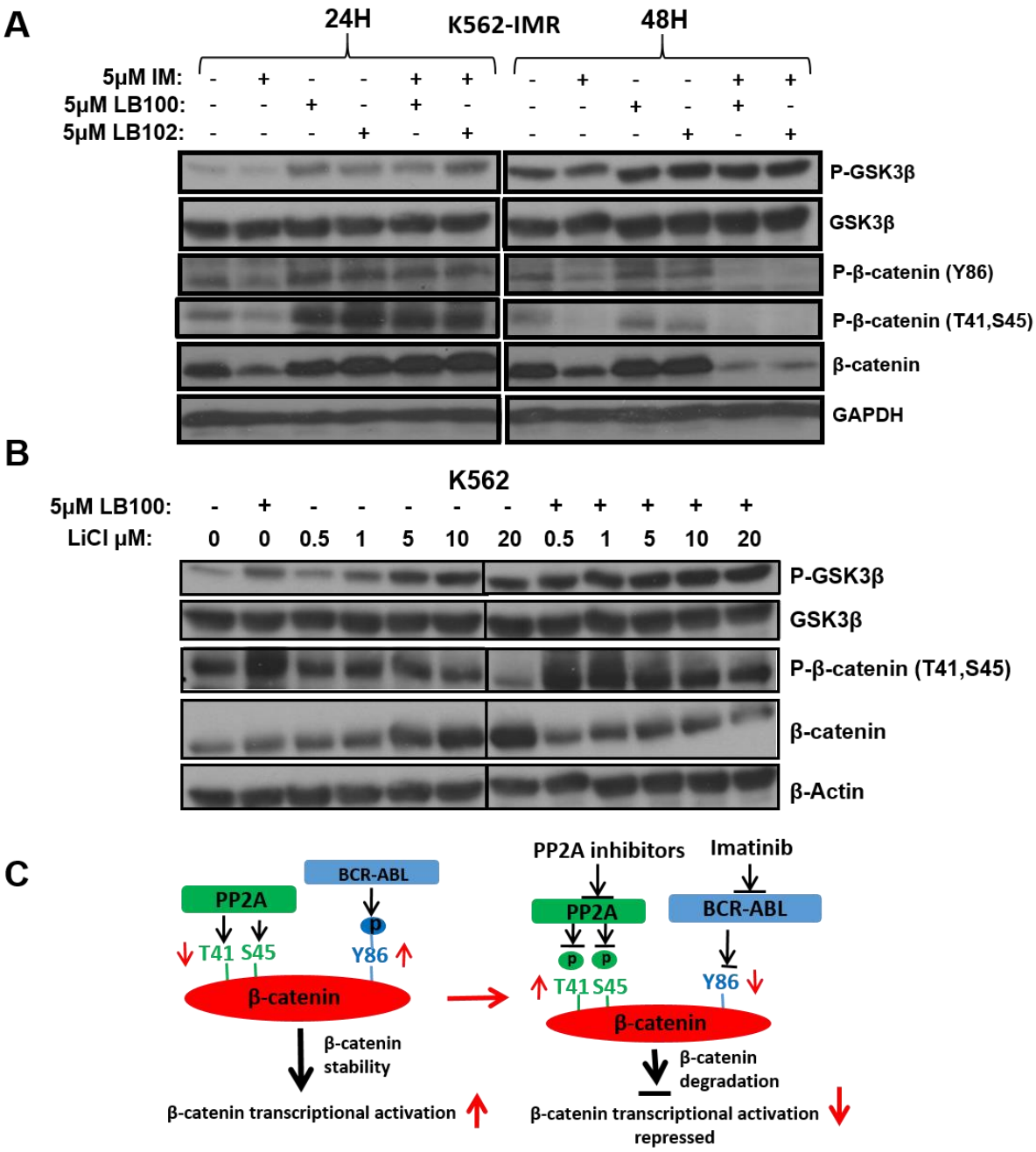



**Figure 17: Dual Inhibition of BCR-ABL and PP2A Modulates Phosphorylation and Protein Expression of β-catenin. (A)** K562 IM-resistant cells (K562-IMR) were treated with the indicated inhibitors, either alone or in combination for 24 hours and 48 hours. Protein expression and phosphorylation of GSK3β and β-catenin were analyzed by western blotting. Data shown are representative blots from three independent experiments. **(B)** K562 cells were treated with varying concentrations of Lithium Chloride (LiCl) as well as 5μM LB100 for 24 hours. Protein expression and phosphorylation of GSK3β and β-catenin were analyzed by western blotting. Data shown are representative blots from three independent experiments. **(C)** Schematic diagram of how BCR-ABL-mediated tyrosine phosphorylation (Tyr86) and PP2A-mediated threonine and serine dephosphorylation (Thr41, Ser45) changes in response to TKIs and PP2A inhibitors in CML cells.

## Chapter 4: Discussion

This study was originally initiated with the goal of identifying a potential AHI-1 specific compound to target and eradicate the TKI-insensitive CML stem and progenitor cells. The drug screen also allowed for the identification of a compound that could potentially be used in combination with TKIs to enhance their effectiveness on these resistant cells. Through this approach Cantharidin (CAN) was identified, leading to this investigation into the role of PP2A in CML and the therapeutic potential of inhibiting this phosphatase in combination with TKIs. Preliminary studies assessing the potency of CAN indicated that PP2A inhibition alone could induce cell death and prevent the proliferation of CML cells. However, significant toxicity issues meant that CAN was limited in its potential as a clinical drug. These studies indicated that PP2A was a potential druggable target and so two new, selective inhibitors, LB100 and LB102, were identified to further this investigation.

While there are several studies investigating the use of PP2A inhibitors in cancer, they were mainly conducted in solid tumour models (130, 131, 133, 137, 139, 148). Both LB100 and LB102 were shown to reduce PP2A activity but it was important to demonstrate their efficacy in hematologic leukemic cells. Three cell lines originally derived from CML patients were used: K562 cells are myeloid cells while BV173 cells are B-lymphoid blast crisis cells. An IM resistant cell line generated from K562 cells was also used. Importantly, the two inhibitors specifically inhibited PP2A activity in all three cell lines (Figure 9B). Furthermore, similar to treatment with CAN, both LB100 and LB102 induced cell death in the K562, K562 IM-resistant, and BV173 cells (Figure 9A). While a low dose of IM (0.5μM) is sufficient to reduce viability of K562 cells to 50% after 48 hour treatment (Figure 11A), a 10-fold increase to 5μM only reduced viability of the K562 IM-resistant cells to 80% (Figure 11A). In contrast, following 48 hours treatment with the two PP2A inhibitors, the difference in sensitivity between K562 IM-resistant cells and parental K562 cells to treatment was not as great with IC50s of approximately 5μM versus 3μM

respectively. This suggests that the reduction in viability induced by PP2A inhibition alone could, at least partially, occur through a BCR-ABL-independent mechanism.

In this study, extensive biological assays assessing viability, proliferation, and apoptosis were performed in CML cell line models to determine whether combination of PP2A inhibitors with IM is more effective to eradicate CML cells, including IM-resistant cells. Interestingly, the combination treatment significantly suppressed cell proliferation and induced apoptosis compared to treatment with IM alone. The enhanced potency of this combination treatment was also observed in K562 IM-resistant cells and BV173 cells (Figures 11 and 12). These results compare well to other studies using these PP2A inhibitors in combination with chemotherapeutic drugs like doxorubicin (130, 134, 137), cisplatin (131, 132, 148), and temozolomide (130, 135). LB100 has also been used in combination with radiation treatment in nasopharyngeal carcinomas (136), pancreatic cancer (139), and glioblastomas (149). These studies support PP2A inhibition as a highly potent means to sensitize cancer cells to chemotherapy, molecularly targeted therapies or radiation treatment (140).

To further assess the potential of this drug combination, primary samples obtained from the blood or bone marrow of CML patients and healthy donors were acquired. While the samples were taken from patients at diagnosis, the samples were specifically chosen from patients who would go on to be classed as TKI-nonresponders. In addition $CD34^+$ cells were isolated from these samples in order to specifically test the drugs on the more primitive cells as this population has been shown to be insensitive to TKI treatment alone (42, 43, 45, 46, 150). Demonstrating the potency of this combination treatment to suppress the growth of primitive $CD34^+$ cells derived from TKI-resistant patients emphasizes the clinical relevance of these results. Indeed, viability assays on $CD34^+$ CML samples showed that combination treatments of IM or DA with the PP2A inhibitors were significantly more effective than the single agent treatments (Figure 13A). CFC assays were also performed to assess the ability of the inhibitors

to prevent myeloid differentiation and colony formation. Consistent with the results obtained in the CML cell line models, the combination treatment was significantly more effective at preventing colony growth compared to the single agents (Figure 13B). Furthermore, this combination treatment was selective in targeting CML stem/progenitor cells compared to the same cells purified from normal bone marrow samples (Figure 13A and 13B). Importantly, CFC assays on normal bone marrow samples demonstrated that these new PP2A inhibitors are drastically less toxic than CAN, highlighting that both LB100 and LB102 have the potential to overcome toxicity issues associated with CAN (Figure 13C). In fact LB100 is now in phase 1 clinical trials as a chemotherapy sensitizer for the treatment of solid tumours (NCT01837667). To further assess the ability of this combination treatment to specifically target the leukemic stem cells, long-term culture initiating cell (LTC-IC) assays will be more reflective of the ability of the combination treatment to effectively eradiate leukemic stem cells and their myeloid progeny. In addition, given the importance of the niche microenvironment in leukemia development and maintenance, *in vivo* experiments to assess the ability of the drugs to block leukemia development in mice would show stronger evidence for the potential of these drugs combinations to treat CML.

Having demonstrated the potency of dual inhibition of BCR-ABL and PP2A in CML cell lines as well as primary samples derived from CML patients, the next question to be addressed was whether this was a synergistic or additive combination effect. A wide range of concentrations of single treatments and combinations were assessed in both K562 IM-resistant and BV173 cell lines at a constant ratio. The compusyn software, based on Chou-Talalay's combination index theorem (144), was used to determine whether the combination treatment was synergistic. Based on the ED50, the combination treatment was determined to be synergistic in both cell lines (Figure 12B). The synergism of combining TKIs and PP2A inhibitors could be further supported by repeating this experiment in primary samples.

Nevertheless, the synergistic response of these cells to the drug combination suggests that there is an intersection of the signalling pathways that BCR-ABL and PP2A are involved in. As discussed earlier, AHI-1 is a potential candidate given its role in mediating BCR-ABL signalling and the PP2A inhibitors were identified in AHI-1-overexpresssed CML cells. An important question was what role AHI-1 played in this. Depletion of AHI-1 in K562 cells was previously shown to increase sensitivity to IM treatment (59). The same AHI-1 depleted K562 cells were used to assess how manipulation of AHI-1 might affect the sensitivity to treatment with the PP2A inhibitors. Similar to IM treatment, the AHI-1 depleted cells were significantly more sensitive to treatments of 2.5uM of LB100 or LB102 alone (Figure 10A). AHI-1 is thought to affect sensitivity to TKI treatment through its critical role in regulation of a protein interaction complex involving the two kinases BCR-ABL and JAK2 (84). Since, like IM treatment, PP2A inhibition more effectively reduced viability when AHI-1 was depleted, PP2A could be affected by or potentially have a role in this protein complex. Further investigation into how manipulation of AHI-1, particularly overexpression of AHI-1, affects sensitivity to PP2A inhibition would provide additional evidence for this potential link. In addition, a recent investigation into AHI-1 interacting partners identified the regulatory PP2A subunit PR55α to be a potential interacting protein with AHI-1 by IP-mass spectrometry (unpublished data from my lab). To confirm this interaction, IP experiments were performed in Ahi-1/AHI-1-transduced BaF3 and 293T cells. Since parental BaF3 and 293T cells express little AHI-1, these cell lines provided a clean background to test the interaction between AHI-1 and PR55α. The parental cell lines also represent a good negative control. Indeed, the results confirmed that AHI-1 and PR55α interact physically (Figure 10B). These two cell line models provide an opportunity for further investigation into this protein interaction network involving AHI-1, PP2A PR55α, BCR-ABL and other key protein interacting partners. For example, overexpressing mutant constructs of AHI-1 and testing for their interaction with PR55α would determine which domain of AHI-1 is involved in this interaction. Given that the WD40 domain of AHI-1 interacts with BCR-ABL and the N-

terminal domain interacts with JAK2 (84), identifying which domain PR55α specifically interacts with could potentially establish whether PR55α has a role in the AHI-1-mediated interaction complex in CML.

PP2A has an important role in cell cycle regulation and manipulation of PP2A activity can affect how cells transition through this highly regulated process (97, 109, 126, 151). In particular, it has been shown that the PR55 family of PP2A has a specific role in the regulation of the G2/M transition point between interphase and mitosis (151-153). The cyclin B-Cdk1 kinase complex is a key regulator of M phase (154). When activated, this kinase complex phosphorylates proteins that are involved in the breakdown of the nuclear envelope, chromosome condensation and remodelling of the actin and microtubule cytoskeletons, leading to the formation of the mitotic spindle (155, 156). The cyclin B-Cdk1 complex is present at the G2/M phase border in its inactive state. At the onset of M phase, this complex is rapidly activated through an autoregulatory loop. Cyclin B-Cdk1 kinase activity also inhibits PP2A-PR55, which normally promotes inhibition of cyclin B-Cdk1 (153, 157). The cyclin B-Cdk1 kinase complex drives PP2A-PR55 inhibition by activation of α-endosulfine (ENSA), an inhibitor of PP2A PR55 (152, 157-159). PP2A-PR55 is thus inactivated in M phase. In addition, the PR55α subunit in particular is thought to direct PP2A phosphatase activity downstream of Cdk1 with a role in cyclin B-Cdk1 substrate dephopshorylation (108). It is involved in mitotic exit through its role in mitotic spindle breakdown and reassembly of the nuclear envelope, both important processes that were initiated by cyclin B-Cdk1 activity (111). Through its role in the antagonization of cyclin B-Cdk1 substrate activity, depletion of PP2A PR55α accelerates entry into mitosis while its reactivation promotes exit from mitosis. Failure to inhibit PP2A-PR55α causes arrest of the cell cycle in G2 phase preventing entry into mitosis (100). Based on its role in mitotic entry and exit it could be predicted that continuous inhibition of PP2A, including PP2A-PR55α, would lead to aberrant entry and arrest in mitosis.

In this study, inhibition of PP2A with LB100 and LB102 led to G2/M phase arrest in a dosage dependent manner in both K562 and K562 IM-resistant cells (Figure 14). Treatment with IM alone did not affect the distribution of the cell cycle and combining the PP2A inhibitors with IM did not lead to increased G2/M arrest. This strongly suggests that the change in cell cycle distribution is solely due to the inhibition of PP2A activity. These findings are supported by other studies that demonstrated that inhibition of PP2A with CAN also induced G2/M arrest and cell death in gastric cancer (91) and pancreatic cancer (93) models. The G2/M phase arrest reflects what is understood about the role of PP2A PR55 in mitosis. However the PI cell cycle assay is not able to differentiate between the G2 and M phases since PI staining allows for determination of the cell cycle phase by assessing DNA content. An alternative method would be needed to determine if the cells were arrested in M phase rather than G2. Arrest in M phase is associated with mitotic catastrophe and identification of cells undergoing mitotic catastrophe could confirm the M phase arrest. Mitotic catastrophe has been described as an aberrant form of mitosis (160). It usually occurs when stressed cells enter prematurely or inappropriately into mitosis often due to a combination of deficient cell cycle checkpoints and accumulated cellular damage (161). During mitosis, formation of the mitotic spindle is essential for accurate segregation of chromosomes. The mitotic spindle is a complex assembly of highly dynamic microtubules, which are able to continuously grow and shrink. Mitotic catastrophe can be triggered by agents that affect the stability of microtubules, leading to defects in the assembly of the mitotic spindle (160). One of the hallmarks of mitotic catastrophe morphologically is multinucleated cells with increased size and DNA content and these cells ultimately die (162).

To determine if inhibition of PP2A with LB100 and LB102 leads to mitotic catastrophe in CML cells, K562 cells were synchronized in S phase and treated with PP2A inhibitors for 18 hours. Notably, an average of 40% of the treated K562 cells showed features of mitotic

catastrophe including disordered microtubules as well as multiple nuclei assessed by confocal microscopy (Figure 15). At this time point there were no dividing cells observed in the untreated control cells. This is likely because the first round of mitosis after cell synchronization was already complete. In contrast, the treated cells observed to be undergoing mitotic catastrophe were likely unable to exit mitosis properly and arrested in M phase. Interestingly, two other studies investigating the potential of inhibiting PP2A to treat glioblastoma also found that the use of PP2A inhibitors induced mitotic catastrophe (130, 149). Further investigation into the mechanism of how inhibition of PP2A, including PR55α, contribute to induction of mitotic catastrophe may lead to other discoveries regarding cell cycle regulation in CML. However, since this disruption of the cell cycle seems to be due to PP2A inhibition alone, M phase arrest and consequent mitotic catastrophe are unlikely to explain the synergism of the dual inhibition of BCR-ABL and PP2A.

To investigate the mechanism of how dual inhibition of BCR-ABL and PP2A synergized to suppress growth and eradicate leukemic cells, western blots were run to examine the activity and protein expression of relevant proteins that could be affected by this combination treatment. Since the idea of targeting PP2A originated through an AHI-1 drug screen, and sensitivity to both drugs can be affected by manipulation of AHI-1, the phosphorylation and expression of proteins involved in the BCR-ABL-AHI-1-JAK2 complex were analyzed. Previous studies of this protein complex have shown that treatment with IM as well as combination treatments of IM with JAK2 inhibitors results in a reduction in the phosphorylation of BCR-ABL, JAK2, and STAT5 but not much change is observed at the total protein expression level of these proteins (84, 85). In K562 IM-resistant cells, 48 hour treatment with 5μM IM similarly reduced the amount of total tyrosine phosphorylated BCR-ABL (4G10), P-JAK2, and P-STAT5 (Figure 16). However the phosphorylation of the tyr177 residue of BCR-ABL that allows BCR-ABL to activate the PI3K/AKT and RAS pathways through its association with a GRB2/GAB2 complex was not

affected, and these downstream pathways were not affected either. IM treatment alone also slightly reduced the total protein expression of AHI-1 and β-catenin. In contrast PP2A inhibition with 5μM LB100 or LB102 did not affect the phosphorylation or expression of any of these proteins. Interestingly there was also no change in PP2A-C or PR55α expression, agreeing with other studies that these drugs block PP2A activity competitively, rather than inducing their protein degradation (133, 136, 139, 140, 149). Remarkably, the expression of these proteins activated by BCR-ABL signalling was drastically different after the combination treatments. Phosphorylation of JAK2 and STAT5 was greatly reduced, consistent with IM treatment alone. 4G10 indicated that there was even less total tyrosine phosphorylation of BCR-ABL with the combination treatment compared to IM alone. This was reflected in a reduction in phosphorylation of the tyr177 residue of BCR-ABL after combination treatment whereas there was no reduction with any of the single treatments. The tyr177 residue allows BCR-ABL to activate the PI3K/AKT and RAS/MAPK pathways and consequently the levels of phosphorylation and total protein expression of AKT and P38 as well as total JNK were reduced with the combination treatment as well. Furthermore, the potency of the combination treatment to suppress the BCR-ABL-AHI-1-JAK2 protein complex was more obvious when looking at total expression of these proteins. The combination treatment led to an obvious suppression of the BCR-ABL, JAK2, STAT5, and AHI-1 proteins. In addition, total expression of β-catenin was greatly reduced after combination treatment compared to single agents. These results indicate that the expression of proteins both directly involved in, as well as closely related downstream to this complex were greatly affected by this combination treatment. While treatment with IM alone is able to moderately suppress the proliferation of these cells through its effect on the phosphorylation levels of BCR-ABL, JAK2 and STAT5, the synergistic potency of the combination treatment could be explained by the observed dramatic reductions in the total expression of these key proteins. Furthermore, the drastic reduction in the levels of proteins directly involved in or affected downstream by the BCR-ABL-AHI-1-JAK2 complex suggests that

dual inhibition of BCR-ABL and PP2A could actually destabilize this complex. The significant reduced levels of AHI-1 after the combination treatment supports this notion given its vital role in holding this complex of proteins together (84). Put together with the evidence that AHI-1 and PR55α can interact (Figure 10), and that the observed mitotic arrest and catastrophe after PP2A inhibition (Figure 14 and 15) could potentially be driven by a reduction in PR55α-mediated PP2A activities, PR55α could be an important component of the BCR-ABL-AHI-1-JAK2 complex.

To further investigate the synergistic potency of the dual inhibition of BCR-ABL and PP2A, it made sense to study proteins that may be affected by both BCR-ABL kinase and PP2A phosphatase activities. It has been shown that β-catenin can interact with both BCR-ABL (61) and PP2A (78). While there is also some unpublished data suggesting that AHI-1 and β-catenin may also directly interact, β-catenin's potential role in the BCR-ABL-AHI-1-JAK2 complex has yet to be fully established. PP2A is thought to have a role in the stabilization of β-catenin by antagonizing the kinase activities of GSK3β and CKIα in the β-catenin degradation complex (78, 163). These kinases normally phosphorylate the thr41 and ser45 residues of β-catenin, signalling it for degradation via the ubiquitin pathway. Consistent with this role, inhibition of PP2A with LB100 or LB102 for 24 hours led to increased phosphorylation of the thr41 and ser45 residues of β-catenin (Figure 17A). These residues were also more highly phosphorylated after combination treatment while there was no change observed after treatment with IM only. This result indicates that the increased phosphorylation of these specific residues of β-catenin was due to PP2A inhibition rather than IM treatment. The increase in phosphorylation of β-catenin at thr41 and ser45 residues could have been attributed to GSK3β activity or reduced phosphatase activity of PP2A. Missplicing of GSK3β has been implicated in CML leading to enhanced stability of β-catenin, which can then drive the self-renewal characteristics of the blast cells in blast crisis CML (51, 62, 77, 79, 164). To determine if the observed thr41/ser45

phosphorylation of β-catenin was dependent on GSK3β activity, the cells were treated with LiCl, an inhibitor of GSK3β (Figure 17B). Treatment with LiCl resulted in a dosage dependent accumulation of inactive phosphorylated (ser9) GSK3β while phosphorylated β-catenin at thr41 and ser45 residues decreased and total β-catenin protein levels increased. This confirmed that LiCl prevented GSK3β kinase activity leading to decreased phosphorylation of β-catenin and its consequent accumulation. Next, combining LiCl with LB100 showed that when GSK3β kinase activity was inhibited, phosphorylation of β-catenin at thr41 and ser45 was still increased and there was no accumulation of β-catenin that was observed with LiCl treatment alone. These results suggest that the increased phosphorylation of the thr41 and ser45 residues of β-catenin after PP2A inhibition is not dependent on GSK3β activity. Interestingly, there was also an increase in the phosphorylated inactive (ser9) form of GSK3β after PP2A inhibition as well as after the combination treatments. This further supports that the increased phosphorylation of β-catenin is not due to increasing GSK3B kinase activity. Further experiments to confirm these observations in the K562 IM-resistant and BV173 cell lines are needed to draw further conclusions. Furthermore, it would be interesting to determine if this thr41/ser45 phosphorylation is dependent on CKIα kinase activity (163).

BCR-ABL has been shown to phosphorylate β-catenin on its tyr86 residue (61). After 24 and 48 hours treatment with IM alone, there was an observable decrease in the phosphorylation of the tyr86 residue of β-catenin (Figure 17A). This indicated that suppression of BCR-ABL kinase activity by IM was able to prevent phosphorylation of this residue of β-catenin. Interestingly treatment with IM alone is able to reduce the levels of total β-catenin, though not as drastic as the combination treatment observed at 48 hours. BCR-ABL and PP2A activity have been separately implicated to have a role in the stabilization of β-catenin. In this study PP2A inhibition alone affected the phosphorylation of β-catenin but this did not result in reduction of the total protein, while IM alone was able to slightly reduce β-catenin expression. Since the

increased phosphorylation of the thr41 and ser45 residues induced by PP2A inhibition at 24 hours was observed in the combination treatment prior to the drastic reduction of total β-catenin at 48 hours, it is possible that this phosphorylation change primed β-catenin for degradation but required the inhibition of BCR-ABL by IM to trigger the drastic reduction. These results strongly suggest that dual inhibition of BCR-ABL and PP2A could converge on β-catenin through the mediation of the phosphorylation status of the tyr86, thr41, and ser45 residues (Figure 17C). Further investigation into this potential mechanism of dual inhibition is needed to validate this model. One experiment could be to introduce mutations to these specific residues to determine if this reverses the reduction of total β-catenin induced by the combination treatment. Interestingly, the PR55α subunit of PP2A has also been suggested to regulate PP2A-mediated dephosphorylation of β-catenin (146). It would be fascinating to deduce the relationship between AHI-1, PR55α, and β-catenin as well as the relevance of the AHI-1-mediated protein complex to this signalling network. One important approach could be siRNA or shRNA-mediated silencing of specific subunits of PP2A, like the PR55α regulatory subunit or the catalytic subunit. This would serve to mimic the effect of using the drugs, as well as provide a clearer picture of how these signalling pathways combine to drive the disease.

These results make an interesting comparison with studies promoting the use of PP2A-activating drugs (PADs). It was reported that these PADs could reduce survival and self-renewal of quiescent CML stem cells through targeting a JAK2-PP2A-β-catenin network (106). These PADs increased PP2A activity by inhibiting SET, an endogenous inhibitor of PP2A. It has been reported that increasing PP2A activity resulted in suppression of β-catenin expression as well as its downstream transcriptional activity. Interestingly, GSK3β activity was shown to be essential for the PADs to target CML cells. In this study treatment with IM and the PP2A inhibitors alone or in combination did not affect the protein levels of SET (Figure 16). It is interesting that increasing PP2A activity in their case, and inhibiting PP2A in this study, both led

to degradation of β-catenin. It is likely that the two approaches target different stages and pathways in the mechanism of regulating β-catenin degradation. Furthermore it is possible that these two approaches could potentially target different stages of disease progression of CML. While studies with PADs primarily investigated their potential effects in blast crisis CML (106), patient samples in this study were derived from TKI-resistant patients in the chronic phase. Finally, the major difference is the dual inhibition of IM and PP2A is an approach to target the AHI-1-mediated protein-protein interaction network.

Further investigation into the role of PR55α in CML is needed but these findings come together to suggest PP2A may play a role, through its regulatory subunit PR55α, in regulating the stability of the BCR-ABL-AHI-1-JAK2 complex. Ultimately, this study emphasizes the importance of AHI-1 as a mediator of protein-protein interactions and the results indicate that dual inhibition of BCR-ABL and PP2A has great promise as a novel approach to treat CML through its potential to destabilize the AHI-1-mediated protein complex.

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