Novel Antagonists of the SH2 Domain of GRB2 Decrease Chronic Myeloid Leukemia (CML) Cell Proliferation via the JAK/STAT Pathway

A Thesis

By



Summer 2019

APPROVED BY INTERIM DEAN OF GRADUATE STUDIES

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# Novel Antagonists of the SH2 Domain of GRB2 Decrease Chronic Myeloid Leukemia Cell Proliferation via the JAK/STAT Pathway

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A Thesis

Presented

To the faculty of

California State University, Chico

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In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

in

Biological Sciences

_____

By

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ABSTRACT

Novel Antagonists of the SH2 Domain of GRB2 Decrease Chronic Myeloid Leukemia Cell Proliferation via the JAK/STAT Pathway

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Master of Biological Sciences

California State University, Chico

Summer 2019

Chronic myeloid leukemia (CML) is a disease that affects the normal growth of myeloid cells, which are blood cells that protect the body against foreign invaders in the blood and bone marrow. In humans, 95% of CML cases are caused by a chromosomal translocation that inappropriately links the breakpoint cluster region (BCR) to Abelson murine leukemia viral oncogene-1 (ABL1), forming a mutant oncogene called *BCR-ABL1*. A protein that physically interacts with BCR-ABL1 is growth receptor bound protein-2 (GRB2), an intracellular adaptor protein involved in cell growth and differentiation. Specifically, BCR-ABL1 binds to a region of GRB2 known as the SRC homology-2 (SH2) domain. This interaction transforms hematopoietic stem and progenitor cells, initiating leukemic transformation. The current frontline therapy to treat CML is a tyrosine kinase inhibitor, imatinib. Some patients have developed a resistance to imatinib, and thus the demand for additional anticancer drugs are needed. To prevent growth of CML cells, Dr. Arpin and her students of the CSU Chemistry and Biochemistry department created two novel SH2 antagonists (NHD2-15 and NHD2-114) and we tested their ability to prevent cell proliferation in the human BCR-ABL1$^{+}$ K562 myelogenous leukemia cell line. The most significant growth reduction was observed 72 hours after the addition of 30 μM of NHD2-15.

Furthermore, adding drugs combinatorially (60 μM NHD2-15, 30 μM NHD2-114, and 1 μM imatinib) to K562 cells showed over 2-fold growth reduction than with imatinib alone. To assess if these compounds are toxic to living organisms, we added the two compounds individually to the water of healthy adult zebrafish, and found that NHD2-15 was non-toxic. After using an enzyme-linked immunosorbent assay (ELISA) we also found that these two novel drugs exhibited prominent binding affinities to GRB2; NHD2-15 with $K_d = 119 \pm 2$ μM, and NHD2-114 with $K_d = 440 \pm 7$ μM (Lewis et al., in revision, 2019). Western blots were performed to determine the pathway these novel antagonists influence, and to ultimately indicate if these drugs stop cancerous cell proliferation via the Janus kinase signal transducer and activators of transcription (JAK/STAT) pathway, as well as the mitogen-activated protein kinase and phosphoinositide 3-kinase (MAPK/PI3K) pathway. Results of western blotting indicated that the combinatorial treatments of both novel drugs reduces expression of proteins involved in both the JAK/STAT and MAPK/PI3K pathways, suggesting these compounds inhibit different target proteins within BCR-ABL+ cells to decrease leukemic cell proliferation. This research should provide an additional alternative treatment for patients who develop imatinib resistance.

# INTRODUCTION

## Myelopoiesis

Hematopoiesis, the production of blood cells and platelets that occurs in the bone marrow of humans, first begins during embryonic development and is important for replenishing blood within the body (Spangrude et al., 1988;Jagannathan-Bogdan et al., 2013). During hematopoietic development, there are progenitors that arise from hematopoietic stem cells, one of which is a common myeloid progenitor (CMP). CMPs give rise to myeloid cells that are types of white blood cells that function as the initial response to infected tissue as part of the body's innate immune system. Myeloid cells include granulocytes (neutrophils, eosinophils, basophils, and mast cells) as well as monocytes. Mature myeloid cells originate from a CMPs that are derived from hematopoietic stem cells (HSCs) that reside in bone marrow (Hobbs et al., 2003). An HSC will either generate more copies of itself, or will differentiate into a multipotent progenitor cell which either becomes a common lymphoid progenitor (CLP) or a CMP, through specific signaling via cytokines. These CMPs either become megakaryocyte erythroid progenitor (MEP) cells, or granulocyte macrophage progenitor (GMP) cells. Myeloid cells arise from GMPs, and eventually become neutrophils, eosinophils, basophils, mast cells, and monocytes in the bloodstream.

Granulocytes, such as eosinophils/basophils/neutrophils, are those cells that release toxic granules during an immune response containing enzymes that destroy pathogens in the body. Neutrophils, eosinophils, basophils, and mast cells are the cell types that encompass the granulocyte subcategory of mature myeloid cells (Borregaard et al., 1997). The primary role of these myeloid cells is to initiate an inflammatory response against pathogens, and act as first responders to fight these pathogens. Myeloid cells, such as macrophages, that reside is tissue-specific regions of the body can function as antigen-presenting cells that present antigens on their surface to adaptive immune cells. Myeloid cells play a significant role in both the innate (neutrophils/eosinophils/basophils) and adaptive (macrophages) immune systems, and are vital for complete destruction of foreign pathogens and infection.

These mature myeloid cells are vital for the immune system to respond to foreign pathogens and disease. As myeloid cells differentiate into neutrophils, they travel through the bloodstream to consistently replace dying cells. Myeloid cells have short lifespans, and neutrophils (making up 50-70% of myeloid cells) need to be replaced every 1-5 days (Mayadas et al., 2014). In CML, myeloid cells differentiate into mutated CMPs, that do not give rise to mature cells. This causes the bloodstream to be overwhelmed with immature, malfunctioning myeloid cells.

BCR-ABL+ Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a malignant blood disease that affects the growth and development of myeloid cells. Most cases of CML are caused by a chromosomal translocation where the breakpoint cluster region (BCR) gene is linked to Abelson-murine-leukemia-viral-oncogene-1 (ABL1). The DNA from 19 CML patients was observed for the rearrangements of chromosome 22, and 17 of these patients demonstrated a chromosomal breakpoint within a limited region of 5.8 kilobase pairs that was thus determined to be the "breakpoint cluster region" (BCR) (Groffen et al., 1984). The binding of the BCR gene to the ABL1 oncogene creates a fusion protein known as BCR-ABL, and the newly formed chromosome 22 is called the Philadelphia chromosome. The Philadelphia chromosome was the first consistent neoplasia-associated, or uncontrollable cell growth in tissues that was discovered, and thus was a major milestone in cancer cytogenetics (Heim et al., 2015). This translocation of the BCR gene located on chromosome 22, and the ABL1 gene located on chromosome 9 was first identified in 1960 on a patient with this modified chromosome 22 (Kang et al., 2016). When cells are BCR-ABL+, growth receptor bound protein-2 (GRB2) binds to the ABL side of this fusion oncogene via the src-homology-2 (SH2) domain (Lugo et al., 1990). This fusion oncogene expresses deregulated protein tyrosine kinase (PTK) activity in these cells, ultimately causing leukemic transformation (Deininger et al., 2000). Deregulation of PTK activity in these BCR-ABL+ cells causes certain signaling pathways to be continuously activated. These signaling pathways are involved in increased cellular growth and proliferation, as well as decreased regulation of apoptosis (Cortez et al., 1996). As the ABL side of the fusion protein is constantly

phosphorylated by this deregulated PTK activity, the protein continues to activate these signaling pathways responsible for leukemic proliferation.

The *Abl* gene is ubiquitously expressed in all cells, and contains three Src-homology domains. The SH1 domain of *Abl* is involved in tyrosine kinase activity. The major function of the *Abl* gene is directing extracellular and intracellular signals to other proteins that affect cell growth and apoptosis (Deininger et al., 2000). The BCR gene is also ubiquitously expressed in cells, but can be phosphorylated on multiple tyrosine residues. When BCR is phosphorylated on Tyrosine 177 (T177), it is able to bind to the adaptor protein GRB2 (Wu et al., 1998).

The phosphorylation of BCR-ABL allows the oncogene to bind to GRB2 through the SH2 domain. Once bound to the oncogene, GRB2 increases the activation of signal transduction pathways that lead to increased cell growth and proliferation, while decreasing apoptosis (Pawson and Scott, 1997). Specifically, the oncogene causes the constant activation of Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase (RAS/MAPK/ERK) pathway, phosphoinositide 3-kinase (PI3K) pathway, and the Janus kinase signal transducer and activator of transcription (JAK/STAT) pathway. These pathways ultimately increase CML cell proliferation, and inhibit apoptosis and autophagy (Skorski et al., 1995). The binding of GRB2 to BCR-ABL initiates leukemic transformation (Million et al., 2000).

GRB2 and the JAK/STAT Pathway

The intracellular adaptor protein growth receptor-bound protein-2 (GRB2) is ubiquitously expressed in cells and structurally contains an SH3 domain flanked by SH2 domains. The SH2 domain of GRB2 recognizes phosphotyrosine and is activated by phosphorylation; this initiates upstream cell signaling of the adaptor protein (Papaioannou et al., 2016). GRB2 ultimately links receptor tyrosine kinases (RTKs) to downstream cell signaling that influences cell growth and differentiation (**Fig. 1**). The

flanking of SH3 domains on GRB2 allows the linkage of RTKs to targets like the SOS guanine nucleotide exchange factor (GEF) which promotes activation of the Ras/MAPK pathway (Frelin et al., 2017).The binding of BCR-ABL to GRB2 ultimately leads to the activation of these cellular pathways that increase leukemic cell proliferation. In normal cells, this fusion protein is not present, so GRB2 activation can be turned on and off. The interaction between BCR-ABL and GRB2 acts as a permanent activation of the adapter protein, which signals other proteins responsible for cell growth, cell proliferation, and decreased apoptosis.

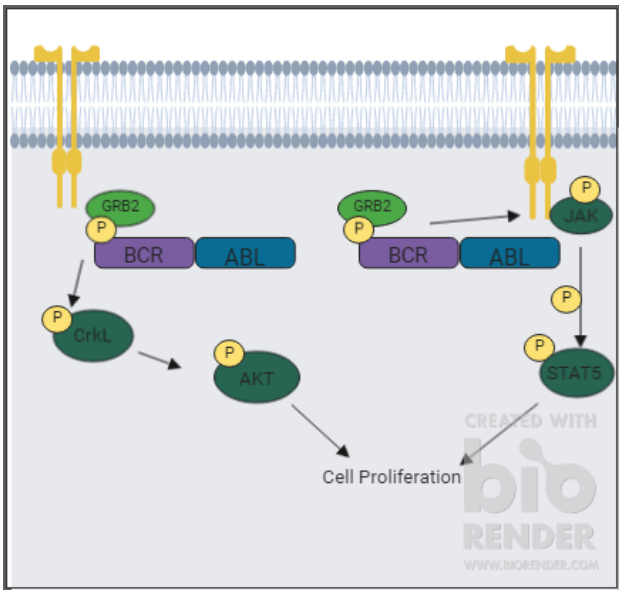


**Figure 1: The phosphorylation and binding of GRB2 to BCR-ABL initiates downstream signaling involved in leukemic cell growth and proliferation.** GRB2 binds to the phosphorylated tyrosine (Y177) on the BCR side of the fusion protein BCR-ABL. This initiates downstream signaling via RAS/MAPK/PI3K pathway increasing BCR-ABL+ cell proliferation. This linkage also initiates the JAK/STAT pathway, ultimately increasing cell proliferation of BCR-ABL+ cells.

The activation of the Janus kinase signal transducer and activator of transcription (JAK/STAT) pathway is one of the signaling pathways that directly leads to increased cell proliferation (Jiang et al., 2007). The JAK/STAT pathway consists of three families of genes; JAKs, STATs, and the suppressor of cytokine signaling (SOCS/CIS) family which downregulates activity of the JAK/STAT pathway. The JAK/STAT pathway involves cytokine signaling that initiates transcription in the nucleus (Steelman et al., 2004). Activation of JAK/STAT leads to cell proliferation, differentiation, and apoptosis, which is important for hematopoiesis. The activation of JAKs is initiated by ligand-mediated receptor multimers, where two JAKs are brought together in close proximity, permitting transphosphorylation. Then the JAKs phosphorylate signals downstream, like STATs, that are reside in cytoplasm until activated (Rawlings et al., 2004).

Treatments for CML and Imatinib Resistance

A successful treatment for CML is the oral drug imatinib (Gleevec™, or STI571). Imatinib is a tyrosine kinase inhibitor that hinders the ATP activity of the ABL side of the BCR-ABL oncogene, and reduced myeloid proliferation in BCR-ABL+ cells. This occurs by the stabilizing of the inactive form of BCR-ABL oncogene (Druker et al., 1996). In order to activate the fusion oncogene, the phosphorylation of tyrosine residue, Y242, must occur; imatinib will only bind to the ATP binding site when Y242 is not phosphorylated, and thus prevents further phosphorylation (Schindler et al., 2000). As BCR-ABL switches back from active to inactive, imatinib renders the oncogene in the inactive state.

Though imatinib has been an effective anticancer drug against CML, there have been relapses amongst patients who initially receive treatment due to mutations of the ABL side of the BCR-ABL fusion protein (Willis et al., 2005). These relapses are caused by mutations including elevated kinase activity, or increase of cytokines, such as interleukin-3 (IL-3) and granulocyte colony-stimulating factor (G-CSF), in primitive chronic myeloid leukemia stem cells (Jiang et al., 2007). Residual BCR-ABL+ cells remain in circulation for those patients treated with imatinib, leading to disease progression for those that develop resistance to this treatment. Some newly diagnosed patients do not achieve a complete recovery of normal red and white blood cell counts, and eventually stop responding to imatinib treatment

(Apperley, 2007). About 50% to 90% of patients who acquire resistance to imatinib experience a mutation in the kinase domain of the BCR-ABL oncogene. Because imatinib inhibits the phosphorylation of the ABL protein on the BCR-ABL oncogene mutations of the kinase domain cannot be controlled (Willis et al., 2005). Though the mechanism is currently unknown, typically an increase in dosage of imatinib permits these patients to end the relapse (Hochaus et al., 2002). These mutations could be increasing the activity of BCR-ABL, ultimately overpowering the efficacy of imatinib. Thus, the demand for additional studies are needed to establish more efficient treatment options for imatinib resistant patients of BCR-ABL+ CML.

Novel SH2 Antagonists

A better method of treatment is needed for CML patients that relapse after becoming resistant to imatinib. Dr. Carolynn Arpin, an Assistant Professor in the Chemistry and Biochemistry Department at CSU Chico, and her student, Natalie Holmberg-Douglas, synthesized several small molecules with structures based off of a known SH2 antagonist of growth receptor-bound protein 7 (GRB7). Though a majority of the protein is structurally different, GRB7's SH2 domain binds phosphotyrosine motifs similar to GRB2's SH2 domain (Pawson et al., 1997; Porter et al. 2007). The SH2 antagonist was able to bind to the SH2 domain of GRB2 with high affinity ($K_d = 3.1 \pm 0.8$ μM), and GRB2 and GRB7 possess very similar SH2 domains, several small molecules were created as highly specific GRB2 SH2 antagonists (Lewis et al., 2019). Two of the novel antagonists also displayed high binding affinity to the SH2 domain of GRB2, leading us to believe the drugs inhibit phosphorylation of GRB2 via the SH2 domain; NHD2-15 with $K_d = 119 \pm 2$ μM, and NHD2-114 with $K_d = 440 \pm 7$ μM (Lewis et al., in revision 2019).

The main objective of this project was to investigate what signaling pathways these novel SH2 antagonists influence, to ultimately determine the method of action of these compounds. Before

investigating the method of action of these novel compounds, I first performed various assays to ensure that these drugs were effectively decreasing CML cell proliferation, non-toxic to healthy living tissue, and could decrease CML cell proliferation on an imatinib-resistant CML cell line.

## MATERIALS AND METHODS

### CellTiter 96 Metabolic Assay

The K562 human myelogenous leukemia cell line were maintained in RPMI 1640 media with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine at 37℃ and 5% $CO_2$. K562 cells were resuspended at 5 x $10^5$ cells/mL, and 100 µL of cell suspension was placed into individual wells of a 96-well plate, either treated with one of the novel SH2 antagonists, DMSO vehicle only, or imatinib. The cells were cultured for 48 hours at 37℃ in 5% $CO_2$, and 15 µL of CellTiter dye was added and incubated for 2 hours, and then 100 µL of stop solution was added to end the reaction. Cells were incubated for an additional 24 hours at 37℃, and absorbance at 570 nm was assessed.

### Cell Proliferation Assays

K562 cells were maintained in RPMI 1640 media with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine at 37℃ and 5% CO2. Cells were plated at 10 x $10^4$ cells/well in a 12-well-plate and incubated for 72 hours after drug addition of either compound at the following concentrations: NHD2-15 at 120, 60, 30, and 0 uM, and NHD2-114 at 120, 60, 30, 0 uM. After incubation with molecules at 72 hours, K562 cells were counted again. Imatinib was used as a positive control for the individual novel SH2 antagonist cell proliferation assays, as the compound is known to decrease BCR-ABL+ cell proliferation at 1 µM.

In the combination treatments, imatinib was added with each novel antagonist at 1 µM. From the individual proliferation assays, the concentrations that demonstrated the highest reduction in K562 cell growth were used; 60 µM of NHD2-15, and 30 µM of NHD2-114. After 72 hours post-drug addition, cells were counted again.

Western Blotting of JAK/STAT and MAPK/PI3K Pathways

After cell proliferation assays, cell lysates were obtained from those K562 cells treated with novel antagonists and/or imatinib. Protein was isolated from cell lysate, and run on a 4-20% acrylamide gels (Mini-Protean GTX, BioRad). Protein was transferred to nitrocellulose paper via iBlot 2 Dry dry transfer system, and then blocked with milk for 1 hour. Blots were probed for phosphorylated BCR-ABL, phosphorylated STAT5, and phosphorylated CrkL using a BCR-ABL Activity Assay (Cell Signaling Technology). To ensure that protein was properly loaded and transferred, nitrocellulose paper was stained with Ponceau to visualize loaded protein levels.

Toxicity Assays

To test if the novel SH2 antagonists were toxic against healthy tissue, each drug was added to the water of four healthy, adult zebrafish, and survival rate/health was assessed at 24, 48, and 72 hours post-drug addition. The compounds appear to be water soluble. Fish were placed in a 100 mL water tank and 15 μM of antagonists were added. Additionally, zebrafish were subjected to 30 μM of antagonist NHD2-15. The novel antagonists were also added at 15 μM to the water tank, and two hours post-addition of drugs, fresh water was replaced in the tanks. Zebrafish survival was then observed 72 hours post-addition of drugs.

Imatinib-Resistant Cell Proliferation Assays

An imatinib-resistant cell line was created by adding 1 uM of imatinib to the K562 cell line weekly for six weeks. Cells were maintained in RPMI 1640 media with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine, with 1 uM of imatinib at 37℃ and 5% $CO_2$. Cells were plated at 10 x $10^4$ cells/well in a 12-well plate and incubated for 72 hours after drug addition of either compound at the following concentrations: NHD2-15 at 120, 60, 30, and 0 uM, and NHD2-114 at 120, 60, 30, 0 uM. After incubation with molecules for 72 hours, K562 cells were counted again.

## DATA AND DISCUSSION

Effects on BCR-ABL+ K562 cells

We exposed K562 cells, a BCR-ABL+ immortalized leukemic cell line, to each of the two novel SH2 antagonists: NHD2-114 and NHD2-15. To determine if the addition of these novel antagonists would reduce the amount of K562 cell growth in comparison to the negative control (DMSO), as well as the positive control (1 μM of imatinib), we first performed a CellTiter 96 assay, which measures the metabolic activity of the cells. Exposing K562 cells to NHD2-15 decreased metabolic activity 4-fold (**Fig. 2**), within the 15-120 μM range. The addition of NHD2-114 to K562 cells did not decrease K562 metabolic activity as prominently as NHD2-15. The vehicle control, DMSO, had no effect in metabolic activity of the K562 cells (data not shown), and imatinib (the positive control) did effectively decrease growth of K562 cells nearly 10-fold with the addition of 1 μM (**Fig. 2**). Ultimately, NHD2-15 had an inhibitory effect on the metabolic activity of K562 cells.

To assess if these novel SH2 antagonists inhibited K562 cell proliferation, we added NHD2-15 to K562 cells and after 72 hours of incubation in the drug we observed a significant decrease in cell proliferation. When NHD2-114 was added to K562 cells individually, there was little to no change in cell proliferation when compared to the controls. We decided to combine each antagonist with imatinib to see if the combination treatments improved reduction of K562 cell proliferation. To do this, K562 cells were plated at $10 \times 10^4$ cells/well, exposed to either one of the antagonists and imatinib, and then incubated for 72 hours. After the 72-hour incubation, cells were counted to assess reduction in K562 cell proliferation in comparison to the controls.

After 72 hours, we saw a significant decrease in K562 cell proliferation with the addition of varying concentrations of antagonist NHD2-15, and 1 μM of imatinib. At 60 μM and 120 μM of NHD2-15, we saw the increased reduction in K562 cell proliferation (**Fig. 3**). The combinatorial treatment of imatinib with NHD2-15 ultimately demonstrated a 2-fold decrease in K562 cell proliferation in comparison to the novel antagonist added individually. With the addition of NHD2-114 and imatinib, we saw a decrease in K562 cell proliferation when compared to single addition of the novel antagonist (**Fig.**

**4**). With these cell proliferation assays, these novel antagonists significantly decreased BCR-ABL+ CML cell proliferation when added with imatinib.

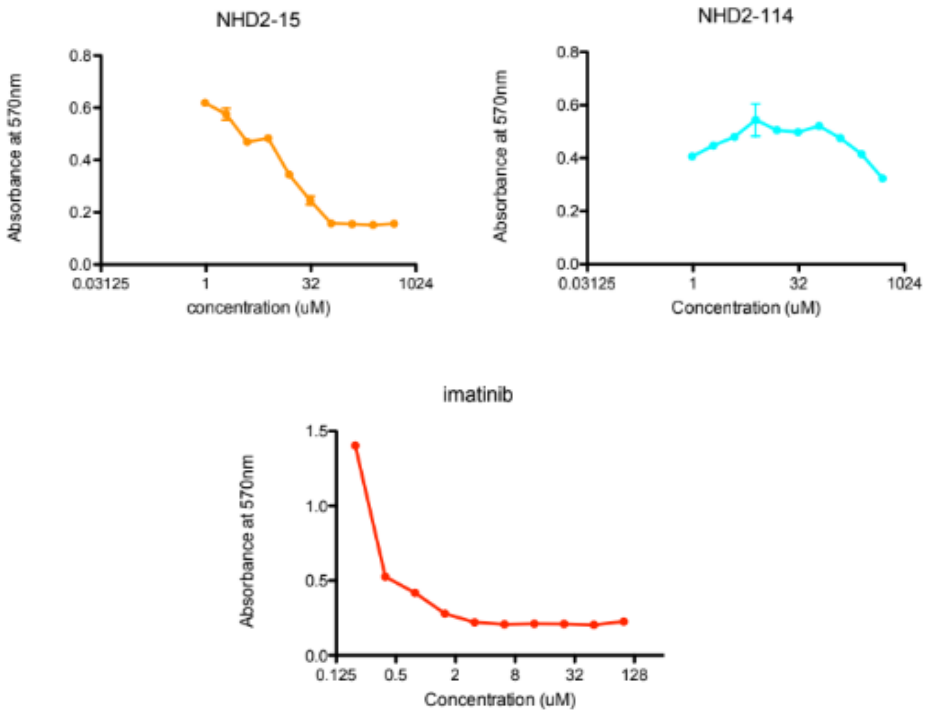


**Figure 2. Treatment with NHD2-15 antagonist reduces metabolic activity of K562 cells in dose-dependent manner.** K562 cells ($1x10^5$ cells) were incubated with either novel SH2 antagonist, or imatinib, at increasing amounts. After 72 hours, a CellTiter 96 Assay was performed and absorbance at 570 nm was assessed. Filled circles represent the mean, and error bars represent the standard deviation; n = 3 for all trials.

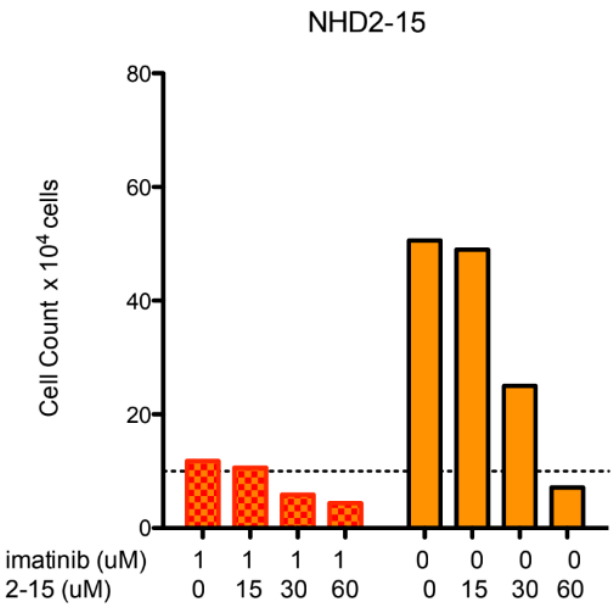


**Figure 3. Exposure to novel SH2 antagonist NHD2-15 with imatinib significantly decreases K562 cell proliferation.** The addition of 1 μM of imatinib with increasing concentrations of NHD2-15 (red and orange bars), reduced cell proliferation at 60 μM of NHD2-15. Combinatorial drug treatments in comparison to NHD2-15 individually (yellow bars) showed a decrease in K562 cell proliferation.

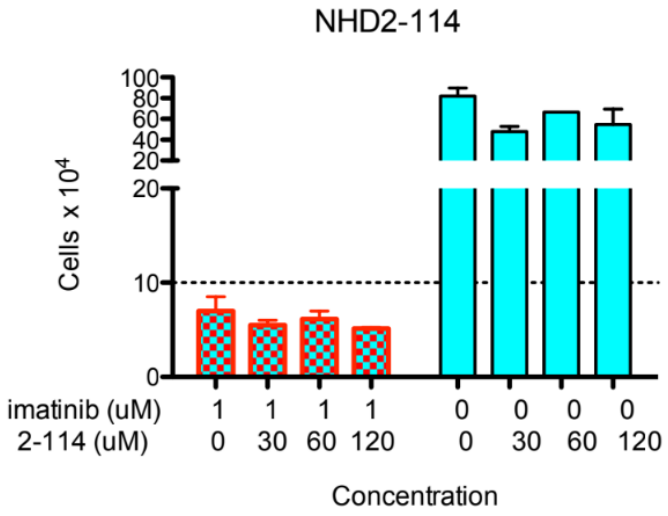


**Figure 4. Exposure to novel SH2 antagonist NHD2-114 with imatinib decreases K562 cell proliferation, when compared to individual addition of NHD2-114.** The addition of 1 μM of imatinib with varying concentrations of NHD2-114 (red and blue bars), demonstrated a decrease in K562 cell proliferation. When NHD2-114 was added to K562 cells individually (blue bars), there was no difference in cell proliferation.

Synergistic Treatments on BCR-ABL+ K562 cells

Since the combination treatments with each novel antagonist and imatinib together proved to significantly reduce BCR-ABL+ CML cell numbers, we decided to create synergistic treatments that combined the two novel antagonists, and also added imatinib. For these synergistic treatments, we took the most efficient concentrations in which there was significant reduction in K562 cell proliferation; NHD2-15 at 60 μM and NHD2-114 at 30 μM.

Treatment of BCR-ABL+ CML cells with both NHD2-15 and NHD2-114 resulted in a 8-fold decrease in leukemic cell proliferation. In comparison to each individual novel SH2 antagonist added with imatinib (**Fig. 3 & Fig. 4**), at 30 μM of NHD2-114 and 60 μM of NHD2-15 decrease K562 cell proliferation the best out of the imatinib combined treatments. When 1 μM of imatinib was added to both novel SH2 antagonists, at 10-fold reduction in BCR-ABL+ CML cell proliferation was seen (**Fig. 5**).

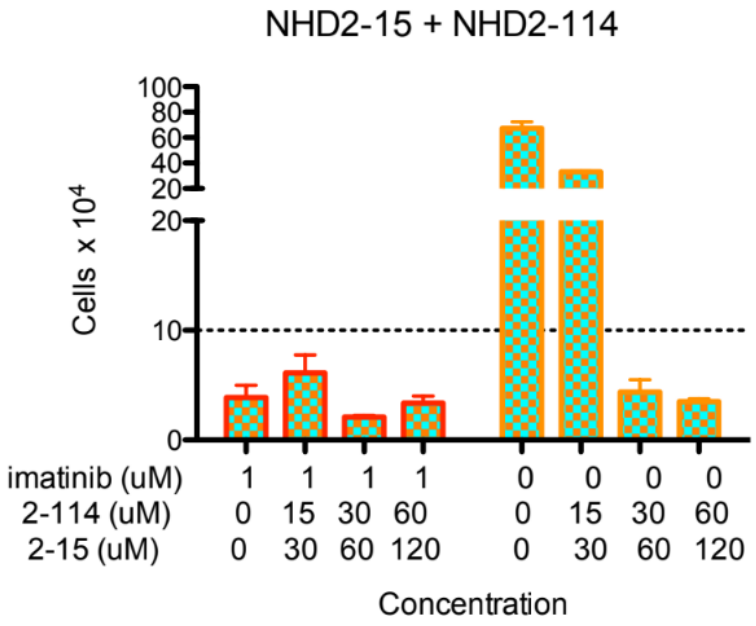


**Figure 5. Synergistic treatments on BCR-ABL+ CML cells resulted in most significant decrease in K562 cell proliferation.** At varying concentrations, NHD2-15 plus NHD2-114 (blue and orange bars) decreased K562 cells more efficiently then when added individually. When both novel SH2 antagonists and 1 μMof imatinib were added (blue, orange, and red bars), there was a decrease in K562 cell proliferation.

NH2-15 is non-toxic to healthy adult zebrafish

Zebrafish were used to assess toxicity of these novel antagonists as they were the most available vertebrate model organism for these experiments, and exhibit similar hematopoiesis function as humans. When healthy adult zebrafish were exposed to 15 μM of each antagonist, NHD2-15 had a survival rate of 100% after 72 hours post drug addition (**Fig. 6**). When zebrafish were subjected to 30 μM of NHD2-15, there was a sharp decrease in survival immediately within the first 2 hours after drug addition, ultimately leading to 0% survival at 6 hours (**Fig. 7**). We added these novel antagonists at 15 μM and then two hours post drug addition, we replaced the tanks with fresh water (**Fig. 8**). When adding each novel antagonist, including NHD2-92 and NHD2-107, the two least successful at decreasing BCR-ABL+ leukemic cell proliferation, there was 100% survival rate for all drugs with this two hour exposure.

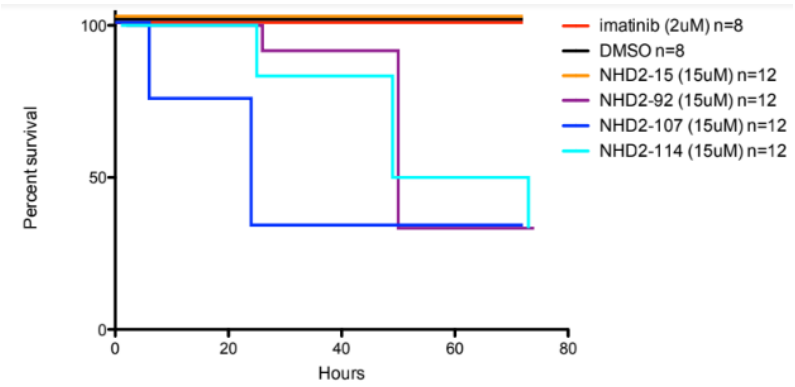


**Figure 6. NHD2-15 is non-toxic to healthy adult zebrafish at 15 µM.** When each novel antagonist was added at 15 µM, NHD2-15 was the most non-toxic drug to healthy zebrafish. Imatinib and DMSO demonstrated no effect on fish health after 72 hours post-drug addition. NHD2-114 and NHD2-92 saw gradual decreases in survivability of fish; NHD2-107 was the most toxic to fish.

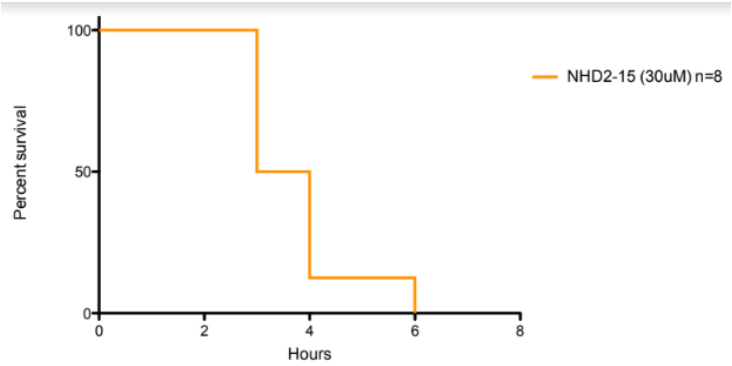


**Figure 7. NHD2-15 at 30 µM is toxic to healthy adult zebrafish at 3 hours incubation.** When 30 µM of NHD2-15 was added to the water of healthy adult zebrafish, survivability started to decrease at 3 hours post drug addition. By 6 hours post-addition of drugs, there was zero survivability of fish.

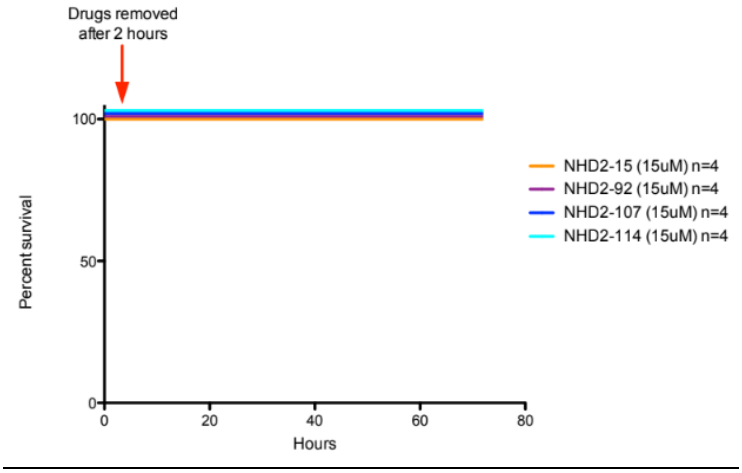


**Figure 8. All four novel SH2 antagonists are non-toxic when washed out after 2 hours of incubation.** After 2 hours of incubation with each novel antagonist at 15 μM, water was replaced. Fish were still monitored for 72 hours, and all fish involved in the 2-hour drug trial demonstrated one-hundred percent survivability.

Imatinib-Resistant BCR-ABL+ Cell Proliferation Assays

After developing an imatinib-resistant BCR-ABL+ cell line through the weekly addition of 1 μM of imatinib to K562 cells for one month, the SH2 novel antagonists were added in a similar fashion to the original K562 cell proliferation assays. This imatinib-resistant cell line was created to determine if these novel antagonists could be a useful alternative treatment for those BCR-ABL+ CML patients that had developed a resistance to imatinib.

When novel antagonist NHD2-15 was added to the imatinib-resistant (IMR) cell line. After 72 hours post-addition of the drug there was a 9-fold decrease in IMR-K562 cells starting at a concentration of 30 μM (**Fig. 9**). Noticeably, there was a decrease in cell proliferation of the IMR-K562 cell line; this could be attributed to the weakened proliferation rate of the imatinib-resistant cell line due to the constant addition of imatinib. Though the IMR-K562 cell line is resistant to imatinib, the proliferation rate of the cells is slowed as a result of the presence of imatinib.

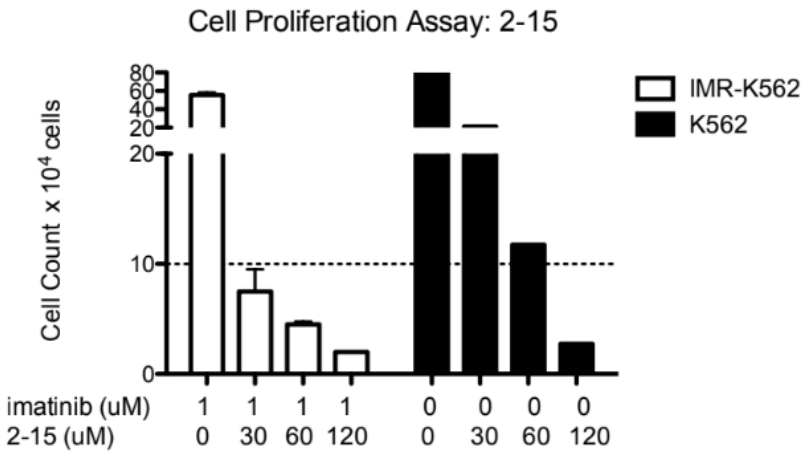


**Figure 9: Novel SH2 antagonist NHD2-15 decreased IMR-K562 cell proliferation at 30 µM.** Novel antagonist NHD2-15 successfully decreased IMR-K562 cell proliferation at 30 µM, and as concentration of the drug increased, proliferation consequently decreased. Comparing the IMR-K562 and K562 cell proliferation with NHD2-15 addition reveals that the imatinib-resistant cell line had a stronger decrease in proliferation.

When novel antagonist NHD2-114 was added to the imatinib-resistant BCR-ABL+ cell line, there was no decrease in cell proliferation when compared to NHD2-15 (**Fig. 10**). Although, different to the pattern of NHD2-15 added to the IMR-K562 cell line, the addition of imatinib did slow the cell proliferation rate down, and consequently there's a notable difference in proliferation when comparing the two BCR-ABL+ cell lines.

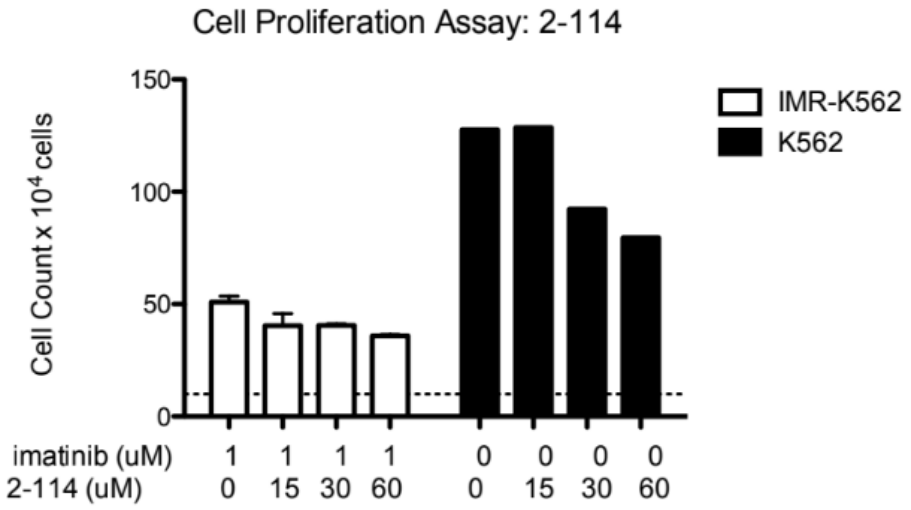


**Figure 10. Novel SH2 antagonist NHD2-114 does not decrease IMR-K562 cell proliferation.** The decrease in IMR-K562 cell proliferation is no different between 15 and 30 μM of NHD2-114. Altogether, there's no decrease in IMR-K562 cell proliferation with the addition of novel antagonist NHD2-114 when added individually.

Based on the results from the initial K562 cell proliferation assays, it was assumed that NHD2-114 would not perform as well as NHD2-15 when added individually. However, when both novel SH2 antagonists were added together against the imatinib-resistant cell line, there was a decrease in proliferation at 30 μM of NHD2-114, and 60 μM of NHD2-15 (**Fig. 11**). This synergistic treatment to the IMR-K562 cell line reveals that both novel antagonists, when combined, work efficiently at decreasing imatinib-resistant BCR-ABL+ leukemic cell proliferation.

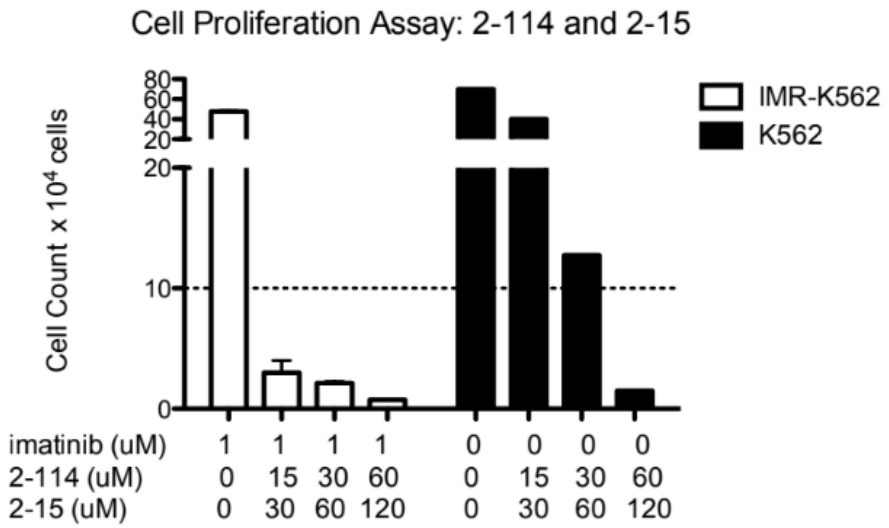


**Figure 11. Synergistic treatment with both novel antagonists decreases imatinib-resistant BCR-ABL+ cell proliferation.** When both NHD2-15 and NHD2-114 are added at varying concentrations together against both BCR-ABL+ cell lines, there are significant decreases in cell proliferation.

These cell proliferation assays against the developed imatinib-resistant BCR-ABL+ cell line show that the novel SH2 antagonists successfully decrease cell proliferation. Similar to the other cell proliferation assays, novel antagonist NHD2-15 decreased BCR-ABL+ K562 cell proliferation when individually added. Individual addition of NHD2-114 did not result in a significant decrease in leukemic cell proliferation. The synergistic combination of both novel antagonists results in a significant decrease of BCR-ABL+ cell proliferation. These synergistic treatments of novel antagonists potentially provides an alternative treatment for those BCR-ABL+ CML patients that have developed a resistance to tyrosine kinase inhibitors, like imatinib.

Effects of SH2 Antagonists on JAK/STAT and MAPK/PI3K Pathways in BCR-ABL+ K562 cells

Western blotting revealed that these novel SH2 antagonists decrease the amount of phosphorylated-ABL, as well as phosphorylated-STAT5 protein when treated with the most effective concentrations of each antagonist, or in combination with 1 μM of imatinib. When individually added to K562 cells, NHD2-15 and NHD2-114 reduce the upregulation of phosphorylated-ABL and phosphorylated-STAT5. However, when the two novel antagonists were added together they decreased both phosphorylated proteins involved in the JAK/STAT pathway completely (**Fig. 12**). When NHD2-15 and imatinib are added together, there's less expression of the phosphorylated BCR-ABL, phosphorylated STAT5, and phosphorylated CrkL (**Fig.13 & 14**). When NHD2-114 was added with imatinib, the activated proteins involved in the JAK/STAT and MAPK/PI3K pathways were downregulated (**Fig. 12 & 14**). These results highlight that the novel antagonists when added in combination or in synergistic treatments decrease BCR-ABL+ CML cell proliferation via the JAK/STAT pathway, as well as the MAPK/PI3K pathway.

These findings reveal that these novel SH2 antagonists when added together or in combination with imatinib not only efficiently decrease BCR-ABL+ CML cell proliferation, but do so using both the JAK/STAT and MAPK/PI3K cell proliferation pathways. These data also indicate that these novel antagonists are inhibiting the binding of BCR-ABL to the SH2 domain of GRB2, as there's less expression of proteins downstream of activated ABL; such as phosphorylated STAT5. This supports our previous claim that these novel SH2 antagonists inhibit the binding of BCR-ABL to the SH2 domain of GRB2.

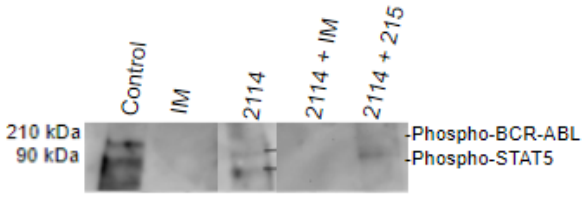


**Figure 12. Phosphorylated-STAT5 is downregulated when IM or NHD2-15 is combined with NHD2-114.** The addition of imatinib (1 μM) to NHD2-114 (30 μM) demonstrates reduced expression of phosphorylated-ABL and phosphorylated-STAT5. Additionally, the combination of NHD2-114 (30 μM) and NHD2-15 (60 μM) reveals reduced expression of both proteins involved in the JAK/STAT cell proliferation pathway. (Control = K562 cells)

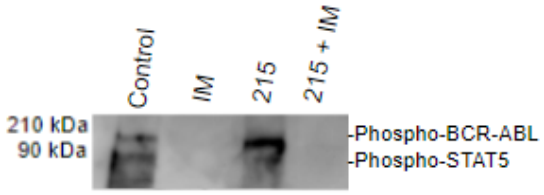


**Figure 13. Phosphorylated-STAT5 is downregulated when IM is added to NHD2-15.** When NHD2-15 (60 μM) is added individually, there is reduced expression of phosphorylated-STAT5, however no significant reduction in expression of phosphorylated-ABL occurs. When imatinib (1 μM) is combined with NHD2-15 (60 μM), there is a reduction in expression of phosphorylated-ABL and -STAT5. (Control = K562 cells)

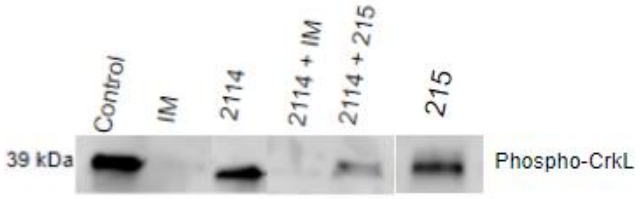


**Figure 14. pCrkL is downregulated when NHD2-114 and NHD2-15 are combined.** When NHD2-114 (30 μM) is added individually, there is a slight reduction in expression of phosphorylated-CrkL. The addition of imatinib (1 μM) to NHD2-114, results in reduction of activated-CrkL; and the combination of both antagonists results in reduced expression of activated-CrkL. This highlights that these novel antagonists are influencing a different cell signaling pathway than imatinb in BCR-ABL+ CML cells. (Control = K562 cells)

## CONCLUSION AND FUTURE DIRECTIONS

Based on the preliminary experiments on these novel SH2 antagonists, NHD2-15 decreases the growth of both BCR-ABL+ and imatinib-resistant BCR-ABL+ cells; is non-toxic to healthy adult zebrafish; and significantly downregulates the expression of proteins involved in leukemic cell proliferation when added individually, and combined with NHD2-114 or imatinib. From the previous research done on these novel SH2 antagonists, we know that these small molecules effectively inhibit binding of GRB2 to BCR-ABL (Lewis et al., in revision 2019).

Comparing the CellTiter 96 metabolic assays with the cell proliferation assays on K562 cells, not only are these novel antagonists decreasing metabolic activity, they are also decreasing cell proliferation. NHD2-15 most effectively decreases BCR-ABL+ K562 cell proliferation when added at 30 μM individually; when combined with 1 μM of imatinib, there's a more prominent decrease in cell proliferation at lower concentrations of the SH2 antagonist. NHD2-114 does not decrease K562 cell proliferation when added individually, however when 60 μM of the antagonist is added with 1 μM of imatinib there are significant decreases in cell growth. These cell proliferation assays ultimately determined the concentrations to add of each novel antagonist, and treatments against K562 cells for the western blotting experiments.

Prior to the western blotting experiments, toxicity of the novel antagonists was assessed by adding each drug to small water tanks of healthy, adult zebrafish. Both novel antagonists were added at 15 μM to assess survival after 72 hours post-drug addition. NHD2-15 had a 100% survival rate of adult zebrafish after 72 hours at 15 μM; however when 30 μM of drug was added, fish were deceased within the first 6 hours. The addition of 15 μM of NHD2-114 demonstrated a 25% decrease in survivability at each 24-hr time point. The other two novel antagonists, NHD2-107 and NHD2-92 did not have nearly as efficient non-toxic qualities as NHD2-15, and were ultimately discontinued in further studies due to their lack of binding-affinity to GRB2 in previous ELISA assays (Lewis et al., in revision 2019). When each novel SH2 antagonist was added at 15 μM and incubated for 2 hours, and then replaced with fresh water,

there was 100% survivability for all zebrafish through 72 hours post initial drug addition. This 2-hour wash out trial highlights that the novel compounds are non-toxic when added for a typical treatment (about 1-4 hours, depending on the type of therapeutic drug used) period for patients receiving chemotherapy. CML patients are not completely immersed in therapeutic drugs for 72 hours straight, so it was important to demonstrate that these novel SH2 compounds were non-toxic at a normal drug treatment period.

While these novel antagonists were able to decrease BCR-ABL+ cell proliferation, we needed to determine if these drugs could successfully decrease an imatinib-resistant K562 cell line. After a month of subjecting 1 μM of imatinib to the existing K562 cell line, an efficient imatinib-resistant BCR-ABL+ cell line was created. This IM-resistant cell line was subjected to each novel antagonist at increasing concentrations and resulted in significant decreases in cell proliferation at 60 μM of NHD2-15. The single addition of NHD2-114 did not notably decrease cell proliferation at any concentration; however when both novel antagonists were synergistically added to the imatinib-resistant cell line resulted in a strong decrease in cell growth. Specifically, when 30 μM of NHD2-114 and 60 μM of NHD2-15 were added to the IM-resistant cell line, there was a significant decrease in cell proliferation. In comparison to other drug studies tackling imatinib-resistant mutations of the BCR-ABL oncogene, specific E255V and Y253H mutations were inhibited by a compound of the 4,6-pyrimidine class, created through several compound screenings. These two mutations, E255V and Y253H, account for 30% of mutations detected at relapse of CML from imatinib treatment (Adrián et al., 2006). Ideally, if our imatinib-resistant K562 cell line specifically possessed these mutations, we could compare the efficacy of our novel compounds in relation to those created by Adrián et al., 2006. These findings ultimately support that the small molecules NHD2-15 and NHD2-114 not only decrease BCR-ABL+ cell proliferation, but also inhibit cell growth on an already imatinib-resistant cell line. The implications of these findings indicate potential future treatments with the use of these drugs for imatinib-resistant CML patients.

To determine if the novel SH2 antagonists were in fact inhibiting the binding of the fusion protein BCR-ABL to GRB2, thus triggering increased cell growth and proliferation, western blotting was used to

probe specific proteins downstream of the oncogene and adaptor protein interaction. Probing for phosphorylated (or activated) BCR-ABL and STAT5 determined that these two novel antagonists had an effect on cell proliferation via the JAK/STAT pathway. More specifically, when NHD2-15 is added at 60 μM with 1 μM of imatinib; or when added with 30 μM of NHD2-114, there is a decreased upregulation of both phosphorylated-BCR-ABL (210 kDa) and phosphorylated-STAT5 (90 kDa). Based on the K562 cell proliferation assays, and the western blot results, my hypothesis that these novel antagonists inhibit cell proliferation via the JAK/STAT pathway is supported.

However, the novel antagonist and/or imatinib-treated K562 lysates were also probed for phosporylated-CrkL. This protein is involved in another cell proliferation pathway, altered by the upstream activation of BCR-ABL, that results in the MAPK/PI3K signaling cascade. This pathway is important for both increased cell growth and proliferation, as well as decreased apoptosis. The individual treatments of either novel SH2 antagonists exhibited slight reduction in expression of phosphorylated-CrkL; however, in all treatments involving the addition of 1 μM imatinib, or the combined treatments of NHD2-15 and NHD2-114, there is an apparent decrease in CrkL activation.

Not only do these novel SH2 antagonists decrease expression of phosphorylated-STAT5 in the JAK/STAT signaling pathway, but they also reduce expression of phosphorylated-CrkL in the MAPK/PI3K pathway. These initial findings indicate that the treatment of K562 cells with the combination of either antagonist with 1 μM of imatinib, or both antagonists added together, effectively downregulates expression of proteins involved in cell proliferation pathways in CML oncogenesis. Based on previous ELISA assays that revealed the high binding affinities of these novel antagonists to GRB2, and the downregulation of phosphorylated proteins involved in cell proliferation pathways, lends support to the hypothesis: these novel antagonists decrease CML cell proliferation via the JAK/STAT pathway. These findings also indicated that combinatorial treatments of both novel antagonists together, or added with 1 μM of imatinib, downregulated the expression of phosphorylated-CrkL; revealing that they decrease activation of the MAPK/PI3K proliferation pathway.

Determining the signaling pathways that are inhibited by the addition of these novel SH2 antagonists ultimately brings us closer to discovering the method of action these drugs take to stop CML cell proliferation. Already we've established that NHD2-15 effectively decreases BCR-ABL+ CML cell proliferation when added individually or in combination with NHD2-114, or imatinib. Not only are these novel SH2 antagonists efficient at decreasing BCR-ABL+ CML cell proliferation, but they are also non-toxic to healthy adult zebrafish. These novel drug compounds inhibit BCR-ABL+ cell proliferation by blocking the binding of BCR-ABL to GRB2 via the SH2 domain; ultimately decreasing both MAPK/PI3K and JAK/STAT signaling pathways. The novel antagonists successfully decrease imatinib-resistant BCR-ABL+ cells; highlighting the significance of these drugs as an alternative form of treatment for those CML patients resistant to imatinib.

Ideally, the next steps to investigating the efficacy of these novel compounds would be to treat a leukemic zebrafish model with the drugs to assess if these drugs could therapeutically treat BCR-ABL+ zebrafish. There are no known leukemic zebrafish models at this time; as CML is a disease that is not hereditary and 95% of cases occur via the fusion of the BCR-ABL oncogene. In order to develop this leukemic zebrafish line, there would need to be a transplantation of these BCR-ABL+ cells, as there is no known orthologous oncogene that naturally mutates in zebrafish. By treating a BCR-ABL+ zebrafish model, we would be able to assess if these novel drugs effectively decrease metastasis of CML, and ensure they're non-toxic.

To better establish the influence of these novel antagonists on specific signaling pathways within BCR-ABL+ CML cells, the imatinib-resistant K562 cell line should ideally be probed for those phosphorylated proteins involved in both the MAPK/PI3K and JAK/STAT pathways. Western blot experiments on the imatinib-resistant cell line could demonstrate whether or not our novel compounds effectively reduce expression of phosphorylated-CrkL, -STAT5, and -ABL; supporting that these drugs work at a different target of the BCR-ABL oncogene than imatinib.

In essence, these preliminary findings indicate that these novel SH2 antagonists could be used as an additive therapy to stop CML cell proliferation, especially in those imatinib-resistant patients. These

novel drugs when combined additively against a BCR-ABL+ CML cell line reduced expression of proteins involved in both the MAPK/PI3K and JAK/STAT cell proliferation pathways. This ultimately suggests that these novel compounds inhibit the binding of GRB2 to the BCR-ABL oncogene, in order to inhibit CML cell proliferation.

Literature Cited


Adrián, Francisco J., et al. "Allosteric Inhibitors of Bcr-Abl–Dependent Cell Proliferation." *Nature Chemical Biology*, vol. 2, no. 2, Feb. 2006, pp. 95–102.

Apperley, Jane F. "Part I: Mechanisms of Resistance to Imatinib in Chronic Myeloid Leukaemia." *The Lancet Oncology*, vol. 8, no. 11, Nov. 2007, pp. 1018–29.

Borregaard, N., and J. B. Cowland. "Granules of the Human Neutrophilic Polymorphonuclear Leukocyte." *Blood*, vol. 89, no. 10, May 1997, pp. 3503–21.

Cortez, D., et al. "The BCR-ABL Tyrosine Kinase Inhibits Apoptosis by Activating a Ras-Dependent Signaling Pathway." *Oncogene*, vol. 13, no. 12, Dec. 1996, pp. 2589–94.

Deininger, Michael W. N., et al. "BCR-ABL Tyrosine Kinase Activity Regulates the Expression of Multiple Genes Implicated in the Pathogenesis of Chronic Myeloid Leukemia." *Cancer Research*, vol. 60, no. 7, Apr. 2000, pp. 2049–55.

Deininger, Michael W. N., et al. *The Molecular Biology of Chronic Myeloid Leukemia*. Vol. 96, no. 10, 2000, p. 15.

Druker, Brian J., et al. "Effects of a Selective Inhibitor of the Abl Tyrosine Kinase on the Growth of Bcr–Abl Positive Cells." *Nature Medicine*, vol. 2, no. 5, May 1996, pp. 561–66.

Frelin, Catherine, et al. "Grb2 Regulates the Proliferation of Hematopoietic Stem and Progenitors Cells." *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, vol. 1864, no. 12, Dec. 2017, pp. 2449–59.

Groffen, J., et al. "Philadelphia Chromosomal Breakpoints Are Clustered within a Limited Region, Bcr, on Chromosome 22." *Cell*, vol. 36, no. 1, Jan. 1984, pp. 93–99.

Heim, Sverre, and Felix Mitelman. *Cancer Cytogenetics: Chromosomal and Molecular Genetic Aberrations of Tumor Cells*. John Wiley & Sons, Incorporated, 2015.

Helgason, Gudmundur, et al. *Helgason GV, Karvela M, Holyoake TL.. Kill One Bird with Two Stones: Potential Efficacy of BCR-ABL and Autophagy Inhibition in CML. Blood 118: 2035-2043*. Vol. 118, 2011.

Hobbs, Josie A. R., et al. "Myeloid Cell Function in MRP-14 (S100A9) Null Mice." *Molecular and Cellular Biology*, vol. 23, no. 7, Apr. 2003, pp. 2564–76.

Hochhaus, A., et al. "Molecular and Chromosomal Mechanisms of Resistance to Imatinib (STI571) Therapy." *Leukemia*, vol. 16, no. 11, Nov. 2002, pp. 2190–96.

Jiang, X., et al. "Chronic Myeloid Leukemia Stem Cells Possess Multiple Unique Features of Resistance to BCR-ABL Targeted Therapies." *Leukemia*, vol. 21, no. 5, May 2007, pp. 926–35.

Jagannathan-Bogdan, Madhumita, and Leonard I. Zon. "Hematopoiesis." *Development (Cambridge, England)*, vol. 140, no. 12, June 2013, pp. 2463–67.

Kang, Zhi-Jie, et al. "The Philadelphia Chromosome in Leukemogenesis." *Chinese Journal of Cancer*, vol. 35, May 2016.

Lewis, T., et al. "Novel Antagonists of Growth Factor Receptor Bound Protein 2 (GRB2) Inhibit Leukemic Proliferation." **In revision.**

Lin, J., and R. Arlinghaus. "Activated C-Abl Tyrosine Kinase in Malignant Solid Tumors." *Oncogene*, vol. 27, no. 32, July 2008, pp. 4385–91.

Lugo, T. G., et al. "Tyrosine Kinase Activity and Transformation Potency of Bcr-Abl Oncogene Products." *Science*, vol. 247, no. 4946, Mar. 1990, pp. 1079–82.

Mayadas, Tanya N., et al. "The Multifaceted Functions of Neutrophils." *Annual Review of Pathology*, vol. 9, 2014, pp. 181–218.

Million, R. P., and R. A. Van Etten. "The Grb2 Binding Site Is Required for the Induction of Chronic Myeloid Leukemia-like Disease in Mice by the Bcr/Abl Tyrosine Kinase." *Blood*, vol. 96, no. 2, July 2000, pp. 664–70.

Pawson, T., and J. D. Scott. "Signaling through Scaffold, Anchoring, and Adaptor Proteins." *Science (New York, N.Y.)*, vol. 278, no. 5346, Dec. 1997, pp. 2075–80.

Porter, Corrine J., et al. “Grb7 SH2 Domain Structure and Interactions with a Cyclic Peptide Inhibitor of Cancer Cell Migration and Proliferation.” *BMC Structural Biology*, vol. 7, Sept. 2007, p. 58.

Papaioannou, Danai, et al. “Structural and Biophysical Investigation of the Interaction of a Mutant Grb2 SH2 Domain (W121G) with Its Cognate Phosphopeptide.” *Protein Science: A Publication of the Protein Society*, vol. 25, no. 3, Mar. 2016, pp. 627–37.

Rawlings, Jason S., et al. “The JAK/STAT Signaling Pathway.” *Journal of Cell Science*, vol. 117, no. 8, Mar. 2004, pp. 1281–83.

Schindler, T., et al. “Structural Mechanism for STI-571 Inhibition of Abelson Tyrosine Kinase.” *Science (New York, N.Y.)*, vol. 289, no. 5486, Sept. 2000, pp. 1938–42.

Skorski, T., et al. “Phosphatidylinositol-3 Kinase Activity Is Regulated by BCR/ABL and Is Required for the Growth of Philadelphia Chromosome-Positive Cells.” *Blood*, vol. 86, no. 2, July 1995, pp. 726–36.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science. *241*, 58–62.

Steelman, L. S., et al. “JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in Cell Cycle Progression and Leukemogenesis.” *Leukemia*, vol. 18, no. 2, Feb. 2004, p. 189.

Willis, Stephanie G., et al. “High-Sensitivity Detection of BCR-ABL Kinase Domain Mutations in Imatinib-Naive Patients: Correlation with Clonal Cytogenetic Evolution but Not Response to Therapy.” *Blood*, vol. 106, no. 6, Sept. 2005, pp. 2128–37.

Wu, Y., et al. “Requirement of Two Specific Tyrosine Residues for the Catalytic Activity of Bcr Serine/Threonine Kinase.” *Oncogene*, vol. 16, no. 1, Jan. 1998, pp. 141–46.

Ziegler-Heitbrock, Loems. “The CD14+ CD16+ Blood Monocytes: Their Role in Infection and Inflammation.” *Journal of Leukocyte Biology*, vol. 81, no. 3, Mar. 2007, pp. 584–92.