ANALYSIS OF NOVEL ANTAGONISTS OF THE GRB2 SH2 DOMAIN THAT DECREASE PROLIFERATION IN CHRONIC MYELOID LEUKEMIA (CML)

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ANALYSIS OF NOVEL ANTAGONISTS OF THE GRB2 SH2 DOMAIN THAT DECREASE PROLIFERATION IN CHRONIC MYELOID LEUKEMIA (CML)

A Thesis

by

Tina R. Hanson

Summer 2018

APPROVED BY THE INTERIM DEAN OF GRADUATE STUDIES:

_____

Sharon Barrios, Ph.D.

APPROVED BY THE GRADUATE ADVISORY COMMITTEE:

_____ _____

Christopher Ivey, Ph. D. David Stachura, Ph. D.

_____

David Keller, Ph. D.

_____

Jeffery Bell, Ph. D.

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ABSTRACT

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THAT DECREASE PROLIFERATION

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95% of the cases of chronic myeloid leukemia (CML) are caused by a chromosomal translocation linking the breakpoint cluster region (BCR) gene to the Abelson murine leukemia viral oncogene-1 (ABL1). Downstream of BCR-ABL is growth receptor bound protein-2 (GRB2), which binds to BCR-ABL via its src-homology-2 (SH2) domain. This binding constitutively activates growth pathways while down regulating apoptosis leading to an over proliferation of immature and dysfunctional myeloid cells. Four novel SH2 antagonists were developed with three of the four showing a significant reduction in proliferation of a BCR-ABL^{+} leukemia cell line. To elucidate the mechanism of action, an enzyme-linked immunosorbent assay (ELISA), a surface plasmon resonance (SPR) assay, and a cellulose nitrate (CN) filter assay were performed. These assays indicated that one of the three effective molecules, NHD2-15, antagonized the SH2 domain

of GRB2 with a K_d value of 119 ± 2 μM, bringing us closer to developing a new treatment for CML.

CHAPTER I

INTRODUCTION

Myelopoiesis

Myeloid cells are a type of white blood cell whose major role is to fight pathogens invading the body as part of the innate immune system. These cells develop through a strictly regulated process known as myelopoiesis. All blood cell types, including myeloid cells, originate from a multipotent hematopoietic stem cell (HSC) that differentiates once exposed to a specific set of cytokines. HSCs are able to divide into two daughter cells; these daughter cells individually have the ability to become a further differentiated cell or remain as an HSC. HSCs destined to be in the myeloid lineage will differentiate into a common myeloid progenitor (CMP), but unlike HSCs, which can differentiate into any blood cell type, CMPs are limited to differentiating into erythrocytes, megakaryocytes, mast cells, or myeloblasts (a myeloid cell precursor). Once a CMP differentiates into a myeloblast, it will still require further cytokine signaling to become one of several fully functional mature myeloid cell types (Dzierzak, 2008).

Mature myeloid cell types are grouped into two categories, granulocytes and monocytes. Granulocytes consist of basophils, eosinophils, neutrophils, and mast cells and are named after the granules present in their cytoplasm. These granules are released during an immune response and contain proteins and enzymes designed to kill bacteria (Borregaard, 1997). Monocytes are precursors of macrophages and dendritic cells and are one of the links between the adaptive and innate immune systems. Both dendritic cells and

macrophages present antigens to cells of the adaptive immune system with macrophages also phagocytosing invaders (Zeigler-Heitbrock, 2007).

Once myeloid cells are fully differentiated, they migrate out of the bone marrow and travel, via the bloodstream, to replace dying cells throughout the body. While the exact lifespan of each myeloid cell type is not known, neutrophils make up 50-70% of all of the circulating leukocytes in humans and are believed to have a lifespan of about 5 days (Mayadas, 2013; Pillay, 2010). This means that the 10^{11} to 10^{12} neutrophils existing in an adult human body will need to be replaced on a constant basis (Mayadas, 2013).

BCR-ABL^{+} Chronic Myelogenous Leukemia

Chronic myelogenous leukemia (CML) is a malignant blood disease affecting the normal growth and development of myeloid cells. Leukemic HSCs will differentiate into CMPs but are not able to progress into fully functional myeloid cells, thus inundating the blood system with large amounts of immature malfunctioning cells. An astounding 95% of the incidences of CML are caused by a chromosomal translocation, which links the breakpoint-cluster-region (BCR) gene on chromosome 22 to the Abelson-murine-leukemia-viral-oncogene-1 (ABL1) gene on chromosome 9 (Lugo et al., 1990).

Normally, the ABL protein is a ubiquitously expressed non-receptor tyrosine kinase which, when signaled, triggers cell proliferation, differentiation, or apoptosis (Wang, 2014). The function of BCR is less clear but two roles are known. First, BCR was found to act as a GTPase activating protein (GAP) by activating Ras-related C3 botulinum toxin substrate 1 (RAC1), a GTPase in the RAS superfamily that is involved in cell proliferation regulation (Diekmann, 1991). Second, BCR has also been found to act as a kinase by phosphorylating, and thereby activating, a wide range of proteins (Maru, 1991).

The fusion of these two genes causes a change in phenotype by activating pathways such as Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase (RAS/MAPK/ERK) pathway, phosphoinositide 3-kinase (PI3K) pathway, and Janus kinase signal transducer and activator of transcription (JAK/STAT) pathway. These pathways increase proliferation and inhibit apoptosis (intentional cell death of unhealthy/aging cells) as well as autophagy (recycling of proteins and other cellular parts that are damaged/not needed) (Sawyers et al., 1995; Skorski et al., 1995). BCR-ABL has also been shown to affect cytoskeletal proteins within the cell. Studies show it is able to bind to actin via an actin-binding domain on its -COOH terminus; furthermore, many of the substrates for BCR-ABL's tyrosine kinase activity are cytoskeletal proteins (Salgia, 1997). Therefore, it is believed that BCR-ABL is directly responsible for the physical malformation of CML cells (Sattler et al., 2002b).

There are several possible variants of the fused BCR-ABL gene based on the exact location of the breakpoint in both ABL1 and BCR. The most common variant exists when the translocation occurs between intron 1 or 2 of ABL1 and the major breakpoint cluster region, M-*bcr* (between exons 13 and 14 or 14 and 15), of BCR (Ling et al., 2003). This leads to transcription of a 210-kDa BCR-ABL fusion gene, $p210^{BCR/ABL}$ (Melo, 1996). Less common combinations of the BCR and ABL1 gene exist and involve other exons on the BCR gene, such as between exons 1 and 2 or 19 and 20, but their transcripts have the same phenotypic effect as $p210^{BCR/ABL}$ even though the proteins are different sizes ($p190^{BCR/ABL}$ and $p230^{BCR/ABL}$ respectively) (Ling et al., 2003).

BCR-ABL and GRB2

Growth-receptor-bound protein 2 (GRB2) is an intracellular adapter protein responsible for linking receptor tyrosine kinases (RTKs) to downstream signaling proteins involved in cellular growth and differentiation (Fig. 1; Salesse et al., 2002). Generally, once an RTK binds its substrate on its extracellular domain, it will dimerize (if not already dimerized) and autophosphorylate (Maruyama, 2014). Once phosphorylated, the RTK dimer will bind to an adaptor protein, like GRB2 (Songyang et. al., 1993). The adaptor protein can then bind a catalytic protein, such as Son of Sevenless (SOS), linking it to the RTK and activating it. This is the beginning of a signal cascade where an extracellular substrate can activate change inside the cell without crossing the cell membrane.

As an adapter protein, GRB2 has multiple binding domains. It is one of many proteins to contain a src-homology 2 (SH2) binding domain as well as a src-homology 3 (SH3) binding domain (Lowenstein et. al., 1992). The SH2 binding domain is a highly conserved, approximately 100 amino acid sequence, that binds phosphorylated tyrosines. It is typically found on adapter or other proteins that do not have any catalytic capabilities. The SH3 binding domain on the other hand, binds proline rich areas and is found on proteins with and without catalytic capabilities. It is approximately 50-75 amino acids in length and is also highly conserved (Marengere, 1994). GRB2 is a small protein consisting of one SH2 binding domain and an SH3 binding domain on each side (Maignan et al., 1994).

GRB2's SH2 domain binds to BCR-ABL via a phosphorylated tyrosine residue (Y177) on the BCR side in the same manner that it binds an activated dimerized RTK (Puil, 1994). This BCR-ABL GRB2 binding is what initiates leukemic transformation (Million

et al., 2000). After binding to BCR-ABL, GRB2's SH3 domain can bind to GRB2-associated-binding protein 2 (GAB2), as well as Son of Sevenless (SOS), and many others (Sattler et al., 2002a). This secondary binding causes the newly formed BCR-ABL GRB2 complex to associate with membrane receptors, such as RTKs, cytokine receptors, g-coupled protein receptors and others, by binding to their intracellular domains activating cellular growth, survival, and differentiation pathways while inhibiting apoptosis and autophagy as previously discussed (O'Hare T et al., 2011).

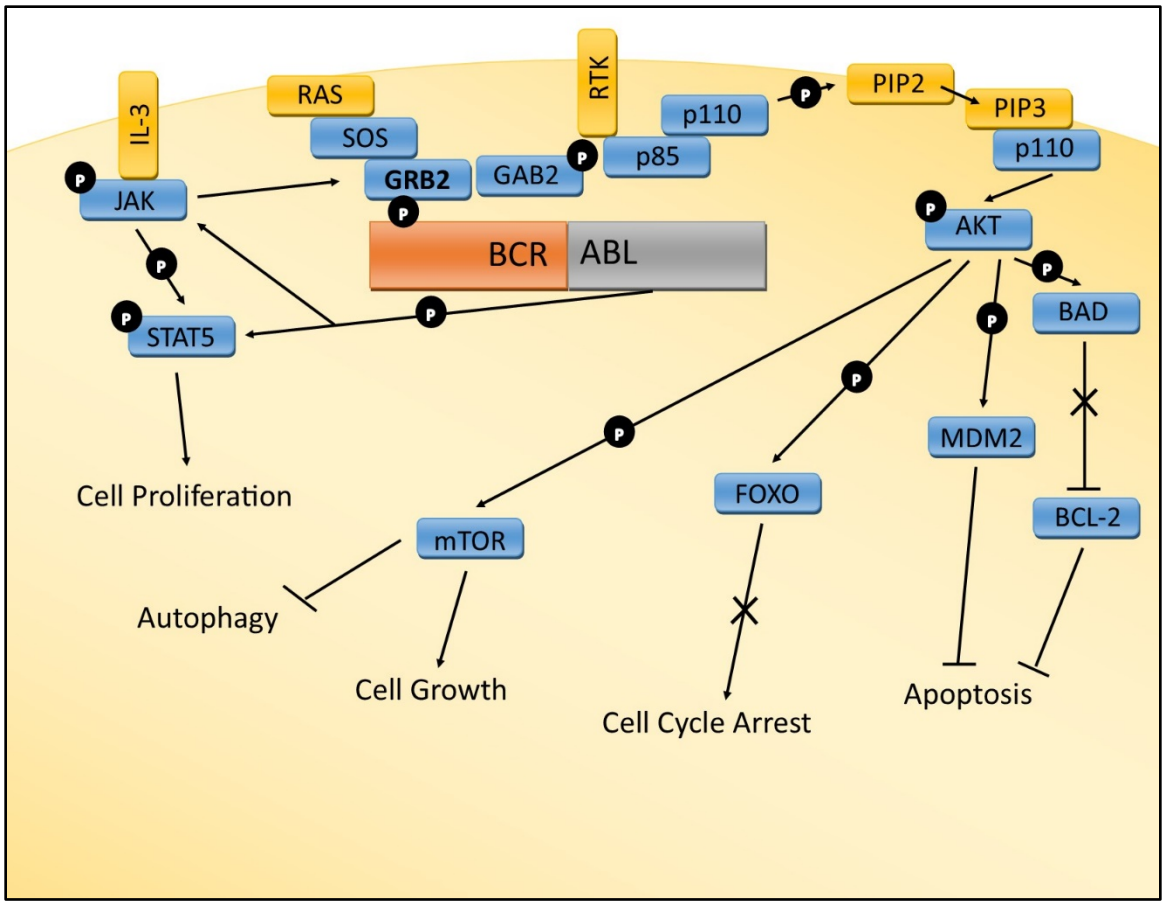

Figure 1. Simplified diagram showing how an increase in GRB2 levels in BCR-ABL^{+} cells can activate cell growth and proliferation while lowering levels of autophagy and apoptosis. When BCR-ABL is present, GRB2 binds to a phosphorylated tyrosine (Y177) on the BCR side. GRB2 activates GAB2 and other proteins leading to lower levels of apoptosis and autophagy, constant cell cycling, and an increase in cell growth due to activation of the PI3K/AKT pathway. Furthermore, ABL is constitutively activated, leading to a constant activation of the JAK/STAT pathway, which further increases cell growth.

Treatments for CML

The common treatment for CML is a drug called imatinib, also known by its brand-name Gleevec, STI571, or CGP57148B. Imatinib is a highly specific ABL tyrosine kinase inhibitor that reduces myeloid proliferation levels in BCR-ABL$^+$ CML cells. It does this by stabilizing the inactive form of BCR-ABL (Deininger et al., 2005, Druker 1996). ABL is inactive when a single proline residue connects its SH3 domain to its catalytic domain. Phosphorylation of a tyrosine residue (Y242) is what opens it back up, leading to activation of ABL's kinase activity (Brasher and Etten, 2000). Imatinib binds to the ATP binding site of BCR-ABL only when Y242 is not phosphorylated and prevents further phosphorylation (Schindler, 2000). In other words, as BCR-ABL fluctuates from inactive to active, imatinib binds the inactivated protein and keeps it that way.

Unfortunately, some patients develop a resistance to imatinib leading to relapse of CML. Resistance is typically caused by one of four mechanisms: point mutations, gene amplification, denial of entry into the cell, and genomic instability. Point mutations that disrupt imatinib's affinity for BCR-ABL are typically found in the ABL kinase domain and are identified in 50-90% of all relapsed patients (Gore et. al., 2001; Hochhaus et. al., 2002). A relapse can also be caused by a drastic increase in BCR-ABL mRNA levels leading to far more BCR-ABL proteins than available imatinib (Le Coutre et. al., 2000). Though the reason and mechanism are not known, an increase in the dose of imatinib is typically enough to end the relapse (Hochhaus et. al., 2002). Furthermore, as with other chemotherapeutic agents, entry into the cell might be restricted, though it is unclear if this is occurring with imatinib specifically (Hegedus et. al., 2002). Lastly, because BCR-ABL is formed during a translocation due to genomic instability, it is more likely that further

mutations can and will occur (Hochhaus et. al., 2002). These mutations might be responsible for an increase in the activity of BCR-ABL, overpowering the effectiveness of imatinib. Because of the resistance some patients develop, further studies are needed to establish better treatment options for people suffering from BCR-ABL$^+$ CML.

Novel SH2 Antagonists

Seeing that a better treatment method was needed for CML, Dr. Carolynn Arpin, a professor in the Chemistry and Biochemistry department at California State University Chico, and her student, Natalie Douglas, synthesized several small molecules with structures based off of a known SH2 antagonist of GRB7, named NSC642056 (Fig. 2a; Ambaye et. al., 2011). GRB7's SH2 domain binds phosphotyrosine motifs like GRB2's SH2 domain but the rest of the protein is structurally different (Pawson, 1995). Instead of the SH2-SH3-SH2 domain configuration of GRB2, GRB7 has a Ras-associating (RA) like domain, a pleckstrin homology (PH) domain, a phosphotyrosine interacting (BPS) region and then a C-terminal SH2 domain (Daly, 1998). Since this SH2 antagonist was able to bind to the SH2 domain of GRB7 with high affinity ($K_d = 3.1 \pm 0.8$ μM) and both GRB2 and 7's SH2 domains are very similar, several small molecules were created in its likeness with the hopes that a highly specific GRB2 SH2 antagonist could be generated (Ambaye et. al., 2011).

To create these SH2 antagonizing molecules, Dr. Arpin constructed an innovative reaction scheme where a quinoxaline and a β-dicarbonyl were used to synthesize di-substituted furo[2,3-*b*]quinoxaline products via a $S_{RN}1/S_NAr$ domino reaction (Fig. 2b). These furo-quinoxaline products resembled the binding region of the known

GRB7 SH2 antagonist with combinations of different R groups (Fig. 2c; Ambaye et. al., 2011).

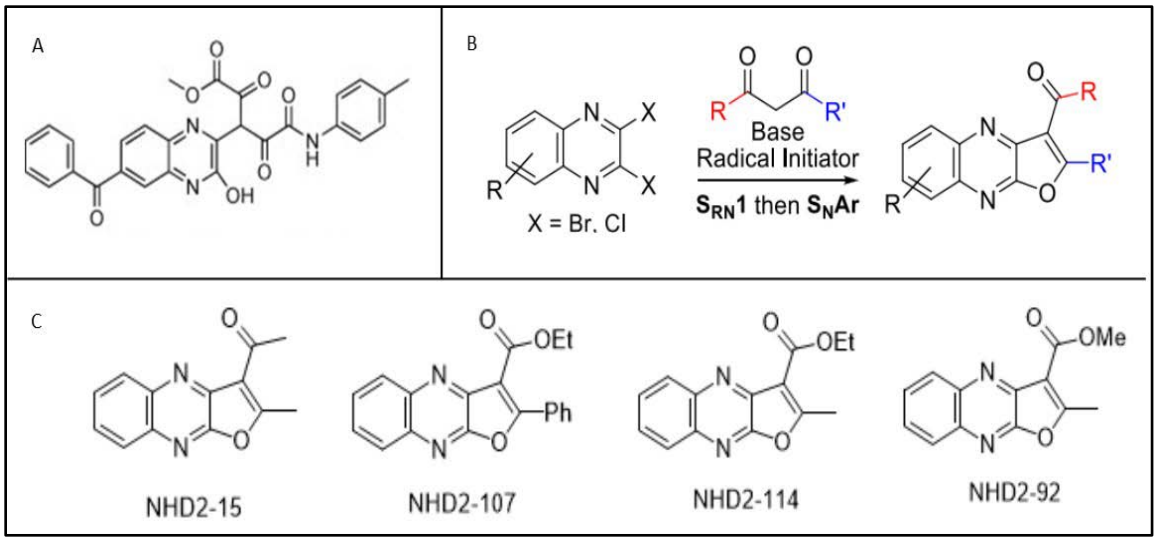

Figure 2. **Novel GRB2 SH2 antagonists were created via an innovative reaction scheme based off a known GRB7 SH2 antagonist.** (A) The model molecule, named NSC642056, is a known SH2 antagonist for GRB7. (B, C) In an attempt to generate an SH2 antagonist for GRB2 a novel reaction scheme involving a $S_{RN}1/S_NAr$ domino reaction, four novel molecules were created.

CHAPTER II

MATERIALS AND METHODS

Cell Proliferation Assay

K562 cells were maintained in RPMI 1640 media with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% L-glutamine at 37º C and 5% CO_2. K562 cells were plated at 10 x 10^4 cells/well in a 96 well plate and incubated for 48 hours before being subjected to the compounds in the following concentrations: 60, 30, 15, 0 μM (control). After the cells were incubated with the molecules for 72 hours, the cells were counted again. Imatinib was used as positive control and dimethyl sulfoxide (DMSO) was used as a negative control.

Enzyme Linked Immunosorbent Assay (ELISA)

Plates coated with streptavidin (Thermo Fisher) were incubated for one hour at 37° C with 100 μl of a 100 nM solution of the peptide (biotin-Aha-PSpYVNVQN) in TBS buffer (Tris 100 mM, NaCl 50 mM, pH 7.5). 800 μl per well of SuperBlock™ Blocking Buffer (Thermo Scientific) was added to each well and incubated for four hours. The antagonists at the desired concentrations (60, 30, 15, and 0μM) were diluted in SuperBlock™ and GST-GRB2 (20 nM, 100 μl per well) and were both added to the desired wells and incubated for one hour at 37° C. The plates were then rinsed four times using SuperBlock™-0.05% tween 20. 100 μl per well of anti-GST antibody with Hrp (Invitrogen, dilution of 1/4000 in SuperBlock™-0.05% tween 20) was added and the plate was then incubated for one hour at 37° C. The plates were then rinsed four times using

SuperBlock™-0.05% tween 20 before being incubated in the presence of 200 µl per well of TMB developing solution at room temperature until a blue color developed. The reaction was stopped by adding 100 µl of 10% (V/V) sulfuric acid per well. The absorbance reading was taken at 450nm and the value read off for each well was then reduced by the control value of an equivalent well with no peptide that was still treated the same as the wells that have a peptide.

BIAcore™ SPR

Surface Plasmon Resonance Assay (SPR) experiments were completed using a BIAcore™ 3000 instrument (GE Healthcare). A CM5 chip surface was activated with 1:1 N-hydroxysulfosuccinimide (NHS)/1-(3-dimethylamino-propyl)-3-ethylcarbodiimide hydrochloride (EDC). GRB2 was immobilized on a CM5 sensor chip following the manufacturer's instructions. We then diluted each antagonist with HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v Surfactant P20) to final concentrations of 125, 62.5, 31.25, 15.63, 7.81, 3.91, and 0 µM in 0.5% DMSO. Then antagonist was injected into the GRB2-immobilized CM5 sensor chips and the response read. Data analysis was carried out using Scrubber 2.0 software.

CN Filter Assay

4 µM of GRB2 was incubated in either 8 µM SH2 domain inhibitor (K_d = 400 nM (Bibbins et. al., 1993)) (Santa Cruz Biotechnology), 16 µM SH3 inhibitor (K_d = 4 µM (Vidal et. al., 1997)) (Santa Cruz Biotechnology), both inhibitors, or neither inhibitor. After incubation, one of the antagonists was added for a final concentration of 25 µM and allowed to incubate before being pushed through a 0.2 µm cellulose nitrate syringe filter (Thermo Fisher). The solution that passed through the filter was then analyzed via a HP

6890 series gas chromatograph (GC) flame ionization detector (FID) with an omegawax 250 column (Sigma Aldrich). A standard curve of each antagonist was made by diluting the antagonist to 100, 75, 50, 25, and 0 μM and then analyzed via GC-FID. A plot of response vs. concentration was made and the resulting linear regression equation was used to calculate the final concentration of that antagonist in the final solution.

Toxicity Assay

Adult zebrafish (>12 weeks) were placed in a tank with the appropriate concentration of compound (60, 30, 15, 0 μM) and incubated for 24, 48, or 72 hours. The fish were then analyzed for any physical signs of toxicity compared to control.

CHAPTER III

DATA AND DISCUSSION

Effects on BCR-ABL$^+$ K562 Cells

As a first step, we wanted to expose a BCR-ABL$^+$ immortalized leukemic cell line, called K562, to each of the four molecules Dr. Arpin created to see if they caused significant proliferation reduction when compared to the control. To accomplish this, K562 cells were plated at $10x10^4$ cells/well, exposed to one of the antagonists, and allowed to grow for 72 hours. After the 72-hour time allotment passed, the cells were counted to see if proliferation was different from the control plate with no antagonist. As a positive control we used imatinib, known to inhibit proliferation of BCR-ABL$^+$ cells, and as a negative control we used the vehicle, DMSO.

After 72 hours, we saw a significant growth reduction, even at low concentrations, with NHD2-15. Higher concentrations, 60 and 120 μM, stopped proliferation completely (Fig. 3). NHD2-107 and NHD2-92 showed less effectiveness but were still able to slow down proliferation, especially at higher concentrations. NHD2-114's results are difficult to interpret as it is much more hydrophobic than the other compounds due to the phenyl (Ph) R group and would not stay in solution; however, when compared to control (0 μM) there was still a significant reduction in proliferation. Therefore, it appears that all four compounds were able to reduce the proliferation levels in BCR-ABL$^+$ K562 cells.

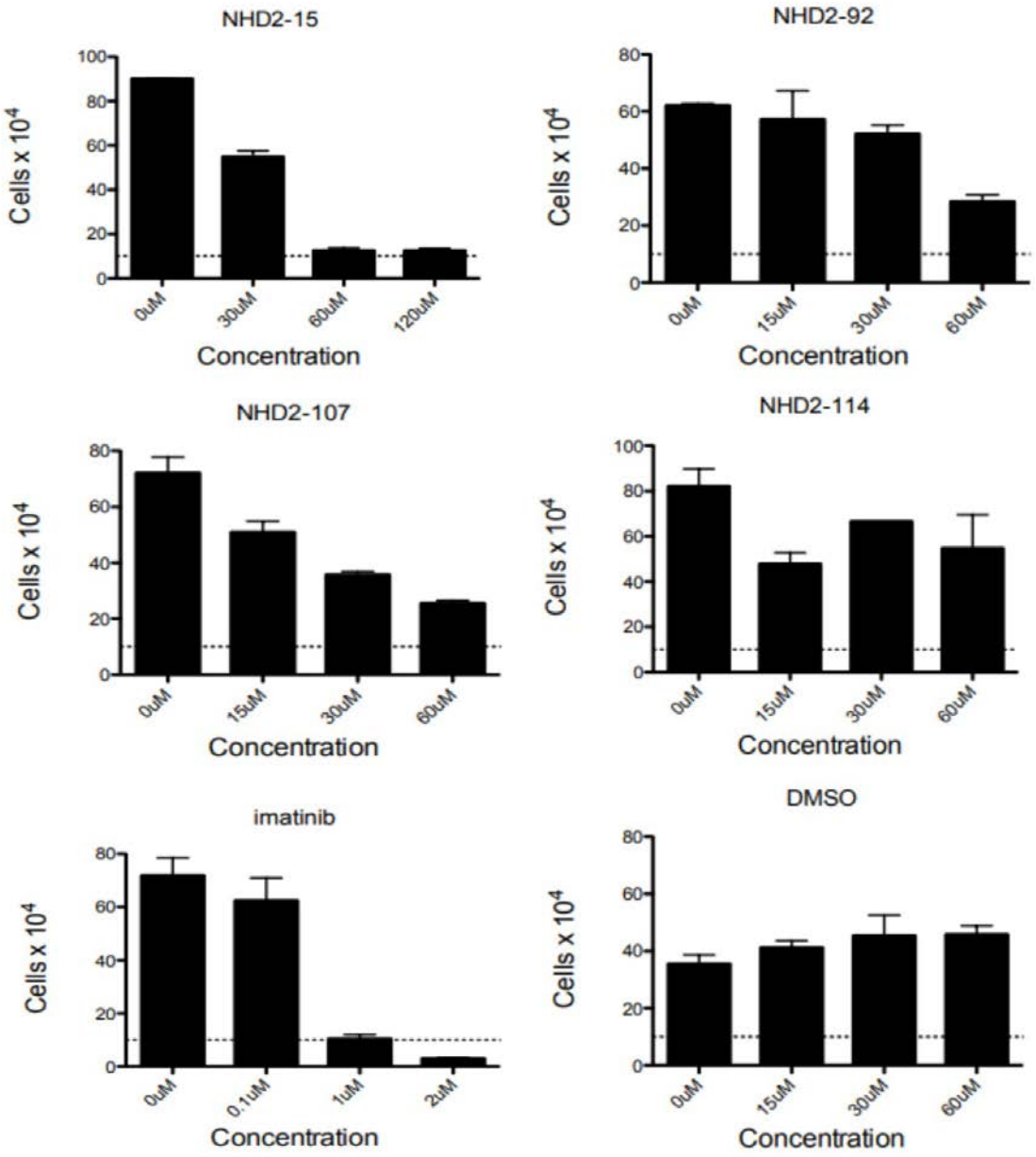

Figure 3. Exposure to the novel antagonists lowers proliferation levels of BCR-ABL$^+$ K562 cells. K562 cells were incubated for 72 hours in the presence of one of the novel SH2 antagonists at increasing concentrations (0, 15, 30, 60, and 120 μM). The cells were counted at t = 0 (10 x 10^4 cells denoted by the dotted line) and again at t = 72 hrs. DMSO was used as a negative control and imatinib was used as a positive control. Data collected by Kallie Griffin and Skylar Tomasetti.

Novel Antagonists Bind GRB2

Once the effectiveness of the antagonists was known, we needed to show that the antagonists were binding to the SH2 domain of GRB2 as expected. We did this by showing each antagonist could out competed the natural GRB2 SH2 ligand. This was done by immobilizing a biotin tagged peptide designed to match the binding region of the natural ligand on the walls of a streptavidin-coated well plate (Fig. 4). Next, we added one of the antagonists and a glutathione s-transferase (GST)-tagged GRB2 to the well. After incubation, the wells were rinsed to wash away any GRB2 that bound antagonist instead of peptide. The amount of GRB2 bound to the peptide attached to the well was then visualized colorimetrically via an anti-GST antibody coupled with horseradish peroxidase (Hrp). The more GRB2 bound to the natural ligand, the lower affinity the drug had for GRB2. We predicted that each one of these antagonists would outcompete the ligand as they were designed after a known SH2 antagonist.

When we examined NHD2-15 and NHD2-114 we saw the same trend as with the positive control; presumably they were outcompeting the peptide for the SH2 domain as desired (Fig. 5). However, when we ran the ELISA on NHD2-107 and NHD2-92 we saw a different trend. Instead of seeing GRB2 leave as we increased the antagonist concentration we observed more GRB2 binding to the peptide at lower concentrations, followed by off-binding at higher concentrations. This result can be interpreted as allosteric binding; however, more information was needed to understand where these molecules were binding.

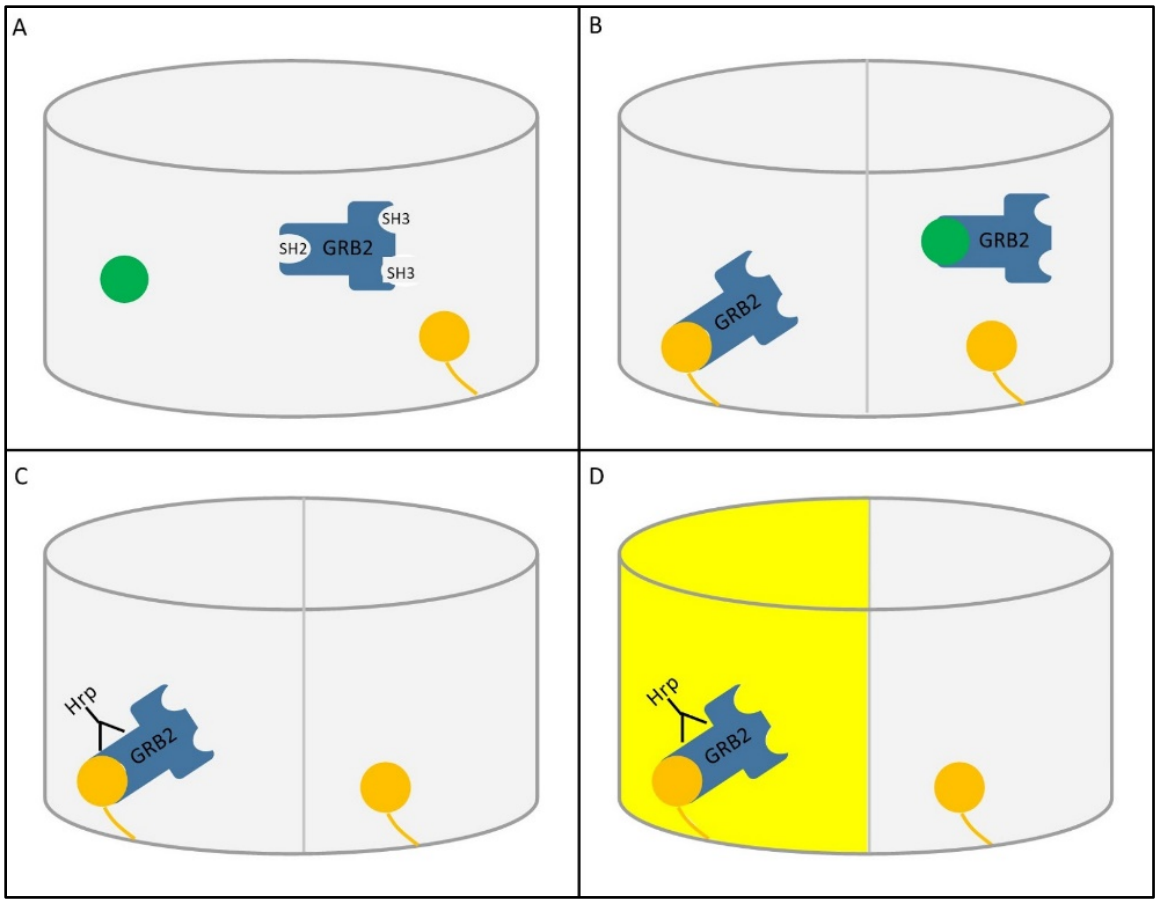

Figure 4. Reaction schematic showing how the Enzyme Linked Immunosorbent Assay (ELISA) was performed. (A) The peptide (orange) with a biotin tag was allowed to incubate in a well that was coated in streptavidin. (B) Once the peptide was adhered to the plate, GRB2 with a GST tag (blue) and one of the antagonists (green) were incubated allowing GRB2 to bind either the peptide or the antagonist. (C) The wells were rinsed to wash away any unbound GRB2 and an anti-GST antibody coupled with horseradish peroxidase (Hrp) was introduced (black). (D) Tetramethylbenzidine (TMB), a substrate for Hrp, was allowed to react until a color developed before being stopped with sulfuric acid and analyzed via UV-Vis at 450 nm.

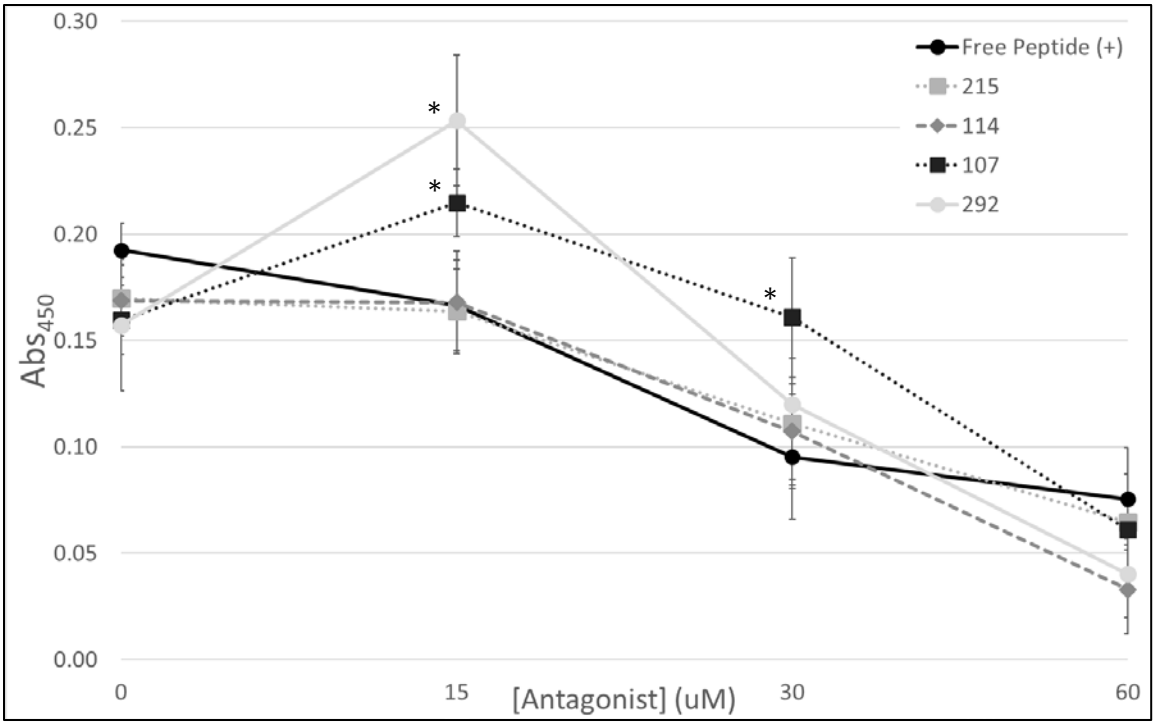

Figure 5. Antagonists outcompete the natural ligand at varying concentrations. The ELISA results show NHD2-15 and NHD2-114 were not significantly different from the positive control (free peptide). This indicates that they outcompete the natural ligand even at low concentrations. NHD2-92 and NHD2-107, however, cause more GRB2 to bind to the natural ligand when present at 15 µM, as seen in a significant increase in absorbance compared to positive control. NHD2-107 also caused significantly more GRB2 to bind at 30 µM versus control but neither NHD2-92 or NHD2-114 were significantly different from the positive control at higher concentrations, showing they do outcompete the ligand at higher concentrations. (Notes: * = $p < 0.05$; n=4; error bars = SD)

Determination of K_d

Because of the unexpected ELISA results, we wanted to verify that all four antagonists were able to bind to GRB2 specifically. This was done by measuring the dissociation constant, K_d, between GRB2 and each of the antagonists. K_d is the concentration of the antagonist at which half of the GRB2 has antagonist bound. This standard allows the comparison of binding affinities between the four antagonists as well as with other small molecules.

To determine the K_d we utilized University of California Davis' BIAcore™ 3000 to perform a BIAcore™ SPR assay. In this assay a small molecule, in our case the GRB2 protein, is bound by the primary amine via a linker molecule to a carbonyl group on a gold sensor chip's surface while another small molecule, the antagonist, flows over the top of the chip at varying concentrations (Fig. 6; GE Healthcare Life Sciences). When the two molecules bind a laser aimed at the bottom of the chip detects it. When the laser is aimed at the reference chip, a gold chip with GRB2 bound but no antagonist flowing over, the light is reflected back at a specific angle. Once the GRB2 binds an antagonist, the electronics of the complex alters the electronics of the gold chip, the angle of reflection changes, and this is seen as a 'response.' The response at different concentrations of antagonists can be plotted and the K_d determined. Considering these molecules were modeled after a known GRB7 SH2 antagonist, and SH2 domains are generally well conserved, we predicted that these antagonists would have decent K_d values (uM-mM range).

The BIAcore™ analysis showed that all 4 antagonists had K_d values in the µM to mM range (Fig. 7; Table 1). NHD2-15 had the highest affinity for GRB2 with a K_d of

119 ± 2μM. NHD2-114 was next with a K_d of 440 ± 7 μM. NHD2-92 and NHD2-107 were similar to each other but in the mM range instead of the μM range (1.00 ± 0.02 mM and 3.4 ± 0.1 mM respectively). This shows that all four molecules bind to GRB2 with K_d values in a desirable range.

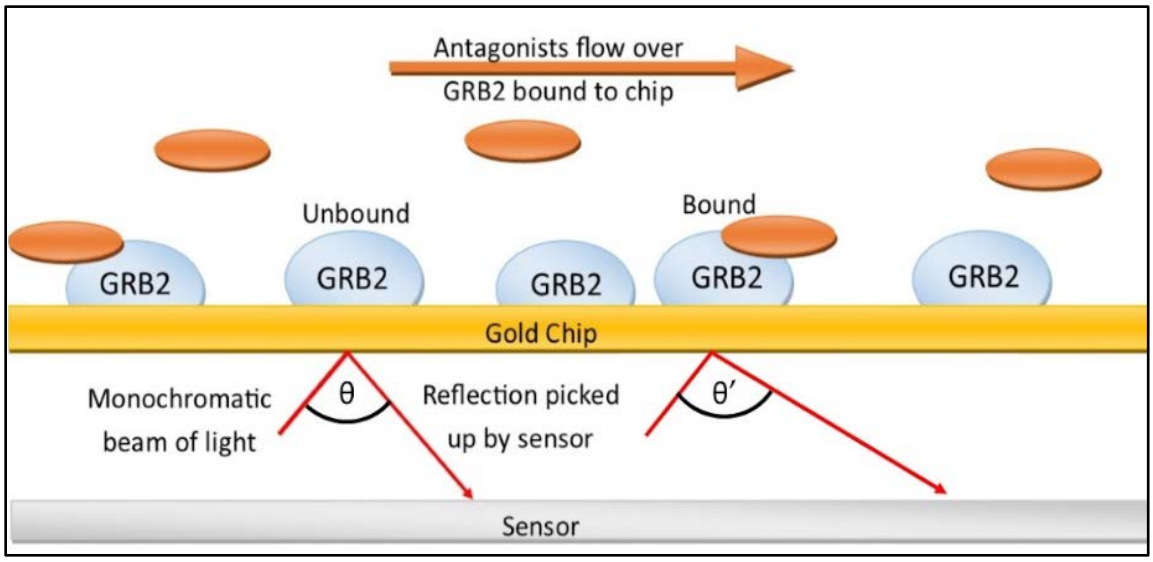

Figure 6. Diagram showing the theory behind BIAcore™ surface plasmon resonance. GRB2 is bound to a gold chip while one antagonist is flowing in solution over the top. When the GRB2 binds an antagonist the electronics of the GRB2-Gold complex changes. A monochromatic beam of light is aimed at the opposite side of the chip and the reflection is picked up by a sensor. When the GRB2 is unbound the light reflects off the opposite side of the gold chip and hits a sensor below at a specific angle (θ), however, when GRB2 complexes with an antagonist the light is bent at a different angle (θ'). This binding response at varying concentrations of antagonists can be measured to determine the K_d.

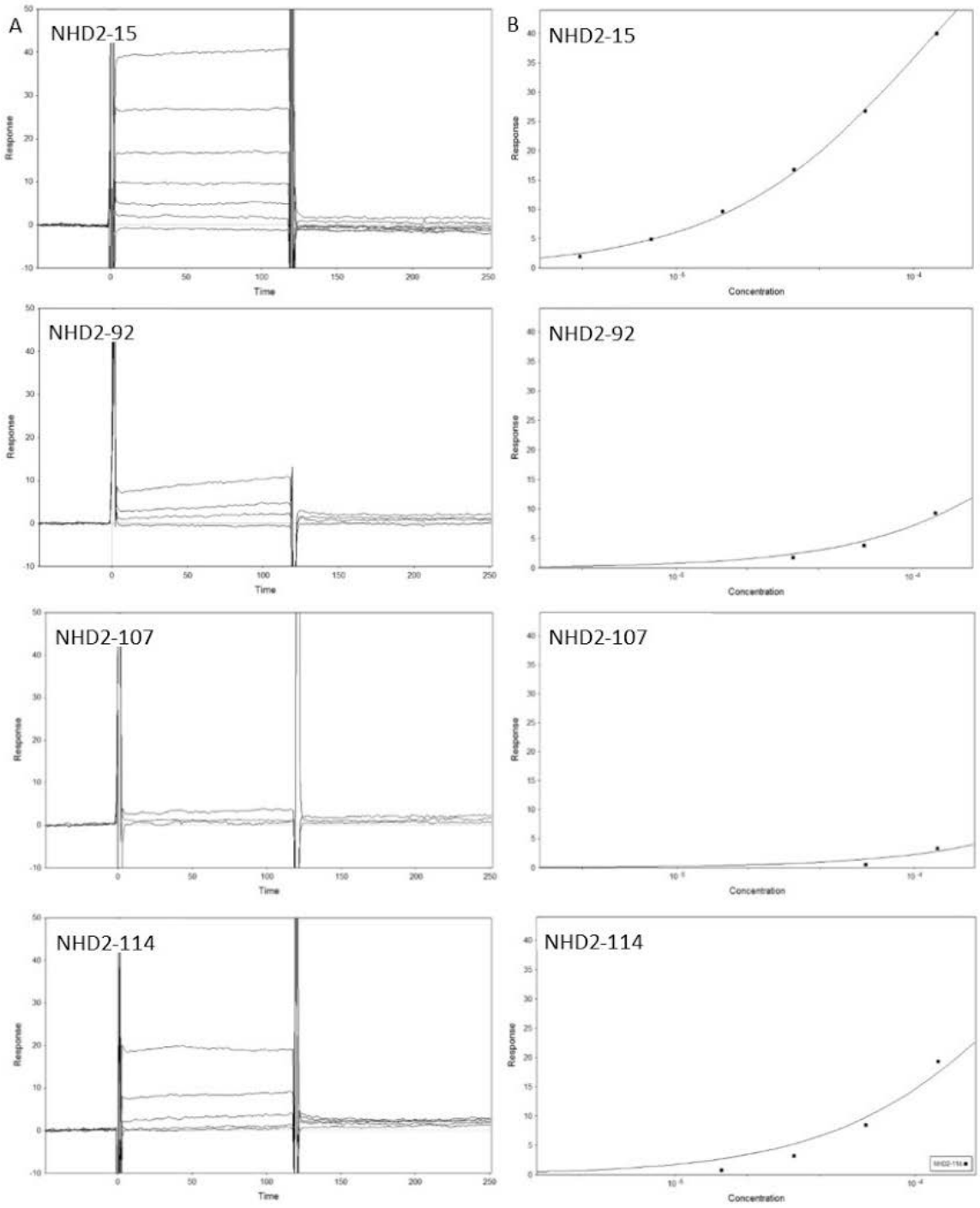

Figure 7. BIAcore™ Assay showing all four novel antagonists bind to GRB2. (A) The response over time was read at increasing concentrations of each antagonist during a BIAcore™ SPR assay (0, 3.91, 7.81, 15.63, 31.25, 62.5, 125 µM). (B) A Scatchard plot of response vs. concentration was constructed for each antagonist to calculate half-maximal response (equivalent to K_d).

Table 1: K_d values for the four antagonists.

Compound Name	**K_d**
NHD2-15	119 ± 2 μM
NHD2-92	1.00 ± 0.02 mM
NHD2-107	3.4 ± 0.1 mM
NHD2-114	440 ± 7 μM

Identification of Binding Location

Lastly, it was important to verify where on GRB2 the antagonists were binding, as there is more than one binding domain and the ELISA results elude to the possibility of allosteric binding. One way to accomplish this was via a cellulose nitrate (CN) filter assay. In this assay, GRB2 was incubated with either an SH2 domain inhibitor, an SH3 domain inhibitor, both, or neither, before being introduced to one of the antagonists (Fig. 8). The solution was then run through a CN filter, which captures proteins, and anything bound to it, and allows small molecules to pass through. The solution that passed through the filter, the flow through, was then collected and ran through a gas chromatography (GC) flame ionization detector (FID). The GC mobilizes the solution into the gas phase and pushes it, via compressed helium, through a column. Any component within the flow through (buffer, antagonist, etc.) would separate by size with the smallest molecules exiting the column first, followed by the next largest, and so on. After exiting the column, the molecules flow through a hydrogen flame that ionizes them right before they hit a detector that recognizes charged particles. The readout tells us how long it took the molecule to go through the

column, retention time (R_t), and a relative readout of how much ionized material hit the detector. Because the molecular weight of each molecule is different they all have specific R_t and can be identified. We can then compare the concentration of the antagonists in the flow through when one, both, or none of the binding domains are blocked to ascertain where these antagonists are binding. We can also construct a calibration curve where we analyze known concentrations of each antagonist to create a linear plot of response vs concentration. Because these plots are linear, we can analyze the graph to obtain a linear regression line and equation. This equation, in the y=mx+b format, can be used to calculate the unknown concentration (x) from the response readout from the GC (y) (Fig. 9). As these molecules are slightly modified versions of a molecule known to bind to GRB7's SH2 domain, we expected most, if not all, of these antagonists to bind to the SH2 domain of GRB2 as well; however, the different R groups might affect where these molecules bound.

The results showed that when the SH2 domain was blocked, less NHD2-15 and NHD2-114 bound to GRB2 as seen by an increase in antagonist concentration in the buffer that flowed through the filter (Fig. 10). Blocking the SH3 domain had no effect. This verified that NHD2-15 and NHD2-114 were binding to the SH2 domain as expected. NHD2-107 showed much less clear results indicating it might be binding to other places on GRB2 or is able to bind SH2 and SH3 under varying circumstances. NHD2-92 appears to be binding to neither the SH2 or SH3 domain; furthermore, when the SH2 inhibitor was used, more (not less) antagonist bound to GRB2 which is consistent with the ELISA data.

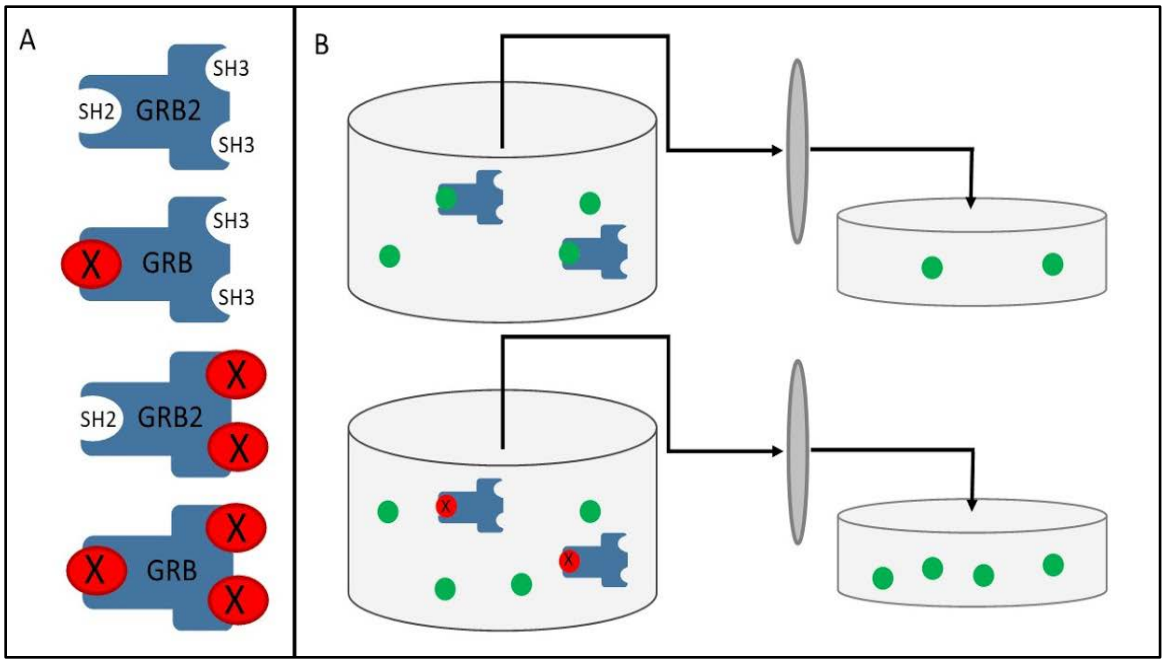

Figure 8. Schematic of the cellulose nitrate (CN) filter assay used. GRB2 (blue) was incubated with either an SH2 inhibitor, and SH3 inhibitor, or both inhibitors (red). An antagonist (green) was added and allowed to incubate before being pushed through a cellulose nitrate syringe filter (dark grey). The flow through was collected and analyzed via GC-FID and against a standard curve to determine concentration.

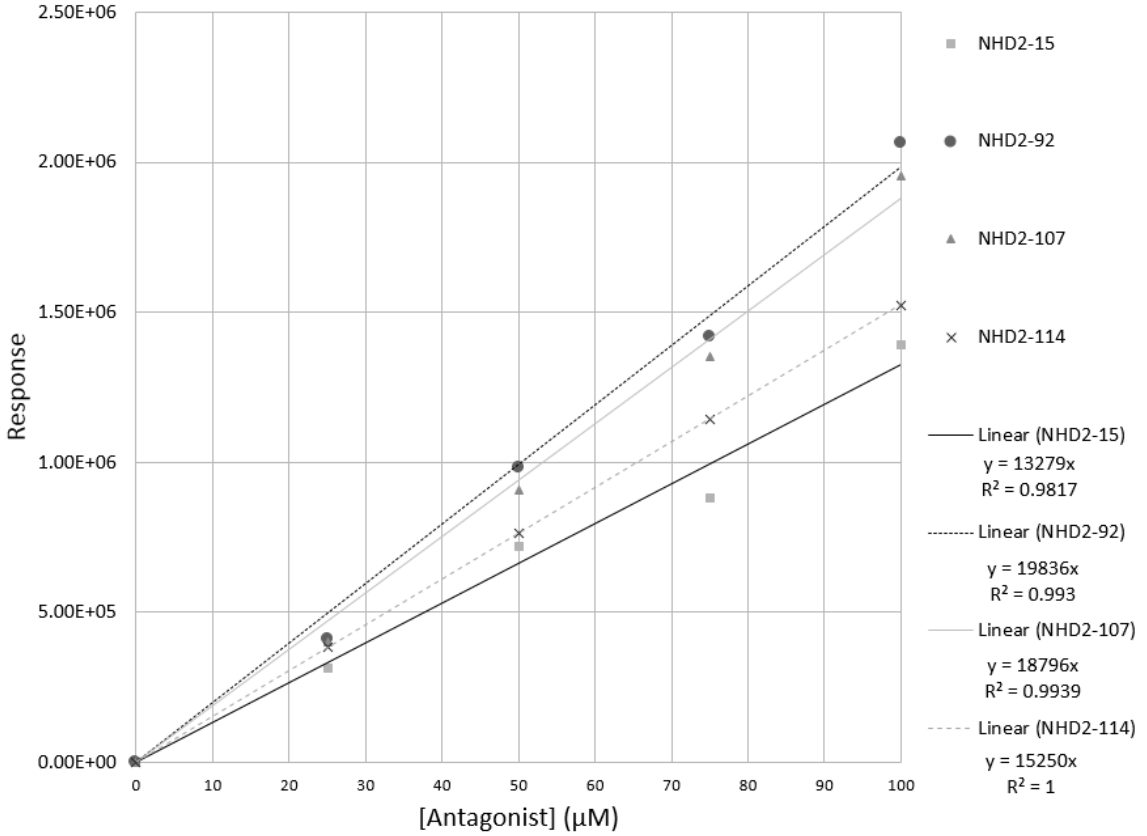

Figure 9. [Antagonist] vs GC-FID response are linearly correlated. Each antagonist was diluted to 100, 75, 50, and 25 µM and run through the GC-FID. The response vs. concentration was plotted and a linear regression line constructed. The equation of the line was used to calculate the concentrations of the antagonists in the final solution of the CN assay.

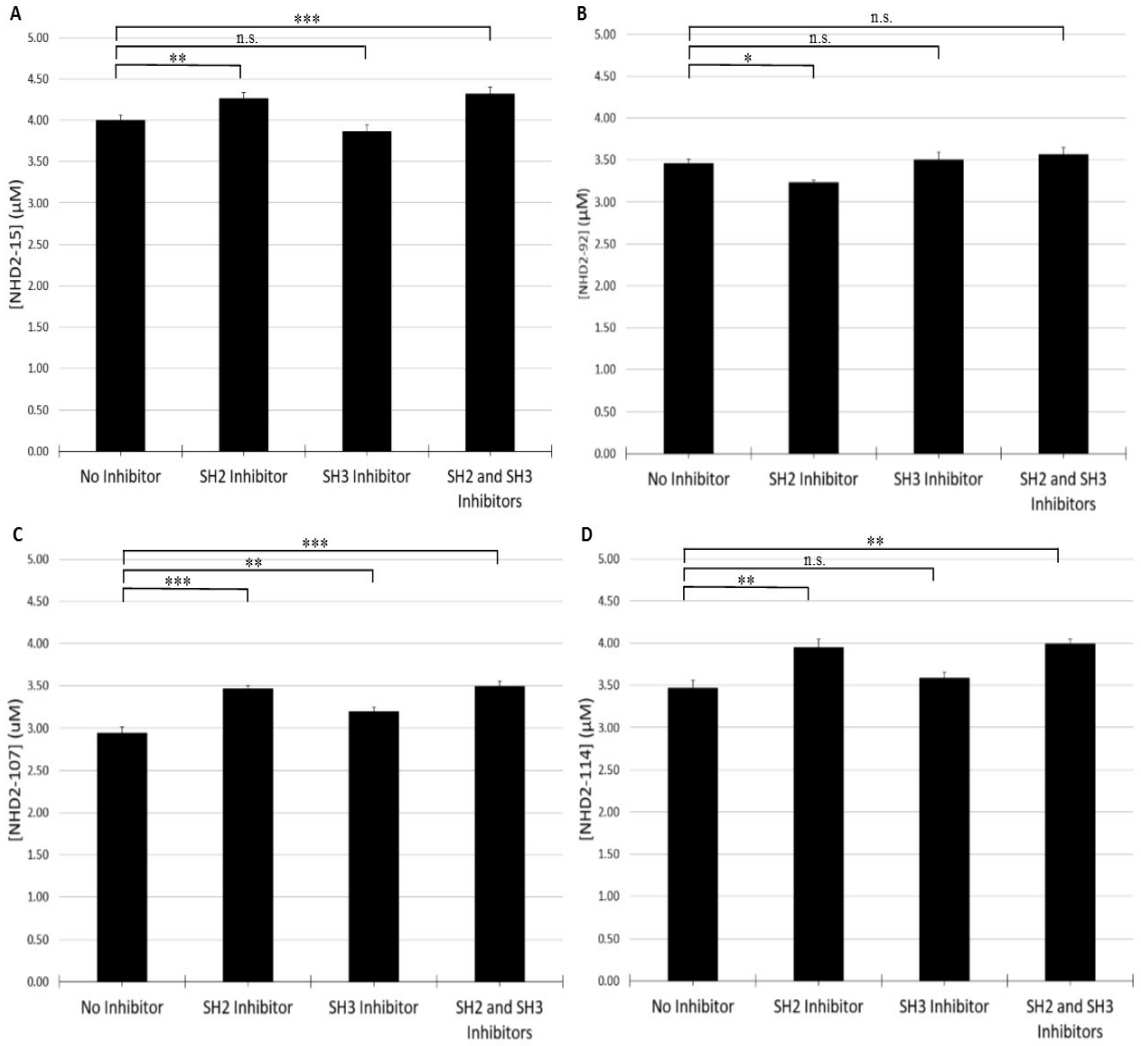

Figure 10. NHD2-15 and NHD2-114 are specific for the SH2 domain on GRB2. (A) NHD2-15 had a higher concentration of antagonist in the flow through in the presence of an SH2 inhibitor compared to the control. The SH3 inhibitor had no significant effect, and the combination of the two inhibitors showed the same effect as the SH2 inhibitor alone. (B) NHD2-92 showed more antagonist binding when the SH2 inhibitor was present. There was no significant difference between the control and SH3 inhibitor or the control and both inhibitors combined. (C) NHD2-107 showed a significant difference between the SH2 inhibitor, the SH3 inhibitor, and both inhibitors vs the control. (D) NHD2-114 showed the same results as NHD2-15. (Notes: * $p < 0.005$; ** $p < 0.0005$; *** $p < 0.01$; n=4; error bars = SD; n.s., not significant)

Allosteric Binding is Likely

GRB2 has two SH2 domains facing out one side and a single SH3 domain in between the two SH2 domains but opening out the other direction. As previously explained, it acts as an adapter protein, binding a phosphorylated tyrosine via its SH2 domain and a proline rich motif with one, or both, of its two SH3 domains. Furthermore, GRB2 has been shown in other circumstances to rely on allostery to change binding affinities (Chook et. al., 1996; Huang et. al., 2017; McDonald et. al., 2013). The results of the CN assay, as well as the ELISA assay, appear to be indicating that two molecules, NHD2-92 and NHD2-107, are able to bind somewhere else entirely and in the case of NHD2-92, influence GRB2's binding via its SH2 domain.

Toxicity in a Whole Animal System

For a molecule to be a potential treatment for any disease, it must not be toxic to the patient. Because of this, we wanted to see if these molecules could be tolerated in adult zebrafish (*Danio rerio).* Zebrafish have advantageous properties that aid in the study of hematopoiesis. Zebrafish develop externally, unlike mice, which allows us to examine development from fertilization all the way through adulthood without disrupting the mother and embryo in the process. Moreover, zebrafish are optically transparent, permitting visualization of the development of internal structures. These features are not explicitly necessary for this particular assay, but future work on these antagonists can utilize zebrafish to try ascertaining a mechanism of action. In addition, because zebrafish live in water, the fish can be exposed to the antagonists simply by adding it to the tank.

To test the toxicity, we put an adult zebrafish into a specific concentration of antagonist (15, 0 μM) in tank water. We allowed the fish to live in the antagonist for 24,

48, or 72 hours before visually determining the health of the fish and comparing it to the control. We used imatinib and DMSO as negative controls. Only one antagonist was found to not be toxic, even over 72 hours of exposure, and it was NHD2-15 (Fig. 11). Both NHD2-92 and NHD2-114 did not appear to be toxic over a 24-hour exposure but at 48 and 72 hours, they proved to be lethal. NHD2-107 was lethal within the first 24 hours.

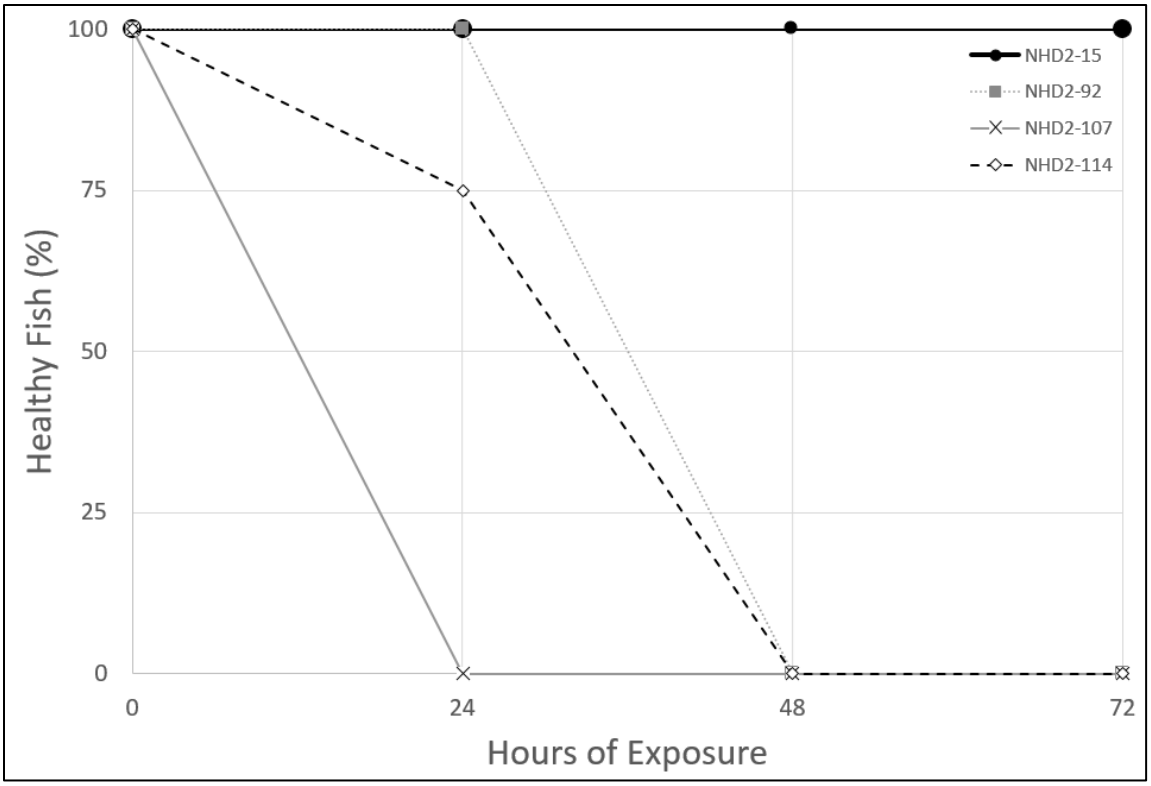

Figure 11. All but NHD2-15 proves to be toxic to adult zebrafish. After either a 24, 48, or 72-hour incubation in one of the antagonists (15 µM; n=4), adult zebrafish were visually assessed for overall health. DMSO and imatinib (15 and 2 µM respectively; n=8) were used as negative controls and showed no effect on health of fish (data not shown). Data collected by Kallie Griffin.

CHAPTER V

CONCLUSIONS AND FUTURE WORK

Looking at the structures of these four molecules, as well as the effects on proliferation, the K_d data, and ELISA data, NHD2-15 stands out with the most drastic proliferation decrease of the four, the lowest K_d, and clear SH2 binding. Additionally, it was also the only molecule that did not exhibit toxic side effects in adult fish at 15 μM. It is the smallest molecule with both R groups consisting of a methyl group and is one of the more amphipathic molecules of the four. As previously stated, the SH3 domain prefers a proline rich moiety, which are generally hydrophobic, non-polar regions. In contrast, the SH2 domain, binding phosphate groups on tyrosine residues, prefers more amphipathic moieties, like NHD2-15.

NHD2-114 had a K_d value only 4 times higher than NHD2-15 and also showed clear SH2 binding. The difference, structurally, is instead of a carbonyl group on the furan ring there is an ethyl acetate group. The addition of an oxygen and two carbons seems to make GRB's SH2 domain have less of an affinity for the antagonist, however, it does not appear to be enough to disrupt the bond completely; this might simply be due to the slight loss of amphipathicity or perhaps it is due to sterics. Furthermore, NHD2-114 did not seem to have a dose-dependent effect on the proliferation of BCR-ABL$^+$ K562 cells and was lethal to zebrafish by 48 hours. The addition of the ethyl group on NHD2-114 did appear to make the molecule less water soluble, as we often had a hard time getting it into solution,

but it also seems to have made the antagonist less effective as a potential treatment for CML.

NHD2-92 had a K_d value an order of magnitude larger than NHD2-15 and was also not as effective at slowing proliferation of K562 cells. Additionally, it is clearly binding the SH3 domain, not the SH2 domain as expected, though this might not keep it from being an effective treatment for CML it might also be the reason NHD2-92 is not as effective as NHD2-15. Interestingly, it is similar structurally to NHD2-114; instead of an ethyl acetate group, NHD2-92 has a methyl acetate. The lack of one CH2 group must be enough of a difference for GRB2's SH2 domain to not bind the antagonist but for the SH3 domain to take it up instead.

NHD2-107 has the same ethyl acetate R group as NHD2-114 with an additional phenyl ring attached directly to the furan ring. NHD2-107 had the lowest affinity for GRB2 and showed inconclusive results from the ELISA and CN assays, indicating that this more hydrophobic antagonist is binding somewhere else on GRB2 besides the SH2 and SH3 binding domains, possibly sticking to the protein to avoid the aqueous solution it is in. Interestingly, NHD2-107 did show significant growth reduction in K562 cells; however, it is possible that this antagonist is simply toxic and not necessarily targeting BCR-ABL+ cells, since it was found to be lethal to zebrafish even after only 24 hours.

Based on this information, NHD2-15 appears to be the best potential candidate for a new treatment for CML. However, more research will have to be conducted to determine the effectiveness of these molecules in vitro and in vivo, such as comparing the proliferation data on K562 cells to results on a BCR-ABL$^-$ cell line as well as a toxicity screen on an animal more similar to humans.

For this study, we only used K562 cells to test effectiveness of the antagonists. We can see a dose response from the molecules, but we do not know if that is due to effectiveness or if the antagonists are toxic. Future work will need to find a negative control that is both BCR-ABL$^-$ and does not have cancerous characteristics. GRB2 is upregulated and has a hand in many pathways that are also upregulated in cancer (Giubellino et. al, 2008; Tari et. al., 2001). Since immortalized cell lines have many characteristics in common with cancer, finding a negative control proved to be difficult.

Furthermore, these antagonists will need to be tested for toxicity in vivo in whole animal systems that more closely resemble humans. Though hematopoiesis is highly conserved between humans and zebrafish, other systems are not. The next logical, and common, step would be to perform a toxicity screen on laboratory rats. We would need to see that the antagonists can be tolerated at moderate doses since the K_d of NHD2-15 is about 120 μM (for comparison, imatinib has a K_d of 0.1 to 0.35 μM (Deininger et. al., 2005)).

Because the specificity of NHD2-15 is relatively low, another future study could center on using imatinib and NHD2-15 concurrently to see if there is an additive effect. Furthermore, several imatinib resistant K562 cell lines could be created, with different causes of the resistance, and NHD2-15 could be tested on them to see if, with or without imatinib, proliferation could be maintained again.

Additionally, finding the exact location of binding would help us better understand the interaction between the antagonists and GRB2. A useful tool for this would be to perform point mutations on the GRB2 gene and produce recombinant proteins with an altered amino acid near or in the SH2 domain. Once an amino acid is altered, K_d can be

analyzed to see if the mutation affected the bond. If it did, then that amino acid is interacting with the antagonist. The end goal would be to find the amino acids involved in binding these antagonists and getting an idea for where and how the antagonist is attaching.

Lastly, a wide range of molecules can be synthesized in an attempt to find an even better antagonist. With these four antagonists, changing the R groups even slightly had profound effects on the binding location, its ability to decrease proliferation, and its toxicity. It appears that the structure of NHD2-15 is on the right track; however, other small (or maybe even large) modifications might prove helpful for making antagonists that are even more effective.

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