Assay of Novel Belactosin A/Carfilzomib Hybrids as Proteasome Inhibitors

by
Conner Moss

A thesis presented to the Honors College of Middle Tennessee State University in partial fulfillment of the requirements for graduation from the University Honors College

Spring 2018

Assay of Novel Belactosin A/Carfilzomib Hybrids as Proteasome Inhibitors

by
Conner Moss

APPROVED:

_____
Dr. Norma Dunlap
Department of Chemistry

_____
Dr. Greg Van Patten
Department of Chemistry

_____
Dr. Kevin Bicker
Department of Chemistry

_____
Dr. John Vile
Dean, University Honors College

Acknowledgements

I cannot say enough in words how much I appreciate the help I have received during this project over the last year. This project has challenged me to dig deeper into applying what I study in biology and chemistry, and what I plan on studying in medicine, to the real world. First, I want to thank my advisor, Dr. Norma Dunlap, for providing a project that applied chemistry to biology and medicine, even though biology was not her field, and for allowing me to work in her lab. She has been very impactful and helpful not only in my research experience, but in my career as a student by teaching me organic chemistry, and in my path to become a physician through her encouragement and letters of support. Next, I would like to thank Dr. Kevin Bicker, whom I would not have been able to complete the project without. Although I was not his research student, he graciously took time out of his busy schedule on a weekly, and sometimes daily, basis to help guide the project with his enzyme kinetics expertise. Although I can ask a lot of questions and can be a slow learner at times, Dr. Dunlap and Dr. Bicker were very patient with me, and I sincerely appreciated that. I would especially like to thank the Honors College for their support through funding of the project and the American Chemical Society National Meeting, and also challenging me to optimize my education through graduation from their program. I would also like to thank Dr. Lynn Boyd for allowing me begin the project in her lab and for providing materials. In addition, thank you to Katie Sampuda and Paola Molina for being my first mentors in research and providing helpful advice. Last, I would like to thank God, my family, and friends for their support and encouragement during the good and also the tough times.

Abstract

Proteasome inhibitors, such as bortezomib and carfilzomib that are used clinically, have proven to be effective against some types of cancer, including multiple myeloma and mantle cell lymphoma. Belactosin A, a naturally occurring inhibitor that is not used clinically has key properties, including a β−lactone that acts as a serine trap. In this project, novel compounds synthesized in Dr. Norma Dunlap's lab based on belactosin A and carfilzomib were tested in an *in vitro* proteasome inhibition fluorescent assay using human 20S proteasome and the fluorogenic substrates Suc-LLVY-AMC and N-OMe-FLF-AMC. Bortezomib and MG-132, both known inhibitors, were also used as controls. In a phenotypic assay performed by Katie Sampuda, a former graduate student at MTSU, it was found that two of the novel compounds, NDMY1 and NDMY2, showed inhibition in an *in vivo* assay in *C. elegans* using confocal microscopy. After testing the novel compounds for this project in an *in vitro* assay, it was concluded that they showed no inhibition of the proteasome compared to the known inhibitors. There are plans to send these compounds to be tested in cancer cell lines to discover more details regarding their effectiveness as proteasome inhibitors.

Table of Contents

List of Tables

List of Figures

CHAPTER I: INTRODUCTION

Background

Proteins are necessary for a cell to survive as they have many functions that include transporting materials, acting as enzymes to catalyze reactions, existing as antibodies in the immune system, providing structure, and controlling the cell cycle (1). Proteins are made of amino acids, which are combined through peptide (amide) bonds and the sequence determines how a protein is structurally folded. Sometimes, when the protein doesn't fold correctly, it becomes inactive or nonfunctional, which can become detrimental to the cell, and the protein needs to be discarded. Other times, as in cancer, it is desirable for certain proteins to be degraded or not expressed in order to halt the cancer progression.

A proteasome is a complex of proteases, which are enzymes that degrade proteins, and is responsible for cleaving the peptide bonds of these proteins and discarding them (2). They can be found in the nucleus and cytoplasm of a cell (3). The 26S proteasome is a 60 subunit particle composed of a 20S core and two 19S caps (Figure 1) (4). The 19S caps are referred to as regulatory because they are not involved with the actual degradation of the protein. Instead, they function to recognize the protein targeted for degradation and unfold the protein so the 20S core can break it down (4). The 20S core has a four-stacked ring cylindrical structure, with each ring having seven subunits for a total of 28 subunits, and is considered the catalytic portion of the 26S proteasome because it is actively involved in protein cleavage (5). The two outside rings are composed of identical alpha subunits and interact with the 19S caps, while the two middle rings are composed of beta subunits (4). Each middle ring is made up of seven

distinct beta subunits. Three in each ring are considered catalytic: the β5 subunit is considered chymotrypsin-like, the β2 trypsin-like, and the β1 caspase-like, or more commonly known as postglutamyl peptide hydrolase-like (PGPH) (4). The hydroxy group of the N-terminal threonine residue in each of these three distinct subunits is considered the active site and acts as a nucleophile to begin cleavage of the peptide bonds in the protein (6). These active sites are common targets for proteasome inhibitors.

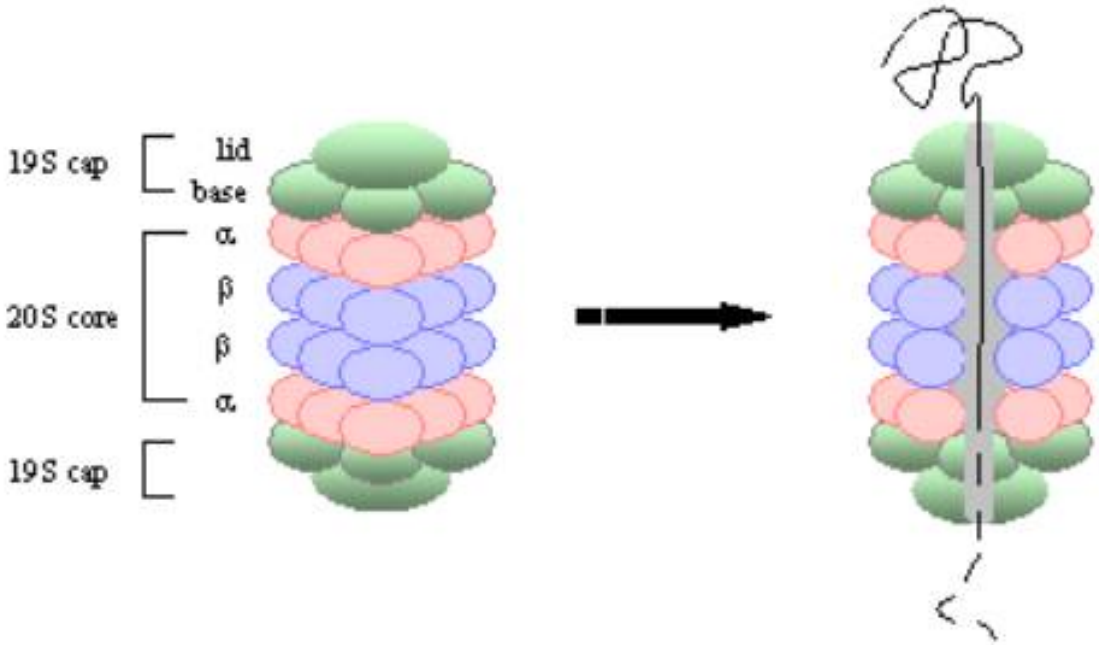

Figure 1: Structure of 26S proteasome (7). 19S regulatory caps and 20S catalytic core shown, with the protein to be cleaved represented as the black line; cleaved fragments shown at the bottom.

For a proteasome to degrade a protein, it first needs to recognize it. This is done through a mechanism called the ubiquitin-proteasome pathway (5) (Figure 2). In this pathway, ubiquitin, a protein found in every eukaryotic cell, recognizes a protein that needs to be degraded and tags it (5). Through a series of complex steps, more ubiquitin is attracted to the newly recognized protein and begins attaching itself, creating a

polyubiquitin chain on the protein (8). The polyubiquitinated protein is targeted to the 26S proteasome, where the 19S regulatory caps recognize the protein, unfold it, and thread it through the inside of its cylindrical shape to the 20S catalytic portion (4). The specific amino acid sequence of the inserted protein determines which beta subunit (chymotrypsin-like, trypsin-like, or caspase-like) will cleave it.

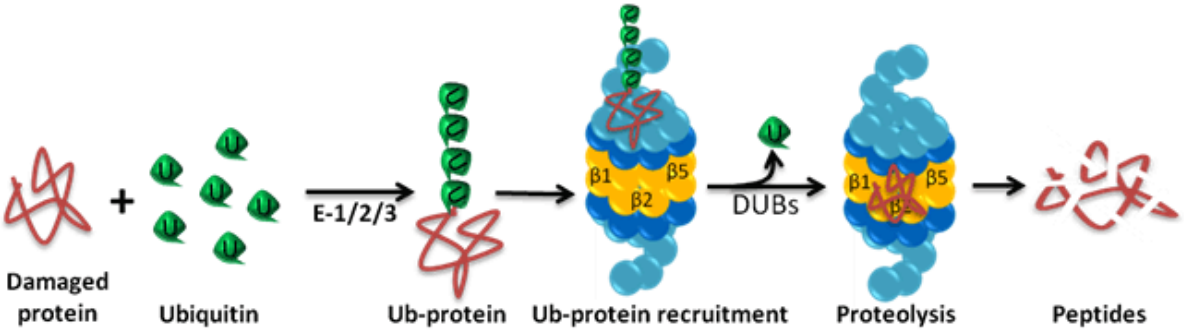

Figure 2: Outline showing the ubiquitin-proteasome pathway (9). Includes polyubiquitination, attachment to the proteasome, and cleavage into peptide fragments.

An example of the detailed mechanism of protein cleavage can be found in Figure 3. The N-terminal threonine of the proteasome adds to the carbonyl of the peptide to be cleaved, leading to a tetrahedral intermediate, and ultimately the cleaved product.

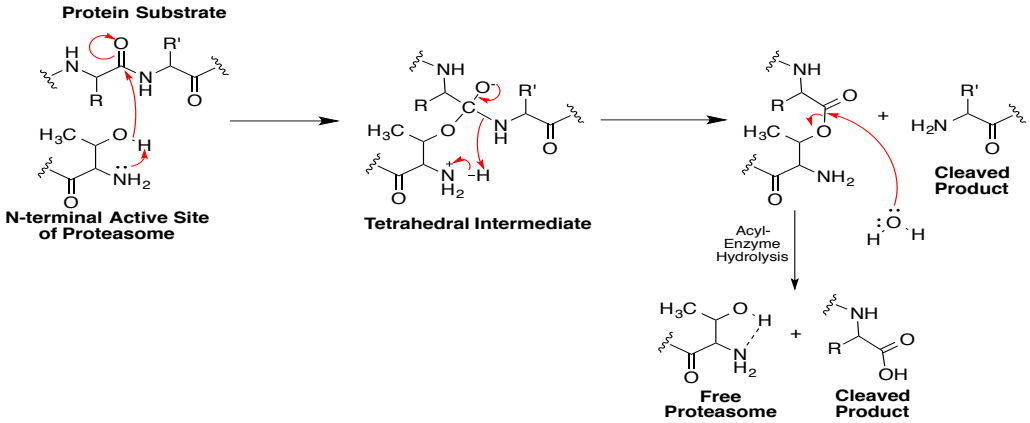

Figure 3: Mechanism of protein cleavage. The N-terminal threonine adds to the carbonyl of the peptide, ultimately resulting in the cleaved product.

Proteasome Inhibitors

Proteasome inhibitors function by binding to the proteasome and blocking its activity. Most known proteasome inhibitors have an electrophilic trap, which is essential to proteasome binding. The N-terminus of the threonine residue in the active site of one of the proteasome's beta rings is nucleophilic and binds to the electrophilic piece of the proteasome inhibitor (2). By disrupting the proteasome's normal function, proteasome inhibitors can affect cellular processes such as cell cycle regulation and apoptosis. Essentially, the proteasome is involved in controlling protein levels, including proteins commonly recognized in cancer. Examples of proteins involved in cancer include p53 (tumor suppressor), p27 (controls cell cycle at G1), and cyclin B (involved in mitotic phase), all of which are important in regulating cell cycle progression (6). Also, the proteasome is regularly involved in degradation of IκBa, which produces NF-κB (transcription factor). When control of NF-κB is not regulated, it has shown to be associated with cancer (6). The use of proteasome inhibitors has been shown to halt the cell cycle at different stages, which is important in controlling rapid proliferation of malignant cancer cells (5). For example, it has been indicated that belactosin A (a known proteasome inhibitor) stops the cell cycle at the G2/M phase (11). Proteasome inhibitors have also been proven to induce apoptosis specific to cancer cells, but not in normal, healthy cells (5). Without control of certain protein levels involved in regulating the cell cycle of cancer cells, pathways for increasing or halting cell growth can conflict and trigger apoptosis (5). Also, under inhibition, the proteasome cannot degrade misfolded proteins, which then accumulate and trigger c-Jun N-terminal kinase (JNK) (stress kinase) to initiate apoptotic events (5). For example, in multiple myeloma, NF-κB is

particularly important in preventing apoptosis and inducing expression of growth factors, so when the proteasome inhibitor disrupts the proteasome's ability to activate NF-κB, a collection of misfolded proteins arise in the cell and activate JNK, leading to apoptosis (10).

Known proteasome inhibitors are either synthetically produced or occur naturally, and are classified in different groups based on the structure of their electrophile. These groups include peptide aldehydes, peptide boronates, peptide epoxyketones, peptide vinyl sulfones, and β-lactones. The first class of proteasome inhibitors discovered was peptide aldehydes, which are synthetic peptides known to enter the cell quickly, have fast dissociation rates, and bind reversibly (5). The major peptide aldehyde known is MG-132 (Z-Leu-Leu-Leu-al), which uses its leucine side chain as an advantage for binding stabilization to the proteasome and has specificity to the β5 subunit (chymotrypsin-like) (Figure 3) (5). Peptide boronates are another class of proteasome inhibitors that are made synthetically, have slower dissociation rates, and are more potent compared to peptide aldehydes (5). The major peptide boronate known is bortezomib (PS-341), which was the first proteasome inhibitor used clinically to treat patients suffering from multiple myeloma and mantle cell lymphoma (Figure 4) (12). It shows specificity to the β5 subunit with less specificity to the β1 subunit (5).

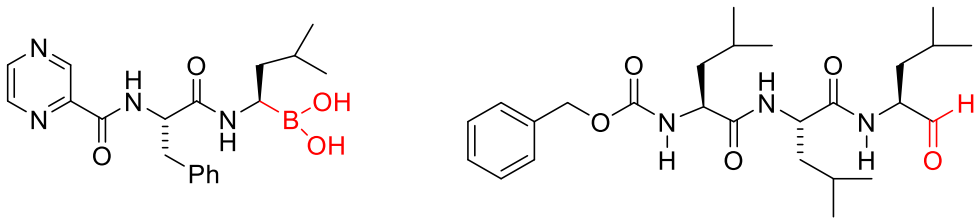

Figure 4: Structures of bortezomib (peptide boronate) and MG-132 (peptide aldehyde). The electrophile is in red.

Peptide epoxyketones are natural peptides that bind irreversibly and have specificity towards the β5 subunit (5). The major inhibitor from this class is carfilzomib. It is the second proteasome inhibitor used on the market to treat multiple myeloma. It is derived from another peptide epoxyketone named epoxomycin that was discovered from a bacterial strain (actinomycete strain Q996-17) (Figure 5) (5).

The last class of proteasome inhibitors is the β-lactones, which include omuralide, salinosporamide A, and belactosin A (5). Omuralide is the active compound produced from lactacystin, which is naturally found in *Streptomyces sp*., and irreversibly inhibits the proteasome through specificity to the β5 subunit (Figure 5) (5). Salinosporamide A (marizomib) is a metabolite found in the bacteria *Salinispora tropica*, and has advanced to clinical trials (Figure 5) (5). It primarily interacts with the β5 subunit. Belactosin A is found naturally in *Streptomyces sp.*, and has proven to inhibit the chymotrypsin-like activity of the proteasome (Figure 5) (5). Belactosin A has important features including a cyclopropane ring and a terminal β-lactone, while it functions by acylating the N-terminal threonine on β5 subunit (5).

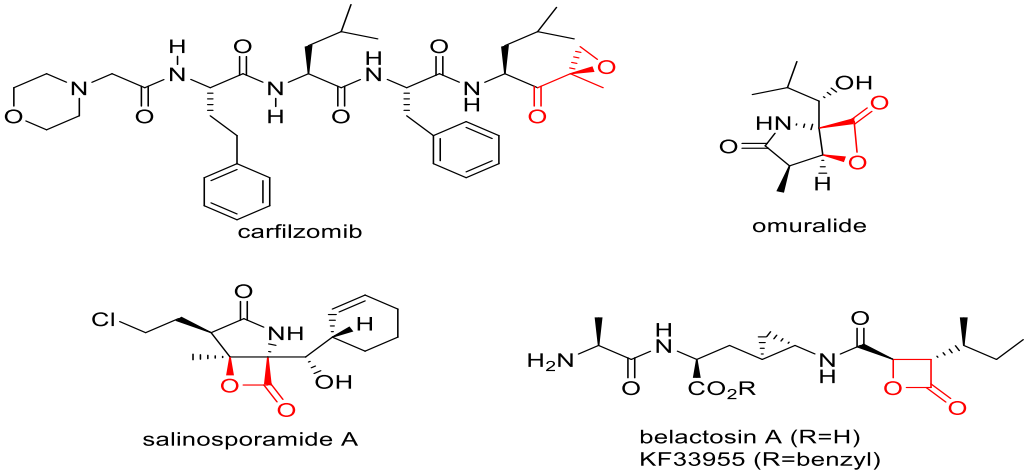

Figure 5: Structures of carfilzomib (peptide epoxyketone), omuralide (β-lactone), salinosporamide A (β–lactone), and belactosin A (β–lactone).

Measurement of Proteasome Inhibition

Measuring proteasome activity can be done by performing assays *in vitro* or *in vivo*. *In vitro* assays help simplify the environment and show if the inhibitor of interest actually affects the intended target, the proteasome. *In vivo* assays using healthy cell lines or cancer cell lines can help determine whether the inhibitor is cell permeable or effective in living cells, and can provide a more realistic outlook of the potential for the compound. In both assays, the basic procedure is to run a standard assay with a proteasome, proteasome substrate, and buffer first. A second run is then done with inhibitor added. *In vivo* assays provide the proteasome in the form of a cell, whereas *in vitro* assays provide the proteasome isolated from a cell. The substrate is a synthetic peptide characterized with a specific amino acid sequence and a fluorescent moiety. The amino acid sequence determines which subunit of the proteasome will cleave it, so substrates will tend to target chymotrypsin-like, trypsin-like, or caspase-like active sites. The proteasome cleaves the substrate at a certain amino acid, which releases the portion that is to become fluorescent. The intensity of this moiety's fluorescence is measured on a plate reader and is ultimately an indicator of proteasome activity. If fluorescence is produced, this signifies that the proteasome cleaved the substrate and is working, meaning that the inhibitor did not effectively inhibit the proteasome. In the presence of an inhibitor, the fluorescence will decrease in comparison to the mixture without inhibitor. Using varying concentrations of the inhibitor will provide K_i, which is the concentration of inhibitor that decreases the activity of an enzyme by half.

Although there are many drugs on the market, cancer is still a disease that the field of medicine is trying to eradicate. There is still much work to do, and proteasome

inhibitors show great potential. For example, since bortezomib was introduced, survival rates for multiple myeloma have increased from 25% to 47% (13). The goal of this project involves synthesis of novel hybrid analogues of belactosin A and carfilzomib and measurement of proteasome inhibition by testing their activity using a fluorescent *in vitro* proteasome assay (Figure 6). My personal contribution to the project involved inhibition assays with minimal synthesis, but the aim was to test these novel compounds and find out how well they inhibit the 20S proteasome with hopes that it will further advance the fight against cancer.

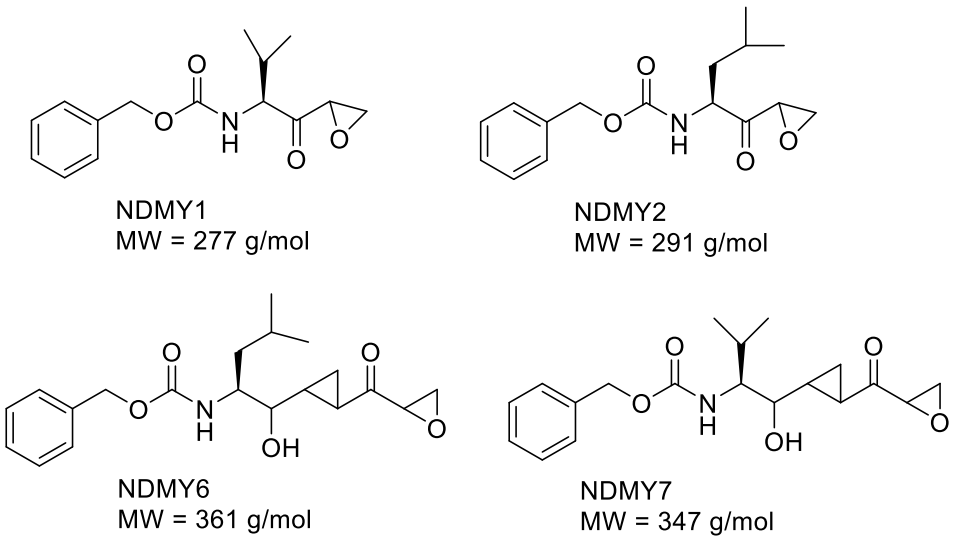

Figure 6: Structures of the novel compounds synthesized, based on belactosin A and carfilzomib.

CHAPTER II: MATERIALS AND METHODS

Materials

Materials used in this project included purified 20S erythrocyte proteasome, proteasome buffer, chymotrypsin-like substrate, trypsin-like substrate, assay buffer, 96-well black, flat bottom plates, pipettes, SpectraMax plate reader, known inhibitors such as bortezomib and MG-132, and the compounds synthesized in Dr. Dunlap's lab (NDMY1, NDMY2, NDMY6, NDMY7). Purchase of proteasome, chymotrypsin-like substrate, trypsin-like substrate, bortezomib, and MG-132 was funded by the MTSU Honors College and was bought from Boston Biochem, Anaspec, Boston Biochem, Selleckchem, and Selleckchem respectively. Materials for the proteasome buffer, assay buffer, 96 well plates, and pipettes were provided by Dr. Kevin Bicker's lab. Access to the plate reader was provided by Dr. Mary Farone's lab.

Preparation of Materials

Proteasome buffer (50 mM HEPES (pH 7.6), 100 mM NaCl, 1 mM dithiothreitol (DTT)) was prepared in a 15 mL centrifuge tube. Deionized water was added to bring the mixture to a volume of 5 mL. Proteasome stock (50 μg/mL) was prepared in a 1.5 mL microcentrifuge tube, then proteasome buffer was added up to bring the mixture to a volume of 100 μL. Aliquots were prepared by transferring 10 μL of proteasome stock into 20 separate 0.5 mL microcentrifuge tubes and were stored in a box at -80˚C. When in use, the proteasome stock was kept in an ice bucket until pipetted into the 96-well plate.

Assay buffer (20 mM Tris (pH 8), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.035% sodium dodecyl sulfate (SDS)) was prepared in a 15 mL centrifuge

tube. Deionized water was added to bring the mixture to a volume of 12 mL, and the assay buffer was stored at room temperature.

Three different substrates were used in this project: Suc-LLVY-AMC (chymotrypsin-like) (Abs/Em = 353/442 nm), N-OMe-FLF-AMC (chymotrypsin-like) (Abs/Em = 380/520 nm) provided by Dr. Lynn Boyd's lab, and Boc-LRR-AMC (trypsin-like) (Abs/Em = 380/460 nm). Suc-LLVY-AMC (5 mg) was prepared as a 50 mM stock using DMSO and stored in a 1.5 mL microcentrifuge tube at -20˚C. The N-OMe-FLF-AMC substrate from Dr. Boyd's lab was a 10 mM stock in DMSO and stored at -20˚C. Boc-LRR-AMC (5 mg) was prepared as a 50 mM stock using methanol and stored in a 1.5 mL microcentrifuge tube at -20˚C. Substrates were kept covered during storage and use for protection from light. Bortezomib and MG-132 were prepared as multiple 10 mM stock aliquots using DMSO and stored in small tubes at -20˚C. The compounds synthesized by Dr. Dunlap's lab were stored in vials at -20˚C and were in an oily form. Stocks were made for each at 20 mM using DMSO and were stored at -20˚C.

Description of Assays

There were two basic assays performed: a substrate optimization assay and an inhibition assay. The substrate optimization assay was performed to find the optimum concentration for each substrate that would give the best results when used in the inhibition assay. In general, both assays were performed by first thawing out the needed components, then spinning each tube in a microcentrifuge, in order to ensure an equal distribution in the mixture. Assay buffer was added first to the correct well, followed by inhibitor if needed, and then substrate, with proteasome added immediately before the

start of the assay. The plate was taken to the plate reader, warmed to 37˚C, proteasome was added, and then the assay began. The assay was setup as a two-hour continuous fluorescent assay with readings every three minutes. The corresponding recommended wavelengths for each substrate and read area were adjusted at the plate reader. The optimum concentration was found to be 25 μM for Suc-LLVY-AMC, 50 μM for N-OMe-FLF-AMC, and 50 μM for Boc-LRR-AMC. The setup for the optimization assay is described below (Table 1).

Table 1: Basic Setup for a Substrate Optimization Assay (Suc-LLVY-AMC, Boc-LRR-AMC, N-OMe- FLF-AMC)

Well #	Assay Buffer (μL)	Substrate (μL)	Proteasome (μL)
1	98	1 (100 μM)	1
2	98	1 (50 μM)	1
3	98	1 (25 μM)	1
4	100	0	0
5	99	1 (100 μM)	0
6	99	1 (50 μM)	0
7	99	1 (25 μM)	0

With the optimized substrate concentrations, inhibition assays were performed first with known inhibitors followed by compounds synthesized in the lab. The setup for the inhibition assay for known inhibitors is found below (Table 2).

Table 2: Basic Setup for an Inhibition Assay with Known Inhibitors (bortezomib and MG-132)

Well #	Assay Buffer (μL)	Substrate (μL)	Inhibitor (μL)	Proteasome (μL)
1	97	1	1 (10 μM)	1
2	97	1	1 (1 μM)	1
3	97	1	1 (0.1 μM)	1
4	97	1	1 (0.01 μM)	1
5	97	1	1 (0.001 μM)	1
6	97	1	1 DMSO (control)	1

*Substrate concentration depended on the type of substrate used

The setup for the inhibition assay for compounds synthesized in lab is found below (Table 3).

Table 3: Basic Setup for an Inhibition Assay with Inhibitors Synthesized in the Lab (NDMY1, NDMY2, NDMY6, NDMY7)

Well #	Assay Buffer (μL)	Substrate (μL)	Inhibitor (μL)	Proteasome (μL)
1	97	1	1 (100 μM)	1
2	97	1	1 (10 μM)	1
3	97	1	1 (1 μM)	1
4	97	1	1 (0.1 μM)	1
5	97	1	1 (0.01 μM)	1
6	97	1	1 DMSO (control)	1

*Substrate concentration depended on the type of substrate used

For all assays, the 7-amino-4-methylcoumarin (AMC) moiety on the substrate becomes fluorescent when the substrate is cleaved by the proteasome. This fluorescence is measured by the plate reader and recorded in SoftMax Pro (microplate data acquisition and analyzing software). The data was then exported and used in Microsoft Excel and GraFit (data analyzing software) to analyze the results and create graphs. Fluorescence indicates that the proteasome is functional, and therefore the proteasome inhibitor is not achieving the intended goal of inhibition. Substrate optimization assays were duplicated

to ensure the optimum concentration and a k_{cat}/K_m value was reported for each substrate. Inhibition assays were first performed with known inhibitors using each substrate to ensure that the assay is working correctly. These results were duplicated for each known inhibitor and k_{inact}/K_i values were reported. Next, each synthesized compound was used for the inhibition assay with each substrate, and results were duplicated. k_{inact}/K_i values were not reported for these compounds.

CHAPTER III: RESULTS

Substrate Optimization Assay

As noted before, substrate optimization assays were performed to find the concentration of the particular substrate to be used for the inhibition assays. After transferring results from the assay into Microsoft Excel, values from wells 5-7 (substrate only, no proteasome present) were subtracted from wells 1-3 (substrate and proteasome both present) at the corresponding concentration to eliminate background fluorescence. These new values were used to plot a relative fluorescent unit (RFU) vs. time (sec). The slope of the line for each concentration represented the rate (RFU sec^{-1}), which was then plotted in an enzyme kinetics graph vs. concentration of substrate [S] (μM). A table of kinetic values found for each substrate can be found below (Table 4).

Table 4: Values Obtained from the Substrate Optimization Assays

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
Suc-LLVY-AMC	0.82	45	1.82×10^4
Boc-LRR-AMC	Not determined	Not determined	1100
N-OMe-FLF-AMC	0.06	35	1.71×10^3

Suc-LLVY-AMC exhibited saturating kinetics, therefore individual k_{cat} (0.82 s^{-1}) and K_m (45 μM) values could be obtained along with the k_{cat}/K_m (1.82×10^4 M^{-1} s^{-1}) (Figure 7). The number of substrate molecules converted into product by an enzyme in a unit time when the enzyme is fully saturated with substrate is represented by k_{cat}. The

substrate concentration that is required for the reaction to occur at half of V_{max} is K_m. This is the Michaelis-Menten constant and indicates how well the substrate binds to the proteasome. The catalytic efficiency of the enzyme is referred to as k_{cat}/K_m. The maximum velocity or rate at which the enzyme catalyzed a reaction is V_{max}. For Suc-LLVY-AMC, the concentration determined for use in inhibition assays was 25 μM, because at this concentration, there is adequate fluorescence signifying that a sufficient amount of substrate is cleaved by the proteasome.

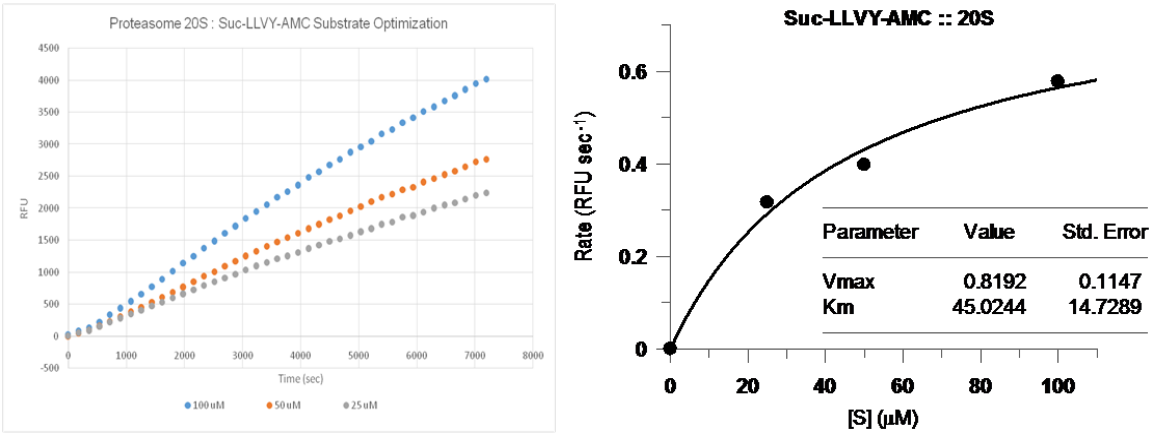

Figure 7: Graphs for Suc-LLVY-AMC optimization assay**.** RFU vs. time graph for each concentration of Suc-LLVY-AMC on left and enzyme kinetics graph on the right providing k_{cat} (0.82 s^{-1}) and K_m (45 μM).

Boc-LRR-AMC exhibited linear kinetics rather than saturating kinetics, therefore only the k_{cat}/K_m (1100 M^{-1} s^{-1}) value could be reported using the slope of line created in the enzyme kinetics graph, but the individual k_{cat} and K_m values could not be determined (Figure 8). For Boc-LRR-AMC, the concentration determined for use in inhibition assays was 50 μM, because at this concentration, there is adequate fluorescence signifying that a sufficient amount of substrate is cleaved by the proteasome.

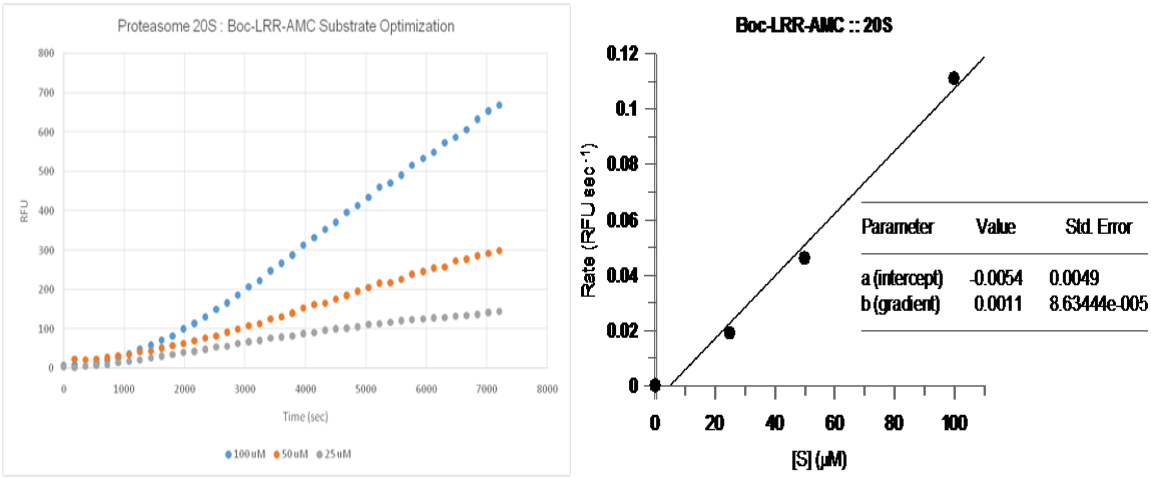

Figure 8: Graphs for Boc-LRR-AMC optimization assay. RFU vs. time graph for each concentration of Boc-LRR-AMC on left and enzyme kinetics graph on the right providing k_{cat}/K_m (1100 M^{-1} s^{-1}).

N-OMe-FLF-AMC exhibited saturating kinetics and the k_{cat} (0.06 s^{-1}), and K_m (35 µM) values were reported along with the k_{cat}/K_m (1.71 x10^3 M^{-1} s^{-1}) (Figure 9). For N-OMe-FLF-AMC, the concentration determined for use in inhibition assays was 50 µM, because at this concentration, there is adequate fluorescence signifying that a sufficient amount of substrate is cleaved by the proteasome.

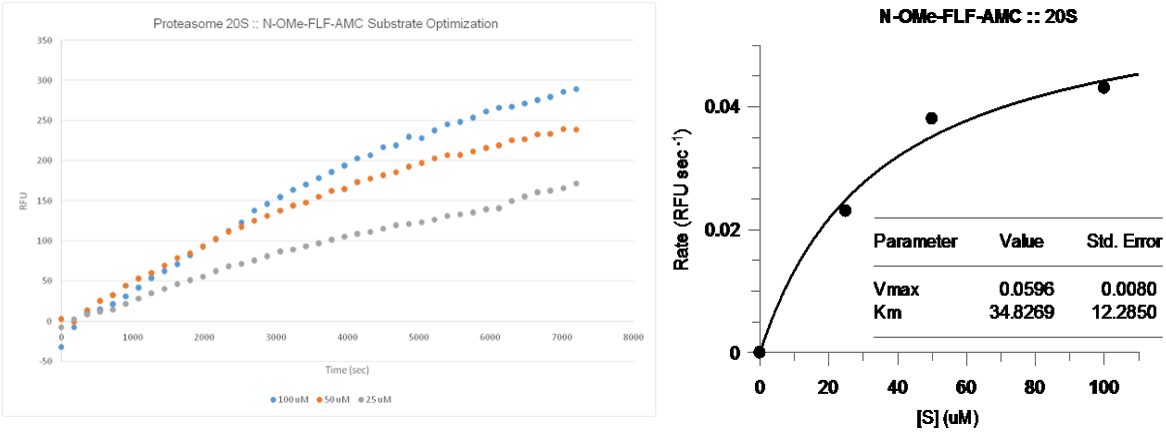

Figure 9: Graphs for N-OMe-FLF-AMC optimization assay. RFU vs. time graph for each concentration of N-OMe-FLF-AMC on left and enzyme kinetics graph on the right providing k_{cat} (0.06 s^{-1}) and K_m (35 µM).

Inhibition Assays

For inhibition assays, results from the assay in RFU over time were transferred to Microsoft Excel and converted to percent activity of the proteasome by dividing each value attained during the first 45 minutes for each inhibitor concentration by the DMSO control for that particular time point. The percent activity values for the first 45 minutes were then transferred to GraFit to create a percent activity vs. time (min) graph. For substrates exhibiting saturating kinetics, the rate constant (k) observed needed to be corrected, so a formula $1 + [S] / K_m$ was used to correct for k_{obs}. These new rate constant values were used in GraFit to create an enzyme kinetics graph plotting k_{obs} vs. concentration of inhibitor, which provided values of k_{inact} and K_i. The rate of enzyme inactivation is k_{inact}, while K_i is the concentration of inhibitor that inhibits the enzyme to half of its activity. A table of values for the inhibition assays can be found below (Table 5).

Table 5: Values Obtained from the Inhibition Assays.

Inhibitor	Substrate	k_{inact} (min^{-1})	K_i (μM)	k_{inact}/K_i ($s^{-1} M^{-1}$)
Bortezomib	Suc-LLVY-AMC	0.07	0.07	16,100
Bortezomib	N-OMe-FLF-AMC	0.10	0.05	32,300
MG-132	Suc-LLVY-AMC	0.11	0.12	16,600
NDMY1, NDMY2, NDMY6, MDMY7	Suc-LLVY-AMC	Not determined	Not determined	Not determined
NDMY1, NDMY2, NDMY6, MDMY7	N-OMe-FLF-AMC	Not determined	Not determined	Not determined

Inhibitors with Suc-LLVY-AMC

Bortezomib was first used with Suc-LLVY-AMC and showed significant inhibition, providing values for k_{inact} (0.07 min^{-1}) and K_i (0.07 μM), and a k_{inact}/K_i (16,100 s^{-1} M^{-1}) (Figure 10). This is evidenced by a decrease in activity over time as compared to the control, with more inhibition as the dose increases. MG-132 was used next with Suc-LLVY-AMC and also showed significant inhibition, providing values for k_{inact} (0.11 min^{-1}) and K_i (0.12 μM), and a k_{inact}/K_i (16,600 s^{-1} M^{-1}) (Figure 10).

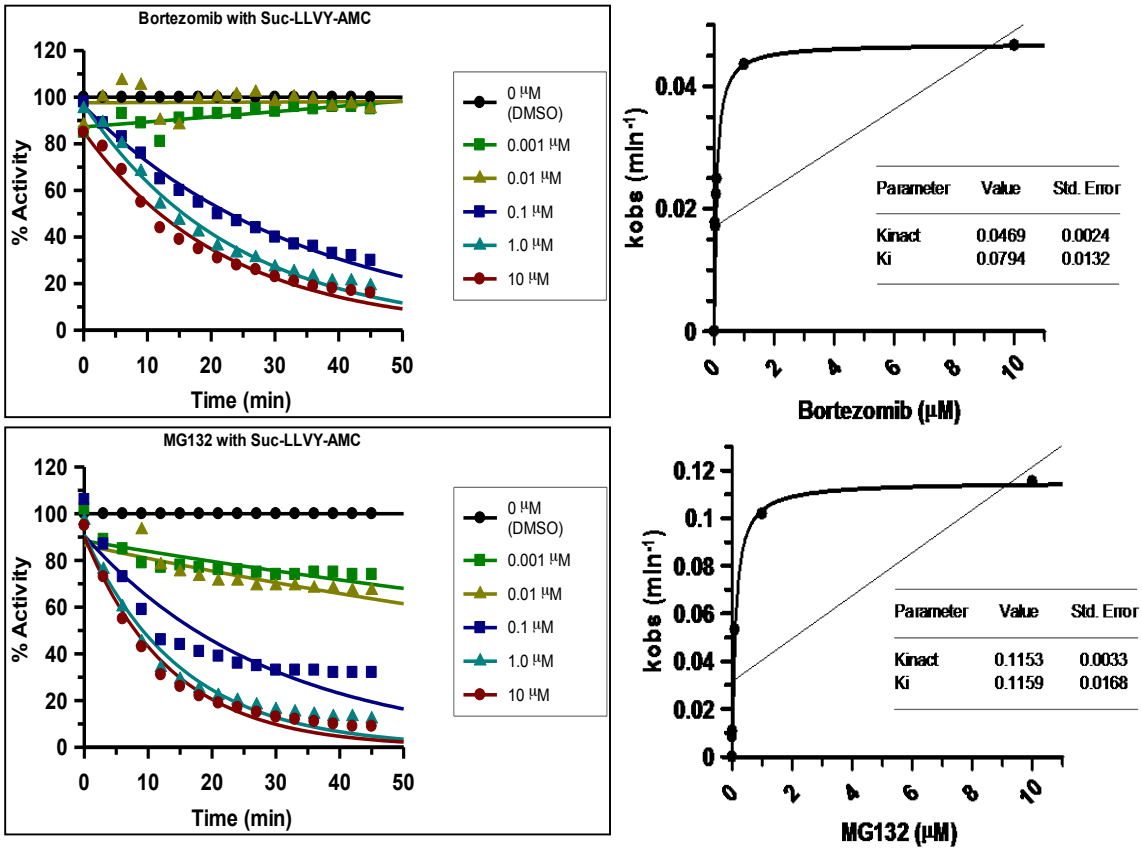

Figure 10: Graphs for bortezomib and MG-132 inhibition assays with Suc-LLVY-AMC**.** Bortezomib percent activity vs. time graph and enzyme kinetics graph on top providing providing k_{inact} (0.07 min^{-1}), K_i (0.07 μM), and k_{inact}/K_i (16,100 s^{-1} M^{-1}). MG-132 percent activity vs. time graph and enzyme kinetics graph on bottom providing providing k_{inact} (0.11 min^{-1}), K_i (0.12 μM), and k_{inact}/K_i (16,600 s^{-1} M^{-1}).

NDMY1, NDMY2, NDMY6, and NDMY7 were next used with Suc-LLVY-AMC and showed no inhibition (Figure 11). Values for k_{inact} , K_i , and k_{inact}/K_i were not calculated due to no inhibition.

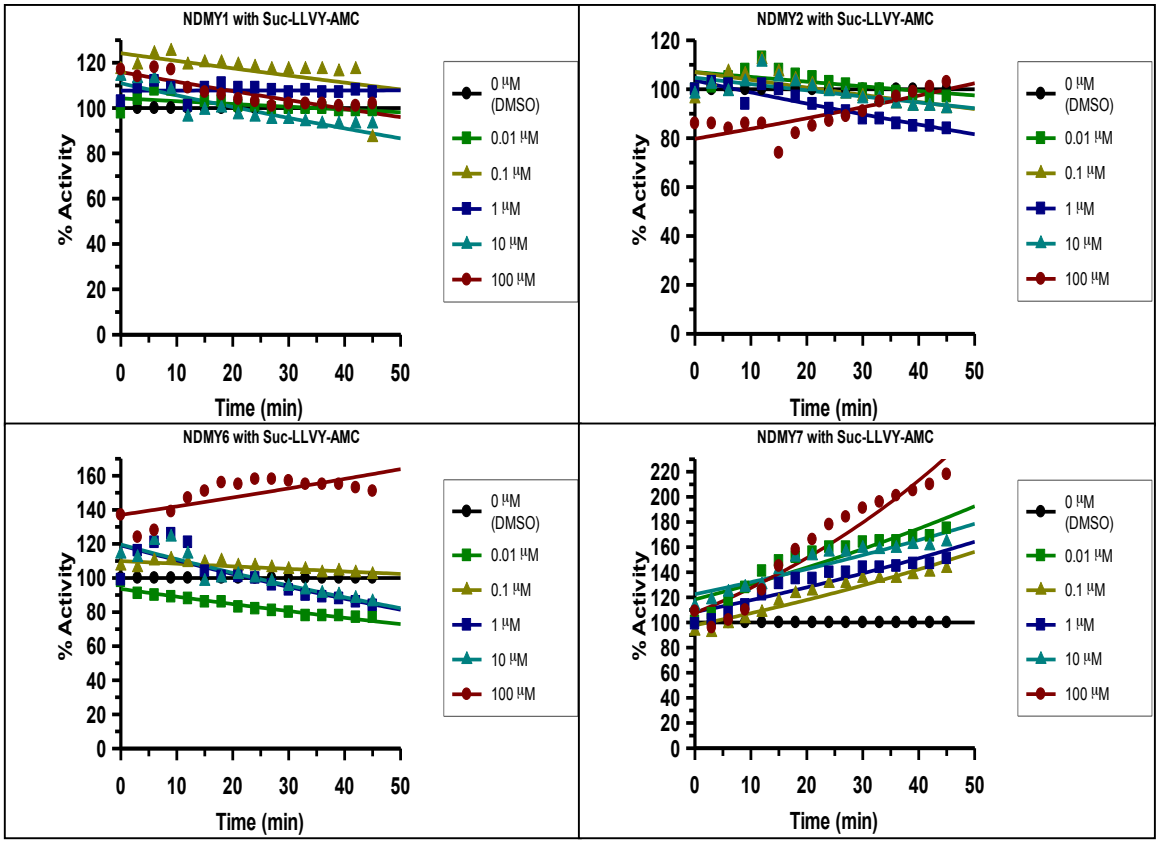

Figure 11: Graphs for NDMY1, NDMY2, NDMY6, and NDMY7 inhibition assays with Suc-LLVY-AMC. Percent activity vs. time graphs showing no inhibition.

Inhibitors with N-OMe-FLF-AMC

Bortezomib was first used with N-OMe-FLF-AMC and showed significant inhibition, providing values for k_{inact} (0.10 min^{-1}) and K_i (0.05 μM), and a k_{inact}/K_i (32,300 s^{-1} M^{-1}) (Figure 12).

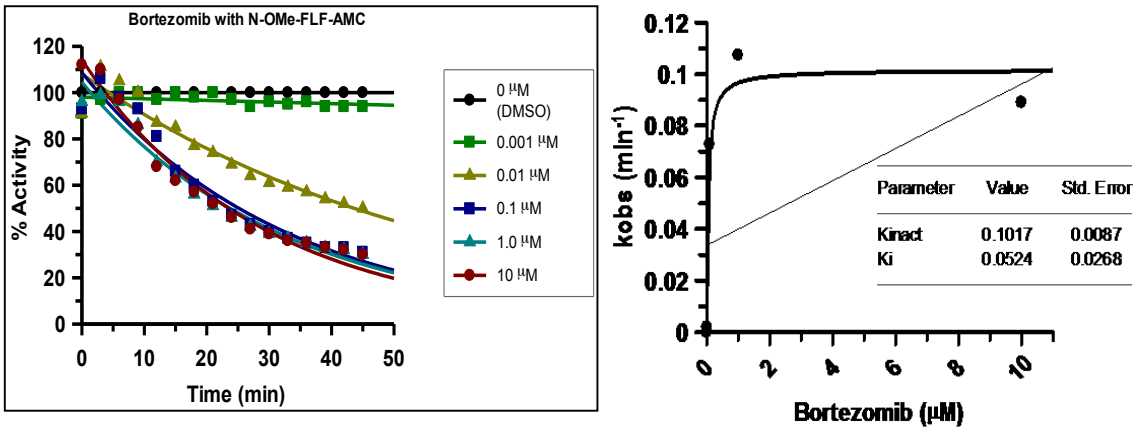

Figure 12: Graphs for bortezomib inhibition assays with N-OMe-FLF-AMC**.** Percent activity vs. time graph and enzyme kinetics graph providing k_{inact} (0.10 min^{-1}), K_i (0.05 µM), and k_{inact}/K_i (32,300 s^{-1} M^{-1}).

NDMY1, NDMY2, NDMY6, and NDMY7 were next used with N-OMe-FLF-AMC and showed no inhibition (Figure 13). Values for k_{inact} , K_i , and k_{inact}/K_i were not calculated due to no inhibition.

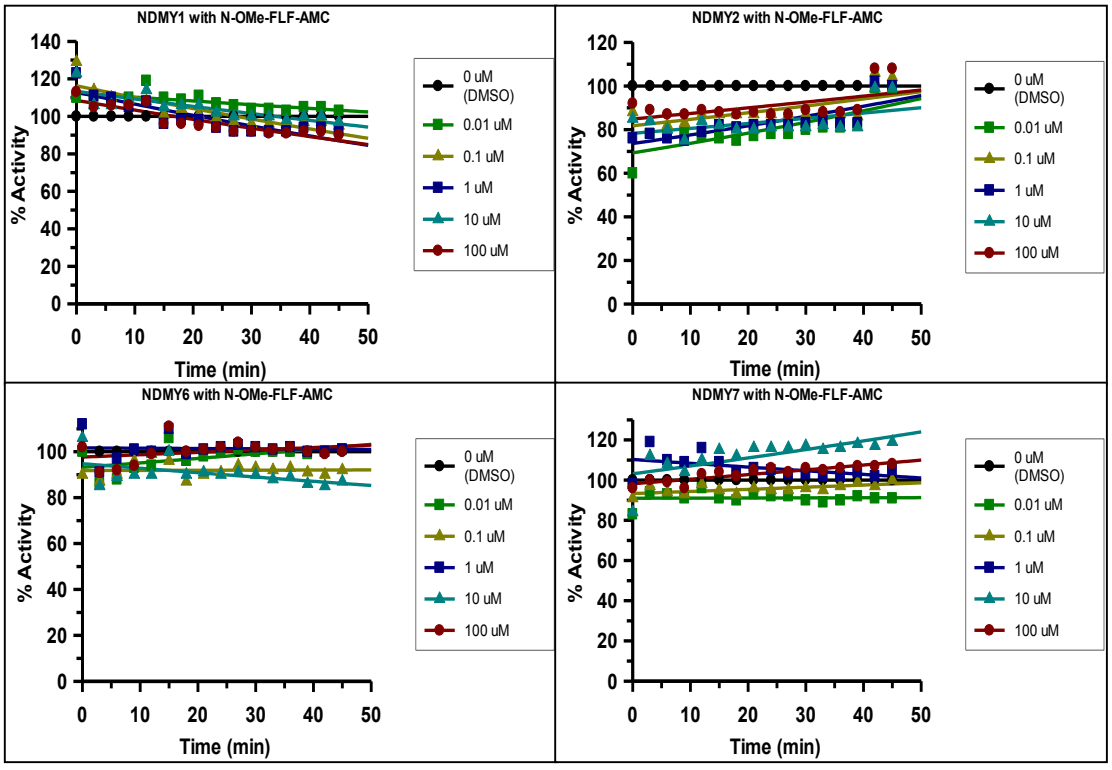

Figure 13: Graphs for NDMY1, NDMY2, NDMY6, and NDMY7 inhibition assays with N-OMe-FLF-AMC. Percent activity vs. time graphs showing no inhibition.

CHAPTER IV: DISCUSSION

As stated previously, the overall goal of this project was to evaluate several novel compounds as proteasome inhibitors in an assay using pure proteasome. These compounds were designed as potential proteasome inhibitors and showed promise in a separate phenotypic assay. In order to carry out the proteasome assay, three proteasome substrates were investigated to determine optimum concentrations. This was followed by incubation of the substrates and proteasome with two known inhibitors, bortezomib and MG-132. Finally, the chosen substrates were incubated with proteasome and the novel inhibitors.

Substrate Optimization

In the substrate optimization assays, three different substrates were tested, all of which have a 7-amino-4-methylcoumarin (AMC) moiety that is released when cleaved (Figure 14).

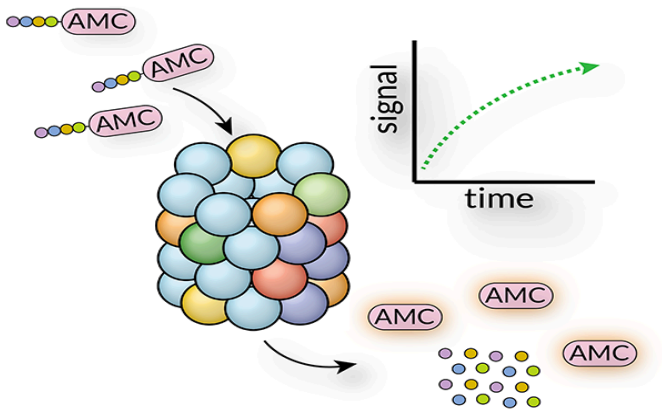

Figure 14: Outline of cleavage of peptide substrate with an AMC moiety (14). The cleaved AMC moiety becomes fluorescent and provides a signal over time.

The first substrate tested was Suc-LLVY-AMC, a substrate that inhibits the chymotrypsin-like sites on the proteasome. This gave a catalytic efficiency of (k_{cat}/K_m = 1.82 x 10^4 M^{-1} s^{-1}).

The second substrate tested was Boc-LRR-AMC, a substrate that inhibits the trypsin-like sites on the proteasome. The proteasome exhibited a much lower catalytic efficiency when paired with Boc-LRR-AMC (k_{cat}/K_m = 1100 M^{-1} s^{-1}) compared to Suc-LLVY-AMC (k_{cat}/K_m = 1.82 x 10^4 M^{-1} s^{-1}). This might be due to a lower efficiency for trypsin-like activity.

A third substrate, N-OMe-FLF-AMC, was also tested. This also inhibits the chymotrypsin-like sites, but is cell permeable, and was used in the phenotypic assay. This exhibited a catalytic efficiency of (k_{cat}/K_m = 1.71 x10^3 M^{-1} s^{-1}), similar to what was found for Suc-LLVY-AMC.

When comparing Suc-LLVY-AMC and N-OMe-FLF-AMC, both chymotrypsin-like substrates, it appeared that the proteasome functioned best when paired with Suc-LLVY-AMC as evidenced by a ten fold higher catalytic efficiency and a slightly higher binding affinity. Both K_m values were slightly similar, signifying that both substrates bound to the proteasome adequately. The number of N-OMe-FLF-AMC molecules converted into product by the proteasome when fully saturated (k_{cat} = 0.06 s^{-1}) was significantly lower than Suc-LLVY-AMC (k_{cat} = 0.82 s^{-1}), which most likely contributed to a lower catalytic efficiency.

Inhibitors

The known inhibitors, bortezomib and MG-132 were first tested in the inhibition assays. Boc-LRR-AMC was not used as a substrate in the inhibition assays because it failed to provide results with bortezomib that could be duplicated. Suc-LLVY-AMC (k_{inact}/K_i =16,600 s^{-1} M^{-1}) and N-OMe-FLF-AMC (k_{inact}/K_i = 32,300 s^{-1} M^{-1}) when paired with bortezomib showed similar characteristics with only a two-fold difference in k_{inact}/K_i, although a higher substrate concentration was used in the inhibition assays for N-OMe-FLF-AMC (50 μM) compared to Suc-LLVY-AMC (25 μM). For this project, the K_i for bortezomib was found to be 0.07 μM when paired with Suc-LLVY-AMC and 0.05 μM when paired with N-OMe-FLF-AMC, which is approximately one hundred-fold different than the expected K_i of 0.6 nM. This expected value was provided on the data sheet from the company bortezomib was purchased from. MG-132 (k_{inact}/K_i = 16,600 s^{-1} M -1) paired with Suc-LLVY-AMC showed an almost identical k_{inact}/K_i value as found for bortezomib (k_{inact}/K_i = 16,100 s^{-1} M^{-1}) paired with Suc-LLVY-AMC. For this project, the K_i for MG-132 was found to be 0.12 μM when paired with Suc-LLVY-AMC, which is approximately thirty-fold different than the expected K_i of 4 nM, which was provided on the data sheet from the company MG-132 was purchased from. These results with the two known inhibitors confirmed that in our hands, the assay gave expected results. Although not identical to reported values, both compounds show strong proteasome inhibition.

The novel compounds, including the simple epoxides (NDMY1 and NDMY2) and the hybrid analogues with the cyclopropyl backbone and epoxyketone (NDMY6 and NDMY7) were tested next in the inhibition assays. These novel compounds showed no

inhibition *in vitro* with any substrate used in this project. This was surprising since earlier, phenotypic assays performed *in vivo* in *C. elegans* in Dr. Boyd's lab by Katie Sampuda gave different results (Figure 15).

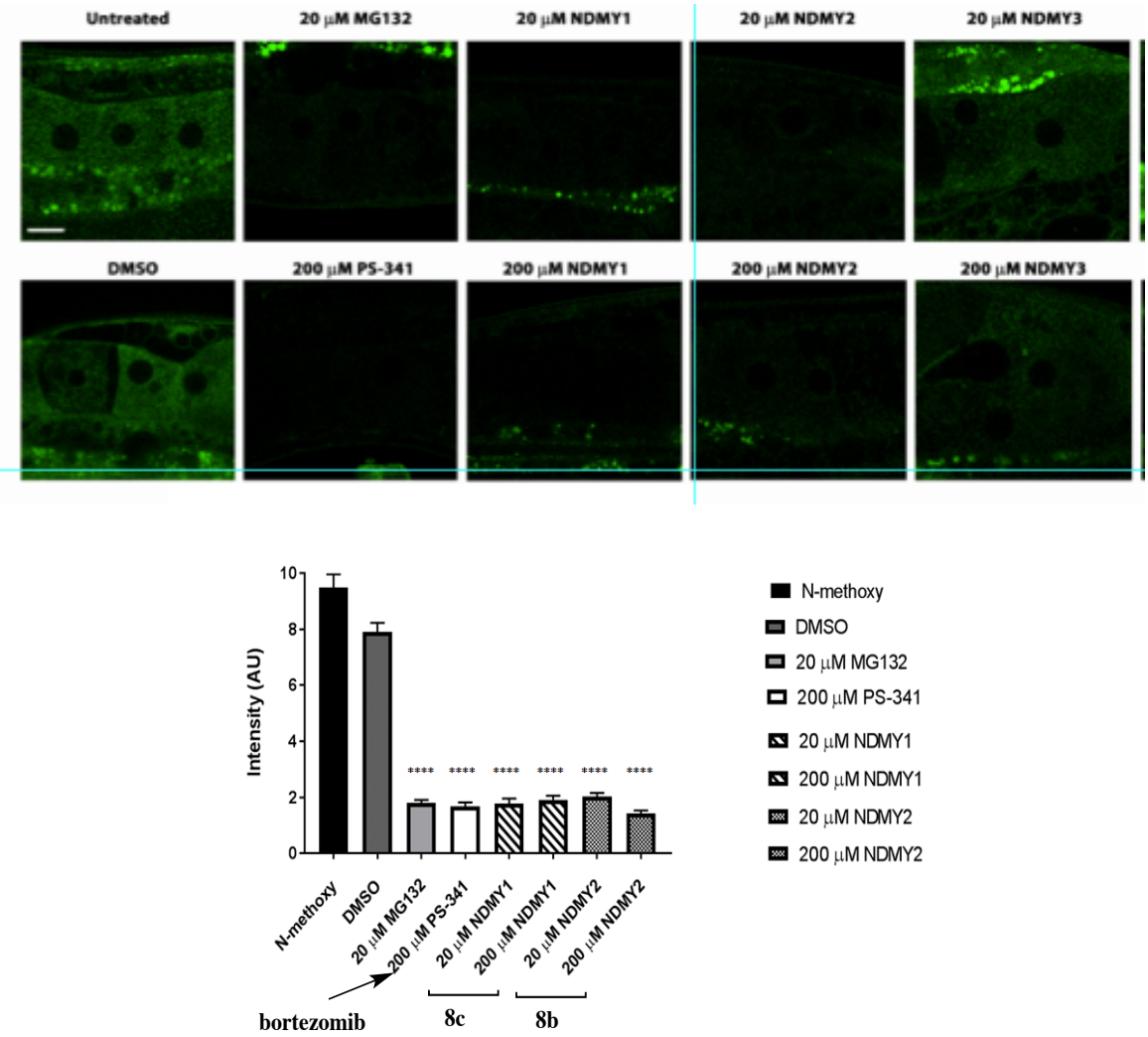

Figure 15: Phenotypic assay in oocytes of *C. elegans*. Fluorescent intensity measure by confocal microscopy using cell permeable fluorescent proteasome substrate N-OMe-FLF-AMC. Simple epoxides NDMY1 and NDMY2 similar in potency to bortezomib at 20 and 200mM.

In the phenotypic assay, inhibition results similar to results from bortezomib and MG-132 were found for NDMY1 and NDMY2 at 20 μM and 200 μM using the N-OMe-

FLF-AMC. Yet, during this *in vitro* project, the same compounds were found to show no inhibition even with the same substrate. The compounds might be showing differing results due to their ability to cause indirect inhibition of the proteasome in a different mechanism for the *in vivo* assay compared to the direct mechanism that is tested for in the *in vitro* assay. To further specify whether the compounds are effective as proteasome inhibitors, the next step is to send the compounds to be tested in cancer cell lines.

REFERENCES

1. "What are proteins and what do they do?" - Genetics Home Reference. (n.d.). Retrieved April 05, 2018, from https://ghr.nlm.nih.gov/primer/howgeneswork/protein.
2. Vivier, M., Rapp, M., Papon, J., Labarre, P., Galmier, M., Sauzière, J., & Madelmont, J. (2008). "Synthesis, Radiosynthesis, and Biological Evaluation of New Proteasome Inhibitors in a Tumor Targeting Approach." *Journal of Medicinal Chemistry,51*(4), 1043-1047.
3. Reidlinger, J., Pike, A. M., Savory, P. J., Murray, R. Z., & Rivett, A. J. (1997). "Catalytic Properties of 26 S and 20 S Proteasomes and Radiolabeling of MB1, LMP7, and C7 Subunits Associated with Trypsin-like and Chymotrypsin-like Activities." *Journal of Biological Chemistry,272*(40), 24899-24905.
4. Kessler, B. M., Tortorella, D., Altun, M., Kisselev, A. F., Fiebiger, E., Hekking, B. G., & Overkleeft, H. S. (2001). "Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping specificities of the catalytic β-subunits." *Chemistry & Biology,8*(9), 913-929.
5. Borissenko, L., & Groll, M. (2007). "20S Proteasome and Its Inhibitors: Crystallographic Knowledge for Drug Development." *ChemInform,38*(22).
6. Nakamura, H., Watanabe, M., Ban, H. S., Nabeyama, W., & Asai, A. (2009). "Synthesis and biological evaluation of boron peptide analogues of Belactosin C as proteasome inhibitors." *Bioorganic & Medicinal Chemistry Letters,19*(12), 3220-3224.

7. AAA ATPases. (n.d.). Retrieved April 05, 2018, from http://www.ebi.ac.uk/interpro/potm/2006_8/Page2.htm.
8. Li, W. & Ye, Y. "Polyubiquitin chains: functions, structures, and mechanisms." (2008). *Cellular and Molecular Life Sciences*. 65: 2397.
9. Rao G et al. (2015). "Ubiquitin-Proteasome System in Neurodegenerative Disorders." *Journal of Drug Metabolism and Toxicology*. 6:4.
10. Goldberg, A. L. (2012). "Development of proteasome inhibitors as research tools and cancer drugs." *The Journal of Cell Biology,199*(4), 583-588.
11. Yoshida, K, et al. (2009). "Three-dimensional structure-activity relationship study of belactosin A and its stereo- and regioisomers: Development of potent proteasome inhibitors by a stereochemical diversity-oriented strategy." *Organic & Biomolecular Chemistry,7*(9), 1868.
12. Dou, Q., & Zonder, J. (2014). "Overview of Proteasome Inhibitor-Based Anti-cancer Therapies: Perspective on Bortezomib and Second Generation Proteasome Inhibitors versus Future Generation Inhibitors of Ubiquitin-Proteasome System." *Current Cancer Drug Targets,14*(6), 517-536.
13. Dou, Q., & Zonder, J. (2014). "Overview of Proteasome Inhibitor-Based Anti-cancer Therapies: Perspective on Bortezomib and Second Generation Proteasome Inhibitors versus Future Generation Inhibitors of Ubiquitin-Proteasome System." *Current Cancer Drug Targets,14*(6), 517-536.
14. Suc-Leu-Leu-Val-Tyr-AMC (LLVY-AMC). (n.d.). Retrieved April 05, 2018, from https://southbaybio.com/products/suc-leu-leu-val-tyr-amc.html.